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Microbial Models: From Environmental to Industrial Sustainability

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Microorganisms for Sustainability

Volume 1

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Microbial Models: From Environmental to Industrial Sustainability

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Preface

In 1987, the Brundtland Report defined sustainable development as "the development that meets the needs of the present without compromising the ability of future generations to meet their own needs." In few words, it means economic growth, environmental protection, and social equality, for now and the future. The environmental, economic, and social developments are thus the three pillars of sustainability. In this scenario, the use of microbes for the industrial production of biomolecules, the manufacture of foods, and the preservation of renewable resource such as the soil, among others, is a matter of sustainable development.

This book volume is dealing with the description of a few microbial genera from the point of view of their environmental and economic sustainable use, focusing on the academic, ecological and industrial relevance of different microbial genera and their contribution to the social sustainability.

Through the different book chapters, the readers will find the description of each genus in terms of their physiology and metabolism and their relevance as microbial models that contribute to climb one step in the pillars of sustainability (environmental and/or economic/industrial sustainability). A few chapters also include worldwide market information and list of patents.

This book is open to academic researchers as well as the industrial sector, which needs information related to the development and market of industrial products that improve the quality of human life. In addition, the chapters also provide information regarding the biological systems or microorganisms that contribute to the human welfare through the conservation of natural resources and the production of foods and eco-friendly biodegradable products. A multidisciplinary picture of microbial sustainability has been captured in this book. It provides a thorough overview of a few microbial models used to withstand sustainability, in terms of their physiology and metabolism, providing information in a historical perspective, including the upcoming news.

The use of *Pseudomonas putida*, *Herbaspirillum seropedicae*, *Saccharomyces cerevisiae*, *Lactobacillus* spp., *Escherichia coli*, *Aspergillus* spp., *Pseudoalteromonas haloplanktis*, and other psychrophilic bacteria as microbial models in industrial sustainability is discussed. The rational uses of these microbes in the production of bioplastics, bioethanol, dairy products and recombinant production of proteins. Environmental sustainability was assessed through the description of a few novel microbial examples (*Flavobacterium*, *Paracoccus* and *Delftia* strains) with

potential uses in bioremediation and other applicative insights. Finally, the third part of the book deals with the description of microbes with many potential uses in agricultural systems (*Streptomyces*, *Rhizobium*, *Azospirillum* and *Pseudomonas* strains), reflecting the global demand for more sustainable agriculture microbial-based technologies.

Montevideo, Uruguay Susana Castro-Sowinski

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Part I

Microbes in Sustainable Industrial Development

Systems and Synthetic Biology Approaches for Metabolic Engineering of *Pseudomonas putida*

1

Pablo I. Nikel

Abstract

Pseudomonas putida is increasingly attracting attention as a bacterial host of reference both for basic and applied research. Over the years, this Gram-negative soil bacterium has been considered a potential agent for environmental bioremediation of industrial wastes and xenobiotic compounds and also a promising colonizer of the rhizosphere. However, the potential biotechnological applications of *P. putida* were enormously multiplied by the advent of contemporary synthetic biology which, together with the wealth of information provided by systems-level analysis of its genome, transcriptome, proteome, metabolome, and fluxome, enabled the implementation of targeted metabolic engineering approaches. This chapter summarizes the main discoveries within this context that mediated the transition of *P. putida* from its humble origin in the soil to modern biotechnology setups, where it excels in a number of practical applications for which other traditional microbial cell factories cannot be used (e.g., in hosting harsh oxidative reactions for the production of valuable chemicals).

1.1 Introduction

Over the years, the application of recombinant DNA technologies to a number of bacterial hosts has mediated the emergence of a phenomenal plethora of biotechnological applications (Oliver 2000; Ladisch and Mosier 2009), which range from the production of novel bioactive molecules to the treatment of xenobiotic chemical

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wastes. Sustainable bioprocesses are among the most sought-after goals (Ruiz et al. 2012). Soil bacteria have been an obvious choice for some of these biotechnological purposes, as the diversity of microorganisms thriving in such environment is certainly enormous. Several attempts have been made to capitalize on microbial soil diversity; these were recently expanded to include the mining of environmental genes in complete bacterial genomes or in metagenomes (Daniel 2004), through the use of functional traps and sophisticated bioinformatic approaches (Medico et al. 2001; Mohn et al. 2006). Historically, the so-called *genetic engineering* was used to accomplish part of these ambitious endeavors, although most strategies often lacked an authentic engineering (i.e., rational) perspective and largely depended on trial-and-error experimental attempts. By contrast, contemporary *synthetic biology* (SynBio) tackles the same type of challenges involving a genuine forward-design engineering approach (Endy 2005; de Lorenzo and Danchin 2008; Kahl and Endy 2013). One of the key components in SynBio is the adoption of a biological *chassis*, into which users can plug-in and plug-out genetic circuits and new-to-nature properties at will. The metaphor of a *chassis* has become commonplace in SynBio (Danchin 2012), the very term evoking the image of a *physical framework* supporting a man-made object (e.g., the carcass containing each of the electronic devices that, together, make a radio equipment).

The last 10 years witnessed a steadily increasing number of SynBio-guided biotechnological applications based on the Gram-negative soil bacterium *Pseudomonas putida* (Nikel et al. 2014a). Its fast growth, low nutrient demand, an extremely versatile metabolism, and the intrinsically high availability of reducing equivalents are only a few natural advantages that make *P. putida* an almost ideal physical and metabolic *chassis* for various industrial and technical applications (Nikel 2012; Loeschcke and Thies 2015). Furthermore, this bacterium is highly robust against harsh environmental and operating conditions such as extreme pH values and the presence of toxic substances and organic solvents (Poblete-Castro et al. 2012a; Kim and Park 2014; Ramos et al. 2015). In addition, *P. putida* is amenable for genetic manipulation and expression of heterologous genes, thus enabling the production of valuable compounds by targeted *metabolic engineering*. Against this background, the present chapter is focused on the most recent attempts to tame the archetypal strain KT2440, a certified biosafety strain *g*enerally *r*egarded *a*s *s*afe (GRAS), using SynBio approaches at the systems level. Rapidly transitioning from a humble origin in the soils to modern and sophisticated biotechnology applications, *P. putida* is becoming the bacterial host of choice for a wide variety of sustainable bioprocesses – the basis of which are discussed in this contribution mainly in terms of the native metabolic properties in this bacterium.

1.2 *Pseudomonas putida* Is an Attractive SynBio *chassis*

1.2.1 Central Metabolism in Pseudomonads as a Treasure Trove for Biotechnology

1.2.1.1 Metabolism, Microbial Lifestyle, and Environment

One of the features of *P. putida* KT2440 that makes this bacterium so appealing for biotechnological applications is its rather flexible metabolism (Clarke 1982; Conway 1992; Nikel et al. 2014a). Yet, the reasons underlying this remarkable plasticity remained obscure thus far. For the sake of historical accuracy, one should not forget that one of the very first phenotypic traits of biotechnological interest associated with *P. putida* was its conspicuous ability to degrade aromatic compounds, later demonstrated to be encoded in the archetypal TOL plasmid pWW0 (Williams and Murray 1974). This metabolic feature alone converts *P. putida* mt-2 into a potentially useful degradation agent of recalcitrant aromatic xenobiotics (e.g., phenol, toluene, benzene, and styrene) in bioremediation processes (de Lorenzo 2008; Nikel et al. 2014b). *P. putida* KT2440 is a spontaneous restriction-deficient derivative of *P. putida* mt-2 which has been cured of the pWW0 TOL plasmid (Bagdasarian et al. 1981). In any case, the broad metabolic scope of these bacterial species is certainly not restricted to strain mt-2 and its close derivatives. Pseudomonads in general have always been considered to be metabolically flexible bacteria from which valuable activities and bioreactions can be outsourced: for instance, den Dooren de Jong (1926) reported that a *Pseudomonas* species would grow on 80 different organic compounds. In the 1950s, another *Pseudomonas* isolate was the source of a newly discovered class of enzymes broadly known as oxygenases (Hayaishi et al. 1955). The huge enzymatic arsenal of *P. putida* encompasses activities such as monooxygenases, aromatic ring-cleaving dioxygenases, and hydroxylases; e.g., as deduced from the genome sequence, wild-type strain KT2440 is genetically equipped with 18 dioxygenases, 15 monooxygenases, and 80 oxidoreductases (Nelson et al. 2002).

Since *P. putida* dwells in environmental niches characterized by harsh conditions (e.g., exposed to oxidative stress), it is to be expected that the whole metabolic complement of this species is geared to withstand extreme physico-chemical situations (Timmis 2002; Martins dos Santos et al. 2004). In particular, many of the compounds that could serve as carbon substrates (e.g., aromatic compounds) are also toxic for the cells, and they can usually cause oxidative stress (e.g., some solvents; see Loza-Tavera and de Lorenzo 2011; Nikel et al. 2013). The central metabolic pathways in pseudomonads are thus prepared to deal with these rather adverse situations. Because of its biotechnological importance, central metabolism in *P. putida* and related species has historically been the subject of a number of several studies, and the most important additions to the body of knowledge that appeared in the last few years are summarized below.

1.2.1.2 The Core Metabolism of *Pseudomonas putida* Is Characterized by a Cyclic Glycolysis

In *P. putida* KT2440, glucose can be either phosphorylated in the cytoplasm by glucokinase (Glk) or oxidized in the periplasm to gluconate and/or 2-ketogluconate by means of glucose dehydrogenase (Gcd) and/or gluconate 2-dehydrogenase (Lessie and Phibbs 1984; Latrach-Tlemçani et al. 2008). Three convergent pathways further transform these intermediates into 6-phosphogluconate (6PG) (del Castillo et al. 2007): [i] the phosphorylative branch, in which glucose-6-*P* (G6P) is the intermediate, via the sequential activity of G6P 1-dehydrogenase (Zwf) and 6-phosphogluconolactonase (Pgl); [ii] the direct phosphorylation of gluconate, mediated by gluconokinase (GnuK); and [iii] the 2-ketogluconate loop, which involves its transport back into the cytoplasm and its conversion into 2-keto-6-phosphogluconate via 2-KG kinase (KguK), later reduced to 6PG via the KguD reductase. The genome of *P. putida* KT2440 also contains all the genes encoding the enzymes that make the three prominent catabolic pathways for hexoses in bacteria: the Entner-Doudoroff (ED) pathway, the Embden-Meyerhof-Parnas (EMP) pathway, and the pentose phosphate (PP) pathway. The EMP pathway in this bacterium is nonfunctional, as the glycolytic enzyme 6-phosphofructo-1-kinase (Pfk) is missing in many pseudomonads, including *P. putida* KT2440. Alas, our attempts to complement this activity by transferring Pfk from *Escherichia coli* into strain KT2440 not only failed to activate an EMP route but also resulted in a very high sensitivity to oxidative stress in the recombinants (Chavarría et al. 2013). Since Pfk is the only activity supposedly missing in a linear EMP pathway, these results suggested that the very architecture of central metabolism in *P. putida* cannot accommodate a linear glycolysis by merely introducing Pfk. A multi-omic approach revealed the reason behind this phenomenon: in strain KT2440, glucose processing occurs through a combination of enzymatic activities from the ED pathway, the incomplete EMP pathway, and the PP pathway in a cyclic fashion (Nikel et al. 2015). This metabolic architecture, which was termed *EDEMP cycle* (Fig. 1.1), mediates the recycling of triose phosphates all the way back to hexose phosphates (i.e., glyceraldehyde-3-*P* → dihydroxyacetone-*P* → fructose-1,6-P_2 → fructose-6-*P* → G6P) through the activity of TpiA (triose phosphate isomerase), Fda (fructose-1,6-P_2 aldolase), Fbp (fructose-1,6-bisphosphatase), and Pgi (G6P isomerase). Under standard growth conditions on glucose, the EDEMP cycle converts about 10 % of the triose phosphates to hexose phosphates. The remaining carbon skeletons stemming from the ED pathway (i.e., glyceraldehyde-3-*P* and pyruvate) are further metabolized through the tricarboxylic acid (TCA) cycle to produce biomass, energy, and other metabolic precursors. Interestingly, the extent of carbon recycling within the EDEMP cycle depends on the environmental conditions. This is an important trait as every turn of the cycle yields one NADPH molecule – thereby directly impacting the overall redox balance of the cell. The central metabolic pathways of *P. putida* KT2440 thus display a remarkable flexibility which is shaped by the characteristics of the growth environment.

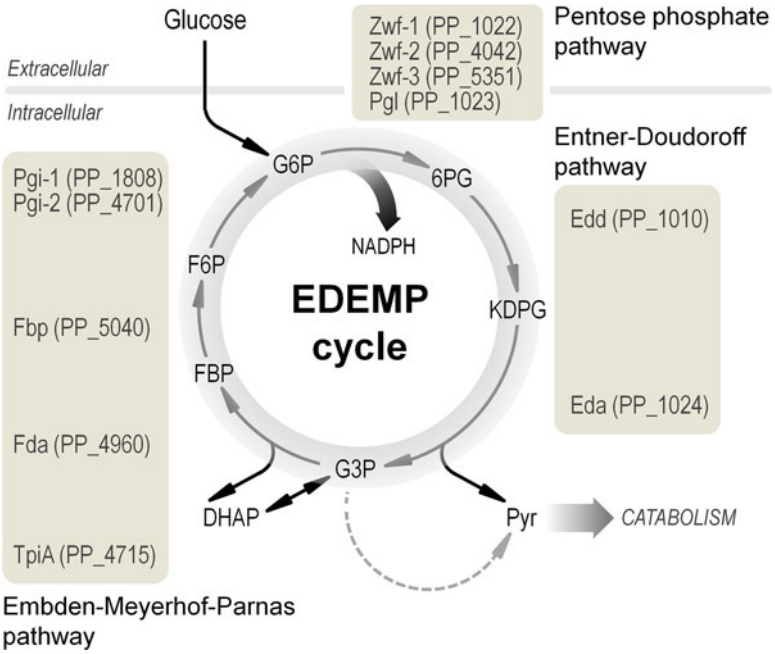

Fig. 1.1 The EDEMP cycle of *Pseudomonas putida* KT2440. The architecture of central carbon metabolism is shown along with the key elements belonging to the Entner-Doudoroff pathway, the (incomplete) Embden-Meyerhof-Parnas pathway, and the pentose phosphate pathway. Note that one *NADPH* equivalent is produced for each turn of the cycle. Reactions of downstream pyruvate are indicated with a wide *shaded arrow*. The abbreviations used for the metabolites are as follows: *G6P* glucose-6-*P*, *6PG* 6-phosphogluconate, *KDPG* 2-keto-3-deoxy-6-phosphogluconate, *G3P* glyceraldehyde-3-*P*, *Pyr* pyruvate, *DHAP* dihydroxyacetone-*P*, *FBP* fructose-1,6-P_2, *F6P* fructose-6-*P*. The enzyme components are indicated along with the *PP* number of the corresponding gene encoding them (Nelson et al. 2002)

1.2.1.3 Redox Metabolism

From the description above, it is clear that *P. putida* can potentially produce elevated amounts of NADPH through the operation of the EDEMP cycle. However, how does NADPH help maintaining the redox balance within the cell? This is a relevant question not only for pseudomonads (Ebert et al. 2011) but for bacteria in general (Nikel and Chavarría 2015). Firstly, let us consider the role of NADPH in counteracting oxidative stress – as this is the type of environmental insult that pseudomonads most commonly face in their native environments. Catalase, superoxide dismutase, and glutathione peroxidase counteract oxidative stress during aerobic respiration (Cabiscol et al. 2000). The effectiveness of these detoxifying enzymes to fight reactive oxygen species (ROS) largely depends on the intracellular NADPH availability. This reduced nucleotide supplies the reducing power necessary to suppress the oxidative damage caused by ROS. The resistance mechanism to quell ROS through the activity of glutathione peroxidase is particularly important in soil bacteria (Lu and Holmgren 2014). Glutathione peroxidase catalyzes the reaction

$2GSH + H_2O_2 \rightarrow GS\text{-}SG + 2H_2O$, where GSH represents reduced monomeric glutathione and GS-SG represents oxidized glutathione (i.e., two GSH molecules linked by a disulfide bridge). In this process, the key metabolite is GSH, which can be found at very high concentrations in several microorganisms. This thiol maintains a strong reducing environment in the cell, and its reduced form is maintained by glutathione reductase using NADPH as a source of reducing power (by means of the reaction $GS\text{-}SG + NADPH + H^+ \rightarrow 2GSH + NADP^+$). Thus, the reducing potential of the cell is highly dependent of NADPH availability, and the production of this reducing agent is an integral part of the microbial metabolic machinery. All these regulatory traits of ROS quenching are particularly relevant when the growth substrate itself causes stress to the cells (Domínguez-Cuevas et al. 2006).

There are two main NADPH sinks in *P. putida* KT2440 during growth on glucose that should be considered when assessing the overall redox balance. The first (and most obvious) fate of NADPH is the anabolic buildup of biomass components (Neidhardt et al. 1990). On the other hand, and as stated above, a considerable part of glucose is converted by most pseudomonads in organic acids, such as gluconate and 2-KG. These intermediates finally converge at the level of 6PG. One of the reducing pathways that feeds this node is catalyzed by 2-keto-6-phosphogluconate reductase (KguD). KguD uses NADPH as the cofactor to reduce 2-keto-6-phosphogluconate to 6PG (Nikel et al. 2015), and it constitutes the second sink of NADPH in the biochemical network operated by strain KT2440 when cells grow on glucose.

Although the metabolic mechanism involving the EDEMP cycle could in principle ensure an appropriate redox balance, there are four key reactions, two of which belong to the PP pathway and two to the TCA cycle, which have been demonstrated to represent the main source of NADPH in a number of bacteria, including pseudomonads (Nikel and Chavarría 2015). G6P dehydrogenase and 6PG dehydrogenase are widely distributed enzymes, from bacteria to humans, and they constitute the nonreversible point of entry to the PP pathway. Particularly, during growth on hexoses, the PP pathway represents a major source of pentose intermediates for nucleotide biosynthesis and NADPH through these two consecutive $NADP^+$-dependent dehydrogenases. Carbon fluxes through the PP pathway can differ considerably between species, and, depending on the demands of reducing power, these reactions may be critical to the regeneration of NADPH. On the other hand, Icd is an enzyme of the TCA cycle that catalyzes the oxidative decarboxylation of isocitrate, thereby producing both 2-oxoglutarate and CO_2. This process involves oxidation of isocitrate to oxalosuccinate, followed by the decarboxylation of the β-carboxyl group to the ketone, forming 2-oxoglutarate. Icd has been reported in all domains of Life, and both NAD^+- and $NADP^+$-dependent enzymes have been described. Finally, the fourth enzyme considered to be relevant for NADPH generation is Mae, which converts malate, an intermediate of the TCA cycle, into pyruvate, the end product of glycolysis and a key metabolite in the split of respiratory and fermentative metabolism, e.g., in *E. coli* (Neidhardt et al. 1990). In *P. putida* KT2440, the activity of Mae can be regarded as part of a metabolic shunt where NADPH is obtained at the expense of one ATP molecule consumed by pyruvate carboxylase and one NADH molecule consumed by malate dehydrogenase (Chavarría et al. 2012).

Redox metabolism is thus one of the most attractive properties of strain KT2440 for the rational design of sustainable bioprocesses. However, considering the tight and complex regulation of the pathways involved (Daniels et al. 2010), the manipulation of this trait requires a dedicate toolbox for targeted genetic engineering. This aspect is covered in the following section.

1.2.2 Tools for Genetic and Metabolic Manipulation of Pseudomonads

Despite the many advantages of soil bacteria as metabolic *chassis*, large-scale industrial applications involving pseudomonads are still comparatively rare (Poblete-Castro et al. 2012a). Many of the (potentially huge) *P. putida*-based biotechnological applications are still in such an early stage of progress due to the current lack of knowledge of genotype/phenotype relationships in this bacterium under conditions relevant for both industrial and environmental endeavors. Over the past decade, new tools and (increasingly refined) techniques in the field of systems biology and SynBio became available and contributed significantly to a true knowledge-based metabolic engineering of *P. putida* strains – thereby facilitating their use in sustainable bioprocesses. Strain taxonomy, gene and protein annotation, and metabolic pathway data quickly emerged after the genome of the parental strain KT2440 was sequenced (Nikel et al. 2014a). The main genetic tools for targeted metabolic engineering manipulations of *P. putida* are summarized in the next sections.

1.2.2.1 Plasmids

The scientific and technical ambition of contemporary SynBio is the engineering of biological entities with the type and degree of predictability expected in electric and industrial manufacturing. For this purpose, biological parts (e.g., DNA pieces) with given specifications are sequence-edited, standardized, and combined into devices, which are assembled into complete systems. Environmental bacteria are considered an optimal source of such biological parts that can be assembled in autonomous DNA molecules bearing the information necessary for the execution of virtually any biological function (Martínez-García et al. 2015). There is a large list of plasmids that were specifically created for *Pseudomonas* species. However, one important addition to the range of vector tools that appeared a few years ago is the so-called *SEVA* initiative. The *S*tandard *E*uropean *V*ector *A*rchitecture (http://wwwuser.cnb.csic.es/~seva/) was conceived as a user-friendly, web-based resource and a material clone repository to assist in the choice of optimal plasmid vectors for deconstructing and reconstructing complex prokaryotic phenotypes (Silva-Rocha et al. 2013; Martínez-García et al. 2014a). Although the SEVA vectors can be used in several Gram-negative bacteria, *P. putida* has been a natural host of choice for these plasmids (Durante-Rodríguez et al. 2014). Under the SEVA standard, every DNA portion (i.e., module) of the vectors is minimized, edited to eliminate any possible flaw in their sequence and/or functionality, and endowed with a specific physical connectivity through three intersegment insulators that are flanked by fixed, rare

restriction sites. Such a physical DNA scaffold enables the exchangeability of multiple origins of replication and diverse antibiotic selection markers to shape a frame for their further combination with a large variety of cargo modules that can be used for a number of different applications. Interestingly, the SEVA format enables the expansion of the collection to virtually any possible combination of individual DNA parts at the user's will – thereby simplifying the often cumbersome and lengthy "classical" genetic engineering procedures.

1.2.2.2 Transposon Vectors

Mini-transposon vectors are available since about 20 years and have proven value for the engineering of diverse Gram-negative bacteria, *P. putida* being a model microorganism for these purposes (de Lorenzo et al. 1990, 1993a, b; de Lorenzo and Timmis 1994). Transpositions can be random (e.g., Tn*5*, *mariner*, and *Mu* transposons) or site specific (e.g., conjugative, AT-rich specific, and Tn*7* transposons). Mini-Tn*5* and mini-Tn*10* transposons are randomly inserted into AT-rich sequences (Martínez-García et al. 2011), whereas mini-Tn*7* targets the downstream region of the *glmS* gene (Schweizer and de Lorenzo 2004; Choi and Schweizer 2006). Tn*5*-derived constructs are particularly attractive because they can be maintained in cells without antibiotic selection and can be reused for different insertions within the same cells. The Tn*5* transposition system requires only the plasmid-borne transposase (that is encoded by *tnpA*) and the terminal ends of the transposon for the system to work independently from the host. Tn*5*-based mini-transposons are not only suitable for generating saturated mutagenesis libraries but also for the chromosomal integration of functional DNA cargos or entire gene clusters (Martínez-García and de Lorenzo 2012; Martínez-García et al. 2014b). Tn*7*-based plasmid vectors have been also designed and applied for genetic footprinting, chromosomal integration of transcriptional and translational fusions, signature-tagged DNA mutagenesis, and transposon-site hybridization and scanner linker mutagenesis (Peters and Craig 2001; Zobel et al. 2015). Some of these synthetic transposons offer the possibility to remove the antibiotic resistance determinants (Nikel and de Lorenzo 2013), therefore leaving a markerless *P. putida* strain that could be used in industrial bioprocesses in which antibiotic addition is to be specifically avoided. Moreover, many transposons follow the SEVA format described in the preceding section, thus enabling the easy interchange of different DNA modules without extensive and time-consuming cloning steps. With all the SynBio tools needed for rational engineering of phenotypes in pseudomonads at hand, the next relevant issue to tackle is the inherent complexity of the bacteria in an attempt to harness their full biotechnological potential.

1.3 From Classical Approaches of Strain Manipulation Toward a Systems-Driven View of *P. putida*'s Biology

The broad field that we presently call *systems biology* had its formal origin more or less at the same time as when *P. putida* mt-2 began to be recognized as a potential microbial platform for biodegradation and industrial catalysis. As the so-called *omic techniques* started to be refined over the next 30 years (Zhang et al. 2010), so

did our understanding of the biology of *P. putida* – thereby drawing the itinerary from basic knowledge of bacterial physiology to targeted biotechnological exploitation. In this section, the most relevant advances in the use and application of systems biology approaches which were instrumental to launch this soil bacterium as a robust microbial cell factory and SynBio *chassis* are summarized. The different levels of regulation of the information encoded in the *P. putida* chromosome will be considered by discussing genomics, transcriptomics, proteomics, metabolomics, and fluxomics/phenomics.

Since the very discovery and characterization of soil-dwelling bacteria, several strategies to improve their biochemical capabilities for several applications were immediately implemented. Most attempts to reach these goals were, however, more an art with experimentation characterized by trial and error. Some papers in the late 1980s and early 1990s advocated the switch from this mostly artistic approach to a more systematic and rational endeavor by focusing on *P. putida* mt-2, which rapidly became a model bacterium for biodegradation studies (Lehrbach et al. 1984; Rojo et al. 1987). This transition mostly involved the use of recombinant DNA technology and a better understanding of cellular physiology to modify intermediary metabolism. Yet the inherent complexity of the microbial metabolism, especially in a bacterium which has evolutionary opted for metabolic diversity rather than efficiency, made the way toward flawless biocatalysis difficult to transit. Fortunately, this situation considerably switched in 2002 with the announcement of the complete genome sequence of the model strain KT2440.

1.3.1 Genomics

The seminal contribution of Nelson et al. (2002), reporting the sequence of the 6.18-Mb genome of *P. putida* KT2440, revealed the existence of diverse transport and metabolic systems hitherto unknown in the domain of pseudomonads. An important trait for industrial purposes was the nonpathogenic nature of *P. putida*, which became immediately clear by analyzing its genome (i.e., no pathogenic determinants were found in the chromosomal DNA). Since the sequence of the KT2440 genome was the only one available for *P. putida* for a long time, the genome landscape of several other *P. putida* strains was compared using an oligonucleotide array of KT2440. Interestingly, depending on the strain tested, 22–99 % of all genetic elements were identified in the genomic DNAs analyzed. The genomotyping of these different pseudomonads using KT2440-based DNA microarrays yielded insights in their phylogenetic relationships and the identification of genes and their distribution over different primary and secondary biological functions.

A recent contribution by Udaondo et al. (2016) aimed at defining the genetic elements of the core genome and the pan-genome among *P. putida* strains. The most abundant genes within the 3,386-gene long core genome in strain KT2440 were found to be those encoding nutrient transporters. Other conserved genes include those related to central carbon metabolism (e.g., components of the ED and the PP pathways), arginine and proline metabolism, and pathways for the degradation of

aromatic carbon sources. Again, this study demonstrated that, although *P. putida* KT2440 shares ca. 85 % of its genetic complement with *P. aeruginosa* PAO1, pathogenicity determinants such as genes encoding exotoxins and type III secretion system are altogether absent.

A further refinement of the sequence reported by Nelson et al. (2002) was recently published by Belda et al. (2016). The authors adopted a suite of state-of-the-art genomic analysis tools to revisit the functional and metabolic information encoded in the chromosomal sequence of strain KT2440. This allowed to identify 242 new protein-encoding genes and to re-annotate the functions of 1,548 genes, which are linked to almost 4,900 PubMed references. The revised *P. putida* genome has 10 additional nucleotides compared to the earlier version (i.e., 6,181,873 bp instead of 6,181,863 bp). New catabolic pathways were predicted for 92 compounds (which were found to be used as carbon, nitrogen, and phosphorus sources) that could not be accommodated by any previously constructed metabolic models (see also next sections). The resulting examination not only accounted for some of the known stress tolerance traits known in *P. putida* but also further emphasized the capacity of this bacterium to perform difficult redox reactions.

All these genome-level studies point to a rather large genomic content of this strain, a situation which asks the question of how the expression of the genes encoded therein is regulated. In order to modulate the > 5,000 genes encoded in its genome, *P. putida* KT2440 is endowed with approximately 600 transcriptional factors and 24 alternative σ subunits of the RNA polymerase which keep the associated transcriptome tightly regulated. The pattern of gene transcription under different growth and environmental conditions (including the presence of alternative carbon sources or stress agents) is discussed below.

1.3.2 Transcriptomics

Any approach aimed at manipulating the many physiological and metabolic capabilities of *Pseudomonas* would require an in-depth knowledge of the regulation of RNA transcripts encoding relevant enzymes and regulatory proteins. In an attempt to understand how the transcriptional network of *P. putida* KT2440 changes under different physiological regimes, Kim et al. (2013) cataloged the high-resolution whole transcriptome when cells are cultured with glucose, fructose, succinate, or glycerol as the sole carbon source. A remarkable feature of the transcriptome, as revealed by means of high-resolution *RNA-Seq* experiments, is that >20 % of the *P. putida* genome is differentially expressed depending on the physiological regime brought about by the carbon source used to grow the cells (ranging from entirely glycolytic to gluconeogenic). Besides the evident changes in the pattern of expression of genes encoding components of metabolic pathways involved in the assimilation of each substrate, other structural and regulatory genes were affected as well. For instance, the expression of the *hupA*, *hupB*, and *hupN* genes, encoding subunits of HU histone-like proteins, was drastically altered among different carbon sources (thus varying the ability of these HU subunits to form heterodimeric combinations).

Two small, noncoding RNAs, *crcZ*, and *crcY*, known to inhibit the Crc protein that mediates catabolite repression in *P. putida* (La Rosa et al. 2015), were found to be downregulated in glucose-grown cells. The raw data has been made available to the scientific community through the *Gene Expression Omnibus* database (http://www.ncbi.nlm.nih.gov/geo, accession number GSE46491).

In a similar study, the ability of *P. putida* mt-2 to metabolize *m*-xylene and related aromatic compounds was exposed at the transcriptional level by applying tiling arrays of densely overlapping oligonucleotides (Kim et al. 2015). Strain mt-2 is known to degrade *m*-xylene through the enzymes encoded by the *xyl* operons of the TOL plasmid pWW0 along with some other chromosomally encoded degradation activities. All the *xyl* genes were found to be actively transcribed in response to exposure to aromatic carbon sources, and the 3′-termini in the mRNAs for both the upper and lower TOL pathways extended beyond the annotated coding regions. Furthermore, the level of the *xylR* transcript, which encodes the master *m*-xylene-responsive transcriptional regulator of the TOL system, was surprisingly decreased by aromatic substrates. RNA-Seq assays confirmed these data at the single nucleotide level and refined the formerly misannotated *xylL* sequence. The chromosomal *ortho* route for degradation of benzoate (i.e., the *ben* and *cat* gene clusters and some genes of the *pca* gene cluster) was found to be transcriptionally activated by this aromatic substrate, but not by toluene or *m*-xylene (i.e., the typical TOL substrates).

Frank et al. (2011) exposed cDNA sequencing as a helpful tool to refine the genome annotation of strain KT2440. This assay identified 36 unknown small, noncoding RNAs, 143 novel open reading frames in 106 intergenic regions, 42 unclassified genes, and 8 highly expressed, leaderless mRNA transcripts. The genome coordinates of 8 genes and the organization of 57 operons were corrected based on this information. Genome-wide transcriptome approaches have also been used to test gene expression under conditions that are relevant for industrial production of a variety of compounds, such as [i] different types of abiotic stresses (Domínguez-Cuevas et al. 2006; Reva et al. 2006), [ii] elevated pressure (Follonier et al. 2013), [iii] nitrogen deficiency (Hervás et al. 2008), and [iv] accumulation of polyhydroxyalkanoates (PHAs) (Poblete-Castro et al. 2012b). In close connection with the pattern of regulation identified at the level of transcript abundance, the associated protein content was also explored in a number of studies as disclosed in the next section.

1.3.3 Proteomics

Soon after the publication of the complete genome sequence of *P. putida* KT2440, a two-dimensional gel protein reference map of this strain was published, describing the pattern of protein abundance for cells growing with glucose as the carbon source (Heim et al. 2003). Peptides were identified by matrix-assisted laser desorption ionization coupled to time-of-flight (*MALDI-TOF*) analysis, in conjunction with an ad hoc computational database deduced from the genome sequence of KT2440. The combination of these techniques enabled the unambiguous identification of ca.200 two-dimensional gel spots.

A staple of bacterial growth in batch cultivations is the transition from exponential growth to stationary, nondividing conditions. This feature was investigated using a proteome-wide, label-free quantification of proteins in *P. putida* F1, revealing that the bacterium adapted to stationary growth in a complex medium by gradually increasing the capacity to use amino acids as the carbon, nitrogen, and energy source (Herbst et al. 2015). This metabolic adaptation started already during the mid-exponential growth phase, and it entailed the upregulation of ABC-type amino acid transporters and the glyoxylate shunt (which allows the processing of C2 compounds, such as acetyl-coenzyme A) and the TCA cycle. The entrance to the stationary phase was accompanied by increased RpoS-mediated oxidative stress protection, as well as a decrease in motility. The data indicate that these metabolic and physiological adaptations take place at the proteome level in a continuous fashion, instead of a sudden physiological switch from exponential growth to stationary phase conditions. Panikov et al. (2015) considered culture and environmental conditions similar to the type of *P. putida* normally encounters in nature. Intensive microbial growth typically observed in laboratory rarely occurs in natural environments. Because of severe nutrient deficiency, bacterial populations in such environments are known to exhibit near-zero growth kinetics. In a continuous culture with cell retention, the proliferating *P. putida* cells attained a steady state, their slow growth balanced by the formation of viable but nonculturable cells. Proteomic analysis revealed upregulation of several proteins (transporters, stress response, self-degrading enzymes, and those involved in the synthesis of extracellular polymers) and downregulation of others (ribosomal, chemotactic, and primary biosynthetic enzymes) in the near-zero growth conditions as compared with cells grown in a traditional batch culture. The authors concluded that near-zero growth conditions require controlled partial self-digestion and deep reconfiguration of the metabolic machinery that results in the biosynthesis of new products and development of broad stress resistance.

Several other studies explored the proteomic response of *P. putida* to stressful conditions, such as [i] presence of solvents (e.g., toluene, naphthalene, styrene, *n*-butanol) (Nikodinovic-Runic et al. 2009; Wijte et al. 2011; Li et al. 2015), [ii] heavy metals (e.g., Cd^{2+}, Ni^{2+}, and Co^{2+}) (Manara et al. 2012), and [iii] shift to low-temperature conditions (Fonseca et al. 2011). The emerging picture of these works – in combination with the evidence gathered through detailed transcriptome analysis – indicates a rather *general* response to stressful conditions (Moreno and Rojo 2013), which encompasses proteins involved in heat shock stress and the universal stress response protein, proteins related to oxidative stress detoxification (e.g., superoxide dismutases and catalase), and a profound rearrangement of the pattern of transporters for metabolites and solvents. All these phenotypic properties observed through omic techniques were further captured in genome-wide metabolic models as detailed below.

1.3.4 Genome-Wide Metabolic Reconstructions

The first metabolic model of *P. putida* KT2440 was published in 2008 (Nogales et al. 2008) and was quickly followed by other three models (Puchałka et al. 2008; Sohn et al. 2010; Belda et al. 2016). The most complete genome-scale metabolic

model currently available, published in 2016, is an extension and improvement of *i*JP962, with additions and corrections based on the latest sequencing data for strain KT2440 (Belda et al. 2016). For each predicted or curated reaction from the functional re-annotation process (i.e., new bioreactions), the original *i*JP962 model was first scanned to search for biochemical transformations involving the same set of metabolites in the newly discovered reactions. If no such biotransformation existed, the new reaction was added to the model. Otherwise, the existing and new reactions were compared in terms of directionalities and associated genes. In particular, whenever a new bioreaction had been manually curated, the corresponding reaction was updated in terms of both directionality and gene associated. In addition, the gene associations of the GSMM reaction were only updated if it was an orphan reaction. The resulting updated metabolic model of *P. putida* KT2440 now spans 1256 reactions comprising 1122 metabolites, related to 1053 fully annotated genes.

Model-based predictions of *in silico* phenotypes were recently refined by the integration of experimental data on stoichiometric demands for anabolism and cellular maintenance (van Duuren et al. 2013), which are crucial to increase the predictive power of any computational design. Additionally, a comprehensive interaction database of *P. putida* KT2440 has been generated from three protein-protein interaction (*Putida*NET) (Park et al. 2009).

1.3.5 Metabolomics and Metabolic Flux Analysis

The nontargeted analysis of the complete set of metabolites in *P. putida* S12 (a solvent-tolerant strain) was implemented through a robust quantitative metabolomics platform that allows the analysis of "snapshot" metabolomes (van der Werf et al. 2008). The metabolite composition was cataloged for cells grown under different physiological conditions with four carbon sources, i.e., fructose, glucose, gluconate, and succinate. Principal component discriminant analysis was applied to identify metabolites specific for each culture conditions, enabling the authors to pinpoint signature metabolic intermediates for either glycolytic or gluconeogenic growth conditions. The thorough quantification of the main metabolites in central catabolic pathways allowed for the in vitro quantification of the main dehydrogenases of *P. putida* KT2440 reproducing quasi in vivo conditions by supplying substrates and cofactors at the concentrations experimentally determined (Nikel et al. 2015). This approach, in turn, enabled to quantify the promiscuity of these dehydrogenases in terms of redox cofactors use. The information from transcriptional patterns and ^{13}C-labeled substrates was used to define central carbon metabolism *bona fide* in strain KT2440 (Sudarsan et al. 2014).

1.4 Multi-omic, Systems-Based Biotechnology Approaches

A recent example on the use of a comprehensive systems biology approach for sustainable biotechnological purposes has been described by Vallon et al. (2015). In this case, the target molecule was *n*-butanol, an attractive biofuel. The cellular responses of strain KT2440 to exposition to *n*-butanol were evaluated at the

transcriptional, proteomic, and metabolic level. Chemostat cultivations, using *n*-butanol either as sole carbon source or together with glucose, were implemented for this purpose. ^{13}C-Based metabolic flux analysis revealed that central metabolism was split into [i] a glucose-fueled combination of the ED and PP pathways and [ii] an *n*-butanol-fueled TCA cycle when both substrates were co-consumed. The hitherto unknown degradation pathway of *n*-butanol of *P. putida* KT2440 was unraveled, thus setting the bases to render *P. putida* KT2440 from an *n*-butanol consumer to an efficient biofuel producer.

Another multi-omic, systems approach aimed at biotechnological purposes entailed the prospection of *P. putida* as a cell factory for bioplastic production (Prieto et al. 2014). PHA metabolism in pseudomonads has been traditionally considered to be a futile cycle for recycling carbon and reducing equivalents (Escapa et al. 2012). In reality, part of that function, PHA granules are dynamic supramolecular structures, termed *carbonosomes* (Jendrossek 2009). Carbonosomes are complexes of biopolyester and several types of structural and regulatory proteins that are essential for granule segregation during cell division and for the functioning of the PHA metabolic route as a continuous cycle linked to cellular demands of carbon and energy (López et al. 2015). This attribute was exposed in *P. putida* as well. Continuous cultures run under different conditions of nutrient limitation revealed further mechanistic insights into PHA accumulation (Poblete-Castro et al. 2012b). The dilution rate was set at $D = 0.1\ h^{-1}$ to ensure PHA formation, and decanoate and NH_4Cl were used as the sole carbon and nitrogen source, respectively. The polyester was accumulated up to 26 %, 62 %, and 81 % of the cell dry weight under conditions of carbon, dual (carbon and nitrogen), and nitrogen limitation, respectively. Dual-limitation continuous cultures resulted in patterns of gene expression, protein level, and metabolite concentrations that substantially differ from those observed under exclusive carbon or nitrogen limitation. Pronounced differences were found in the energy and fatty acid metabolism, as well as in stress proteins and transport systems. One striking dissimilarity between nitrogen and dual-limited chemostat when compared with carbon-only limitation was a sharp increase in the transcription of genes encoding the branched-chain amino acid ABC transporter. The same held true for *phaI* and *phaF*, two granule-associated proteins, and *phaC1* and *phaC2*, encoding two PHA synthases. The expression of porins under both nitrogen and dual limitation correlated well with the uptake of the carbon source – and thus cells adjust the input of carbon in response to nutrient limitation to ensure sufficient carbon needed for each cellular process. Genes involved in ATP generation through the respiratory chain were found to be overexpressed as the amount of PHA accumulated increased, indicating that PHA-forming cells undergo a shortage in energy resources. This study demonstrated that PHA formation and hydrolysis affect a number of cellular processes in *P. putida* and not only, as it may seem obvious, those strictly related to carbon and nitrogen metabolism.

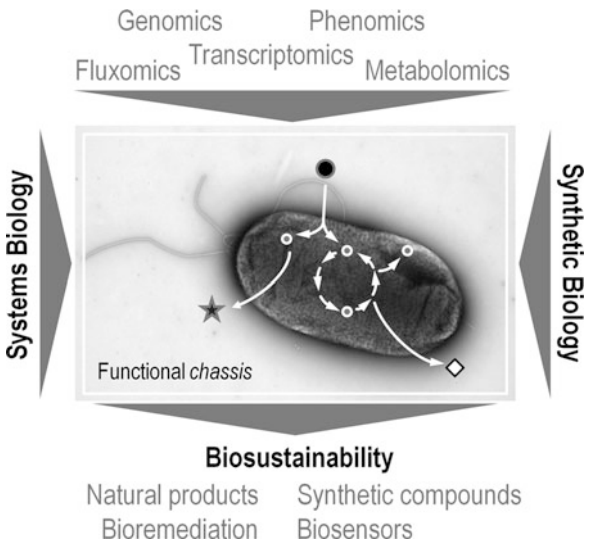

Fig. 1.2 *Pseudomonas putida* as the key microbial player in the roadmap between microbial ecology and the development of sustainable bioprocesses. The wealth of omic techniques and data enabled the emergence of strain KT2440 as a *primus inter pares chassis* not only for the production of a suite of chemicals and bioproducts but also as a biodegradation agent and a whole-cell biosensor. Synthetic and systems biology are the burgeoning fields contributing tools and basic knowledge to mediate the smooth transition of *P. putida* from dirt to industrial bioreactors

1.5 Conclusion

From the examples discussed in the present chapter, there is a clear emerging picture on our way toward efficient and sustainable bioprocesses: *P. putida* (and related soil-dwelling bacterial species) is firmly becoming a veritable standard for both fundamental and biotechnological endeavors (Fig. 1.2). While there remain some gaps in our current knowledge of the complex *P. putida* metabolism, we now have a clear picture of its overall functioning – enabling the implementation of useful, rationally designed metabolic engineering manipulations. The adoption of *P. putida*-based SynBio *chassis* for biotechnological purposes resembling *bacterial cyborgs* (i.e., displaying natural microbial properties merged with artificial implanted traits) is not a difficult-to-reach ambition for the future any longer. As we rapidly transit the road toward a completely programmable *P. putida chassis*, further fundamental research is necessary to expand our biological knowledge of this microorganism in order to enable a fully predictable behavior in industrial setups.

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Potentiality of *Herbaspirillum seropedicae* as a Platform for Bioplastic Production

2

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Abstract

Polyhydroxybutyrate (PHB) is a polyhydroxyalkanoate produced by several bacteria as carbon storage and reducing equivalent sink. The production of PHB is a hot topic in biotechnological research due to its properties similar to oil-based plastics, while PHB is readily degradable in the environment. Therefore, PHB is a bio-sustainable alternative for synthetic plastic materials. The main hurdle for PHB use is the cost of fermentative process in large scale, which is still high when compared to industrial processes based in petroleum. The use of cheap biomass feedstocks and industrial by-products can potentially reduce costs of microbial PHB production. In addition, the application of metabolic engineering to fine-tune metabolic pathways or even create more efficient pathways may yield important gains in PHB production competiveness. In this chapter we will address the potential of the bacterium *Herbaspirillum seropedicae* as a platform for PHB production, analyzing the functions of genes involved in PHB and carbon metabolism to propose strategies for metabolic engineering of new bacterial strains.

2.1 Introduction

The production of polyhydroxyalkanoates (PHA), including poly-3-hydroxybutyrate (PHB), has attracted a substantial attention from researchers, not only because of their importance in bacterial metabolism but also because of their potential

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biotechnological applications, as they have thermoplastic and elastomeric properties, making them an environmentally friendly biodegradable alternative to oil-derived plastics. *Herbaspirillum seropedicae* is an endophytic nitrogen-fixing beta-proteobacterium which has attracted an increasing interest in recent years as a plant-growth-promoting rhizobacteria (PGPR) (Chubatsu et al. 2011; Monteiro et al. 2012). In addition to the relevant plant-growth-promoting characteristic, *H. seropedicae* is a PHA-producing bacterium, being the PHB of the main PHA produced (Catalan et al. 2007). However, the metabolism of PHA in *H. seropedicae* has only recently been subject of deeper investigation. PHB is an aliphatic polyester biosynthesized by several bacteria as a means of carbon storage and source of reducing equivalents (Anderson and Dawes 1990; Madison and Huisman 1999). PHB is usually produced under conditions of carbon oversupply and low levels of other nutrients including nitrogen, phosphate, and oxygen (Steinbuchel and Hein 2001). The polymer synthesis is dependent on, at least, three enzymes, 3-ketothiolase, acetoacetyl-CoA reductase, and PHA synthase, encoded by *phaA*, *phaB* and *phaC* genes, respectively (Anderson and Dawes 1990). The PHB polymer is stored as intracellular insoluble granules coated with proteins which represents approximately 0.5–2 % of the granule weight (Jendrossek 2009). When carbon and/or energy is required, the polymer is mobilized by PHA depolymerase, encoded by *phaZ* (Uchino et al. 2008; Brigham et al. 2012). However, the cell function of PHB is not completely understood. In addition of being a reserve molecule in bacteria, PHB synthesis and degradation has been proposed as an essential mean to regulate the cell redox balance, since NAD(P)H is consumed during its synthesis. Under excess reducing power, PHB is synthesized to reduce NAD(P)H in a way similar to reduction of pyruvate to lactate in lactic fermentation (Trainer and Charles 2006; Trainer et al. 2010). The capacity of PHB production has also been related to bacterial survival under stress conditions or in competitive environments (Kadouri et al. 2003; Kim et al. 2013; Balsanelli et al. 2015).

Genome sequencing of *H. seropedicae* SmR1 revealed 13 genes potentially involved in PHB metabolism (Pedrosa et al. 2011). Recently, the development of genetic, biochemical and physiological studies has expanded the understanding of PHB metabolism in this organism. Furthermore, global transcript profiling has revealed that PHB mobilization is important for fitness during the colonization of both maize (Balsanelli et al. 2015) and wheat (Pankievicz et al. 2016) roots, emphasizing the importance of PHB for the lifestyle of this microorganism. In this chapter, we will discuss important aspects of *H. seropedicae* PHB metabolism and its regulation. We will further explore this knowledge to propose strategies to bioengineer more efficient bioplastic-producing strains.

2.2 Genomic Organization of *pha* Genes in *H. seropedicae* SmR1

Analysis of the *H. seropedicae* SmR1 genome revealed the presence of all genes required for the synthesis and degradation of PHA, especially poly-β-hydroxybutyrate (PHB). These genes are mainly dispersed in different regions of the genome as

observed in Fig. 2.1a. Two genes encoding putative 3-ketothiolases (*phaA1* and *phaA2*), which catalyze the condensation of two acetyl-CoA in the first step of PHB synthesis, were encountered (Fig. 2.1a). Genes encoding the PHA synthase (*phaC1*), acetoacetyl-CoA reductase (*phaB1*), and a transcriptional regulator (*phaR*) are also present (Fig. 2.1a). With the exception of *phaR*, *phaC1*, and *phaB1* which are located in close proximity in the genome, the other genes are spread in different operons (Fig. 2.1a). The protein PhaR is important for regulation of PHA-related gene expression as in other bacteria (Maehara et al. 2002; Potter et al. 2002), while PhaB1 and PhaC1 are required for the second and third steps of the PHB synthesis, respectively (Fig. 2.1b). A second copy of acetoacetyl-CoA reductase coding gene (*phaB2*) and two other copies of PHA synthase coding genes (*phaC2* and *phaC3*) are also present in the genome (Fig. 2.1a).

In addition to genes coding for proteins important in different steps of PHB granule synthesis (Fig. 2.1b), other PHB-related genes coding for granule coating proteins and PHB mobilization enzymes were also found in *H. seropedicae* genome. Genes encoding two phasins (*phaP1* and *phaP2*), which are important for the granule structure and also to avoid premature granule mobilization (Tirapelle et al. 2013), are present in different locations of the genome, as observed in Fig. 2.1a. Finally, two genes encoding PHA depolymerases (*phaZ1* and *phaZ2*) were also found in distinct regions of the genome. The specific genomic localization of the PHA-related genes and other relevant information about the encoded protein function and domain organization are presented in Table 2.1.

2.2.1 *H. seropedicae* SmR1 PhaC Proteins Belong to Different Phylogenetic Groups

Although *H. seropedicae* SmR1 possesses three genes encoding proteins homologous to PHA synthases (Fig. 2.1a), the PHB synthesis is mainly dependent upon the PHA synthase encoded by the *phaC1* gene, since mutation in this gene abolishes PHB accumulation (Tirapelle et al. 2013). In addition, the production of PHB by the *phaC1* mutant strain is not complemented by either *phaC2* or *phaC3*. It is possible that PhaC2 and PhaC3 are not active under the physiological conditions employed for PHB quantification (Tirapelle et al. 2013) or these proteins are dedicated to other catalytic activities.

In silico analyses using the Pfam database (Finn et al. 2014, 2016) of all three PhaC proteins from *H. seropedicae* SmR1 showed that these proteins are all different in length and domain composition (Table 2.1), suggesting that they might have different functions in the PHB metabolism. PhaC1 and PhaC2 proteins share two domains, namely, the PhaC-N domain (Pfam PF07167) and the alpha-beta hydrolase domain (Pfam: PF00561). PhaC2 protein has an additional N-terminal domain identified as PHBC_N (Pfam: PF12551). The PhaC3 protein seems to be the most divergent among the *H. seropedicae* PHA synthases, since it is comprised by a sole abhydrolase_6 (Pfam: PF12697) domain. Among *H. seropedicae* PhaC proteins, Hs-PhaC1 and Hs-PhaC2 share the highest homology (37.8 % of identity and 47.2 %

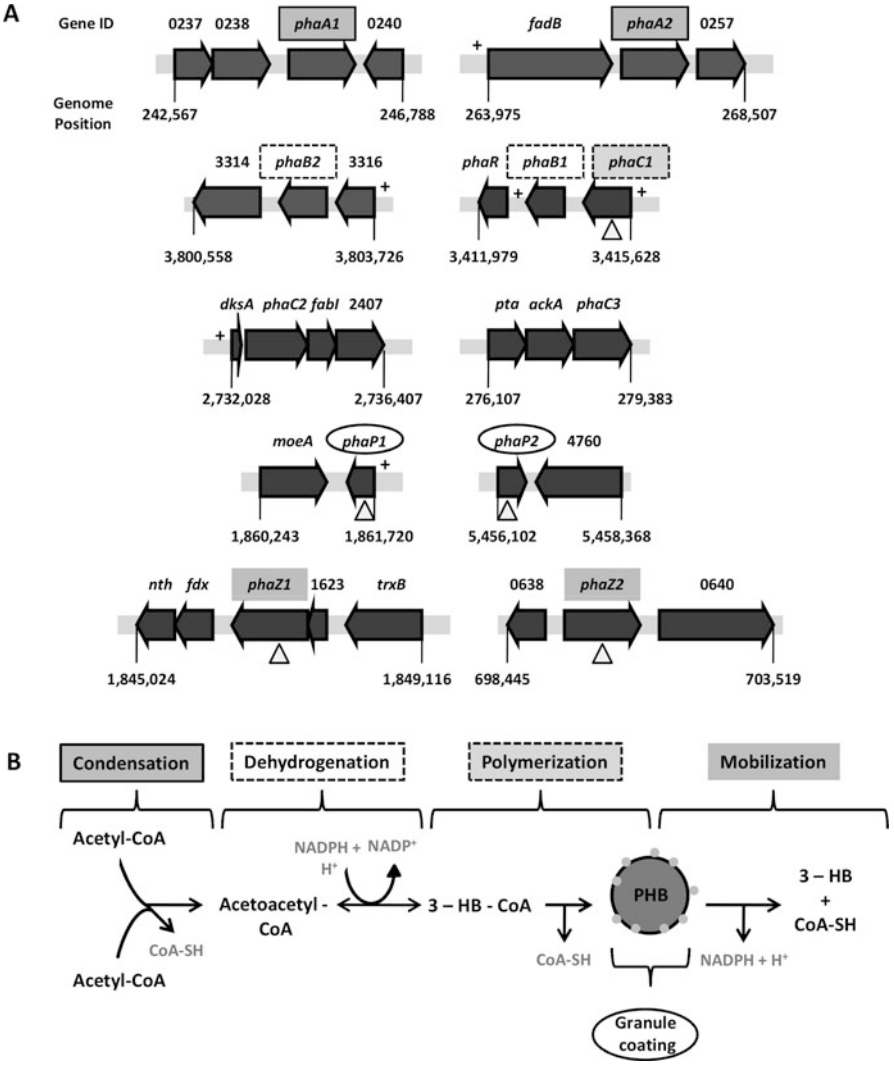
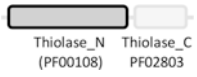
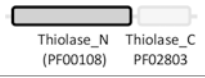
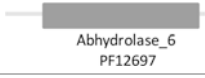
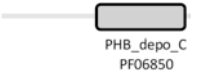
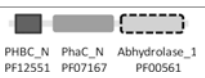


Fig. 2.1 Genome organization of genes potentially involved in the PHB metabolism in *H. seropedicae* SmR1. In (**a**) the diagrams represent the organization of the PHB-related genes within the *H. seropedicae* genome. Genes are represented by *arrows* which are identified (*upper lines*) either by the gene name (*italicized*) or by the numeric part of the respective genomic locus tag. The genome location (*lower lines*) is indicated by the numeric position of the first and last nucleotide in each representation. Plus signs indicate that the gene promoter is bound by PhaR *in vitro*, while pyramid symbols indicate genes for which the deletion was already obtained. The *PHB*-related genes are highlighted in *rectangular* or *elliptic boxes* which correlate to the function of the gene product during the steps of the *PHB* synthesis represented in B. Drawings are not to at scale

Table 2.1 List of all genes related to the PHB metabolism in *Herbaspirillum seropedicae* SmR1 (in attachment)

Genome location	Locus tag	Gene ID	Genbank	COG	Function	Protein length	Domain organization PFAM[a]
244668..245843	Hsero_0239	*phaA1*	ADJ61765.1	I	Acetyl-CoA acetyltransferase	391 aa	
266442..267638	Hsero_0256	*phaA2*	ADJ61782.1	I	Acetyl-CoA acetyltransferase	398 aa	
278253..279383	Hsero_0265	*phaC3*[b]	ADJ61791.1	I	Poly(3-hydroxyalkanoate) synthetase	376 aa	
698712..699938	Hsero_0639	*phaZ2*	ADJ62158.1	I	Poly-beta-hydroxyalkanoate depolymerase	408 aa	
1846523..1847758	Hsero_1622	*phaZ1*	ADJ63135.1	I	Poly-beta-hydroxyalkanoate depolymerase	411 aa	
1861157..1861720	Hsero_1639	*phaP1*	ADJ63152.1	NF	Phasin protein	187 aa	
2732357..2734129	Hsero_2405	*phaC2*[b]	ADJ63904.1	I	Poly(3-hydroxyalkanoate) synthetase	590 aa	
3411979..3412545	Hsero_2997	*phaR*	ADJ64485.1	Q ; T	PHA synthesis regulator protein, binds DNA and PHA	188 aa	
3412873..3413613	Hsero_2998	*phaB1*	ADJ64486.1	I;Q;T	NAD(P)-dependent dehydrogenase	246 aa	

(continued)

Table 2.1 (continued)

Genome location	Locus tag	Gene ID	Genbank	COG	Function	Protein length	Domain organization PFAM[a]
3413862..3415628	Hsero_2999	*phaC1*[b]	ADJ64487.1	I	Poly(3-hydroxyalkanoate) synthetase	588 aa	
3802057..3802818	Hsero_3315	*phaB2*	ADJ64796.1	I;Q;R	NAD(P)-dependent dehydrogenase	253 aa	
5456102..5456671	Hsero_4759	*phaP2*	ADJ66220.1	NF	Phasin protein	189 aa	

[a]Domain drawings are not to scale

[b]Searches using Pfam database release of December 2015(Finn et al. 2016) did not identify the alpha-beta hydrolase domain in PhaC1 and PhaC2 proteins. In addition, the assignment of the type 6 Abhydrolase to the PhaC3 was changed to DUF3141 (Pfam: PF11339), which resembles the Abhydrolase_1 domain (Pfam: PF00561) previously found in other orthologous counterparts. The domain assignments were kept as predicted by the 2014 release of Pfam database (Finn et al. 2014)

of similarity). As already suggested by domain structure comparison, PhaC3 is most dissimilar among PhaC-like proteins, sharing 14.4 % of identity and 23.8 % of similarity with PhaC1 and 13.6 % of identity and 22.9 % similarity with PhaC2.

Alignments of each domain from *H. seropedicae* PhaC1, PhaC2 and PhaC3 with the homologous domains of the PhaC1 and PhaC2 proteins from *Ralstonia eutropha* H16 were performed. The alignments of abhydrolase-like domains from Hs-PhaC and Re-PhaC (Fig. 2.2) revealed conservation of the lipase box (G-X-[S/C]-X-G), which is essential for catalysis (Rehm 2003). As observed in other PhaC proteins, the active serine site in lipase boxes (characteristic of true lipases and esterases) has been replaced by a cysteine (Muh et al. 1999; Steinbuchel et al. 1992).

According to the primary structure, substrate specificities and subunit composition, the PHA synthases are divided in four major classes: I, II, III and IV (Rehm 2003). The phylogenetic tree and domain comparisons revealed that the *H. seropedicae* PhaC1 clustered into a clade together with other recognized members of the Class I group, such as *R. eutropha* H16 PhaC1 (Rehm 2003) and *Bradyrhizobium japonicum* USD110 PhaC3 (Quelas et al. 2013). The Class I members of *H. seropedicae* SmR1, *R. eutropha* H16 and *B. japonicum* USD110 seem to be the main PHA synthases in these organisms (Tirapelle et al. 2013; Rehm 2003; Quelas et al. 2013). The PhaC1 of *H. seropedicae* probably is a scl-PHA synthase (scl, small chain length) able to recognize 3-hydroxybutyryl- and 3-hydroxyvaleryl-CoA as substrates (Catalan et al. 2007). The recognition of other 3-hydroxyalkanoyl-CoA such as 3-hydroxyhexanoyl-CoA or longer remains to be determined.

The *H. seropedicae* PhaC2 protein is grouped in a clade named Unclassified A (Fig. 2.3) together with PhaC2 proteins from *R. eutropha* H16 and *B. japonicum* USD110. The PHA synthases within Unclassified A group comprise three domains. Interestingly, the PhaC2 from *B. japonicum* has no catalytic activity in vitro and is unable to complement a *phaC1* mutation in *R. eutropha* H16 (Quelas et al. 2013), and the *phaC2* gene in *R. eutropha* H16 is not transcribed (Peplinski et al. 2010), suggesting that the contribution of these proteins for PHB production in the host organisms might be not physiologically important under the conditions tested so far. *H. seropedicae* SmR1 PhaC3 is grouped with the PhaC5 from *B. japonicum* USDA 110 (Ref Seq: NP_771188) into the Unclassified B group (Fig. 2.3). The *B. japonicum* USDA 110 *phaC5* gene is expressed at very low levels, and its deletion did not cause a significant reduction in PHB synthesis in *B. japonicum* USD110 (Quelas et al. 2013). The Unclassified B group and Class III proteins are comprised of a sole DUF3141 or abhydrolase_6 domain (Fig. 2.3). Interestingly, the alpha-beta hydrolase domains of Unclassified B and Class III groups are distinct from each other. In the Class III, as well as Classes I, II and IV and Unclassified A groups, the alpha-beta hydrolase domain is identified as PF00561, while the same domain in the Unclassified B group is identified as PF12697, according to Pfam database, which might suggest different functions. Overall, the in silico analyses performed here reveal that probably the PhaC1, PhaC2, and PhaC3 proteins from *H. seropedicae* have different physiological functions as already suggested for other organisms which also have multiple PHA synthases genes. Some of our observations, such as the phylogenetic clustering of *H. seropedicae* PhaC2 with nonfunctional PhaC2

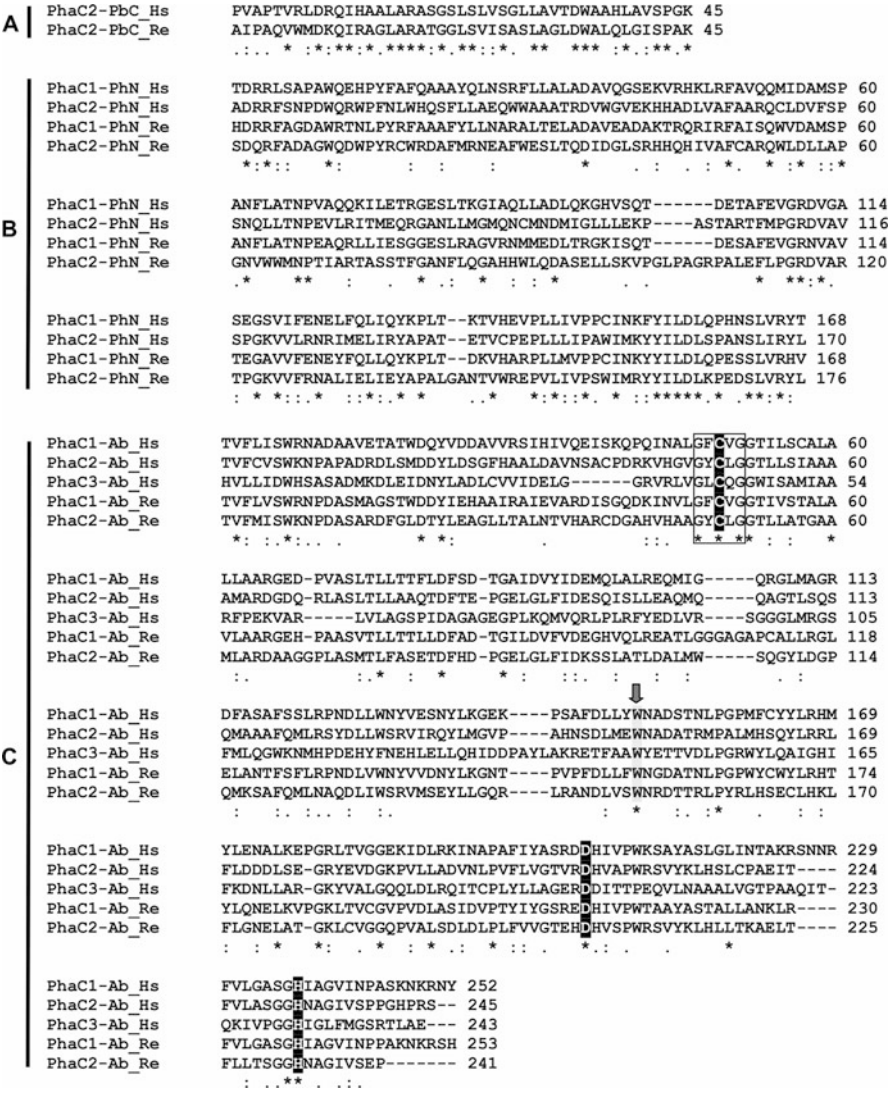


Fig. 2.2 Alignment between *H. seropedicae* and *R. eutropha* PhaC proteins. Each protein was first submitted to Pfam domain identification and then the domains were aligned separately. (**a**) Alignment of PHBC_N domain found in *PhaC2* proteins from *H. seropedicae* (Hs) and *R. eutropha* (Re). (**b**) Alignment of PhaC-N domain from *H. seropedicae PhaC1* and *PhaC2* and *R. eutropha PhaC1* and *PhaC2* proteins. (**c**) Alignment of abhydrolase domains found in all *H. seropedicae* and *R. eutropha* PhaC proteins. In (**c**) the lipase box motif is boxed. The catalytic triad amino acids are highlighted in a *black background*. The tryptophan residue important for the protein-protein interaction is highlighted in a *gray background* and indicated by a *thick arrow*. Other symbols denotes identical amino acids (*), high-similarity amino acids (:) and low-similarity amino acids (.)

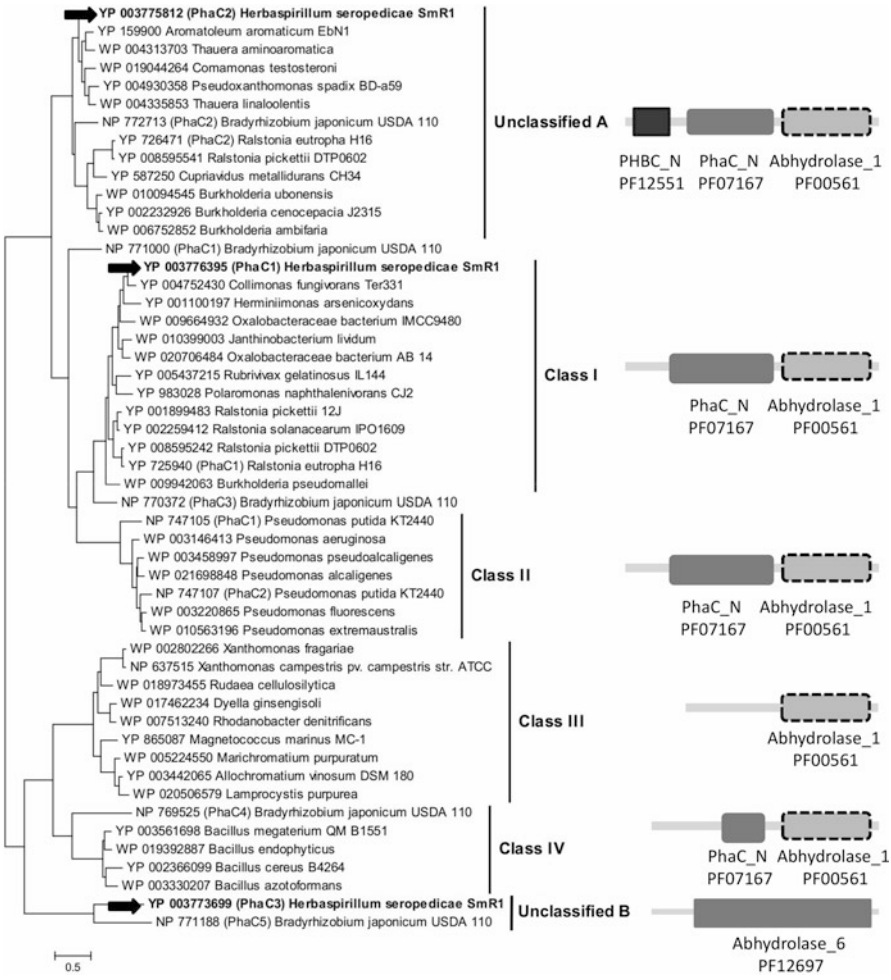


Fig. 2.3 Phylogenetic reconstruction of selected members of different classes of PHA synthases. In the *right* panel, the unrooted, maximum-likelihood tree with the highest log likelihood (−17216,5786) is shown. The proteins are identified by the NCBI Reference sequence identification number followed by the name of the host bacterium. Drawings are to scale, with branch lengths measured in the number of substitutions per site. The *H. seropedicae* PhaC proteins are highlighted in *bold* and with a *thick arrow*. In the *left* panel, the domains found in representative proteins from each taxonomic group are correlated to the phylogenetic affiliations proposed

from *R. eutropha* H16 and also the clustering of *H. seropedicae* PhaC3 with PhaC5 from *B. japonicum*, is in agreement with experimental evidence from the published literature and previous characterization of *H. seropedicae* Δ*phaC1* strain, which suggests that PhaC1 is the main PHA synthase in *H. seropedicae*, while both PhaC2 and PhaC3 are likely to have their functions or expression depend on PhaC1 activity. Interestingly the *phaC2* gene was upregulated in *H. seropedicae* colonizing

wheat roots (Pankievicz et al. 2016). Furthermore, both *phaC2* and *phaC3* expressions are activated by Fnr under low-oxygen conditions (Batista et al. 2013). Further physiological characterization is needed to precisely determine the roles of PhaC2 and PhaC3 proteins in *H. seropedicae*.

2.3 The Role of Phasins PhaP1 and PhaP2 on PHB Granule Formation in *H. seropedicae*

Proteins associated with PHB granules in *H. seropedicae* SmR1 were identified by proteomic analysis (Tirapelle et al. 2013). PhaP1 (encoded by Hsero_1639 – *phaP1*) is the main phasin coating PHB granules in *H. seropedicae*, while PhaP2 (Hsero_4759 – *phaP2*) was not observed. An isogenic mutant strain Δ*phaP1* was constructed by deletion of *phaP1*, in order to verify if PhaP2 could substitute PhaP1 in coating PHB granules. The Δ*phaP1* mutant produced PHB, indicating that *phaP1* deletion does not completely impair PHB production. Proteomic analysis of granule-associated proteins on the PHB granules from Δ*phaP1* revealed a high abundance of PhaP2, indicating that, in the absence of PhaP1, the second phasin PhaP2 is expressed and associated to PHB granules. Accordingly, transcriptome analysis by RNA-seq of the wild-type strain (SmR1) indicated that *phaP1* expression was 5.7-fold higher than *phaP2* (Tirapelle et al. 2013). Other organisms have been reported to carry more than one phasin coding gene. *R. eutropha* possesses seven genes encoding to phasins (Kuchta et al. 2007; Pfeiffer and Jendrossek 2012). Although all the *R. eutropha* phasin genes are expressed during PHB synthesis, the PhaP1 seems to be the main protein covering PHB granules (Kuchta et al. 2007). On the other hand, in *Sinorhizobium meliloti* two phasins are the main proteins associated with PHB granules (Wang et al. 2007).

2.4 Transcriptional Regulation of *pha* Genes in *H. seropedicae*

PhaR (formerly PhbF) was shown to bind at 11 promoters of genes and operons predicted to be related to PHA metabolism. A consensus binding site for PhaR was predicted in silico (Bailey et al. 2009) and further confirmed by in vitro DNase I footprinting assays (Kadowaki et al. 2011). A region containing the partially palindromic sequence TGC-N_3-GCA was found protected by PhaR in the regulatory region of *phaR*, and the same sequence motif was found in putative regulatory regions of other *pha* genes by using computational motif search analysis (Kadowaki et al. 2011). Accordingly, a similar motif was found in the binding sites of the PhaR from *Rhodobacter sphaeroides* FJ1 (Chou and Yang 2010) and from *Paracoccus denitrificans* (Maehara et al. 2002). In addition, it was shown that *H. seropedicae* PhaR is able to bind to the regulatory region of *phaP1* and to repress its transcription in an *Escherichia coli* background (Kadowaki et al. 2011). Based on that, it seems that the regulatory function of PhaR in *H. seropedicae* is similar to that

proposed for PhaR from *R. eutropha* and *P. denitrificans* (Potter et al. 2002; Maehara et al. 2002), whereby early synthesized PHB interacts with PhaR, preventing its inhibitory interaction with the DNA at certain promoters which contains a PhaR binding site. The isogenic strain Δ*phaR* of *H. seropedicae* SmR1 was recently obtained. This strain has a significant reduction in PHB production (3.2-fold lower in comparison to the wild type), suggesting that PhaR is regulating other genes important for PHB synthesis and accumulation. Since PHB metabolism is inextricably linked to metabolism of carbon, nitrogen, oxygen and redox balance, it is likely that other transcriptional regulators such as Crp, Fnr, SoxR and NtrC, among others, could be acting either directly or indirectly in the control of PHB synthesis.

2.5 Metabolic Engineering Strategies to Improve PHA Production in *H. seropedicae*

The previous report has shown that *H. seropedicae* strain Z69 is able to produce PHB (and PHB-*co*-PHV, upon addition of nonanoic acid) during growth on organic acids such as succinate and monosaccharides such as glucose, xylose, galactose and mannitol (Catalan et al. 2007). *H. seropedicae* cannot naturally metabolize disaccharides; however, the transference of *lacZ* (beta-galactosidase-encoding gene) and *lacY* (lactose permease gene) from *Escherichia coli* to *H. seropedicae* allowed it to metabolize lactose and produce PHB up to 36 % of cell dry weight (Catalan et al. 2007). Results from our lab with the strain *H. seropedicae* SmR1 showed a high production of PHB using organic acids (malate, succinate, citrate, and lactate) and monosaccharides (glucose, galactose, xylose and fructose), and remarkably a very high production (~70 % of PHB/cell dry weight) was observed when grown using glycerol as sole carbon source (unpublished data). These results demonstrate the potentiality of *H. seropedicae* to produce PHB from several types of carbon sources, including monosaccharides which are quite abundant in biomass feedstock (Saratale and Oh 2015) and glycerol which can be obtained for a low price as a by-product from biodiesel synthesis (Yoneyama et al. 2015). In addition, the available genome information of *H. seropedicae* SmR1 turns this bacterium as a potential model to study PHB metabolism and to apply metabolic engineering aiming to improve polymer yield.

A genomic inspection in the carbohydrate metabolic pathways from *H. seropedicae* revealed that all genes for the Entner-Doudoroff (ED) pathway are present (Pedrosa et al. 2011). However, the oxidative branch of the pentose phosphate pathway (oxPPP) lacks the 6-phosphogluconate dehydrogenase (6-PGD, E.C. 1.1.1.44) (Fig. 2.4). Likewise, the Embden-Meyerhof-Parnas (EMP) pathway lacks the classical 6-phosphofructokinase (PFK, E.C. 2.7.1.11), suggesting that metabolism of D-glucose, D-fructose, or D-mannose to pyruvate in *H. seropedicae* SmR1 proceeds via the ED and the nonoxidative branch of PPP. The metabolism of L-arabinose, an abundant pentose in biomass feedstock, was previously investigated in *H. seropedicae* (Mathias et al. 1989). The strain Z78 (parent of SmR1) metabolizes L-arabinose by a pathway producing non-phosphorylated intermediates and 2-oxoglutarate as

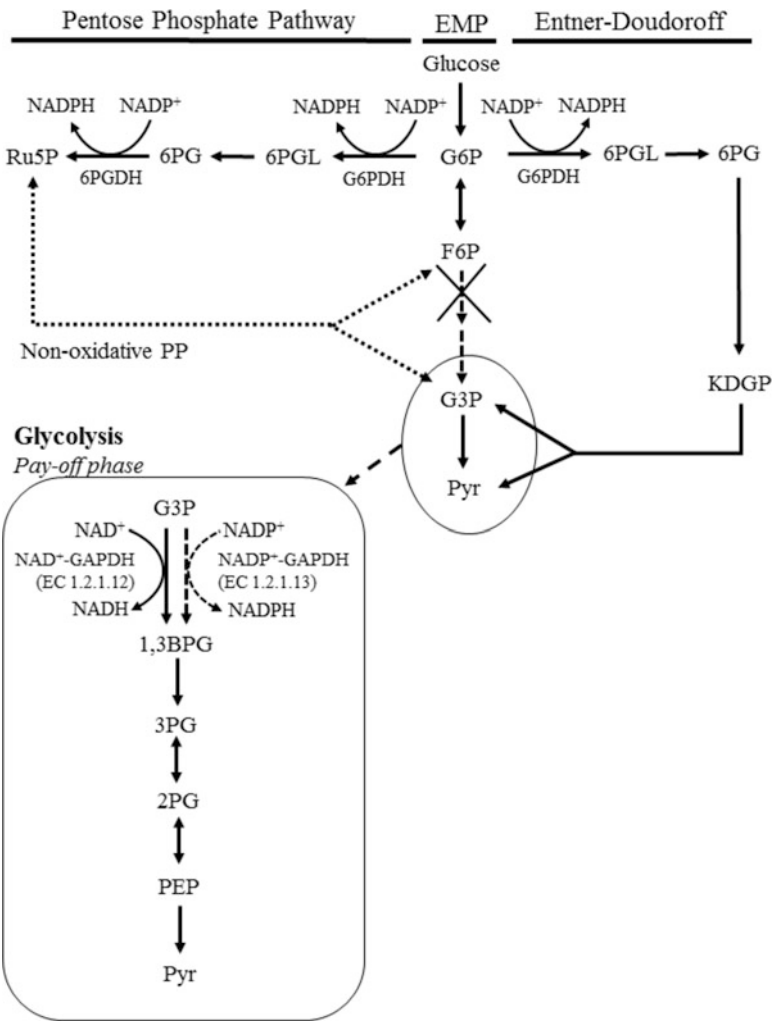


Fig. 2.4 Schematic representation of glucose catabolism pathways in *H. seropedicae* SmR1. The predictions were made based on analysis of the *H. seropedicae* SmR1 genome (NC_014323). Accordingly, this bacterium is unable to catabolize glucose via the Embden-Meyerhof-Parnas (*EMP*) glycolytic pathway due to the absence of the PFK (6-phosphofructokinase 1) enzyme – represented by the crossed-reaction in the middle panel. Instead, it can catabolize glucose either via the Entner-Doudoroff pathway (*left panel*) or via the pentose phosphate pathway (*right panel*). In the lower *right panel*, the payoff phase of glycolysis is represented. In the first step, the reaction catalyzed by the alternative $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (*EC 1.2.1.13*) is represented by *dashed arrows*. Abbreviations are as follows: *1,3BPG* 1,3-bisphosphoglycerate, *2PG* 2-phosphoglycerate, *3PG* 3-phosphoglycerate, *6PG* 6-phosphogluconate, *6PGDH* 6-phosphogluconate dehydrogenase, *6PGL* 6-phospho-D-glucono-1,5-lactone, *F6P* fructose-6-phosphate, *G3P* glyceraldehyde-3-phosphate, *G6P* glucose-6-phosphate, *G6PDH* glucose-6-phosphate dehydrogenase, *EMP* Embden-Meyerhof-Parnas, KDGP 2-keto-3-deoxy-6-phosphogluconate, *PEP* phosphoenolpyruvate, *Pyr* pyruvate, *Ru5P* ribulose-5-phosphate

final product (Mathias et al. 1989). The genome annotation of *H. seropedicae* SmR1 corroborated the pathway determined by enzymatic activity analysis (Pedrosa et al. 2011; Mathias et al. 1989). The catabolism of D-xylose, another important pentose in plant biomass feedstock, probably follows the Weimberg pathway (Stephens et al. 2007), since a gene coding the xylose kinase was not identified.

2.5.1 Engineering NADPH Generation as a Strategy to Improve PHB Production in *H. seropedicae*

The synthesis of PHB is dependent on acetyl-CoA and reductive power in the form of NADPH. Therefore, the accumulation of NADPH in bacteria is, in part, diverted to PHB synthesis. Thus, PHB has an important role as carbon reserve, but also it is an important electron sink, contributing to maintain the redox balance. The ED and oxidative PP pathways are important targets to be engineered, since high carbon flux through these pathways are often correlated with high levels of NADPH generation (Spaans et al. 2015). The overexpression of the NADPH-generating enzymes, namely, glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH), was already addressed in other organisms. The overexpression of G6PDH has been successfully applied to improve the NADPH/$NADP^+$ ratio in bacteria, yeast, and fungi (Lim et al. 2002; Verho et al. 2003; Poulsen et al. 2005; Becker et al. 2007; Lee et al. 2007; Wang and Zhang 2009; Lee et al. 2011; Tang et al. 2011; Fang et al. 2013; Shi et al. 2013). The overexpression of the oxPPP enzyme 6PGDH also yields high NADPH/$NADP^+$ ratio (Wang and Zhang 2009; Lim et al. 2002). Since in *H. seropedicae* the glucose metabolism follows exclusively the ED pathway, it is likely that fructose-6-phosphate and glyceraldehyde-3-phosphate are rerouted into the non-oxPPP to produce ribulose-5-phosphate (Fig. 2.4). Therefore, one NADPH is generated per glucose catabolized via ED, since the 6PGDH activity is absent in *H. seropedicae*. The overexpression of a 6PGDH gene in *H. seropedicae* would divert part of the 6-phosphogluconate to the oxidative branch of PP, yielding two NADPH per glucose catabolized. Consequently, the higher production of NADPH could improve the synthesis of PHB.

Another important target is the glyceraldehyde-3-phosphate dehydrogenase reaction. *H. seropedicae* has only one gene coding a NAD^+-dependent GAPDH (EC: 1.2.1.12), as revealed by the genome annotation. However, several organisms express a $NADP^+$-dependent GAPDH (EC: 1.2.1.13), which generates NADPH as by-product (Tamoi et al. 1996; Verho et al. 2002; Martinez et al. 2008). The overexpression of $NADP^+$-dependent GAPDH or replacement of the native NAD^+-GAPDH with the NADP variant was used to improve the production of NADPH-dependent compounds, such as lycopene, ε-caprolactone, L-ornithine, or coenzyme Q10 (Martinez et al. 2008; Huang et al. 2011; Jiang et al. 2013; Wang et al. 2013). To the best of our knowledge, few works targeted the overexpression of *gapN*, the non-phosphorylating $NADP^+$-dependent GAPDH (EC: 1.2.1.9) to improve the PHB yield in *E. coli* (Centeno-Leija et al. 2014). Thus, the overexpression of a

$NADP^+$-dependent GAPDH is equally an important strategy for enhancing PHB production in *H. seropedicae*.

2.6 Conclusions

H. seropedicae is a versatile PHB-producing bacterium, reaching high levels of polymer production when metabolizing organic acids, glycerol and monosaccharides as sole carbon sources. The availability of its genome sequence and the recently growing knowledge on its physiology make *H. seropedicae* as an emergent model for studying PHB metabolism and applying metabolic engineering to develop strains that overproduce PHB from cheap substrates such as industry by-products. In addition to the biotechnological interest, recent studies suggested that PHB production and accumulation are directly correlated with the fitness of *H. seropedicae* for plant colonization in both maize and wheat. These findings suggest that *H. seropedicae* strains overproducing PHB may also take competitive advantage during the rhizosphere colonization, making such engineered strains also interesting for the generation of better plant-growth-promoting bacteria.

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Engineering Hemicellulose-Derived Xylose Utilization in *Saccharomyces cerevisiae* for Biotechnological Applications

3

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Abstract

Saccharomyces cerevisiae has been used for thousands of years for alcoholic beverage and bread production. Today, it is also used in biotechnological processes with a major focus on bioethanol production. This yeast is nonpathogenic and classified as GRAS (generally regarded as safe). *S. cerevisiae* is the most intensively studied eukaryotic microorganism and has been instrumental in establishing our current wealth of information in biochemistry, genetics and cell biology. Studies over many years have resulted in refined methods to manipulate and analyze its biology, biochemistry, and genetics. Highly efficient methods for transformation and construction of gene knockouts are only two examples of a plethora of methodologies widely available. These advances together with having the genome sequence have made *S. cerevisiae* an ideal model for both basic and applied research. Metabolic engineering has been used for optimization of specific production processes, including extending the range of compounds either assimilated or synthesized by a given pathway or process, blocking the synthesis of by-products and improvement of productivity and yield.

The rational use of lignocellulosic biomass for second-generation bioethanol/biofuel production and other chemicals with added value will clearly increase in the coming years. The importance of developing second-generation bioethanol is due to limitations associated with production of first-generation bioethanol and especially the decline in availability and devastating consequences of continued fossil fuel use. Fermentable sugars present in plant lignocellulosic biomass are

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mainly glucose, xylose and arabinose. Wild-type strains of *S. cerevisiae* are unable to consume the pentoses. Construction of recombinant strains able to assimilate lignocellulosic xylose for ethanol production is a developing area of investigation that we describe below. Metabolic and evolutionary engineering has been successfully used in some cases, although there is still no strain capable of producing ethanol from xylose or arabinose at industrial levels. Production of ethanol as well as other valuable chemicals from lignocellulosic xylose and arabinose will require further investigation.

3.1 Introduction

The origins of words used to refer to yeasts in different languages have a relationship with their abilities to ferment different substrates (Mortimer 2000). This illustrates an old and intimate alliance between yeasts and the evolution of human civilization. Alcoholic beverages like wine and beer were probably the first yeast fermentation products. Strong evidence for wine production in Iran's Northern Zagros Mountains was found to be between 5400 and 5000 B.C. (McGovern et al. 1996). Industrial production of wine in Egypt was initiated during the Third Dynasty. There is also evidence for various types of alcoholic beverage production, including rice and grape wine, beer, and various liquors including baijiu in China, ca. 7000 B.C. (Legras et al. 2007).

Wine appears to have been the first yeast fermentation product because an inoculum is not necessary. During these early times, the concept of microorganisms as being responsible for fermentative processes was probably not yet conceived. In time however, producers noticed that leaving crushed grapes with grape juice resulted in the mixture being transformed to wine (Mortimer and Polsinelli 1999). Analysis of residues recovered from Egyptian wine jars (3150 B.C.) established the presence *Saccharomyces cerevisiae* DNA (Cavalieri et al. 2003).

The use of yeasts for bread production dates back to 500 B.C. in Egypt. The addition of wine or cross-contamination by insects probably resulted in production of the first yeast leavened bread which had improved characteristics compared with unleavened bread. Production of leavened bread also extended to Greek and Roman civilizations. Much later in 1874, a specific method was developed using well-adapted yeasts for the production of Vienna bread. In general yeasts used for production of wine and bread are previously selected, but are still usually considered as "natural" organisms (Legras et al. 2007). In 1856 Louis Pasteur developed the scientific concept of fermentation by yeasts and then the idea of selecting and improving the use of different yeast strains for specific applications.

3.1.1 *Saccharomyces cerevisiae*

Budding yeast, with a genome of about 12 Mb, is one of the most important model organisms in biochemistry, genetics and molecular biology. The budding yeast

genome sequence was the first to be determined for a eukaryote (Goffeau et al. 1996). These yeasts are also easy to use for genetic analysis and are safe as well as simple to grow and manipulate (Nevoigt 2008). *S. cerevisiae* has been used intensively for improved production of specific metabolites using metabolic engineering strategies (Da Silva and Srikrishnan 2012). In addition, it has been used as a recombinant organism for production of new chemicals and the metabolism of new carbon sources. This chapter focuses on advances in production of different compounds including bulk chemicals and fuels by genetically modified yeasts using carbon sources like xylose.

3.2 Bioethanol Production

Bioethanol is generated by microbial fermentation. Current industrial production is based on crops such as sugar cane, corn, sorghum, and others which are also used for human and animal food production. Compounds used for microbial production of ethanol are sugars such as hexoses generally occurring in plants as disaccharides and starch. Microbial biofuel produced from renewable sources is part of a closed CO_2 cycle because when used as fuel CO_2 is produced which can then used by plants in photosynthesis.

The production of this biofuel termed first-generation bioethanol will continue to increase and is estimated that by 2022 more than 100 billion liters will be produced per year (Goldemberg and Guardabassi 2010). This model for production, however, has numerous disadvantages (Naik et al. 2010). The generation of raw material requires extensive areas for plant cultivation, competing with land areas needed for food production. The sugar content in crops is also relatively limited such that areas needed for production of bioethanol are currently insufficient. Increasing the size of cultivation areas for biofuel production by displacing natural habitats, e.g., has a negative impact on biodiversity and on balancing CO_2 emission versus photosynthetic CO_2 assimilation (Ros et al. 2010).

These problems have prompted investigation oriented for production of second-generation bioethanol, i.e., from other raw material and specifically from materials not used for food production. These materials include lignocellulose and other sources like agricultural residues, wood, paper and municipal solid waste. Lignocellulose has been a primary focus of attention since it is the most abundant plant-derived organic component in the biosphere (Claassen et al. 1999; Wi et al. 2015).

3.2.1 Lignocellulosic Biomass

About half of plant biomass is composed of lignocellulose, which is also considered to be the most abundant renewable resource in soil. This material consists of three types of polymers: cellulose, hemicellulose and lignin. Cellulose, the most abundant fraction, is composed of glucose-based monomers (Peters 2006). Hemicellulose contains diverse sugars, including xylose, glucose and arabinose. Lignin is a diverse cross-linked polymer containing phenolic compounds and is of complex

composition and structure. Many microorganisms are able to consume and degrade cellulose and hemicellulose, and some filamentous fungi can degrade lignin. Generation of lignocellulosic residuals results in pollution and is a waste of resources that could be used to produce other materials with added value.

Lignocellulosic sugars are generated by chemical, mechanical and thermal treatments involving steps designed to eliminate the structural rigidity of plant material and separate sugars from lignin, e.g., hydrolysis results in depolymerization of cellulose and hemicellulose into fermentable sugars. A mixture of sugars containing mainly glucose, xylose, and arabinose is obtained by this process (Maity 2015; Jordan et al. 2012). In general, microorganisms currently used in the production of bioethanol and other bio-products are able to very efficiently ferment glucose but not xylose. Commonly, *S. cerevisiae* is used in fermentation because it is a robust microorganism with relatively high production yields. This yeast also has relatively good tolerance to high ethanol concentrations and inhibitors present in treated lignocellulose (Olofsson et al. 2008).

Unfortunately, native strains of *S. cerevisiae* are usually unable to ferment pentoses to ethanol (Batt et al. 1986). In order to consume all the sugar present in lignocellulosic biomass for second generation bioethanol production, scientists have engineered various lab strains as well as strains already selected and used for industrial production. A large number of combinations have been attempted, but actually there is still no strain capable of producing ethanol from xylose at industrial levels.

Compared with studies on metabolism of glucose and other hexoses, investigation of pentose metabolism in microorganisms is relatively fragmentary. Gene databases are reduced and bioprospecting new organisms able to utilize lignocellulosic sugars are clearly needed. This knowledge will help to develop improved strains with better industrial capabilities, either by developing new microorganisms or using metabolic engineering to modify strains already in use (Dumon et al. 2012).

3.2.2 Xylose Metabolism in Microorganisms

Different pathways have been described for xylose catabolism. The routes of Weimberg (Weimberg 1961) and Dahms (Dahms 1974) are found in some bacteria (Stephens et al. 2007) and archaea (Johnsen et al. 2009). Both routes involve a xylose dehydrogenase and other enzymes to form the intermediate 2-keto-3-deoxy-xylonate, which can then be converted to α-ketoglutarate by the Weimberg pathway or to pyruvate and glycolaldehyde by the Dahms route (Stephens et al. 2007). In some bacterias, a periplasmic PQQ-dependent glucose dehydrogenase can oxidize D-xylose to D-xylonolactone. After this, a periplasmic gluconolactonase is involved in the synthesis of D-xylonate that can be excreted or internalized so to continue degradation by Weimberg or Dahms routes (Zhang et al. 2013) (Fig. 3.1).

Another route is the oxo-reductive pathway which is commonly found in yeasts and filamentous fungi. This route converts D-xylose to xylitol by a NAD(P)H-dependent xylose reductase (XR). Then, xylitol is oxidized to D-xylulose by a

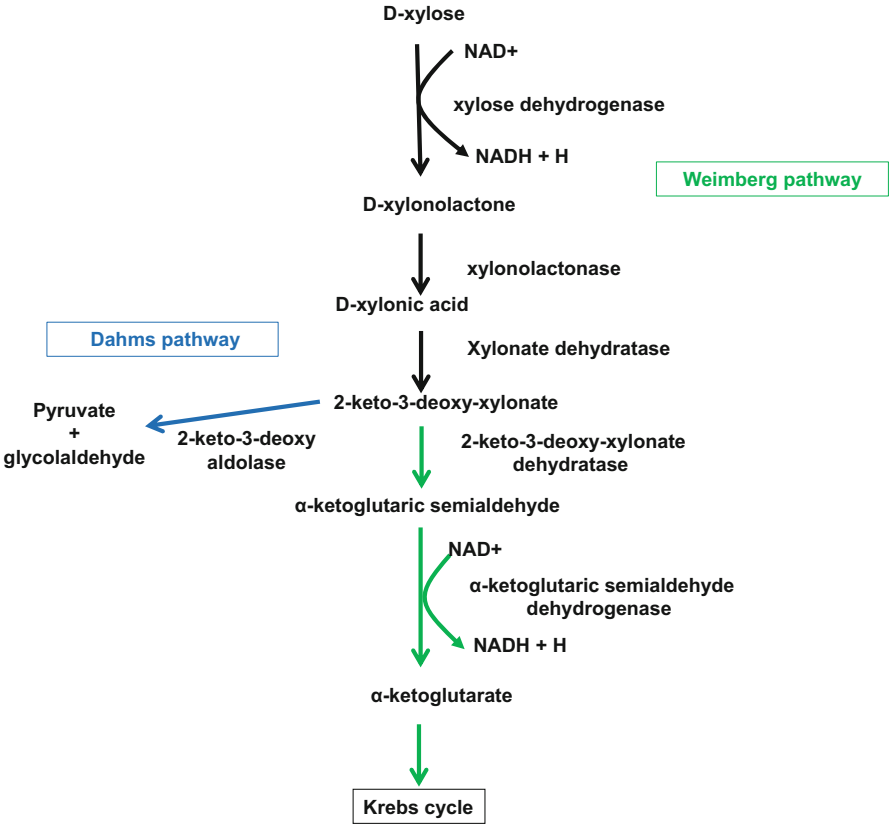

Fig. 3.1 Weimberg and Dahms pathways. Shared reactions are shown in *black*. Specific reactions for Weimberg pathway are shown in *green* and the specific reaction for Dahms pathway in *blue*

NAD^+-dependent xylitol dehydrogenase (XDH). In the last step, D-xylulose is phosphorylated by a xylulose kinase (XK) generating D-xylulose 5-phosphate, which enters the pentose phosphate pathway (PPP) (Jeffries 2006) (Fig. 3.2). Finally, the D-xylose isomerase route which is the most commonly found pathway in bacteria converts D-xylose to D-xylulose, mediated by a D-xylose isomerase (XI) (Jeffries 2006) (Fig. 3.2).

3.2.3 Production of Ethanol by Engineering *S. cerevisiae* Utilizing Xylose as Sole Carbon Source

S. cerevisiae wild-type strains are unable to consume D-xylose despite having a xylose-inducible GRE3 gene encoding a reductase for synthesis of xylitol from D-xylose, a constitutive XDH gene encoding xylitol dehydrogenase and a XKS1 gene that encodes xylulose kinase. This oxo-reductive route is found in native

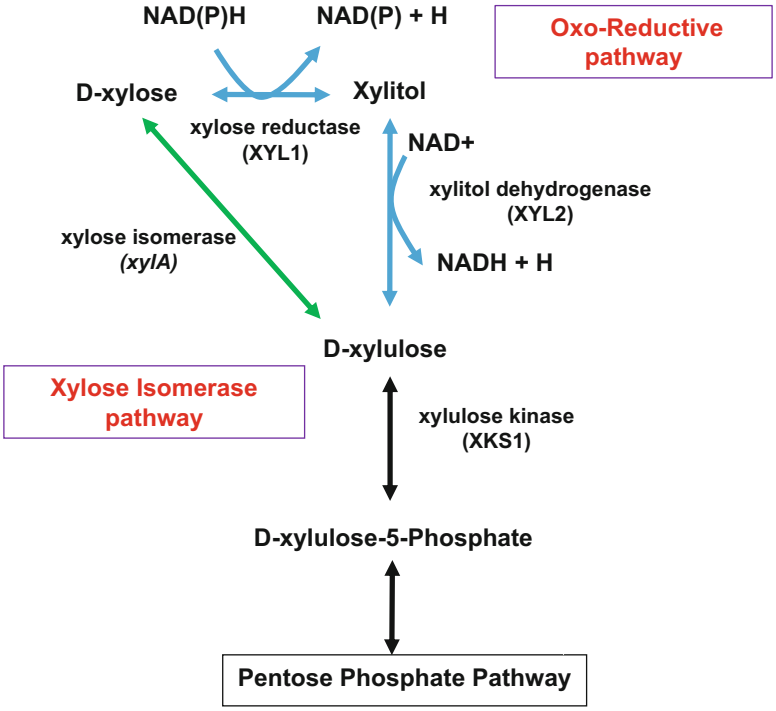

Fig. 3.2 Route of xylose isomerase and oxo-reductive pathway. Shared reactions are shown in *black*. Specific reaction for xylose isomerase pathway is shown in *green* and specific reactions for oxo-reductive pathway in *blue*. Xylose reductase (*XR*) is encoded by XYL1, xylitol dehydrogenase (*XDH*) by XYL2, and xylulose kinase (*XK*) by XKS1. In bacteria, xylose isomerase (*XI*) is encoded by the gene xylA

strains of *S. cerevisiae* and the enzymes are expressed. These strains, however, are unable to grow on xylose and primarily produce xylitol (Van Vleet and Jeffries 2009). This inability to grow is due to an imbalance of redox cofactors, whereas GRE3 principally uses NADPH as cofactor, XDH preferentially uses NAD^+ (Jeffries 2006).

Studies on bioethanol production from xylose by *S. cerevisiae* have focused on one of two heterologously expressed pathways: oxo-reductive route (XR/XDH) or the XI pathway. Since the first recombinant strains were constructed, there have been numerous attempts to generate an ideal strain for industrial production of second-generation bioethanol. Heterologous expression of genes encoding for the oxo-reductive pathway (XR/XDH) from *Scheffersomyces stipitis* (*Pichia stipitis*) (Eliasson et al. 2000; Sonderegger and Sauer 2003) and the XI route from *Piromyces* sp. have been the most commonly attempted strategies (Harhangi et al. 2003; Chu and Lee 2007).

The metabolism of xylose for entry to the PPP in *S. stipitis* (*P. stipitis*) involving the XR/XDR route has been successfully expressed in *S. cerevisiae*.

The incorporation of XR/XDH from *S. stipitis* in *S. cerevisiae* also requires overexpression of the xylulokinase (XK) and the non-oxidative PPP (Moniruzzaman et al. 1997; Gancedo and Lagunas 1973; Kotter and Ciriacy 1993; Fiaux et al. 2003).

Cofactor specificity for XR from *S. stipitis* could be modified by substituting the lysine at position 270 with other amino acids like arginine or methionine. The resulting modified XR enzymes were able to use NADH with a higher affinity compared with unaltered XR (Kostrzynska et al. 1998; Xiong et al. 2011). Recombinant strains of *S. cerevisiae* expressing these modified XRs had improved ethanol production (Table 3.1) (Bengtsson et al. 2009). It was also found that overexpression of an additional XDH under control of strong constitutive promoters in a xylose-fermenting strain not only reduced xylitol accumulation but also increased ethanol yield (Kim et al. 2012).

Cofactor specificity of XDH in *S. stipitis* was also modified in order to control the resulting redox imbalance inherent in this pathway. Watanabe et al. (2005) constructed novel $NADP^+$-dependent XDH mutant(s) by multiple mutagenesis using a sorbitol dehydrogenase as model enzyme. They also introduced a structural zinc atom to increase thermostability and catalytic activity of this enzyme with $NADP^+$ as cofactor. A recombinant strain (MA-R5) derived from *S. cerevisiae* IR-2 expressing one of these $NADP^+$-dependent XDHs (ARSdR) had an improved xylose consumption rate and ethanol yield, compared with reference strain MA-R4 as shown in Table 3.1. Furthermore, MA-R5 was able to effectively coferment sugars present in lignocellulosic hydrolysate to produce ethanol with high yield (0.48 g/g of consumed sugar) (Matsushika et al. 2009).

Xylose isomerase (*xylA*, XI) catalyzes the conversion of xylose to xylulose, eliminating the problem of redox cofactor imbalance (Matsushika et al. 2009). Heterologous expression of bacterial XI in *S. cerevisiae* has several other problems. Relatively good expression levels, however, were obtained when XI genes from *Clostridium phytofermentans* (Brat et al. 2009; Walfridsson et al. 1996; Demeke et al. 2013) or *Thermus thermophilus* (Karhumaa et al. 2005) were transferred. Also, the XI (*xylA*) gene from *Piromyces* sp. E2 was functionally expressed in *S. cerevisiae* (Harhangi et al. 2003; Kuyper et al. 2003; Chu and Lee 2007). *S. cerevisiae* strains carrying the *xylA* gene from *Piromyces* sp. E2 and overexpressing genes for XK and non-oxidative PPP were also constructed (Kuyper et al. 2005). Some of these constructions exhibited improved xylose utilization only when evolutionary engineering was used as strategy to select xylose-consuming strains (Qi et al. 2015).

The bio-prospection of new XI genes was also done using a bovine rumen metagenomic library functionally expressed in *S. cerevisiae*. The XI activities found using this approach were comparable with those of *Piromyces* sp. (Hou et al. 2016). An alternative strategy was also developed using metagenomic analysis of three soils differing in plant vegetation and geographical location. In this study an amplicon pyrosequencing approach was used employing novel primer sets to capture XI gene sequences not previously described (Nurdiani et al. 2015).

The oxo-reductive and XI pathways both generate xylulose-5-phosphate, which is then metabolized by the non-oxidative branch of the PPP as shown in Fig. 3.2. A consequence of overexpressing a heterologous pathway is that the immediately

Table 3.1 Ethanol production by *S. cerevisiae* recombinant strains cultured under anaerobic conditions or with oxygen limitation. Cells were grown in batch flasks for 72 h of incubation

Genotype	Substrate	Ethanol ($g.l^{-1}$)	Ethanol yield ($g_{EtOH}.g_{sugars}^{-1}$)	Productivity ($g.l^{-1}.h^{-1}$)	Xylitol yield ($g_{xylitol}.g_{sugars}^{-1}$)	Glycerol yield ($g_{glycerol}.g_{sugars}^{-1}$)	References
SXA-R2P-E (BY4741 xylA*3/TAL1/XKS1/ΔGRE3/ΔPHO13/evolved)	70 g/L glucose and 40 g/L xylose	50	0.43				Ko et al. (2016)
BY4741X expressing XYL1 and XYL2 from *S. stipitis* and XKS1 from *S. cerevisiae*	80 g/l xylose	25.4	0.279	0.826			Fujitomi et al. (2012)
BY4741X/ΔPHO13 expressing XYL1 and XYL2 from *S. stipitis* and XKS1 from S. *cerevisiae*	80 g/l xylose	33.9	0.357	2.184			Fujitomi et al. (2012)
XR-0 (YC-DM MATa ADH1p-XYL1(K270R)-ADH1t/PGK1p-XYL2-PGK1t/PGK1p-XKS1-PGK1t)	55.6 g/l xylose	18.8	0.34	3.28	0.15	0.06	Xiong et al. (2013)
XR-1 (YC-DM MATa ADH1p-XYL1(K270R)-ADH1t/PGK1p-XYL2-PGK1t/PGK1p-XKS1-PGK1t)	40.2 g/l glucose and 41.6 g/l xylose	36.4	0.44	3.20	0.04	0.06	Xiong et al. (2013)
IR-2, AUR1::AUR1-C-PGKp-XK-PGKt, PGKp-XDH-PGKt, PGKp-XR-PGKt	45 g/l xylose	15.3	0.34	0.36	0.048	0.101	Matsushika et al. (2009)
IR-2, *AUR1::*[*PGK1p-XKS1-PGK1t*, *PGK1p-ARSdR-PGK1t*, *PGK1p-XYL1-PGK1t*]	45 g/l xylose	16	0.37	0.50	0.038	0.076	Matsushika et al. (2009)

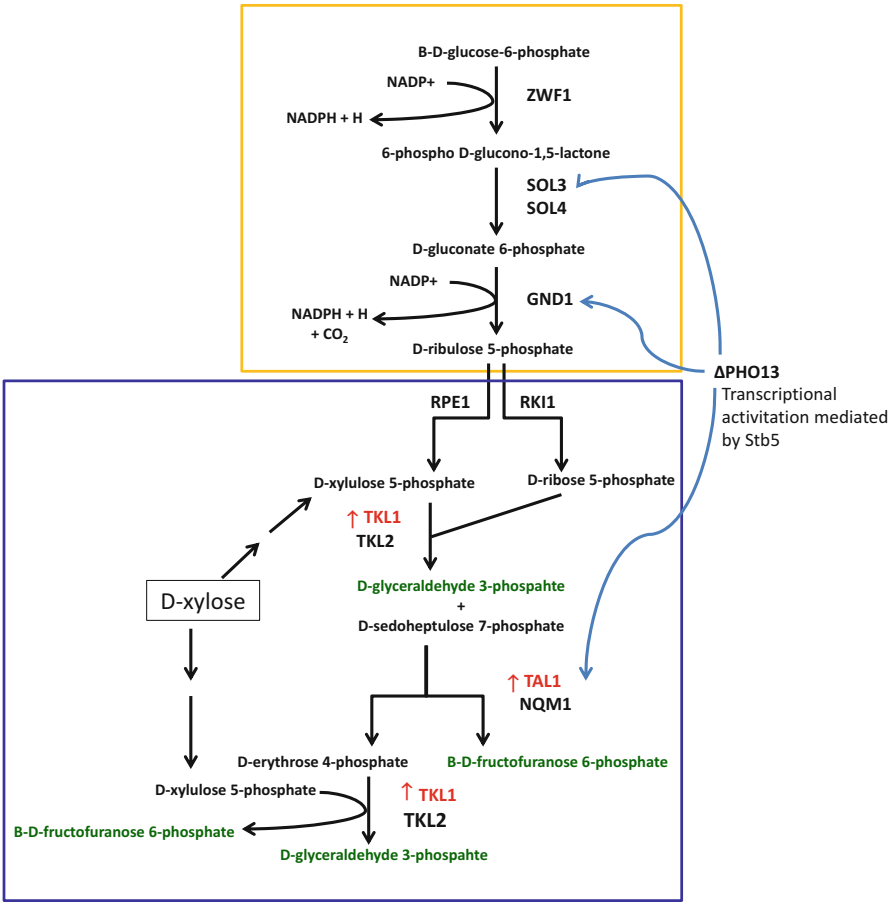

Fig. 3.3 Pentose phosphate pathway. The oxidative branch is in the yellow square and the non-oxidative branch, where D-xylose catabolic intermediates are incorporated, is in the blue square. Glycolytic and gluconeogenic intermediates are written in *green*. Overexpression of TAL1 and TKL1 can improve ethanol production in recombinant strains expressing heterologous paths for D-xylose catabolism. Deletion of PHO13 activates the transcription of genes SOL3, GND1 and TAL1, and this contributes to improve ethanol production in recombinant strains (*arrows in light blue*). Genes encoding enzymes are indicated: *ZFW1* glucose-6-phosphate dehydrogenase, *SOL3* and *SOL4* 6-phosphogluconolactonase, *GND1* 6-phosphogluconate dehydrogenase, *RPE1* D-ribulose-5-phosphate 3-epimerase, *RKI1* ribose-5-phosphate ketol-isomerase, *TKL1* and *TKL2* transketolase, *NQM1* and *TAL1* transaldolase

adjacent pathway reactions may limit metabolic flux. In this case, it was found that overexpression of the TAL1 gene encoding for transaldolase and the TKL1 gene encoding a transketolase can help overcome this kind of limitation by increasing flux through the PPP and thus improve growth on xylose and ethanol production (Matsushika et al. 2009) (Fig. 3.3). However, overexpression of GND1, which encodes 6-phosphogluconate dehydrogenase of the oxidative branch of PPP, did not

result in improved growth on xylose or ethanol production (Xu et al. 2016). The inactivation of PHO13, encoding a para-nitrophenyl phosphatase (pNPPase), improved growth and ethanol yields in xylose-utilizing recombinant strains. Apparently, PHO13 deletion results in upregulation of PPP genes (GND1, SOL3 and TAL1) and thus catabolism of xylulose-5-phosphate (Fig. 3.3). This deletion also results in modulation of redox balance by transcriptional activation of NADPH-producing enzymes (GCY1, GOR1 and YEF1). The process is mediated by a transcription factor (Stb5), which is activated under NADPH-limiting conditions. This seems to be a response to oxidative stress conditions generated when xylose is metabolized and expressing the oxo-reductive pathway thereby leading to imbalance in redox cofactors (Kim et al. 2015). The PHO13 deletion did not affect growth when glucose was used as carbon source and when xylose was used, trans-activation of NADPH-producing enzymes was still observed (Kim et al. 2015). The PHO13 deletion also prevents accumulation of sedoheptulose and sedoheptulose-7-phosphate (S7P) intermediates in the non-oxidative PPP. This in turn contributes to upregulate TAL1 (S7P is substrate for TAL1). The TAL1 reaction step was identified as a bottleneck for flux in recombinant strains expressing heterologous pathways for D-xylose catabolism (Xu et al. 2016; Van Vleet et al. 2008). Finally, recombinant strains expressing the oxo-reductive pathway and having the PHO13 deletion showed increased tolerance to inhibitors produced by hydrolysis of lignocellulose including acetic acid, formic acid and furfural (Fujitomi et al. 2012).

3.3 Expression of Weimberg-Dahms Pathways and Production of Alternative Metabolites

Heterologous expression of some genes of Weimberg-Dahms pathways in *S. cerevisiae* has been used for production of D-xylonate. This compound has potential applications in chemical industry for production of chelators, dispersants and clarifying agents (Toivari et al. 2012a). Some reports describe heterologous expression of xylose dehydrogenase (*xylA*) and xylonolactonase (*xylC*) from *Caulobacter crescentus* and another with the *xylA* gene from *Trichoderma reesei* (Toivari et al. 2012a, b). One of the main problems involves the need to introduce more than five bacterial genes for the expression of these heterologous pathways.

S. cerevisiae has been analyzed for potential production of carboxylic acids including succinic, malic and lactic acids (Liu and Jarboe 2012; Otero et al. 2013). These compounds can be used for synthesis of other valuable chemicals using enzymatic or chemical methods. The most recent studies involve the use of metabolic engineering for the design of new metabolic pathways. In general, analyses are focused using the metabolic network of glucose consumption as a starting point. The evaluation of alternative carbon sources like xylose, consumed using heterologous routes, should be developed in future studies.

We estimated the maximum yield for succinate utilizing xylose as carbon source taking into account expression of different pathways in *S. cerevisiae* by flux balance analysis (FBA). COBRA toolbox (Orth et al. 2010) was applied using a genome

scale reconstructed metabolic network (Aung et al. 2013) under anaerobic conditions. Results showed that when the Weimberg pathway is expressed, the maximum yield for succinate is $Y_{succ/xyl} = 0.79$ g/g. Evaluating the expression of oxo-reductive or XI routes resulted in maximum yields of 0.66 g/g in both cases. Likewise, the same analysis using the wild-type genomic background and glucose as sole carbon source, $Y_{succ/gluc} = 0.66$ g/g was the maximum yield. This indicates that production of succinate is higher when cells are expressing the Weimberg route compared with those expressing the oxo-reductive or XI pathways. This analysis should be evaluated, considering the expression of alternative routes for xylose metabolism in *S. cerevisiae* under aerobic conditions.

3.4 Transport of Xylose

S. cerevisiae contains 18 genes identified as encoding for permeases involved in hexose transport (Hxt1-17 and Gal2), but only seven (Hxt1 to Hxt7) recognize glucose as substrate. Hxt1, Hxt4, Hxt5, Hxt7 and Gal2 permeases can transport xylose, but their affinities are 200-fold lower compared with those determined for glucose (Moysés et al. 2016). Permeases with high affinity for pentoses have not been identified in this organism. In the presence of xylose and glucose, cultures exhibit a diauxic growth profile first consuming glucose. For this reason, overexpression of individual transport genes in *S. cerevisiae* strains without HXT and GAL2 genes was analyzed. Results showed that Hxt1 permease allows for efficient glucose/xylose consumption and ethanol production during cofermentation, but xylose is not transported when added as sole carbon source. Hxt2 can transport xylose even during glucose consumption, but fermentations are incomplete. Hxt5 is a poor permease for glucose and xylose. Hxt7 is an efficient permease for xylose, but with higher affinity for glucose. It seems that there is not a unique permease that could improve industrial fermentations using lignocellulosic hydrolysates. Hxt1 could be suitable for cofermenting glucose and xylose, while Hxt7 could be an option for the fermentation of substrates rich in xylose (Gonçalves et al. 2014).

Various xylose transport genes from diverse organisms have been expressed in a strain of *S. cerevisiae* deficient in Hxts and Gal2 transporters but carrying XR and XDH genes from *S. stipitis* (Moysés et al. 2016). XUT1, HXT2.6 and QUP2 were identified by transferring a genomic DNA library from *S. stipitis* to a *hxt*-null mutant strain of *S. cerevisiae* (de Sales et al. 2015). These three genes were able to partially restore the ability of the mutant to grow on xylose. GXF1 from *Candida intermedia* expressed in *S. cerevisiae* TMB 3057 improved affinity (Km) for xylose transport compared with the wild-type strain. The specific growth rate was improved only when low concentrations of xylose were used (Runquist et al. 2009). AraEp from *Corynebacterium glutamicum* improved transport capacity of xylose compared with endogenous transporters Hxt7 and Gal2 (Wang et al. 2013). Finally, Mgt05196p from *Meyerozyma guilliermondii* was also expressed in a *hxt*-null mutant of *S. cerevisiae*, and xylose transport capacity was found to be comparable with that exhibited by a strain expressing the GAL2 gene (Wang et al. 2015).

In addition, construction of mutations of endogenous transport genes from *S. cerevisiae* with improved ability to transport xylose has also been accomplished. Farwick et al. (2014) analyzed a collection of mutants in GAL2 and HXT7 transport genes. Substitution of asparagine at position 376 with phenylalanine in the GAL2 gene (Gal2-N376F) was the most effective modification in that it abolished glucose uptake and had an increased affinity for D-xylose (Farwick et al. 2014).

3.5 Strategies to Reduce the Effect of Fermentation Inhibitors in Lignocellulose Hydrolysates

Lignocellulosic hydrolysates not only contain fermentable sugars (glucose, xylose and arabinose) but also contain inhibitors like furfural, 5-hydroxy-methyil-furfural, acetic acid, and phenolic compounds. To overcome problems associated with these inhibitors, various strategies have been developed including evolutionary engineering in hydrolysate media or in the presence of specific inhibitors and also mutagenesis and genome shuffling (Snoek et al. 2015; Gong et al. 2009). Recombinant *S. cerevisiae* strains overexpressing enzymes for resistance to specific inhibitors as well as for modifying redox cofactor imbalance were also investigated (ZWF1, ADH7, ADH1) (Parawira and Tekere 2011). Overexpression of transcription factors SFP1, ACE2, ATR1, FLR1, or YAP1 was successfully used to increase tolerance to acetic acid, furfural, HMF and phenolic compounds (Chen et al. 2016; Alriksson et al. 2010). Expression of RNA-binding protein, lsm6, also improved tolerance to acetic acid (Gao and Xia 2012). As an alternative strategy, expression of heterologous laccases in *S. cerevisiae* can also help diminish the toxic effect of phenolic compounds (Parawira and Tekere 2011).

3.6 Conclusions

Sustainable development involving use of lignocellulosic residues for production of biofuels and other products has continued to gain increased attention in recent years. Treatment of these residues involves several steps that must be optimized in order to generate economically viable and sustainable processes. Construction of *S. cerevisiae* strains metabolizing xylose has been established as a highly competitive area of investigation and has become an integral part of studies for recycling lignocellulose residues. Design of *S. cerevisiae* strains able to efficiently metabolize xylose has several limiting steps, and different strategies have been developed and tested to address these limitations. The availability of an expanded database of genes encoding enzymes involved in the metabolism of pentoses is a challenge that can be investigated using classical microbial methodologies as well as use of high-throughput sequencing approaches.

The study of recombinant strains expressing heterologous pathways as well as overexpressing or deleting endogenous genes requires the integration of various methods including whole genome engineering approaches for the accelerated

improvement of a phenotype. In addition, metabolic engineering strategies should be used to facilitate optimizing a bioprocess of interest.

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Lactobacillus in the Dairy Industry: From Natural Diversity to Biopreservation Resources

4

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Abstract

Biopreservation, defined as the extension of shelf life and enhanced safety of foods by the use of natural or controlled microbiota and/or antimicrobial compounds, is an innocuous and ecological approach to the problem of food preservation and has gained increasing attention in recent years. Fermentation of food by lactic acid bacteria (LAB) is one of the oldest forms of biopreservation practiced by mankind, not only to enhance the hygienic quality but also to minimize the impact of the nutritional and organoleptic properties of perishable food products. *Lactobacillus* spp. are Gram-positive rod bacteria belonging to the LAB group. Their phenotypic traits, such as homo-/heterofermentation abilities, play a crucial role in souring raw milk and in the production of fermented dairy products such as cheese, yogurt, and fermented milk (including probiotics). Either as starter, as adjunct cultures, or as probiotics, *Lactobacillus* strains are used as food preservatives not only to prevent the development of food spoilage but also to give consumers a health benefit. Some lactobacilli produce bacteriocins, proteins active against other bacteria. In recent years, the interest in these compounds has grown substantially due to their potential usefulness as natural substitute for chemical food preservatives in the production of foods with enhanced shelf life and/or safety. Bacteriocins can be incorporated directly into fermented foods, or indirectly by using a bacteriocin-producing strain, as a starter or adjunct culture. As the consumers' interest in natural and healthy foods increases, LAB are currently playing a key role in the development of new products that may respond to this demand.

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4.1 Introduction

Foods are plants or animals (or their products) that contain essential nutrients, such as carbohydrates, fats, proteins, vitamins, or minerals, and are ingested and assimilated by an organism to produce energy, stimulate growth, and maintain life. Foods often contain many types of microorganisms that may show properties involved in the production or conversion of foods. The preservation of foods, including the use of otherwise perishable raw materials, has been used by man since the Neolithic period (around 10,000 years BC) (Prajapati and Nair 2008). The role of a single bacterium, "*Bacterium lactis*" (*Lactococcus lactis*), in fermented milk was described in 1877 by Sir John Lister (Santer 2010).

One of the most common forms of food biopreservation is fermentation, a process based on the growth of microorganisms in foods, whether natural or added. These microorganisms mainly comprise lactic acid bacteria, which produce organic acids and other compounds that, in addition to antimicrobial properties, also confer unique flavors and textures to food products. Traditionally, a great number of foods have been protected against spoiling by natural fermentation.

Food fermentations not only have a great economic value, but it also has been accepted that these products contribute in improving human health. The beneficial properties of fermented foods by lactobacilli were postulated at the beginning of the twentieth century by Metchnikoff (1908). This author correlated the longevity of Caucasians to the consumption of fermented milk products containing *Lactobacillus bulgaricus*. Since then, these microorganisms have been used to produce a new generation of healthy foods for human nutrition. These kinds of foods were termed as *probiotic*, which means "alive microbial feed supplement that beneficially affects the host by improving the intestinal microbial balance." Members of lactic acid bacteria (LAB) are widely used probiotics in fermented food and beverage industries, but also contribute to the sensory qualities of the food and in the prevention of spoilage.

Lactic acid bacteria comprise an ecologically diverse group of microorganisms that produce lactic acid as the primary metabolite of sugar catabolism. They are Gram-positive, non-spore-forming, and facultative anaerobic prokaryotes. LAB acid tolerance provides the ability to outcompete other bacteria in fermentation processes. The classification of LAB includes members of six families: *Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Lactobacillaceae*, *Leuconostocaceae*, and *Streptococcaceae* (Bourdichon et al. 2012; Erten et al. 2014). LAB utilize glucose by either homofermentative (producing 85 % lactic acid) or heterofermentative (producing lactic acid, CO_2, ethanol, and/or acetic acid in equimolar amounts) pathway (Kandler and Weiss 1986).

Among LAB, *Lactobacillus* spp. (family *Lactobacillaceae*) have common taxonomical features such as rod shape (under certain growth conditions, they can look almost coccoid-like) and the ability to produce lactic acid either as single or major end product (Batt 2014). Lactobacilli are strictly fermentative and have complex, sometimes very fastidious, nutritional requirements for carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives, and vitamins.

However, they grow in a variety of habitats. They are aciduric or acidophilic, as they endure the slowdown pH produced during their growth, inhibiting the growth of other microorganisms (Sharpe 1981). These bacteria are involved in the fermentation of a wide range of substrates like milk, vegetables, cereals, and meat. They are also natural inhabitants of the gut of many mammals. The fact that they can survive in many different habitats is a clear indication of their metabolic diversity.

From a biochemical point of view, fermentation is a metabolic process that produces energy from organic compounds, but without a change of the redox state of substrate and products. Fermentation plays different roles in food processing, such as:

1. Preservation of foods through the production of inhibitory metabolites such as organic acid (lactic acid, acetic acid, formic acid, propionic acid) and decrease of water activity (by drying or use of salt) (Gaggia et al. 2011)
2. Improvement of food safety due to the production of metabolites that inhibit the growth of pathogens or due to the removal of toxic compounds (Adams and Nicolaides 1997)
3. Improvement of the nutritional value, increasing the bioavailability of some molecules (Van Boekel et al. 2010)
4. Production of desirable organoleptic end products that improve the quality of the food (Lacroix et al. 2010)

Currently, more than 2000 different fermented foods are consumed by humans worldwide; many are ethnic and produced in small quantities to meet the demand of a minor population in a particular region. Some are produced commercially, but only a few products are manufactured by large food processor companies. As consumers have shown an increased interest in natural and healthy foods, the development of fermented foods has been increased significantly worldwide (Doyle and Meng 2006), and the production of fermentative microorganisms (starter cultures) has to meet this demand.

Yogurt production is a well-controlled industrial process that utilizes milk, milk powder, sugar, fruit, flavors, coloring, emulsifiers, stabilizers, and pure cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. These microorganisms exhibit a symbiotic relationship during the processing of yogurt. During fermentation, *S. thermophilus* grows quickly at first, utilizing essential amino acids produced by *L. bulgaricus. S. thermophilus*, in return, produces lactic acid, which reduces the pH to an optimal level for *L. bulgaricus* growth. Although lactic acid is the main end product in yogurt fermentation, compounds such as acetaldehyde, acetone, diacetyl (2,3-butanediol), and acetoin are also produced at very low concentrations, contributing to the final flavor (Caplice and Fitzgerald 1999; Chaves et al. 1999). In addition, the exopolysaccharides produced by LAB change the structural properties of the fermented dairy products, increasing the apparent viscosity. In recent years, a few yogurt products have been reformulated, including now alive strains of *L. acidophilus* and *Bifidobacterium* spp., as well as a mixed culture of *L. acidophilus* and *L. bulgaricus* for the production of acidophilus milk.

Lactobacillus species are also used as probiotics. For example, the addition of *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. casei*, *L. rhamnosus*, *L. fermentum*, *L. plantarum*, and/or *L. reuteri* to foods, or their use as starters in food fermentations, provides beneficial health effects to men and animals (Rosenberg 2013). Well-known commercial probiotic strains are *L. casei* Shirota (Sugita and Togawa 1994) and *L. rhamnosus* GG (Saxelin et al. 1996).

Among probiotic foods, kefir is an acidic and low-alcoholic fermented dairy product prepared by inoculation of cow, goat, or sheep milk with "kefir grains" (a combination of LAB and yeasts, in a matrix of proteins, lipids, and sugars) (Fig. 4.1). Kefir grains have a complex microflora that includes heterofermentative and homofermentative LAB, acetic acid bacteria, and yeasts (Marshall et al. 1984; Toba et al. 1987, Pidoux et al. 1990), including *Lactobacillus acidophilus* (Angulo et al. 1993), *Lactobacillus brevis* (Simova et al. 2003), *Lactobacillus paracasei* subsp. *paracasei* (Simova et al. 2003), *Lactobacillus delbrueckii* (Simova et al. 2003; Witthuhn et al. 2004), *Lactobacillus helveticus* (Angulo et al. 1993; Lin et al. 1999; Simova et al. 2003), *Lactobacillus kefiri* (Angulo et al. 1993; Takizawa et al. 1998; Garrote et al. 2001), *Lactobacillus kefiranofaciens* (Takizawa et al. 1998), *Lactobacillus plantarum* (Garrote et al. 2001), *Leuconostoc mesenteroides* (Lin et al. 1999; Garrote et al. 2001; Witthuhn et al. 2004), *Lactococcus lactis* (Garrote et al. 2001; Simova et al. 2003; Witthuhn et al. 2004), and *Streptococcus thermophilus* (Simova et al. 2003). Kefir contains alive active probiotic strains that control the development of pathogenic organisms, repopulate the digestive tract, and aid in the digestion and absorption of macromolecules, as well as metabolize lactose. Thus people with lactose intolerance can consume kefir.

Cheese and cheese-based products are among commonly commercialized foods fermented by LAB. A wide variety of bacterial cultures are used to produce characteristics cheese flavor and texture, preventing the growth of spoilage organisms and pathogens. Typical starter bacteria include *Lactococcus lactis* subsp. *lactis* or *cremoris*, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, and *Lactobacillus helveticus. L. helveticus* is used in the production of Swiss-type cheese, Parmesan, Romano, provolone, and mozzarella, but it is also used as an adjunct culture to prevent bitterness in Cheddar cheese. Adjunct cultures are used to provide or enhance the characteristic flavors and textures of cheese. Other common adjunct cultures are *Lactobacillus casei* and *Lactobacillus plantarum* (flavor in Cheddar cheese) or *Propionibacterium freudenreichii* (eye formation in Swiss-type cheese).

Non-starter lactic acid bacteria (NSLAB) are also used during the production of traditional cheese, giving up the characteristic ripening flavor of a few varieties of cheeses. NSLAB are not part of the normal starter flora but develop in the product, particularly during maturation as a secondary flora, and include wild strains of the *Lactobacillus casei* group. These bacteria probably arise from the raw milk (surviving pasteurization), or the cheese-making environment, showing proteolytic, lipolytic, and esterolytic activities that contribute to the cheese flavor (Gobbetti and Minervini 2014).

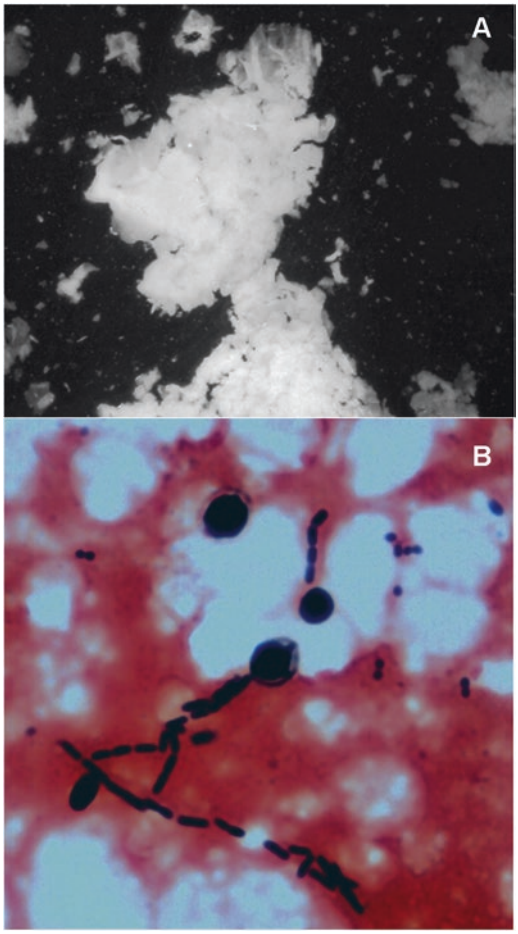

Fig. 4.1 Photomicrographs of kefir grains obtained at the unmagnified level (**a**) and Gram staining showing Gram-positive rods, cocci, and yeast at 100× magnification (**b**)

LAB also produce antimicrobial peptides (AMPs) called bacteriocins (Dimov et al. 2005; Cotter et al. 2013). The addition of *Lactobacillus* spp. as starters, or the addition of purified or semipurified bacteriocin preparation into a food product, is also a common procedure that may prevent pathogen growth during the manufacturing of dairy products (Batt 2014) (Fig. 4.2).

All the examples presented above show the relevance of the incorporation of microorganisms or their metabolites in our diet. The characterization of microorganisms present in traditional fermented foods consumed by mankind for centuries, and the improvement of LAB starter cultures properties, opens a new horizon in the field of food microbiology.

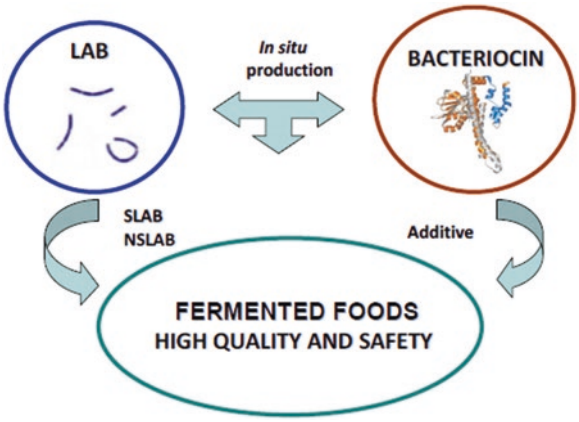

Fig. 4.2 Overview of potential applications of LAB as biopreservants

4.2 Non-starter Lactic Acid Bacteria

Non-starter lactic acid bacteria (NSLAB) are adventitious bacteria that gain access to cheese via milk and/or the dairy environments/factory, including cheese-making equipment, mainly because bacteria survive and remain in biofilm structures, even after cleaning and disinfection (Sheehan 2013; Van Hoorde et al. 2010; Donnelly 2013). NSLAB are a mixture of facultative heterofermentative mesophilic lactobacilli (*L. casei*, *L. plantarum*, *L. paracasei*, *L. pentosus*, *L. curvatus*, and *L. rhamnosus*), obligate heterofermentative lactobacilli (*L. brevis*, *L. fermentum*, *L. buchneri*, *L. parabuchneri*, and *L. farciminis*), as well as *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Enterococcus faecium*, *Enterococcus faecalis*, *Enterococcus durans*, and *Leuconostoc* spp. (Sheehan 2013; Hickey et al. 2015).

NSLAB survive the heating treatments, and do not contribute to the acidification during the early stages of the cheese manufacturing process, but play an important role during ripening (Kołakowski et al. 2012). They survive the pasteurization even at low cell number and stay at low concentration in curd. These bacteria are commonly found at concentrations below 10^2 cfu/g in pasteurized milk, due to the metabolic antagonism of starter lactic acid bacteria (SLAB) that prevent or reduce their proliferation (Broadbent et al. 2013, Bezekova 2013).

Generally, starter and nonstarter populations exhibit opposite growth kinetics. SLAB populations dominate during early cheese manufacture (10^8–10^9 cfu/g cheese), but decrease during the ripening process, in which dead starter culture cells and debris are used as nutrients by nonstarter microflora (O'Sullivan et al. 2013; Bove et al. 2012). NSLAB thus begin to grow as secondary microflora (reaching 10^6–10^8 cfu/g cheese depending on the ripening time and temperature) and become the dominant microflora (Burns et al. 2012; Sgarbi et al. 2013; Castro et al. 2015).

4.3 Role of NSLAB in Cheese Ripening

The process of cheese making produces a stressful environment for microorganisms (heat-related, osmotic, oxidative, and acidic stresses) (Gatti et al. 2014). NSLAB are highly adapted to these hostile conditions of cheese, which at the endpoint is typically an anaerobic, salty (4–6 % salt in moisture), low moisture (32–39 %), and acidic (4.9–5.3) environment at temperatures between 5 °C and 13 °C. In addition, cheese is a rich source of carbohydrates and nitrogen, but with low bioavailability (Settanni and Moschetti 2010; Neviani et al. 2013). Upon these stress conditions, NSLAB modulate at genetic level several regulons related to different stress responses, adopting a physiological state characterized by a downregulation of nucleic acid and protein synthesis, accompanied by a simultaneous upregulation of protein degradation and amino acid synthesis. This adaptative response is efficient and varies among species and strains (Bove et al. 2012, Al-Naseri et al. 2013, Gobbetti et al. 2015).

NSLAB increase the expression of enzymes involved in nitrogen metabolism, especially catabolism of free amino acids, and utilize peptides and amino acids derived from the proteolytic activity of SLAB (Bove et al. 2011; Neviani et al. 2013). Deamination and decarboxylation are the principal pathways of catabolism of free amino acids. The oxidative deamination of glutamate generates α-ketoglutarate and NH_3, and the catabolism of α-ketoacids into aldehydes, ketones, alcohols, or acids improves the cellular redox state and/or the synthesis of ATP. Additionally, NH_3 protect the cells against the acid stress, increasing intra- and extracellular pH (Bove et al. 2012).

Unlike SLAB, certain NSLAB metabolize citrate, a carbon source commonly found in the cheese matrix, during cheese ripening. The pathway of citrate catabolism varies among lactobacilli; for example, *L. plantarum* and *L. rhamnosus* use citrate as the sole nutrient source, but usually NSLAB co-metabolize citrate and fermentable carbohydrates (Hassan et al. 2012). The concomitant utilization of hexoses, pentoses, and citrate produces an excess of pyruvate that is converted into acetoin, 2,3-butanediol, and diacetyl which protect the cells against low pH as well as contribute to cheese flavor, as has been shown in a few number of LAB (Laëtitia et al. 2014).

In contrast with the well-known role of SLAB in the cheese-making process, the effect of NSLAB is not completely understood. However, it is known that they increase the content of peptides, free amino acids, and free fatty acids in the cheese matrix that contributes to flavor intensity, increasing aroma and reducing bitterness and harshness, as well as accelerates cheese ripening (Hati et al. 2013; Tulini 2014). As a consequence of NSLAB use, raw milk cheese has more intense and typical flavor and a higher concentration of amino acids, in comparison with pasteurized milk cheese (Gobbetti et al. 2015), suggesting that NSLAB play an important role in production of a full-flavored cheese (Skelin et al. 2012).

Contribution of NSLAB in the development of good organoleptic properties of dairy products is mainly due to their proteolytic and lipolytic activities (Van Hoorde et al. 2010; Ricciardi et al. 2015). NSLAB degrade proteins and peptides using

endopeptidases and N-terminal aminopeptidases, dipeptidases, and tripeptidases with proline-specific exopeptidase activity (Yarlagadda 2014). The catabolism of the free amino acid generates volatile aroma compounds aldehydes, indoles, alcohols, amines, sulfur and phenolic-containing moieties, and carboxylic acids that bring the characteristic taste of cheese (Bove et al. 2012). The production of CO_2 by NSLAB is responsible for cheese eye formation, but in a few cases, this CO_2 splits and cracks the cheese matrix as in Cheddar Dutch- and Swiss-type cheeses. These defects can also be attributed to obligate and facultative heterofermentative lactobacilli overgrowth and the production of CO_2 as end product of glucose metabolism (Milesi et al. 2010). As biotypes of NSLAB vary among dairy plants or within the same dairy plant, the final food products may have different organoleptic properties whichever is the processing plant (Solieri et al. 2012; Tan et al. 2012). However, a few faults in cheese making are usually attributed to NSLAB (post-acidification, gas formation (not desired), production of off-flavors, and lactate racemization) (Burns et al. 2012; Ristagno et al. 2012). Certain NSLAB produce racemization of L(+)-lactate to D(-)-lactate. Although both isomers can precipitate with calcium ions, leading to the formation of calcium lactate crystals, Ca-D-lactate is less soluble than Ca-L-lactate. This defect may be detected by the presence of white specks (calcium lactate crystals) in the cheese (Hassan et al. 2012).

In order to prevent quality inconsistencies and defects in cheese, NSLAB have been selected and used as adjunct cultures (Burns et al. 2012). Their incorporation in the cheese-making process accelerates ripening, but also enhances proteolysis (increasing the concentration of free amino acids and volatile compounds) contributing to cheese safety and preventing spoilage (Ciprovica and Mikelsone 2011; Irmler et al. 2013).

Nonstarter lactobacilli isolated from raw milk cheese with good qualities have been used as adjunct culture to accelerate cheese ripening in soft and semihard cheese like Manchego, Roncal, Cheddar, and Gouda (Jokovic et al. 2011; Singh and Singh 2014). These adjunct cultures consist mainly of mixed species and biotypes of *L. casei*, *L. paracasei*, *L. rhamnosus*, *L. plantarum*, and *L. curvatus* (Barouei et al. 2011; Gobbetti et al. 2015). These microorganisms are described in the following subsection.

4.3.1 *Lactobacillus casei* Group

For a long time, the taxonomy of bacteria belonging to the *Lactobacillus casei* group was debated mainly due to the difficulty of differentiating *L. casei* and *L. paracasei* strains, even by molecular techniques. Currently, the members of this group are as follows: *L. casei* (type strain, ATCC 393™), *L. paracasei* subsp. *tolerans* (type strain, ATCC 25599™), *L. paracasei* subsp. *paracasei* (type strain, ATCC 2530 ™), and *L. rhamnosus* (type strain, ATCC 25599 ™). These bacteria may be found in milk, dairy products, fermented sausages, wine, vegetables, occasionally sourdough, raw meat, cured meat products, silage, sewage, human reproductive and gastrointestinal tract, and stools (Felis et al. 2009; Gobbetti and Minervini 2014).

Some strains are commonly used as adjunct cultures for intensification and acceleration of flavor development in dairy products. During cheese ripening, strains of the *L. casei* group metabolize a variety of amino acids, such as methionine, leucine, and aspartic acid, producing compounds that improve cheese flavor. In addition, they are used as probiotic that promote human and animal health (Felis et al. 2009; Gobbetti and Minervini 2014; Solieri et al. 2015).

Lactobacillus rhamnosus (formerly *L. casei*) is an obligate heterofermentative microorganism that uses rhamnose as carbon source. *L. rhamnosus* is commonly used during the production of yogurt and other dairy products, such as fermented and unpasteurized milk, and semihard cheese, being *L. rhamnosus* GG (isolated from human feces) the most used commercial probiotic strain.

Lactobacillus paracasei comprises two subspecies: *Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus paracasei* subsp. *tolerans*; both species are commonly found in dairy products (Hammes and Hertel 2009; König and Fröhlich 2009). *L. paracasei* subsp. *paracasei* is used as adjunct cultures for the production of cheese varieties such as Cheddar, Edam, and feta, but also in Danbo cheese (Barouei et al. 2011). *L. paracasei* strains also show antimicrobial activity against *C. sporogenes*, *C. tyrobutyricum*, *C. beijerinckii*, and *C. butyricum*. Antimicrobial cultures of *L. paracasei* are expected to produce antimicrobial substances at the beginning of ripening, but also to inhibit the growth of *Clostridium* in cheese during ripening (Christiansen et al. 2005; Tuma et al. 2008) (Fig. 4.3).

4.3.2 *Lactobacillus plantarum* Group

This group comprises four species: *L. plantarum*, *L. paraplantarum*, *L. pentosus*, and *L. fabifermentans. L. plantarum* is a facultative heterofermentative microorganism, representative of this group, and comprises two subspecies: *L. plantarum* subsp. *plantarum* and *L. plantarum* subsp. *argentoratensis* strains are encountered in a variety of environmental niches, as dairy, meat, and bakery products, fish, vegetables, as well as intestinal tract of animals and humans (Todorov and Franco 2010).

L. plantarum strains have been isolated from fermented milks, such as kumis and kefir, and from different varieties of cheese such as Cheddar, Pecorino Romano, Belgian soft cheese, Stilton, Gouda, Greek cheese, Kopanisti, feta, mozzarella cheese, ovine cheeses, Alberquilla cheese, Manchego cheese, Danbo cheese, and Moroccan soft white cheese (Jben) (Todorov and Franco 2010). They usually dominate in the latter stage of fermentation of sauerkraut, pickles, olives, sourdough, and kimchi, presumably due to the low pH they endure, and improve the organoleptic properties of the final product (Siezen and van Hylckama 2011). They are used alone or in combination with pediococci (and/or micrococci) as starter to process fresh and fermented meat, inhibiting the growth of pathogenic and spoilage bacteria. *L. plantarum* strains produce bacteriocins with antimicrobial activity against several microorganisms such as LAB, *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium sporogenes*, and some members of *Enterobacteriaceae* family (Ortolani et al. 2010).

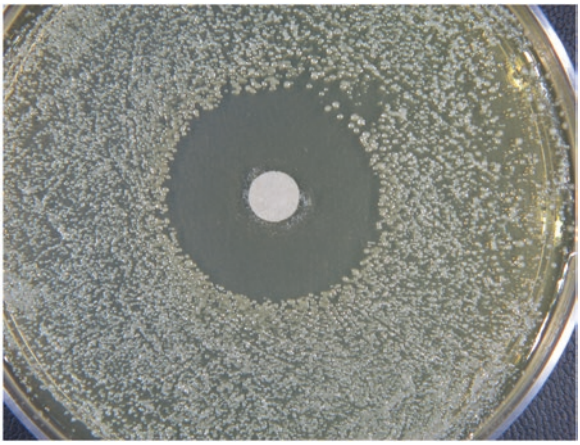

Fig. 4.3 Anticlostridial activity of bacteriocin-like inhibitory substance produced by a NSLAB strain

Lactobacillus pentosus belongs to the *L. plantarum* group. In Europe, this bacterium is commonly used as starter culture in sausage manufacture. Rodríguez-Gómez et al. (2014) reported the ability of *L. pentosus* TOMC-LAB2 (isolated from olive processing) as a potential probiotic strain that survives under packing conditions at room temperature for long periods. In the fermentation of three kinds of oyster mushrooms (*Pleurotus sajor-caju*, *P. cornucopiae*, and *P. ostreatus*), *L. pentosus* was able to control the growth of pathogenic and spoilage microorganisms and improves the organoleptic characteristics of the final products (Liu et al. 2016). A few bacteriocin-producing strains of *L. pentosus* have been reported, exhibiting inhibitory activity against *Clostridium* spp., *L. innocua*, and *Lactobacillus* spp. (Todorov and Dicks 2007; Dover et al. 2008, Muhialdin et al. 2012). Although *L. pentosus* strains can inhibit the development of spoilage bacteria, their use may also have detrimental effects on starter cultures. Thus, the use of these bacteria has to be analyzed in each case.

4.3.3 *Lactobacillus curvatus*

Lactobacillus curvatus is a facultative heterofermentative lactobacilli with lactate racemase and proteolytic activities that has been isolated from cow dung, milk, silage, grape must, wine, pressed yeast, sauerkraut, sourdough, prepacked finished dough, and meat products (Hammes and Hertel 2009; König and Fröhlich 2009). *L. curvatus* along with *L. sakei* are the typical dominant populations in raw fermented sausage and pasteurized emulsified meat products, mainly due to their high tolerance to salts (Olaoye and Ntuen 2011). This species and *L. sakei*, *L. plantarum*, *L. pentosus*, *L. casei*, *Pediococcus pentosaceus* and *Pediococcus acidilactici*, *Staphylococcus xylosus*, and *Staphylococcus carnosus* are the most common meat starter cultures. In fermented meat products, LAB produce a fast reduction of pH that enhance the firmness and prevent the growth of spoilage and pathogenic microorganisms.

In addition, their proteolytic and lipolytic activities produce intense color and aroma (Marty et al. 2012). *L. curvatus* is also used as biopreservative culture in fresh and processed meat, fish, and ham. Many antifungal and bacteriocin-producing strains have been reported (Ahmadova et al. 2013; Beshkova and Frengova 2012). It is noteworthy that bacteriocins have low activity in meat products (rather than in in vitro studies) because these molecules bind to the fat matrix and/or other food components, masking their antimicrobial activity against the target bacteria (Olaoye and Ntuen 2011; Campos et al. 2013).

L. curvatus have been found as adjunct culture in Cheddar and semihard cheese (Mannu et al. 2000), but their presence is not entirely desirable due to their lactate racemase activity. Cheddar cheese manufactured with *L. curvatus* rapidly developed a mixture of D-/L-lactate during ripening, and as the temperature rises up during ripening, the rate of D-lactate racemization increases. Unfortunately, when the cheese is stored at a low temperature, calcium lactate crystallizes producing white specks (Chou et al. 2003). On the other hand, some *L. curvatus* strains, isolated from cheese, have a good potential as preservative due to its antifungal and strong anti-*Listeria* activity (Ahmadova et al. 2013).

4.4 *Lactobacillus* as Biopreservation Resource

The current demand for safe, natural, and minimally processed foods, with a fresh taste and appearance, is worldwide rising. Among alternatives, the combination of different preservation agents and processes to reduce or to inhibit microbial growth is in vogue and includes changes in temperature (heating or cooling), water activity, pH, and redox potential, but also includes the use of chemical preservatives, vacuum packaging, modified atmosphere, and competitive microbiota, such as LAB producing antimicrobial compounds (Ananou et al. 2007).

So, LAB are "biopreservative" or inhibitory agents against other microorganisms because they may compete for nutrients, but also produce antagonistic compounds such as organic acids (lactic, acetic, formic, propionic and butyric acids), ethanol, fatty acids, hydrogen peroxide, diacetyl, bacteriocins, and non-bacteriocin antibacterial compounds.

4.4.1 Organic Acids

End products of both homo- and hetero-LAB fermentation include organic acids such as lactic, acetic, and propionic acid that reduce environment pH and prevent the growth of many pathogens and spoilage bacteria. Organic acids are thought to function as antimicrobials by interfering with the maintenance of cell membrane integrity, inhibiting active transport, reducing intracellular pH, and affecting a variety of metabolic functions (Ross et al. 2002). They have a very broad mode of action and inhibit both Gram-positive and Gram-negative bacteria as well as yeast and molds (Ross et al. 2002).

Lactic acid [CH3-CH(OH)-COOH] production by LAB has a long history in food industry. It is present in many foods, both by the addition and as a product of "in situ" production by microbial fermentation. It is used as an acidulate, flavoring, buffering agent that inhibits bacterial spoilage in a wide variety of processed food such as candy, bakery products, soups, dairy products, beer, and jams, among others (Datta and Henry 2006). About 90 % of the literature regarding lactic acid production is focused on bacterial fermentation by *Lactobacillus*, *Streptococcus*, *Leuconostoc*, and *Enterococcus* species.

Acetic acid (CH3-COOH) and its salts inhibit growth and reduce the viability of bacteria, yeasts, and fungi. Generally, it has a bacteriostatic effect at 0.2 %, while the bactericidal effect is present only above 0.3 %, being more effective against Gram-negative bacteria (Ryssel et al. 2009). The production of acetic acid by heterofermentative lactobacilli contributes to the aroma and prevents spoilage of fermented foods (Messens and De Vuyst 2002).

Benzoic acid (C6H5-COOH) is the most commonly used preservative in food industry. Benzoic acid and sodium benzoate are used as antifungal agents. LAB are able to transform some acids naturally present in milk into benzoic acid, including *L. acidophilus*, *L. casei*, *S. thermophilus*, and *L. helveticus* (Garmiene et al. 2010).

Propionic acid (CH3-CH2-COOH) is used as a fungistatic agent in foods, but it is also effective against Gram-positive and Gram-negative bacteria in cheese, butter, bakery products, syrup, and apple sauce (Reis et al. 2012). Traditionally, propionic acid bacteria (*Propionibacterium* spp.) are known for their ability to convert lactate to propionate, but a few heterofermentative LAB can also produce propionic acid at traces.

4.4.2 Diacetyl and Acetaldehyde

LAB produce flavor compounds like diacetyl and acetaldehyde that could exert antimicrobial activity; however, these flavor compounds are produced at low concentration that probably do not achieve inhibition of microorganisms, limiting their use as food preservative (Patel and Shah 2014). Diacetyl (2,3-butanediol) is produced during citrate metabolism and is responsible for the aroma and flavor of butter and some other fermented milk products. Many LAB, including strains of *Leuconostoc*, *Lactococcus*, *Pediococcus*, and *Lactobacillus*, may produce diacetyl. Gram-negative bacteria, yeasts, and molds are more sensitive to diacetyl than Gram-positive bacteria.

4.4.3 Hydrogen Peroxide (H_2O_2)

In the presence of oxygen, LAB produce hydrogen peroxide by several mechanisms (Vuyst and Vandamme 1994). The antimicrobial effect of H_2O_2 is due to the oxidation of sulfhydryl groups causing denaturation of a number of enzymes and also the

peroxidation of membrane lipids that increases membrane permeability. Production of H_2O_2 by lactic acid bacteria can prevent the growth of psychrotrophic and pathogenic microorganisms at refrigeration temperatures (Ito et al. 2003).

4.5 Bacteriocins

Bacteriocins are ribosomal-synthesized peptides, classified as biologically active proteins or protein complexes with antimicrobial activity. Produced during the primary phase of growth, bacteriocins usually have low molecular weight and undergo posttranslational modification. Bacteriocins can be easily degraded by proteolytic enzymes especially by the proteases of the mammalian gastrointestinal tract, which makes them safe for human consumption. Genes related to bacteriocin biosynthesis are generally clustered and are encoded on plasmids, chromosome, and/or transposons (Klaenhammer 1993).

LAB bacteriocins form a heterogeneous chemical group of molecules with different molecular sizes, physical and chemical properties, stabilities, antimicrobial spectrum, and mode of action. *Lactococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Carnobacterium*, *Streptococcus*, *Enterococcus*, and *Bifidobacterium* are the most common bacteriocin-producing genus (Cavera et al. 2015, Vuyst and Vandamme 1994).

The classification of bacteriocins is continuously changing. As consensus, three classes of bacteriocins (I, II, and III) have been described based on their chemical structure, molecular size, and thermal stability (Klaenhammer 1993).

4.5.1 Class I: Lantibiotics

Class I, the lantibiotics, are small peptides (< 5 kDa) that contain polycyclic thioether amino acids as lanthionine or methyllanthionine, as well as the unsaturated amino acids dehydroalanine and 2-aminoisobutyric. The LAB lantibiotics were initially divided into two subclasses based on structural similarities: (1) subclass Ia includes relatively elongated, flexible, and positively charged peptides that generally disrupt the cytoplasmic membranes forming pores, being nisin and lacticin 3147 the major representatives of this group, and (2) subclass Ib are globular peptides with a rigid structure that interfere with the normal cellular enzymatic metabolism (Deegan et al. 2006).

4.5.2 Class II: Non-lantibiotics

Class II, the non-lantibiotic bacteriocins, are also small (<10 kDa) relatively heat-stable, non-lanthionine-containing peptides. A number of different subclasses of class II bacteriocins have been suggested (Diep and Nes 2002; Nes et al. 1996), but

their heterogeneous nature makes subclassification difficult. According to Drider et al. (2006), three subdivisions are proposed:

1. Subclass IIa – pediocin-like or *Listeria*-active bacteriocins, with a consensus N-terminal sequence Tyr-Gly-Asn-Gly-Val-Xaa-Cys. These bacteriocins are synthesized with a leader peptide which is removed by proteolytic processing. Pediocin PA-1 and sakacin A are representative class IIa bacteriocins (Patton and Don 2005).
2. Subclass IIb – are bacteriocins that require two peptides to work synergistically in order to have an antimicrobial activity. By itself these peptides have little or no activity. Lactacin F and lactococcin G are members of this group (Nissen-Meyer et al. 1992).
3. Subclass IIc – bacteriocins that have a covalent bond between C and N terminals, resulting in a cyclic structure (Kawai et al. 2004). Enterocin AS-48, circularin A, and reutericin 6 are representatives of this subclass.

4.5.3 Class III: Large Thermolabile Bacteriocins

Class III are large thermolabile bacteriocins (>30 kDa) that have complex activity and protein structure. They promote lysis of the cell wall of the target microorganism, probably due to their N-terminal endopeptidase activity, while the C-terminal portion is responsible for the recognition of the target cell. Helveticin I produced by *Lactobacillus helveticus* is the representative of this group.

LAB bacteriocins are inherently tolerant to high thermal stress (class I and II) and to a wide pH range. These antimicrobial peptides are also colorless, odorless, and tasteless that may not change the organoleptic characteristics of the food; thus they have a potential use as food preservant. LAB bacteriocins are naturally considered food grade. In fact, the US Food and Drug Administration (FDA) classified LAB and its by-products as generally recognized as safe (GRAS) (U.S. Food and Drug Administration 1988). These compounds may be used in three ways: (i) as purified or semipurified antimicrobial additives, (ii) as bacteriocin-based ingredients from fermented foods, and (iii) through bacteriocin-producing starter cultures.

Among these antimicrobial compounds, nisin is largely applied in the food industry. Nisin is a class Ia bacteriocin or lantibiotic peptide composed of 34 amino acid residues, with a molecular mass of 3.5 kDa (Hurst 1981). It is produced by *Lactococcus lactis* subsp. *lactis* isolated from milk and vegetable products and is effective against many Gram-positive microorganisms, including lactic acid bacteria, but also against foodborne pathogens like *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, and *Clostridium botulinum*. Nisin does not have activity against Gram-negative bacteria because of their outer membrane, but these bacteria may become susceptible to nisin after a heat shock or when this is coupled with the chelator EDTA (Patel and Shah 2014). Members of the class I (or lantibiotic) bacteriocins, such as nisin, have been shown to have a dual mode of action. They can bind to lipid II, the main transporter of peptidoglycan subunits

from the cytoplasm to the cell wall, preventing correct cell wall synthesis and leading to cell death. Furthermore, nisin can also form a docking molecule with lipid II and to initiate a process of membrane insertion and pore formation that leads to a rapid cell death. Nisin presents two variants (A and Z), which differ by a single amino acid substitution (histidine at position 27 in nisin A and asparagine in nisin Z). This modification has no effect on the antimicrobial activity, but it gives nisin Z higher solubility and diffusion characteristics rather than nisin A (a relevant characteristics for food applications) (Arauz et al. 2009; Laridi et al. 2003). The use of nisin as a biopreservative has been widely investigated, and it has been admitted into the European food additive list (number E234; EEC 1983). Currently, this is the bacteriocin that has been approved by the World Health Organization for use as a food preservative, and it is commercialized as a dried concentrated powder.

4.6 Most Important *Lactobacillus* spp. Bacteriocins

Bacteriocins isolated from *Lactobacillus* species usually have a broad antimicrobial spectrum and can inhibit common pathogenic bacteria that are responsible for food spoilage or human diseases (Table 4.1).

4.6.1 Sakacin

Sakacin is a bacteriocin produced by food-associated species of *L. sakei* (Vaughan et al. 2003). *L. sakei* is a potential biopreservative LAB that is naturally found in meats, and it has been used in the production of fermented meat products (D'Angelis et al. 2008). Sakacin A, produced by *L. sakei* Lb706, belongs to class IIa bacteriocins and has been characterized as an active agent against *Listeria* species.

4.6.2 Plantaricins

Plantaricins are *L. plantarum* bacteriocins. *L. plantarum* strains produce at least 6 peptides with synergistic antibacterial effect, classified as class IIb bacteriocins. Plantaricins inhibit the growth of a broad number of LAB including their natural competitor *L. plantarum* and other bacteria like *Pediococcus*, *Carnobacteria*, *Clostridium*, and *Propionibacterium* (Deegan et al. 2006).

4.6.3 Helveticin

Helveticin J is a class III large (over 10 kDa) bacteriocin produced by *L. helveticus* 481 that displays a narrow range of activity and exhibits bactericidal activity against *L. helveticus*, *L. delbrueckii* subsp. *bulgaricus*, and *L. lactis* (Joerger and Klaenhammer 1986). This characteristic and its heat instability are the main

Table 4.1 *Lactobacillus* bacteriocin-producing species reported (up to April 2016)

Lactobacillus producer species	Numbers of bacteriocins reported in each class						Example
	I	II a	II b	II c	III	ND*	
L. acidophilus	–	1	1	1	2	3	Lactacin B (Barefoot and Klaenhammer 1983)
L. amylovorus	–	–	–	–	–	2	Amylovorin L471 (De Vuyst et al. 1996)
L. bavaricus	–	1	–	–	–	–	Bavaricin A (Larsen and Nørrung 1993)
L. brevis	–	–	–	–	–	3	Brevicin 37 (Rammelsberg et al. 1990)
L. casei	–	–	–	–	1	1	Caseicin 80 (Rammelsberg et al. 1990)
L. coryniformis	–	–	–	–	–	1	Lactocin MXJ32 (Lü et al. 2014)
L. crispatus	–	–	–	–	–	1	Crispacin A (Tahara and Kanatani 1997)
L. curvatus	–	1	–	–	–	2	Curvacin A (Tichaczek et al. 1992)
L. delbrueckii	–	–	–	–	2	–	Lacticin A (Toba et al. 1991)
L. fermentum	–	–	–	–	–	1	Bacteriocin 466 (De Klerk and Coetzee 1961)
L. gasseri	–	–	2	1	–	1	Gassericin A (Kawai et al. 1998)
L. helveticus	–	–	–	–	2	1	Helveticin J (Joerger and Klaenhammer 1986)
L. johnsonii	–	–	1	–	–	–	Lactacin F (Allison et al. 1994)
L. paracasei	–	–	–	–	–	4	Paracin 1.7 (Ge et al. 2016)
L. pentosus	–	–	–	–	–	1	Pentocin TV35b (Okkers et al. 1999)
L. paraplantarum	–	–	1	–	–	–	Paraplantaricin C7 (Lee et al. 2007)
L. plantarum	1	1	4	–	–	1	Plantaricin EF (Anderssen et al. 1998)
L. reuteri	–	–	–	1	–	–	Reutericin 6 (Kabuki et al. 1997)
L. sakei	1	2	1	–	–	7	Sakacin T (Vaughan et al. 2003)
L. salivarius	–	1	–	–	–	–	Bacteriocin L-1077 (Svetoch et al. 2011)

**ND* Not determined*

impediment to use this bacteriocin as a food preservative. However, helveticin J certainly contributes to the ability of *L. helveticus* 481 to compete efficiently during processing of fermented dairy products (Vuyst and Vandamme 1994).

4.7 *Lactobacillus reuteri* Antimicrobial Compounds

L. reuteri is a heterofermentative LAB, dominant microflora in cereal fermentations, that occurs as an adjunct culture in long ripened cheese and is a common inhabitant of the gastrointestinal tract of humans and animals (Leser et al. 2002; Tungjaroenchai et al. 2001; Walter et al. 2003). The ability of *L. reuteri* strains to produce powerful antibacterial compounds is unique among LAB. *L. reuteri* isolates produce: bacteriocin reutericin 6 (Kabuki et al. 1997), reutericyclin (tetramic acid)

(Gänzle et al. 2000), and reuterin (3-hydroxy-propionic aldehyde, 3HPA) (Talarico and Dobrogosz 1989). However, the production of two or more of these antimicrobial compounds by a single *L. reuteri* strain has so far not been described.

4.7.1 Reutericin

Reutericin 6 is produced by *L. reuteri* LA6, a microorganism isolated from the feces of a human infant. It is a high-molecular-weight, cyclic, class IIc bacteriocin that exhibits both bactericidal and bacteriolytic activity against other LAB species (Kabuki et al. 1997).

4.7.2 Reutericyclin

Reutericyclin is an inhibitory compound produced by sourdough isolates of *L. reuteri* that is structurally related to naturally occurring tetramic acids ($C_{20}H_{31}NO_4$). It shares chemical properties with bacteriocins from LAB, although its chemical structure is different. It is an amphiphilic molecule that is used to form aggregates in aqueous solution. Generally, Gram-positive bacteria are sensitive to reutericyclin, as well as the pathogenic bacteria *S. aureus*, *L. innocua*, *Streptococcus* sp., and *C. difficile* (Hurdle et al. 2011). Gram-negative enteric bacteria are resistant to reutericyclin; yeasts and fungi are not inhibited, indicating intrinsic resistance (Gänzle 2004). A few studies suggest that the cytoplasmic membrane is the cellular target of this compound (Gänzle and Vogel 2003). The structural similarity of reutericyclin and several strong cytotoxic tetramic acids raises the question of its toxicity to humans and animals, an answer that still remains.

4.7.3 Reuterin

Reuterin (3-hydroxypropionaldehyde) is an antimicrobial compound produced by *L. reuteri* as an intermediate in the conversion of glycerol to 1,3-propanediol (Lüthi-Peng et al. 2002). Reuterin is active against a wide range of Gram-positive and Gram-negative bacteria, including spore formers (*Bacillus*, *Clostridium*), LAB (*Enterococcus*, *Lactobacillus*, *Streptococcus*, *Pediococcus*), as well as several foodborne pathogens (*Listeria*, *Staphylococcus*, *Escherichia*, *Salmonella*, *Shigella*, and *Campylobacter*) (Arqués et al. 2004; Bian et al. 2011; Spinler et al. 2008). In addition, reuterin is a water-soluble molecule, active at a wide range of pH, which resists proteolytic and lipolytic cleavage. It has been reported that the addition of reuterin to milk and dairy products (Arqués et al. 2004, 2011) and the in situ production of reuterin by *L. reuteri* strains in dairy products (Langa et al. 2013) prevent the growth of pathogenic microorganisms.

4.8 The Use of Bacteriocins in the Dairy Industry

Bacteriocins can be used to control spoilage and microbial degradation of dairy products by either addition of purified or crude extracts or in situ production by the introduction of a bacteriocin-producing strain (starter culture). In addition, bacteriocins could be combined with other antimicrobial compounds such as sodium acetate and sodium lactate, resulting in enhanced inactivation of pathogens or spoilage bacteria. Bacteriocins can also be used to improve food quality and sensory properties, for example, increasing the rate of proteolysis or in the prevention of gas blowing defect in cheese caused by *Clostridium* spp. Another industrial use of bacteriocins is during the bioactive packaging, a process that can protect the food from external contaminants. Bioactive packaging can be prepared by immobilization of the bacteriocin in the food packaging, or by addition of a sachet containing the bacteriocin into the packaged food, which will be released during storage. The gradual release of bacteriocins from a packaging film on the food surface may have an advantage over dipping and/or spraying bacteriocins on foods. The absorption of bacteriocin into the food may reduce the antimicrobial activity (Lopez-Rubio et al. 2006). A new strategy to maintain the antimicrobial properties is the use of an immobilized bacteriocin packaging. As an example, polyethylene film containing immobilized bacteriocin 32Y from *L. curvatus* was able to reduce viable counts of *L. monocytogenes* in packaged pork steak and ground beef during storage (Mauriello et al. 2004).

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Antarctic Psychrophilic Microorganisms and Biotechnology: History, Current Trends, Applications, and Challenges

5

Luis Andrés Yarzábal

Abstract

The potential use of psychrophilic (i.e., cold-loving) microorganisms as biotechnological tools remained a promise for decades. This vision changed in the early 1990s when scientists began to seriously guess on their usefulness for the industry and the medical field, among many others. Nowadays, most of these expectations have been confirmed, and psychrophiles are considered as sustainable and invaluable resources for the development of diverse biotechnological processes and/or products, many of which have already been patented or are protected by industrial secrecy. Besides, some new and unexpected applications of psychrophiles are starting to emerge as scientists discover new species in the most extreme environments. Antarctica is such an extraordinary place to prospect for this kind of microorganisms: it is the coldest and driest continent on Earth, with different climatic regions and many different ecosystems (both terrestrial and aquatic). Antarctic microbes have been investigated for more than a century. Currently, more than two hundreds patents have been filed related to actual or potential commercial biotechnological applications based on Antarctic genetic resources; many of them concern microorganisms and their metabolites. This chapter highlights recent reports dealing with products and/or processes derived from the study of Antarctic psychrophilic or psychrotolerant microorganisms, with emphasis on their applications in the fields of medicine, nanotechnology,

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energy production, and agriculture. It also focuses on the specific problems of bioprospecting in Antarctic pristine environments, under the light of past and present experiences

5.1 Introduction

Earth is a planet of extremes. Arid deserts, subsurface environments, glaciers, hot springs, shallow submarine hydrothermal systems, alkaline or acidic soils, soda lakes, and deep-sea sediments, among many others, are all natural environments which exhibit one or more extreme conditions including high temperatures, pressures, and salt concentrations or low pH, nutrient concentration, or water availability. Common live beings – those we consider "normal" because they inhabit or colonize familiar environments – are actually the exception rather than the rule. In fact, the great majority of organisms, the unseen majority mainly composed of microorganisms, thrive under the effect of at least one extreme condition. Not surprisingly these microorganisms are known as "extremophiles."

Strikingly, the dominant environment of the biosphere is cold: the *cryosphere* currently comprises more than 33 million km^3 of frozen water (Williams and Ferrigno 2012), including the terrestrial ice sheets of Antarctica and Greenland, mountain glaciers, polar marine ice shelves, and sea ices. But the cryosphere also includes the mesosphere and stratosphere and, to a lesser extent, man-made habitats such as refrigerators and freezers (Boetius et al. 2015). Adding to this list, more than 90 % of the oceans' volume is 5 °C or colder. Therefore, it can be concluded without doubt that Earth is – actually – a cold planet.

Cold temperatures impose one of the most serious threats and constraints to life. Indeed, cell integrity, water viscosity, solute diffusion rates, membrane fluidity, enzyme kinetics, and macromolecular interactions are all negatively influenced by low temperatures (D'Amico et al. 2006; Rodrígues and Tiedje 2008). That is why some extremophiles have evolved a number of strategies to endure – and even thrive – in the cold. *Psychrophiles* (i.e., cold-loving organisms) and *psychrotolerants* (i.e., cold-tolerant organisms) are the most abundant living beings in terms of biomass, diversity, and distribution (Margesin and Feller 2010).

If there is a place where psychrophiles proliferate, this place is Antarctica: the coldest and driest continent on Earth. Most of Antarctica's territory is perennially covered by glaciers (the Antarctic ice sheet); however, some regions – like the low latitude peninsula and the high latitude dry valleys which represent almost 0.35 % of the continent – contribute ice-free zones for part or all of the year (Hopkins et al. 2006). In any case, the Antarctic climatic conditions are harsh: besides low temperatures throughout the year (sometimes as low as −95 °C), the continent's ecosystems are characterized by low water availability, frequent freeze–thaw cycles, high UV radiation, strong winds, and high sublimation and evaporation rates, among others. Therefore, it is no surprise that the development of life, particularly of higher forms (i.e., plants and animals), is severely limited in these latitudes. On the contrary,

Antarctic microbes are much better adapted to survive – and to multiply – under these extreme conditions.

The study of Antarctica's microbial biodiversity and its biotechnological applications is a tale of great scientists that share an amazing history of exploration of the unknown and great discoveries. In the following pages, I will explore some aspects related to the history of psychrophiles and microbiology research in Antarctica, the microbial biodiversity of the continent, and some important biotechnological applications of Antarctic psychrophilic microorganisms, with an emphasis on recent discoveries and developments. I will also focus on the specific problems of bioprospecting in Antarctic pristine environments, under the light of past and present experiences.

5.2 Psychrophiles and Psychrophilic Adaptations to Low Temperatures

The first report concerning psychrophilic microorganisms was published almost 130 years ago, in 1887, by a German scientist named J. Forster. But it was not until 1902 that the term psychrophile (from the Greek words *psychros*, cold, and *philos*, loving) was coined by Schmidt-Nielsen to refer to bacteria with the ability to grow actively (or *rapidly*) at 0 °C. Since their discovery, most of the initial studies on this type of microorganisms were related to food spoilage, and for many decades a large proportion of the literature was concerned almost exclusively with food microbiology.

However, during the 1960s, true psychrophiles (and not psychrotolerants) were isolated from natural environments (particularly Antarctica), and some fundamental aspects of their mode of life at low temperatures began to be studied. This scientific effort was well echoed on several reviews dealing with the physiology and the ecology of psychrophiles (Stokes 1963; Rose 1968; Inniss 1975; Morita 1975; Baross and Morita 1978; Inniss and Ingraham 1978; Gounot 1986; 1991), the molecular bases of psychrophily (Herbert 1986; Russell 1990), and the biotechnological potential of psychrophiles (Sharp and Munster 1986; Gounot 1991).

Without entering the debate concerning the very definition of the word psychrophile (which is well beyond the aim of this chapter), there is no doubt on the serious threats cold temperatures impose to live beings. For instance, cellular membranes become more rigid as temperature decreases, thus limiting their ability for up-taking nutrients and releasing by-products of cellular metabolism; additionally, cell integrity and functioning might also be severely compromised due to the formation of water crystals which could damage cellular membranes. Moreover, the diffusion of solutes is also markedly limited by an increase in water viscosity (D'Amico et al. 2006; Rodrígues and Tiedje 2008).

To avoid the deleterious effect of freezing conditions, several adaptations evolved and are shared by almost all psychrophiles. These include (1) the synthesis of increased amounts of unsaturated fatty acids to maintain membrane fluidity (Russell 1997); (2) the production of antifreeze proteins which bind to ice crystals, creating

thermal hysteresis and inhibiting their growth and recrystallization (Jia and Davies 2002); (3) the production of cryoprotectants – like trehalose – to increase the cytoplasmic viscosity, inhibit the formation of intracellular ice crystals, and prevent protein inactivation and aggregation (Elbein et al. 2003); and (4) the production of carbohydrate-based extracellular polymeric substances (EPS), which creates a thick layer of cryoprotection outside the cells, retain water, and protect some exoenzymes from cold denaturation (Mancuso Nichols et al. 2005).

Nonetheless, by far the most important constraint psychrophiles must face is the strong inhibition of enzyme-catalyzed reactions caused by low temperatures. According to the Arrhenius law, the activity of any enzyme is exponentially dependent on temperature. Accordingly, for every 10 °C decrease, the rate of the reaction drops by a factor of 2–3. In the case of mesophilic enzymes (enzymes produced by mesophilic organisms), shifting the temperature from 37 °C to 0 °C results in 20–80 times lower activity (Feller 2013). To compensate for this significant decrease, psychrophilic enzymes (enzymes produced by psychrophilic organisms) have up to tenfold higher specific activities at cold temperatures as compared with their mesophilic counterparts (Bakermans 2012). How this is achieved is currently unknown. However, the most accepted hypothesis is related to an increased flexibility of psychrophilic enzymes – either affecting the entire polypeptide or some particular regions involved in catalysis – which compensates the strong slowdown of molecular motions at low temperatures (D'Amico et al. 2006). This form of adaptation explains the low stability and thermolability of psychrophilic enzymes, which are easily denatured by exposure to higher temperatures (Feller 2013).

5.3 Antarctic Microbiology

Initial studies on Antarctic microbiology, particularly marine bacteria, were the result of expeditions conducted between 1901 and 1907: the French Antarctic Expedition (also known as Charcot's Expedition, 1904–1907), the Swedish Antarctic Expedition (1901–1904), and the Scottish National Antarctic Expedition (1902–1904) (Tsiklinsky 1908; Ekelöf 1908; Gazert 1912). However, it is generally assumed that the study of Mc Lean's (1918) on bacteria isolated from snow and frozen algae collected near the South Pole, by the Australian Antarctic Expedition of 1911–1914, represents the pioneer work on Antarctic microbiology.

As time passed the initial interest on Antarctic microbes vanished rapidly and remained low for more than two decades, until Darling and Siple (1941) isolated bacteria from Antarctic ice, snow, water, plant debris, and soil samples. This report was followed by many others concerning bacteria in Antarctic marine and terrestrial environments (reviewed by Friedmann 1993).

For many years, though, the prevailing paradigm was that the microorganisms isolated from Antarctic environments were exogenous contaminants, transported by winds from other regions of the planet (Horowitz et al. 1972). Furthermore, it was also believed that some regions of the continent – particularly the ice-free cold deserts – were almost sterile. This idea was challenged by Vishniac and Mainzer (1972)

who proposed the existence of indigenous microorganisms in Antarctica. Seven years later, the isolation of previously unknown psychrophilic yeasts from Dry Valley soils (Vishniac and Hempfling 1979) confirmed this assumption, which was further reinforced by the discovery of a new actinomycete in ice cores collected in central Antarctica (Abyzov et al. 1983). The final point to this debate arose when it was finally demonstrated that endolithic autotrophs were the primary producers in the dry deserts of Antarctica, able to sustain other life forms (Friedmann and Ocampo 1976).

5.3.1 Microbial Biodiversity in Antarctica

Besides glacial and soil environments, the Antarctic continent and the surrounding Southern Ocean also include other habitats like marine waters, coasts, lakes (both surface and subglacial with much variation in geochemistry), anoxic brines, lake sediments, deep-sea sediments, and deep-sea thermal vents, among others. Therefore, it is no surprise that a diverse range of microbial physiologies and phylotypes evolved in different regions throughout the continent, thus contributing to its biological diversity. The increasing interest in studying and depicting this biodiversity – well reflected on the numerous reports published during the past decade on this matter – was reinforced by the realization that the very understanding of the biological diversity on Earth requires exploration of polar regions (Convey et al. 2014; Chown et al. 2015).

For decades, the use of traditional microbiological methods allowed the successful isolation, culture, and detailed characterization of many microbial strains. These studies, conducted during the second half of the past century mainly with samples collected from soil habitats, demonstrated the presence of a narrow range of bacterial families, dominated by the Gram-positive *Firmicutes* (Cowan 2014), a discovery which might reflect the selective pressure imposed by the harshness of the Antarctic environment. The prospection of other less familiar environments – like the rhizosphere of two vascular plants, the sediments of subglacial lakes, the subsurface glacial ice, or the saline waters of brines – significantly expanded this narrow view to other taxonomic groups. However, the microbial diversity remained restricted to the very small culturable fraction of microorganisms (Smith et al. 2006).

With the advent of modern molecular phylogenetic methods and next-generation sequencing technologies, the real diversity of Antarctic microbial communities and their true complexity began to emerge. Several independent studies conducted around the continent have shown that, in contrast to what happens with higher eukaryotes, the taxonomic and phylogenetic diversity of Antarctic microbes is not only substantial, but subjected to local and regional spatial patterns (Cowan 2014; Chong et al. 2015). This high degree of local or regional heterogeneity is particularly evident in soil microbial communities, whose diversity reflect their geographical isolation and seems to be driven by local microclimate and landscape history (Cowan et al. 2014; Chong et al. 2015).

As is typical of many soil habitats elsewhere, *Proteobacteria* and *Actinobacteria* appear to be the most prominent groups on Antarctic environments (Babalola et al. 2009; Makhalanyane et al. 2013). On the other hand, at the specific level, it seems that there has been a strong selection toward a specific subgroup of microorganisms, highly adapted to the prevailing conditions (Niederberger et al. 2008). In addition, even though the structures of these communities – at least at the phylum or class levels – are similar to soils elsewhere and relatively consistent, their diversity is lower than in many temperate soil systems (Chong et al. 2015). Aquatic systems exhibit similar characteristics with relatively lower levels of diversity as compared to their temperate counterparts (Wilkins et al. 2013).

By means of a high-throughput ribosomal gene sequencing strategy, Teixeira et al. (2010, 2013) showed that the bacterial diversities of the Antarctic vascular plants *Deschampsia antarctica* and *Colobanthus quitensis* rhizospheres are higher than expected. Furthermore, their results revealed similar patterns of bacterial diversities between plant specimens collected from different locations, with *Firmicutes* and *Actinobacteria* as the most abundant groups. They also highlighted the strong influence exerted by vascular plants and birds in shaping the microbial communities of Antarctic soils. Similar results were obtained by Kim et al. (2012) while comparing the abundance and diversity of microbial communities colonizing mineral soils and ornithogenic soils on King George Island. Both angiosperms were previously shown to be natural reservoirs of endophytic fungal communities whose species are well adapted to the extreme conditions in Antarctica (Rosa et al. 2009, 2010).

In the Antarctic marine context, sponges are an important component of benthic communities, serving as accumulation spots for particular microbial communities which include members from the three domains of life. In a recently published work, massive sequencing of ribosomal RNA genes revealed that Antarctic sponge-associated communities are more diverse than the surrounding planktonic communities and significantly different to other tropical–temperate ecosystems, particularly in terms of their microbial eukaryote component (Rodríguez-Marconi et al. 2015).

Still, the majority of these Antarctic microbes have not yet been cultured and their ecological roles remain obscure. However, this has not hampered the growing interest in studying these exciting forms of life to understand the molecular and cellular bases of their striking adaptation to the extreme conditions they face. On the other hand, the potential use of this vast genetic resource for the development of biotechnological processes and/or products of high value is –perhaps – the strongest incentive for public and private institutions to continue with their research efforts.

5.4 Antarctic Microorganisms and Biotechnology

With the exception of a well-known study on the degradative ability of psychrophilic microorganisms carried out in 1974 by Westlake and co-workers, the potential use of psychrophilic microorganisms as biotechnological tools remained a promise for decades. In fact, they were better known for the damage they caused (e.g., refrigerated food spoilage). However, starting from the early 1990s, scientists

began to seriously guess on their usefulness for the food and mining industries or the genetic bioengineering field, among others (Gounot 1991). Today, most of these expectations have been confirmed, and some new and unexpected applications of psychrophilic microorganisms – many of which were isolated from Antarctic environments – became evident. Indeed, psychrophiles (and their derived metabolites) are nowadays considered sustainable and invaluable resources for the development of diverse biotechnological processes and/or products, many of which have already been patented or are protected by industrial secrecy (Margesin and Feller 2010; Feller and Margesin 2012; Feller 2013).

The main areas of research and development of new processes and/or products related with Antarctic psychrophilic microorganisms include (1) psychrophilic enzymes (for industrial processes, molecular biology, and medicine), (2) other psychrophilic biomolecules (for therapeutic use, cosmetic development, molecular biology, nutrition, and other uses), (3) whole psychrophilic microorganisms (for processes like nanoparticle synthesis, agriculture production, energy production, bioremediation, and wastewater treatment, among others), and (4) genetic elements (for instance, to develop cold-adapted expression systems and live vaccines).

Some of these issues have been already reviewed in a thorough way by different authors (see, for instance, Lohan and Johnston 2005; Leary 2008; Margesin and Feller 2010; Cavicchioli et al. 2011; Feller and Margesin 2012; Martínez-Rosales et al. 2012; Buzzini et al. 2012; Feller 2013; Buzzini and Margesin 2014). In the following sections, we will focus specifically on recent reports dealing with products and/or processes derived from the study of Antarctic psychrophilic or psychrotolerant microorganisms, with a particular emphasis on their applications in the fields of medicine, nanotechnology, energy production, and agriculture.

5.4.1 Medicine

5.4.1.1 Antimicrobials

Many free-living microorganisms are able to colonize a particular environment by killing, inhibiting, or detracting their competitors and/or predators. These antagonistic interactions rely, mainly, on the synthesis and excretion of secondary metabolites most of which are soluble and many volatile. By using either co-culture or dual culture assays, which usually include an unknown isolate and a well-known pathogen, antagonistic microorganisms with potential antagonistic abilities can be detected and further explored as novel sources for antimicrobials, some of which can even elicit unknown mechanisms of action (Mojib et al. 2010). In recent years, an increasing number of Antarctic microorganisms have been shown to exhibit such antimicrobial activities (Table 5.1).

This is the case, for example, of spore-forming and cold-adapted bacterial strains, able to inhibit growth of methicillin-resistant *Staphylococcus aureus* and *Candida albicans* (Vollú et al. 2014). Another example of this kind of studies is the use of crude extracts of different Antarctic microorganisms to test their potential antimicrobial activities. For instance, Goncalves and co-workers (2015) recently

Table 5.1 Antagonistic activities of Antarctic microorganisms and their metabolites against pathogenic microbes

Species	Phylum/division	Active compound	Source of isolation (locality)	Antagonistic to	References
Bacteria					
Bacillus aryabhattai	*Firmicutes*	Unidentified (overlay method)	King George Island	Methicillin-resistant *Staphylococcus aureus*	Vollú et al. (2014)
Nocardioides sp. A-1	*Actinobacteria*	Glycolipids and lipopeptides (antibiotics)	Casey Station, Budd Coast, Wilkes Land	*Bacillus subtilis*, *Staphylococcus aureus*, *Sarcina lutea*, *Candida tropicalis*, and *Xanthomonas oryzae*	Gesheva and Vasileva-Tonkova (2012)
Streptomyces spp.	*Actinobacteria*	Unidentified bioactive metabolites (diffusion agar plug method)	Livingston Island, Antarctica	*B. subtilis* and the phytopathogens *Clavibacter michiganensis*, *Xanthomonas euvesicatoria*, *Xanthomonas gardneri*, *Xanthomonas perforans*, and *Xanthomonas vesicatoria*	Encheva-Malinova et al. (2014)
Brevibacterium sp., *Gordonia* sp.	*Actinobacteria*	Unidentified bioactive metabolites (96-well plate cultures with supernatants)	Barrientos Island	*Candida albicans*, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, and/or *Pseudomonas aeruginosa*	Lee et al. (2012)
Janthinobacterium sp. Ant5-2	*Proteobacteria*	Violacein (purple violet pigment)	Proglacial Lake Podprudnoye, Schirmacher Oasis, East Antarctica	*Mycobacterium smegmatis* and *M. tuberculosis*	Mojib et al. (2010)

Flavobacterium sp. *Ant342*	*Bacteroidetes*	Flexirubin (yellow-orange pigment)	Land-locked freshwater lake L49	*Mycobacterium smegmatis* and *M. tuberculosis*	Mojib et al. (2010)
Janthinobacterium sp. Ant5-2	*Proteobacteria*	Purple violet pigment (colorimetric resazurin assay)	Proglacial Lake Podprudnoye, Schirmacher Oasis, East Antarctica	*S. aureus (multidrug-resistant strains)*	Huang et al. (2012)
Janthinobacterium sp.	*Proteobacteria*	Ethanolic extract	King George Island, Fildes Peninsula	Multiresistant strains of *Serratia marcescens*, *Escherichia coli*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*	Asencio et al. (2014)
Pseudoalteromonas spp.	*Proteobacteria*	Microbial volatile compounds	Ross Sea	Multiresistant *Burkholderia cepacia* complex isolates (cystic fibrosis pathogens)	Maida et al. (2015), Papaleo et al. (2012, 2013), and Romoli et al. (2011, 2014)
Fungi					
Geomyces spp.	Fungi/*Ascomycota*	Methanol crude extracts	Fildes Bay, King George Island	*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Xanthomonas campestris*, and *Clavibacter michiganensis*	Henríquez et al. (2014)
Pseudogymnoascus sp.	Fungi/*Ascomycota*	Ethanolic extracts, asterric acid nitro derivatives	Fildes Bay, King George Island	No biological activity detected	Figueroa et al. (2015)

(continued)

Table 5.1 (continued)

Species	Phylum/division	Active compound	Source of isolation (locality)	Antagonistic to	References
Penicillium sp.	Fungi/*Ascomycota*	Crude extracts	Elephant Island, King George Island, and Deception Island, Antarctic Peninsula	*Cladosporium sphaerospermum* and *Trypanosoma cruzi*	Godinho et al. (2013)
Guehomyces pullulans, *Metschnikowia australis*, *Dipodascus australiensis*, *Pseudogymnoascus* sp.	Fungi/*Basidiomycota* (Gp), *Ascomycota* (Ma, Da, P sp.)	Crude extracts	Elephant Island, King George Island, and Deception Island, Antarctic Peninsula	*Candida albicans*, *C. krusei*, and/or *Cladosporium sphaerosperum*	Furbino et al. (2014)
Penicillium steckii	Fungi/*Ascomycota*	Ethanolic extract	Elephant Island, King George Island, and Deception Island, Antarctic Peninsula	Yellow fever virus	Furbino et al. (2014)
Alternaria sp., *Antarctomyces* sp., *Cadophora* sp., *Davidiella* sp., *Helgardia* sp., *Herpotrichia* sp., *Microdochium* sp., *Oculimacula* sp., *Phaeosphaeria* sp.	Fungi/*Ascomycota* (Al, An, C, D, Hel, Her, M, O, P)	Ethanolic extracts	Admiralty Bay, King George Island, South Shetland Islands	*Leishmania amazonensis*	Santiago et al. (2012)
Penicillium spp., *Pseudogymnoascus* spp.	Fungi/*Ascomycota*	Ethanolic extracts	Admiralty Bay, King George Island, and Deception Island, Antarctic Peninsula	*Paracoccidioides brasiliensis*	Goncalves et al. (2015)

Purpureocillium lilacinum	Fungi/*Ascomycota*	Ethanolic extract		*Trypanosoma cruzi*, *Paracoccidioides brasiliensis*, and *S. aureus*	Goncalves et al. (2015)
Aspergillus sydowii, *Penicillium allii-sativi*, *Penicillium chrysogenum*, and *Penicillium rubens*	Fungi/*Ascomycota* (A, P)	Organic extracts	Ellsworth Mountains, Continental Antarctica	*E. coli*, *S. aureus*, *P. aeruginosa*, *C. albicans*, *C. krusei*, *C. sphaerosperum*, *T. cruzi*, *L. amazonensis* (antibacterial, antifungal, antitumoral, antiprotozoal, and herbicidal activities)	Godinho et al. (2015)
Penicillium brevicompactum	Fungi/*Ascomycota*	Organic extract	Ellsworth Mountains, Continental Antarctica	*Colletotrichum gloeosporioides*, *C. fragariae*, *C. acutatum* (phytopathogenic fungi)	Godinho et al. (2015)
Penicillium nalgiovense Laxa	Fungi/*Ascomycota*	Amphotericin B (methanol extracts)	Paulette Island	*Candida albicans*, *Escherichia coli*, and *Staphylococcus aureus*	Svahn et al. (2015)
Mrakia frigida 2E00797	Fungi/*Basidiomycota*	Cold-tolerant killer toxin (LMW proteins or glycoproteins)	Antarctic sea sediments (unidentified site)	*Metschnikowia bicuspidate*, *Candida tropicalis*, and *Candida albicans*	Hua et al. (2010) and Liu et al. (2012)
Lichens					
Stereocaulon alpinum	Fungi/*Ascomycota*	Lobaric acid, lobastin	Unspecified	*B. subtilis* and *S. aureus*	Bhattarai et al. (2013)

demonstrated the antimicrobial properties of ethanolic extracts of diverse Antarctic fungi. A similar result was obtained by Godinho and co-workers (2015) when evaluating organic extracts of fungi isolated from Antarctic oligotrophic soils.

Most of the microbial bioactive compounds discovered so far, including many currently used worldwide, are produced by actinomycetes, the most economically important and biotechnologically useful microorganisms (Baltz 2007). Bacteria belonging to this group are widespread in soils and produce diverse secondary metabolites of medical importance such as antibiotics, antifungal, antiprotozoal, antiviral, and antihelminthic compounds, among others.

For instance, a halophilic Antarctic actinomycete, *Nocardioides* sp. A-1, produces several antimicrobial/antifungal compounds – mainly glycolipids and lipopeptides – which inhibit Gram-positive bacteria (*Bacillus subtilis*, *S. aureus*, and *Sarcina lutea*), *Candida tropicalis* (a pathogenic yeast), and *Xanthomonas oryzae* (the etiologic agent of bacterial blight, a major disease of rice) (Gesheva and Vasileva-Tonkova 2012).

By using high-throughput screening methods, Lee et al. (2012) demonstrated that actinobacterial strains isolated from soil samples produce bioactive metabolites which inhibit growth of *C. albicans*, methicillin-resistant *S. aureus*, and/or *Pseudomonas aeruginosa*. Another group of actinomycetes, *Streptomyces* spp., were also shown to exhibit a wide range of antagonistic activities toward both Gram-positive and Gram-negative phytopathogens (Encheva-Malinova et al. 2014). The genomes of these *Streptomyces* sp. isolates harbored genes involved in the synthesis of aromatic polyketides, glycopeptides, and polyenes, all of which are well-known antibiotics.

However, considering the urgent need of new antibiotics for therapeutic use, and having in mind the scarcity of new products or antibiotic classes in the pharmaceutical industry (Spizek et al. 2010; Lo Giudice and Fani 2015), the new psychrophilic-derived compounds have to be properly identified, purified, and tested. In the context of Antarctic research, this has already started. Amphotericin B, for example, is an antifungal metabolite secreted by *Penicillium nalgiovense* Laxa, a fungi isolated from a soil sample collected at a penguin's nest (Svahn et al. 2015). This metabolite strongly inhibits *C. albicans* growth and, to a lesser degree, *Escherichia coli* and *S. aureus*.

Other unique antimicrobial metabolites are produced by Antarctic lichens, apparently as a strategy to survive under extreme conditions. In 2013 Bhattarai et al. reported the isolation of lobaric acid and lobastin, a structurally new pseudodepsidone-type metabolite, while prospecting a collection of Antarctic *Stereocaulon alpinum* lichens. These compounds were active against Gram-positive bacteria (*B. subtilis* and *S. aureus*).

A cold-tolerant killer toxin, produced and excreted by the psychrotolerant yeast *Mrakia frigida*, was shown to be active against *Metschnikowia bicuspidata* (a crab pathogenic yeast), *C. tropicalis*, and *C. albicans* (Hua et al. 2010). Although several fungal species have been shown to produce these kinds of killer toxins – i.e., low-molecular-mass protein or glycoproteins that kill sensitive cells of the same or related yeast genera – the purified *M. frigida* toxin has a much lower optimal

temperature (16 °C) than any other known enzymes produced by other psychrophilic Antarctic yeasts (Liu et al. 2012). Together with other unique characteristics, this discovery opens the gate to new potential applications and special uses of this toxin.

Pigmented bacteria have also been recognized as producers of antimicrobial compounds for decades (Duran et al. 2007; Soliev et al. 2011). For example, bacteria belonging to the *Janthinobacterium* genus, originally isolated from Antarctica in 1991 (Shivaji et al. 1991), synthesize a violet pigment named *violacein* which exhibits multiple therapeutic activities including antitumoral, antibacterial, antiulcerogenic, antileishmanial, and antiviral activities (Durán et al. 2007). Violacein, an indole type of compound derived from tryptophan metabolism, is intracellular and poorly soluble in water and biological fluids. In 2010 a violacein-like purple violet pigment (PVP), produced by an Antarctic *Janthinobacterium* sp. Ant5-2, was shown to inhibit *Mycobacterium smegmatis* and *M. tuberculosis* growth (Mojib et al. 2010). In 2012, the same pigment was shown to induce a steady decrease in viability of multiple drug-resistant and methicillin-resistant clinical strains of *S. aureus*, at very low doses (Huang et al. 2012). Similarly, an ethanolic extract of another soil-borne *Janthinobacterium* strain was shown to inhibit eight multiresistant clinical strains of Gram-negative bacilli (Asencio et al. 2014). This inhibitory activity was not related to the presence of violacein alone and was attributed to other compounds that might act synergistically with this pigment.

As can be seen, the great majority of studies concerning antimicrobial compounds have been conducted with microorganisms isolated from Antarctic soils. However, in recent years the antimicrobial activity of marine microorganisms also came to light confirming that, besides representing an unexplored reservoir of a vast biodiversity, they might also be an ideal source for isolating still undescribed bioactive molecules. These studies involved both free-living and symbiotic marine microorganisms.

For example, Antarctic *Pseudoalteromonas* strains isolated from seawater or sediments collected at the Ross Sea have been shown to completely inhibit growth of opportunistic cystic fibrosis pathogens of the *Burkholderia cepacia* complex (Maida et al. 2015; Papaleo et al. 2012, 2013). This activity was related mainly to the production of microbial volatile organic compounds (mVOCs) (Romoli et al. 2011, 2014), i.e., low-molecular-weight lipophilic compounds responsible for intra- and inter-microorganism interactions. Quite interestingly, it has been demonstrated that the same mVOCs are synthesized by Antarctic bacteria belonging to different taxonomic groups, which suggest that they should have an ecological significance (Lo Giudice and Fani 2015). In order to shed some light on this aspect, the genomes of several sponge bacteria have been sequenced (Fondi et al. 2012, 2014; Papaleo et al. 2013; Orlandini et al. 2014; Maida et al. 2014, 2015). The comparative analysis of these genomes did not allow the identification of the genes and/or (still unknown) metabolic pathways involved in the biosynthesis of the inhibitory mVOCs; however, it permitted to identify a few genes belonging to the core genome involved in the production of other non-volatile secondary metabolites.

Sponge-associated fungi are also promising sources of novel secondary metabolites, including antimicrobials (Chávez et al. 2015). In 2014 Henríquez et al. reported the isolation of 101 phenotypically distinct fungal strains from 11 sponge samples, half of which showed antimicrobial activity against *S. aureus*, *P. aeruginosa*, *Xanthomonas campestris*, and *Clavibacter michiganensis*. In a follow-up of this study that highlights their importance as sources of new natural products with unusual structural features, Figueroa et al. (2015) purified four asterric acid nitro derivatives bearing an unusual nitro group from cultures of *Pseudogymnoascus* sp.

Antarctic microorganisms with potential pharmaceutical applications have also been isolated from endemic and cold-adapted macroalgae (Loque et al. 2010). In 2013 Godinho et al. isolated 148 fungal isolates from eight macroalgae (four Phaeophyceae, three Chlorophyta, and one Rhodophyta) and demonstrated that some of them displayed high and selective antimicrobial activities. For example, extracts from two *Penicillium* sp. isolates inhibited growth of *Cladosporium sphaerospermum* – a cosmopolitan dematiaceous fungus that causes serious problems in patients with respiratory tract disease. The extracts also inhibited growth of *Trypanosoma cruzi* trypomastigotes – the causal agent of Chagas disease – with low MIC and IC_{50} values.

Similarly, Furbino et al. (2014) isolated 239 fungi from two endemic Antarctic macroalgae – *Monostroma hariotii* and *Pyropia endiviifolia*. Ethanolic extracts of some of these fungal isolates displayed biological activities against *C. albicans*, *C. krusei*, and/or *C. sphaerosperum*. Additionally, the extract of one particular fungal isolate, *Penicillium steckii*, inhibited 96 % of yellow fever virus, a much better performance than the one exhibited by the currently recommended treatment, interferon alpha (IFN-α) (68 %).

Fungal isolates exhibiting some unexpected antimicrobial characteristics have also been isolated from the leaves and roots of the only two vascular plants found in the Antarctic Peninsula, namely, *D. antarctica*, the Antarctic grass, and *C. quitensis*, the Antarctic pearlwort (Santiago et al. 2012). Extracts of some of these fungi inhibited the proliferation of *Leishmania amazonensis*, the etiologic agent of a deadly tropical disease, and of *Paracoccidioides brasiliensis*, a dimorphic fungus and the causative agent of the disease paracoccidioidomycosis (Goncalves et al. 2015).

5.4.1.2 Antioxidants, Antitumorals, and Other Potential Therapeutic Compounds

Besides from their use as antimicrobials, microbial secondary metabolites might also be fundamental for developing other important therapeutic agents to treat non-infectious, chronic diseases like cancer, diabetes, and neurodegenerative and cardiovascular diseases, among many others. Some of these diseases are characterized by an enhanced state of oxidative stress, which may result from the overproduction of reactive species and/or a decrease in antioxidant defenses (White et al. 2014). Considering some encouraging results showing that long-term consumption of antioxidant-enriched foods can retard or avoid the occurrence of such diseases, many research groups have focused on the antioxidant properties of natural products. In this context, the search of new microbial metabolites with enhanced

antioxidant activities from Antarctic biological resources has been the focus of research efforts in recent years (Table 5.2).

Lobaric acid, a member of the depsidone family of chemical compounds, was isolated from the Antarctic lichens *S. alpinum* and *Cladonia* sp. and has been shown to reduce DPPH (1-diphenyl-2-picrylhydrazyl) free radicals in a dose-dependent manner, without any toxic effects against brine shrimp larvae (Bhattarai et al. 2013). Besides, lobaric acid was also shown to act as a gastroprotectant, by counteracting gastric mucosal damage in mice, through mechanisms unrelated to inhibition of acid secretion (Sepúlveda et al. 2013).

Another very potent antioxidant compound, ramalin [γ-glutamyl-N′-(2-hydroxyphenyl)hydrazide], was isolated from methanol–water extracts of the Antarctic lichen *Ramalina terebrata* (Paudel et al. 2011). Ramalin presented both in vitro and in vivo antioxidant activities and was also able to inhibit the tyrosinase enzyme activity with very little cytotoxicity in human keratinocyte and fibroblast cells.

Another group of antioxidants, namely, ergosterol, torularhodin, torulene, β-carotene, and CoQ_{10}, have been purified from the Antarctic yeast *Sporobolomyces salmonicolor* AL_1 isolated from Antarctic soil lichens (Dimitrova et al. 2010, 2013a). A model emulsion system, which included these bioactive metabolites in combination with exoglucomannan (as emulsifier), showed UV-A protection properties and could, therefore, be used to develop sunscreens.

Photoprotectors are another group of compounds of fundamental importance for the chemoprevention of skin cancer, caused by the cumulative damage of the skin cells due to UV-B radiation. Carotenoids and mycosporines, for example, are synthesized by Antarctic yeasts (isolated from different sources including *D. antarctica* rhizosphere, ornithogenic soils, lake water, seawater, and marine sediments) when exposed to high doses of UV radiation (Libkind et al. 2009; Vaz et al. 2011). From a total of 89 yeast isolates, 12 produced carotenoid pigments and 8 produced mycosporines (including species of the *Dioszegia*, *Cryptococcus*, *Exophiala*, *Microglossum*, and *Rhodotorula* genera). Another photoprotective pigment, melanin, was recently purified from water extracts of an oligotrophic, cold-tolerant bacterium, *Lysobacter oligotrophicus*, isolated from freshwater samples containing microbial mats (Fukuda et al. 2013; Kimura et al. 2014). UV-B irradiation of *L. oligotrophicus* cells induced melanogenesis; furthermore, suspensions of *E. coli* DH5a cells were protected from the lethal effect of UV irradiation by melanin solutions.

Some microbial enzymes, like photolyases, have been shown to revert UV-induced photoproducts (e.g., pyrimidine dimers) to normal bases by using blue light as an energy source. Photolyase-containing sunscreens diminish the number of UV-B radiation-induced dimers by 45–50 % and significantly reduce UV radiation damage when applied to the human skin (Stege et al. 2000; Berardesca et al. 2012). A gene encoding a class II CPD photolyase (PHR-2) from an Antarctic microalgae, *Chlamydomonas* sp., was recently overexpressed in *E. coli* cells (Li et al. 2015). When performing in vivo photolyase activity assays, the survival rate of the

Table 5.2 Antarctic microorganisms and their metabolites exhibiting potential therapeutic activities

Species	Phylum/division	Active compound	Source of isolation (locality)	Therapeutic activity	References
Bacteria					
Lysobacter oligotrophicus	*Proteobacteria*	Melanin	Skarvsnes region, East Antarctica	UV protection	Fukuda et al. (2013) and Kimura et al. (2014)
Janthinobacterium sp.	*Proteobacteria*	Violacein (purple violet pigment)	Proglacial Lake in Dronning Maud Land of East Antarctica	Antitumoral (fibrosarcoma cell growth inhibition), UV protection, cryoprotection	Mojib et al. (2011, 2013)
Fungi					
Sporobolomyces salmonicolor AL_1	Fungi/*Basidiomycota*	Ergosterol, torularhodin, torulene, β-carotene, and CoQ_{10}	Livingston Island, South Shetland Islands	Antioxidants and UV protection	Dimitrova et al. (2010, 2013a)
Dioszegia, *Cryptococcus*, *Exophiala*, *Microglossum*, *Rhodotorula*	Fungi/*Basidiomycota* (D, C, R), *Ascomycota* (E, M)	Carotenoids and mycosporines	Admiralty Bay, King George Island; Port Foster Bay, Deception Island	UV protection	Vaz et al. (2011)

Lichens					
Stereocaulon alpinum, *Cladonia* sp.	Fungi/*Ascomycota*	Lobaric acid	Unspecified	Antioxidant	Bhattarai et al. (2013)
Stereocaulon alpinum, *Cladonia* sp.	Fungi/*Ascomycota*	Lobaric acid	Unspecified	Gastroprotectant	Sepúlveda et al. (2013)
Ramalina terebrata	Fungi/*Ascomycota*	Ramalin	Barton Peninsula, King George Island	Antioxidant	Paudel et al. (2011)
Ramalina terebrata	Fungi/*Ascomycota*	Usimine-like compounds	Barton Peninsula, King George Island	Antiaging (fibroblast cell proliferation)	Lee et al. (2010)
Microalgae					
Chlamydomonas sp.	Protista/Chlorophyta	Photolyase	Larsemann Hills of Princess Elizabeth Land, East Antarctica	UV protection (revert UV-induced pyrimidine dimers)	Li et al. (2015)

recombinant *E. coli* strain was much higher than the one exhibited by the PHR mutant after photoreactivation.

Prevention of skin cancer is also possible by means of a completely different type of compound: violacein (see above). In 2011 Mojib et al. elucidated the chemotherapeutic activity of a violacein-like purple violet pigment (PVP) – purified from extracts of an Antarctic *Janthinobacterium* sp. strain. PVP inhibited growth of murine fibrosarcoma cells and also induced their cell cycle arrest and apoptotic cell death via mitochondrial pathway. PVP also exhibited antiproliferative effect on melanoma cells without any adverse effect on normal human fibroblasts or keratinocytes suggesting that it could be used as a potent chemotherapeutic agent against skin cancers. In a follow-up of this work, the same pigment protected *Janthinobacterium* sp. cells against the lethal effect of high doses of UV-B and UV-C radiation (Mojib et al. 2013). Unexpectedly, apart from UV protection, PVP seemed also to function as cryoprotectant, preventing the deleterious effect of repeated freeze–thaw cycles on *Janthinobacterium* sp. cells.

As a final example of the unexpected therapeutic potential of Antarctic microbe secondary metabolites, usimine-like compounds derived from *R. terebrata* (a lichen) induced cell proliferation of human dermal fibroblasts, type I procollagen synthesis (Lee et al. 2010). These results suggest a potential use of these compounds in anti-aging therapy.

5.4.2 Nanotechnology

A currently active and promising area of research in nanoscience (i.e., the synthesis and use of small structures and small-sized materials of dimensions in the range of few nanometers to less than 100 nm) deals with nanoparticles (NPs). These nanometer-size particles exhibit some unique properties (chemical, physical, and biological) compared to bulk of the same chemical composition, due to their high surface-to-volume ratio and some of its derived properties (Daniel and Astruc 2004). The synthesis of NPs of different morphologies and sizes is at present an important area of research which has attracted the attention of many research groups and is generating a great number of applications in different fields such as cosmetics, electronics, catalysis, chemical and biological sensing, tissue and tumor imaging, environmental remediation, agriculture, and medicine. For instance, many NPs are useful as antimicrobial agents, vehicles of drug delivery or gene transfer, optical receptors, and bioprobes (Salata 2004).

An eco-friendly, cost-effective, sustainable, and efficient way to achieve the synthesis of NPs is by making profit of the biosynthetic abilities of living cells, particularly bacteria, fungi, and yeasts (Sastry et al. 2003; Korbekandi et al. 2009; Iravani 2014). Besides growing at fast rates and yielding high biomasses, most bacteria are able to produce extracellular nanoparticles, facilitating their recovery from the liquid culture (Singh et al. 2015). Therefore, a wide number of bacterial species have been used in *green* nanotechnology to search for alternative methods for the

synthesis of monodispersed, water-soluble, biocompatible, highly stable, and low-cost NPs in both controlled and up-scalable processes.

The great majority of reports dealing with NP synthesis, either biological or chemical, involve the use of processes conducted between 35 °C (when using mesophilic microorganisms) and 350 °C (when chemical synthesis is used). However, it is becoming more and more evident that lower temperatures may improve the chemical process of NP biosynthesis (Meixner et al. 2001), thus reorienting the interest of researchers in this field toward psychrophilic microorganisms.

The first report dealing with low-temperature biosynthesis of silver NPs (AgNPs) used *Morganella psychrotolerans* cultures – a psychrotolerant species isolated from fresh tuna, cold-smoked tuna, and garfish – and silver nitrate as substrate (Ramanathan et al. 2011). In the same year, the synthesis of 6–13 nm diameter AgNPs was achieved using cell-free culture supernatants of five Antarctic psychrophilic bacteria (*Pseudomonas antarctica*, *P. proteolytica*, *P. meridiana*, *Arthrobacter kerguelensis*, and *A. gangotriensis*) and silver nitrate as substrate (Reddy et al. 2004; Shivaji et al. 2011). These particles were synthesized at temperatures as low as 4 °C. More interestingly, the AgNPs showed antibacterial activity at low concentrations (i.e., between 2 μg and 10 μg ml^{-1}) against three Gram-positive bacteria (*Arthrobacter kerguelensis*, *A. gangotriensis*, and *Bacillus indicus*) and three Gram-negative bacteria (*P. antarctica*, *P. proteolytica*, and *E. coli*). This bactericidal activity was attributed to the ability of the AgNPs to anchor and penetrate the bacterial cell wall and to modulate cellular signaling.

The abovementioned nanoparticles were produced rapidly, but they were not stable in time, particularly when maintained under light. Besides, the size range of these AgNPs was too wide (e.g., from 1 to 400 nm). For these reasons, other bacterial strains – namely, *Psychrobacter* sp., *Aeromonas salmonicida*, *Pseudomonas veronii*, and *Yersinia kristensenii* – were assayed for their ability to produce AgNPs at low (4 °C) and medium (30 °C) temperatures (Siamak et al. 2015). Depending on the bacterial strain and temperature, NPs were spherical with sizes 5.0–11.1 nm, stable after several months of incubation at 4 °C under light, and active mainly toward Gram-positive pathogens (*Staphylococcus epidermidis*).

Antarctic bacteria have also been used to produce tellurium-containing nanostructures (TeNS) (Pugin et al. 2014). Oxidized forms of Te – tellurite (TeO_3^{2-}) and tellurate (TeO_4^{2-}) – are extremely toxic to most bacteria, and for that reason Te-based compounds have been used for a long time as antimicrobial and therapeutic agents. A tellurite-reducing isolate, *Pseudomonas* sp. strain BNF22, produces TeNS from tellurite at 25 °C by means of a tellurite reductase, identified as a glutathione reductase (GOR) (Arenas et al. 2014). By using a purified recombinant GOR produced by *E. coli* cells, the authors were able to produce TeNS which exhibited antibacterial properties against *E. coli*, with no apparent cytotoxicity against eukaryotic cells.

In another *tour de force* concerning the use of Antarctic microorganisms, the first report describing the biosynthesis of semiconductor fluorescent nanoparticles (quantum dots, QDs) at low temperature was published in 2014 by Gallardo et al. These nanocrystals exhibit unique optical and electronic properties with

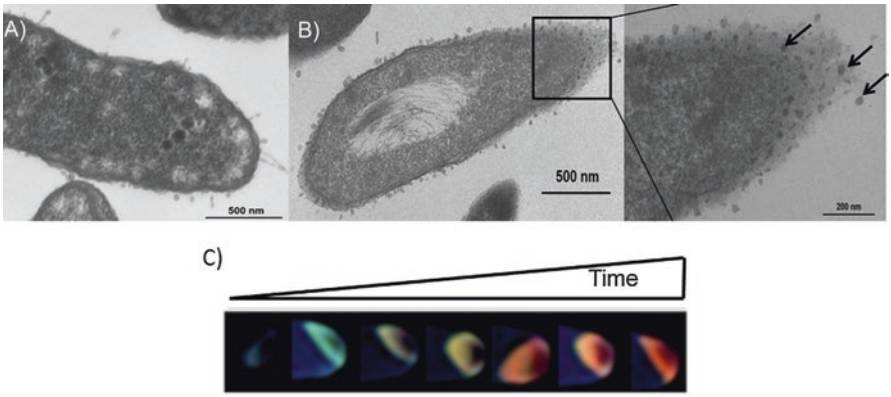

Fig. 5.1 CdS nanoparticle biosynthesis by *Pseudomonas* sp. strain. Ultrathin section of bacterial cells exposed for 3 days to nanoparticle biosynthesis conditions. (**a**) A general view of untreated cells. (**b**) Cells grown under biosynthesis conditions. The inset shows a bacterial pole in which nanometric structures are accumulated (*black arrows*). (**c**) *Pseudomonas* GC01 was grown under biosynthesis conditions and the fluorescence when exposed to UV was evaluated every 24 h (7 days total incubation) (Reprinted from Journal of Biotechnology, 187, C. Gallardo et al. Low-temperature biosynthesis of fluorescent semiconductor nanoparticles (CdS) by oxidative stress resistant Antarctic bacteria, 2014, Pages No. 108–115, with permission from Elsevier)

fluorescence emission wavelengths depending on particle size. Thus, they have potential uses in solar cells, optoelectronics, transistor components, biomedicine, and imaging, among many other applications. Since the biological production of QDs is related to the microbial antioxidant defenses and the redox status of cells, a few oxidative stress-resistant *Pseudomonas* isolates were able to biosynthesize fluorescent semiconductor QDs at 15 °C after exposure to $CdCl_2$.

When excited with UV light, the cell pellets exhibited a fluorescent emission which evidenced intracellular nanoparticle formation (Fig. 5.1). Changes of fluorescence color upon time – from green to red – confirmed the generation of QDs. Therefore, once again, the use of Antarctic strains for the synthesis of relevant biotechnological NPs was confirmed, highlighting the potential of Antarctic microorganisms for the green production of improved NPs, at low temperatures and at low cost.

5.4.3 Energy Production

Due to the numerous environmental problems caused by greenhouse gas emissions and to the limited availability of fossil fuels, it is expected that their complete replacement by renewable, sustainable, and less pollutant *biofuels* will occur in the next few decades. These biofuels are produced through natural biological processes both directly and indirectly. In the first case, biofuels are produced by plants and oleaginous microorganisms through photosynthesis; in the second case, biofuels are

the end product of the biotransformation of either agricultural, commercial, domestic, or industrial wastes (Singh et al. 2011).

Biodiesel is one of the most common biofuels. From a chemically point of view, it consists mainly of fatty acid methyl (or ethyl) esters (FAMEs), which makes it similar to fossil/mineral diesel. Biodiesel is produced using plant or animal oils as substrates, by transesterification with low-molecular-weight alcohols. During the past 25 years, microalgae emerged as a very promising and efficient source of feedstock for biodiesel production (Mata et al. 2010). However, although microalgae offer several advantages over terrestrial plants, the high production cost of biodiesel production still limits its large-scale application. This explains why researchers are actively looking for better oleaginous microalgal species or strains – high lipid containing and high lipid producing – in order to reduce the cost of biomass harvesting, improve the biomass and lipid productivities, and maximize the values of by-products. As stressful conditions induce the synthesis and accumulation of lipids inside algal cells (Chen et al. 2012a), scientist are bioprospecting Antarctica and other cold regions around the world searching for new sources of lipid-rich microalgae.

An Antarctic ice alga *Chlamydomonas* sp. ICE-L (Fig. 5.2), originally isolated by Liu and co-workers (2006) from floating ice, was shown to accumulate high amounts of lipids (up to 84 μL L^{-1} of algal suspensions) inside intracellular bodies during the late stationary phase when grown at 6 °C (Mou et al. 2012). The analysis of its fatty acid composition showed that polyunsaturated fatty acids (PUFAs) were dominant, which was consistent with the up-regulation of genes encoding for fatty acid desaturases (An et al. 2013). Furthermore, the amount of PUFAs was higher at 0 and 5 °C as compared to 15 °C. Therefore, besides offering a great potential as a source of lipids for biofuel production, the growth of strain ICE-L at low temperatures can reduce the cost of algal biomass production.

Another oil-rich, freshwater Antarctic microalga, *Scenedesmus* sp. NJ-1, was identified by Chen et al. (2012b) as a suitable candidate to produce biodiesel. This strain was able to grow at temperatures ranging from 4 to 35 °C and accumulated large amounts of triacylglycerols composed of saturated and monounsaturated fatty acids at the late growth phase. Altogether, these characteristics make *Scenedesmus* sp. NJ-1 a very promising strain for biodiesel production.

Cold-adapted Antarctic yeasts belonging to the *Mrakia* and *Mrakiella* genera, identified in soil samples, were also shown to accumulate high amounts of intracellular lipids (Thomas-Hall et al. 2010). Similarly, Zlatanov et al. (2010) reported on the high lipid content and composition of selected Antarctic yeasts isolated from soil samples. More recently, Pereyra et al. (2014) reported the identification of two oleaginous psychrotrophic yeasts isolated from Antarctic soils. The isolates, namely, *Leucosporidium scottii* At17 and *Rhodotorula mucilaginosa* At7, accumulated high amounts of intracellular lipids, with levels exceeding 40 % of cell dry weight, which underlines the potential use of Antarctic yeasts as lipid-producing biofactories for the synthesis of biofuels.

Not only are Antarctic yeasts good sources of lipids suitable for biodiesel production; they are also important sources of lipases able to catalyze the esterification

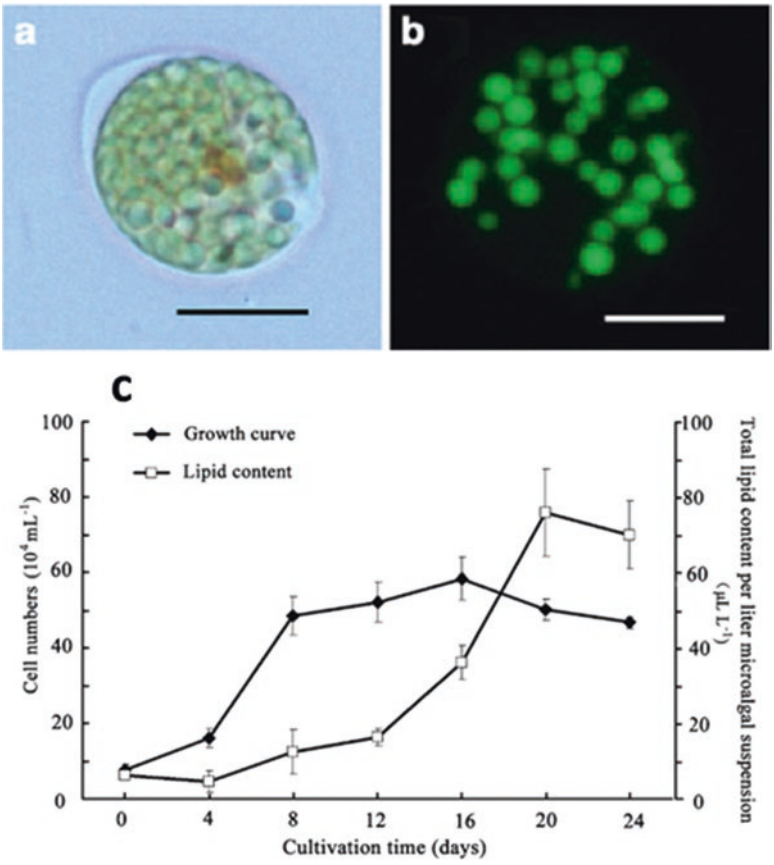

Fig. 5.2 *Chlamydomonas* sp. ICE-L. (**a**) Light micrograph. (**b**) Fluorescent confocal micrograph of algae stained with BODIPY 505/515. Scale bars = 10 μm. (**c**) Growth curve and total lipid content over 24-day cultivation (Reprinted from Journal of Applied Phycology, Rapid estimation of lipid content in an Antarctic ice alga (*Chlamydomonas* sp.) using the lipophilic fluorescent dye BODIPY505/515, 24, 2012, 1169–1176, Mou et al. with permission of Springer)

of fatty acids or transesterification of oils and fats with short-chain alcohols in mild conditions (Tan et al. 2010). Perhaps the best examples are the lipases A and B produced by *Pseudozyma antarctica* (former *Candida antarctica*). Incidentally, and even though this is beyond the scope of this chapter, *P. antarctica* lipase B – commercially known and sold under the name of Novozym 435 – is the most common employed lipase to catalyze biodiesel production from various sources of oils such as lard, soybean oil, cottonseed oil, and waste oil. Lipase-producing yeasts were also isolated from Antarctic marine and terrestrial samples by Carrasco et al. (2012), Duarte et al. (2013), and Vaca et al. (2013).

Another widely used biofuel is ethanol fuel (ethyl alcohol). Bioethanol has been made from biomass of agricultural feedstocks such as corn or sugarcane, by industrial fermentation using *Saccharomyces cerevisiae*. According to the Global

Renewable Fuels Alliance (GRFA), in 2014 the worldwide ethanol fuel production surpassed 90 billion liters. However, due to serious concerns – i.e., increased food prices due to the large amount of arable land required for crops and the energy and pollution balance of the whole cycle of ethanol production, especially from corn – researchers are still investigating new methods to produce bioethanol from waste plant dry matter, such as cellulose and lignocellulose. However, since these polymers are not fermentable by most microorganisms, an additional step of enzymatic hydrolysis is required to release fermentable monomers. This so-called *saccharification* step is usually accomplished through cellulose hydrolysis by microbial cellulases.

Cellulase-producing Antarctic bacteria belonging to the *Firmicutes*, *Actinobacteria*, *Gammaproteobacteria*, and *Bacteroidetes* have been isolated by Soares Jr. et al. (2012), Loperena et al. (2012), Melo et al. (2014) and Herrera et al. (2016). The analysis of the genome sequence of one of these strains, namely, *Bacillus* sp. CMAA 1185, revealed the presence of 17 gene copies related to hydrolases, mainly glycosyltransferases, polysaccharide lyase, peptidoglycan chitinase A, and amylase (Santos et al. 2015).

Fungi are also very well known for their ability to degrade lignocellulosic substrates very efficiently (Payne et al. 2015). From a total of 20 fungal strains isolated from Antarctic soils, one *Verticillium* sp. strain was shown to produce high amounts of a cold-active cellulase (Wang et al. 2013). Thirty-eight cellulase-producing fungi, including 20 *Geomyces* and two *Pseudeurotium* isolates, were also isolated by Krishnan et al. (2011, 2016) from ornithogenic, undisturbed, and human-impacted soils.

Cellulase-producing psychrotolerant yeasts were also isolated by Loperena et al. (2012), Vaca et al. (2013) and Herrera et al. (2016) from diverse Antarctic environments, including marine sponges and worms. Several extracellular enzymes were also shown to be produced and excreted by a total of 78 yeast strains isolated from soil and water samples collected by Carrasco et al. (2012). Extracellular cellulases are also produced by *Leuconeurospora* sp., *Cryptococcus* sp., *Dioszegia* sp. and *Mrakia* spp. isolates. In fact, Antarctic *Mrakia*, considered as the dominant culturable fungi in East Antarctica (Thomas-Hall et al. 2010; Tsuji et al. 2013a), is able to ferment typical sugars such as glucose, sucrose, maltose, raffinose, and fructose at cold temperatures (Tsuji et al. 2012). For example, *M. blollopsis* SK-4 was shown to ferment glucose to produce ethanol at 10 °C (Tsuji et al. 2013b). More importantly, this strain produced ethanol from lignocellulosic biomass also at 10 °C, through a technique known as direct ethanol fermentation (i.e., the enzymatic hydrolysis of cellulose to glucose and simultaneous fermentation to produce ethanol) (Tsuji et al. 2013c, 2014). Even though this process remains to be optimized, it is considered a good technique for the production of ethanol from woody biomass under cold temperature conditions.

Among renewable energy sources, hydrogen stands as a very attractive option. This is mainly due to its clean combustion, which does not pollute the environment (yielding only water and heat as products), and its high energy yield of 122 kJ/g (Kapdan and Kargi 2006). Since it can be produced by microbial fermentation,

many research groups are prospecting all kinds of natural environments to find highly efficient hydrogen-producing strains. Antarctica is not an exception: recently, Alvarado-Cuevas et al. (2015) published the first report on hydrogen-producing psychrophilic Antarctic bacteria. The maximum production yield (253.3 ml) and production rate (16.64 ml/l/h) were attained by *Janthinobacterium agaricidamnosum* GA051; on the other hand, the maximum biohydrogen yield (1.57 mol H_2/mol glucose) was obtained when culturing *Polaromonas jejuensis* GA024 in biofermentors at 25 °C. Even though more studies are necessary to explore this issue, this work clearly shows another exciting facet of Antarctic microorganisms as versatile producers of renewable energy sources.

5.4.4 Agriculture

The use of plant growth-promoting microorganisms (PGPM) in the field of agriculture is nowadays a well-established technology. PGPM can act as *biofertilizers* (e.g., by mobilizing mineral nutrients in the rhizosphere), *biocontrollers* (e.g., by counteracting and inhibiting growth of phytopathogens), *biostimulants* (e.g., by directly promoting plant growth and development through excretion of phytohormone-like metabolites), and *bioelicitors* (e.g., by activating the plant defense systems) (Glick 2012). Sometimes, the same microorganism may exhibit several of these traits simultaneously.

Antarctic environments have been prospected during the past years to isolate and characterize potential psychrophilic or psychrotolerant PGPM in order to develop new biotechnological products to increase crop yield in different regions of the world. Even though some of these microorganisms may be found in the bulk soil, they are mostly associated with plant roots. This explains why several research groups have focused on the rhizosphere microbiology of the two vascular plants able to withstand the extreme conditions prevailing in the Antarctic Peninsula: *D. antarctica* and *C. quitensis*. Studies on the rhizosphere microbiome of these plants have been performed by traditional (culture-dependent) techniques and by more recent (culture-independent) methods, particularly making use of the next-generation sequencing technologies.

Bacteria colonizing the rhizosphere of *D. antarctica*, mainly belonging to the *Pseudomonas*, *Flavobacterium*, and *Arthrobacter* genera, were isolated and further characterized by Barrientos-Díaz et al. (2008). Authors reported both Gram-positive and Gram-negative isolates that produced and excreted phosphatases, esterases, and lipases. One of these isolates, a psychrotolerant *Pseudomonas* sp. strain able to solubilize mineral phosphate and to produce phytohormones (e.g., indole-3-acetic acid), promoted growth of *D. antarctica* seedlings under in vitro conditions at 13 °C (Berríos et al. 2013). Scanning electron microscope analyses revealed an intimate association between the bacterium and plant root hairs, suggesting that its promoting effect is exerted on close proximity to the roots.

The potential use of Antarctic microorganisms for biocontrolling phytopathogens has also been the subject of several reports (Fig. 5.3). For instance, Gesheva (2010)

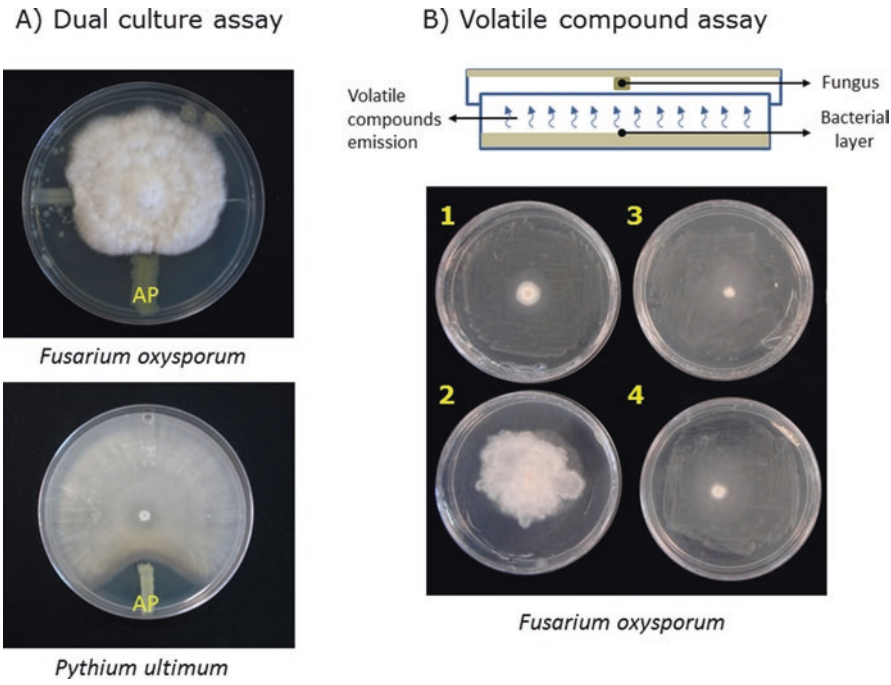

Fig. 5.3 (**a**) Antagonism assays of an Antarctic *Pseudomonas* sp. isolate (AP) against *Fusarium oxysporum* and *Pythium ultimum*. (**b**) Effect of volatiles produced by Antarctic bacteria on the growth of *Fusarium oxysporum. 1 Pseudomonas protegens* CHA0 (positive control of inhibition); *2 F. oxysporum* without exposure to bacterial volatiles (negative control of inhibition); *3* and *4 F. oxysporum* exposed to Antarctic *Pseudomonas* spp. volatiles (L.A. Yarzábal (unpublished))

showed that two actinomycetes (*Streptomyces* sp. 5 and *Micromonospora* sp. 18) isolated from soil samples produced extracellular substances able to inhibit growth of three well-known phytopathogenic fungi, namely, *Cladosporium cladosporioides*, *Fusarium graminearum*, and *Botrytis cinerea.* Another actinomycete, *Streptomyces anulatus* 39 LBG09, synthesized several extracellular antibiotics which inhibited growth of the phytopathogenic bacteria *Erwinia amylovora* and *Pseudomonas syringae* pathovar *syringae* when tested in vitro (Dimitrova et al. 2013b). Similarly, Encheva et al. (2013) isolated from soil samples several *Streptomyces* sp. strains able to inhibit *Xanthomonas euvesicatoria*, *Burkholderia gladioli*, and *Erwinia amylovora*. Extracts of three of these *Streptomyces* strains were further shown to be active against the causative agents of bacterial spot of pepper, *X. euvesicatoria*, *X. vesicatoria*, and *X. gardneri*, both in vitro and in vivo (Encheva et al. 2015).

Another well-established use of biocontrol microorganisms is the biological control of postharvest diseases (Nunes 2012). Indeed, postharvest losses of stored fruits, vegetables, and grains due to decay by fungal pathogens are considered as a big problem in the agriculture field. Yeasts have been proved to be particularly

useful as biocontrol agents of postharvest pathogens (Droby et al. 2009). In line with this, Vero and co-workers (2013) isolated a psychrotrophic strain of *Leucosporidium scottii* from soil samples and showed its ability to control blue and gray molds of two apple cultivars caused by *Penicillium expansum* and *Botrytis cinerea*, respectively. Besides having the capacity to form biofilms – considered an important attribute of postharvest antagonists to successfully colonize wounds and intact fruit surfaces – *L. scottii* synthesized both soluble and volatile antifungal substances which inhibited growth of the pathogens. On the other hand, the strain was resistant to commonly used postharvest fungicides, which allow their combined use in an integrated management practice.

5.5 Present and Future Challenges in Sustainable Use of Antarctic Microorganisms

The abovementioned achievements have been, mostly, the result of an increasing activity of *bioprospection* in Antarctica and the surrounding Southern Ocean. According to the most recent definition proposed – only for working purposes – during the XXXVII Antarctic Treaty Consultative Meeting held in Brasilia in 2014, bioprospection defines "any activity of search, identification, description, collection, survey, monitoring, cultivation, replication, or any other scientific investigation processes, performed on indigenous biological species…with the initial intention to consider potential industrial or commercial derived products or applications, notably through the development of patentable material or process."

As can be deduced from this definition, there is – indeed – a considerable growing interest in accessing Antarctica's biological resources and derived data for commercial purposes. Alas, as highlighted by several reports, the absence of clear rules governing the use and ownership of these resources permits their appropriation by those who find them and – consequently – develop a patentable invention (Lohan and Johnston 2005; UNU-IAS 2007; UNU-UNEP 2009; Jabour 2010; UNEP 2012; Puig-Marcó 2014).

As noticed by the Scientific Committee on Antarctic Research (SCAR) in 2009, it is not an easy matter to retrieve information or scientific literature clearly related with biological prospecting activities which, on the other hand, take place largely undetected and may even predate non-commercially oriented research efforts. In some cases there is also a significant lag between the initial sampling and product development. Whatever the case, SCAR concluded in 2010 that the bioprospecting activity "…in the Antarctic region and/or involving Antarctic organisms is extensive and widespread." (ASOC 2013)

In order to deal with this topic, the Antarctic Treaty Committee launched, in 2008, a central online database on biological prospecting in Antarctica, with the aim to allow access to up-to-date information about the level and outcomes of bioprospecting in Antarctica. The database also includes details of research and commercialized products arising from biological samples that were sourced from the Antarctic region. By 2012 the database contained a total of 218 records related to

actual or potential commercial applications based on Antarctic genetic resources (UNEP Report 2012). While many of these patent applications related to krill, many others dealt with microbes and their metabolites with potential biotechnological applications. Evidently, this increasing patenting activity confirms that Antarctic microbes are, indeed, an invaluable source of tremendous potential benefits.

Nevertheless, one of the main challenges to guarantee a sustainable use of Antarctic biological resources is still related on how to allow positive benefits from bioprospecting without incurring significant harm to the Antarctic environment. Uncontrolled prospecting activities, including the logistical efforts necessary to access the natural resources, and the impact associated with them could prove – in fact – to be unsustainable and to severely compromise Antarctic microbial habitats (Hughes et al. 2015).

On the other hand, commercially oriented research activities and the secrecy surrounding them may compromise one of the main pillars of the Antarctic Treaty System (Article III), namely, the free and public exchange of information and cooperation among all the parties. Further, the growing commercial interest in Antarctic biological resources raises some key policy, ethical, and moral questions: Who owns these biological/genetic resources? How should they be used? And how should the benefits of this research be distributed? Some of these questions, which also concern the impact of the human presence in Antarctica and its consequences for Antarctic life, were included in the 2014 SCAR1-sponsored Antarctic and Southern Ocean Horizon Scan (Kennicutt et al. 2014, 2015). They need to be properly addressed considering the current uncertainty and lack of policies governing the access and use of Antarctic biological resources. The debate should be channeled through SCAR, as the international body responsible for Antarctic science policy, to the Antarctic Treaty nations. As previously stated, the ATS has already initiated some discussion on bioprospecting. However, there are still important territorial and commercial sensitivities that prevent the international community to reach essential agreements to safeguard Antarctica's environment.

5.6 Conclusions

Twenty-five years ago, psychrophiles were regarded as promising tools for the development of different biotechnologies. Nowadays, a big part of this potential has turned into reality, thanks – among others – to the study of Antarctic microorganisms. Psychrophile-derived products and metabolites are currently used in the industrial sector, with many patents filed to protect these products or processes and many other still waiting for their approval. Other fields of knowledge – from agriculture to nanotechnology and from medicine to energy production – can benefit from the sustainable and rational use of these biological and genetic resources. Antarctic psychrophilic microorganisms have proven – indeed – to be invaluable resources for the discovery of new compounds or metabolites which can find important applications in these fields. Consequently, the interest in conducting further research into Antarctica's useful biological and genetic resources will continue to grow.

Nonetheless, the sustainable use of these resources demands the development of new policies to prevent the environmental impact that can be caused by uncontrolled prospecting activities. Furthermore, and considering that Antarctic resources may be considered as common heritage of mankind, the international community should clarify many important ethical and moral questions – including the ownership of these resources and the equitable access to any possible benefits – before it is too late.

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Escherichia coli: The Leading Model for the Production of Recombinant Proteins

6

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Abstract

Proteins are a diverse group of biomolecules that have many different biological activities and structures. Both in the industrial and research laboratory, proteins are used for very diverse purposes and are produced at different scales. Main fields of application of proteins are human and animal prophylactics, therapeutics, and diagnostics, as well as the food processing industry. Besides, proteins are employed as additives to generate a diverse range of products. Pectinases are employed in the food industry since 1930, in fruit juice manufacturing; other enzymes like papain, bromelain, pepsin, rennin, lipases, cellulases, and amylases have several industrial applications. Due to their relevance in medicine, proteins used as biopharmaceuticals are the best characterized. This chapter addresses the production of recombinant proteins as aligned with the concept of industrial sustainability and *Escherichia coli* as a model for heterologous protein production. It describes and updates the main production strategies and the current available tools for the protein expression system. The main disadvantages, as well as the improvements done to this model, are detailed. A final summary that includes industrial uses and fields of applications of recombinant proteins points out the relevance of this prokaryotic system.

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6.1 Sustainability of Recombinant Protein Production

For a very long time, proteins were extracted and purified from their natural source, such as plants, microorganisms, animals, or human cells. However, this approach has important limitations in the availability of the product and standardization of the production quality, and presents important risks of contamination and elevated production costs. This can be illustrated through the following examples:

- Insulin. Insulin is a peptide hormone produced in the pancreas. It regulates carbohydrate and fat metabolism by promoting the absorption of glucose from blood to skeletal muscles and fat tissue and by causing fat to be stored rather than used as energy. Patients with type 1 diabetes mellitus do not produce this hormone and therefore it must be regularly administered. For many years, insulin was obtained by standard purification from cows and pig pancreas. Although the procedure was expensive and difficult and the hormone could cause allergic reactions, this was the only type of insulin available to treat diabetes between the 1920s and 1980s. During the 1980s, the development of the recombinant protein technology achieved a product identical to human insulin (Keefer et al. 1981).
- Human growth hormone (GH, also known as somatotropin). This peptide hormone is produced by cells within the anterior pituitary gland. It is important for human development and its deficiency during childhood leads to a slow growth speed. In adults, growth hormone is needed to maintain the proper amounts of body fat, muscle, and bones. Growth hormone deficiency is treated by replacing this hormone with daily subcutaneous or intramuscular injections. Since 1958 and for 25 years, therapeutic growth hormone preparations were derived from human cadaver pituitaries. But, after decades of treatment with pituitary-derived GH, some patients developed the degenerative Creutzfeldt–Jakob disease (Rudge et al. 2015). Thus, this hormone preparation strategy was withdrawn for safety concerns (Hardin 2008). Since 1985, recombinant human GH has provided a readily available and safe drug that greatly improved the management of children with GH deficiency and other growth disorders (Hardin 2008).
- Chymosin or rennin (Emtage et al. 1983). This enzyme, a protease found in the rennet of ruminant mammals, is widely employed in the production of cheese. The native substrate of chymosin is kappa-casein, and the enzyme specifically cleaves the peptide bond between Phe 105 and Met 106 residues. Newborn calves curdle the ingested milk by producing chymosin in the lining of the fourth stomach, thus slowing the digestive transit and allowing a better absorption. Chymosin secretion is maximal during the first few days after birth and declines thereafter, being produced together with other proteases with different specificities. Rennet-derived natural chymosin is commonly extracted from abomasum from newborn animals and used for the production of cheese. However, some microorganisms and plants also synthesize enzymes that can be employed for this purpose, but these enzymes show less specificity and produce different peptides which can give rise to bitter taste cheeses. In this context, the production of recombinant chymosin has allowed the substitution of animal sourced chymosin

by a genetically modified microorganism source, such as bacteria, fungi and yeasts.

Taken all together, the aforementioned examples illustrate how the extraction of proteins from their natural sources has important industrial limitations, consisting of difficult and expensive production protocols, safety risks of the end product, and some ethical implications. In this regard, the production of recombinant proteins by microorganisms appeared as a powerful technological tool, and the Gram-negative bacterium *Escherichia coli* remains the most versatile host for the production of heterologous proteins. In the 1980s, the first recombinant proteins produced in *E. coli* – human insulin and growth hormone – were registered and commercialized. Since then, a permanently increasing number of proteins have been produced in *E. coli* for different purposes, and at present more than 50 proteins expressed in this system are employed as therapeutics (Leader et al. 2008). The production of recombinant proteins presents many advantages related to increased and reproducible product quality, lower biosecurity risks, lower production costs, and sustainability as a renewable resource.

6.2 *Escherichia coli* as a Host for Heterologous Protein Expression

E. coli is a Gram-negative, rod-shaped bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms. Most *E. coli* strains are harmless, and as part of the intestinal flora, this microbe can benefit their hosts by producing, for example, vitamin K2 (Bentley and Meganathan 1982) and queuine, a hypermodified nucleotide base essential for protein synthesis, produced exclusively by eubacteria and used by animal, plant, and several fungal species (Fergus et al. 2015).

The *E. coli* cellular architecture is characterized by an organized cytoplasm, which includes nucleoid and ribosomes, surrounded by inner and outer membranes, the periplasm, and a capsule. Three types of appendages project from the cell surface: flagella, involved in locomotion, organelles involved in material transfer, and other organelles acting as adherence factors involved in colonization (Fig. 6.1).

A particular advantage of *E. coli* is the availability of huge expertise and deep knowledge on its genetics, metabolism, and physiology. In this sense, there are excellent books and reviews describing main characteristics of *E. coli*, and therefore, those aspects are not developed in this chapter. For a complete cellular and molecular description of *E. coli*, see *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology (Neidhardt et al. 1996). Concerning the use of *E. coli* as a cellular host for protein production, the leading position of this expression system is due by the availability of many commercial vectors and constructs, as well as different modified *E. coli* strains adapted to each particular use (see below).

An industrial advantage of *E. coli* as a microbial model for protein production is that cells grow rapidly, bearing most wild-type strains cultured in a rich medium a

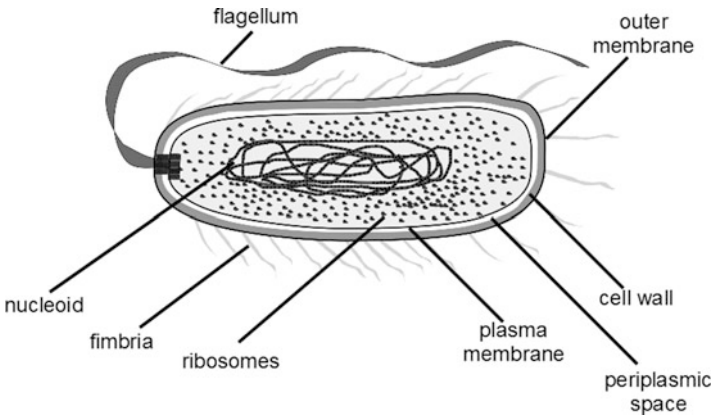

Fig. 6.1 *E. coli* cellular organization showing chromosome, nucleoid, and ribosomes in the cytoplasm, plasma membrane, periplasm, cell wall, and outer membrane. Flagellum and fimbriae projected structures are indicated

doubling time (at the log phase) of 20–30 min. This means that in a relatively short time, high cell mass can be obtained. In addition, reagents and culture medium, as well as the general equipment required for bacteria growing and protein production, are common and have low cost.

6.2.1 Protein Synthesis in *E. coli*

All proteins in *E. coli* are initially synthesized in the cytoplasm. This environment is extremely crowded due to the presence of high concentrations of macromolecules (Kuznetsova et al. 2014). The resulting macromolecular crowding is predicted to strongly favor intermolecular interactions. The protein quality control system – composed of chaperones and proteases – and the protein aggregation and disaggregation processes are part of a regulated cellular response that maintains protein homeostasis (reviewed in Tyedmers et al. 2010). Protein synthesis initiated in *E. coli* cytoplasm then follows a pathway that depends upon their ultimate cellular destination. Secreted proteins follow different pathways and are secreted directly or indirectly to the extracellular environment, i.e., through the periplasm (reviewed in Yoon et al. 2010). Proteins destined for the periplasm are synthesized as precursors, carrying an N-terminal signal peptide that directs them to the general secretion machinery at the inner membrane. Using alternative pathways, proteins can be translocated in a folded or unfolded state (Baneyx and Mujacic 2004; Georgiou and Segatori 2005; Choi and Lee 2004). In the latter case, after translocation and signal peptide cleavage, the newly exported mature proteins are folded and assembled into the periplasm. Maintaining quality control over these processes relay on chaperones, folding catalysts, and proteases present in both compartments (cytoplasm and periplasm) (Baneyx and Mujacic 2004; Miot and Betton 2004). In this way, proteins

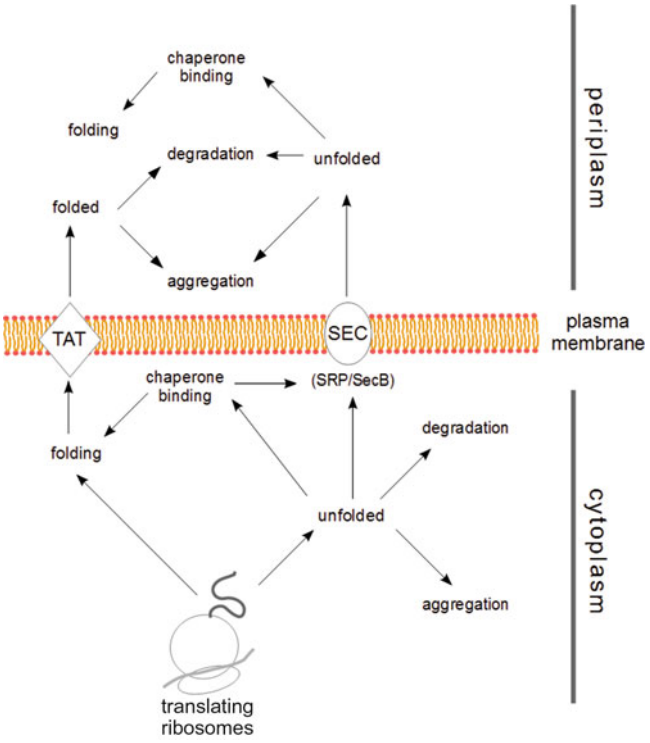

Fig. 6.2 Protein synthesis, folding, and translocation to the periplasm. Proteins are synthesized at ribosomes from the cytoplasm. Polypeptides can fold in the same compartment, assisted by chaperones, and translocate through the twin-arginine translocation (Tat) pathway. Unfolded polypeptides are transported to periplasm mediated by SRP or SecB-SEC pathways (Adapted from Miot and Betton 2004)

can be properly folded, aggregated, or degraded at both compartments (Fig. 6.2) (Arie et al. 2006).

Cytoplasm and periplasm are compartments with different redox environment; while the former is kept under reducing conditions, the latter is an oxidizing environment, where the formation of disulfide bonds is favored. Recombinant proteins can be directed for their expression to the cytoplasm, periplasm, or membrane by fusing specific signal peptides at the N-terminal end of the protein (see Sect. 6.3.4).

6.2.2 Limitations and Disadvantages

The successful production of proteins in *E. coli* mainly depends on the protein itself and the purposes of the specific protein to be produced. When posttranslational modifications are required for protein activity or for particular production purposes, the selection of other host cells, rather than *E. coli*, may be necessary. Proteins

carrying many disulfide bonds or functional glycosylation, for example, are often preferentially produced in eukaryotic systems. Limitations of *E. coli* as protein production system are intensively investigated and constitute a rapidly evolving field of active research. *E. coli* strains are currently being developed in order to overcome some of these limitations. In this sense, there are several commercial strains designed for the overexpression of enzymes with disulfide bond (see Sect. 6.3.4).

E. coli also has a limited use for the production of glycosylated proteins. The characterization of the first bacterial N-linked glycosylation system in the human enteropathogen *Campylobacter jejuni* has opened a field of research with the aim to produce N-glycosylated proteins in *E. coli*. In this context, the *C. jejuni pgl* gene clusters, involved in the synthesis of various glycoproteins, were successfully transferred to *E. coli* (Wacker et al. 2002). Much investigation on glycosylation pathways is in progress and future work is focused in the development of engineered *E. coli* strains able to produce humanized glycoproteins (Cuccui and Wren 2015).

Another disadvantage of *E. coli* as expression host has been the accumulation of lipopolysaccharides and toxins that have to be carefully eliminated during the protein purification procedure, and hence increasing the production costs. A recently developed endotoxin-free strain of *E. coli* (ClearColi™ Expression Technology) was genetically modified for avoiding the endotoxin signal production that is normally part of the capsular lipopolysaccharide. This has been accomplished by radically modifying the synthesis of LPS through the incorporation of seven genetic deletions, which remove all of the carbohydrate moieties usually attached to LPS (Mamat et al. 2015).

Finally, an important obstacle frequently found during the production of recombinant proteins in *E. coli* is the accumulation of the protein in an insoluble form, as aggregates in structures called "inclusion bodies." For industrial purposes, this may represent a disadvantage, but, in some cases, it could also be considered as an attractive strategy to easily get pure proteins. This question will be discussed later (see Sect. 6.4).

6.3 Designing the Expression System for Recombinant Protein Production in *E. coli*

An "expression system" includes the expression plasmid containing the coding sequence of the protein to be produced and the *E. coli* strain selected for expression and production. The expression system design summarizes the scheme defined for every step of the procedure and hence many aspects need to be taken into account from the beginning.

1. Structure and properties of the protein: monomeric/multimeric, homomeric/heteromeric, the presence of disulfide bonds, posttranslational modifications and transmembrane domains
2. Coding sequence and verification of the sequence information

3. Codon optimization and cloning of the coding sequence
4. Choice of cellular location for protein accumulation: cytoplasm, periplasm, membrane, and the addition of signal peptides to direct the polypeptide
5. The protein expression induction form (IPTG, Arabinose, temperature, autoinduction)
6. Purification strategy and addition of purification tags
7. Addition of sites for tag cleavage
8. Choice of the selection marker (antibiotic resistance) according to the objective of protein production (ampicillin, kanamycin, other)
9. Choice of the *E. coli* strain for protein expression

The knowledge of protein structure and the requirements of modifications in the final recombinant product is determinant in the choice of the host expression system (bacteria, yeast, cell lines, other). For heterodimers, for example, different alternatives should be analyzed: (i) co-expression of two different polypeptides in tandem into the same vector (same promoter), or expression controlled by different promoters, (ii) use of different vectors in the same cell, and (iii) production of each polypeptide in a different expression system and protein mix after purification.

Another aspect to be explored carefully is the verification of the protein sequence information; is it complete? Is the methionine really the initial one or an internal residue? Comparative sequence analyses of protein families bring relevant information in this sense. The encoding nucleotide sequence can be obtained from its natural source, or synthetically produced, but in any case codon usage has to be analyzed previously, considering codon optimization or choosing a particular *E. coli* strain for expression (Sect. 6.3.6).

The main elements present in an expression plasmid construct are shown in Fig. 6.3. Detailed experimental procedures have been recently described in Burgess and Deutscher 2009 and Lorsch 2015.

6.3.1 Antibiotic Resistance

The addition of antibiotics to the culture medium ensures the growth of cells carrying plasmids with resistance element exclusively and not those without plasmid. Resistance to ampicillin is conferred by the expression of the gene *bla* which encodes β-lactamase, a periplasmic enzyme that inactivates β-lactam antibiotics. Other antibiotics frequently employed as selective markers are kanamycin, chloramphenicol, and tetracycline.

It is worth mentioning that the use of antibiotics as selective markers is under revision. For some large-scale production and particularly for some purposes such as therapeutics, it is strongly advised to avoid the use of any kind of antibiotics in bacterial culture. The main concern regards to the propagation of antibiotic resistance genes to pathogenic microorganisms in the environment or to commensal flora, through horizontal genetic transfer. Besides, a widespread use of antibiotics, for example, penicillin and streptomycin, can produce hyperreactivity in patients

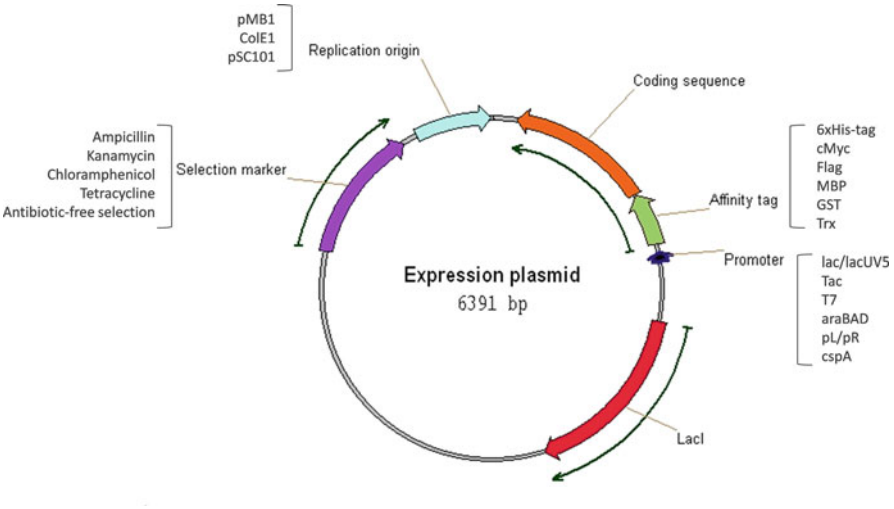

Fig. 6.3 Diagram of an expression vector construct. The figure resumes the major features present in most expression vectors. Main components are described in the text

allergic to β-lactam family antibiotics. When antibiotics are used at industrial scale, specifications of their level in the final product should be established. It is expected that in the future the expression vectors should not contain antibiotic resistance genes (Peubez et al. 2010).

The search for alternative selective markers is currently in progress. A promising solution is the development of antibiotic-free plasmid systems, in which cells survive only if they contain a plasmid encoding a "life-saving molecule" (Peubez et al. 2010). Different systems are being developed, based on poison/antidote systems, metabolism-based systems, and operator repressor titration systems, among others (reviewed in Kroll et al. 2010), and some of them have been assayed in large-scale fermenters as well (Peubez et al. 2010).

6.3.2 Replication Origin

For autonomous replication, plasmids include a genetic element called replication origin. It determines plasmid incompatibility group and controls the copy number of plasmids per cell. Plasmid copy number can be high, medium, or low, ranging from 500 to 700 copies/cell in pMB1 origin-containing vectors (pUC), 15 to 20/cell in ColE1 origin-derived pQE vectors and pBR322 origin-derived pET plasmids, and about 5 copies in pSC101 origin vectors. In general, the propagation of different plasmids carrying the same replication origin fails because they compete for the same replication machinery. This defines the incompatibility groups (Austin and Nordstrom 1990). For the co-expression of two proteins in *E. coli*, from two different plasmids, vectors should belong to different incompatibility groups.

6.3.3 Promoters

The promoter is the DNA region specifically recognized by RNA polymerase to start transcription. Located just upstream the gene to be expressed, it often contains information for its regulation by specific factors. In bacteria, promoters play a crucial role in determining when and how the gene of interest has to be expressed. Regarding the frequency of transcription initiation, and hence the transcriptional level of the gene, promoters can be defined as strong or weak. Promoters can show a different level of basal expression, which can be dramatically increased by a specific inducer or inhibited by repressors. For the expression of recombinant proteins, strong promoters, easily inducible and with low basal expression level, are particularly appreciated. For large-scale production, the cost of inducer has also to be taken into account and can influence the promoter and vector of choice. Frequently employed promoters in commercial vectors are (Fig. 6.3):

- *lac* promoter from the *E. coli* lactose operon. If glucose (the preferred energy source) is not available, the transcription of genes under the control of *lac* promoter is highly induced by lactose, the natural inducer, or by the non-hydrolyzable analog isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fig. 6.4). This promoter regulation is mediated by: (1) lac repressor (LacI), a protein that binds DNA and represses transcription only in the absence of lactose (or IPTG); the presence of lactose leads to repressor inactivation and hence to transcriptional derepression;

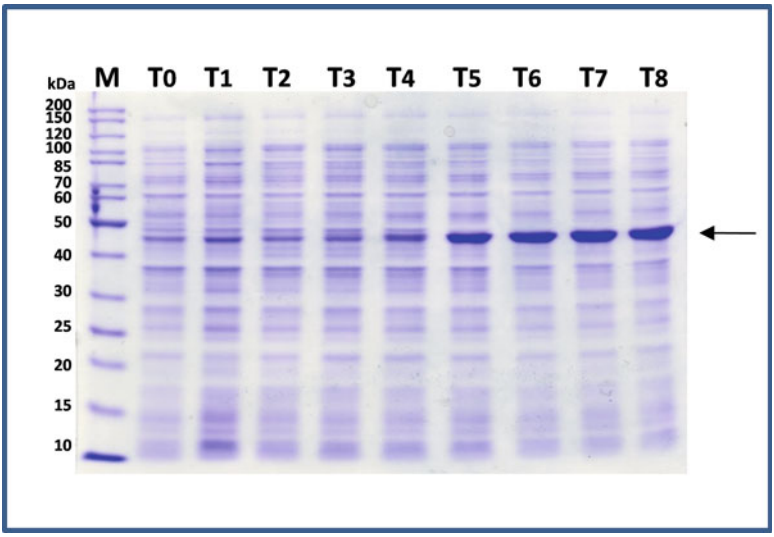

Fig. 6.4 Time course profile of recombinant protein expression in *E. coli*. Time course of the expression of a recombinant protein (45 kDa) in *E. coli*, before and after IPTG induction (at T3, 3 hs from culture start). Target protein is indicated (*black arrow*). T0, T1, T2, T3, T4, T5, T6, T7, and T8: *E. coli* cell extracts obtained at 0, 1, 2, 3, 4, 5, 6, 7, and 8 hs of culture. *M* molecular weight marker. 12 % SDS-PAGE, Coomassie blue staining

(2) protein CAP (also called CRP) mediates the inhibitory effect produced by glucose availability; CAP binds to promoter and activates transcription only in the absence of glucose. When *lac* promoter is present in multicopy plasmids, high level of basal gene expression can be seen, even in the absence of inducer. Lac repressor, synthesized from a single copy in the bacterial chromosome, is present at low levels (about 10 molecules/cell), which is not enough to completely repress transcription from high copy plasmids. For this reason, some vectors include a *lacI* gene in their sequence to allow for a tighter regulation of basal expression. For the expression of proteins that can be toxic for the bacterial cell host, a high basal expression level can lead to production failure.

- *tac* promoter. A hybrid synthetic promoter, constructed by combining *trp* (promoter from the tryptophan operon) and the aforementioned *lac* promoter. *tac* promoter shows the strength of the *trp* promoter (the recognition −35 region) and conserves advantages of *lac* promoter (centered at −10 region) described above. In this way, *tac* promoter is about ten times stronger than *lac* promoter. This promoter has been included in pMAL (NEB) and pGEX (GE Healthcare Life Science) vectors.
- Promoter of the *araBAD* operon from *E. coli*. This promoter is activated in the presence of arabinose and absence of glucose. The expression of a gene fused to the *araBAD* promoter is under the control of arabinose concentration. In the absence of arabinose, there is no expression of the gene, and the addition of arabinose (in the absence of glucose) induces the expression. Interestingly, the induction is strongly controlled by arabinose, which is a safe and inexpensive inducer that can be employed for industrial purposes. pBAD series of vectors (Invitrogen, Thermo Fisher Scientific) include this promoter.
- *T7* promoter. *T7* promoters are very strong promoters recognized by the T7 RNA polymerase (T7 RNApol) and not by the *E. coli* RNA polymerase. If this promoter is selected for protein expression in *E. coli*, T7 RNApol has to be expressed and regulated to assure the protein expression in the host strain. *T7* promoters include both constitutive and negatively regulated promoters that can be turned off by a repressor protein. pET vectors (Novagen) include the *T7* promoter system. *E. coli* strain BL21(DE3) contains a λ-DE3 lysogen on the chromosome which includes the T7 bacteriophage gene I, encoding T7 RNAPol under the control of a *lacUV5* promoter (a mutated version of *lac* promoter, stronger than the non-mutated version). The addition of IPTG induces the expression of T7 RNApol from the bacterial chromosome, which turns on transcription from vector *T7* promoter. This promoter yields a robust transcription, so it normally results in a high basal expression. As this could be detrimental for the host, BL21(DE3) strain has been modified to allow for a controlled basal expression. BL21(DE3)pLysS host strain (from Novagen) expresses T7 RNApol and also contains the pLysS plasmid, which constitutively expresses the bacteriophage T7 lysozyme. T7 lysozyme inhibits T7 RNApol and then suppresses the basal expression of toxic target proteins prior to induction.
- For industrial purposes and large production scales, particularly interesting is the induction of protein expression by temperature shifts, without addition of

chemicals as inducers. Even though thermal-inducible systems were developed in the late 1970s, studies on their usage at bioreactor scale are in progress. The temperature-inducible expression system, based on the pL and/or pR phage lambda promoters, is regulated by the thermolabile cI857 repressor which is unstable at temperatures higher than 37 °C. In this expression system, therefore, bacterial growth is carried out at 30 °C, and when high cell density is reached, the induction of the heterologous protein is achieved by increasing the culture temperature up to 40 °C (reviewed in Valdez-Cruz et al. 2010). Similarly, the major *E. coli* cold-shock promoter *cspA* has been employed in guiding the synthesis of recombinant proteins at low temperatures in batch fermentations (Vasina et al. 1998). The vectors based on cold temperature shift are derived from pUC18 and contain this *cpsA* promoter (Hayashi and Kojima 2008).

A high number of expression vectors, including diverse selection markers, different inducible promoters, tags, and other features, are available. Main characteristics of around 90 commercial expression vectors can be found in EMBL Heidelberg website, at https://www.embl.de/pepcore/pepcore_services/cloning/choice_vector/ecoli/vectorfeatures. The commercial supplier, the main features, and a short description of the main genetic elements included in each vector are described in this website. On the other hand, noncommercial available expression vectors with thousands of inserts are offered to the research community from Add-gene at https://www.addgene.org/.

6.3.4 Targeting of Proteins to Cellular Compartments in *E. coli*

Although for most applications it is desirable to achieve maximal production within the cytoplasm, targeting the protein to periplasm or extracellular milieu may offer an interesting alternative, especially for proteins containing disulfide bonds or when cytoplasmic production results in cellular toxicity or improper folding. Besides, periplasm contains a reduced level of proteases, and the purification of proteins from the periplasm, as from the culture medium, is simpler. Some disadvantages of periplasmic expression of proteins is the reduced space (20–40 % compared to cytoplasm) that leads to a saturated bacterial translocation systems during the recombinant protein overexpression.

The presence of disulfide bonds in recombinant proteins requires special attention. Disulfide bonds play an important role in protein stability, folding, and function (reviewed in Salinas et al. 2011). The reduced redox state of the cytoplasm of eukaryotic and prokaryotic cells is due to the presence of numerous disulfide bond reductases (e.g., thioredoxins and glutaredoxins). As such, any disulfide bond formed between two cysteines may be quickly and efficiently reduced back to thiolate state into the cytoplasm. To form stable disulfide bonds within proteins, the protein has to be segregated to other compartments. In *E. coli* and other Gram-negative bacteria, the required machinery is located in the periplasm (Denoncin and Collet 2013), and hence those proteins are usually targeted to this compartment (Salinas et al. 2011).

The pathway responsible for the oxidation of disulfide bonds in the bacterial periplasm comprises DsbA and DsbB proteins and quinones. The first step of disulfide bond formation consists in the oxidation of the recombinant protein Cys residues by DsbA with the consequent reduction of DsbA cysteines. Next, DsbA is turned back to its reduced state by DsbB, a periplasmic protein anchored to the inner membrane. As needed, DsbC modifies the incorrectly formed disulfide bonds by isomerization of mispaired disulfide bonds. Finally, DsbC is reduced to its active form by DsbD, also an anchored to the inner membrane protein (reviewed in Salinas et al. 2011).

As mentioned above, different transport systems allow protein translocation to the periplasm as either unfolded proteins or already folded in the cytoplasm (Georgiou and Segatori 2005; Choi and Lee 2004). In order to drive protein translocation to the periplasm, specific signal peptides located at the N-terminal end of the protein are employed. Many vectors include signal peptides to direct the target protein to periplasm, using the SEC-dependent pathway or the twin-arginine translocation (TAT) system – employed for secretion of folded proteins (Choi and Lee 2004). Commercial expression vectors such as pET22 (Novagen) or pBAD/gIII (Invitrogen, Thermo Fisher Scientific) carry signal peptides that allow targeting to the periplasm.

Alternatively, in order to improve the production in the cytoplasm of proteins carrying disulfide bonds, *E. coli* strains have been modified, leading to an oxidative cytoplasmic environment. An example of those strains is the Origami host strain (commercialized by Novagen), which contains deletions for glutathione (*gor*) and thioredoxin (*trxB*-) reductase genes, responsible for maintaining the reducing environment in the cytoplasm. Another example is SHuffle strain (commercialized by New England Biolabs), which presents the mentioned deletions and constitutively expresses a chromosomal copy of the *dsbC* gene, thus showing a proofreading mechanism of incorrect disulfide bonds in the oxidative cytoplasm.

6.3.5 Targeting Proteins for Detection and Purification

It is advisable to include a fusion tag to a recombinant protein, for three main reasons: (1) to follow up the protein expression – if the expression level is low and not easily detectable by SDS electrophoresis, the protein could be detected by Western blot using commercial anti-tag antibodies; (2) to facilitate fusion protein purification by employing a one-step affinity chromatography; and (3) to increase protein solubility.

Two main types of tags are frequently used: fusion proteins or short peptides tags. The former requires the production of a soluble protein containing the native conformation of the protein tag to achieve efficient purification. Moreover, in most cases correct folding of the protein tag influences positively the proper folding of the protein of interest. So, these tags are generally used to promote protein folding and to design simpler purification schemes. Peptide tags do not have generally folding requests, and this type of tags is preferred if recombinant protein is insoluble

(accumulates in inclusion bodies). Many commercial vectors include different peptide tags to be fused to the recombinant protein at the N-terminal or C-terminal ends. 6xHis-tag, cMyc, and FLAG are commonly used. A deeper description of tags and the associated purification procedure are described in Rosano and Ceccarelli (2014), Burgess and Deutscher (2009) and Lorsch (2015). The most frequently used tags are briefly described below.

6.3.5.1 Histidine Tag

His-tag (6xHis-tag), the peptide tag most frequently used in recombinant proteins, can be located at the N-terminal or C-terminal ends of the polypeptide chain. Proteins fused to this tag can be easily purified by IMAC (immobilized metal ion affinity chromatography), as His-tag binds with high affinity to metal ions bounded to resins (Ni^{+2} or Co^{+2}) (Spriestersbach et al. 2015). The elution of immobilized proteins can be performed using buffers with imidazole (100–500 mM) or low pH (4.5–6). The simplicity of this purification procedure, together with the fact that in most cases the tail tertiary structures neither influence the purification process nor interfere with the folding of the fused protein, justifies its success. Since binding of His-tag to resin is independent of protein conformation, it can be employed to bind denatured proteins and to perform on-column refolding by buffer exchange. This is the case of proteins recovered from inclusion bodies after solubilization with chaotropic agents (urea or guanidinium-HCl). Indeed, binding to IMAC resins is stronger under denaturing conditions as the His-tag becomes more exposed. IMAC medium has high binding capacity (5–40 mg of His-tagged protein/ml of media), is relatively low cost, and can be easily reused by stripping metal ions and recharging the resin. If necessary, His-tag can be removed by proteases if a protease cleavage site is included between the tail and the recombinant protein, but for many purposes this tag can be maintained (Carson et al. 2007). Particularly, due to its low immunogenicity, it is generally not necessary to remove it for antibody production.

6.3.5.2 Maltose-Binding Protein (MBP)

Recombinant maltose-binding protein produced in *E. coli* is a 42 kDa polypeptide chain carrying a 20-amino acid linker. This MBP-tag allows the purification of the protein of interest by affinity chromatography using a dextrin or amylose sepharose resin (Duong-Ly and Gabelli 2015). Purification is carried out in all commonly used aqueous buffers and mild elution is performed with maltose: an ideal situation to preserve target protein activity. This purification method generally achieves a 70–90 % purification index after a single purification step. The addition of MBP might also provide an increased expression level, proper folding, and higher solubility probably due to its hypothetical activity as molecular chaperone (Kapust and Waugh 1999; Duong-Ly and Gabelli 2015). MBP fusion proteins can be directed to the periplasm where the redox environment favors the formation of disulfide bonds. The most popular commercial vectors containing MBP fusion tag are the pMAL vectors (NEB), pMAL-c5X and pMAL-p5X, for cytoplasmic or periplasmic protein expression, respectively.

6.3.5.3 Glutathione S-Transferase (GST)

Glutathione S-transferase is a 26 kDa protein produced by the parasitic helminth *Schistosoma japonicum*. The addition of a GST-tag to the N-terminal end of the recombinant protein increases its solubility, probably as consequence of GST chaperone activity. The expressed fusion protein can be purified by affinity chromatography using glutathione covalently bound sepharose resins under mild elution conditions (glutathione 10–40 mM) (Smith and Johnson 1988; Schafer et al. 2015).

For proper GST binding to glutathione, GST has to be correctly folded. Besides, due to its large size, it is prone to degradation by proteases; thus, GST fusion proteins need to be quickly purified to minimize sample loss. As GST has four solvent-exposed cysteines, reducing conditions must be controlled (Kaplan et al. 1997); otherwise, GST can form homodimers. pGEX vectors include the GST-tag (GE Healthcare Life Science).

6.3.5.4 Thioredoxin (Trx)

Thioredoxin tag is a thermostable 12 kDa intracellular oxide-reductase from *E. coli* that can be overexpressed up to 40 % of the total cellular protein (LaVallie et al. 1993) and remains on the soluble form. Its ability to function as a solubility-promoting agent of the fused protein also may be due to its putative chaperone activity. One of the advantages of thioredoxin is that it accumulates at cytoplasmic membrane adhesion sites (Bayer et al. 1987), thus allowing Trx fusion proteins to be released from the cell by simple osmotic shock or freeze/thaw cycles. Furthermore, thanks to its high thermostability, it can tolerate many lysis conditions. Although Trx is not a purification tag per se, Trx variants were developed to provide more generic, efficient, and high-throughput purification scheme. Thus far, purification can be performed by affinity chromatography using avidin or streptavidin matrices to capture thioredoxin variants that have been modified to allow in vivo biotinylation (BIOTRX) or by using engineered forms of thioredoxin compatible with available affinity matrix (Lu et al. 1996). An example of the latter is a modified form of thioredoxin that comprises the Trx fused to a His-tag (called "His-patch Trx"). This tag allows an easy purification procedure by using metal chelate resins in a single step. Commercial vectors that include Trx tag are pTRXFUS and hpTRXFUS (Invitrogen).

6.3.5.5 Other Tags

6.3.5.5.1 Stimulus-Responsive Tags

A different group of fusion tags are stimulus responsive, i.e., some tags induce the reversible precipitation of fusion protein out of microbial culture medium when subjected to the proper conditions. The addition of β roll tags to a recombinant protein allows for its selective precipitation in the presence of calcium. The final products present a high purity and the precipitation protocol only takes a couple of minutes (Shur et al. 2013).

6.3.5.5.2 Cleaving and Self-Cleaving Tags

If the tag could interfere with protein activity or structure, after expression it needs to be removed from the final product. This step requires the use of a protease cleaving at a specific site located between the tag and the fusion partner. As mentioned, many commercial expression vectors include sites for protease cleavage. The most frequently employed are enterokinase, thrombin, factor Xa, and the tobacco etch virus (TEV) protease. In general, depending on the scale production, this is a relatively expensive step during protein production which generally requires further purification steps to eliminate both protease and tags. A promising alternative are "self-cleaving" tags, a special group of fusion tags that possess inducible proteolytic activity. Combined with appropriate affinity tags, they enable fusion protein purification, cleavage, and target separation to be achieved in a single step (reviewed in Li 2011).

6.3.6 Adapting Recombinant Coding Sequence to Host Codon Usage

The genetic code refers to the correspondence between the codons in the mRNA sequence and the amino acid residues in the polypeptide chain, and hence only concerns the coding region within the mRNA molecule. Most of the 20 amino acids included in protein biosynthesis are encoded by more than one codon. "Synonymous codons" are different codons that code for the same amino acid but in fact, they are not used at random or similarly. The usage of synonymous codons varies significantly inter- and intraspecies from bacteria to mammals and even within a specific gene. In *E. coli*, tRNA species have been quantified in different growth conditions. It was shown that frequently used synonymous codons present in highly expressed proteins are those corresponding to more abundant tRNAs. Instead, the so-called rare codons correspond to tRNAs found at low concentration in the cell and concomitantly are rarely present in mRNAs encoding highly expressed proteins. During protein biosynthesis, the presence of rare codons can lead to frameshifts, misreading, aborted peptide synthesis, or to a very low expression (Kurland 1991; Deana et al. 1998; Cortazzo et al. 2002). Then, for the expression of heterologous proteins in *E. coli*, codon usage needs to be considered in plasmid construct designing. Two approaches can be followed: (a) overexpression of rare tRNAs and (b) substitution of rare codons in the coding sequence by synonymous frequent ones:

(a) The first approach allows solving the different codon usage by expressing the recombinant construct in *E. coli* strains that overexpress rare tRNAs. For this purpose, several commercial strains are available. Two versions of BL21-CodonPlus strains (Agilent), RIL or RP strains, are recommended for expressing proteins according to their original source: RIL for proteins produced from AT-rich genomes and RP strain for proteins from GC-rich genomes. Similarly, Rosetta host strains (Novagen) are BL21 derivatives designed to overexpress tRNAs for AGG, AGA, AUA, CUA, CCC and GGA codons.

(b) The second approach involves the identification of rare codons related to *E. coli* codon usage present in coding sequence, the analysis of their distribution, and their substitution by synonymous frequent ones. Substitution could be carried out by directed mutagenesis, but the general trend is to obtain the synthetic coding sequence, "optimized" to include frequent synonymous codons according to *E. coli* codon usage. Besides rare codon substitution, the analysis of RNA secondary structures or nuclease-sensitive sites, GC content, and other elements is also taken into account in "coding sequence optimization" for improving translation efficiency and mRNA stability. Software is available to design an optimized sequence for expression in a particular host (OPTIMIZER, Puigbo et al. 2007 or OptimumGene from Genscript). Several companies offer the possibility of gene "optimization" taking many regulatory elements into account, to provide gene synthesis and cloning in an expression vector (IDT, GenScript, GeneArt Thermo Fisher Scientific).

Employing such strategies for codon optimization, the expression level of the recombinant protein can be increased hundreds of times, therefore increasing the production yield and significantly reducing the production cost (Fig. 6.5).

It is worth mentioning that the expression of some proteins from codon-optimized sequence can lead to a higher insolubility of the protein (Rosano and Ceccarelli

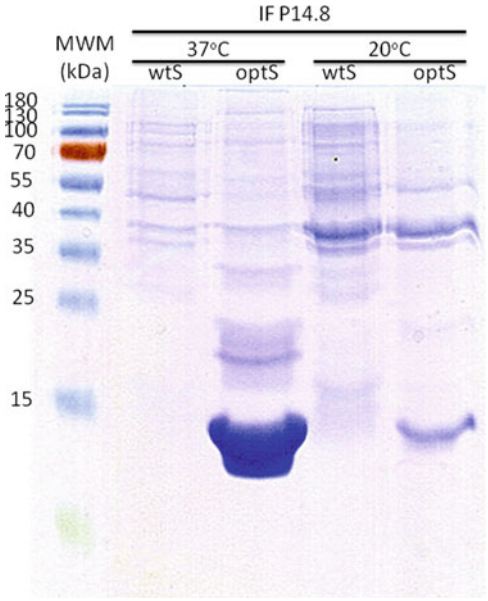

Fig. 6.5 Coding sequence optimization and protein solubility. Comparative analysis of P14.8 protein expression level, encoded by wild-type (wt) sequence vs optimized sequence (opt), both accumulated in the insoluble fraction (IF) at 20 or 37 °C induction temperatures. As shown, the higher yield was obtained when producing P14.8 protein encoded by the optimized sequence, at 37 °C in the insoluble fraction

2009) and its accumulation in inclusion bodies (IBs). This can be seen as unsuitable, and in this case, experimental conditions might be explored to avoid IBs formation. Alternatively, it can be seen as an advantage, and an opportunity to recover almost pure proteins, included in such structures. Inclusion bodies are described in Sect. 6.4.

The change of recombinant protein solubility suggests that a conformational change can occur after the modification of codon usage. Conservation of rare codons at a specific location in mRNAs can be related to folding events and protein secondary structure. In particular, in bacterial mRNAs, rare codons appear preferentially located toward the 5′ end, in β-sheets, loops, and disordered structures, whereas α-helices are encoded by frequent codons. As well, N-terminal regions in proteins are generally translated slower than C-terminal regions, whereas a clear decrease in translation speed is found at the start of secondary structures. Interestingly, in sequences encoding signal peptides for protein translocation to the periplasm, the presence of rare codons is relevant for protein translocation and proper folding in the periplasm (Zalucki et al. 2010, 2011). Therefore, it should not be "optimized."

Another recent example illustrating a role of rare codons in mRNAs is shown in Hess et al. (2015). Centered on epoxide hydrolases, the authors used structure-based homology to identify structural domains and then introduced slow codons in the linking domain sequence. These synonymous codon substitutions dramatically improved the solubility of two epoxide hydrolases in *E. coli* (Hess et al. 2015).

6.4 Inclusion Bodies: Structures to Avoid or to Recover?

During the production of recombinant proteins in *E. coli*, structures called inclusion bodies (IBs) frequently appear in the insoluble fraction. In fact, the formation of IBs is currently detected when analyzing the composition of soluble and insoluble protein fractions by electrophoresis. After bacterial growth, cells are recovered and lysed, and total proteins in the extract are fractionated by centrifugation, determining soluble and insoluble protein fractions (Figs. 6.5 and 6.6).

During bacterial growth, IBs are mainly localized in the cell poles. Accumulated in these structures, the recombinant protein can have a native-like structure, even maintaining enzymatic activity, or can have a misfolded inactive structure. Predictive software has been developed in order to predict aggregation propensity of proteins, which take into account the primary amino acid sequence of the polypeptide, experimental proteome data including information about cellular localization, and structural data (AGGRESCAN, de Groot et al. 2012). The aggregation propensity of bacterial proteins appears to be associated with their length, conformation, location, function, and abundance (Castillo et al. 2011; Zambrano et al. 2015). Other factors that affect the aggregation of recombinant protein expression in bacteria are temperature and growth rate, fusion to soluble protein tags, specific codon usage, tRNA availability, and general codon optimization in the heterologous expressed sequence (Cortazzo et al. 2002; Rosano and Ceccarelli 2009) as described above.

Experimental approaches are currently employed trying to avoid IB formation during protein production. Generally a solubility improvement is seen by

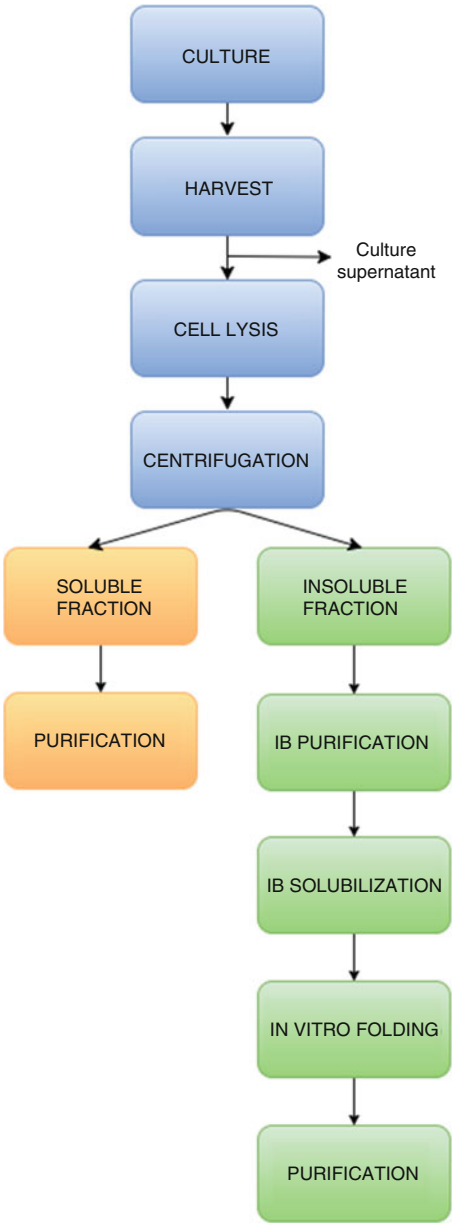

Fig. 6.6 Main steps of recombinant protein production in *E. coli*, from soluble or insoluble fractions

decreasing the temperature during bacterial growth after expression induction. With the same purpose, simultaneous expression assays are carried out employing different vectors, promoters, induction conditions, medium composition, temperatures, etc. The evaluation of hundreds of conditions requires the use of high-throughput cloning and screening methods, and allows determining the conditions for a higher

yield of soluble recombinant protein production (Correa and Oppezzo 2011, 2015; Saez and Vincentelli 2014) (Fig. 6.5).

However, for some purposes, the formation of IBs can also be considered as an advantage. They can be easily extracted, purified from cellular extracts, and are an almost pure source of recombinant protein. Recent information about their composition and structure, their use as an attractive approach to produce low-cost proteins, and other promising applications was reviewed in Ramon et al. (2014). For example, based on their particular structural and functional characteristics, IBs can be potentially exploited as naturally immobilized enzymes or as nanomaterials. Moreover, production of proteins as aggregates in IBs opened a new interesting perspective for diverse applications in Biomedicine, for example, they were used as an experimental screening model for the evaluation of protein aggregation inhibitors (Garcia-Fruitos et al. 2011).

Protein recovery from IBs requires their extraction and purification, followed by their solubilization, refolding (in vitro folding), and purification of the renatured protein. Different procedures have been established for different proteins, and for each one methods have to be experimentally adapted. Denaturation conditions can be mild or strong, and frequently use urea or guanidinium as denaturant. Renaturation is performed following different approaches, which comprise dilution (slow or quick) of the denaturant in a refolding buffer, dialysis, renaturation in the presence of BMe, or redox pairs, among others. The website "Refold database" (Chow et al. 2006) describes successful refolding procedures for more than 300 proteins and constitutes a useful tool when designing protein recovery from IBs. Other recent refolding strategies are described in Burgess (2009) and Ramon et al. (2014).

6.5 Industrial Production of Recombinant Proteins

Getting functional and pure proteins is a main goal for research and industrial laboratories. As mentioned, the natural sources of proteins are of limited access, imply bioethical considerations, or do not fulfill the current requirements of quantity or purity. With the modern advances in genomics, proteomics, and bioinformatics, the number of proteins being produced using recombinant techniques is exponentially increasing and seems to guarantee an unlimited supply of valuable proteins, so recombinant production is the method of choice for a growing number of proteins (Papaneophytou and Kontopidis 2014). Indeed, the demand of recombinant proteins has increased as more applications in several fields become a reality. Commercial recombinant proteins are applied in human and animal health and diagnostics, food processing industry, and industrial manufacture of detergents, textiles, leather, paper, pulp, polymers, and plastics. In that context, *E. coli* is widely used for massive production of many commercialized proteins. Other systems available for recombinant protein expression include Gram-positive bacteria, yeast, molds, mammalian, insect or plant cell lines, and transgenic animals or plants. Protein quality, functionality, posttranslational modifications, production speed, and yield are the

most important factors to consider when choosing the right expression system (Demain and Vaishnav 2009).

6.5.1 Recombinant Proteins in Human Medicine

Biopharmaceuticals are a group of therapeutics that include mainly human proteins, monoclonal antibodies, and engineered chimeric proteins obtained by recombinant technology. There are various classifications of these molecules, but for the purpose of this chapter, a functional classification is proposed (adapted from Leader et al. 2008):

1. A first group of proteins with enzymatic or regulatory activity, composed by two subgroups with different functions, those that replace a protein that is deficient or abnormal and those that augment an existing pathway. Some examples of therapeutical proteins of this group are used in endocrine and metabolic disorders and during the potentiation of immune responses and hematological pathways.
2. The second group consists of proteins with special targeting activities, which have been further categorized as: proteins interfering with a molecule or organism and proteins delivering other compounds to a specific target. Several generations of monoclonal antibodies, with a broad spectrum of therapeutic uses, are members of this group.
3. The third group is integrated by recombinant protein vaccines, which are usually used to protect against a deleterious foreign agent or to treat autoimmune diseases or cancer.
4. The last group comprises engineered proteins that provide a novel function or activity. The development of this group of proteins is driven by the rational use of the available research information to generate innovative products to treat human diseases.

As a whole, between 180 and 200 biopharmaceuticals have been authorized for commercialization by the FDA and EMA (regulatory agencies) from the USA and European Union, respectively (Walsh 2014; Baeshen et al. 2015).

Biopharmaceutical market has been evaluated in 160.5 billion dollars in 2014, and it represents about 20 % of the pharmaceutical market. This market is expected to grow at an annual growth rate of 9.6 % during 2015–2020, to reach global revenues of about 278 billion dollars by 2020 (www.psmarketresearch.com/market-analysis/biopharmaceuticals-market).

*E. coli i*s one of the preferred hosts for the expression of therapeutic proteins, as around 30 % (more than 50 products) of the approved biopharmaceuticals are currently being produced in this system. In Table 6.1 relevant examples of therapeutic proteins expressed in *E. coli* are presented and classified according to their function. At least one representative of each functional group of biopharmaceuticals is currently expressed in *E. coli*.

Table 6.1 Examples of biopharmaceuticals produced in *E. coli*

Functional classification	Protein	Product	INN	Therapeutic indication
Proteins with enzymatic or regulatory activity: replacing a protein that is deficient or abnormal	rh insulin	Humulin	Insulin human	Diabetes mellitus
	rh insulin analog (long acting)	Lantus	Insulin glargine	Diabetes mellitus
	rh insulin analog (fast acting)	Humalog	Insulin lispro	Diabetes mellitus
	rh growth hormone	Humatrope	Somatropin	GH deficiency in children
Proteins with enzymatic or regulatory activity: augmenting an existing pathway	G-CSF	Neupogen	Filgrastim	Neutropenia
	PEG-G-CSF (PEGylated G-CSF)	Neulasta/Neupopeg	Pegfilgrastim	Neutropenia
	GM-CSF	Leukine	Sargramostim	Autologous bone marrow transplantation
	IFN-α2b	IntronA	Interferon alfa-2b	Cancer, chronic hepatitis C
	PEG IFN-α2a (PEGylated IFN)	Pegasys	Peginterferon alfa-2a	Hepatitis C
	IFN-β1b	Betaseron/Betaferon	Recombinant interferon beta-1b	Relapsing/remitting multiple sclerosis
	rh tPA	Rapilysin	Reteplase	Acute myocardial infraction
Proteins with special targeting activity: interfering with a molecule or organism	rh IL-1 receptor antagonist	Kineret	Anakinra	Rheumatoid arthritis
	PEGylated rh GH analog (antagonist)	Somavert	Pegvisomant	Acromegaly
	Anti-TNF-α humanized and PEGylated antibody Fab´ fragment	Cimzia	Certolizumab pegol	Crohn's disease, rheumatoid arthritis
	Humanized IgG fragment that binds and inactivates VEGF-A	Lucentis	Ranibizumab	Neovascular (wet) age-related macular degeneration

(continued)

Table 6.1 (continued)

Functional classification	Protein	Product	INN	Therapeutic indication
Proteins with special targeting activity: delivering other compounds to a specific target	r IL-2-diphtheria toxin fusion protein that targets cells displaying a surface IL-2 receptor	Ontak	Denileukin diftitox	Cutaneous T-cell lymphoma
Recombinant protein vaccines	*Neisseria meningitidis* group B multicomponent subunit vaccine	Bexsero	Meningococcal group B vaccine, rDNA component, absorbed	Immunization against invasive meningococcal disease
Proteins providing a novel function or activity	Bacterial recombinant carboxypeptidase	Voraxaze	Glucarpidase	Treatment of toxic methotrexate concentrations in patients with impaired renal function
	Dimeric fusion protein with each monomer consisting of two thrombopoietin receptor binding domains and the Fc region of hIgG-1	Nplate	Romiplostim	Thrombocytopenia

Functional classification adapted from Leader et al. (2008). Data collected from Walsh (2014)
INN International Nonproprietary Name, *rh* recombinant human, *G-CSF* granulocyte-macrophage colony-stimulating factor, *PEG* polyethylene glycol, *GM-CSF* granulocyte-macrophage colony-stimulating factor, *GH* human growth hormone, *IFN* interferon, *tPA* tissue plasminogen activator, *IL* interleukin, *TNF* tumor necrosis factor, *VEGF* vascular endothelial growth factor

This expression system was the prevalent platform when the biopharmaceutical sector emerged in the 1980s, and it was followed by the implementation of *Saccharomyces cerevisiae* as an alternative expression host. Nowadays, mammalian cell lines (CHO, BHK, NS0, Sp2/0, and human cells) are the prevailing systems (55 % of the biopharmaceutical approved products), due to their ability to produce glycosylated proteins. Quantitatively, microbial production of biopharmaceuticals still predominates, and it has been estimated that from a total market of more than 26.500 kg of pure protein (active pharmaceutical ingredient), up to 68 % are produced in microbial systems. Insulin constitutes the bulk of biopharmaceuticals produced from microbial systems (*E. coli* and *S. cerevisiae*), whereas monoclonal antibodies (mAbs) constitute the principal products manufactured in mammalian systems (Walsh 2014).

In this area, both prokaryotic and eukaryotic systems are constantly evolving and competing to improve their properties to be selected as the platform of choice for protein drug production.

6.5.2 Other Commercial Applications

One biopharmaceutical example for animal health is bovine somatotropin (bST). The gene of this protein was first cloned in *E. coli* by Genentech Inc. This technology was licensed to Monsanto Corporation in 1981, and a commercial product named POSILAC was developed. This recombinant protein has the same amino acid sequence as the natural occurring hormone, with the exception of a methionine residue added to the N-terminus of the molecule. This protein is used in dairy cattle to increase milk production and serves as a tool to improve the efficiency and profitability of dairy producers. Although the use of bST is controversial and not worldwide approved, bST production facilities are one of the largest pharmaceutical-grade protein manufacturing plants in the world (Hammond 2007).

In addition to human health, the use of vaccines has had great impact on the ability to control and prevent infectious diseases in the veterinary field. The first vaccines consisted of whole pathogens, killed or attenuated, but today the recombinant subunit approach, i.e., to use only a defined subunits of the pathogen, is dominating the vaccine research in the search for new effective prophylaxis. The ability to use small, defined parts of a pathogen and produce it in a nonpathogenic host will increase the safety of future vaccines. The identification of antigens involved in protective immunity, the isolation of the encoding genes, and their recombinant production are driving the development of animal health biotechnology. Subunit vaccine candidates typically consist of surface proteins or exotoxins (for a review, please refer to Jackwood et al. 2008). Approved veterinary biopharmaceuticals are mainly vaccine-based products; that means 47 out of 52 products registered in the USA or Europe until 2012. Among these, three subunit vaccines and three proteins with enzymatic or regulating activities are produced in *E. coli* (Ryan and Walsh 2012).

In food processing industry, proteins expressed in *E. coli* are not so common, and other expression systems are normally chosen, particularly filamentous fungi such as *Aspergillus niger* or *Aspergillus oryzae* (Olempska-Beer et al. 2006). Nevertheless, bovine chymosin produced in *E. coli* was the first recombinant enzyme approved for use in food processing by the FDA (http://www.ecfr.gov/ Electronic Code of Federal Regulations Title 21, Chapter I, Subchapter B, Part 184.1685).

Other relevant area is diagnostics, where various recombinant proteins are used both for in vivo or in vitro techniques. Imaging agents include a broad group of labeled proteins that can be used in vivo to identify the presence or localization of a pathological condition. Besides, there are numerous in vitro diagnostics that use recombinant proteins or antibodies. Examples are HIV, hepatitis C, hepatitis B, syphilis, and Chagas disease immunoassays. These assays use recombinant antigens of the pathogen that are recognized by specific antibodies present in the serum of infected patients (Leader et al. 2008). Most of these proteins are produced using *E. coli* as expression host. The reason of this preference is that the use of recombinant antigens for these assays avoids pathogen culture and manipulation.

For each application described, expression systems must guarantee not only protein functionality but also a cost-effective industrial fabrication. For biopharmaceuticals and vaccines either for human or animal use, as well as for industrial enzymes employed in food processing, the expression platform must warrant the absence of hazardous contaminants and meet the requirements of regulatory agencies. For commercial vectors and cell lines used for industrial production, generally some royalties apply and each case should be accorded with the provider.

6.5.3 Industrial Process Development

In this section we briefly describe the process for recombinant protein production, from an industrial perspective. Once vector and gene design has ended, well characterized and documented, the production cell banks must be generated and stored below −20 °C. That is important to guarantee a consistent source of contaminant-free production cells throughout the life of the product. The storage is commonly accepted as two-tiered cell banks: (1) the master cell bank (MCB), composed of several vials, is made first, usually directly from an initial clone or from a preliminary cell bank derived from an initial clone, and (2) the working cell bank (WCB) is derived from one or more vials of the MCB. The WCB is typically used to provide cells for the manufacturing process. WCBs are generated from the MCB according to the producer's needs. When evaluating the production process, the amount of MCB and WCB vials has to be defined to assure a continuous product supply.

An industrial biotechnological production process normally includes a fermentation protocol (upstream) and one or more purification steps (downstream). The upstream process is defined as the process of culture expansion of the cells of the WCB until final harvest of the live cell batch at a desired optical density. This stage should be optimized for each protein, in order to achieve a product with an

acceptable yield that fulfills industrial requirements of purity and biological activity. Parameters affecting yield and quality are culture media composition, induction strategy, incubation temperature, agitation, oxygen availability, among others. The downstream section of a bioprocess refers to the part where the cell mass obtained in upstream stage is processed to meet established quality requirements. Downstream processing is usually divided into three main sections: cell disruption, a purification section, and a polishing step. After that, the protein has to be formulated in a buffer condition that improves the product shelf life. Downstream processes are generally more difficult to optimize, as each protein has different physicochemical and biological characteristics, so chromatographic methods should be defined case by case.

Production yield is critical for commercial-scale manufacturing bioprocesses. This parameter reflects recombinant protein production efficiency and determines the cost of a particular process. Although it is highly dependent of the target protein, the current average reported commercial-scale yield is 2.56 g/L (Rader and Langer 2015). Yield has been steady incrementally increasing since the 1980s, when values of 0.2 g/L were common. Some examples of yields achieved for heterologous protein expression in *E. coli* are 4.2 g/L for human granulocyte colony-stimulating factor (Baeshen et al. 2015) and 5.5 g/L for a consensus alfa-interferon (Demain and Vaishnav 2009).

6.6 Conclusions and Perspectives

The production of recombinant proteins is aligned with the concept of industrial sustainability, defined as "… a process of change in which the exploitation of resources, the direction of investments, the orientation of technological development, and institutional change are all in harmony and enhance both current and future potential to meet human needs and aspirations." Basically, this definition implies meeting the needs of the present generation without compromising the ability of future generations to meet their own needs (Gavrilescu and Chisti 2005). In fact, the recombinant production of proteins solves the problem of the source availability, is considered a bio-safe and green process, and confers the availability to modify amino acid sequences to design a new or a chimeric protein that better adjust to a desired function (Sanchez-Garcia et al. 2016).

Human medicine, as well as other important economic fields of activity, is full of examples of valuable recombinant proteins with a variety of functions and uses. This situation, currently very common, started 30 to 40 years ago with the recombinant human insulin production. *E. coli* as the leading model of recombinant protein production was the first system to get an approved biopharmaceutical and continues being prevalent in most fields of application based on its easy manipulation and cost-effective production method. As some of the principal limitations of the model are being solved, it is expected that in the next years, the spectrum of proteins produced in *E. coli* becomes wider, improving the availability of recombinant proteins.

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Heterologous Protein Expression in the Aspergilli: Overcoming Obstacles in the Secretory Pathway

7

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Abstract

Some *Aspergillus* species are widely used as cell factories for the production of heterologous proteins, showing, among other advantages, an exceptional secretion capacity. Due to the complexity of the translation and maturation of secretory proteins, several bottlenecks in this process are at the basis of low product yields. In this work, we review the most recent strategies aiming to improve the use of the aspergilli for recombinant protein production, centering on those which target the translation and secretion process.

7.1 Introduction

Aspergillus is a genus of filamentous molds belonging to the subphylum Pezizomycotina of the phylum Ascomycota. It comprises more than 230 species of ubiquitous fungi, many of which have a tremendous impact in human life, both positive and negative (Fig. 7.1). They are saprophytic fungi, being soil or decaying organic matter their primary ecological niche. Notwithstanding, they are able to colonize a wide spectrum of substrates at a wide range of conditions (10–50 °C; pH 2–11; up to 34 % salt concentration; Wheeler et al. 1991; Kis-Papo et al. 2003; Singh et al. 2014). This outstanding versatility is related to a prodigious ability to

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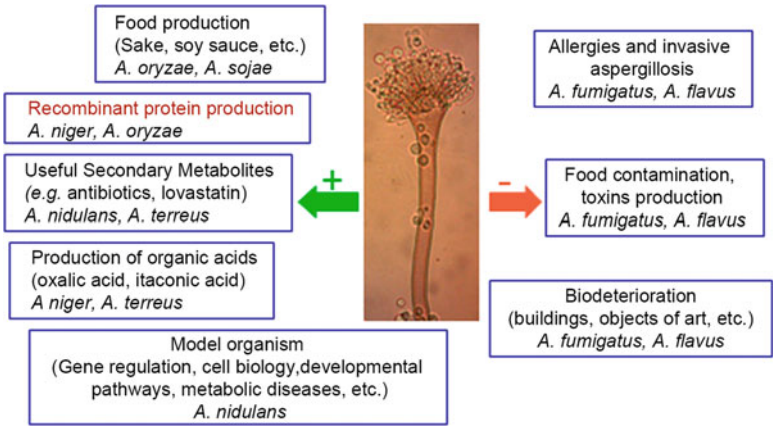

Fig. 7.1 Main positive and negative aspects for humans of different *Aspergillus* species. See main text for references (Photograph: an *A. nidulans* conidiophore)

produce and excrete high levels of enzymes capable of degrading pectic polysaccharides, cellulose, xylan, etc.

Many *Aspergillus* species are allergenic and opportunistic pathogens of plants and animals (including men) (Gugnani 2003; Pasqualotto 2009; Scazzocchio 2009). Invasive aspergillosis is a serious and difficult-to-treat condition, formerly caused mainly by *A. fumigatus* and to a lesser extent *A. flavus.* In the last two to three decades, the number of cases of invasive aspergillosis (now also by other *Aspergillus* species) has shown an alarming rise accompanying the advent of transplantation techniques and the concomitant immunosuppression as well as the emergence of AIDS. Plants do not escape to the scourge (Gugnani 2003). Some species, like *A. aculeatus*, *A. flavus* and *A. niger*, cause important economic losses for a number of common crop species including corn, cotton and peanuts. But the concern about food crop infection is not only economical: when growing on stored food, these aspergilli (especially *A. flavus* and *A. parasiticus*) secrete extremely toxic chemical substances as secondary metabolites, like aflatoxin B1, which is one of the most toxic and carcinogenic compounds known (Williams et al. 2004; Wild and Gong 2010). The aspergilli are also among fungi which cause biodeterioration to buildings and objects, being of great concern, for example, in museums and libraries (Sterflinger 2010).

Notwithstanding, the aspergilli have also been useful for humans for thousands of years. Apart from aflatoxins, other fungal metabolites are beneficial to humankind, like organic acids (e.g., citric acid, lactic acid, itaconic acid) (Magnuson and Lasure 2004; Papagianni 2007; Scazzocchio 2009; Steiger et al. 2013) or other secondary metabolites like antibiotics and pigments (Demain and Fang 2000; Scazzocchio 2009; Gunatilaka 2010). Various *Aspergillus* species *(A. oryzae*, *A. sojae*, *A. usami*, *A. awamori*, *A. kawachii*, etc.) have also been for more than 2000 years at the basis of the preparation of oriental foods like soy sauces, miso, fermented black soybeans and grain-based wines like sake (Campbell-Platt and

Cook 1989; Scazzocchio 2009). When grown on solid or semisolid substrates like the cooked grains or legumes used as feedstocks for these products, these molds produce and secrete many enzymes, including amylases, proteases, lipases and tannases, that hydrolyze macromolecules like starches, proteins, and fats into their constituent parts (glucose, dextrin, peptides, amino acids, and fatty acid chains). These are then utilized by yeast and bacteria as substrates for fermentation, which then yield the final products. It is worth mentioning that these fungi are classified as "generally recognized as safe" (GRAS), which means apt as a food additive (http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/).

A. nidulans as a model organism has allowed for the study of diverse phenomena, ranging from the control of gene expression at various levels, metabolic diseases, cell biology, developmental pathways, fungal pathogenicity, secondary metabolism, etc. (Davis 2003; Scazzocchio 2009).

Since 2005, when the genomes of *A. nidulans*, *A. fumigatus* and *A. oryzae* became available (Galagan et al. 2005; Machida et al. 2005; Nierman et al. 2005), a rapidly growing number of *Aspergillus* genomes have allowed for the development of an extensive number of comparative, functional and evolutionary studies, which may lead to the identification of genes putatively involved in the production of secondary metabolites, engineering of pathways to produce new metabolites, etc. (Kim et al. 2008; Andersen and Nielsen 2009; Wright et al. 2009; Liu et al. 2014).

The aforementioned capacity of the aspergilli to produce and excrete proteins, as well as their ability to grow by simple fermentation processes at high rates and to high biomass densities, on low-cost substrates, has led to their use as cell factories for the production of enzymes destined to the food, detergent, textile and paper industries (Fleissner and Dersch 2010; Meyer et al. 2011). However, even if successful in the production of fungal enzymes, the use of *Aspergillus* species for the heterologous expression of mammalian proteins is still modestly fruitful. The list of commercially produced enzymes in *Aspergillus* species included in Table 7.1 reflects this fact.

Hence, considerable effort has concentrated in developing strategies to raise production yields of correctly folded, active products by selecting mutants with improved characteristics or genetically engineered strains or expression plasmids. There are excellent, very comprehensive works which review the numerous strategies explored (Lubertozzi and Keasling 2009; Fleissner and Dersch 2010; Meyer et al. 2011; Ward 2012), many of which aim to increase heterologous gene expression. Even if many of these strategies have focused on expression at the level of transcription, those targeting the complex processes of translation and export of secreted proteins are far less explored. Along this complex process, secreted proteins must be translated, translocated to the endoplasmic reticulum (ER), properly folded and modified, and pass a series of controls so that only if properly assembled will the functional protein be exported to the Golgi. Any of these events may impose a bottleneck in the production process.

In this work, we review the most recent reports on strategies aiming to improve the use of the aspergilli as cell factories for recombinant protein production, centering on those which target the translation and secretion process. When possible, we

Table 7.1 List of industrially applied enzymes produced in *Aspergillus* species and their main uses

Enzymes of fungal origin produced in Aspergillus species			
Enzyme	Origin	*Aspergillus* host species	Industrial applications
Aminopeptidase	*Aspergillus* sp.	*A. melleus*, *A. oryzae*, *A. niger*	Food, pharmaceutical
AMP deaminase	*Aspergillus* sp.	*A. melleus*	Food
α-Amylase	*Aspergillus* sp.	*A. oryzae*, *A. niger*	Food, feed, pharmaceutical, textile, paper, detergent
Arabinofuranosidase	*Aspergillus* sp.	*A. niger*	Food
Asparaginase	*Aspergillus* sp.	*A. oryzae*, *A. niger*	Food
Carboxypeptidase	*Aspergillus* sp.	*A. niger*	Food
Catalase	*Aspergillus* sp.	*A. niger*	Food, textile, pharmaceutical
Cellulase	*Aspergillus* sp.	*A. niger*	Food, feed, paper, detergents
α-Galactosidase	*Aspergillus* sp.	*A. niger*	Food, pharmaceutical, detergents, paper
Glucanase	*Aspergillus* sp.	*A. niger*	Food, feed, textile
	Thermoascus sp.	*A. oryzae*	
Glucoamylase	*Aspergillus* sp.	*A. niger*	Food, feed, pharmaceutical, paper, textile
	Talaromyces sp.		
	Rhizomucor sp.		
Glucose oxidase	*Aspergillus* sp.	*A. oryzae*, *A. niger*	Food, pharmaceutical, textile
	Penicillium sp.		
Glucosidase	*Aspergillus* sp.	*A. niger*	Food, feed, biofuel
Glucosyltransferase	*Aspergillus* sp.	*A. niger*	Food
Hemicellulase	*Aspergillus* sp.	*A. niger*	Food, paper, textile
Inulase	*Aspergillus* sp.	*A. niger*	Food, paper, pharmaceutical
Laccase	*Myceliopthora* sp., *Polyporus* sp.	*A. oryzae*	Food, textile, pharmaceutical, biofuel
Lactase (β-galactosidase)	*Aspergillus* sp.	*A. oryzae*	Food, feed, biofuel
Lipase triacylglycerol	*Aspergillus* sp., *Candida* sp.	*A. niger*	Food, biofuel, detergents
	Fusarium sp.	*A. oryzae*	
	Rhizomucor sp.		
	Thermomyces sp.		
Mannanase (endo-1,4-β)	*Aspergillus* sp.	*A. niger*	Food, feed, paper
Pectin lyase	*Aspergillus* sp.	*A. niger*	Food, feed
Pectinesterase	*Aspergillus* sp.	*A. niger*	Food, feed

(continued)

Table 7.1 (continued)

Enzymes of fungal origin produced in Aspergillus species			
Enzyme	Origin	*Aspergillus* host species	Industrial applications
Pentosanase	*Aspergillus* sp.	*A. niger*	Food
Peroxidase	*Marasmius* sp.	*A. niger*	Food
Phospholipase A2	*Fusarium* sp.	*A. oryzae*	Food
Phospholipase B	*Aspergillus* sp.	*A. niger*	Food
Phytase	*Aspergillus* sp.	*A. niger*	Food, feed
	Peniophora sp.	*A. oryzae*	
Pectinase	*Aspergillus* sp.	*A. niger*	Food, wood preservation, water treatment
Protease	*Aspergillus* sp.	*A. niger*, *A. melleus*	Food, feed, detergent, leather pharmaceutical
	Rhizomucor sp.	*A. oryzae*	
Tannase	*Aspergillus* sp.	*A. niger*	Food, feed, pharmaceutical, ink, leather, water treatment
Xylanase	*Aspergillus* sp.	*A. niger*, *A. oryzae*	Food, feed, paper textile
	Talaromyces sp.	*A. niger*	
	Thermomyces sp.	*A. oryzae*	
Enzymes of mammalian origin produced in Aspergillus species			
Chymosin	*Calf*	*A. niger*	Food (cheese)
Lactoferrin	*Human*	*A. oryzae*, *A nidulans*	Food, feed, pharmaceutical
Phospholipase A2	*Pig pancreas*	*A. niger*	Food

have introduced a very concise revision of what is known about the underlying molecular mechanisms, with the hope that this will be of use for the rational design of novel improvement strategies.

7.2 Going Through the Secretory Pathway: Not an Easy Journey

Once achieving high levels of stable and translationally competent mRNA of the gene encoding for the heterologous protein of interest, a series of steps have to be fulfilled to achieve its correct secretion (reviewed in Delic et al. 2013). These steps include (1) targeting and translocation to the ER, (2) protein folding and maturation in the ER, (3) ER exit (i.e., ER to Golgi transport), (4) Golgi to cell surface transport and (5) plasma membrane fusion of secretory vesicles from where heterologous proteins are secreted. In the case of fungi, the protein of interest also has to go through the cell wall to reach the extracellular media. A variety of quality control mechanisms operate in the ER and downstream compartments of the secretory

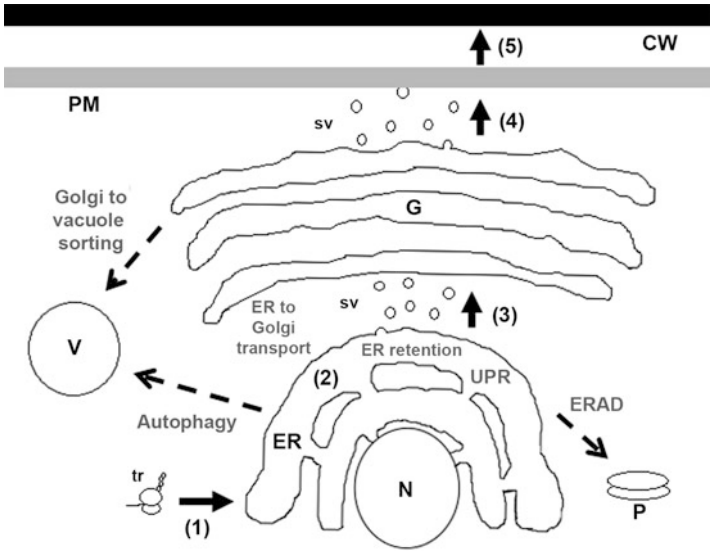

Fig. 7.2 Simplified overview of the fungal secretory pathway and associated bottlenecks in heterologous protein production. *Full arrows* indicate the different steps in the secretory pathway (*1*) ER translocation, (*2*) protein folding and maturation in the ER, (*3*) ER to Golgi transport, (*4*) Golgi to PM transport, (*5*) fusion of secretory vesicles with the PM for secretion to the extracellular media (passing through the CW). *Dotted arrows* indicate the different degradation routes followed by proteins that cannot overcome quality controls. *N* nucleus, *ER* endoplasmic reticulum, *P* proteasome, *V* vacuole, *G* Golgi compartment, *sv* secretory vesicles, *tr* translating ribosome, *PM* plasma membrane, *CW* cell wall

pathway to ensure the fidelity and regulation of protein expression. As a rule, only proteins that pass a stringent selection of quality control are transported to their final destination. If proper maturation fails, the aberrant products are degraded (Ellgaard et al. 1999). These quality controls are the major obstacles to be overcome by heterologous proteins. Taking this into account, we briefly review the different steps of the secretory pathway, the quality control mechanisms underlying it, and the strategies undertaken to overcome them (Fig. 7.2 and Table 7.2).

7.2.1 Targeting and Translocation to the ER: Entering the Secretory Pathway

The first step on the secretory pathway is ER translocation. The nascent peptide of a secretory protein is translocated into the ER through the recognition of a signal sequence. Most of the secreted proteins are targeted to the ER in a co-translational manner, which is usually mediated by the signal recognition particle (SRP). Proteins possessing signal sequences are recognized at the ribosome by SRP while they are translated. This triggers their delivery to the ER protein translocation channel, to be directly translocated into the ER (reviewed in Akopian et al. (2013); Nyathi et al.

Table 7.2 Summary of key processes, potential bottlenecks and solutions for proper heterologous protein secretion

	ER translocation	ER maturation	ER to Golgi transport	Golgi to PM transport	Cell wall passage
Key processes	Secretory signal sequence recognition and protein translocation	Proper protein folding and posttranslational modifications	Recognition by specific ER cargo receptors for efficient ER export	Loading onto secretory vesicles on route to the PM	Release onto the extracellular media
Potential bottlenecks	Inefficient recognition by ER translocation machinery (e.g., SRP)	Protein misfolding, leading to activation of the UPR leading to degradation by ERAD or autophagy	Increased retention rate by ER cargo receptors	Rerouting to the vacuole	Cell wall adhesion
Solution	Fusion to known secreted proteins	Constitutive induction of the UPR pathway	Deletion of ER cargo receptors	Deletion of Golgi vacuolar-sorting receptors	Addition of 0.1 M phosphate buffer to the culture medium
	Fusion to known signal sequences	Overexpression of UPR-related genes and deletion of genes encoding ERAD components			
		Deletion or conditional expression of autophagy-related genes			
		Deletion of endogenous highly expressed secretory proteins to diminish the ER protein burden			

See main text for references

(2013)). Little is known about this signal sequences in the aspergilli. A recent work by Madhavan and Sukumaran (2015) reported seven signal peptides from highly secreted proteins in *A. niger*, *A. nidulans*, *A. terreus* and *A. awamori* based on these species secretome data (Han et al. 2010; Ferreira de Oliveira et al. 2011; Saykhedkar et al. 2012). These were evaluated in the yeast *Kluyveromyces lactis* for secretion of enhanced green fluorescent protein (EGFP) and recombinant human interferon. The authors found that all secretion signals were effective for the secretion of heterologous protein in *K. lactis* and, interestingly, codon optimization (see below) of these signal sequences and incorporation of Kex2 protease cleavage site resulted in improved transcriptional and secretion efficiency. Heterologous signal sequences have also been successfully used in *A. oryzae*. It has been reported that when a 28-amino-acid region within the propeptide of *Rhizopus oryzae* lipase (N28, Hama et al. 2008) was fused to a llama-variable heavy-chain antibody fragment (V_{HH}), secretion yield of V_{HH} in *A. oryzae* was about 3.5-fold higher compared with the non-fused construction (Okazaki et al. 2012). These findings highlight the importance of studying signal sequences, in order to develop expression strategies to improve extracellular production of heterologous proteins. The efficient secretion of heterologous proteins fused to carriers may also be explained by the correct translocation in the ER of the latter, which leads the heterologous protein into this compartment.

It is worth mentioning that most of the current knowledge regarding SRP refers to mammalian and yeast SRP, but the study of this key ribonucleoprotein complex has been left behind in filamentous fungi. This is quite curious considering the great biotechnological importance of this key mechanism for secretory proteins. A deeper understanding of this mechanism will be of great advantage for the design of new strategies to improve co-translational ER translocation for secretory protein production. Our group is currently addressing an in silico study of the SRP composition in 21 sequenced aspergilli (Veyga M, Ramon A, Sanguinetti M, Iriarte A, unpublished results). We have found that SRP composition seems not to be completely conserved in the genus. SRP54 (the most conserved component of SRP), SRP68 and SRP72 are present in all of the species. On the contrary, SRP14, SRP21, and SRP65 are lacking in some of the genomes. SRP54, SRP65, SRP68, and SRP72 constitute, together with the central region of the 7SL RNA, the S domain which is responsible for the recognition of the signal sequence in the proteins destined to the secretory pathway. SRP21 (SRP9 in mammals) and SRP14 are part of the Alu domain, together with the terminal portion of the 7SL ARN. This domain imposes a translational arrest which is believed to be necessary for the efficient translocation of proteins to the ER (Hann et al. 1992; Rosenblad et al. 2004; Akopian et al. 2013). We still don't know which are the functional relevance of these differences. We have determined experimentally that SRP54, the most conserved unit of the SRP complex, is essential for cell function (Veyga M, Sanguinetti M, Ramon A, unpublished results).

7.2.2 Protein Folding and Maturation, a Prerequisite for ER Exit: Alleviating ER Stress

Correct protein folding and maturation is an essential step for proper ER exit. Several chaperones and foldases participate in these processes. Foldases catalyze covalent changes, such as disulfide bond formation and proline isomerization, which are essential for obtaining a functional conformation. Molecular chaperones assist proteins that transiently and noncovalently bind to nonnative proteins to prevent nonproductive protein–protein interactions and thus promote correct folding. Folded proteins can then undergo distinct modifications such as glycosylation, disulfide bridge formation, phosphorylation, and subunit assembly (Conesa et al. 2001).

Improper folding and maturation lead to ER stress, which activates different quality control mechanisms to alleviate this stress and reestablish protein homeostasis. This can be considered the main bottleneck on the secretory pathway for heterologous protein production. Quality control mechanisms include ER retention, the activation of the unfolded protein response (UPR) (Heimel 2015) and/or the ER-associated degradation (ERAD) (Nishikawa et al. 2005) and autophagy (Pollack et al. 2009).

The accumulation of potentially toxic misfolded proteins in the ER triggers UPR activation and the induction of genes important for protein folding in the ER, ER expansion and transport from and to the ER. Along with this adaptation, the overall capacity for protein secretion is markedly increased by the UPR. In the aspergilli, various approaches to employ the UPR for improved production of homologous and heterologous proteins have been investigated by modulating the expression of genes induced during UPR (reviewed in Heimel 2015). As an example, the constitutive induction of the UPR pathway in *A. niger* var. *awamori* was achieved by expressing the activated form of the transcription factor HacA (which induces expression of UPR-related genes by binding to a specific region of their promoters). This induction enhanced the production of *Trametes versicolor* laccase by up to 7-fold and of bovine preprochymosin (prepro-CHY) by up to 2.8-fold (Valkonen et al. 2003). Furthermore, increased production and secretion of heterologous proteins in *A. niger* was observed when UPR-related genes were overexpressed and genes encoding ERAD components (see below) were in addition deleted (Sagt et al. 2008; Heimel 2015).

If UPR is not sufficient to relieve stress, the ERAD pathway is activated (Nishikawa et al. 2005; Vembar and Brodsky 2008). This pathway is a conserved mechanism to remove misfolded proteins from the ER by targeting them to the proteasome for degradation. The downregulation of ERAD components (such as the ubiquitin–proteasome system) has proven to be a successful strategy to enhance recombinant protein production. Carvalho et al. (2011) observed a positive effect of downregulating ERAD components in the intracellular production of a glucoamylase–glucuronidase (GlaGus) fusion, increasing yields up to 6-fold. This positive effect might be due to an increase in the time window for proper protein (re)folding in the ER before being targeted for degradation, as proposed by Heimel (2015).

Notwithstanding, Carvalho et al. (2011) observed that extracellular production levels were not improved and proposed that other mechanisms besides ERAD might be of importance in the clearance of misfolded proteins to help the cells cope with ER stress. Regarding this, autophagy (Pollack et al. 2009) has been reported to serve as an alternative/complementary route for ERAD and appears to degrade misfolded proteins that accumulate in the ER lumen (Kario et al. 2011). The autophagic process consists of autophagosome formation, fusion to vacuoles and degradation of autophagic bodies (Pollack et al. 2009). The study in *A. oryzae* of Kimura et al. (2011) provided the first evidence of a role for autophagy in the degradation of misfolded secretory proteins from the ER to vacuoles in filamentous fungi. Moreover, Yoon et al. (2013) examined the production of bovine chymosin (CHY) in several autophagy gene disruptants of *A. oryzae* and found that the production levels of this protein increased up to 3-fold compared to the control strain, demonstrating that the disruption of autophagy-related genes enhances the production levels of heterologous proteins.

Another approach to alleviate ER stress due to overexpression of heterologous proteins is the reduction of highly expressed endogenous secretory proteins, which can compete with heterologous proteins within the secretory pathway, resulting in a reduced production of the latter. In addition, endogenous proteins secreted in the culture medium interfere with the purification of the produced heterologous proteins. This strategy was applied in *A. oryzae* through the silencing of three α-amylase genes by RNA interference (RNAi) in an *A. oryzae* CHY-producing strain, resulting in a reduction in α-amylase activity and an increase in CHY production in the culture medium (Nemoto et al. 2009). Recently, Kitamoto et al. (2015) deleted the amylase genes in *A. oryzae* strain KBN616, completely eliminating all detectable α-amylase activity.

7.2.3 ER Exit/ER to Golgi Transport: Keeping Moving Forward

In spite of the relative success of the approaches mentioned before, secretion of heterologous proteins in recombinant strains remains far below the secretion of endogenous proteins, indicating that other bottlenecks for heterologous protein production and secretion must be overcome before reaching maximal production capacity (Hoang et al. 2015). Inefficient transport of heterologous proteins between the ER and the Golgi compartment is believed to be one of these. Cargo proteins are transported between these compartments in coat protein complex II (COPII)-coated vesicles which transport secretory proteins in the anterograde direction (Geva and Schuldiner, 2014). Protein-sorting receptors participate in the selection of folded cargo for COPII-mediated ER export. After incorporation into COPII transport vesicles, protein-sorting receptors release bound cargo in pre-Golgi or Golgi compartments, and receptors are then recycled back to the ER for additional rounds of cargo export (Dancourt and Barlowe 2010).

Little is known in filamentous fungi about these receptors and their role in the ER to Golgi export. Recently Hoang et al. (2015) addressed the role of cargo receptors

in the heterologous protein production in *A. oryzae*. They studied the involvement of two putative lectin-like cargo receptors, AoVip36 and AoEmp47, in the secretion of heterologous proteins (proCHY and EGFP) expressed in fusion with the endogenous glycosylated enzyme α-amylase as the carrier (AmyB-proCHY and AmyB-EGFP). It was determined that AoVip36 and AoEmp47 interfere with the secretion of carrier-fused heterologous proteins by increasing the ER retention rate, a conclusion that is consistent with the increased secretion of carrier-fused proCHY and EGFP in the *Aovip36* and *Aoemp47* deleted strains. Strikingly, overexpression of the cargo receptors has different effects on AmyB-proCHY and AmyB-EGFP secretion. As expected, overexpression of both cargo receptors hampered the increased secretion of AmyB-proCHY observed in the *Aovip36* and *Aoemp47* deleted strains. Notwithstanding, overexpression of AoEmp47 significantly decreased AmyB-EGFP secretion, whereas AoVip36 had the opposite effect.

7.2.4 Golgi to Cell Surface Transport: Almost There!

Once proteins leave the ER and enter the Golgi, additional modifications can take place such as further glycosylation, peptide processing and phosphorylation, among others. Then they are packed again in secretory vesicles and directed to the plasma membrane from where they are secreted (Prydz et al. 2013). In some cases, proteins do not reach the extracellular space, but are targeted to intracellular compartments such as the vacuole, either to become resident proteins or to undergo proteolytic degradation. This routing of protein to the vacuole is mediated by vacuolar-sorting receptors. In *Saccharomyces cerevisiae*, this process is mediated by Vps10p, a transmembrane protein that cycles between the late Golgi and the endosome (Marcusson et al. 1994). Most importantly, this receptor has also been implicated in the targeting and delivery of recombinant proteins from the late-Golgi compartments to vacuoles for degradation (Hong et al. 1996).

Thus, rerouting of these proteins from the Golgi to the vacuole could also hinder heterologous protein secretion in filamentous fungi. To address this, Yoon et al. (2010) deleted the homologue of Vps10p in *A. oryzae* and investigated the extracellular production levels of recombinant proteins CHY and human lysozyme (HLY) in this background. They found that the *Aovps10* deletion increased the extracellular production levels of CHY and HLY by 3- and 2.2-fold, respectively. This suggested a role for *Aovps10* in the regulation of heterologous protein secretion in *A. oryzae* and its possible participation in the vacuolar protein degradation through the Golgi apparatus.

Finally, the a priori easy step of a heterologous protein going through the cell wall is not always such. In the late stage of a submerged culture of *A. oryzae*, the disappearance of α-amylase activity has been reported, probably due to the adsorption of the enzyme onto a cell wall component, most likely chitin (Sato et al. 2011). Hence, heterologous proteins may adsorb to the cell wall, resulting in an apparently reduced production level. In the case of α-amylase, the addition of 0.1 M phosphate buffer liberated the adsorbed enzyme from the cell wall (Sato et al. 2011; Tanaka and Gomi 2014).

7.3 Fusion to Carrier Proteins or to Modified Control Sequences: It Works, But Why?

The fusion of partial or complete carrier proteins to the N-terminus of heterologous proteins, as mentioned before, is a commonly used strategy to successfully obtain recombinant products in *Aspergillus*. These carriers are usually abundantly secreted fungal enzymes, such as glucoamylase, α-amylase and cellobiohydrolase. Examples are presented in Table 7.3. In most cases, KEX2 endopeptidase sites are included in linkers between the carrier and the protein of interest, so that the carrier can be excised by in vivo cleavage of the desired product.

This approach seems to be efficient in alleviating many of the aforementioned bottlenecks encountered in the secretion process. As mentioned above, the fusion of heterologous proteins to carrier N-terminal peptides holding signal sequences (Hama et al. 2008; Okazaki et al 2012), or also to whole proteins, may improve the recognition by SRP and hence their targeting to the ER. As proposed by Hoang et al. (2015), a complex interaction may also exist between different cargo receptors and the carrier-fused heterologous proteins they direct to the vesicular traffic (see above). Carrier proteins seem to interact with folding and quality control mechanisms in the ER, as well. A DNA microarray analysis comparing gene expression patterns of two *A. oryzae* strains expressing CHY, either alone or fused to α-amylase (AmyB) as a carrier, revealed that the carrier-fused version had an enhanced

Table 7.3 Examples of recombinant proteins fused to carrier peptides or 5′UTR-modified sequences for efficient expression in *Aspergillus* species

Fusion to carrier peptides			
Recombinant protein	**Carrier peptide**	**Host**	**References**
Neoculin	*A. oryzae* α-amylase AmyB + KEX2 cleavage site	*A. oryzae*	Nakajima et al. (2006)
Hepatitis B surface antigen	*A. niger* glucoamylase catalytic domain GlaA G2	*A. niger*	James et al. (2012)
Bovine chymosin	*A. awamori glaA*	*A. awamori*	Ward et al. (1990)
A. awamori and *A. fumigatus* phytases	*A. awamori* glucoamylase *glaA* + KEX2 cleavage site	*A. awamori*	Martin et al. (2003)
Human lactoferrin	N-terminus *A. awamori* glucoamylase	*A. awamori*	Ward et al. (1995)
Rhizopus oryzae ROL lipase	N28 of *R. oryzae* ROL lipase propeptide	*A. oryzae*	Hama et al. (2008)
Fusion to modified 5′UTRs			
Recombinant protein	**5′UTR**	**Host**	**References**
E. coli uidA (GUS) gene	*Hsp12* 5′UTR	*A. oryzae*	Koda et al. (2006)
E. coli uidA (GUS) gene	Engineered P-No8142/*enoA* 5′UTR	*A. oryzae*	Koda et al. (2004)
A. niger l accase-like multicopper oxidases	Engineered *glyA 5′UTR*	*A. niger*	Tamayo-Ramos et al. (2013)

production level of ~2-fold (Ohno et al. 2011). The expression of the carrier-fused protein led to an upregulation of genes involved in ER protein folding and secretion, including those known to be upregulated by UPR, like ER chaperones and foldases (*bipA*, *pdiA*, *clxA*, etc.). An upregulation of genes involved in ER protein folding was also reported in other works, both for noncarrier-fused CHY in *A. nidulans* (Sims et al. 2005) and for carrier-fused tPA (tissue plasminogen activator) in *A. niger* (Guillemette et al. 2007). This would suggest that the expression of a non-fused heterologous protein would induce the UPR (which is easy to accept, if high amounts of a nonproperly recognized polypeptide needs to be traduced, folded, modified and secreted along the ER), but when the same peptide is carrier-fused, the UPR induction would be stronger, which would then help to attain the proper folding of the protein and its release from the ER.

Most expression systems for the production of recombinant proteins imply an inducible promoter and a 5′UTR after which the coding region of the protein of interest is placed. As mentioned above in *Aspergillus* systems, the molecular mechanisms involved in the regulation at the level of transcription of the promoters have been extensively studied and exploited to achieve better protein yields (Fowler et al. 1990; Gouka et al. 1996; Ganzlin and Rinas 2008; Bando et al. 2011). Much less is known about the function of 5′UTRs in these systems and how they could exert a positive role in the translational process. Notwithstanding, some works report evidence that the use of 5′UTR sequences from specific endogenous genes (*A. oryzae* heat shock protein *Hsp12;* Koda et al. 2006) or the creation of synthetic 5′UTRs by combining sequences from *Aspergillus* strongly expressed genes (Koda et al. 2004) or even introducing sequences which are known to efficiently enhance translation from plant 5′UTRs (Tamayo-Ramos et al. 2013) may result in augmented recombinant protein yield. An engineered *gly*A 5′UTR was recently reported (Tamayo-Ramos et al. 2013), which improves the production levels of five endogenous laccases in *A. niger.* The authors introduced in the *gly*A 5′UTR sequences which are known to efficiently enhance translation from plant 5′UTRs, like GAA or CAA repeats, to find that these modifications in the 5′UTR of the *gly*A expression system effectively improved yields at the level of protein activity and protein secretion.

7.4 Tailoring Glycosylation: Not All Is About Yield

There are two aspects that make glycosylation an important step in the secretion of heterologous proteins in fungi. On one hand, glycosylation is one of the processes which take place during the secretory pathway in the ER (Cabral et al. 2001; Wujek et al. 2004) and has been proven to be critical to achieve high yields of stable, functional protein (van den Brink et al. 2006; Gasser and Mattanovich 2007). It is believed that this may be related to the fact that erroneously glycosylated heterologous proteins might lack some of the characteristics needed to be recognized as genuine secreted proteins. On the other hand, adequate glycosylation is fundamental in the case of heterologous proteins to be used in therapeutics (Gemgross 2004;

Solá and Griebenow 2010; Liu 2015) and must mimic as much as possible the mammalian kind, so that the recombinant product is biologically active, stable and non-immunogenic. In this sense, filamentous fungi present some advantages with respect to yeast, where protein hyperglycosylation usually occurs (Piirainen et al. 2016). Notwithstanding, in contrast to mammals, glycosylation in filamentous fungi is of the high-mannose type and glycans do not undergo terminal sialylation (Deshpande et al. 2008; Kainz et al. 2008).

In this sense, strategies aiming to modify glycosylation patterns or levels have been developed, so that proteins are not only efficiently produced and secreted but also are apt and competent for its use in therapeutics. The methods used to attain these objectives include the improvement of glycosylation sites by mutagenesis (van den Brink et al. 2006) and the "humanization" of fungal glycosylation pathways by introducing enzymes capable of rendering glycans more similar to those found in mammals or by eliminating enzymes involved in the synthesis of typically fungal glycans (Kasajima et al. 2006; Kainz et al. 2008).

It is also worth mentioning that the most commonly used carrier proteins are glycosylated proteins. It has been proposed that the success in carrier-fused protein secretion may be in part due to the fact that glycosylation of carriers may promote their interaction with lectin-type cargos, which may then facilitate the transport of the carrier-fused proteins through the secretory pathway (Hoang et al. 2015).

7.5 Codon Optimization: Adapting to the Host's Codes

Because synonymous codons do not change a protein's sequence, synonymous changes have long been considered "silent" or "neutral" at the level of protein function. However, there is growing evidence that each organism has a nonrandom preferred codon usage, which influences every stage in protein biogenesis, including transcription, translation, co-translational folding, secretion and posttranslational modifications (for a review, see Chaney and Clark 2015). For each organism, both eukaryotic and prokaryotic, there is a set of "rare" and "frequent" codons which correlates with the genome % GC bias (Ermolaeva 2001; Chen et al. 2004). For several species, it has been demonstrated that highly expressed genes tend to use a subset of codons which usually match the most abundant tRNAs (Ikemura and Ozeki 1983; Elf et al. 2003; Guo et al. 2012; Iriarte et al. 2012). This bias in codon usage and tRNA content seems to control local translation rate, which in turn controls protein folding (Cortazzo et al. 2002; Fedyunin et al. 2012; Chaney and Clark 2015; Buhr et al. 2016). As an indirect effect, translational pauses would allow the time for co-translational interaction with chaperones or other molecular partners. Following these premises, codon optimization (i.e., changing codons in the native sequence of the target protein by their most frequently used counterparts in the host species) is a commonly employed method in heterologous protein expression (Dong et al. 2015; Gao et al. 2015; Ben Azoun et al. 2016). Notwithstanding, this technique may fail if slow codons are necessary, for example, for proper protein folding. Alternatively, the method of codon harmonization (Angov et al. 2011; Hu et al. 2013)

aims to imitate codon usage frequencies from the native host, changing the DNA sequence so that in the heterologous system, these frequencies will be maintained. It is worth mentioning that there are a number of free codon optimization tools available online (Fuglsang 2003; Grote et al. 2005, Chin et al. 2014).

Synonymous codon usage could also alter protein synthesis not because of the decoding of the mRNA sequence by the ribosome but because the mRNA structure or processing is changed. Since a synonymous change implies a difference in RNA sequence, the appearance of stable loops could cause a slowing down of translational rate, a change in splice sites, a difference in posttranslational modifications, etc. (for excellent reviews, see Shabalina et al. 2013; Fahraeus et al. 2015).

Codon optimization as a method to improve the heterologous expression of foreign genes was successfully employed in *Aspergillus* in a number of cases. When the gene coding for alpha-galactosidase of the plant *Cyamopsis tetragonoloba*, expressed in *A. awamori* under the control of 1,4-beta-endoxylanase A (*exlA*) promoter, protein mRNA became detectable when the gene with an optimized *S. cerevisiae* codon preference was expressed (Gouka et al. 1996). Koda et al. (2005) successfully expressed a synthetic version of the *Solanum tuberosum* alpha-glucan phosphorylase (GP) gene in *A. niger*. In this case, 39 % of the codons were optimized, adapting codon usage and A + T content to those of the host. This resulted in a significant improvement in the level of the GP mRNA and a dramatic increase in the quantity of GP protein produced, which accounted for approximately 10 % of the total soluble protein. Lichtenberg et al. (2011) also report the successful production of a phytase from the opportunistic pathogen *Citrobacter braakii* in a safety-improved strain of *A. oryzae.* The enzyme was produced from two synthetic codon-optimized genes, which shared 85 % identity on DNA level and 70 % and 73 % identity to the wild-type sequence.

What do we know about codon usage in the genus and its effects at a functional level? We have determined the codon usage patterns of eight completely sequenced genomes of members of the genus (Fig. 7.3) (Iriarte et al. 2012) and found that there is a set of 21 optimal codons conserved among all members of the genus. The great majority of preferred triplets match the respective cognate tRNA with more copies in the each genome. This suggests that the triplets that we detected are effectively translationally optimal but also that they are highly conserved across the studied species.

The effect of codon optimization at the level of mRNA stability was investigated by Tokuoka et al. (2008) by expressing the mite allergen Derf7 as a model protein in *A. oryzae*. Wild-type and codon-optimized sequences were expressed under the control of a strong promoter, with their own signal sequences or fused to a carrier (see below), the *A. oryzae* glucoamylase GlaA. The authors found a notorious increase in both mRNA and protein levels for the codon-optimized version, while for wild-type sequences, they detected the occurrence of truncated, unstable transcripts. It was proposed that in the native messengers, some sequences would be interpreted in *A. oryzae* as cryptic polyadenylation signals, thus leading to the production of aberrant mRNAs lacking termination codons. These mRNAs were unstable and presumably eliminated by the eukaryotic nonstop mRNA degradation

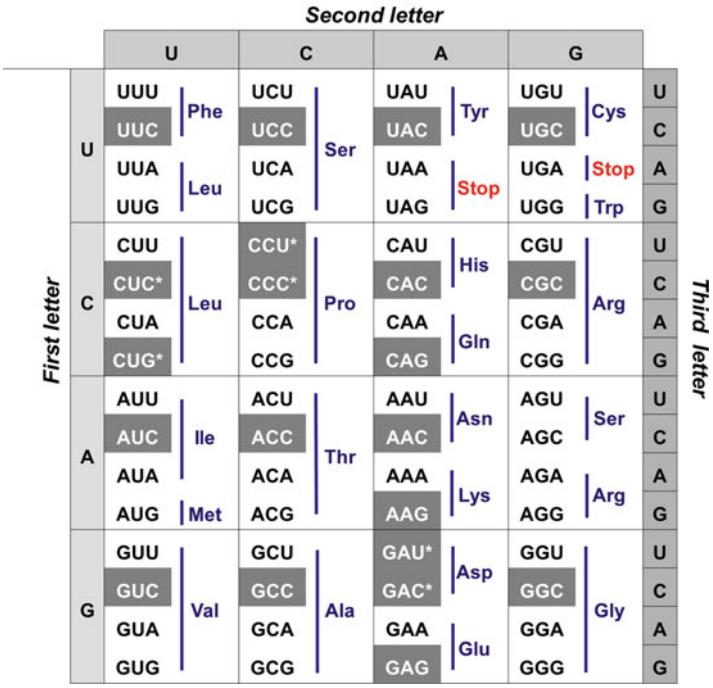

Fig. 7.3 Codon usage in the genus *Aspergillus*. Preferred codons are shown in *white* letters over *dark* background. When preferred codon usage is different in different species, codons are marked with a * (See Iriarte et al. 2012 for details)

mechanism. In a later work, the authors reviewed theirs and others' results which signaled an increase of mRNA levels as a result of codon optimization (Tanaka et al. 2012). In brief, they point out that most low-frequency codons in the aspergilli contain A or U in the third position (Iriarte et al. 2012). So, when optimizing the sequence, the % GC would increase in the modified sequence, and as a consequence, AU-rich sequences typical in polyadenylation signals would be abolished. Alternatively, the presence of slow codons in non-optimized sequences could cause ribosome stalling, leading to mRNA degradation by a nonstop decay mechanism (Tokuoka et al. 2008).

To summarize, in the aspergilli (and other filamentous fungi), codon optimization could improve heterologous gene expression by acting on mRNA stability, as well as improving translational efficiency. Even if there are no direct proofs of the role of codon usage at the level of local rate of translation and protein folding in *Aspergillus*, it is worth mentioning some interesting recent results (Zhou et al. 2013, Yu et al. 2015) showing that protein folding and function may be affected by codon usage in *Neurospora crassa*, a close relative of the aspergilli. By using a *N. crassa* cell-free translation system to monitor the rate of translation elongation, they show a direct relationship between preferred or rare codons and the rate of translation elongation, as well as an effect of codon usage on co-translational protein folding.

7.6 Conclusion

The production of secreted heterologous proteins in the aspergilli, as in other organisms, has proven to be a road full of bottlenecks. Most of them are linked to the complex process of translation and export of these proteins, but the strategies developed to surpass these barriers usually regard each step in this complex process as an isolated phenomenon. Notwithstanding, in this review, we have intended to show how the physiological processes that are at the basis of these bottlenecks are deeply interconnected. This means that when one of them is engineered to improve protein yields, one cannot be sure of the final results. It would be interesting to combine different genetic tools, e.g., simultaneously (and conditionally) overexpressing the heterologous gene of interest and/or the genes involved in the UPR, while repressing those involved in ERAD and/or the autophagic process. A deeper understanding of the physiology and the metabolism of the aspergilli (and other fungi) is required to develop methods that will efficiently improve heterologous protein yields. Greater efforts should be undertaken to further understand the physiology of intracellular trafficking, uncovering new proteins that participate both in the secretory pathway and in the quality control mechanisms underlying it. Furthermore, the development of new gene engineering tools and the use of system biology approaches, which are most suitable to study complex cellular processes such as protein secretion, should enable a more rational design of strategies for the optimization of heterologous protein production strains and product yield.

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8 Recombinant Antibody Fragment Production in the Antarctic Marine Bacterium *Pseudoalteromonas haloplanktis* TAC125

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Abstract

Within the biopharmaceutical industry, the antibody market is one of the fastest-growing segments, due to wide applications of recombinant antibodies and antibody fragments in research, diagnostics and therapy. Large-scale production of this protein class requires the use of a platform characterised by low costs, accessible for genetic modifications and easily scaled up. Although their production in prokaryotic hosts can significantly lower production costs, recombinant antibody production in conventional bacterial hosts, such as *Escherichia coli*, may result in formation of inclusion bodies. As protein solubility (and consequently its correct folding) may be enhanced by lowering of the expression temperature, a novel process for recombinant antibody fragment production at low temperatures was set up using *Pseudoalteromonas haloplanktis* TAC125 as recombinant expression host. To test the versatility of the new process developed in the Antarctic Gram-negative bacterium, three model proteins, corresponding to the most common formats of antibody fragments, were produced: Fab, scFv and VH. Several critical aspects were considered in the construction of an ad hoc genetic expression system for each model protein, including the selection of molecular signals for periplasmic protein translocation and the choice of an optimal gene-expression strategy. For instance, an artificial operon was designed and constructed for Fab fragment production in fully heterodimeric form. Furthermore, a novel defined minimal medium was made up to maximise

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bacterial growth parameters and recombinant production yields. All antibody fragments were produced in soluble and biologically competent form. The observed ability of the Antarctic bacterium to produce recombinant antibody fragments was justified by the observation that *P. haloplanktis* TAC125 genome contains an unusually high number of genes encoding peptidyl-prolyl *cis-trans* isomerases, making this bacterium the host of choice for the recombinant production of this protein class.

8.1 Introduction

According to recent reports (Ecker et al. 2015), the monoclonal antibody market represents the fastest-growing segment within the biopharmaceutical industry, and this trend will probably accelerate in future because of the demanding structural features of the emerging antibody-based reagents such as antibody drug conjugates (ADCs) and immunotoxins (Kochuparambil and Litzow 2014; Kim et al. 2013). Indeed, therapeutic monoclonal antibodies and antibody-related products today are approved for the treatment of a variety of diseases.

Recombinant antibodies and antibody fragments are widespread tools for research, diagnostics and therapy (Joosten et al. 2003), and their large-scale production requires a suitable expression system which has to be cheap, accessible for genetic modifications, easily scaled up for greater demands and safe for use in consumer applications. Therefore, an increasing variety of recombinant production systems have been developed, ranging from Gram-negative and Gram-positive bacteria, yeasts and filamentous fungi, insect cell lines and mammalian cells to transgenic plants and animals. Smaller antibody fragments, including bispecific antibodies without any glycosylation, for example, classic monovalent antibody fragments (Fab, scFv) and engineered variants (diabodies, triabodies, minibodies and single-domain antibodies) can be successfully produced in bacteria. While many bacterial strains have been tested for recombinant antibody production with different extents, most experience has been gathered with *Escherichia coli* (Ferrer-Miralles et al. 2009). Incentives for the use of *E. coli* expression systems include simple fermentation conditions, ease of genetic manipulation, ease of scale-up, relatively short duration between transformation and protein purification, no concerns about viruses that are harmful to humans and relatively low capital costs for fermentation (Arbabi-Ghahroudi et al. 2005). However, several obstacles to the production of quality proteins limit its application as a factory for recombinant pharmaceuticals. Indeed, heterologous protein overexpression in *E. coli* often results in insoluble aggregate production as cytoplasmic or periplasmic inclusion bodies (Baneyx and Mujacic 2004). Inclusion body (IB) proteins need elaborate and cost-intensive solubilisation/refolding procedures to recover functionally active products (Vallejo and Rinas 2004). Since protein aggregation is mainly driven by hydrophobic interactions (Carrio et al. 2005), it is weakened by a decrease of process temperature; moreover lowering the temperature has a pleiotropic effect on the folding process.

Although in some cases this approach has been reported to increase yields of soluble and active recombinant protein products, the major drawback in *E. coli* cultivation at suboptimal temperatures is the decrease in biomass production. A rational alternative to improve the quality of recombinant products is to explore the potential of naturally cold-adapted bacteria as hosts for protein production at low temperature (even at around 0 °C). Therefore the production of recombinant proteins in psychrophilic bacteria could represent an interesting model to improve the quality/solubility of protein products. In this context, a few cold-adapted bacterial species are under early but intense exploration as cold cell factories, among them *Pseudoalteromonas haloplanktis* TAC125 being a representative example.

P. haloplanktis TAC125 is a Gram-negative bacterium isolated from an Antarctic coastal seawater sample, and it was the first Antarctic Gram-negative bacterium from which the genome was fully sequenced and carefully annotated (Medigue et al. 2005). *P. haloplanktis* TAC125 genomic and metabolic features, which were discovered by in silico and in vivo analyses, explain its notable versatility and fast growth compared with other bacteria from aqueous environments. Several traits make *P. haloplanktis* TAC125 an attractive host as cell factory for proteins. (1) It is well adapted to protection against reactive oxygen species (ROS) under cold condition by the development of a novel anti-ROS and anti-reactive nitrogen species (RNS) strategy using a two-on-two haemoglobin (Parrilli et al. 2010). Moreover it produces a relevant number of classical oxidative stress-protecting enzymes (such as the superoxide dismutase SodB, the thioredoxin reductase TrxB, the thioredoxin-dependent peroxide reductase AhpCB and the catalase) (Wilmes et al. 2010). (2) *P. haloplanktis* TAC125 in silico proteome composition revealed a specific bias that provides a way to resist to the protein ageing features involving asparagine cyclisation and deamidation (Medigue et al. 2005). (3) The Antarctic bacterium genome is characterised by a quite high number of rRNA genes and of tRNA genes which justify its relevant capacity for translation at low temperatures. (4) Moreover, to create genetically engineered bacterial strains with improved features as protein factories, an efficient genetic scheme for the construction of genome insertion/deletion mutants was set up (Parrilli et al. 2010; Giuliani et al. 2012). (5) *P. haloplanktis* TAC125 was also the first Antarctic bacterium in which an efficient gene-expression technology was set up (Tutino et al. 2001). Several generations of cold-adapted gene-expression vectors allow the production of recombinant proteins either by constitutive (Duilio et al. 2004) or inducible profiles (Papa et al. 2007), to address the product towards any cell compartment or to the extracellular medium (Parrilli et al. 2008).

Furthermore, it was demonstrated that a *P. haloplanktis* TAC125 fed-batch (Wilmes et al. 2010) and chemostat (Giuliani et al. 2011) fermentation strategies could be established, which is feasible to be used in lab-scale or for industrial purposes.

Beneficial effects in using this cold-adapted protein production platform were validated by the successful production of difficult proteins (Vigentini et al. 2006; Papa et al. 2007) and biopharmaceuticals (Corchero et al. 2013; Dragosits et al. 2011; Giuliani et al. 2011).

Statement that insoluble aggregates of recombinant protein have never been found in *P. haloplanktis* TAC125 (although production yields in some cases reached hundreds of mg protein per litre of culture (Papa et al. 2007)) suggests that its cellular physicochemical conditions and/or folding processes are rather different from those observed in mesophilic bacteria.

A recent differential proteomic analysis carried out on *P. haloplanktis* TAC125 demonstrated that ribosome-bound trigger factor is the main upregulated protein at low temperature (Piette et al. 2010). Virtually all nascent polypeptides interact with this chaperone and can be regarded as the primary folding factor for the growth of *P. haloplanktis* TAC125 (Piette et al. 2010). Furthermore, one of the often observed adaptations to cold lifestyle in bacteria genome is the amplification of the number of genes coding for peptidyl-prolyl *cis-trans* isomerases (PPIases) and/or the reduction of the proline distribution among psychrophilic proteins (Feller 2013). Indeed, the *P. haloplanktis* TAC125 genome contains 15 genes encoding a set of PPIases (Giuliani et al. 2014). Since it is widely accepted that scFv and Fab antibody fragment folding rely on the activity of peptidyl-prolyl *cis-trans* isomerases (PPIases) (Feige et al. 2010), *P. haloplanktis* TAC125 resulted to be a naturally optimised host for the recombinant production of antibody fragments.

In this review, we summarise our recent results on successful production in soluble and biologically competent form of aggregation proteins of biopharmaceutical interest, such as antibody fragments. In particular:

- The scFv anti-oxazolone single-chain antibody (Giuliani et al. 2014)
- The camelid VHHD6.1 fragment (Giuliani et al. 2015)
- The Fab 3H6 fragment (Dragosits et al. 2011; Giuliani et al. 2011)

8.2 The Psychrophilic Genetic Expression System for the Recombinant Production of Antibody Fragments in *Pseudoalteromonas haloplanktis* TAC125

Since the correct folding of antibodies and antibody fragments needs disulphide bond formation, the work was initially focused on the choice of a proper molecular signal (signal peptide) allowing the delivery of the recombinant product into the periplasmic space, the cell district where these post-translational modifications are accomplished in Gram-negative bacteria. There are two main routes directing an unstructured protein towards the Sec translocation system, which is located in the inner membrane of Gram-negative bacteria. The features of the different existing signal peptides (i.e. amino acidic sequence and distribution) determine which one of these two pathways is followed by a preprotein.

The most frequently used system is the SecB-dependent translocation (Wicknerw and Leonard 1996), through which the polypeptide is post-translationally translocated into the periplasmic space, which means its delivery takes place after protein complete synthesis (Dalbey and Chen 2004). The protein recognition by the SecYEG complex requires that the polypeptide is in an unfolded state. As a

consequence, there could be some translocation problems for those proteins whose folding kinetics is faster than their recognition by the export system. In fact, if a protein folds in any three-dimensional structure, it becomes an unsuitable substrate for the translocation machinery and is therefore destined to remain in the cytosol where it is generally proteolytically degraded (Chatzi et al. 2013). *P. haloplanktis* TAC125 genome analysis revealed that it contains a classical SecB-dependent translocation system (Medigue et al. 2005).

On the other hand, bacteria have also a translocon which is equivalent to the eukaryotic signal recognition particle (SRP) which transports preproteins using a co-translational mechanism. During protein synthesis, the bacterial SRP recognises the hydrophobic signal sequence of the emerging polypeptide, so that its translation and translocation are simultaneous. In *E. coli*, the SRP (Speed et al. 1996) is composed of the Ffh protein interacting with the small 4.5 S RNA, whereas its receptor is FtsY, a protein which is partially associated with the inner membrane (Grudnik et al. 2009). *P. haloplanktis* TAC125 genome was analysed looking for the genes encoding proteins which are homologous of Ffh and FtsY. Such analysis led to the identification of two genes (PSHAa0942 and PSHAa0354) encoding the SRP-dependent translocation system present in the psychrophilic bacterium.

In order to define which of the two translocation pathways is more effective for the production of antibody fragments in *P. haloplanktis* TAC125, two different scFvOx protein variants bearing distinct signal peptides were produced. One preprotein harboured a SecB-dependent signal sequence (the PsA signal peptide deriving from the psychrophilic α-amylase of the bacterium *P. haloplanktis* TAB23), while the other one had a SRP-dependent peptide (the PsD sequence from the DsbA protein of the bacterium *P. haloplanktis* TAC125). It is worth noting that, according to the data reported by Thie and co-workers (Thie et al. 2008), both the translocation pathways are effective for the production of scFvs in the most conventional host for recombinant protein production, *E. coli*. However, our results show that only the signal for the co-translational translocation allows the correct production of the antibody fragment, its periplasmic translocation and its accumulation in a totally soluble and active form (Giuliani et al. 2014). Furthermore, the total absence of the protein in the cytoplasmic fraction certifies its perfect recognition and its very effective translocation. On the basis of these data, the PsD signal peptide was selected for the production of other antibody fragments in the psychrophilic bacterium.

Once the most suitable sequence for the periplasmic targeting had been chosen, we selected a specific psychrophilic expression system to be used in all the production experiments in *P. haloplanktis* TAC125. The selected expression vector is pUCRP (Papa et al. 2007), as it is characterised by the presence of a psychrophilic regulated promoter, whose activation is induced by the presence of L-malate in the growth medium. Moreover, this promoter shows the highest transcriptional efficiency in minimal media among all the currently available psychrophilic promoters. In the specific case of the cloning of scFvOx, the gene *psD-scFvOx-c-myc* was inserted in frame with a 6xHis-tag coding sequence.

The above-mentioned expression system allowed us to obtain a fusion recombinant protein presenting the N-terminal PsD peptide needed for its periplasmic

secretion and two contiguous C-terminal fusion tags, the c-myc used for the protein detection and a 6xHis tail for its purification (Fig. 8.1a).

In order to achieve the production of VHHD6.1, the same expression vector was properly modified (Fig. 8.1b), replacing the scFvOx coding gene with the *vhh*D6.1 gene amplified by PCR.

As for the Fab 3H6 production, particular attention was devoted to the design of a synthetic operon (Fig. 8.1c) where the two genes coding for Fab 3H6 heavy and light chains were properly spaced. In fact the correct assembly of a light chain with a heavy chain in a 1:1 stoichiometric ratio is needed to obtain a functional protein. In the natural prokaryotic operons, the balanced translation of co-transcribed genes

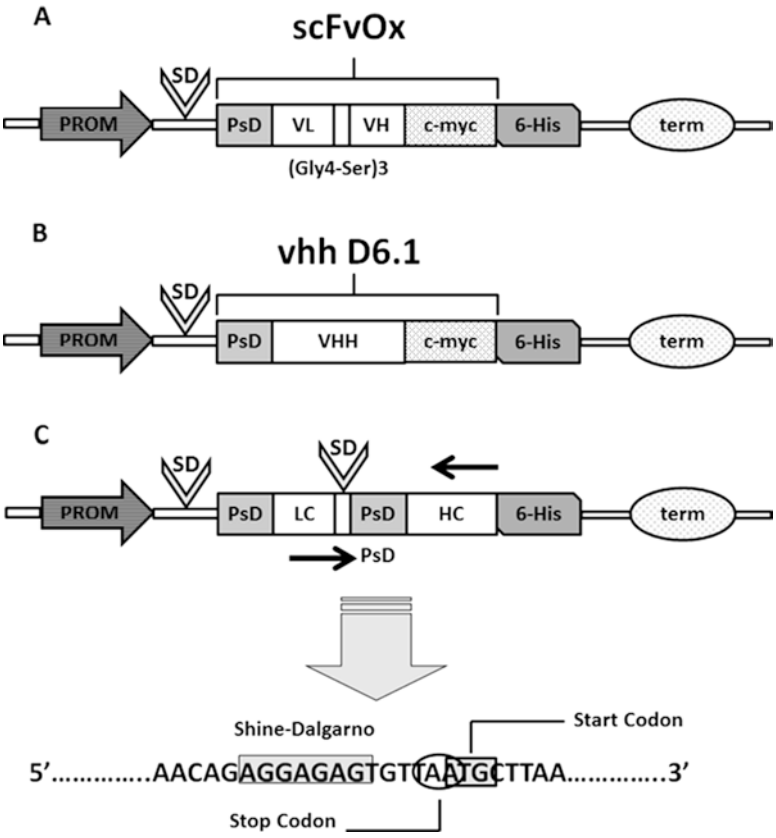


Fig. 8.1 Scheme of expression cassettes used for the recombinant production of antibody fragments in *P. haloplanktis* TAC125. *Panel A* expression cassette for the production of scFvOx, *Panel B* expression cassette for the production of VHHD6.1, *Panel C* expression cassette for the production of Fab 3H6. Prom: psychrophilic promoter inducible L-malate, *Term* transcription terminator from *P. haloplanktis* TAC125 *aspC* gene, *SD* Shine-Dalgarno sequence, *PSD* the signal peptide coding sequence of the *P. haloplanktis* TAC125 *dsbA* gene, *c-myc* sequence encoding a tag used for immunodetection, *6xHis* sequence encoding six histidine residues, used for the purification of the recombinant protein

is achieved through the so-called translational coupling, according to which the complete translation of the upstream gene is required for the effective translation of the distal gene. Translationally coupled genes are characterised by a peculiar structure of their intercistronic region, which was identified for the first time in *E. coli* tryptophan operon (Oppenheim and Yanofsky 1980). Considering this bacterial feature in gene expression, an in silico analysis about the gene structural organisation of operons in *P. haloplanktis* TAC125 was carried out. Most of the analysed operons showed the same structural organisation according to which the start codon of the second gene of the cluster overlaps the stop codon of the upstream gene, so that the two coding sequences share a base pair. As shown in Fig. 8.1c, this structure was replicated in the design of the artificial operon used for the Fab 3H6 production. Furthermore, two copies of the same Shine-Dalgarno sequence were placed upstream both the *lc* gene and the *hc* gene. In fact, both the first and the distal gene need a ribosome binding site for a satisfying expression.

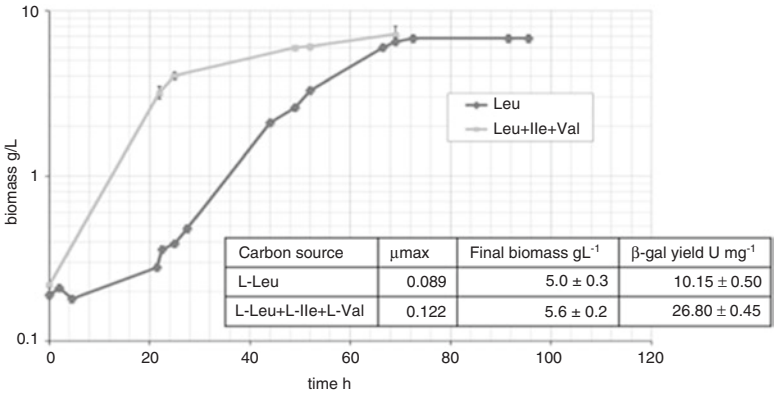
8.3 Optimisation of the Growth Medium for the Recombinant Protein Production in *Pseudoalteromonas haloplanktis* TAC125

The understanding of cellular physiology and the optimisation of cultivation strategies are essential factors to obtain a microbial recombinant protein production with high yields. In fact, protein productivity of cell-associated products is related to the level of biomass obtained during the process. In this case, the optimisation of protein production is strictly related to the composition of the growth medium; therefore it also depends on the selection of the most suitable cultivation strategy. An efficient, completely soluble and catalytically competent production of several thermolabile or aggregating proteins was often obtained in defined culture media, in which induction levels and production yields were optimised even compared to those obtained with common rich and complex culture media (Papa et al. 2007).

In order to increase production yields of recombinant proteins of industrial interest by the psychrophilic expression system, the influence of medium composition, final biomass concentration, growth rate and protein production were investigated (Giuliani et al. 2011).

Moreover, *P. haloplanktis* TAC125 recombinant protein production was evaluated in a synthetic medium optimised for this purpose.

In several studies it has been reported that the psychrophilic bacterium of our interest shows nutritional preferences for amino acids as carbon and nitrogen source. For this reason, bacterial growth parameters were estimated in synthetic media composed of a mineral base (Schatz salts) with the addition of several amino acids (Giuliani et al. 2011). Obtained results suggested the use of L-leucine, in combination with L-isoleucine and L-valine, as carbon and nitrogen source, and led to the formulation of LIV medium, which was then used in all the subsequent attempts to perform a recombinant protein production both in batch and in chemostat cultivations. In detail, LIV medium contains the three selected amino acids L-leucine,



Carbon source	μmax	Final biomass gL^{-1}	β-gal yield U mg^{-1}
L-Leu	0.089	5.0 ± 0.3	10.15 ± 0.50
L-Leu+L-Ile+L-Val	0.122	5.6 ± 0.2	26.80 ± 0.45

Fig. 8.2 Comparison of the growth curves of the bacterium *P. haloplanktis* TAC125 in Schatz-defined synthetic media containing L-leucine (*diamonds*) and a mixture of L-leucine, L-isoleucine and L-valine in a 1:1:2 molar ratio (*filled squares*)

L-isoleucine and L-valine in a 1:1:2 mole ratio. As shown in Fig. 8.2, the psychrophilic bacterium growth in LIV medium is characterised by a brief lag phase and a modest but significant increase of specific growth rate and biomass yield, compared to the growth in the medium with only L-leucine (Giuliani et al. 2011).

8.4 Examples of Recombinant Production of Antibody Fragments in *Pseudoalteromonas haloplanktis* TAC125

8.4.1 scFv Anti-oxazole Single-Chain Antibody Fragment

An scFv (single-chain variable fragment) is the smaller fragment (~30 kDa) of immunoglobulins that still retains the active antigen-binding site. Clinical applications of this kind of molecules require low cost production of large amounts of functional scFvs. However, the recombinant production of this protein family in the conventional bacterial host *E. coli* entails several problems which are related to scFvs' tendency to form insoluble aggregates when produced in this host (Somerville et al. 1994). In order to overcome the above production issues, several experimental approaches were tested, such as the scFvs co-expression with molecular chaperones (Levy et al. 2013; Feige et al. 2010) or the lowering of expression temperature.

The latter approach is often applied when recombinant products accumulate as inclusion bodies (Feige et al. 2010), since, as previously asserted, temperature lowering minimises hydrophobic interactions (Yang et al. 1992), which are the driving force involved in inclusion body formation (Carrio et al. 2005).

However, *E. coli* cultivation at suboptimal temperatures generally involves a decrease in biomass productivity, due to the temperature-lowering effect on bacterial specific growth rate and so to the cold shock response (Coleman et al. 2003).

Therefore it is clear that the use of naturally cold-adapted bacteria as host for recombinant protein production may represent an effective alternative to improve conformational quality and solubility of these pharmaceutical protein products.

In order to evaluate the effectiveness of the psychrophilic expression system in recombinant antibody fragment production, the anti-2-phenyl-5-oxazolone single-chain variable fragment, scFvOx (Fiedler and Conrad 1995), was chosen as model. scFvOx is the typical example of single-chain variable fragment that forms aggregates. In fact it has been used for years as model for the development of refolding protocols of inclusion bodies (Patil et al. 2008). Recombinant production was addressed towards the periplasmic space to allow the formation of the antibody disulphide bridges. Recombinant protein production and cellular localisation were evaluated by Western blot analysis, and a lab-scale fermentation was set up. As shown in Fig. 8.3, the protein was fully translocated into the periplasmic space and produced in soluble form. By suitably combining the cellular translocation signals, the gene-expression system and the fermentation strategy, a production yield of 4.69 ± 0.12 mg/L of soluble scFvOx was obtained. This is the highest yield of this specific scFv reported until now for a conventional prokaryotic expression system, even after the inclusion body refolding (Patil et al. 2008). Moreover also the recorded product yield ($Y_{P/X}$) was very high ($Y_{P/X} = 0.94 \pm 0.03$ mg gX^{-1}). This aspect suggests that a further increase of specific biomass yield would allow to obtain higher levels of scFvOx production (Giuliani et al. 2014).

8.4.2 Camelid VHHD6.1 Fragment

Since their first description (Hamers–Casterman et al. 1993), IgG antibodies from Camelidae (camels, dromedaries and llama) had attracted scientists' interest. These antibodies are naturally devoid of light chains, making them significantly smaller than conventional antibodies, and are therefore called "heavy-chain antibodies" or HCAbs. Their binding domains consist only of the heavy-chain variable domains, referred to as VHHs (Muyldermans and Lauwereys 1999) to distinguish them from the conventional VHs. VHH is the smallest available intact antigen-binding

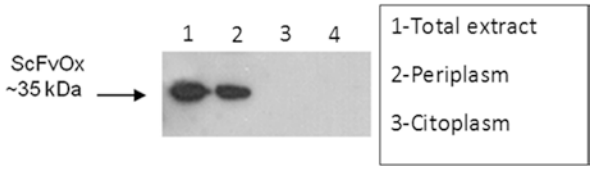


Fig. 8.3 Production and cellular localisation of the antibody fragment scFvOx produced in the bacterium *P. haloplanktis* TAC125. The recombinant cells were recovered, and their total extract (*lane 1*), the two subfractions of periplasmic extract (*lane 2*) and the cytoplasmic extract (*lane 3*) were analysed by Western blotting, using the anti-c-myc primary antibody. Negative control corresponds to the total extract of nonrecombinant Antarctic cells

fragment (~15 kDa), and it has a great potential in therapeutic and diagnostic applications as multi-specific fusion product (Joosten et al. 2003).

Thanks to their low structural complexity, VHHs are successfully produced in conventional microbial hosts, such as *E. coli*. However, a small VHH fraction shows a low solubility in some recombinant production systems, preventing the evaluation of their binding capacity. Although their great potential, conventional microbial hosts are not always suitable or optimised for VHH production, and this makes their application not immediately accessible.

To validate *P. haloplanktis* TAC125 capacity to successfully produce a soluble camelid antibody fragment, an antihuman fibroblast growth factor receptor1 (FGFR1) VHHD6.1 was chosen as model protein. It was selected by phage display from a naïve llama library (Monegal et al. 2009), but its large-scale production in conventional *E. coli* expression systems was unsatisfactory due to inclusion body formation (De Marco A., personal communication). A new production process leading to the improvement of soluble production of VHHD6.1 was therefore required for its further characterisation.

The antihuman fibroblast growth factor receptor 1 (FGFR1) VHHD6.1 production was performed by *P. haloplanktis* TAC125 (pUCRP-*vhh*) batch cultivation in LIV medium in optimised conditions. Analysis of production and cellular localisation of recombinant anti-*h*FGFR1 VHHD6.1 was carried out by semi-quantitative Western blotting analysis on total soluble protein extracts and periplasmic fractions of samples collected at different times of cultivation (Fig. 8.4). Immunodetection performed with anti-*c*-myc antibodies revealed VHHD6.1 soluble production during all fermentation and its correct periplasmic localisation during early (22 h) and middle (29 h) exponential growth phase. In contrast, no recombinant VHHD6.1 was found in periplasmic fraction extracted from samples collected at late exponential growth phase (42 h) and stationary phase (60 h), while production titres seem to increase during exponential growth reaching the highest yield at late exponential phase (42 h). Furthermore, another specific signal showing an apparent molecular weight of about 30 kDa was detected in total soluble protein extracts probably corresponding to VHHD6.1 dimers. The appearance of VHH dimers is not surprising

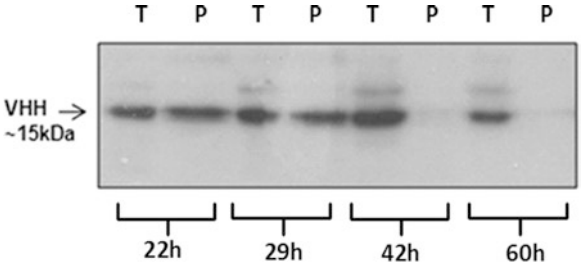


Fig. 8.4 Production and cellular localisation of the antibody fragment VHHD6.1 produced in *P. haloplanktis* TAC125. Aliquots of recombinant cells were recovered at the indicated growth times and their total protein extract (T) and the corresponding periplasmic fraction (P) were analysed by Western blotting using the anti-c-myc primary antibody

as a strong tendency of multimerisation has been reported for this, and other formats of antibody fragments in vivo when their local concentration in recombinant host cells reach a critical value (Hollinger and Hudson 2005). It is worth noting that as far as the high molecular weight signal relative intensity increases, the secretion efficiency of recombinant product into the periplasmic space seems to decrease. One explanation can be found in VHH dimerisation kinetics that could be faster than the product recruitment by the periplasmic secretion system (Giuliani et al. 2015).

8.4.3 H6 Fab Antibody Fragment

The anti-idiotypic antibody Ab2/2H5 Fab is able to recognise the 2 F5 antibody, which is able to neutralise a wide spectrum of type 1 human immunodeficiency virus (HIV-1) strains, thus representing a potential key component in anti-HIV vaccine formulation (Kunert et al. 2002). Moreover, it was chosen as a protein model in the framework of a European project for comparative analysis of the protein recombinant production in prokaryotic and eukaryotic microbial hosts, since it was able to induce the unfolded protein response (UPR) in yeast cells (Gasser et al. 2007).

Fab fragments have a heterodimeric structure containing each a light and a heavy chain. For this reason, an artificial operon was constructed for the co-expression of Fab 3H6 light- and heavy-chain coding genes, in order to achieve a balanced translation of the two Fab 3H6 chains through translational coupling (Fig. 8.1) in *P. haloplanktis* TAC125.

The analysis of Fab 3H6 production was performed by ELISA experiments on total soluble protein extracts from *P. haloplanktis* TAC125 (pUCRP-*fab*) samples collected at different times of a batch cultivation, in optimised condition at 15 °C.

Results show that the recombinant Fab 3H6 nicely accumulates during fermentation reaching the highest production yield of 3.99 ± 0.11 mg L^{-1} in late exponential phase corresponding to about 48 h of cultivation. At the end of the process, recombinant product yield is of 0.89 ± 0.15 mg $gbiomass^{-1}$, expressed as cellular dry mass. The yield value is increased up to fivefold compared to *Pichia pastoris* production and up to 50-fold compared to *E. coli* and *Saccharomyces cerevisiae* production, as reported in a recent paper (Dragosits et al. 2011).

Analysis of Fab 3H6 cellular localisation and quaternary structure was performed by Western blotting experiments on periplasmic and cytoplasmic extracts from samples collected at different times of cultivation, using specific anti-light-chain antibodies in nonreducing condition (Fig. 8.5). The analysis revealed a specific signal corresponding to Fab 3H6 in heterodimeric form (≈50 kDa) exclusively in periplasmic fractions, demonstrating that soluble Fab 3H6 is not only effectively produced but also correctly and totally translocated into the periplasmic space. Moreover, the apparent molecular weight of the detected signal (≈50 kDa) and the complete absence of signals corresponding to free light chains (≈25 kDa) suggest that the recombinant product is fully assembled in heterodimeric quaternary structure (Giuliani et al. 2011).

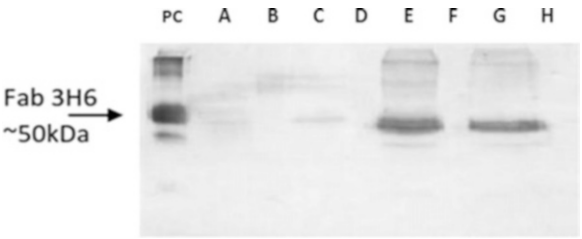


Fig. 8.5 Production and cellular localisation of the antibody fragment Fab 3H6 produced in the bacterium *P. haloplanktis* TAC125. Aliquots of recombinant cells were recovered in four different stages of cell growth; their periplasmic protein extract (*lanes A, C, E* and *G*) and the corresponding cytoplasmic fractions (*lanes B, D, F* and *H*) were analysed by Western Blotting using the anti-c-myc primary antibody. PC, positive control corresponding to a sample of Fab 3H6 produced in recombinant *Pichia pastoris* cells. The SDS-PAGE electrophoretic analysis was carried out under nonreducing conditions; therefore the immunodetection analysis highlights the Fab 3H6 in heterodimeric form (~50 kDa)

8.5 Conclusion

In this review, we summarised results obtained in our laboratories on the production of recombinant antibody fragments in the Antarctic bacterium *P. haloplanktis* TAC125. This microorganism resulted to be a host of choice for the synthesis of this class of valuable proteins. Consistently, all three antibody fragments selected for recombinant production in the Antarctic host were produced in soluble and correctly assembled form, also in virtue of the choice of the SRP-dependent co-translational periplasmic secretion pathway. In fact, only in the case of the camelid VHHD6.1 antibody production the translocation across the inner membrane resulted to be somehow ineffective when growth reached high cell density. In all three processes, however, the psychrophilic host was able to produce the antibody fragments with yields comparable, if not largely superior, to those obtained in conventional expression systems, such as *E. coli, S. cerevisiae* or *P. pastoris*. The peculiar *P. haloplanktis* TAC125 proficiency in producing high-quality recombinant antibody fragments prompted us to further investigate on its cellular features. In this regard, numerous studies on protein folding processes of the antibody molecules highlighted the critical role played by PPIases, a class of enzymes which involved *cis-trans* isomerisation of X-Pro bond in polypeptides. In a recent work, Levy and co-authors (Levy et al. 2013) demonstrated that co-expression of FkpA, an *E. coli* periplasmic PPIase, resulted in a considerable increase in the periplasmic secretion of functional scFv fragments, arising both from Vk chains (and, therefore, containing proline in the *cis* conformation) and Vl chains, not equipped with a *cis*-proline. These results also suggested that the effect of FkpA is linked to either its PPIase enzymatic activity or to that as molecular chaperone. The speed of the *cis-trans* isomerisation reaction of X-Pro bond can become a limiting step during protein folding when the temperature approaches the freezing point of water. It was recently observed that when shifted to low growth temperatures (4 °C), *P. haloplanktis* TAC125 mainly upregulates the production of the trigger factor, the molecular

chaperone that interacts with the ribosome and is also endowed with PPIase activity. This observation points towards the central role of this protein (and likely of PPIase activity) in the adaptation of this organism to growth at low temperatures (Piette et al. 2010). Another interesting observation on adaptive strategies to psychrophilic lifestyle comes from the in silico study of polar bacterial genomes, in which it has often reported an increase in the number of genes coding for PPIases that may be also accompanied by a reduction of the distribution of the proline residues in the encoded proteome (Feller 2013). For this reason, the *P. haloplanktis* TAC125 genome was analysed for genes encoding PPI enzymes. The results of this analysis are summarised in Table 8.1, which also shows the ten PPIase encoding genes in mesophilic bacterium *E. coli*. In good agreement with the previously reported trend, the Antarctic bacterium genome contains a higher number (15) of genes encoding PPIases, ten of which encode the psychrophilic counterparts of mesophilic proteins. The other five genes found in *P. haloplanktis* TAC125 genome encode a duplication

Table 8.1 Peptidyl-prolyl *cis-trans* isomerase encoding genes in *Pseudoalteromonas haloplanktis* TAC125

Gene name	*Ph*TAC125	MAGE gene annotation[a]	Localisation
slyDB	***PSHAa0292***	Peptidyl-prolyl *cis-trans* isomerase, FKBP family	Cytoplasm
slyDA	***PSHAa0721***	FKBP-type peptidyl-prolyl *cis-trans* isomerase (rotamase)	Cytoplasm
fkpB	***PSHAa0920***	FKBP-type peptidyl-prolyl *cis-trans* isomerase (rotamase)	Cytoplasm
	PSHAa1034	Peptidyl-prolyl *cis-trans* isomerase, FKBP type	Cytoplasm
fklB	***PSHAa1414***	FKBP-type 22KD peptidyl-prolyl *cis-trans* isomerase (rotamase)	Cytoplasm
ppiC	***PSHAa2488***	Peptidyl-prolyl *cis-trans* isomerase C (rotamase C)	Cytoplasm
ppiC	***PSHAb0308***	Peptidyl-prolyl *cis-trans* isomerase C (rotamase C)	Cytoplasm
tig	***PSHAa2063***	Peptidyl-prolyl *cis-trans* isomerase (trigger factor), molecular chaperone involved in cell division	Cytoplasm
ppiB	***PSHAa2066***	Peptidyl-prolyl *cis-trans* isomerase B (rotamase B)	Cytoplasm
ppiD	***PSHAa2058***	Peptidyl-prolyl *cis-trans* isomerase D (PPIase D) (rotamase D)	Inner membrane associated
fkpA	***PSHAa2901***	Putative periplasmic peptidyl-prolyl *cis-trans* isomerase	Inner membrane associated
surA	***PSHAa2633***	Peptidyl-prolyl *cis-trans* isomerase (PPIase)	Periplasm
ppiA	***PSHAb0347***	Putative peptidyl-prolyl *cis-trans* isomerase	Periplasm
	PSHAa1981	Putative peptidyl-prolyl *cis-trans* isomerase (PPIase)	Periplasm
	PSHAa2247	Putative peptidyl-prolyl isomerase	Periplasm

The genome of the Antarctic bacterium (https://www.genoscope.cns.fr/agc/microscope/mage/viewer.php) was searched for PPI-encoding genes. For each retrieved gene, gene name, MAGE gene annotation and predicted cellular localisation of the gene product are also reported. PSHAa, *Ph*TAC125 chromosome 1; PSHAb, *Ph*TAC125 chromosome 2

[a]https://www.genoscope.cns.fr/agc/microscope/mage/viewer.php

of cytoplasmic SlyD and PpiC and three other PPIases, predicted to be localised one in the cytoplasm and two in the periplasmic space (Table 8.1) (Giuliani et al. 2014). In summary, in the light of the comparison of the distribution of PPIase enzymes between the two bacteria, it can be concluded that the Antarctic bacterium has three cytoplasmic PPIases and two periplasmic PPIases more than *E. coli*, an observation that can justify the peculiar properties of *P. haloplanktis* TAC125 as naturally optimised host for recombinant production of proteins or protein fragments stabilised by disulphide bridges (Giuliani et al. 2014). More generally, our results definitively show that the production of recombinant proteins in this psychrophilic bacterium is not only a reliable technology but can be a valid strategy to mitigate the solubility or incorrect folding issues that are sometimes experienced in conventional expression systems, such as *E. coli*. Furthermore, *P. haloplanktis* TAC125 and the gene-expression strategy developed have shown an interesting biotechnological potential as an unconventional production platform of recombinant proteins of significant value, such as antibody fragments.

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Part II

Microbes in Environmental Sustainability

The *Flavobacterium* Genus in the Plant Holobiont: Ecological, Physiological, and Applicative Insights

9

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Abstract

Members of the *Flavobacterium* genus are widely distributed in nature where they are often associated with the capacity to degrade complex organic compounds. A myriad of recent studies indicate that the class *Flavobacteria*, and specifically the genus *Flavobacterium*, represent a significant fraction of root- and leaf-associated microbiomes in a broad range of plant species. Several of these studies have shown that the relative abundance of members of this genus increases substantially along the soil, rhizosphere, and rhizoplane continuum, indicating a specialized capacity to proliferate in plant environments and suggesting a role in plant functioning. Unlike other plant-associated genera such as *Pseudomonas* and *Bacillus* that have been exhaustively documented, little is known about the ecology of *Flavobacterium* strains in plant environments. This chapter summarizes current knowledge of *Flavobacterium* strains in plant habitats. It explores their abundance and diversity in the rhizosphere and the

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phyllosphere of a large range of plant species, elucidates the potential role of unique flavobacterial gliding-motility and gliding-secretion mechanisms in plant-*Flavobacterium* interactions, and explores the potential role of *Flavobacterium* strains in plant growth and protection.

9.1 Introduction: The Plant Microbiome

9.1.1 The Root-Associated Microbiome

The root ecosystem is often divided into three microenvironments: the rhizosphere, the thin layer of soil that surrounds the root that was first introduced by Hiltner in 1904 (Hartmann et al. 2008); the rhizoplane, the ecosystem directly bound to the root surface; and the endosphere, the ecosystem within plant roots that is colonized by specialized microorganisms coined endophytes (Compant et al. 2010; Hardoim et al. 2015). Certain endophytic microorganisms may be vertically inherited through seeds (Wiewióra et al. 2015), but for the most part, root-associated microorganisms are actively recruited by plants from surrounding soil (Berg and Smalla 2009; Bulgarelli et al. 2013). Therefore, while the microbial diversity of the bulk soil is an important factor in shaping the rhizosphere microbiome (Garbeva et al. 2008; Lundberg et al. 2012), the root environment selects for specific soil strains. The fact that root exudates differ between different plant species (Rovira 1969) and physiological stages (Chaparro et al. 2014) explains observed differences in rhizosphere microbiomes of different plant species (Ofek et al. 2014).

It is estimated that up to 40 % of carbon that is fixed by plants is passively or actively transported to the rhizosphere in the form of either root biomass or carbon exudates (Jones et al. 2009). This immense influx of organic matter relative to the generally sparse surrounding soil creates an environment around the root that is analogous to a desert oasis, which is characterized by higher microbial biomass and activity relative to the surrounding bulk soil (Hacquard et al. 2015). Plant roots not only increase biologically available carbon and nitrogen in the rhizosphere but also modify physiochemical characteristics such as oxygen availability and pH (Hinsinger et al. 2009). This results in the establishment of unique root-associated microbial communities (Carvalhais et al. 2015; Lebeis et al. 2015; Rudrappa et al. 2008) that are generally characterized by increased abundance and reduced diversity relative to the surrounding bulk soil microbiome (Hacquard et al. 2015; Ofek et al. 2014).

Deciphering the microbial ecology of the root is crucial for understanding plant health, and the current state of the art has only touched the tip of the iceberg regarding the composition and function of the root microbiome (Hacquard et al. 2015). For centuries, the root-associated microbial community was studied individually as single species interactions. However, recent development in next-generation sequencing technologies has enabled scientists to determine the composition of

complex root-associated microbial communities, and thereby apply these methodologies to better understand the microbial ecology of the root as well as root-microbe interactions (Hacquard et al. 2015, Mendes et al. 2013; Zilber-Rosenberg and Rosenberg 2008; Rosenberg et al. 2010, Hacquard et al. 2015). Despite the fact that soil represents one of the richest microbial ecosystems on earth (Gans et al. 2005), culture-independent approaches have clearly demonstrated that relatively few bacterial phyla actually colonize the rhizosphere. Most notable are members of the *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* phyla, which are significantly enriched in the rhizosphere compared to the adjacent bulk soil (Fierer et al. 2009; Kolton et al. 2011; Bulgarelli et al. 2012; Mendes et al. 2011; Coleman-Derr et al. 2015; Shi et al. 2015; Peiffer et al. 2013; Zarraonaindia et al. 2015). Although these phyla appear to be ubiquitous in a broad range of plant species, there is significant evidence indicating that individual plant hosts harbor specific communities within these lineages (Aleklett et al. 2015; Ofek et al. 2014; Ofek-Lalzar et al. 2014; Mark et al. 2005; Berg et al. 2002; Smalla et al. 2001). Selection of the root-associated microbiome by plants has been previously demonstrated in several studies (Bais et al. 2006; Rudrappa et al. 2008; Chaparro et al. 2014; Lebeis et al. 2015; Carvalhais et al. 2015; Bai et al. 2015). However, the initially recruited microbiome shifts significantly as a function of plant physiological age and changes, e.g., during the flowering period (Chaparro et al. 2014; Reinhold-Hurek et al. 2015), and the root-associated microbial community is also influenced by complex inter-microbial interactions in the rhizosphere.

9.1.2 The Phyllosphere Microbiome

The phyllosphere refers to the total aboveground or aerial surface of plants, and it is colonized by both epiphytically and endophytically naturally occurring microbial communities (Lindow and Brandl 2003; Ruinen 1956). Based on plant morphology and specific plant compartments, the structure, abundance, and general occurrence of bacteria in the phyllosphere are highly variable (Hunter et al. 2010). In addition, numerous environmental determinants, including, among others, wind, precipitation and solar radiation, have been shown to shape the structure of microbes indigenous to the phyllosphere (Kinkel et al. 2011). Next-generation sequencing approaches shed new light on phyllosphere microbiota and revealed diverse and abundant communities of microorganisms where multipartite interactions occur. Although the degree of host specificity is still only partly characterized, epiphytic fitness including the resistance to abiotic stresses and antimicrobial compounds of phyllosphere-colonizing bacteria seems to be a prerequisite for colonization and persistence (Berg and Smalla 2009; Leff and Fierer 2013; Vorholt 2012). The functional roles of leaf-associated microbiomes are estimated to be immense given the size of this habitat which has been estimated to encompass a global surface of 4×10^8 km^2, supporting about 10^{26} cells (Morris et al. 2002; Whipps and Lynch 1986).

9.2 The Genus *Flavobacterium*

The name *Flavobacterium* was originally proposed in 1923 for a genus of the family *Bacteriaceae* encompassing the rod-shaped, non-endospore-forming, chemoorganotrophic, Gram-negative eubacteria (Stanier 1947). Currently (February 2016), 165 *Flavobacterium* species are described (www.dsmz.de). Members of the genus *Flavobacterium* are widely distributed in nature and are present in very different environmental habitats ranging from fresh and marine waters, soil to ocean sediments, foods and food-processing plants, and clinical environments, most notably in fish where they are associated with several diseases (Bernardet and Bowman 2006, 2011; Bernardet and Nakagawa 2006). They frequently harbor an arsenal of extracellular macromolecular-degrading enzymes such as amylase, cellulase, chitinase, peptidases and diverse glycoside hydrolases (GH) that enable the bacteria to digest easily degradable polymers like starch as well as recalcitrant biopolymers like chitin. For this reason, they are well known to have a pivotal and important role in the turnover of various types of organic matter (carbohydrates, amino acids, proteins and polysaccharides) (Bernardet and Bowman 2006, 2011; Bernardet and Nakagawa 2006; Christensen 1977; Cottrell and Kirchman 2000). Their robust assemblage of extracellular enzymes is believed to be associated with the degradation of bacteria, fungi, insect and nematode constituents in the environment (Bernardet and Bowman 2006; Peterson et al. 2006).

Flavobacterial colonies are commonly characterized by a yellow-orange color, attributed to the pigment flexirubin (Bernardet and Bowman 2006, 2011; Reichenbach et al. 1980). In addition, they generally harbor a unique *Bacteroidetes*-specific gliding-motility system that enables rapid gliding movement over solid surfaces (average gliding speed of 2 μm/s) (Shrivastava and Berg 2015; Jarrell and McBride 2008; McBride 2004; Nakane et al. 2013; Nan et al. 2014). The gliding-motility system is tightly associated with a recently described type IX secretion system (T9SS) that is responsible for the secretion of several characterized extracellular hydrolytic enzymes (Sato et al. 2010; McBride and Zhu 2013; Nakane et al. 2013). The T9SS secretes the cell-surface motility adhesins SprB and RemA and the chitinase ChiA, while additional proteins including GldK, GldL, GldM, GldN, SprA, SprE, and SprT are involved in secretion by the T9SS (McBride and Nakane 2015; McBride and Zhu 2013) (Kharade and McBride 2015) resulting in circular movement analogous to the motion of a snowmobile tread.

9.2.1 Abundance of Plant-Associated *Flavobacterium* strains

The first inclusive evidence of Flavobacterial abundance in plant ecosystems was from a comprehensive culture-based study of the barley rhizosphere microbiome that screened close to 5000 isolates on general media (Johansen and Binnerup 2002). Members of the *Flavobacterium* genus were the most abundant of the characterized isolates (25 %) followed by fluorescent *Pseudomonas* (10 %). Interestingly, at early stages of plant growth, 70 % of the rhizosphere isolates carrying enzymes

involved in carbon turnover were characterized as *Flavobacterium*, suggesting that they play a crucial metabolic role during initial stages of plant growth. Early plant growth-stage abundance was also supported by a subsequent culture-based study conducted by the same authors (Johansen et al. 2009).

Culture-based estimations of microbial communities in complex ecosystems are often bias, and therefore, microbial ecology has shifted to molecular-based tools that target the 16S rRNA gene to determine the relative abundance of bacterial phyla in natural environments (Hugenholtz et al. 1998). For example, culture-independent 16S rRNA amplicon sequencing determined that *Flavobacterium* was also highly abundant in the rhizosphere of cucumbers (8.6 % of defined genera). Contrary to other genera, Flavobacterial levels were not dictated by soil composition or compost amendment, suggesting that their abundance is strongly dictated by plant-associated factors (Tian and Gao 2014). Additional studies also indicated that they represent a major component of the rhizosphere microbiomes of peppers (Graber et al. 2010), lettuce (Cardinale et al. 2015), peanuts (Haldar et al. 2011), maize (Li et al. 2014), tomatoes (Kolton, unpublished) and *Arabidopsis thaliana* (Bodenhausen et al. 2013; Bulgarelli et al. 2012; Lundberg et al. 2012).

Although most studies to date focus primarily on rhizosphere communities, a recent 16S rRNA amplicon sequencing-based study (Qin et al. 2016) determined that *Flavobacterium* sp. was among the most dominant taxa in the phyllosphere (2.1–41.4 % of the total defined genera) as well as the rhizosphere (0.9–8.5 % of the total defined genera) of wheat, contrary to most of the identified genera that proliferated in either the phyllosphere or the rhizosphere. The relative abundance of Flavobacteria in both environments decreased at later stages of plant growth, suggesting a stronger influence of this genus in early and intermediate growth stages, similar to results from culture-based analyses described above.

Several studies have specifically assessed the spatial relations of bacterial communities in plant ecosystems. Two comprehensive 16S rRNA amplicon sequencing-based assessments of *Arabidopsis thaliana* microbiomes determined that while Flavobacteria were detected in bulk soil and rhizosphere, their relative abundance substantially increased in the rhizoplane where they were among the most abundant detected taxa (Bodenhausen et al. 2013; Bulgarelli et al. 2012; Lundberg et al. 2012). A recent study assessed endophytic in addition to epiphytic bacterial communities associated with the leaves and roots of *Arabidopsis thaliana*. Similar to previously described results, *Flavobacterium* was among the most dominant genera on both leaves and roots, but interestingly they represented a higher proportion of the endophytic bacterial communities in both ecosystems (9.8 *vs.* 3.9 % and 5.9 *vs.*10 % of the leaf-associated and root-associated relative abundance, respectively).

Amplicon sequencing-based analyses may generate a skewed portrayal of bacteria communities in complex environments due to PCR biases, and therefore, some studies have shifted to direct metagenomic approaches (Kuczynski et al. 2012). Metagenome analysis of the *Eruca sativa* revealed that members of the *Flavobacterium* genus were highly abundant in both rhizosphere and phyllosphere microbiomes (Erlacher, unpublished). Classification to species level revealed that

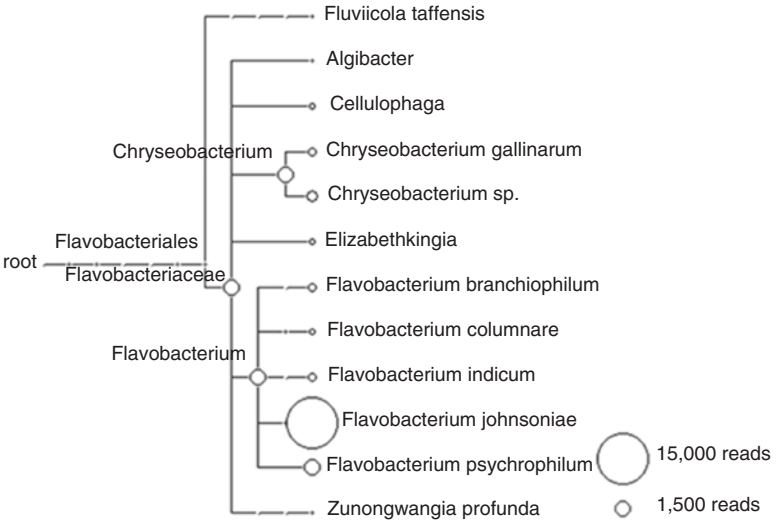


Fig. 9.1 Taxonomical structure of *Flavobacterium* based on BLASTN assembled metagenomes from *E. sativa* whole plant including rhizosphere and phyllosphere. Only taxa >100 hits are considered

the vast majority of strains resembled *Flavobacterium johnsoniae*, although strains associated with *F. psychrophilum*, *F. branchiophilum*, *F. columnare* and *F. indicum* were also present (Fig. 9.1). This is consistent with culture-based analyses, which have demonstrated that *F. johnsoniae*-like strains are ubiquitous in terrestrial environments strongly influenced by plants, whereas *F. psychrophilum*, *F. branchiophilum*, *F. columnare* and *F. indicum* are generally associated with aquatic environments (Kolton et al. 2013).

Microbial community structure in the rhizosphere and phyllosphere may be strongly dictated by temporal dynamics (Butler et al. 2003). In a time-lapse experiment monitoring bacterial dynamics on tomato roots using next-generation sequencing (Illumina) of 16S rRNA gene amplicons, we found that different strains of *Flavobacterium* had different relative abundance patterns in the rhizosphere as a function of plant age (Fig. 9.2; Kolton, unpublished). This suggests that the root microbiome comprises multiple *Flavobacterium* strains that coexist and can fluctuate in response to temporal dynamics, potentially by means of different physiological and metabolic characteristics.

The data presented above indicates that *Flavobacterium* strains frequently constitute a significant fraction of epiphytic and endophytic root and leaf microbiomes. The abundance of these strains in these environments can vary considerably as a function of plant strain and environmental conditions (Table 9.1), and currently, the specific factors that dictate their profusion in these environments are predominantly unknown. One factor associated with Flavobacterial abundance is pH. *Flavobacterium* growth is often restrained to narrow pH ranges (5.5–8.5) (Yoon et al. 2006, Kolton et al. 2013), and their relative abundance in soil and roots has

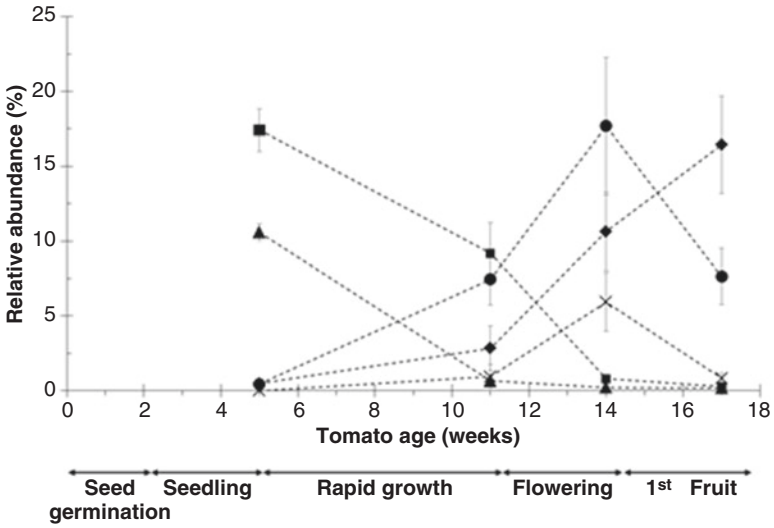


Fig. 9.2 Temporal dynamics of Flavobacterial taxa in the tomato rhizosphere. Analysis (Kolton, unpublished) implemented Illumina MiSeq-based amplicon sequencing targeting the V4–V5 region of the 16S rRNA gene. Clustering of operational taxonomic units (OTUs) based on a 97 % sequence similarity cutoff (characterized as species level) revealed six abundant Flavobacterial taxa (annotated by *triangles*, *squares*, *circles*, and x's). Analyses were conducted using the QIIME 1.9 pipeline (http://qiime.org/)

been found to significantly decrease in acidic soils (Kumar personal communication) (Rousk et al. 2010). This could explain their low relative abundance in plants growing in peat bogs and other acidic environments.

9.3 Role of Flavobacteria in Plant Growth and Protection

Although *Flavobacterium* strains were not directly associated with plant growth promotion and protection in the past, several studies have demonstrated that their abundance in the rhizosphere is positively correlated to plant biomass (Manter et al. 2010), plant resistance to pathogens (Kolton et al. 2014; Alexander and Stewart 2001; Gunasinghe and Karunaratne 2009; Hebbar et al. 1991; Sang et al. 2008; Sang and Kim 2012), and stimulation of fruit maturation (Sang and Kim 2012). One study demonstrated that the volatile-producing *Flavobacterium johnsoniae* strain GSE09 confers enhanced biocontrol activity against *Phytophthora capsici* in pepper (Sang et al. 2008), and the authors of this study hypothesized that this may have been stimulated by the production of biofilms, indolic compounds, biosurfactants, and 2,4-di-tert-butylphenol that are produced by this strain (Sang and Kim 2012).

Certain *Flavobacterium* strains have been found to possess H_2-oxidizing and ACC deaminase activities that have been directly correlated to plant growth promotion, and these strains were indeed found to induce plant biomass (Maimaiti et al.

Table 9.1 Relative abundances of Flavobacteria in selected metagenomes. Read counts are based on MG-RAST assignments within all bacterial rRNA gene sequences in the specified sample

	Plant habitat	MG Rast accession	Reads bacteria	Reads Flavobacteria	% of all bacteria	Author	Country
Eruca sativa (Mill.) Thell.	Phyllosphere	4551355.3	18454843	695837	3.8	Erlacher et al.[a]	Austria
Arabidopsis thaliana (L.) Heynh.	Phyllosphere	4447810.3	1211215	52764	4.4	Knief et al. (2012)	Philippines
Trifolium L.	Phyllosphere	4447811.3	1079498	25602	2.4	Knief et al. (2012)	Switzerland
Oryza sativa L.	Phyllosphere	4450328.3	2343660	16532	0.7	Knief et al. (2012)	Philippines
Eriophorumvaginatum L.	Whole plant (beat bog)	4551107.3	21452578	113134	0.5	Bragina et al.[a]	Austria
Calluna vulgaris (L.) Hull	Whole plant (beat bog)	4551108.3	16989172	95575	0.6	Bragina et al.[a]	Austria
Pinusmugo Turra	Whole plant (beat bog)	4551110.3	5229582	23735	0.5	Bragina et al.[a]	Austria
Andromeda polifolia L.	Whole plant (beat bog)	4551111.3	13162046	73802	0.6	Bragina et al.[a]	Austria
Eruca sativa (Mill.) Thell.	Rhizosphere	4447811.3	21946485	590015	2.7	Erlacher et al.[a]	Austria
Oryza sativa L.	Rhizosphere	4449956.3	1075595	8777	0.8	Knief et al. (2012)	Philippines
Brassica Napus L.	Rhizosphere	4574757.3	39494127	448590	1.1	Müller et al.[a]	Germany
Brassica Napus L.	Rhizosphere	4574758.3	33932035	663598	2.0	Müller et al.[a]	Germany

Brassica Napus L.	Rhizosphere	4574759.3	30301702	455525	1.5	Müller et al.[a]	Germany
Brassica Napus L.	Rhizosphere	4574760.3	31843071	580172	1.8	Müller et al.[a]	Germany
Matricariachamomilla L.	Rhizosphere	4555162.3	27529235	328753	1.2	Köberl et al.[a]	Egypt
Solanumdistichum Schumach.&Thonn.	Rhizosphere	4555633.3	29208669	451906	1.5	Köberl et al.[a]	Egypt
Bulk soil (*E.sativa*)	Soil	4551574.3	16864838	181175	1.1	Erlacher et al.[a]	Austria
Agricultural soil (corn)	Soil	4508939.3	7334405	103534	1.4	Smith et al.[b]	USA
Agricultural soil (soy)	Soil	4508940.3	10494734	107094	1.0	Smith et al.[b]	USA
Leafy wood soil	Soil	4508941.3	6247099	48226	0.8	Smith et al.[b]	USA
Bulk soil *(Brassica Napus* L.)	Soil	4574761.3	39494127	448590	1.1	Müller et al.[a]	Germany
Bulk soil *(Brassica Napus* L.)	Soil	4574762.3	35279695	340448	1.0	Müller et al.[a]	Germany

[a]The authors had exclusive access to these unpublished datasets to extract required taxonomic information for this book chapter
[b]Unpublished study with publicly available data

2007). In an additional study, a significant increase in wheat and barley canopy Rubisco expression associated with plant biomass accumulation was observed in field studies when H_2-oxidizing strain *Flavobacterium* JM162a was applied (Flynn et al. 2014). Other studies that screened *Flavobacterium* isolates for ACC deaminase activities indicated the presence of genes, but in some cases, the enzymatic activity could not be confirmed biochemically (Kolton et al. 2013; Soltani et al. 2010). Additionally, members of *Flavobacterium* genus can synthesize growth-stimulating phytohormones. For example, plant growth promotion was observed by plant-associated *Flavobacterium* strains producing auxins, gibberellins and cytokinins (Tsavkelova et al. 2007a, b; UmaMaheswari et al. 2013).

Despite the beneficial traits of certain plant-associated *Flavobacterium* strains, their commercial application as biocontrol agents or biostimulants in the future may be questioned due to their capacity as fish (Sudheesh et al. 2012) and opportunistic human (Berg et al. 2005) pathogens.

9.4 Elucidating Mechanisms Associated with Plant-Flavobacterial Interactions

The capacity of a microorganism to effectively colonize, proliferate and persist in the highly competitive root ecosystem must have unique characteristics that are collectively addressed by the term "rhizosphere competence" (Lugtenberg et al. 2001). Over the past decades, a myriad of studies have explored the genetic, physiological and biochemical characteristics that facilitate rhizosphere competence. These rhizosphere competence-associated traits include mobility, chemotaxis, carbon metabolism, antibiotic production, iron assimilation, and nitrogen metabolism (Ahmad and Baker 1987; Lugtenberg and Dekkers 1999). Despite the array of evidence demonstrating the high abundance of *Flavobacterium* strains in the rhizosphere microbiomes and their significant propagation along root surfaces relative to bulk soil, there is only limited data regarding the mechanisms that enable them to colonize and persist in this highly competitive environment. Terrestrial and plant-associated members of this genus are generally characterized by fast growth rates, which are essential in the highly competitive root environment; however, this alone cannot explain their high abundance in the rhizosphere, and other mechanisms are inevitably essential. Below, we discuss recent findings that have shed light on specific*Flavobacterium*-associated traits that are potentially associated with rhizosphere competence.

Metabolism The capacity to utilize carbon compounds that are readily available in the rhizosphere is a prerequisite for rhizosphere competence. It has been suggested that by having the capacity to utilize macromolecules, root-associated *Flavobacterium* strains do not compete with members of the phylum *Proteobacteria* that prefer to degrade low-molecular-mass substances such as plant exudates (Olsson and Persson 1999), a phenomenon previously defined as metabolic niche partitioning (Georges et al. 2014). This hypothesis is supported by the fact that in

addition to their high abundance in root environments, Members of the genus *Flavobacterium* are also highly profuse on and within plant roots (described above) where exudates do not exist. Although a certain level of niche partitioning may occur, these strains undoubtedly compete with other macromolecule-degrading bacteria (i.e., *Bacillus* and *Actinobacteria*) and fungi in the highly complex root ecosystem, necessitating additional rhizosphere competence-associated mechanisms. For example, a *Flavobacterium* sp. was found to outcompete *Bacillus* species by metabolizing the peptidoglycan component of the Gram-positive cell wall (Peterson et al. 2006).

A comprehensive comparative whole-genome analysis of soil and aquatic *Flavobacterium* strains revealed that plant-associated terrestrial *Flavobacterium* strains were characterized by a significantly higher abundance and diversity of genes involved in metabolism of plant cell wall-associated carbohydrates such as xylose, arabinose and pectin, whereas aquatic strains possessed higher numbers of genes involved in protein degradation. Interestingly, genes encoding glycoside hydrolase (GH) families GH78 and GH106, responsible for rhamnogalacturonan utilization (exclusively associated with terrestrial plant hemicelluloses), were only present in terrestrial clade genomes (Kolton et al. 2013). The abundance of plant carbohydrate-degrading glycoside hydrolases in these terrestrial *Flavobacterium* genomes was also significantly higher than in the genomes of root-associated *Proteobacteria* (i.e., *Pseudomonas*), supporting the metabolic niche partitioning hypothesis described above. This study clearly demonstrates that the genomes of plant-associated *Flavobacterium* strains are strongly influenced by their environment and indicates that they have specifically evolved to metabolize complex sugars (glycans) that are constituents of the plant cell wall (Kolton et al. 2013).

We targeted the metagenome of the *Eruca sativa* rhizosphere described above (Erlacher, unpublished) to specifically assess the relative abundance of specific carbohydrate-utilizing mechanisms that are enriched in *Flavobacterium* strains relative to the rest of the rhizosphere microbiome (Fig. 9.3). Interestingly, genes encoding beta-galactosidases and glucosamine-6-phosphate deaminases were significantly higher in *Flavobacterium* strains relative to the whole rhizosphere metagenome, whereas endo-1,4-beta-xylanase-encoding genes were significantly lower. In legumes, galactosides were found to be present in patches around zones of lateral root initiation and around root hairs but not around root tips (Bringhurst et al. 2001). Collectively, this may indicate both spatial and metabolic niche differentiation in the rhizosphere which may support the coexistence of multiple taxa in the rhizosphere.

Motility The migration of soil bacteria toward the root and their adhesion to root surfaces are a prerequisite for rhizosphere competence and are key attributes of bacterial root surface colonization (de Weert et al. 2002; Rodriguez-Navarro et al. 2007; Sessitsch et al. 2012). Plant roots are highly dynamic, and growth and temporal and spatial fluctuations in root-exudate composition and concentration undoubtedly demand that microorganisms are not only capable of migrating from bulk soil

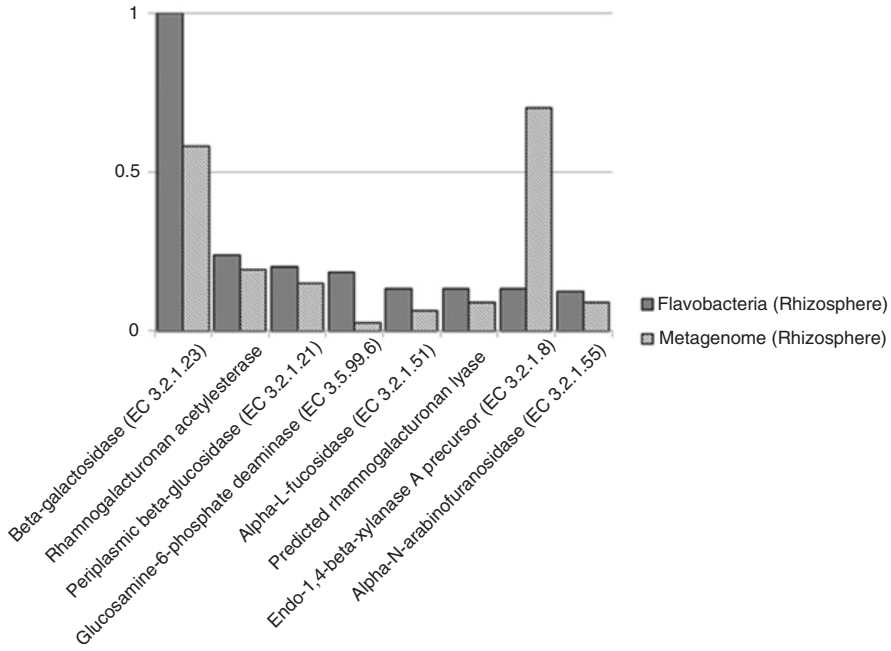
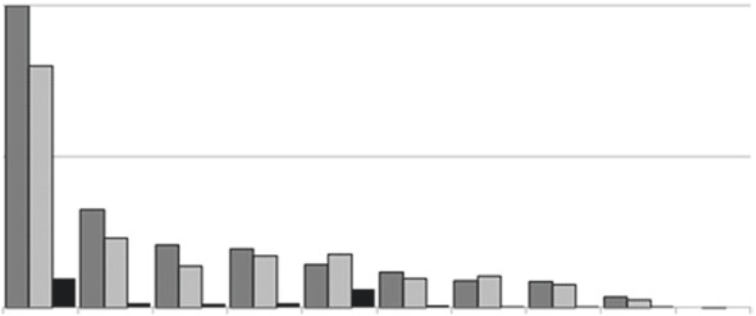


Fig. 9.3 Selected *Flavobacterium* carbohydrate metabolism-associated genes derived from the rhizosphere metagenome compared to the whole rhizosphere metagenome of *E. sativa* based on the BLASTX algorithm. Relative abundance was normalized to 1

to the root but also possess the capacity to move along the plant root surface. Different bacterial taxa have developed highly diverse mechanisms of motility (Jarrell and McBride 2008; Kearns 2010), and several of these mechanisms have been linked to both of the above modes.

As detailed above, members of the genus *Flavobacterium* are also characterized by a unique form of gliding motility (linked to a novel-type IX secretion system) that allows it to rapidly move over solid surfaces (McBride and Zhu 2013; McBride and Nakane 2015). We previously hypothesized that this gliding-secretion complex may be essential for colonization and persistence on plant roots and therefore constructed gliding-secretion mutants from a *Flavobacterium* sp. isolated from sweet pepper (*Capsicum annuum*) roots (Kolton et al. 2012). Disruption of the gliding-motility-type IX secretion system complex had a significant impact on seed adhesion capacity, plant root colonization, and rhizosphere persistence but showed no differences relative to wild-type strains when grown under planktonic conditions (Kolton et al. 2014).

We further assessed the role of the T9SS in plant biomes by screening for selected T9SS-associated genes in the phyllosphere, rhizosphere and surrounding bulk soil *E. sativa* metagenomes (Fig. 9.4). Abundance of all of the targeted T9SS-associated genes was significantly higher in the rhizosphere and phyllosphere than in the bulk



	SprA	SprE	GldM	GldK	SprB	GldN	GldL	SprT	RemA	ChiA
Phyllosphere	507	146	87	108	112	61	67	49	16	1
Rhizosphere	634	205	131	123	90	74	57	55	22	0
Soil	62	9	7	9	39	4	3	3	3	1

Fig. 9.4 GenBank abundance hits of T9SS-associated genes in the phyllosphere, rhizosphere and the corresponding bulk soil *E. sativa* metagenomes

soil. This was especially true for *sprA* which encodes a protein required for secretion of the cell-surface gliding-motility adhesins SprB and RemA (Shrivastava et al. 2013). While RemA has a lectin domain that binds exo-polysaccharides, and therefore may be associated with binding to plant glycans and to motility along plant surfaces, the binding domain of SprB is currently unknown.

9.5 Summary and Conceptual Model of Root-Flavobacterial Interactions

A multitude of data presented here clearly indicates that *Flavobacterium* strains constitute a significant fraction of the root and leaf microbiomes of many plant species, where they may be associated with plant health. Nonetheless, the current state of the art is only beginning to shed light on the potential mechanisms that allow members of this genus to colonize and compete in these highly competitive ecosystems, and even less is known about how and to what extent these populations interact with different plant strains. Based on current knowledge, we infer that they are specialized in the metabolism of complex plant-associated carbohydrates and that their unique gliding-motility complex is associated with colonization and propagation on root surfaces (Fig. 9.5).

To further understand the role of *Flavobacterium* strains in plant ecosystems and terrestrial primary production, future research needs to focus on elucidating several questions including: (A) what mechanisms are associated with their observed enrichment along root surfaces relative to bulk soils (chemotaxis); (B) does the *Flavobacterium* gliding motility play a role in rhizosphere competence; (C) which carbohydrates are most readily metabolized by *Flavobacterium* strains in root and

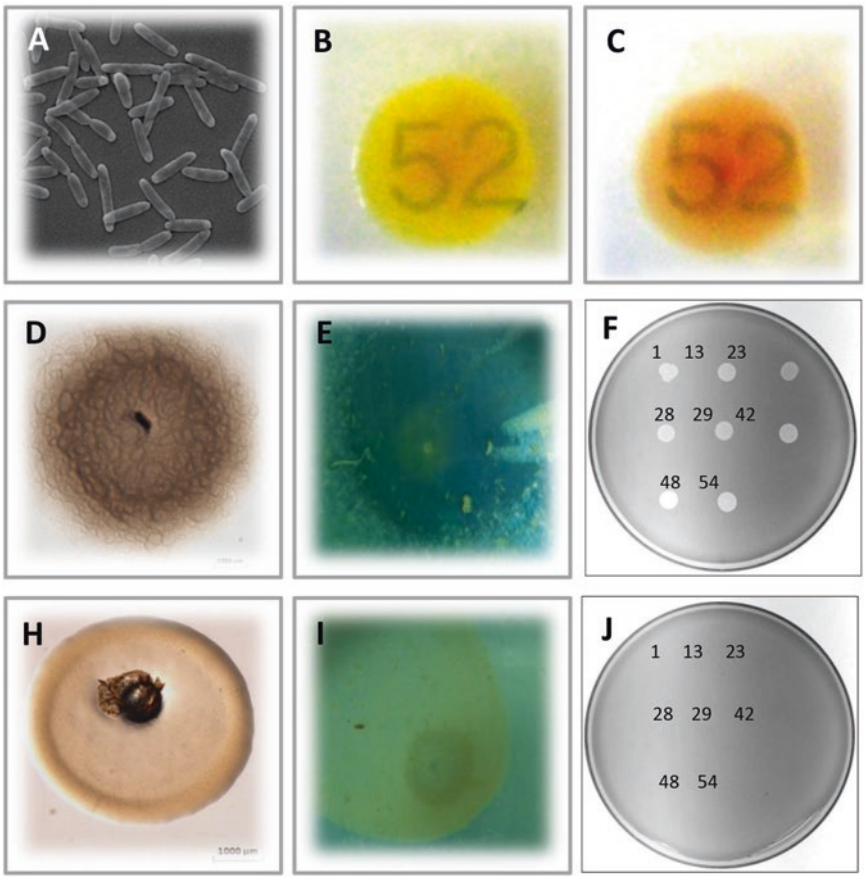


Fig. 9.5 Phenotypic characterization of the sweet pepper root isolate *Flavobacterium* sp. F52 (*top row*): Scanning electron micrograph (**A**) and flexirubin pigment of colony before (**B**) and after addition of KOH (**C**). Comparison of *Flavobacterium* sp. F52 wild type (*middle row*) and gldJ gliding-secretion mutant strains (*bottom row*): gliding-motility colony phenotype (**D** and **H**); extracellular chitinase activity (**E** and **I**); and phage sensitivity assay (**F** and **J**). Phage infection test was conducted by Prof. Mark McBride (University of Wisconsin)

leaf environments and how are these carbohydrates related to plant species and physiology; (D) what is the role of the *Flavobacterium*-associated T9SS in the plant environment, and specifically, which extracellular enzymes are secreted through T9SS and what plant components may be transported into the cell by this system; (E) how are *Flavobacterium* strains distributed on root and leaf surfaces and is their distribution linked to the availability of specific carbohydrates in these microenvironments; and finally (F) how do *Flavobacterium* strains interact with other dominant constituents of root- and leaf-associated microbiomes. The combined use of

environmental metabolic modeling based on state-of-the-art molecular, metagenomic, metatranscriptomic, and proteomic analyses coupled to comprehensive physiological and biochemical (including metabolomics) analyses should contribute to elucidating these questions in the future.

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Heterotrophic Denitrification and *Paracoccus* spp. as Tools for Bioremediation

10

Gastón Azziz, Gabriela Illarze, and Pilar Irisarri

Abstract

Denitrifiers comprise a metabolically diverse group of microbes used as a resource for environmental engineering, due to their ability to perform anaerobic respiration. The main use of denitrification in environmental sustainability is the removal of nitrate and nitrite in water treatment plants.

Heterotrophic denitrifiers are those that use organic molecules as C sources, including pollutants, a trait that makes them as a potential tool for many bioremediation processes. A notorious advantage of denitrifiers over other microorganisms is that they are able to degrade pollutants in anaerobic environments, which extend their potential usefulness.

In this chapter, recent advances regarding the use of heterotrophic denitrifiers in environmental sustainability will be discussed. We end the chapter discussing the singularities of denitrifying strains of the genus *Paracoccus* and their potentiality in environmental sustainability.

10.1 Introduction

Denitrification is a biological process where nitrogen oxides (i.e., NO_3^-, NO_2^-) are converted to reduced forms. The main products of denitrification are N_2O and N_2, which are in gaseous state in all environmental conditions. Microorganisms capable of performing denitrification are collectively called denitrifying microorganisms or denitrifiers. These microorganisms comprise a phylogenetically broad and diverse

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group, with representatives in the domains Bacteria, Archaea, and Eukarya. Most well-known and characterized denitrifiers, however, belong to the Bacteria domain (Long et al. 2015). In the Eukarya domain, a few fungi species are able to denitrify (Shoun et al. 1992).

Not every biological N oxide reduction is considered to be denitrification. The term denitrification is restricted to the use of N oxides as electron acceptors in a respiratory chain, coupled with ATP synthesis for energy conservation. Most denitrifying microorganisms are facultative anaerobes and therefore can use either O_2 or certain N oxides as electron acceptors (Zumft 1997). With a few exceptions, such as *Bacillus azotoformans* (Nielsen et al. 2015) that is unable to use O_2 as electron acceptor, no obligate denitrifiers are known. Thus, denitrification is a process that is favored in anaerobic or microaerobic environments.

As a very diverse group of microorganisms, denitrifiers can use different compounds as electron sources. Regarding their preferred electron source, denitrifiers can be separated into heterotrophic and autotrophic denitrifiers. Heterotrophic denitrifiers use organic compounds as electron source and comprise a metabolically diverse group. The process by which organic molecules serve as electron donors in a denitrification process is called heterotrophic denitrification. On the other hand, autotrophic denitrifiers use inorganic molecules as electron donors. Methane can also be used as electron donor by a group of denitrifiers called denitrifying methanotrophs (He et al. 2015). Autotrophic denitrification is widely used in environmental sustainability; however, to discuss such examples is beyond the scope of this chapter.

The complete denitrification pathway comprises the conversion of NO_3^- to N_2 (Fig. 10.1). This reduction occurs in a stepwise manner with the following intermediates: NO_2^-, NO, and N_2O:

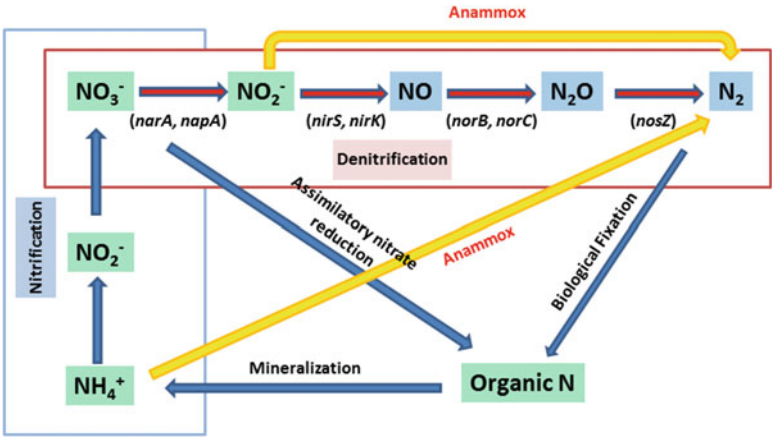

Fig. 10.1 Overview of the microbiological nitrogen cycle. The denitrification pathway is outlined in a *red square* and the names of the genes that code for the different enzymes active sites are included. Gaseous products are depicted as *blue boxes*

(i) The conversion of nitrate to nitrite (NO_2^-) is catalyzed by either a periplasmic or a membrane-bound respiratory nitrate reductase (Bell et al. 1990), whose catalytic subunits are encoded by the *napA* and *narG* genes, respectively. Interestingly, *narG* has also been found in non-denitrifying microorganisms such as *Escherichia coli* (Philippot 2002).

(ii) Nitrite is then reduced to nitric oxide (NO) by a nitrite reductase. Nitrite reductases have long been considered the step-committed enzymes of the denitrification processes, due to the fact that in this step the reduction of N oxides becomes exclusively committed to energy conservation. There are two kinds of nitrite reductases, a copper and a cytochrome cd1-containing nitrite reductase, encoded by the *nirK* and *nirS* genes, respectively. Most information support that *nirK* and *nirS* genes do not coexist in the same organism (Jones et al. 2008), but a few exceptions have been found recently (Graf et al. 2014). Denitrifying microorganisms can be classified into *nirK*- and *nirS*-type denitrifiers (Jones et al. 2014).

(iii) Nitric oxide is reduced to nitrous oxide (N_2O) by the nitric oxide reductase, a heterodimeric enzyme encoded by the *norB* and *norC* genes (Hino et al. 2010).

(iv) Finally, N_2O is converted to N_2 by the nitrous oxide reductase, encoded by the *nosZ* gene (Orellana et al. 2014).

According to the set of genes present in the microorganism, denitrifiers can be divided into two groups: (1) microorganisms with a functional entire denitrifying pathway (complete denitrifiers) and are therefore able to perform the stepwise reduction of NO_3^- or NO_2^- all the way to N_2 and (2) microorganisms lacking some of the genes involved in the reduction pathway to N_2, but that still use N oxides as electron acceptors in a respiratory chain. Examples of these incomplete denitrifiers are those that have either *nirK* or *nirS* genes but lack *nosZ*; consequently their denitrification product is N_2O rather than N_2. After the analysis of many denitrifier genomes, the following observation was done: *nirK*-type denitrifiers are more prone to lack the *nosZ* gene than *nirS*-type denitrifiers (Jones et al. 2008). A few microorganisms carry only the *nosZ* gene (Sanford et al. 2012), suggesting that they just reduce N_2O to N_2.

Denitrifiers are practically present on every habitable environment on earth, and denitrification occurs in a myriad of environments (anaerobic and microaerobic milieus). Extremophilic denitrifiers have been recently described, and most of them belong to the Archaea domain (Offre et al. 2013). The denitrification in extreme environments opens up new possibilities and challenges for the study of this process in novel conditions and environments.

Most denitrifying organisms are neutrophilic, but also acidophilic (Huang et al. 2014a) and alkaliphilic (Sorokin et al. 2001) denitrifiers have been reported, with a prevalence of N_2O over N_2 production as final product at low pH (Huang et al. 2014b).

The temperature also plays an important role in the regulation of denitrification rates. Although denitrification activity increases with temperature, it can still occur at near 0 °C, with optimal activity in soil at the mesophilic range (Palmer et al. 2010).

Thermophilic denitrification (over 50 °C) is carried out mainly by spore-forming gram-positive bacteria and archaea, with a few known thermophilic gram-negative bacteria (Courtens et al. 2014).

The aim of this chapter is to analyze the current knowledge on denitrification and denitrifying organisms, with focus in heterotrophic denitrification processes and their use as biological tools to improve environmental sustainability, mainly as mediators of bioremediation. Current uses, pilot-scale experiences, and perspective of future applications, as well as limitations of the different cases, will be discussed. The chapter ends with a section dedicated to the genus *Paracoccus*, which has gained attention recently due to its metabolic versatility.

10.2 Heterotrophic Denitrification in Bioremediation Strategies

10.2.1 Nitrate and Nitrite Contamination of Waters

Nitrate and nitrite produce several environmental and human health risks (methemoglobinemia and gastrointestinal cancer; Powlson et al. 2008). The excess of these molecules in water bodies can trigger an accelerated growth of certain microorganisms. This often results in algae or cyanobacterial blooms with an excessive biochemical oxygen demand (BOD), which can be harmful for the resident normal biota. Besides, some cyanobacterial strains release toxins that can be detrimental to human health (Sivonen 1996).

The WHO guideline values of NO_3^- and NO_2^- in drinking water are 50 mg/l and 3 mg/l, respectively (World Health Organization 2011), and the "Nitrates Directive" of the European Commission states that all surface freshwater or groundwater with more than 50 mg/l of nitrate is considered polluted or at risk of pollution (European Commission).

Nitrate pollution derives mainly from agricultural practices and from sewage. As the worldwide food demand continuously increases, the usage of fertilizer in agriculture is not foreseeable to decrease; therefore the development of environmental friendly techniques to efficiently remove N from water is becoming necessary.

The use of denitrification to remove NO_3^- and NO_2^- from sewage and water in treatment plants certainly was the first use of denitrifying microorganisms in environmental sustainability and arguably is among the first experiences in the use of microorganisms as bioremediation agents. The idea of designing an anoxic zone to enhance denitrification in an activated sludge with the aim of N-removal from water dates back to 1962 (Lofrano and Brown 2010). In the following subsections, we summarize the current knowledge and recent innovations of water N-removal technologies that use denitrification and denitrifying organisms, with emphasis in the N-removal performed by heterotrophic denitrification.

Due to its applicability in many different scenarios and the need to improve wastewater treatments, N-removal by heterotrophic denitrifying microorganisms is a dynamic field of research. Denitrification is mainly used to remove excess nitrate or nitrite from groundwater or from wastewater, as described in the following sections.

10.2.1.1 N-Removal from Groundwater

Groundwater contamination with nitrate and nitrite has different sources. Although the most common cause of nitrate contamination is agricultural practices and inadequate wastewater management, other sources such as mining, aquaculture, landfill leachate, and industrial wastes (Wakida and Lerner 2005) also play an important role on groundwater N-pollution.

As part of a natural biogeochemical cycle, naturally occurring denitrification should be sufficient to reduce nitrate and nitrite levels in a pristine groundwater. However, anthropogenic activities have often led to increased concentrations of these molecules, and usually the native resident microbiota of aquifers is not sufficient to remediate pollution. As explained earlier, denitrification rates are subject to environmental conditions, mainly to oxygen and nutrient concentrations (Rivett et al. 2008). As heterotrophic denitrifiers use organic molecules as source of energy and these organic molecules are far from an optimum concentration for denitrification in natural conditions, usually the denitrification rates are slow, even when denitrifying microorganisms are present in the groundwater.

Strategies to alleviate nitrate or nitrite contamination in groundwater include the modification of the environmental parameters to enhance the denitrification process by the resident microbiota by the addition of organic molecules or by the inoculation of the groundwater with denitrifying microorganisms.

In groundwater, the most limiting factor for denitrification is often the energy source which in the case of heterotrophic denitrification is organic molecules. One way to tackle this limitation is to add organic material to the environment. For example, as the efficient denitrification takes place in riparian buffer zones (Burt et al. 1999), water purification systems have been designed to simulate these zones as shown in Fig. 10.2. A denitrification wall (a permeable reactive barrier or matrix, composed of organic material that has been dug in a trench to the groundwater) is enriched in resident bacteria, and as the groundwater passes through this wall, the denitrification process takes place, and the nitrate levels are reduced. Denitrification walls are relatively inexpensive and require little maintenance. It has been shown that a wall constructed with sawdust as C source may remove NO_3^- through denitrification after 14 years of operation (Long et al. 2011). In a field trial conducted to demonstrate the utility of sawdust as amendment in a denitrification wall, the nitrate concentrations were tenfold reduced (from 5 to 16 mg of N L^{-1} in the incoming side to 0.6 and 2 mg of N L^{-1} in the outgoing site) (Schipper and Vojvodić-Vuković 1998), even after a year of operation without subsequent C-source additions.

A critical point to consider in the implementation of denitrification walls is the permeability of the matrix. Depending on the subsoil quality of the aquifer, some materials used to construct the wall can force the water to flow underneath the wall (Schipper et al. 2004), avoiding the purification process. Different flow rates through the wall can also influence the nitrate removal rate; therefore, the porosity of the material should be considered. In addition, the microorganisms that proliferate on the matrix can alter its porosity, either by forming biofilms or by degrading the matrix that decrease or increase the porosity, respectively (Scherer et al. 2000). The material used in the construction of the matrix, which provides physical support and acts as C source, has also to be evaluated. Many materials show good nitrate removal

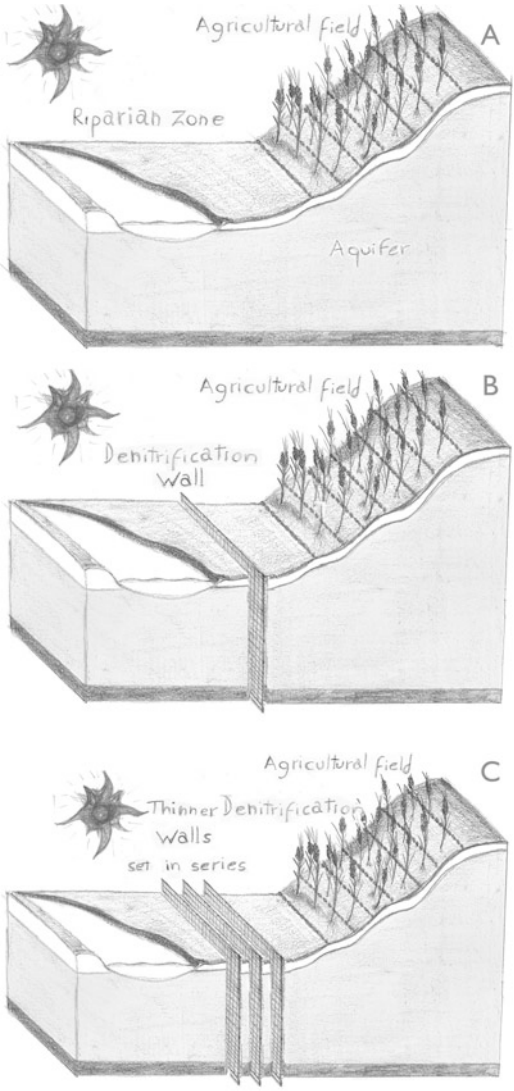

Fig. 10.2 Riparian zones (**a**) provide a natural way to remove nitrate and nitrite from groundwater. To improve and enhance this natural process, as well as to mimic them, nitrification walls can be constructed (**b**). A series of thinner denitrification walls can be more efficient than a single but broader one (**c**) (Drawing by Juan José Marizcurrena)

rates, such as softwood (Gibert et al. 2008) or dried cornstalks, cardboard, wood chips saturated with soybean oil, and wood chips (Greenan et al. 2006). Currently, the installation of a series of walls over the groundwater, instead of a single but broader wall, is recommended (Schmidt and Clark 2012) (Fig. 10.2c).

The addition of organic substrates to groundwater by direct injection also improves the denitrification process. A natural attenuation of nitrate contamination in a uranium mining site in Arizona was observed in microcosm experiments, but the addition of ethanol or methanol increased the nitrate removal rate over an order of magnitude (Borden et al. 2012; Carroll et al. 2009). Moreover, the nitrate

concentration in the groundwater remained low even months after the injection. Ethanol may have served as a primer for denitrifiers proliferation; meanwhile, the decay of the microbial population may have provided nutrients for the following generations of denitrifiers, eliminating the need of further ethanol applications.

The addition of glucose and acetate in a lab-scale study to enhance natural denitrification of an aquifer in Spain showed that both molecules increased nitrate removal rates, but the addition of acetate produced nitrite accumulation (Calderer et al. 2010). Under anaerobic conditions, most glucose (99 %) was used for energy source in denitrification, suggesting that the addition of this sugar is a promising strategy to enhance in situ denitrification of aquifers, but optimum parameters in a full-scale operation have still to be set up.

The inoculation of groundwater or the use of submerged filters embedded with microorganisms (a single strain or a consortium of microorganisms) may increase the denitrification rates. Classically, submerged filters are inoculated with activated sludge that typically consists in a diverse and uncharacterized population of microorganisms, but usually these microbes (denitrifiers and other ones) form biofilm that occludes the filter (Gómez et al. 2000). The inoculation with axenic cultures may circumvent this disadvantage. A study comparing the effect of the inoculation with activated sludge or *Hydrogenophaga pseudoflava* showed the use of *H. pseudoflava* gives a better quality of the treated water (Moreno et al. 2005).

Biofilm-based filters used to treat groundwater have a limited efficiency regarding removal of nitrates. Membrane reactors, for instance, are prone to fouling; aeration can be used to control fouling, but it disrupts the anaerobic environment needed for denitrification. Currently, many efforts have been made to decrease fouling by using different polymers in the construction of the filters (Yamato et al. 2006). Submerged filters are usually primed with activated sludge and are limited by the number of bacteria they can sustain (de la Rúa et al. 2008). One variant of biofilm filters are fiber-based biofilms, in which fiber threads are used to construct the matrix of the biofilms. Lab-scale studies that evaluated the performance of a fiber-based biofilm reactor found that nitrate removal rates were above 99 % with nitrate loads of 148.66 ± 10.64 mg L^{-1} of NO_3^- (Wang et al. 2009). In the first runs, nitrite was present in the effluent, but the problem could be solved by adjusting retention times.

The use of constructed wetlands for pollution control is yet another strategy to remove nitrate from groundwater. This depends on both plant uptake and denitrification to remove nitrate and other contaminants from groundwater. Although at first it was thought that plant uptake was the main driver of nitrate removal in constructed wetlands, an experiment evaluating different macrophyte species (Lin et al. 2002) found that between 89 and 96 % of the nitrate was removed by denitrification.

Denitrifying microorganisms have played a paramount role on nitrate and nitrite removal from groundwater. These microorganisms constitute the natural way in which nitrogen is returned to the atmosphere. As showed above, the use of denitrifiers represents a promising and environmentally sound alternative to remove nitrate and nitrite from groundwater, either as inoculants or by enhancing the denitrifying activity of resident populations.

10.2.1.2 N-Removal from Wastewater

Industrial and municipal wastewaters often contain high levels of nitrates that must be diminished before discharge to the environment, and the approaches used to promote denitrification in groundwater can also be applied to wastewater treatment. However, due to the different nature of both environments, different bioremediation strategies have to be performed.

Sequencing batch reactors (SBRs) are the most used approach to treat wastewater. They have been used since the 1920s, and for a long time, they were employed without full understanding of the biological processes that occurred. These systems are used to remove both C and N pollutants, and many variants have been developed (Wang and Li 2009). Usually the tanks containing the wastewater are filled, at the bottom, with sludge containing microbes that can use pollutants as nutrients, and as wastewater comes in contact with the sludge, the pollutants start being removed. The rationale behind this operation is to alternate aerobic and anaerobic conditions in such a way that couples nitrification with denitrification to remove both ammonium and nitrate. Ammonium removal can also be anaerobically done due to the activity of anaerobic ammonium-oxidizing (Anammox) microorganisms, which in turn produce nitrate further used by denitrifiers. The main difference between a SBR and a typical activated sludge is that SBR occurs following sequential steps in a single tank.

In this subsection we will focus on the recent uses and research on the role of the heterotrophic denitrification process and heterotrophic denitrifying microorganisms in SBR treatment of wastewater.

Using the SBR technology, Lyles et al. (2008) treated shrimp aquaculture wastewater, obtaining fully reusable water. Authors showed that removal of ammonia, nitrate, and nitrite was achieved after 7 days of treatment in the pilot-scale SBR and confirmed that the sequential processes of nitrification and denitrification were responsible for N-removal. However, the key microorganisms were not identified, and the population responsible for such performance was not characterized.

An alternative to the classic nitrification-denitrification approach is the process called nitrification-denitrification via nitrite accumulation (Ruiz et al. 2006). It consists of the sequential process of nitritation (conversion of ammonium to nitrite, carried out by autotrophic ammonia-oxidizing microorganisms) and denitritation (reduction of nitrite to N_2) that creates a shortcut in the metabolic pathway in the N cycle (Fig. 10.1), which allows the oxidation of ammonia to nitrite and further denitrification (Fig. 10.3). The main advantage of this alternative is that it spares aeration costs for ammonia oxidation and reduces C input needs for the heterotrophic nitrate reduction. However, during this process, it is important to maintain a low O_2 pressure during nitrification and denitrification. Interestingly, it was found that once the reactor is stabilized, increasing aeration does not restore nitrite oxidation to nitrate (Yongzhen et al. 2007).

This system has been used mostly to treat wastewaters with relatively low nitrogen levels such as domestic wastewaters. The nitrification-denitrification process via nitrite accumulation is commonly influenced by environmental factors. At temperatures above 20 °C, the development of ammonia-oxidizing bacteria (AOB) over

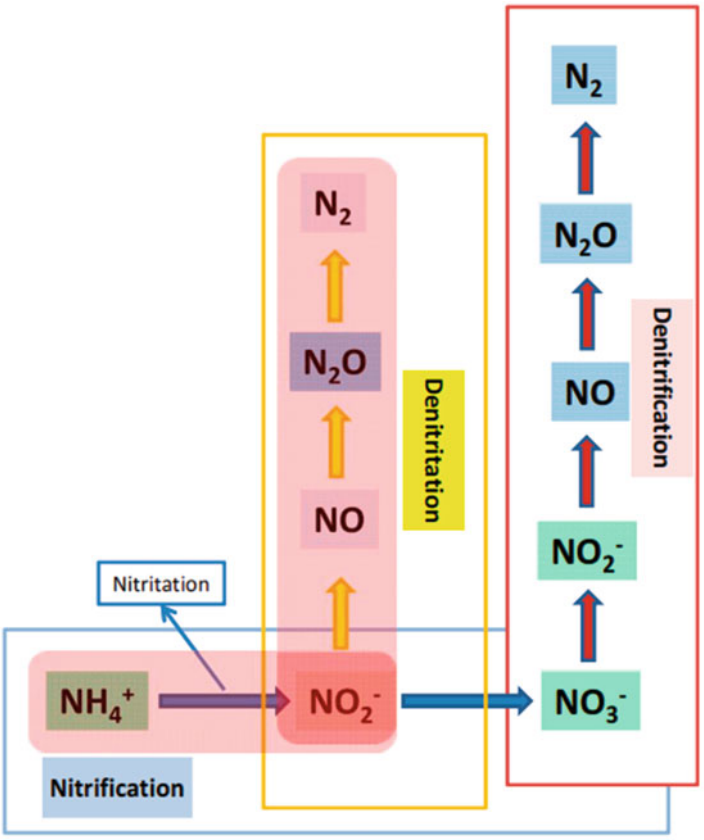

Fig. 10.3 Nitrification-denitrification via nitrite accumulation couples the processes of nitritation and denitritation (outlined in a *yellow square*) and creates a shortcut in the nitrification-denitrification process. Nitrification-denitrification via nitrite accumulation is highlighted in *pink*

nitrite-oxidizing bacteria (NOB) guarantees sufficient nitrite accumulation (Wu et al. 2007). Aeration also increases the development of AOB over NOB in temperate temperature ranges (Blackburne et al. 2008). Unfortunately, this strategy is unaffordable in cold environments, but the enrichment and posterior acclimation with AOB, together with NOB washout by aeration control, have shown promising results (Guo et al. 2010).

The use of SBRs for piggery wastewater treatment has operational limitations to many farmers, but Shipin et al. (2007) developed a system that takes the advantages of the nitritation-denitritation coupled process and works on separate tanks containing floating filters.

Contamination with both nitrate and ammonium is a common situation in wastewater. The SBR systems embody the most ancient use of denitrifiers in environmental sustainability and one of the most studied and improved N-removal strategies. However, the use of SBR systems has many limitations. The development of

systems that take advantage of nitrification-denitrification via nitrite accumulation can represent a leap forward in optimizing wastewater treatment systems. It is expectable that future researches would be focused in improving these systems.

10.2.2 Polyaromatic Hydrocarbon (PAH) Degradation

PAHs are polyaromatic hydrocarbon molecules (Wilcke 2000) with potential carcinogenic effects. They occur naturally as a consequence of wildfires and volcanic activity, but are also produced by plants (Edwards 1983). However, anthropogenic sources are the main matter of concern. Bioremediation is currently the most promising strategy to solve contamination by PAHs, in terms of feasibility. Soil is the main sink of this pollutant and the target of most bioremediation strategies. In the present subsection, we will focus on the use of denitrifying microorganisms as bioremediation agents to treat PAH pollution.

PAHs are naturally degraded by many microorganisms mainly through mineralization. However, naturally occurring degradation is insufficient to cope with the amounts of PAHs at polluted sites. Therefore, different strategies that involve the utilization of PAH-degrading microorganisms are being developed.

Bioaugmentation is the inoculation to polluted sites with microorganisms capable of pollutant degradation. It has been shown that this is a good strategy to remediate PAH-contaminated areas. Aerobic degradation of PAHs is a widespread trait among microorganisms (Habe and Omori 2003). However, if in situ bioremediation is intended for environments where O_2 availability is spatially or temporally scarce, denitrifying microorganisms provide a promising resource for the degradation of PAHs.

The degradation of three PAHs present in polluted aquifers was successful (Durant et al. 1995), but it was limited by the environmental conditions. The authors evaluated whether nitrate amendment could enhance PAHs degradation in anaerobic conditions. It was found that nitrate and phosphorus were limiting the anaerobic degradation. This study was one of the first to show that indigenous heterotrophic denitrifying bacteria present in polluted aquifers were able to degrade PAHs, but this degradation was limited by the environmental conditions of the aquifer. It also opened the possibility of in situ enhancement of anaerobic degradation through the addition of limiting nutrients.

Following the rationale of providing nutrients to optimize denitrifying activity, Wang et al. (2012) conducted a microcosm experiment in an anthracene-contaminated aquifer sediments amended with nitrate (80 days of anaerobic incubation at 25 °C) showing that only 1.2 % of anthracene remained, probably due to the decontaminating activity of *Paracoccus*, *Herbaspirillum*, *Azotobacter*, and *Rhodococcus* strains. These results clearly show that given appropriate conditions, populations of anaerobic PAH-degrading microorganisms can be enriched, and nitrate addition is a feasible strategy to provide those conditions. Also mangrove forest sediments supplemented with phenanthrene showed an enrichment of PAH-degrading bacteria, including *Paracoccus versutus* (Guo et al. 2005). Therefore, mangrove sediments are an auspicious source of PAH-degrading microorganisms, particularly PAH-degrading denitrifiers.

Denitrification is an interesting alternative to bioremediate PAH-polluted sites. Particularly, denitrifying organisms may play a crucial role in degrading PAHs in these environments where aerobic conditions are not strict. Soil is the most common environment where PAH contamination occurs, and most soils have fluctuating oxygen dynamics. Thus, the development of systems that take advantage of denitrifiers to degrade PAHs in situ is probably one of the most promising approaches to solve this problem.

10.2.3 Bioremediation of Other Organic Pollutants

Heterotrophic denitrifying microorganisms have been used to decontaminate environments polluted with different organic molecules and are the main alternative of bioremediation when aerobic degradation of the pollutant is impracticable.

Among recalcitrant products, it has been shown the degradation of petroleum hydrocarbons (Hasinger et al. 2012), toluene (Martínez et al. 2007), ethylbenzene, and m-xylene (Kao and Borden 1997), by denitrification. In most cases of groundwater contamination with benzene, toluene, ethylbenzene, and xylene (BTEX), nitrate is a limiting factor for denitrification, but nitrate injection did enhance BTEX degradation in the aquifer as shown by da Silva and Corseuil (2012). Probably, nitrate alleviates the BOD which resulted in the conservation of microaerophilic niches that supported the activity of aerobic degraders that may have contributed with the increase in BTEX degradation. Recently, a strain of *Pseudomonas thivervalensis* was found to degrade BTEX under denitrifying conditions at 10 °C, showing their potential use at low temperatures (Qu et al. 2015).

10.3 *Paracoccus*

Bacteria of the genus *Paracoccus* are gram-negative Alphaproteobacteria that exhibit a great range of metabolic flexibility. The number of species classified as *Paracoccus* has been rapidly growing. In 1991 only two species were recognized, but by the year 2006, 14 species were proposed (Kelly et al. 2006). Currently, 51 different species of *Paracoccus* are registered into the taxonomy browser of the NCBI, being *P. denitrificans* the type specie, a denitrifying bacterium. Due to their metabolic diversity, many researchers analyzed the potential of *Paracoccus* strains in environmental sustainability, mainly by the use of their denitrifying abilities. The following examples reflect the diversity of potential uses that encompass the genus.

Organophosphate insecticides are widely used in agriculture due to its effectiveness in battling economically important plagues. However, they are highly toxic compounds that persist in the environment and commonly reach groundwater through lixiviation. During the isolation of monocrotophos-degrading microbes (a kind of organophosphate), Jia et al. (2006) found a *Paracoccus* strain with promising traits as organophosphate degrader. *Paracoccus* sp. M-1 degrades monocrotophos both aerobically and anaerobically, and interestingly, the anaerobic degradation

occurred via denitrification. Thus, these abilities point out this microbe as an excellent candidate for in situ bioremediation of soils, where O_2 concentration varies greatly. Remarkably, it was also found that M-1 may degrade a variety of monocrotophos-related compounds, a useful trait that may be exploited for the bioremediation of co-contaminated soils.

Paracoccus sp. T231, a strain isolated from activated sludge samples collected in a wastewater treatment plant, showed the ability to perform the anaerobic degradation of trimethylamine, at a rate that was found to be dependent on nitrate concentration (Kim et al. 2001). This trait supports the hypothesis that denitrification was responsible for the anaerobic degradation.

According to the NCBI genome database, the genomes of three strains of *Paracoccus denitrificans* are currently available. The genome analysis of *P. denitrificans* strain SD1, an isolate that uses *N,N*-dimethylformamide (DMF) as sole carbon and nitrogen source, showed the presence of nitrate and nitrite reductase genes and also coding sequences for enzymes probably involved in DMF degradation (Siddavattam et al. 2011). The analysis of the complete genome of *P. denitrificans* strain TRP has shown the presence of at least 25 coding sequences for the degradation of xenobiotic compounds (Li et al. 2011).

As describe above (Sect. 10.2.1.2), the classic biological approach to remove both nitrate and ammonium from water bodies is coupling the nitrification and denitrification processes, typically in SBRs. A recent alternative to SBR has been brought by the discovery of microorganisms that can perform both nitrification and denitrification under similar environmental conditions. Contrasting to conventional denitrification, which takes place in anaerobic environments, aerobic denitrification occurs in the presence of O_2, a condition that promotes nitrification. *P. denitrificans* (Ludwig et al. 1993) (formerly *Thiosphaera pantotropha*; Robertson et al. 1988) was the first bacterium described as an heterotrophic nitrifier and aerobic denitrifier (HNAD) microbe. The HNAD process is also performed by *P. versutus* strain LYM that simultaneously removes ammonium and nitrate under aerobic conditions (Shi et al. 2013). Contrary to many anaerobic denitrifiers, the end product of HNAD was mainly N_2 (Takaya et al. 2003).

Due to their ability to denitrify under aerobic conditions, it is not surprising that *Paracoccus* spp. are found as dominant bacterial population in environments where O_2 concentrations fluctuate. This hypothesis was proved by Neef et al. (1996). The authors, using DNA probes targeting *Paracoccus*, found that *Paracoccus* spp. were the dominant genus in the sand bed of a methanol feed reactor, in a wastewater treatment system. In this system, aeration was used to prevent clogging by the sludge, but aeration is a tricky solution. Low aeration would allow clogging, but excessive aeration may inhibit the denitrification process. Thus, *Paracoccus* strains could be used during the decontamination of N-polluted environments and avoid aeration-derived problems.

The members of the genus *Paracoccus* give insight to a myriad of opportunities for different bioremediation prospects. In Table 10.1, examples of *Paracoccus* strains and the results in the degradation of pollutants are shown. Besides denitrifying strains, non-denitrifiers *Paracoccus* are also included in the table.

Table 10.1 List of *Paracoccus* spp. and their use during the degradation of environmental pollutants

Strain	Pollutant degraded	Potential environments where it can be used	Denitrifying conditions	References
Paracoccus sp. BW001	Pyridine	Activated sludge/wastewater	Yes	Bai et al. (2008)
Paracoccus sp. TOH	Piperazine	Activated sludge/wastewater	No	Cai et al. (2013)
Paracoccus sp. TRP	Chlorpyrifos/3,5,6-trichloro-2-pyridinol/pyridine/methyl parathion/carbofuran	Activated sludge/wastewater/soil	No	Xu et al. (2008)
Paracoccus denitrificans M-1	Pyrene	Sediments	Yes	Yang et al. (2013)
P. denitrificans M-1	Monocrotophos	Activated sludge/wastewater	Yes	Jia et al. (2006)
Paracoccus sp. SKG	Acetonitrile	Wastewater	No	Santoshkumar et al. (2011)
Paracoccus versutus LYM	Nitrate/ammonium	Water treatment plants	No	Shi et al. (2013)
P. denitrificans SD1	*N,N*-dimethylformamide	Wastewater/coal mine	Yes	Siddavattam et al. (2011)
Paracoccus sp. XF-3	Chlorothalonil	Soil	No	Yue et al. (2015)
Paracoccus sp. MKU1 and MKU2	*N,N*-dimethylformamide	Activated sludge/wastewater	No	Nisha et al. (2015)
P. denitrificans GH3	Isopropanol	Soil	No	Geng et al. (2015)
Paracoccus pantotrophus SAG_1	Melanoidin	Soil/wastewater	No	Santal et al. (2016)

10.4 Final Considerations

The potential use of denitrifying microorganisms as pollutant removal agents is mainly based on its metabolic diversity. These microorganisms are active at both anaerobic and aerobic environments, simultaneously removing N oxides and organic or inorganic molecules by their use as electron acceptors and donors, respectively.

A single strain or a community of denitrifying microorganisms could be used to remove the pollutant. Contaminant removal techniques that rely on a single microorganism face the challenge of the removal efficiency. Some isolates that effectively remove a contaminant in certain environmental conditions are unable to do so in the prevailing or fluctuating conditions of a more complex contaminated site. Moreover, many contaminated sites present a mixture of contaminants rather than a single pollutant. Often, to find a microorganism that efficiently removes all pollutants present at certain sites is certainly impossible. However, advances in metabolic engineering have opened the possibility of improving the bioremediation abilities of bacterial isolates (Singh et al. 2008). Thus, specific enzymatic activities or pathways for the degradation of many pollutants can be optimized or simply introduced into other bacteria by genomic engineering.

Alternatively, the use of microbial communities can circumvent some of the drawbacks. Usually there is a lack of information about the composition and identity of the microbial community, but next-generation sequencing approaches may allow the full description of the communities, giving insight to the metabolic pathways involved in the degradation of pollutants. To understand the phylogenetic diversity and the metabolic profile of a community that efficiently remove pollutants may help to design the novel strategies of N-removal. In this scenario, *Paracoccus* strains may play a relevant role.

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The Sustainable Use of *Delftia* in Agriculture, Bioremediation, and Bioproducts Synthesis

11

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Abstract

Bacteria of genus *Delftia* are environmental microorganisms with a wide geographical distribution. They are versatile microbes with diverse metabolic capacities that easily adapt to several environments. This chapter provides an overview of various aspects and potential biotechnological applications of *Delftia* spp. as microbial model of sustainability, including their use in bioremediation and bioconversion of contaminants, their use in production of bioproducts, and their use as inoculant to increase plant yield. As a few *Delftia* spp. have been found in clinical samples, their potential role as opportunistic pathogenic agents is also discussed.

11.1 Introduction

Heterotrophic bacteria of the genus *Delftia* are known for their metabolic versatility. They are gram-negative, straight to slightly curved rod-shaped, strictly aerobic, non-glucose-fermenting, oxidase-positive, and mainly motile bacteria (Wen et al. 1999; Han et al. 2005). *Delftia* spp. are environmental microorganisms with a wide geographic distribution that have been found in fresh and marine water, soil, rhizosphere, plants, clinical samples, and activated sludge (Wen et al. 1999).

The genus *Delftia* belongs to the family *Comamonadaceae* within the order *Burkholderiales* of the class *Betaproteobacteria* and was described in the year 1999, after the reclassification of *Comamonas acidovorans*, mainly due to their

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phylogenetic and phenotypic analyses. *Delftia* is a genus that includes only five recognized species: *Delftia acidovorans* (Wen et al. 1999), *Delftia tsuruhatensis* (Shigematsu et al. 2003), *Delftia lacustris* (Jørgensen et al. 2009), *Delftia litopenaei* (Chen et al. 2012), and the recently described *Delftia deserti* (Li et al. 2015).

Bacteria of the genus *Delftia* have been characterized by their ability to transform or degrade a wide variety of organic and inorganic compounds including anilines, the herbicides linuron and diuron, and metallic ions, among others (Benndorf and Babel 2002; Patil et al. 2006; Bazot et al. 2007; Morel et al. 2011; Yan et al. 2011, among others). In addition, they have the ability to promote plant growth by direct and indirect mechanisms (Han et al. 2005; Morel et al. 2011; Ubalde et al. 2012; Prasannakumar et al. 2015, among others) and to produce and accumulate biocompatible and sustainable polymers like polyhydroxyalkanoates, commonly used for manufacturing petrochemical-free bioplastics (Kimura et al. 1992; Hsieh et al. 2004; Mothes and Ackermann 2005; Loo and Sudesh 2007, among others). All these metabolic abilities have attracted the attention of several researchers and companies to developing new sustainable biotechnological processes using bacteria belonging to this genus.

Furthermore, the genome sequence of some *Delftia* strains has recently been published showing that *Delftia* spp. genomes have sizes of 6–6.7 Mb and an approximate GC content of 66 %. The analysis of these genomes allowed the identification of the genetic elements involved in diverse biodegradation pathways (Shetty et al. 2015; Wu et al. 2016), heavy metal resistance (Wu et al. 2016; Morel et al. 2016), phytohormone and siderophore production (Morel et al. 2016), and antimicrobial compound production (Hou et al. 2015). As a whole, the genetic information evidenced that bacteria of genus *Delftia* are versatile microbes with diverse metabolic capacities that easily adapt to different environments.

This chapter provides an overview of various aspects of *Delftia* spp. including bioremediation and biosorption of contaminants, production of bioproducts, beneficial interaction with plants, as well as their role in human infection as opportunistic agents. Finally, a list of patent based on the use of *Delftia* isolates registered to date is described.

11.2 Uses and Potential Applications in Biotechnology

This section provides an overview of potential uses and applications of *Delftia* spp. as microbial model of sustainability. A wide variety of biotechnological applications and the programs affecting bioremediation and bioconversion of toxic organic compounds, biosorption of metals, bioproduct production, and market opportunities, as well as their use in agriculture as plant growth-promoting bacteria are discussed. The potential applications are summarized in Fig. 11.1.

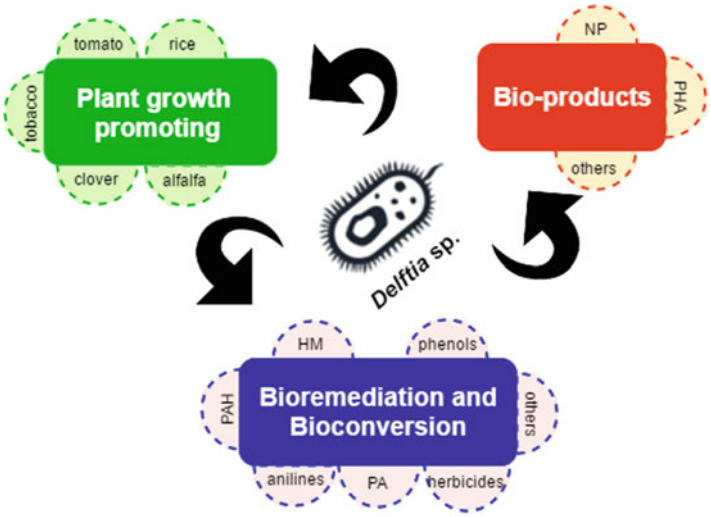

Fig. 11.1 Potential biotechnological applications of *Delftia*. Meanings are as follows: *NPs* nanoparticles, *PHAs* polyhydroxyalkanoates, *HMs* heavy metals, *PAHs* polycyclic aromatic hydrocarbons, *PA* phthalates

11.2.1 Bioremediation and Bioconversion

As the global agricultural and industrial development is in progress, pollution by chemicals has become a serious concern to human and environmental health. Over the last years, diverse physical and chemical treatments to remediate pollution have been developed; however, most of these treatments exhibit low specificity and produce non-desirable secondary chemical compound (Megharaj et al. 2011).

The use of microorganisms in removing or detoxifying pollutants which may otherwise threaten public health is known as bioremediation (Talley 2005; Wasi et al. 2008). Furthermore, microbial bioremediation is an efficient strategy for pollution treatment since it has low cost and high efficiency/specificity, and it is an environmentally friendly approach (Kulkarni and Chaudhari 2007; Rayo et al. 2012; Krastanov et al. 2013; Wołejko et al. 2016).

Environmental contaminants can be found as organic as well as inorganic compounds, and if the pollutant is not excessively toxic or inhibitory for the structural and functional integrity of the microbial cells, they can be biologically transformed and/or immobilized by microorganisms. In this regard, inorganic and organic compound transformation occurs most efficiently in aqueous environments and solid matrices, respectively (Lloyd et al. 2005). Among other microbes, *Delftia* spp. isolates have been reported to be useful for cleaning up organic compounds as well as heavy metal ions. Some examples are described below.

11.2.1.1 Phenols

Phenolic compounds are normally present in effluents from several industries such as wine and olive oil production, pharmaceutics, and textile and paper manufacturing, among many others (Annachhatre and Gheewala 1996; Cerezo et al. 2008, Nalewajko-Sieliwonjuk et al. 2008). These compounds are considered as environmental pollutants due to their high toxicity at low concentrations (Krastanov et al. 2013). The capacity of *Delftia* strains to degrade some phenolic compounds is well known. For example, the growth of *D. tsuruhatensis* on catechol was reported by Sheludchenko et al. (2005). Additionally, *Delftia* strains could transform various aromatic compounds (such as aniline, vanillin, ferulic acid, 2,4-dichlorophenoxyacetic acid, and terephthalate) into catechol or protocatechuic acid and further complete degradation (Kahng et al. 2000; Plaggenborg et al. 2001; Shigematsu et al. 2003; Hoffmann and Müller 2006).

In addition, *D. tsuruhatensis* BM90 (isolated from water samples collected in the Tyrrhenian Sea) was selected for its ability to degrade a pool of mixed phenols, including derivatives of benzoic acid, cinnamic acid, and phenolic aldehydes among other phenolic compounds (Juarez-Jiménez et al. 2010). Immobilized cells of BM90 showed a high biodegradation potential, reducing 90 % of phenols in each round of repeated batch processes. These results suggest that *Delftia* spp. could be used as an efficient degrading microbe for phenols containing effluent decontamination (Juarez-Jimenez et al. 2012).

11.2.1.2 Aniline and Chloroanilines

Aniline and its derivative compounds are toxic and recalcitrant molecules that have been broadly used for the synthesis of chemical products such as herbicides and dyes. Liu et al. (2002) informed the isolation from activated sludge of a *Delftia* sp. strain (named AN3) that uses aniline or acetanilide as sole carbon, nitrogen, and energy sources. Similar levels of aniline degradation were reported for *D. tsuruhatensis* AD9 isolated from the soil surrounding a textile dyeing plant (Geng et al. 2009). Other aniline-degrading *Delftia* strains have also been described (Kahng et al. 2000; Urata et al. 2004; Sheludchenko et al. 2005; Chengbin et al. 2009).

Chloroanilines (CAs) are also toxic and recalcitrant compounds widely used in the production of polyurethanes, rubber, dyes, pharmaceutics, photographic chemicals, varnishes, and pesticides (Zhang et al. 2010). The widespread use of these compounds has resulted in their ubiquity in industrial effluents, sludge, and agriculture soils. Zhang et al. (2010) described that *D. tsuruhatensis* H1 is able to degrade several CAs, using 3-CA and 4-CA as growth substrates, along with the concomitant metabolization of 2-CA and other CA compounds. The strain could also efficiently degrade a mixture of the three CA compounds.

11.2.1.3 Herbicides

The 2,4-dichlorophenoxyacetic acid (2,4-D) is a phenoxy herbicide widely used for postemergence control of broad-leaved weeds of crops, pastures, forests, and noncropland. This herbicide is highly water soluble and has a low tendency to accumulate inorganic matter; therefore, 2,4-D enters as contaminant into streams, rivers, or

lakes directly from drainage of agricultural lands (Gonzalez et al. 2012). Biodegradation is the main process involved in the breakdown of 2,4-D in soil and water. A few *Delftia* strains exhibit high potential for the degradation of this herbicide (Hoffmann et al. 1996; Müller et al. 1999), including the successful biodegradation of 2,4-D at a removal rate of 21.7 g/m^3 day in a continuous-flow fixed-bed reactor as described by González et al. (2012).

Diuron, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, is a herbicide belonging to the phenylamide family widely used on many agricultural crops. Due to the long-term dispersion of this compound and its great persistence, it has been considered as a priority hazardous substance (Tomlin 1997; Tixier et al. 2001). Several microorganisms degrade diuron, producing the highly toxic compound 3,4-dichloroaniline (3,4-DCA) as an end product (Tixier et al. 2002; Widehem et al. 2002; Giacomazzi and Cochet 2004; Sharma et al. 2010). However, it has been shown that microbial consortiums, including microbes that degrade diuron and mineralize 3,4-DCA, are efficient systems for diuron bioremediation (You and Bartha 1982; Dejonghe et al. 2003; Sørensen et al. 2008). In this regard, Bazot et al. (2007) achieved total mineralization of diuron using a coculture of an *Arthrobacter* sp. strain (degradation of diuron to 3,4-DCA) and the 3,4-DCA-degrading *D. acidovorans* W34 strain (isolated by Dejonghe et al. 2003).

11.2.1.4 Micropollutants

A large fraction of biologically active compounds such as pharmaceuticals reach wastewater treatment plants (WWTPs) after human consumption and excretion (Castiglioni et al. 2006). In general terms, WWTPs have not been designed for the elimination of pharmaceutical micropollutants, and new strategies for their removal are essential. In this sense, an acetaminophen (paracetamol, APAP)-degrading *D. tsuruhatensis* strain was isolated from a membrane bioreactor that successfully removed APAP from wastewater (De Gusseme et al. 2011).

11.2.1.5 Phthalates (PAs)

Phthalates are chemicals synthesized in extremely large volumes and mainly used in plastic matter industries (imparts flexibility to polyvinyl chloride resins and other polymers) that have been widespread in different environments (Liang et al. 2008). PAs and their metabolites are hepatotoxic, teratogenic, and carcinogenic compounds and thus are considered harmful for human as well as for the environment (Matsumoto et al. 2008). Many bacteria have been isolated for their ability to degrade PAs, including *Delftia* sp. strain TBKNP-05 (degradation of di-n-butylphthalate) as described by Patil et al. (2006). In addition, a terephthalate-assimilating *D. tsuruhatensis* (strain T7T) was isolated from an activated sludge collected from a domestic wastewater treatment plant (Shigematsu et al. 2003).

11.2.1.6 Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs are a large group of organic compounds, with two or more fused aromatic rings, produced during the incomplete combustion of organic fuels. They are widely distributed environmental contaminants that have detrimental biological effects

(toxicity, mutagenicity, and carcinogenicity). A few examples of PAHs are naphthalene, anthracene, and phenanthrene, which can be degraded by microbes as an important means of PAHs-contaminated soil remediation (Cerniglia 1992; Johnsen et al. 2005; Haritash and Kaushik 2009).

Among others, *D. lacustris* strain LZ-C degrades naphthalene and 2-methylnaphthalene as shown by Wu et al. (2016). In addition, a *D. acidovorans* strain (formerly *Delftia* sp. Cs1-4) capable of degrading phenanthrene was isolated from a PAH-contaminated soil in Wisconsin (Vacca et al. 2005). Curiously, Cs1-4 produce novel bacterial organelles termed "nanopods" that can project outer membrane vesicles (OMVs) in long distances from the cell (Shetty et al. 2011). Usually, OMVs contain a broad range of macromolecules that mediate diverse functions, enabling bacterial endurance and regulating microbial interactions (Schwechheimer and Kuehn 2015). Shetty et al. (2011), Shetty and Hickey (2014) reported that nanopod formation by *D. acidovorans* Cs1-4 is an essential characteristic for the phenanthrene degradation process. Nanopod production appears not to be an exclusive characteristic of *Delftia* strains, since evidence of nanopod production in *Acidovorax* and other genera of the family Comamonadaceae has been found (Shetty et al. 2011).

11.2.1.7 Anionic Surfactants

Anionic surfactants are the major class of surfactants used in detergent formulations. Sodium dodecyl sulfate (SDS), one of the major representatives of linear alkyl sulfate class, has large-scale industrial applications, and thus SDS has been widely released to the environment (Liwarska-Bizukojc and Bizukojc 2006). SDS is used in industrial cleaners and household detergents as well as in other industries such as textile and fiber, plastic, paint, leather production, and metal industries (Yilmaz and Icgen 2014). The consequences arising from SDS overuse and subsequent disposal in waterways are of serious concern. Selecting effective microorganisms for SDS treatment is a big challenge. Yilmaz and Icgen (2014) reported the isolation and identification of an effective SDS-degrading *D. acidovorans* isolate from detergent-polluted river waters.

11.2.1.8 Heavy Metals (HMs)

Through history, diverse human activities have contaminated the environment with toxic HMs and metalloids (Singh and Steinnes 1994; Järup 2003), and microorganisms have been extensively used for detoxification/bioremediation of HMs (Lovley and Coates 1997; Gupta et al. 2000; Garbisu and Alkorta 2003; Wasi et al. 2011, 2013). They have the ability to remove, immobilize, and/or detoxify metals and radionuclides through various mechanisms (Ji and Silver 1995).

Several studies have shown that *Delftia* strains resist HMs such as Zn(II), Hg(II), Pb(II), and Cr(VI) (Morel et al. 2011; Ubalde et al. 2012; Bautista-Hernández et al. 2012). They can transform HM into less toxic compounds as shown by Caravaglia et al. (Caravaglia et al. 2010) and Morel et al. (2011). The authors described the reduction of the highly toxic hexavalent chromium ion [Cr(VI)] to the less toxic Cr(III).

The immobilization (or biosorption) of metals and radionuclides is an effective approach for the remediation of industrial effluents as well as the recovery of metals (Volesky and Holan 1995; Gavrilescu 2004; Das 2010; Michalak et al. 2013). The process involves the immobilization of metals and radionuclides by biological components that act as bioadsorbents (Volesky 2001; Michalak et al. 2013). The zinc (Zn) and lead (Pb) biosorption was successfully achieved by a *D. tsuruhatensis* strain isolated from mine tailings (Bautista-Hernández et al. 2012). In addition, Ubalde et al. (2012) reported the bioaccumulation of Pb(II) by *Delftia* sp. JD2 (isolated from a Cr-contaminated soil) as well as other *Delftia* strains (isolated from a HM-contaminated soil). These results are consistent with those of Jackson et al. (2009) and Bestawy et al. (2013) who reported a multiple HMs resistance of *Delftia* sp. strains isolated from HMs-contaminated sources.

11.2.2 Cell Factory for the Production of Bioproducts

11.2.2.1 Polymers

Bacteria of the genus *Delftia* are able to degrade hydrocarbons and to produce polyhydroxyalkanoates (PHAs) (Sabirova 2010). The ability to accumulate large amounts of PHAs under stress conditions by a number of bacterial strains has been widely reported (Kadouri et al. 2005), including *Delftia* spp. strains isolated from root nodules of leguminous plants (Kumbhakar et al. 2014), freshwater shrimp culture pond (Chen et al. 2012), palm oil mill effluent (Gumel et al. 2012), or activated sludge (Saito and Doi 1994).

The ability to produce and accumulate PHAs has been deeply studied in *D. acidovorans* by Kimura et al. (1992), Saito and Doi (1994), Hsieh et al. (2004, 2009), Mothes and Ackermann (2005), Loo and Sudesh (2007), San et al. (2012), Ch'ng et al. (2012), and Romanelli et al. (2014). The huge potential of this polymeric biodegradable, compostable, biocompatible, and sustainable material (Ng et al. 2011; Romanelli et al. 2014) lies on the advantage of using PHAs as renewable plastics instead of petrochemical plastics. However, microbial PHAs production has a high price due to the high production costs, as compared with petrochemical-based plastics (polyethylene) (Ng et al. 2011). In this regard, the development of cost-effective biosystems to produce PHAs has increased the research in this area, focusing the work in the isolation of highly PHA-producing microbes, mainly using cheap and renewable carbon sources (Tan et al. 2014). This may reduce the environmental footprint of plastic production (Hassan et al. 2013). Particularly, among renewable carbon sources, *D. acidovorans* is able to synthesize and accumulate PHAs directly from cheap fatty waste materials as carbon source (Romanelli et al. 2014).

The improvement of PHAs production by *Delftia* spp. (growing at different carbon sources, pH, temperature, and aeration, among others) was analyzed by Sudesh et al. (1999), Loo and Sudesh (2007), and Lee et al. (2004). These works proved the ability of some *Delftia* strains to produce PHAs with valuable properties for medical and industrial applications, by growth at diverse and inexpensive sources. The

studies published until now have shown that *Delftia* are very promising strains for the future cheap production of PHAs with specific compositions (more biodegradability and biocompatibility) (Ch'ng et al. 2012; Hsieh et al. 2009).

The genome sequencing and analysis showed that *Delftia* strains carry a PHA biosynthetic single operon, similar to the gene organization found in *Comamonas testosteroni* and *Acidovorax* sp. strains (Yee et al. 2012). However, the information for the biosynthesis and depolymerization of polyhydroxybutyrate (PHB) is dispersed in the genome of *Delftia* sp. JD2 (Morel et al. 2016). The attempts to understand the genetic mechanisms involved in PHA biosynthesis are wide and include several studies such as the deletion of the PHA synthase gene (Saito and Doi 1994; Tsuge et al. 2004), the increase of the copy number of genes related to PHA synthesis and accumulation (Sudesh et al. 1998, 1999), and the heterologous expression of PHA synthase from *Delftia* strains in *Escherichia coli* (Hiroe et al. 2013).

Nowadays there is a commercial patent for the production of PHA based on the use of a microbial consortium that includes a novel PHA-producing *D. acidovorans* strain (Smith et al. 2014) (see Sect. 11.4).

11.2.2.2 Nanoparticles (NPs)

Bio-directed synthesis of metal NP using different noble metals is gaining importance in view of their biocompatibility, low toxicity, and eco-friendly characteristics. NPs could be used in a wide range of biomedical applications such as drug delivery, but also in cancer therapy and related diseases, molecular imaging, gene therapy, magnetic resonance imaging, and as antibacterial agents, among others (Salata 2004).

Johnston et al. (2013) reported gold biomineralization using delftibactin, a novel metallophore secreted by a gold-associated *Delftia acidovorans*. Delftibactin facilitates the complexation (Au^{3+}) and deposition of solid gold (Au^{0}), in a manner similar to Fe^{3+} chelation, enhancing the cell survival in the presence of toxic gold ions. Once contacting Au^{3+}, delftibactin complexes, reduces, and detoxifies soluble gold ions, resulting in the formation of gold nanoparticles. Based on these reports, Kumar et al. (2014) studied the synthesis of gold nanoparticles (AuNPs) by using the culture supernatant of *Delftia* sp. strain KCM-006 and their use as a promising delivery vehicle for cancer therapy. The biogenic NPs were successfully synthetized and exhibited good stability (6 months at room temperature). Subsequently, the AuNPs were conjugated with resveratrol (RSV) for synthesizing RSV-AuNP, and their use as cytotoxic agent on a lung carcinoma cell line was examined. The RSV-AuNPs were stable at physiological conditions and exhibited a pH-responsive release behavior of the bioactive agent. In addition, it was observed that AuNPs enhanced the cytotoxicity of resveratrol in the tumor cells. These results suggest that AuNPs produced by *Delftia* strains could find applications as convenient nanomaterials for drug delivery system.

Silver nanoparticles (AgNPs) have also been produced using the cell-free supernatant of *Delftia* sp. strain KCM-006, and their application as antifungal agents and drug carrier has been proposed. AgNPs are known to exhibit a broad spectrum of biocidal activity toward many bacteria, fungi, and viruses (Zachariadis et al. 2004). From a therapeutic application perspective, these biosynthesized AgNPs find use as

antimicrobial, anti-inflammatory, anti-angiogenic, and antiviral agents (Nadworny et al. 2008; Rogers et al. 2008; Gurunathan et al. 2009). The synthetized AgNPs exhibited very good antifungal activity against various pathogenic *Candida* strains, and their effectiveness was increased by conjugation with the antifungal drug miconazole. In addition, the cell viability and immunocytochemistry analysis against different normal cell lines demonstrated that these nanoparticles were non-toxic (up to 20 μM) and showed good biocompatibility with all normal cell lines assayed (Kumar and Poornachandra 2015). These works pointed out the potential use of *Delftia* strains for the production of pharmaceutical products.

11.2.2.3 Others

3-Hydroxyaspartate and its derivatives have attracted attention due to their biological activities as competitive blockers against glutamate transporters in the mammalian central nervous system (Shimamoto et al. 1998; Shimamoto 2008). Selective and potent inhibitors are required to identify the physiological roles of transporters in the regulation of synaptic transmission as well as in the pathogenesis of neurological diseases (Shimamoto et al. 2004). Matsumoto et al. (2015) reported a novel approach for the production of optically pure 3-hydroxyaspartate by using a purified enzyme (D-threo-3-hydroxyaspartate dehydratase) isolated from *Delftia* sp. HT23, instead of the high-cost chemical synthesis.

Production of economically important chiral carboxylic acids and their amide derivatives by biotransformation of nitrile using nitrile-converting enzymes (like amidases) has become a promising chiral synthesis method (Wang et al. 2010). Biotransformations of amides could be carried out by either purified or partly purified amidase or by amidase harboring microbial cells. Wang et al. (2010) described the use of immobilized cells of *D. tsuruhatensis* CCTCC M 205114, an R-amidase-producing strain, in the asymmetric hydrolysis of (R)-2,2-dimethylcyclopropane carboxamide (R-1) from the racemic (R,S)-2,2-dimethylcyclopropane carboxamide and the accumulation of (S)-2,2-dimethylcyclopropane carboxamide (S-1). This is a key intermediate of cilastatin synthesis, a renal dehydropeptidase inhibitor administered with the antibiotic imipenem to prevent their degradation in the kidney, thus prolonging its antibacterial effect.

11.2.3 Plant Growth Promotion

Plant growth-promoting bacteria (PGPB) are microorganisms associated with plants that fulfill several important functions involved in growth and physiological state of plants (Hayat et al. 2010; Morel and Castro-Sowinski 2013). PGPB show several physiological mechanisms that explain their plant growth-promoting abilities. These mechanisms are directly based on their ability to produce phytohormone (auxin, cytokinin, gibberellin) or by their ability to enhance nutrient acquisition (solubilizing phosphates, iron uptake from the environment by siderophores) and biological nitrogen fixation (Glick et al. 1999; Podile and Kishore 2006; Hayat et al. 2010). There are also indirect mechanisms of plant promotion as the suppression of phytopathogens by competition by niches or nutrients or the production of

antimicrobial substances against plant pathogens (biocontrol) (Han et al. 2005; Hayat et al. 2010; Maisuria and Nerurkar 2015; Prasannakumar et al. 2015).

Some species of the genus *Delftia* have been reported as PGPB (Table 11.1). These bacteria were described as growth promoters of rice (Han et al. 2005), canola (Banerjee and Yesmin 2004; Berg 2009), tomato (Prasannakumar et al. 2015), tobacco (Hou et al. 2015), and some legumes (Han et al. 2005; Morel et al. 2011, 2015; Ubalde et al. 2012). *Delftia* spp. have the ability to fix atmospheric nitrogen as free-living bacteria, as well as to produce phytohormones and siderophores, cooperating during the rhizobia-legume interaction (Han et al. 2005; Berg 2009; Morel et al. 2011, 2015; Ubalde et al. 2012).

D. tsuruhatensis WGR-UOM-BT1, isolated from the rhizosphere of *Rauvolfia serpentina*, has the ability to improve early growth of tomato and to produce a broad spectrum of antifungal compounds (nitrogen-containing heterocyclic compound) that explains the positive effect of this bacterium in plant yield (Han et al. 2005; Prasannakumar et al. 2015). Under in vitro conditions, WGR-UOM-BT1 suppressed the growth of phytopathogenic fungi, colonized tomato rhizosphere, produced indole-3-acetic acid (IAA), showed deaminase activity (ACC), and also solubilized phosphate.

D. tsuruhatensis strain MTQ3 was isolated from the rhizosphere of tobacco, showing antimicrobial activity against pathogens such as *Phytophthora nicotianae* and *Ralstonia solanacearum* (Hou et al. 2015). The analysis of the MTQ3 genome showed the presence of many genes related with its antimicrobial activity (bacteriocin and phenazine biosynthetic genes, among others) as well as genes with relevance to rhizosphere competence like quercetin dioxygenase-coding gene (involved in the degradation of plant-produced antimicrobial root exudates) (Hou et al. 2015).

Table 11.1 *Delftia* strains reported as PGPB. Crops and potential mechanisms involved in the plant growth-promoting phenotype

Microbe	Strain	Crop	Proposed mechanisms	References
D. tsuruhatensis	BT1	Tomato	Diazotroph, IAA, ACC activity, phosphate solubilization	Prasannakumar et al. (2015)
	HR4	Rice	Diazotroph, antifungal activity	Han et al. (2005)
	MTQ3	Tobacco	Antimicrobial activity against plant pathogen	Hou et al. (2015)
D. acidovorans	RAY209	Canola	Diazotroph, IAA, phosphate solubilization, sulfur oxidizing	Berg (2009) and Banerjee and Yesmin (2004)
Delftia sp.	JD2	Alfalfa and clover	Diazotroph, IAA, phosphate solubilization	Ubalde et al. (2012), Morel et al. (2011, 2015) and Han et al. (2005)

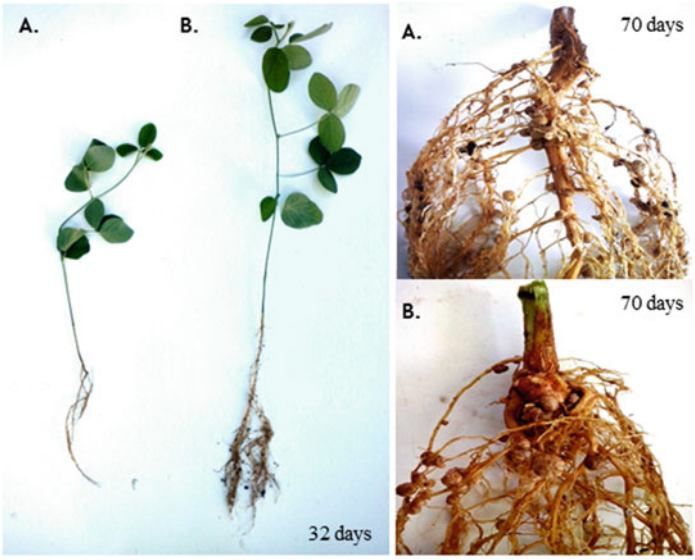

Fig. 11.2 Effect of *Delftia* sp. JD2 on soybean plants. *Left picture*: plants inoculated with *Bradyrhizobium elkanii* (**A**) and co-inoculated with *B. elkanii* and JD2 (**B**), both plants collected 32 days after inoculation. *Right picture*: roots of soybean plants inoculated with *B. elkanii* (**A**) and co-inoculated with *B. elkanii* and JD2 (**B**) (Cagide, C.; unpublished data)

Among *Delftia* strains, it has been described that JD2 may assist during the nodulation of leguminous plants (alfalfa, clover, and soybean) by rhizobia strains, increasing the nodulation rate, root development, and final plant yield (Morel et al. 2011; Ubalde et al. 2012; Morel et al. 2015) (Fig. 11.2). This bacterium has the ability to produce phytohormones (IAA) and siderophores and to change the pattern of plant-secreted flavonoids and other molecules involved in the microbe-plant association (Morel et al. 2011, 2015).

Currently, an inoculant containing *D. acidovorans* strain RAY209 as active ingredient has been commercialized for increasing production of canola (BioBoost® from BrettYoung™) (Banerjee and Yesmin 2004). Another formulation (BioBoost® + Soybean) containing RAY209 and *Bradyrhizobium* strains is also currently at the market, for improving soybean yields.

11.3 Is *Delftia* a Pathogenic Microorganism?

Human infections by *Delftia* strains have been sporadically reported; however, these infections commonly occur in immunocompromised patients as a result of underlying diseases. Some reports of cases in immunocompetent individuals are mainly related to invasive treatment procedures.

D. acidovorans has been isolated from keratitis lesions (Lema et al. 2001; Lee et al. 2008), ocular infections (Stonecipher et al. 1991), otitis media (Reina et al.

1991), urinary tract infections (Ojeda-Vargas et al. 1999), bacteremia (Castagnola et al. 1997; Kam et al. 2012; Hagiya et al. 2013), and skin lesions (Khan and Krishnan 2015). They have also been associated to empyema (Chun et al. 2009; Khan et al. 2012), endocarditis (Horowitz et al. 1990; Mahmood et al. 2012), pneumonia (Taş et al. 2012; Bilgin et al. 2015), and intravascular dispositive-related bloodstream infections (Castagnola et al. 1994 and 1997; Ender et al. 1996; Chotikanatis et al. 2011; Kawamura et al. 2011; Lang et al. 2012). In addition, Preiswerk et al. (2011) and Tabak et al. (2013) informed two cases of catheter-related bacteremia in immunocompromised patients infected by *D. tsuruhatensis*. Meanwhile *D. lacustris* has been isolated from keratitis lesions (Sohn et al. 2015) and bacteremia (Sohn and Baek. 2015). There have not been clinical reports of infection caused by *D. litopenaei* and *D. deserti* in humans, yet.

The sources of *Delftia* infection, determined in some cases, were river water (Reina et al. 1991), devices and equipment from hospitals and clinics (Weinstein et al. 1976; Stampi et al. 1999; Miño de Kaspar et al. 2000), and other contaminated stuff (Horowitz et al. 1990; Perla and Knutson 2005; Mahmood et al. 2012), demonstrating the ubiquitous distribution of those microorganisms.

The ability of *Delftia* strains to develop biofilm may explain their presence and persistence in invasive devices, accounting for their potentially pathogenic effects in immunocompromised and immunocompetent individuals. Thus, this low virulent environmental microorganism may become as an opportunistic pathogen (Khan and Krishnan 2015). As *Delftia* isolates used to have intrinsic resistance to aminoglycosides (Lipuma et al. 2011) and some cases of β-lactamics and quinolone-resistant *Delftia* spp. have been described (Ravaoarinoro and Therrien 1999; Kang et al. 2015), therefore, their rapid detection and identification is encouraged.

11.4 Patents

Recently, many patents related to the use of *Delftia* sp. isolates have been published, mainly in the areas of bioremediation, biopolymer production, chemical synthesis, and plant growth promotion (Table 11.2).

The ability of certain *Delftia* spp. for the degradation or transformation of various pollutants has been exploited. This fact has been reflected in several patents registered for bioremediation of diverse compounds. For example, Xiang (2007) patented a process for the biological denitrification of wastewater under aerobic conditions, using the isolate *D. tsuruhatensis* WXZ-9. This strain can be seeded in conventional nitrifying aerobic sludge systems, wherein nitrogen-containing wastewater can be dealt to achieve simultaneous nitrification and denitrification. The degradation of chloroaniline pollutants by *D. tsuruhatensis* strain H1 in aerobic conditions was patented by Zhang et al. (2011). Also, a method for the remediation of soil or groundwater contaminated with volatile organic chlorine compounds or mineral oils by using a consortium of microorganisms including bacteria of the genus *Delftia* was patented by Tetsuaki et al. (2013 and 2014). Recently, Jianmeng et al. (2015) reported an invention using *D. tsuruhatensis* LW26 for the

Table 11.2 Patents based on the use of *Delftia* isolates

Used for	Patent no.	Applicant	Inventors
Bioremediation	CN101016525A	Beijing Technology and Business University (China)	Xiang (2007)
	CN101993838A	Zhejiang University of Technology (China)	Zhang et al. (2011)
	JP2011274874	Kokusai Environmental Solutions Co., Ltd. (Japan)	Tetsuaki et al. (2013)
	JP2014239656A	Kokusai Environmental Solutions Co., Ltd. (Japan)	Tetsuaki et al. (2014)
	CN105039222A	Zhejiang University of Technology (China)	Jianmeng et al. (2015)
	CN104531585A	China National Rice Research Institute (China)	Xiaoyan et al. (2015)
PHA production	US8722383	Micromidas Inc. (United States)	Smith et al. (2014)
Chemical synthesis	WO2004055172A	Helmholtz Center for Environmental Research (Germany)	Müller et al. (2004a)
	WO2004055218A	Helmholtz Center for Environmental Research (Germany)	Müller et al. (2004b)
	WO2006030909	Yuki Gosei Kogyo Co., Ltd. (Japan)	Sasaki and Matsumoto (2006)
PGPB	EP2239318A1	BrettYoung Seeds Limited (Canada)	Banerjee and Yesmin (2010)
	CN103980026A	China National Rice Research Institute (China)	Yikay and Defeng (2014)

decontamination of chlorobenzene-polluted areas. Moreover, *Delftia* isolates are also used to remove HM from contaminated soil and water bodies, as published by Xiaoyan et al. (2015).

In addition, a few commercial patents point out the use of *Delftia* strains for the synthesis of bioproducts, such as the production of polyhydroxyalkanoate (PHA) using the PHA-producing *D. acidovorans* MM01 (Smith et al. 2014), or the production of the highly pure S or R enantiomers of 2-phenoxypropionic acid by *D. acidovorans* MC1-R and MC1-S (Müller et al. 2004a, b), and the production of 2-hydroxy-4-substituted pyridine by a consortium including *Delftia* sp. strains (YGK-A649 and YGK-C217) (Sasaki and Matsumoto 2006).

The ability of *Delftia* isolates to promote plant growth has been used to design new biofertilizers. In this regard, Banerjee and Yesmin (2010) patented an invention related to the use of a sulfur-oxidizing *D. acidovorans* (strain RAY209) as a biofertilizer to increase canola performance. In addition, Yikay and Defeng (2014)

patented a microbial fertilizer that promotes the growth of rice seedling roots. The microbial fertilizer contains a consortium of microorganisms including bacteria of the genus *Delftia*, which promotes rice seedling root development and besides resistance to adverse environments.

11.5 Concluding Remarks

Members of the genus *Delftia* are highly adaptable and metabolic versatile bacteria with potential uses in bioremediation of organic and inorganic compounds; in synthesis of some enantiomeric molecules, polymers, and nanoparticles with industrial and clinical applications; and, finally, in the promotion of plant growth to improve crop yield. There are few reports on the potential risks of some *Delftia* strains as opportunistic pathogens, but other PGPB microorganisms different from *Delftia* have also been described as opportunistic human pathogen, and it seems to be that the rhizosphere is a reservoir for opportunistic pathogenic bacteria (Berg et al. 2005; Tyler and Triplett 2008; Berg et al. 2013; Mendes et al. 2013). The increasing number of patents that have recently been published reveals the relevance of this genus as a sustainable model for the development of industrial and agronomic products.

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Part III

Microbes in Sustainable Agriculture

Streptomyces for Sustainability

12

Patricia Vaz Jauri, Nora Altier, and Linda L. Kinkel

Abstract

Streptomyces is a genus of gram-positive bacteria with a mycelial growth habit and the ability to produce spores. Due to their unparalleled ability to produce antibiotics, most of the early research carried out on *Streptomyces* was antibiotic discovery-driven, with over two thirds of antibiotics used for medical purposes originally isolated from *Streptomyces*. However, their ubiquity, high capacity of adaptation to different niches and rich secondary metabolite production, make them an invaluable source of solutions in diverse human activities, including medicine, agriculture, industry and toxic waste remotion. In addition to the ability to culture and produce *Streptomyces* and *Streptomyces*-derived metabolites, knowledge on how to manipulate natural populations of *Streptomyces* will likely improve our ability to make environmentally sustainable decisions.

12.1 Introduction

The *Streptomyces* are a large and diverse group of microorganisms that have long captured the attention of researchers. They have been studied for diverse reasons, including their unique morphology; their apparently endless source of secondary metabolites, especially antibiotics and other chemicals for medical use; their pathogenicity; and their symbiotic associations with other organisms, including insects and plants. There is substantial literature on *Streptomyces*, and, overall, research on

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Streptomyces has been extremely fruitful, especially in relation to clinical applications of *Streptomyces* secondary metabolites. In this chapter, we aim to present an up-to-date overview of this genus, with an emphasis on their use in agricultural settings.

12.1.1 Life History Traits

The *Streptomyces* are filamentous, spore-forming bacteria. They are ubiquitous in soil and have been more recently recognized to be common and widespread in marine and freshwater sediments (Maldonado et al. 2005; You et al. 2005; Jensen et al. 2005). Most species are mesophilic, aerobic, and saprophytic organisms (Kieser and John Innes Foundation 2000). However, some isolates have been found in extreme environments (Pathom-Aree et al. 2006; Ribbe et al. 1997). Beyond their roles as saprophytes, some *Streptomyces* live as endophytes (Cao et al. 2005; Misk and Franco 2011; Gangwar et al. 2014; Qin et al. 2015), as pathogens of plants (Takeuchi et al. 1996; Hiltunen et al. 2005) or immunocompromised humans (Dunne et al. 1998), and as beneficial symbionts with a wide variety of higher organisms, including insects, plants, and sponges (Haeder et al. 2009; Loria et al. 2006; Kaltenpoth 2009; Kaltenpoth and Engl 2014; Khan et al. 2011; Hulcr et al. 2011; Seipke et al. 2012; Book et al. 2014). *Streptomyces* are gram positive, and their genomes have high G + C content, typically above 70 %. *Streptomyces* genomes are generally large compared to other actinobacteria and eubacteria in general (Gao and Gupta 2012; Myronovskyi et al. 2013; Rückert et al. 2013). However, their genome size is variable (~5.2–12.7 Mbp) and is thought to be related to their life history. Species that have a restricted or highly circumscribed niche width, such as within a host, have a tendency to have smaller genomes than those that are free living and coexist with high densities of other bacteria (Kirchman 2012). *Streptomyces* possess a linear chromosome, which has been observed in only three other actinobacterial genera (*Rhodococcus*, *Gordonibacter*, and *Kineococcus*; Gao and Gupta 2012). Transposons, or highly mobile elements, have also been found in *Streptomyces* genomes (Kieser and John Innes Foundation 2000).

One striking feature of this genus is their mycelial growth, which is uncommon among bacteria. When grown in solid medium in the laboratory, *Streptomyces* form an aerial mycelium after vegetative growth, which is followed by spore formation at the hyphal tips. Spores are resistant to desiccation and heat (Flärdh and Buttner 2009) and are pigmented, which provides protection against UV radiation (Funa et al. 2005). These characteristics are believed key for dispersal in wind and water as well as for survival in natural environments.

Streptomyces are especially notable as antibiotic producers. Secondary metabolites produced by *Streptomyces* have been used in medicine and agriculture for over 60 years. Production of diverse bioactive compounds is perceived to be crucial to *Streptomyces* life history in soil and in symbiotic associations, where antibiotics as weapons or signaling molecules are believed to provide a fitness advantage (Linares et al. 2006; Martínez 2008; Fischbach 2009; Vaz et al. 2013).

12.1.2 Taxonomy and Phylogeny

The genus *Streptomyces* lies within the family *Streptomycetaceae*, order *Actinomycetales* and class *Actinobacteria* (http://www.bacterio.net/-classifphyla.html). *Streptomyces* spp. have been identified based on their cultural and physiological characteristics, including spore and hyphal shapes, and by cell-wall fatty acid content (Wellington et al. 1992). More recently, ribosomal RNA sequences, specifically partial or total 16S rRNA sequences, have become the most common means of identification (Takeuchi et al. 1996; Davelos et al. 2004; Anzai et al. 2008; Kanini et al. 2013; Schlatter and Kinkel 2014; Qin et al. 2015). However, high numbers of 16S rRNA gene copies may be present in each genome and difficulties in distinguishing among closely related isolates based on 16S gene sequence have driven researchers to evaluate phylogenetic relationships using other genes. For example, substantial variation in sporulation-associated genes within the *Streptomyces* (Hsiao and Kirby 2007) led Girard et al. (2013) to propose to classify actinomycetes based on sporulation-associated Ssg-A and Ssg-B. Another strategy involves the sequencing of several conserved or "housekeeping" genes or multilocus sequencing analysis (MLSA) (Guo et al. 2008; Rong et al. 2009). This approach gives more detailed information but is highly demanding in effort and resources. Within the last decade, with the advent of new sequencing technologies, entire genomes have become available, allowing whole-genome comparisons. Thus, phylogenetic studies of *Streptomyces* and, more broadly, of actinobacteria have been based on 16S rRNA, individual or multiple genes, concatenated sequences of several proteins, Ssg-A and Ssg-B, and whole-genome comparisons, depending on the availability of technology and the goals of the study (Takeuchi et al. 1996; Egan et al. 2001; Kim et al. 2004; Guo et al. 2008; Gao and Gupta 2005, 2012; Chater and Chandra 2006; Manteca et al. 2006).

Although there have been changes throughout the years (Anderson and Wellington 2001), as of December 2015, the family *Streptomycetaceae* has a total of ten genera (http://www.bacterio.net/), many of which have been occasionally considered within the genus *Streptomyces*. Genera closely related to *Streptomyces* include *Frankia*, *Kitasatospora*, and *Thermobifida*, which are organisms present within a wide range of habitats and exhibiting diverse functions. Studies based on morphology, chemical taxonomy, and 16S rRNA support the idea that *Kitasatospora* is closely related to *Streptomyces*, sharing characteristics such as excellent soil colonization, mycelial growth habit, and secondary metabolite production with the *Streptomyces* (Chung et al. 1999; Groth et al. 2004; Hsiao and Kirby 2007). In addition, combined approaches using whole genomes and several conserved gene sequences (Alam et al. 2010) showed that the *Streptomyces* spp. group was closest to *Frankia* spp. and also to *Thermobifida fusca* YX, a moderately thermophilic soil bacterium that belongs to the order *Streptosporangiales*. It is likely that future whole-genome sequencing will bring substantial modifications to the taxonomy of members of the *Streptomyces* and related genera and will enhance our understanding of the ecological and evolutionary relationships among these taxa.

12.1.3 Sequenced Genomes: What We Have Learned

New technologies have reduced the costs of sequencing, and thus the number of fully sequenced *Streptomyces* genomes has increased dramatically in recent years (Davis et al. 2013; Tarkka et al. 2015; Gomez-Escribano et al. 2015; Zhai et al. 2015; Deng et al. 2015; Rückert et al. 2013; Ortseifen et al. 2015; Tian et al. 2015; Thibessard et al. 2015; Nanthini et al. 2015). As of December 2015, over 250 *Streptomyces* spp. genome sequencing projects are underway in the GenBank (http://www.ncbi.nlm.nih.gov/bioproject/browse). Much has been learned from the assembly and annotation of the genomes.

Among *Streptomyces* genomes found in the GenBank, the median genome size is 8.2 Mb with 72 % GC content. A few species have particularly small genomes compared to others within the genus [e.g., *S. somaliensis* 5.2 Mb (Kirby et al. 2012), *S. violaceusniger* 6.4 Mb (Chen et al. 2013)]. Most *Streptomyces* carry one or two plasmids, either linear or circular (Gomez-Escribano et al. 2015; Myronovskyi et al. 2013).

Whole-genome sequence data has revealed a wealth of secondary metabolites beyond expected. Work by Zhou et al. (2012) comparing genomes of five *Streptomyces* spp. found 3096 gene families in all 5 genomes, which represented 18 % of the pangenome or the total complement of genes in the group. These may represent a possible "core genome" for this group. All of the fully sequenced *Streptomyces* genomes (n > 20 to date) have genes that confer the potential to produce bioactive secondary metabolites, with more than thirty distinct pathways evident in some isolates. For example, bioinformatic analyses reveal 22 gene clusters predicted to be involved in the synthesis of secondary metabolites in the genome of *S. coelicolor* (Bentley et al. 2002), 30 in the genome of *S. avermitilis* (Ikeda et al. 2003), 32 in the genome of *S. fulvissimus* (Myronovskyi et al. 2013), and 35 in the genome of *S. leeuwenhoekii* (Gomez-Escribano et al. 2015). Such analyses highlight the potential of finding new compounds from organisms, even those that have been thoroughly studied, bringing optimism to the natural products community. Identifying the conditions for expression of such genes and their roles in nature provides important challenges for researchers to solve.

12.2 Plant-Associated *Streptomyces*

Bacteria are commonly associated with plants in all natural settings, composing a portion of what is known as the microbiome (Berendsen et al. 2012). These associations vary in specificity and in relative cost or benefit to the plant. For this reason, plants in natural settings have been seen as part of a holobiont (Hartmann et al. 2014; Lebeis 2015; Smith et al. 2015). While it seems obvious that a diseased plant is infected by microorganisms (bacterium, virus, or fungus), it is still not widely recognized that a healthy, vigorous plant may owe its splendor to the activities of microorganisms. These beneficial associations, just like detrimental ones, vary in specificity. Some associations occur in the rhizosphere, others in the phyllosphere,

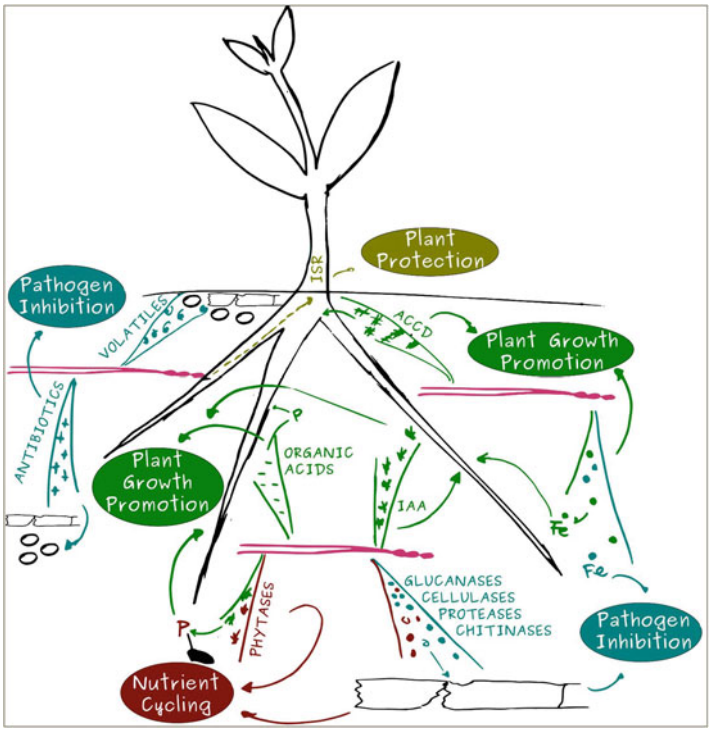

Fig. 12.1 Secondary metabolites produced by *Streptomyces* spp. and processes in which they participate in soil and plants

or even in the inside of the plants (endophytic microorganisms). These interactions have been explored from many standpoints, but of most interest is the potential to utilize these naturally occurring relationships as alternatives to the use of chemicals in crop production.

12.2.1 Plant Growth Promotion

One of the many types of interactions *Streptomyces* spp. may have with plants is as growth-promoting bacteria (Gopalakrishnan et al. 2014, 2015; Jog et al. 2014). *Streptomyces* spp. found in the rhizosphere, on plant surfaces, and living endophytically have traits that are characteristic of plant growth-promoting bacteria (Noumavo et al. 2015; Vorholt 2012; Rungin et al. 2012). Among plant growth-promoting endophytic actinobacteria, most isolates have been reported to belong to the genus *Streptomyces* (Qin et al. 2011, 2015; Kim et al. 2012). Colonizing *Streptomyces* may promote growth in the plant host through direct or indirect mechanisms (Fig. 12.1). The production of hormones and enzymes that interact with plant molecules is an example of a direct mechanism of plant growth promotion by *Streptomyces*, while indirect mechanisms are those involved in nutrient acquisition.

12.2.1.1 Hormones and Enzymes

Some *Streptomyces* have been shown to produce the auxin indole-acetic acid (IAA). Auxins are plant hormones involved in cell division and elongation, whose general effect on plants is the stimulation of growth. Thus, IAA-producing bacteria, including *Streptomyces*, are thought to induce growth through the direct effect of IAA (Barbieri and Galli 1993; Shao et al. 2014). Several studies suggest IAA-producing *Streptomyces* are relatively common among rhizospheric strains (Palaniyandi et al. 2013a, 2014; Qin et al. 2015; Jog et al. 2014), which has been suggested to be a consequence of their potential signaling activity among bacteria (Spaepen et al. 2007). However, Palaniyandi et al. (2013a) showed that IAA production is not always accompanied with plant growth promotion. The influence of IAA production on growth promotion for each plant-microbe pair is difficult to predict, due to potentially different gene expression among isolates in culture vs. *in planta*, variation in microbial colonizing abilities, the potential for IAA metabolism by microbial competitors, and the likelihood of other molecules acting in the process of growth promotion observed in the experiments.

Ethylene is another plant hormone which participates in stress signaling and decreases in production or availability to enhance plant growth. The enzyme 1-ami nocyclopropane-1-carboxylate (ACC) deaminase degrades the precursor of ethylene in plants, ACC, into ammonia and α-ketobutyrate, thereby preventing ethylene production. In a screening of rhizospheric actinobacteria isolated from yam roots, Palaniyandi et al. (2013a) found that of 24 *Streptomyces* spp. isolates, six produced ACC deaminase. These six isolates producing ACC deaminase were subsequently tested for plant growth promotion by inoculation onto *Arabidopsis* plants, but only four increased root biomass. Among these, three *Streptomyces* inoculants induced greater seedling and root length, seedling fresh weight, and leaf total area than non-inoculated plants. In another report, screening endophytic bacteria from *Jatropha curcas*, Qin et al. (2015) found that 19 of 257 (7.4 %) of isolated endophytes were positive for ACC deaminase, 16 of which were *Streptomyces* spp. Similarly to results with IAA-producing strains, plant growth promotion activity was not necessarily found in strains which produced both ACC deaminase and IAA. Instead, plant growth seemed to be stimulated by a variety of factors, including ACC deaminase, IAA, phosphate solubilization, and probably others not evaluated. Information on the identity of these additional factors, as well as understanding the elements that trigger their expression, will aid the achievement of consistent results with PGP bacteria.

12.2.1.2 Facilitation of Nutrient Uptake

An alternative mechanism of plant growth promotion is based on facilitation of nutrient uptake. Bacteria may increase plant nutrient uptake indirectly through the stimulation of root growth or by modifying root architecture and increasing root hair formation. These modifications are driven by hormones and enzymes, including those mentioned above. The increased root surface area can enable higher

efficiency of water and ion uptake into the root system. Alternatively, bacteria may facilitate phosphorus (P) uptake, mediating phosphate solubilization or mineralization through modifications of P-containing molecules in the rhizosphere (Rodríguez et al. 2006 and Richardson and Simpson 2011). Similarly, iron (Fe) acquisition by plants may be mediated through the activities of bacterial siderophores. Both P and Fe are highly abundant in soil but are often found in chemicals that plants cannot access, and bacterial activities can make these more accessible to the host plant.

Inorganic phosphorus in soil is generally bound to calcium, iron, or aluminum [$Ca_3(PO_4)_2$, $FePO_4$ and $AlPO_4$, respectively], and none of these are easily accessible for plant use. Although P is added to soil in fertilizers in soluble forms, it becomes rapidly insoluble. Inorganic P may subsequently be solubilized by rhizospheric bacteria through the secretion of organic acids. Tricalcium phosphate solubilization has been observed in *Streptomyces* spp. (Oliveira et al. 2009; Jog et al. 2014; Qin et al. 2015), and such activity has been associated with promotion of growth of several plant species (Guiñazú et al. 2009; Mamta et al. 2010; Shahid et al. 2012). Secretion of malic acid and a derivative of gluconic acid has also been associated with the mobilization of inorganic P by *Streptomyces* (Jog et al. 2014). Phosphorus mineralization, the process of separating P from larger, organic molecules, is dependent on enzymes (phosphatases and phytases) which have been found in several *Streptomyces* spp. and other microorganisms (Richardson and Simpson 2011; Jog et al. 2014).

Despite the fact that Fe is the second most abundant metal on Earth, both plants and microorganisms must find ways to acquire it from soil. Among *Streptomyces*, siderophore production is one of the most widely distributed plant growth-promoting traits (Jog et al. 2014). Siderophores are typically non-ribosomally produced small peptides that are secreted to the outside of the cell and later reintroduced back to the bacterial cell when bound to an Fe molecule, thus introducing Fe by highly specific transport systems. Plants have been shown to utilize iron from microbially produced siderophores (Crowley et al. 1988; Bar-Ness et al. 1992). Thus, the proliferation in the rhizosphere of bacteria that produce siderophores which may be taken up by the host plant increases the ability of the plant to utilize Fe. Siderophores have diverse chemical structures, and *Streptomyces* may produce more than one type; Gangwar et al. (2014) found siderophores of both catechol and hydroxamate types being produced by single *Streptomyces* isolates. Genome analyses support these observations, revealing that more than one gene associated to the synthesis of siderophores are commonly found in individual strains (Chen et al. 2013; Tarkka et al. 2015; Zhai et al. 2015), suggesting the potential significance of the trait to the ecology of the genus. Lautru et al. (2005), using a genome-mining approach, found a tris-hydroxamate tetrapeptide siderophore coelichelin in the genome of *S. coelicolor*. Information on phylogenetic, niche, and functional variation in the presence and utilization of siderophores is needed.

12.2.2 Plant Protection

Biological control may be used as alternative to pesticides, a complement to existing disease management, or a solution when there is no other type of available control. The management of soils to control plant diseases by addition of green manures or crop rotation may be considered one form of biological control, but here we focus first on "traditional" or inundative biological control. There are several mechanisms by which *Streptomyces* protect plants from pathogens, which include direct growth inhibition through production of antibiotics or cell-wall-degrading enzymes, indirect inhibition through competition for nutrients, and induced systemic resistance (ISR) in the plant (Mandeel and Baker 1991; van Loon et al. 1998; Pieterse et al. 2014).

12.2.2.1 Antibiotics

The capacity of *Streptomyces* to suppress other microbes was first noted nearly a century ago by Greig-Smith (1917). Since then, their extraordinary capacity to produce antibiotics has captured the attention of a broad range of scientists, including natural products chemists, pharmaceutical researchers, plant pathologists, and biochemists. The widespread ability among *Streptomyces* to produce pathogen-inhibiting secondary metabolites (Watve et al. 2001; Kim et al. 2012; Palaniyandi et al. 2013b) makes them well suited to protect crops from pathogens. In fact, soil *Streptomyces* with plant protection capacities have been reported in diverse settings. Among the best studied are antibiotic-producing *Streptomyces* spp. that act as antagonists of bacterial pathogens such as *S. scabies* and *S. turgidiscabies*, causal agents of potato scab, which have been found on potato tubers (Liu et al. 1995) and in soil (Hiltunen et al. 2009; Kobayashi et al. 2015). Soil *Streptomyces* isolates have been shown to protect alfalfa and soybean plants from oomycete infections, such as root rot caused by *Phytophthora medicaginis* and *P. sojae*, respectively (Fig. 12.2; Xiao et al. 2002). Even *Streptomyces* isolated from desert soils have shown antagonism toward soil phytopathogens, such as the nematode *Meloidogyne incognita* and the bacterium *Ralstonia solanacearum* (Köberl et al. 2013).

Endophytic *Streptomyces* with the ability to inhibit pathogens have been found surprisingly often. *Streptomyces* endophytes from cucumber protected the plants from *Colletotrichum orbiculare* (Shimizu et al. 2009). Nematicidal activity has also been reported in *Streptomyces* spp. by production of inhibitory compounds (Kun et al. 2011; Ruanpanun et al. 2011; Ruanpanun and Chamswarng 2016). Abundance, distribution, and ecology of these activities have not been widely studied and would be greatly valued.

Antibiotics are usually produced at the onset of the stationary growth phase when grown in liquid culture and are often associated with aerial hyphae and spore formation on solid medium (Bibb 2005). The biochemical pathways and genes involved in the synthesis of many antibiotics have been studied thoroughly, and polyketide synthases (PKS) and non-ribosomal peptide synthases (NRPS) are often involved (Núñez et al. 2003; Karray et al. 2007; Pulsawat et al. 2007; Laureti et al. 2011). As an example, virginiamycin M is an antibiotic produced by a PKS-NRPS

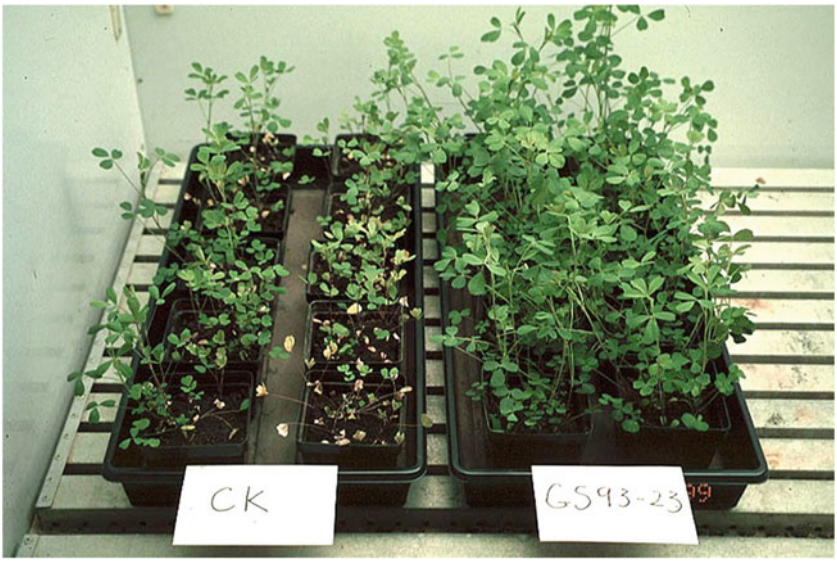

Fig. 12.2 Alfalfa plants grown in field soil naturally infested with *Phytophthora*. Plants on the *left* are non-inoculated; plants on the *right* are inoculated with a single pathogen-suppressive *Streptomyces* isolate (Xiao and Kinkel unpublished)

hybrid by *S. virginiae*, and has inhibitory activity against gram-positive bacteria (Pulsawat et al. 2007). The production of more than one antibiotic substance by *Streptomyces* is also commonly found, leading to a broad range of inhibited targets and in some cases greater inhibition due to the synergistic effect of multiple antibiotics. Production of multiple antibiotics is also desirable in order to minimize the development of resistance in the pathogens.

12.2.2.2 Volatile Compounds

Streptomyces produce many volatile compounds that inhibit growth of potential competitors (Audrain et al. 2015). These compounds are believed to be particularly important in soil settings, where diffusion through a liquid phase may sometimes be impaired.

A compound of 27 volatiles produced by *Streptomyces alboflavus* TD-1 inhibits growth of several plant pathogens, including *Fusarium moniliforme*, *Penicillium citrinum*, and several *Aspergillus* species in vitro (Wang et al. 2013). Volatile-producing *Streptomyces* spp. have the potential to be used as biocontrol agents, for instance, of *Botrytis* postharvest infections (Li et al. 2012) and fruit rot of strawberry (Wan et al. 2008). *S. platensis* F-1 produces volatiles that protect rice from leaf and seedling blight caused by *Rhizoctonia solani* and leaf blight of oilseed rape caused by *Sclerotinia sclerotiorum* (Wan et al. 2008). Further researches on bioactive volatiles from *Streptomyces* are likely to provide compounds with potential applications in medicine and agriculture.

12.2.2.3 Cell-Wall-Degrading Enzymes

Fungal and oomycete plant pathogens have cell walls made of chitin, glucans, and cellulose, respectively. These structures may be subject to degradation by lytic enzymes, such as chitinases, glucanases, and cellulases. Most terrestrial *Streptomyces* species are prolific producers of such extracellular enzymes (Chater et al. 2010), contributing to growth inhibition of fungal and oomycete pathogens.

Anthracnose biocontrol in pepper by *S. cavourensis* SY224 has been associated with glucanase production (Lee et al. 2012). Glucanases were also found responsible for the protection of several plant species from oomycete-caused diseases. As an example, *Streptomyces* spp.-derived reduction of root rot in raspberry caused by *Phytophthora* (Valois et al. 1996), and infection of cucumber by *Pythium aphanidermatum* (El-Tarabily et al. 2009), was dependent on the production of glucanases. Thus, glucanase production is one phenotypic trait worth evaluating while searching for biological control by *Streptomyces*.

Chitinase production and biocontrol potential of endophytic *Streptomyces* isolates were positively correlated, as determined in in vitro inhibition of fungal growth (Quecine et al. 2008). Furthermore, endochitinase production by *S. violaceusniger* XL-2 inhibits the growth of several wood-rotting fungi (Shekhar et al. 2006). In both studies, the *Streptomyces* isolates had to be incubated in chitin-containing medium in order to induce chitinase synthesis, indicating that enzyme production is a tightly regulated process. Chitinase production is apparently widespread among this genus (Kim et al. 2012; Jog et al. 2014), which is in accordance with the ability of *Streptomyces* to colonize varied habitats, such as insects, compost, and soil.

Extracellular proteases are another group of enzymes produced by *Streptomyces* that have been shown to inhibit plant pathogens. Secretion of proteases gives *Streptomyces* the ability to incorporate N from proteinaceous sources, some of which may be quite recalcitrant. Several *Streptomyces* isolates with protease activity have been studied (Chater et al. 2010; Kim et al. 2012). The isolate ExPro138 produces several proteases that inhibit the pathogen *Colletotrichum coccodes* at several stages of development (Palaniyandi et al. 2013c), and another isolate, *Streptomyces* sp. A6, produces a protease which inhibits the wilt-causing pathogen *Fusarium udum* (Singh and Chhatpar 2011).

In plant litter, cellulose is the most abundant organic molecule, making cellulose-degrading enzymes, or cellulases, fundamental for nutrient cycling in soil. *Streptomyces* produce both endo- and exo-cellulases (Book et al. 2014), which have been mostly associated with the turnover of plant material. However, the secretion of cellulases by *Streptomyces* spp. has also been associated with their ability to inhibit some plant pathogens, particularly those with cellulose-containing cell walls, such as *Oomycetes* (van Bruggen and Semenov 2000).

The widespread ability of *Streptomyces* spp. to degrade large organic molecules through the activity of extracellular enzymes provides them with the possibility of colonizing diverse habitats. In addition, these enzymatic activities make them good antagonists of plant pathogens. The ample diversity of enzymes with similar activities that are present in each genome needs to be further studied in order to understand their role in nature and their possibilities as tools for agronomic and industrial purposes.

12.2.2.4 Competition

Niche competition is another potential mechanism of pathogen control. *Streptomyces* spp. have the ability to utilize a broad range of nutrients, which likely contribute to their success in soil (Kieser and John Innes Foundation 2000). Their diverse arsenal of hydrolytic enzymes and siderophores likely make them superb competitors. Iron competition acting as a mechanism of biocontrol by endophytic *Streptomyces* has been suggested against *Fusarium* wilt on banana roots (Cao et al. 2005). It is likely that in many cases, competition acts as a combined factor in disease suppression, and the extent of the importance of competition on antagonistic interactions should be evaluated for each system.

12.2.2.5 Induced Systemic Resistance

Induced systemic resistance (ISR) is another mechanism of plant protection from pathogens. One striking feature about this process is that the beneficial microorganism does not need to be in indirect physical contact with the pathogen for successful disease suppression. ISR is characterized by the readiness with which the plant responds to a pathogen attack. After being exposed to an ISR-inducing microorganism, a latent state of resistance is acquired, which is expressed upon exposure to a pathogen. Generally, the range of pathogens to which the ISR will bring protection is quite broad, although that is specific for each plant-microbe interaction (van Loon et al. 1998; Pieterse et al. 2014).

Most of the research on ISR has been carried out on *Pseudomonas* and *Arabidopsis thaliana* systems; however, other microorganisms including *Streptomyces* have been shown to induce ISR. Induction of systemic resistance on *A. thaliana* by an endophytic *Streptomyces* sp. strain was demonstrated, shifting in gene expression profiles upon exposure to the pathogens *Erwinia carotovora* and *Fusarium oxysporum* with respect to non-primed plants (Conn et al. 2008).

Rhododendron plants were protected from *Pestalotiopsis sydowiana* by endophytic *Streptomyces*. The isolates showed no in vitro antagonism to the pathogen but induced systemic resistance in the plants (Shimizu et al. 2006). More recently, RNA sequence analyses of oak responses to *Streptomyces* priming showed a variety of defense mechanisms being activated by the resistance-inducing isolate; these mechanisms were different from those reported for *Pseudomonas* (Kurth et al. 2014).

Streptomyces isolates with antibiotic production, but also with plant growth-promoting traits or ISR-inducing activity, are currently sought. Table 12.1 shows a list of currently available commercial *Streptomyces*-based inoculants, in which most of the products are based on the antagonistic activity of the isolates. In agronomic settings, where the biotic and abiotic conditions are diverse and vary over time, a combination of plant protection and growth promotion is likely to give *Streptomyces*-based inoculants better chances of success. Combinations of strains in one formulation are also likely to provide enhanced disease protection. In addition, inconsistencies in the efficacy of biological control applications may be improved through a better understanding of the microbial dynamics and species interactions in soil.

Table 12.1 List of *Streptomyces*-containing products for plant care

Product name	Active ingredient	Use (reference)
Actinovate® AG Actinovate® SP	*S. lydicus* WYEC108	Root rot, damping off, foliar and turf diseases (*Fusarium*, *Rhizoctonia*, *Pythium*, *Phytophtora*, *Aphanomyces*, *Armillaria*, *Botrytis*, *Monilinia*, *Xanthomonas perforans*, *X. arboricola* pv. *A. juglandis*, *Gaeumannomyces graminis*, *Lanzia* spp., *Mollerodiscus* spp., *Erysiphe graminis*, *Puccinia* spp., *Colletotrichum graminicola*, *Pynculana grisea*, *Musilaga*, *Physarum*, *Typhula* spp., *Microdochium nivale*) (http://www.monsanto.com/products/pages/actinovate-us.aspx)
Actino Iron®	*S. lydicus* WYEC108	Root rot and damping off fungi (*Pythium*, *Rhizoctonia*, *Phytophthora*, *Fusarium*) (http://www.evergreengrowers.com/actino-iron.html)
Thatch control	*S. violaceusniger* YCED9	Thatch decomposer, prevents turf diseases (http://www.amazon.com/Natural-Industries-LGTC02-Control-Microbes/dp/B0044EK7G0)
Mycostop®	*S. griseoviridis* K61	Root and seed rots, root and stem wilt (*Botrytis*, *Pythium*, *Phytophtora*, *Rhizoctonia*, *Fusarium*, *Alternaria*, *Phomopsis*) (http://www.planetnatural.com/product/mycostop/)
YAN TEN	*S. saraceticus* KH400	Root rots and root-knot nematode (*P. aphanidermatum*, *R. solani* AG-4, *Meloidogyne* sp.) (http://www.yanten.com.tw/products-3_30633-english.html)

Modified from Palaniyandi et al. (2013b). All websites were accessed on January 28, 2016

12.3 Soil Health and *Streptomyces*

The concept of soil health has been evolving in the past few years (van Bruggen and Semenov 2000; Garbeva et al. 2004; Chaparro et al. 2012). Soil health was originally focused on one service of soil, plant productivity, and consequently, was mostly based on soil fertility measurements. However, interest has arisen more recently in a broader range of soil characteristics including sustainability of crop production and human health, adding new factors to the idea of soil health. Recent definitions of soil health involve the idea of soil as a living system that, in addition of enabling productivity, maintains environmental quality and promotes plant, animal, and human health (Larkin 2015). Thus, promoting crop management practices that consider this broader concept of plant health is highly desirable. Some factors, such as soil texture and depth, play a significant role in soil health, but such factors are not within what can be usually managed. Instead, practices that increase organic carbon in soil seem to generally enhance other characteristics related to comprehensive soil health metrics, such as higher water retention and higher microbial activity and density, which comes along with higher pathogen suppression (Altier and Zerbino 2012; Kinkel et al. 2012; Larkin 2015). Managing agronomic soils to increase beneficial *Streptomyces* communities could enhance nutrient cycling in soil, increase crop productivity, and protect crops from disease while reducing chemical inputs.

12.3.1 Nutrient Cycling

A healthy soil may be seen as a stable system in which high internal nutrient cycling is a key factor. Nutrient cycling depends on a microbial community with the ability to metabolize what is being introduced into the soil. Thus, communities with a wide range of nutrient use capacity, and thus able to utilize the diversity of nutrients available in such a complex medium, are desirable. As mentioned previously, most species of the genus *Streptomyces* are saprophytic and have the ability to metabolize a variety of compounds. Their diverse arsenal of secreted molecules includes enzymes that allow the degradation and later utilization of cellulose, chitin, lignin, melanin, and others (Schlatter et al. 2009; Kim et al. 2012), making breakdown products broadly available, which will likely increase total bacterial biomass.

The potential for managing *Streptomyces* antagonistic activities in soil to increase pathogen suppression was explored in a recent study. Following nutrient amendments, *Streptomyces* isolated from soils exposed to different nutrient amendments varied in their antagonistic activity, and populations from soils amended with high doses of nutrients (glucose or lignin) were more inhibitory than isolates from soils with low nutrient doses (Schlatter et al. 2009). Thus, both the type of nutrient and the amount of available nutrients shape *Streptomyces* communities in traits that are particularly important for pathogen suppression and sustainability. In related work, variation in resource use phenotypes within *Streptomyces* genetic groups was significantly associated with the location from which *Streptomyces* were isolated, suggesting that resource use is adapted to local environments (Schlatter et al. 2013). Local adaptation of *Streptomyces* to local nutrient sources highlights once more the relevance of this group in nutrient cycling in diverse environments and the potential for active management of soil populations to achieve specific agronomic goals (pathogen suppression, nutrient cycling).

12.3.2 Suppressive Soils

Suppressive soils are soils in which diseases do not develop on susceptible host plants or do so to a lesser extent than in conducive soils, although the pathogens may be present. Soils showing suppressiveness to several diseases have been reported, including *Fusarium* wilt in carnation and other crops (Cugudda and Garibaldi 1981; Alabouvette et al. 2009), take-all on wheat (Landa et al. 2002), *Phytophthora* on apple (Mazzola et al. 2002), black rot of tobacco (Kyselková et al. 2009), club root disease of Chinese cabbage (Murakami et al. 2000), and scab on potato (Liu et al. 1995). Using metagenomic approaches, *Streptomyces* spp. have been found more abundantly in suppressive than in conducive soils. Furthermore, when suppressive soils were added with *Rhizoctonia solani*, the abundance of *Streptomyces* spp. increased (Mendes et al. 2011), suggesting an important role in the suppressiveness against the pathogen.

In some cases, suppressiveness is achieved through competition for niche use, as was suggested for some *Fusarium* diseases (Alabouvette et al. 2009). Other times, suppressiveness is due to the presence of antibiotic-producing bacteria. In the case of suppressiveness to potato scab, disease reduction was correlated with the presence of high densities of antibiotic-producing, nonpathogenic *Streptomyces* spp. (Liu et al. 1995). However, the fact that antagonists and pathogens share the habitat and nutrients is a key factor in the effectiveness of the suppression of this disease.

12.3.3 Managing Soils for Suppression

In 1926, Sanford investigated the mechanisms by which rye green manure reduced potato scab and noted that *Actinomyces scabies* (now *Streptomyces scabies*) was "very sensitive to the secreted products of many molds and bacteria, some of which prevent its growth." He further suggested green manures favored the antagonistic bacteria that inhibited the pathogen. Subsequently, Millard and Taylor (1927) took the next step in showing that inoculating soil with a saprophytic (nonpathogenic) *Actinomyces* isolate could significantly reduce both disease and pathogen populations. Millard and Taylor concluded that the saprophytic inoculated strain outcompeted the pathogen in soil, thereby reducing plant disease.

It has been widely documented that plants (Micallef et al. 2009; Bakker et al. 2010, 2012) and soil management shape the underground microbial communities (Mazzola 2007; Chaparro et al. 2012; Bakker et al. 2013; Fraser et al. 2015). For instance, the use of green manures has been reported to be beneficial in many cropping systems (Hoagland et al. 2008; Weerakoon et al. 2012). In potato, *Verticillium* wilt was significantly reduced with corn and alfalfa used as green manures. Furthermore, in that experiment, streptomycete inhibitory activity was frequently negatively correlated with plant disease and positively correlated with potato yield (Wiggins and Kinkel 2005). Substantial work has been carried out to find management practices that increase soil health and reduce disease. However, a global understanding of the interactions and dynamics belowground is still evolving.

12.4 Other Notable Lines of Research in *Streptomyces*

The genus *Streptomyces* has been the focus of attention in many and diverse lines of research. Some of these may seem at a first glance to be completely unrelated to their agricultural or sustainability facet. However, the research in signaling among *Streptomyces* within and among strains and species is likely to influence their potential use in agricultural settings. Bioremediation may be a major industrial use for *Streptomyces*, since the accumulation of toxic materials derived from anthropic activities is ubiquitous.

12.4.1 Signaling

Although bacteria are mostly unicellular organisms, they are never alone in natural conditions and have developed the ability to sense signals from others. *Streptomyces* produce signaling molecules that participate in quorum sensing, the gamma-butyrolactones (GBLs) (Takano 2006; Morin et al. 2012). In addition to GBLs, other signaling molecules have been found in *Streptomyces*, such as furans (Corre et al. 2008). The biosynthetic routes for signal production and the receptors involved are well studied for many strains (Kato et al. 2007; Hsiao et al. 2007; Nishida et al. 2007; Sello and Buttner 2008; O'Rourke et al. 2009; Corre et al. 2010). Signaling among kin organisms modulates activities as diverse as sporulation, antibiotic production, entrance to a competent state, biofilm production, and expression of pathogenesis-related molecules (Weinrauch et al. 1991; Kato et al. 2007; Williams 2007).

In nature, the produced signals are likely to participate in sensing the presence of others, integrating information about the outside and the inside and thus synchronize specific activities (Camilli and Bassler 2006; Kato et al. 2007). Eavesdropping, a process in which signals that modulate activities within kin organisms are detected and induce a response by non-intended recipients, may alter the timing or quantity of antibiotic production by the eavesdropper, potentially enhancing the fitness benefit of antibiotic production in the presence of a competitor (Duan et al. 2003; Chandler et al. 2012). Chemical manipulation, when one organism secretes chemicals that modify the metabolism of the target population, usually to benefit the signal emitter (Keller and Surette 2006; Egland et al. 2004), could also significantly alter the phenotype of a *Streptomyces*. Interspecies interactions among *Streptomyces* within a community may shape the phenotype of the coexisting isolates (Vaz Jauri and Kinkel 2014). The compounds mediating such interactions may or may not have evolved for such purposes, as is the case of antibiotics at subinhibitory concentrations (Vaz Jauri et al. 2013, Yim et al. 2006). Nonetheless, these interactions may influence the fitness of the interacting species, and the sum of these and other chemical interactions are likely to shape the structure and function of natural microbial communities.

12.4.2 Bioremediation

Soils and water can be contaminated with a number of substances, ranging from heavy metals, petroleum derivatives, pesticides, and industry effluents. Organisms belonging to the genus *Streptomyces* are valuable also in this area, due to their great metabolic diversity and production of secondary metabolites.

12.4.2.1 Heavy Metals

Soils contaminated with heavy metals are often a consequence of mining activities. However, soils with high concentrations of heavy metals can also be found naturally, due to geogenic activities. These soils may explain the initial existence of

plants and microbes that are able to survive in such extreme conditions, which would be toxic to nonadapted individuals (Kothe et al. 2010). Bacteria cope with heavy metal contamination through adsorption, mineralization, accumulation, chelation, chemical reduction, or simply remotion from the intracellular space through efflux transporters (Schütze and Kothe 2012). Studies on the microbial communities of soils highly contaminated from mining activities have shown that higher proportions of *Streptomyces* and *Bacillus* are found in contaminated soils than in non-contaminated soils, revealing that these organisms are better adapted to contaminated soils than other common inhabitants of soils, such as gram-negative bacteria (Kothe et al. 2010).

The action of siderophores by soil bacteria may protect other organisms, e.g., plants and other microorganisms, by reducing the oxidative damage caused by the presence of heavy metals. Furthermore, secretion of siderophores may reduce the damage to plants by reducing the negative effects of cations on auxin production (Dimkpa et al. 2008). In addition, the presence of heavy metals has been observed to induce the synthesis of siderophores in heavy metal-resistant *Streptomyces* (Schütze et al. 2014).

12.4.2.2 Pesticides

Insecticide, herbicide, and fungicide applications are commonplace in agricultural settings. Some of these compounds are recalcitrant to degradation, leaving a long-lasting toxicity on soils and watercourses where they are drained. In some cases, the compounds are partially degraded, but the degraded products may also have an impact on the health of a plethora of organisms. Thus, finding enzymes and/or organisms that will degrade these compounds is highly valued.

Streptomyces with the ability to degrade insecticides of diverse chemical nature have been found (Fuentes et al. 2010; Chen et al. 2012; Cuozzo et al. 2012; Saez et al. 2012). Furthermore, *Streptomyces* have been found to act as bioremediators in soils with mixed contaminants, such as heavy metals and pesticides (Polti et al. 2014). Given their ability to degrade contaminants and their plant-association capacities, methods have been developed for the combined use of *Streptomyces* and host plants (maize) for the removal of pesticide accumulation (Benimeli et al. 2008; Alvarez et al. 2005).

12.4.2.3 Other Sources of Contamination

Contamination of soil and water with oil is sadly common. Numerous ways to deal with this problem have been searched and are still a matter of concern. As an example, in a work by van Gestel et al. (2003), diesel-contaminated soil was mixed with compost and tested for its capacity to maintain microbial life. Numerous microorganisms were able to live in such conditions, including *Streptomyces* spp., which are common inhabitants of compost. Using another strategy, a strain of *Streptomyces rochei* has been isolated from heavy crude oil with the ability to degrade three- and four-ring compounds (anthracene, fluorene, phenanthrene, and pyrene) (Chaudhary et al. 2011). This finding is encouraging for further efforts to use *Streptomyces* as one more strategy to deal with oil spills.

Although not intuitive, the use of bacteria from sites drastically different from where they will be used is also possible. *S. indianensis* and *S. hygroscopicus* isolated from marine sediments have been tested with enormous success on dairy industry sludge. Effluents treated with each isolate tested alone or a combination of both had improved all the parameters tested, which included chloride and oil content and germination and shoot length of *Vigna radiata* (Sathya Priya et al. 2014). In a similar example, a *Streptomyces* isolated from marine sediments was used to treat soils contaminated with vinase, a waste product in the generation of bioethanol. In this case, the remediation process is not due to the metabolization of a toxic compound, but rather by the production of an emulsifier that reduces the concentration of the waste product (Colin et al. 2016).

12.5 Conclusions

Streptomyces are abundant, diverse, and ubiquitous. Across their evolution, they have developed a number of characteristics that are highly valuable for industry, medicine, and sustainability. The use of *Streptomyces* for industrial and medical purposes has been widely explored, although their high diversity allows for further exploration. The use of *Streptomyces* for sustainable crop production has been less exploited. However, the research already carried out on secondary metabolites production and regulation and signaling should be incorporated to that of their PGP activities and behavior under different environments. Thoughtful analysis of the information rendered by new tools that provide large amounts of information, such as high-throughput sequencing, among others, will greatly accelerate the development of this much-needed area of research. Big companies dedicated to the production of inputs for agriculture have turned to explore this area, carrying out gigantic experiments with over 2000 seed microbial coatings and close to 500,000 field trials (http://www.scientificamerican.com/article/microbes-added-to-seeds-could-boost-crop-production/). This new attitude toward the use of microorganisms reflects the global demand for a more sustainable agriculture but also the great potential microbial-based technologies possess.

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Short-Term Evolution of Rhizobial Strains Toward Sustainability in Agriculture

13

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Abstract

Some rhizobial strains are widely used as biofertilizers substituting inorganic nitrogen fertilization, mainly for legumes. The successful use of rhizobia in agriculture derives from appropriate selection of strains with high capacities to fix nitrogen. However, the selection of more efficient rhizobia is from a limited number of plant growth conditions. In other environments, inoculant strains may exhibit low competitiveness or low nitrogen fixation. We argue here that rhizobial strains are continuously evolving in plants and therefore recommend an approach inspired on experimental evolution studies where strains adapted to particular conditions may be selected. The selection and detection of efficient rhizobial strains should take place under local field conditions in order to obtain superior nitrogen-fixing symbionts. To support our recommendation, we reviewed different examples of experimental evolution in bacteria and summarized results on rhizobial co-inoculation with different microbes.

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13.1 Introduction

Rhizobium is a collective name that refers to nitrogen-fixing nodulating bacteria belonging to different taxonomic groups of alpha- and also betaproteobacteria (Willems 2006; Velázquez et al. 2010; Ormeño-Orrillo et al. 2015). *Rhizobium* and *Bradyrhizobium* have been used for over a hundred years as safe inoculants in legume agricultural fields to enhance associations with legume plants, providing natural substitutes for inorganic nitrogen fertilizers. Previous reviews on agricultural applications of rhizobial inoculants have been published (Lupwayia et al. 2006; Peoples et al. 2009; Sprent et al. 2010; Castro et al. 2016), and there are overviews of general aspects of rhizobial symbiosis (López-López et al. 2010a, b; Remigi et al. 2016). In addition to nodule-forming bacteria, there are nonsymbiotic rhizobia that lack symbiosis plasmids or symbiosis islands (reviewed in López-Guerrero et al. 2012) and that may colonize legume and nonlegume rhizospheres in some cases promoting plant growth (Martínez et al. 2003; Gutiérrez-Zamora and Martínez-Romero 2001).

Recently, many novel rhizobial species have been described based on genomic data that has clearly delineated distinct genomic lineages (Althabegoiti et al. 2012, 2014; Ormeño-Orrillo et al. 2015). New studies on rhizobial diversity are revealing an enormous reservoir of strains and species with a large potential of applications in agriculture. However, even with present knowledge, we are incapable of predicting which strains will show a high capacity to fix nitrogen in the field and be more adequate for certain environmental conditions. Molecular marker-assisted selection of rhizobia strains would be useful if properly developed. The selection of strains to be used in agricultural fields is nowadays still based on empirical analysis, in which different strains are usually tested as inoculants usually first in greenhouses and later in experimental trials and in agricultural soils. Recommendations on how to select high-quality inoculant strains for agriculture have been reviewed by Catroux et al. (2001), Sessitsch et al. (2002), and Hungria et al. (2005). The beneficial effects of rhizobia are then evaluated in comparison to the yields obtained with control non-inoculated plants or with nitrogen fertilizers. Ideal strains considered for inoculant production should show increased crop yields in comparison to non- inoculated controls and similar yields to fields treated with inorganic fertilizers. However, it is not possible to test all strains for all environmental conditions in which rhizobia inoculation is needed, and this may lead to erratic degrees of success under different conditions. The success of the Brazilian inoculant program has been partially due to the continuous isolation and selection of effective native strains from different agricultural sites (Hungria et al. 2000, 2006; Dall'Agnol et al. 2013; Mostasso et al. 2002). Here we provide a conceptual framework that justifies this strategy for inoculant strain selection.

In a few cases, rhizobia strains substitute completely for chemical nitrogen fertilization. In other cases, in spite of not providing all the nitrogen required by the plants, rhizobia may be considered as indispensable for sustainability (Hungria and Vargas 2000; Peoples et al. 2009). Sustainability means attaining productivity without risking future resources and wealth (Young and Burton 1992). Nitrogen is the

most limiting element under natural and agricultural conditions (Graham and Vance 2000); consequently novel paths to improve nitrogen fixation in agriculturally important crops are needed. Herein we discuss a simple strategy based on experimental evolution (that allows the selection of new metabolic traits under experimental conditions): We thus recommend selecting inoculant rhizobia strains from naturally "evolved" rhizobia by re-isolating competitive and efficient bacteria from nodules in field trials. Several examples of the success of experimental evolution trials in microbiology will also be presented in this review.

13.2 Sustainability and Rhizobia

Rhizobial symbionts of plants can provide up to 450 kg of fixed nitrogen per hectare per year (Herridge and Rose 2000), which is equivalent to a heavy application of inorganic nitrogen fertilizer. Therefore, rhizobia are considered the preferred substitutes for chemical fertilizers, with the additional advantage of delivering nitrogen compounds directly into the plant without unwanted leakage into the environment generating pollution. Consequently, commercialization of rhizobial inoculants has increased involving today many companies that use rhizobia all over the world as commercial inoculants. Indeed, nowadays there is a large competition among the agro-industry companies producing commercial inoculants. The effectiveness of the inoculants is highly variable depending on the agricultural sites and type of products. Rhizobial inoculants are produced for soybean, alfalfa, lentils, chickpea, beans, peas, and other legumes, and the recommended strains for many different legumes have been published (Menna et al. 2006). Soil pH and phosphorous limitations seem to be the main abiotic soil factors determining the outcome of rhizobial inoculants in agricultural fields (Vance 2001; Hassen et al. 2014; Vance 2001). In some burkholderias (Ormeño et al. 2012b) and in some rhizobia, the symbiosis plasmid may be easily lost upon subculturing, and this genetic instability has been considered as an undesirable characteristic in legume inoculant strains.

Research on improving formulations to increase survival of bacteria in peat or other substrates has led to better-quality inoculants (Xavier et al. 2004; Catroux et al. 2001). The advantages of liquid presentations of inoculants have been discussed (Albareda et al. 2008). Commercial inoculants have been shown to enhance growth and nitrogen fixation of a promiscuous soybean variety in Africa (Thuita et al. 2012). Different companies producing inoculants use *Bradyrhizobium diazoefficiens* USDA110 (Delamuta et al. 2013) for soybean and *R. tropici* CIAT899 (Ormeño-Orrillo et al. 2012a, b) for *Phaseolus vulgaris* beans, but other strains, mainly isolated from local crops, may be better nitrogen fixers in some conditions. Different rhizobia-legume partnerships show different levels of nitrogen fixation (Schulze 2004), and this variability is not only determined by the inoculant species but also by the plant host. This represents a big challenge in developing effective biofertilizers for a single leguminous species, and perhaps rhizobial inoculants should be cultivar specific. For example, the newly developed variety of *Phaseolus vulgaris*, Negro Jamapa Plus, is poorly nodulated by *R. tropici* strain CIAT899

(unpublished data), whereas the original Negro Jamapa was a good symbiont with this strain.

We are committed to supporting and encouraging the preservation of plant diversity. Plant diversity may drive rhizobial divergence as do microhabitats that locally select for genetically distinct microorganisms of the same species. Even minor plant to plant variations or local differences in roots of a single plant may exert some sort of rhizobial strain selection. When rhizobia diversity has been analyzed in restricted areas, a remarkable variety of strains and/or symbiovars that are classified within a single species has been revealed (Epstein et al. 2012; Kumar et al. 2015). In such a complex framework of locally selected symbiotic associations, we should consider inoculation strategies based upon multiple rhizobial strains to better control natural selection of genetically diversified strains and expand their efficiency in symbiotic association as well as nitrogen fixation.

Commercial inoculants are mainly based on rhizobia but may contain other microorganisms, for instance, *Azospirillum* spp. and *Pseudomonas fluorescens* (Sarig et al. 1986; Parmar and Dadarwal 1999; Remans et al. 2008), that have been found to increase yield or growth of several legumes (Table 13.1). However, it should be noted that stimulation effects may be strain or cultivar specific, and a general prediction of the effects of different partners in the inoculants may not be fully established. In the maize colonization assays that we are performing (Fig. 13.1), we have observed increased maize plant growth with the community tested in comparison to the single-strain rhizobial inoculation in the laboratory.

Rhizobial inoculation of *Phaseolus vulgaris* (common bean) plants may affect other soil bacteria, some of them with potential plant growth-promoting activities (Trabelsi et al. 2011). Furthermore, the benefits that rhizobia confer to plants are not restricted to nitrogen fixation. For example, rhizobia may inhibit fungal pathogens, produce phytohormones, solubilize phosphate and phytate (López-López et al. 2010a), and produce bioinsecticides (in a *Bradyrhizobium* sp. strain from *Phaseolus microcarpus*, Servín-Garcidueñas et al. 2016).

13.3 Experimental Evolution Assays

Given the complex variability of rhizobia-plant interactions we have just discussed, the exploration of experimentally evolved bacteria in plants assumes paramount importance. Studies of experimental evolution have led to novel bacterial symbionts (Marchetti et al. 2010) and may be possible means to obtain rhizobial inoculants with enhanced efficiency of nitrogen fixation. Besides this biotechnological usefulness, experimental evolution studies can reveal new connections between genotype and phenotype in symbiotic bacteria of plants. Rhizobia experimentally selected in agricultural fields could then show a better correlation of genetic changes with specific environmental or abiotic conditions, which may help in determining markers for adaptation to specific conditions in agriculture practice.

Experimental microbial evolution was reviewed by Adams and Rosenzweig (2014). Perhaps the most famous experimental evolution studies are the ones

Table 13.1 Co-inoculation assays of different bacteria and fungi with rhizobia

Strain	Plant and conditions	Effect	References
Mesorhizobium cicer & *Piriformospora indica* & *Pseudomonas argentinensis*	Chickpea (*Cicer arietinum* L.)	Improvement in total N content	Mansotra et al. (2015)
	Field		
Ochrobactrum ciceri & *Mesorhizobium ciceri*	Chickpea (*Cicer arietinum* L.)	More nodules, 62 % more biomass, and 111 % grain yield	Imran et al. (2015)
	Soil		
Bradyrhizobium japonicum & actinomycetes	Soybean (*Glycine max*)	Increased yield	Nimnoi et al. (2014)
	Soil		
Rhizobium sp. & *Micromonas*	Clover plantlets	Increased shoot length	Trujillo et al. (2014)
	Greenhouse		
Rhizobium & *Bacillus megaterium*	Pigeon pea	Increase in plant fresh weight, chlorophyll content, and nodule number	Rajendran et al. (2008)
	Pot experiments		
Bradyrhizobium japonicum & *Bacillus* spp.	Soybean	Plant growth promotion	Bai et al. (2003)
	Greenhouse		
Bradyrhizobium japonicum & *Pseudomonas fluorescens*	Soybean	Increased colonization of roots, nodule number, and acetylene reduction activity	Chebotar et al. (2001)
	Growth chamber		
Rhizobium sp. & *Pseudomonas*; *Rhizobium sp.* & *Bacillus*	*Cicer*	Increased nodule weight, root and shoot biomass, and total plant nitrogen	Parmar and Dadarwal (1999)
	Gnotobiotic conditions and soil		
Rhizobium leguminosarum sv. trifolii & *Bacillus insolitus*; *Rhizobium leguminosarum sv. trifolii* & *B. brevis*	Soybean	Plant growth promotion	Sturz et al. (1997) and Liu and Sinclair (1993)
	Growth room		
Rhizobium etli & *Bacillus* producing IAA	*Phaseolus vulgaris*	Increased nodule number, nodule fresh weight, nitrogenase activity, leghemoglobin content	Srinivasan et al. (1996)
	Gnotobiotic conditions in growth chambers		

(continued)

Table 13.1 (continued)

Strain	Plant and conditions	Effect	References
Rhizobium & *Pseudomonas*	Lentil, pea	Plant growth promotion	Chanway et al. (1989)
	Field plot		
Rhizobium & *Azospirillum*	*Medicago polymorpha* and *Macroptilium atropurpureum*	Increased nodule formation	Yahalom et al. (1987)
	Greenhouse		
Bradyrhizobium japonicum & *Pseudomonas*; *Bradyrhizobium japonicum* & *Bacillus* sp.	Soybean *Glycine max* (L)	Enhanced nodulation and increased dry weight of soybeans. Increased seed yield	Li and Alexander (1988)
	Soil		
Antibiotic producing *Sinorhizobium meliloti* & *Pseudomonas*	Alfalfa (*Medicago sativa* L)	Enhanced nodulation	Li and Alexander (1988)
	Soil		
Rhizobium & *Azotobacter*	Leguminous plants	Enhanced nodulation	Burns et al. (1981)
	Greenhouse		
R. leguminosarum & *Pseudomonas*; *R. leguminosarum* & *Serratia*	Lentil	Increased yield	Zahir et al. (2011)

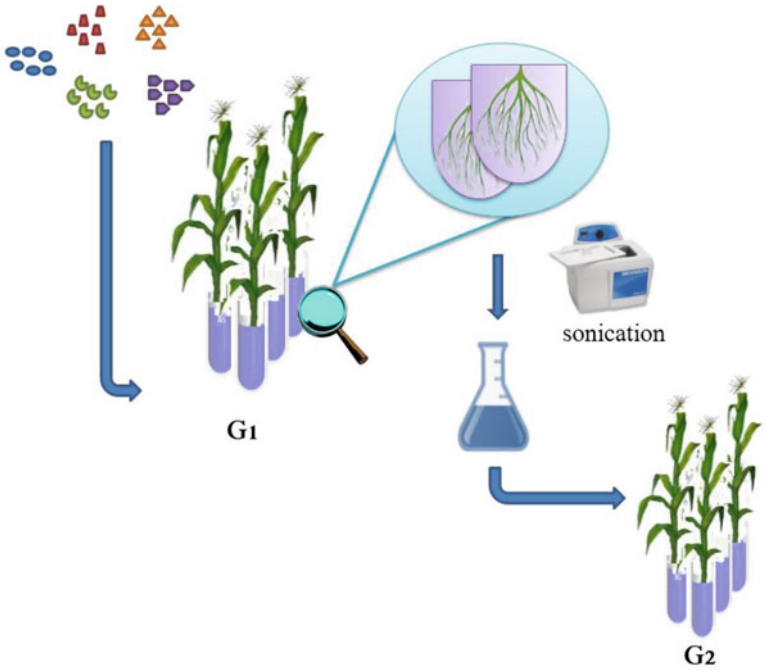

Fig. 13.1 Equal cell numbers of each bacterial species were inoculated in maize plants at the beginning of the experiment. Bacteria were periodically recovered from roots by indirect sonication after each plant cycle and used for inoculating other maize plants for a new cycle. An aliquot from the bacteria recovered from roots from each cycle was grown in culture media in plates to estimate CFU from each species. Aliquots were kept frozen for further genomic analyses. G1 first cycle or generation, G2 second cycle or generation

performed with *Escherichia coli* at Lenski's laboratory over 28 years. There liquid subcultures were made every day for 12 different independent populations that, in January 2016, have attained over 64000 generations of bacteria. Bacteria from the different subcultures were periodically frozen and were found to exhibit different metabolic capabilities and distinct mutations (Barrick et al. 2009). Whole genome sequencing of the evolved strains has subsequently allowed the identification of specific mutations that have arisen in the selection process. Fitness of evolved strains was evaluated in competition with the original ancestor and was found to increase over time (Wiser et al. 2013). Some strains lost genes for ribose catabolism (Cooper and Lenski 2000; Cooper et al. 2001) but gained citrate catabolism (Tenaillon et al. 2016). Besides several thousand mutations, they found a reduction of about 1.4 % of the genome length (Tenaillon et al. 2016). In early cultures, hypermutating strains appeared (Cooper and Lenski 2000), and most of the point mutations were present in the six mutator strains (Sniegowski et al. 1997; Wiser et al. 2013; Blount et al. 2008).

The model bacterium *E. coli* has become a favorite species to evolve in the laboratory, and even in short times (44 days up to 6 months), metabolic capacities that lead to higher growth rates under specific conditions were obtained. For example, bacteria were selected to attain larger growth rates in glycerol and lactate, at 42.2 °C (Herring et al. 2006; Conrad et al. 2009; Tenaillon et al. 2012), under different oxygen concentrations (Puentes-Téllez et al. 2013, 2014) or to have altered carbon fluxes to increase the production of aromatic amino acids or ethanol (Balderas-Hernández et al. 2011).

Besides *E. coli*, other bacteria such as *Lactobacillus* (Bachmann et al. 2012) and *Pseudomonas* (Barrett et al. 2005; Huse et al. 2010) have been used in experimental evolution assays. Out of the laboratory, strains of *Pseudomonas aeruginosa* evolve in the lungs of human cystic fibrosis patients where a high frequency of hypermutable *P. aeruginosa* is found (Oliver et al. 2000). Large chromosomal inversions and different insertions were related to O-antigen and type IV deficiencies and hypermutability (Kresse et al. 2003). Colony morphology variants were isolated from *P. aeruginosa* biofilms in cultures, similar to those from cystic fibrosis biofilms (Kirisits et al. 2005).

In the laboratory, we selected agrobacteria or *Ensifer* transconjugants that were capable of forming nitrogen-fixing nodules in *Phaseolus vulgaris* bean (Martínez et al. 1987; Rogel et al. 2001). We also selected burkholderia variants that were found to produce effective nodules in bean plants (Martínez-Romero 2009). Catherine Boivin and coworkers have carried out detailed studies of experimental evolution to understand the genetic modifications involved in becoming an effective plant symbiont (Guan et al. 2013; Marchetti et al. 2010, 2016). This work has focused on betaproteobacteria, which have similar symbiotic *nod* genes to alphaproteobacterial symbionts (Moulin et al. 2001) but different properties with regard to the energy metabolism sustaining the process of nitrogen fixation (Degli Esposti and Martinez-Romero, 2016).

The plant pathogen *Ralstonia solanacearum* was turned into a legume symbiont by first integrating a symbiosis plasmid from the plant symbiont *Cupriavidus taiwanensis* and then by sequential cycles of nodulation with the plant *Mimosa pudica* followed by strain isolation from nodules. Sequential cycles of selection by the plants allowed the recovery of symbionts with incrementally improved capacity to infect and persist within plant nodules, even if no nitrogen fixation was obtained. Whole genome sequencing allowed the identification of the genetic changes occurring during growth of bacteria in laboratory media and selected by the plants in nodules. An increased mutation rate was observed in the transconjugant *R. solanacearum* strains, which was attributed to an error-prone DNA polymerase that is encoded in the *C. taiwanensis* symbiosis plasmid (Marchetti et al. 2014).

In Brazil soybean crops were introduced in the 1960s, and inoculant strains were found to have undergone a process of evolution in Brazilian agricultural fields (Barcellos et al. 2007; Menna and Hungria 2011). In New Zealand and in Australia, lateral transfer of symbiotic information led to the appearance of native strains with a capacity to form nodules in the introduced crop. Evolution of novel nodulating rhizobia occurred by the acquisition of a 500 Kb symbiosis island that integrated into a phenylalanine tRNA gene (Sullivan and Ronson 1998).

As shown in Table 13.1, most co-inoculation assays involved one or two species in addition to rhizobia and not multispecies trials, and there are no evolution experiments of rhizobia in bacterial communities. Consequently, we are currently performing experimental evolution assays of *Rhizobium phaseoli* Ch24-10 in the presence of four other plant growth-promoting bacterial species in maize roots or exudates in the laboratory, as shown in Fig. 13.1, with the aim to select rhizobial variants better adapted to maize.

13.4 Short-Term Evolution of Rhizobial Strains in Crop Fields

Our hypothesis is that natural evolution of strains is always occurring in crop fields by the different mechanisms depicted in Fig. 13.2. In A-E different possible evolutionary pathways are illustrated because not all strains suffer the same modifications. This scheme provides a conceptual framework that justifies the strategy for a continuous selection of evolving strains.

The simple strategy that we propose for experimentally evolving efficient rhizobial strains as inoculants is to isolate and sequentially re-isolate bacteria from field nodules for the plants and conditions desired. A correlation of the genetic changes and the field conditions may help to determine markers for adaptation to field-specific conditions. One of the field conditions that may be important in the selection of strains is the associated plants in polycultures or crop rotation. We have reported that certain bean-rhizobia strains may be beneficial for companion plant production, for example, for different varieties of maize in the context of traditional polycultures (Gutiérrez-Zamora and Martínez-Romero 2001; Rosenblueth and Martínez-Romero 2004). Furthermore in plant rhizospheres, there are complex bacterial communities, and they may also exert a strong selection on the rhizobial populations. Selection should consider rhizobial function in consortia with other bacteria or fungi. The bacteria-based strategy challenge would be to direct evolution toward more efficient nitrogen fixation with local strains and plants well adapted to their site.

Nitrogen is the most limiting element under natural and agricultural conditions (Graham and Vance 2000), but adequate amounts of phosphorus are required for symbiotic nitrogen fixation (Vance 2001). Phosphate together with micronutrients may also contribute in the selection of rhizobial strains in fields. Providing phosphate in a sustainable manner is a challenge as phosphorus is not renewable.

13.5 Conclusions

Despite many years of research on rhizobia, we still do not know how to select broadly efficient strains for wide diverse conditions (including different plant varieties, soil abiotic characteristics, environments, and bacterial communities in the rhizosphere). A continuous laboratory or greenhouse selection of strains for different plant varieties or species and soil and environmental conditions appears to be a

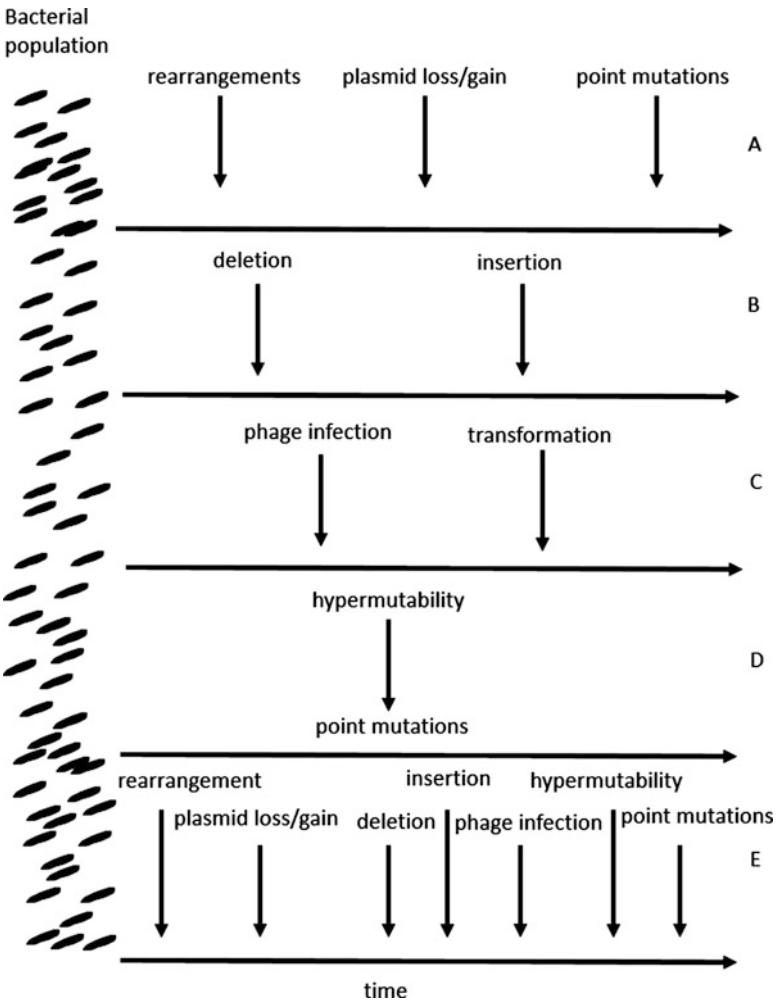

Fig. 13.2 Hypothetical genetic modifications that a single bacterial population may undergo in the field over time. Not all bacterial strains (depicted at *left*) undergo the same modification events

resource- and time-exhausting task. Alternatively, we suggest that in nature and in agricultural fields, there is always a continuous selection of nodulating bacterial strains that grow with the plant nutrients locally available in specific environmental conditions or microbial communities. Re-isolation of bacteria from some plant nodules in fields would allow the selection of bacteria adapted to those conditions. The most current practice, practical and cheap, in inoculant production is to use some few elite strains that have gained the reputation to be good nitrogen fixers. Here we oppose to this practice and encourage a continuous search of adapted novel strains as performed in Brazil that has the most successful program for biofertilization worldwide. From this program (Hungria et al. 2000, 2006), nitrogen efficient strains

such as *R. freirei* PRF81 (Ormeño-Orrillo et al. 2012a, b) and *Bradyrhizobium diazoefficiens* (Delamuta et al. 2013) strains were obtained. The strategy of natural rhizobial selection in short-term assays avoids the use of transgenics that are not well accepted specially in Europe.

A common observation in inoculation assays is that depending on the amounts of indigenous rhizobia in soils, only a small proportion of inoculated strains are recovered from nodules and consequently the effects of superior inoculants is not fully observed (Thies et al. 1991). Competition with indigenous bacteria could act to select some inoculant variants that may be more competitive than the original inoculant strain; thus the strategy to continuously select field nodule bacteria may render more competitive inoculant strains.

There are very few studies of experimental evolution in structured or complex environments. Field is a one of the most complex environments, and therein the tracing of evolved strains would certainly reveal many unexpected genetic events, as those found in New Zealand when the inoculant strains were traced backed revealing the appearance of novel symbionts (Sullivan and Ronson 1998). Thus field rhizobial evolution studies should be encouraged and supported.

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The Contribution of the Use of *Azospirillum sp.* in Sustainable Agriculture: Learnings from the Laboratory to the Field

14

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Abstract

Azospirillum sp. is one of the best-studied genus of plant growth-promoting rhizobacteria at present. These bacteria are able to colonize hundreds of plant species and significantly improve their growth, development, and productivity under field conditions. Different microbial abilities have been described to explain the plant growth regulation by *Azospirillum* sp.; clearly, a single mechanism is mostly not responsible for the full effect observed on inoculated plants, so the bacterial mode of action is currently better explained as an additive and selective effect. The most studied mechanism proposed for *Azospirillum* sp. to explain plant growth promotion of inoculated plants is the ability to produce several phytohormones and other related molecules with the capacity to promote plant growth or enhance the plant response to an environmental stressing condition. One of the most important achievements obtained from the research is the utilization of azospirilla commercial inoculants in approximately 3.5 million ha mainly cultivated with cereal crops in South America. Recently published reports of *Azospirillum* spp. inoculation of dryland crops showed a mean grain response of 10 % with greater benefits in winter (14.0 %) than in summer cereals (9.5 %) or legumes (6.6 %). In general, the increase of crop production could be obtained 70 % of the time, explained in part not only due to the complex interaction between the modes of action of *Azospirillum* sp. and plants but also by the multiplicity of abiotic stress conditions that the microbes help to mitigate. Azospirilla

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behavior leads to the theory of multiple mechanisms acting in sequential or cumulative patterns. Also, part of the variability of the plant response could be related to the different methods of inoculation (farmer or industrial seed treatments, in-furrow, foliar, or soil-sprayed applications) as well as the interaction with crop management practices related with the occurrence of abiotic limitations for crop growth (i.e., irrigation, fertilization, genotypes, combination with other beneficial microbes, etc.). But, under strong stressful growing conditions (i.e., severe droughts, major nutrients limitations, etc.), these responses are barely observed. *Azospirillum* sp. inoculation promotes corn (*Zea mays* L.) productivity, and this response may be related with the increase in the root development that increases the soil volume that the plant uses to explore for nutrients and water acquisition. The crop responses to the inoculation are greater in plant attributes defined during early growth than in those from late reproductive crop development stages. The combined inoculation of legumes with rhizobia and azospirilla, among other beneficial soil microorganisms, could over-improve the performance of the plants, compared with a single inoculation, due to the complementary biological processes of both microbes. Although the contribution of the co-inoculation with rhizobia and azospirilla on the productivity of diverse legume crops and pastures is promising, the available information under large production conditions is still limited.

14.1 Introduction

Soil is a natural environment for plant roots where microorganisms can proliferate usually in a large number. Rhizosphere is the portion of soil in which growth of microorganisms is influenced by the presence of the root system (Hartmann et al. 2008). The rhizosphere bacteria, also named rhizobacteria, can be divided in those which form a symbiotic relationship with the plants and those that do not establish a relationship with the plant. The latter are microbes closely associated with the root surfaces (free-living microbes) or that resides within the roots like the endophytic bacteria (Kloepper et al. 1989). When the presence of rhizobacteria benefits plant growth, they are named plant growth-promoting rhizobacteria or PGPR. The vast group of rhizobacteria was recently divided into different subgroups according to the mechanisms they used during their interaction with plants, such as the PGPR groups proposed by Kloepper and Schroth (1978), the biocontrol-plant growth-promoting bacteria (biocontrol-PGPB) group proposed by Bashan and Holguin (1998), or the plant stress homeostasis-regulating rhizobacteria (PSHR) group proposed by Cassán et al. (2009a).

PGPR, either directly or indirectly, may facilitate or promote plant growth under non-stressful or under biotic (biocontrol-PGPB) or abiotic (PSHR) stressful conditions. The indirect way of action of plant growth-promoting microbes shown by biocontrol-PGPB in biotic stress conditions includes a variety of mechanisms by which bacteria prevent the deleterious effects of phytopathogens on plant growth,

acting as rhizosphere competition (Kapulnik 1991), inducing systemic resistance (ISR) (van Loon et al. 1998; De Vleesschauwer and Höfte 2009), producing stress-related phytohormones like jasmonic acid (JA) (Forchetti et al. 2007) or ethylene (ET), and/or producing antimicrobial molecules (Thomashow and Weller 1996; Berg 2009). The direct growth-promoting mechanisms include the widely studied biological nitrogen fixation; phytohormone production such as auxins, gibberellins (GAs), and cytokinins (CKs) (Boiero et al. 2007; Perrig et al. 2007); iron sequestration by bacterial siderophores (Leon 1986); and phosphate solubilization (Seshadri et al. 2000). Indirect plant growth promotion and direct regulation induced by PSHR in abiotic stress conditions include production of stress-related phytohormones such as abscisic acid (Cohen et al. 2008), jasmonates (Forchetti et al. 2007), or other plant growth regulators such as a polyamine, cadaverine (Cassán et al. 2009a), and the ethylene catabolism-related enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which reduces the level of ethylene production under unfavorable conditions, thus conferring resistance to stress (Glick et al. 1998), and the production of a wide range of osmotically active metabolites.

14.2 *Azospirillum* sp. as Model of Plant Growth-Promoting Rhizobacteria

Azospirillum sp. is one of the best-studied genus of plant growth-promoting rhizobacteria at present. This microorganism is able to colonize hundreds of plant species and significantly improve their growth, development, and productivity under field conditions (Bashan and de Bashan 2010). One of the principal mechanisms proposed for *Azospirillum* sp. to explain plant growth promotion of inoculated plants has been related to its ability to produce and metabolize several phytohormones and other plant growth regulation molecules (Tien et al. 1979). From a historical perspective, many studies detailing the beneficial effects of inoculation with beneficial rhizobacteria, especially *Azospirillum* sp., have been undertaken, and they describe morphological and physiological changes that occur in inoculated plants. However, in many cases, the processes, or the compounds responsible for inducing such responses, have not been unequivocally identified, and therefore the responses are usually considered within a "black box" model which goes beyond the resulting growth promotion due to the presence of only these organisms or active metabolites in the culture medium or plant tissues.

14.3 Mechanisms of Plant Growth Promotion in *Azospirillum* sp.

The first mechanism proposed for the bacterial plant growth promotion ability of *Azospirillum* sp. has mostly been associated with the nitrogen status of inoculated plants (Okon et al. 1983), mainly through their biological nitrogen fixation or nitrate reductase activities; however, these mechanisms have been of less agronomic

significance than initially expected (Kennedy et al. 1997). Other mechanisms of plant promotion have been studied and proposed as relevant for this microbial genus, such as phytohormone and/or siderophore production, phosphate solubilization (Puente et al. 2004), biocontrol of phytopathogens (Bashan and de Bashan 2010), and protection of plants against stress like soil salinity or toxic compounds (Creus et al. 1997). Tien et al. (1979) were the first authors to suggest that *Azospirillum* sp. could enhance plant growth by auxins and particularly indole-3-acetic acid (IAA) production, and subsequent studies showed the capacity of this genus to produce several other phytohormones and plant growth regulators. Although many mechanisms have been described to explain the plant growth promotion by *Azospirillum* sp., one single mechanism is mostly not responsible for the full effect. *Azospirillum* sp. modes of action could be better explained by the "additive hypothesis" which allows explaining the plant growth-promoting effect due to inoculation. This hypothesis was suggested more than 20 years ago (Bashan and Levanony 1990) and considers multiple mechanisms rather than one mechanism participating in the successful association of *Azospirillum* with plants.

One of the main mechanisms proposed to explain the "additive hypothesis" is related to the ability of *Azospirillum* sp. to produce or metabolize phytohormonal compounds (Cassan et al. 2014). Today, after more than eight decades of studies, it is already known that these rhizobacteria positively affect plant growth by the production of auxins (Prinsen et al. 1993), cytokinins (Tien et al. 1979), gibberellins (Bottini et al. 1989), ethylene (Strzelczyk et al. 1994), and other plant growth regulators, such as abscisic acid (Cohen et al. 2008), nitric oxide (Creus et al. 2005), and polyamines like spermidine, spermine, and diamine cadaverine (Cassán et al. 2009a). Several of the biologically active plant regulators produced by *Azospirillum* sp. are summarized in Table 14.1 (adapted from Cassan et al. 2014).

14.4 Available Genomes of *Azospirillum* sp.

At date, five whole azospirilla genomes have been sequenced: *A. brasilense* Sp245 (Wisniewski-Dyé et al. 2011), *A. brasilense* Az39 (Rivera et al. 2014), *A. lipoferum* 4B (Wisniewski-Dyé et al. 2011), *Azospirillum* sp. B510 (Kaneko et al. 2010), and *A. amazonense* Y2 (Sant'Anna et al. 2011). In addition, three other genomes were sequenced, but the information is still unpublished (*A. brasilense* Sp7, *A. brasilense* FP2, and *A. brasilense* CBG497).

A. brasilense Sp245 is one of the most studied strains worldwide and is considered as a type strain for this species. It was isolated from surface-sterilized wheat (*Triticum aestivum* L.) roots in Paraná state from the south region of Brazil (Baldani et al. 1986), and it has been used as a promising strain for wheat inoculation in Brazil during the 1980s. *A. lipoferum* 4B was isolated from the rhizosphere of rice (*Oryza sativa* L.) (Bally et al. 1983), and it was successfully used as inoculant to increase rice yield under field conditions (Charyulu et al. 1985). *Azospirillum* sp. B510 is an endophytic bacterium isolated from surface-sterilized stems of rice plants in Kashimadai, Japan (Elbeltagy et al. 2001). *A. amazonense* Y2 was isolated

Table 14.1 Overview of biologically active plant growth regulators produced in vitro by *Azospirillum* sp.

Class	Hierarchy	Molecules	References
Auxins	1st	IAA, PAA, IBA	Prinsen et al. (1993), Martínez-Morales et al. (2003), and Somers et al. (2005)
Gibberellins	4th	GA_3, GA_1	Bottini et al. (1989) and Piccoli and Bottini (1996)
Cytokinins	3rd	iP, iPr, Z, *t*-Zr	Horemans et al. (1986) and Esquivel-Cote et al. (2010)
Ethylene	5th	Et	Strzelczyk et al. (1994)
ABA	6th	ABA	Kolb and Martin (1985)
Nitric oxide	2nd	NO	Creus et al. (2005)
Polyamines	7th	Cad, Spm, Spd, Put	Cassán et al. (2009a) and Thuler et al. (2003)

The table shows the chemical class (i.e., biologically active molecules identified from *Azospirillum* sp. liquid cultures by unequivocal methodology like HPLC or GC-MS) and its hierarchy (importance or role in the interaction with the plant, as proposed by the authors or available evidence) Adapted from Cassán et al. (2014)
IAA indole-3-acetic acid, *PAA* phenylacetic acid, *IBA* indole-3-butyric acid, *NO* nitric oxide, *iP* isopentenyl adenine, *iPr* isopentenyl adenine riboside, *Z* zeatin, *t-Zr trans*-zeatin riboside, *GA3,1* gibberellins n, *Et* ethylene, *ABA* abscisic acid, *Cad* cadaverine, *Spm* spermine, *Spd* spermidine, *Put* putrescine

from forage grasses in the Amazon region (Brazil), but further studies revealed its broad ecological distribution in association with gramineous plants (Magalhães et al. 1983). Recently, this strain was reclassified as *Nitrospirillum amazonense* Y2 (Lin et al. 2014). *A. brasilense* strain CBG497 was isolated from the rhizosphere of corn plants grown on soils with pH of 8.0 from the northeast region of Mexico (Nelson and Knowles 1978). *A. brasilense* Sp7 (ATCC 29145) was isolated by Johanna Döbereiner from pangola grass (*Digitaria decumbens*) plants from Rio de Janeiro in Brazil and proposed by Tarrand et al. (1978) as the type strain for the species. Finally, *A. brasilense* FP2 is a spontaneous mutant of Sp7 (Nal^r and Sm^r) obtained by Pedrosa and Yates (1984).

14.4.1 Genomic Analysis of *Azospirillum brasilense* Az39

A. brasilense Az39 was isolated in 1982 from surface-sterilized wheat seedlings in Marcos Juarez (Córdoba, Argentina) and selected for inoculant formulation based on its ability to increase crop yield of corn and wheat under agronomic conditions (Diaz-Zorita and Fernandez Canigia 2009). The potential mechanisms responsible for the growth promotion by the strain Az39 have been partially unraveled (Perrig et al. 2007; Rodriguez Cáceres et al. 2008). The genome of *A. brasilense* Az39 is divided in six replicons (one chromosome, three chromids, and two plasmids) and consists of 6311 protein-coding sequences: 2763 on the chromosome, 1605 on AbAZ39_p1, 744 on AbAZ39_p2, 534 on AbAZ39_p3, 557 on AbAZ39_p4, and

108 on AbAZ39_p5. The putative genes involved in plant growth promotion have been related to the following mechanisms: nitrogen fixation; auxin, cytokinin, gibberellin, ethylene, abscisic acid, polyamine, and nitric oxide biosynthesis; biofilm formation; and type I, II, and VI secretion systems. Identical mechanisms and similar genes encoding for homologues proteins were previously identified in *A. brasilense* Sp245 genome (Wisniewski-Dyé et al. 2011) and later in Sp7, FP2, and CBG497 genomes (unpublished). Contrarily, in the case of *A. lipoferum* 4B, *Azospirillum* sp. B510 (Wisniewski-Dyé et al. 2011), and *N. amazonense* Y2 (Sant'Anna et al. 2011), their genome sequences reveal differences at the level of molecules involved in *quorum sensing* and IAA biosynthesis, among others. In the case of *quorum sensing*, the presence of genes encoding for LuxI homologue proteins was not detected in all sequenced strains of *A. brasilense*. For IAA biosynthesis, no evidence has been found for the existence of *ipdC* or aldehyde dehydrogenase genes in the genome sequence of *A. lipoferum* 4B. In the case of *Azospirillum* sp. B510, the genome sequence revealed two candidate genes proposed to be involved in the IAM pathway by Kaneko et al. (2010). However, we question their role in IAA biosynthesis due to the low similarity with known *iaaM* and *iaaH* genes. In addition, the genome sequence of *A. amazonense* did not reveal the presence of genes involved in the IPyA or IAM pathway (*ipdC*, *iaaM*, or *iaaH*) but revealed a gene encoding a protein with about 70 % similarity to nitrilases of *A. thaliana*.

A. brasilense Az39 is the only strain successfully used for agriculture during the last 50 years in diverse soils of Argentina and South America; thus, the full ungapless genome should be considered as a useful tool for molecular studies and an excellent basis for in-depth comparative genome analyses, to understand the specific mechanisms of *Azospirillum*-plant interactions.

14.5 The Sustainable Use of *Azospirillum* spp.

14.5.1 *Azospirillum*-Based Inoculants and Plant Growth Promotion

Due to the positive effects of *Azospirillum* sp. on growth of several plant species, this genus has attracted the attention of researchers to develop specific strains as inoculant for use in agriculture. Most inoculants claim plant growth-promoting effects under suboptimal conditions, such as limited plant-available nitrogen, resulting in a higher final yield compared to non-inoculated treatments. However, in many field experiments around the world, inoculation studies have not been reproducible, therefore questioning the capacity of these inoculants strains. In South America, a flourishing inoculant business is being developed, possibly due to the large surface dedicated to extensive agriculture and the reproducible results under field conditions. The rest of this section will focus in the experience gathered with *Azospirillum*-based inoculants in Argentina, South America, and other parts of the world. From 1981 to 1996, the Microbiology and Zoology Institute for Agriculture (IMYZA) from the National Institute for Agricultural Technologies (Instituto Nacional de

Tecnología Agropecuaria, INTA) based on Castelar (Buenos Aires, Argentina) developed an intensive program with the main objective to select and to identify *Azospirillum* sp. strains and to evaluate their ability to promote plant growth in different crop species. The experiments showed a more pronounced effect with *A. brasilense* as compared to *A. lipoferum* on most of the evaluated plant species and allowed bioprospecting of *A. brasilense* Az39 as the most promising strain for inoculant formulation, based on its ability to increase growth and grain yield of evaluated crops in the range of 13–33 %. Based on this information, the National Service of Agricultural Health (Servicio Nacional de Sanidad Agropecuaria, SENASA) proclaimed a nationwide recommendation of the native strain *A. brasilense* Az39 for inoculant production for corn, wheat, and other non-legume species. From a physiological point of view, the plant growth-promoting capacity of *A. brasilense* Az39 has been agronomically confirmed due to its effectiveness in increasing the productivity of inoculated crops in large number of assays under field conditions, during the last 30 years (Diaz-Zorita and Fernández-Canigia 2009). *A. brasilense* Az39 inoculated alone or in combination with *Bradyrhizobium japonicum* E109 has the capacity to promote seed germination and early growth in soybean [*Glycine max* (L.) Merrill], wheat, and corn. This strain is able to produce and release gibberellic acid (GA_3), zeatin (Z), and also IAA in culture medium (Perrig et al. 2007) at concentrations that produce morphological and physiological changes in treated seeds or seedling of several plant species (Cassán et al. 2009b). *A. brasilense* Az39 possesses the capacity to promote germination and early growth in a phytohormone-concentration-depending way. Bacterial releasing of GA_3, Z, and IAA into culture medium during fermentation showed differences during exponential or stationary growth phase (Cassán et al. 2010). In stationary growth phase, Az39 accumulates more phytohormones than in exponential growth phase, and this accumulation alters the "inoculant" capacity to promote the germination and early plant growth. This action has been defined as the "hormonal effect of inoculation" by Cassán et al. (2010) and could be extended to several plant species and phytohormone-producing microorganisms.

14.5.2 Field Crop Responses to the Inoculation with *Azospirillum sp.*

The crops usually show negative growth responses to different environmental stress conditions that frequently occur simultaneously, and it is recognized that *Azospirillum* sp., among other microbes, ameliorates these conditions and promotes better growth and productivity. The plant's response to abiotic stresses involves the activation of the hormones, redox, nitric oxide, kinase, and calcium signaling pathways (Gassman and Appel 2016), and most of these paths are also triggered by diverse natural soil bacteria. The interplay between the various signaling pathways in response to abiotic stresses is complex, especially in the field, where the plant is exposed to a combination of stressors. The contribution of soil microorganisms on plant productivity is often studied under isolated or controlled growth conditions,

providing information regarding the promising contributions of their use as inoculants and enriching the rhizosphere. But, this approach has limitations for its extrapolation to field production conditions.

Studies performed in large agricultural settings under regular environmental and crop management practices are scarce and limited to a few regions. Díaz-Zorita et al. (2015) reviewed 47 articles worldwide published during the last decade (347 cases of grain crop production responses to the application of diverse *Azospirillum* sp. inoculants in 12 countries) observing that most of the studies were performed in cereal crops (86.7 %), mainly dryland corn (*Zea mays* L.). Most experiments were located in Brazil and in different countries of Asia. Among all the reported crops, the mean grain response to the inoculation with *Azospirillum* sp. was 10 %, with greater yields in winter cereals (14.0 %) rather than in summer cereals (9.5 %) or legumes (6.6 %). The positive responses to the inoculation with *Azospirillum* spp. were also reported by Bashan et al. (1989), working with non-cereal crops, and reviewed by Okon and Labandera-Gonzalez (1994), on many other crops, including both row crops and vegetables grown in diverse environments and production conditions.

There is a common agreement among most of these large studies about the inconsistency of the responses to inoculation. In general, the production response to the inoculation with *Azospirillum* sp. is successful in 70 % of the experiments (Okon and Labandera-Gonzalez 1994; Díaz-Zorita and Fernández-Canigia 2009). The "inconsistent" results in field experiments are one of the main obstacles impeding a widespread use of plant growth-promoting microorganisms like *Azospirillum* sp. at commercial level (Dobbelaere et al. 2001). The interpretation and application of these results are variable and not always conclusive, in part, because of the complex interaction between the different modes of action of *Azospirillum* sp. on plants and also due to the multiplicity in abiotic stress conditions to be mitigated in presence of the microorganisms. Thus, we support the theory of multiple mechanisms acting in sequential or cumulative patterns (Bashan and de Bashan 2010). In addition, the methods of inoculation as well as the diverse crop management practices strongly related with the occurrence of abiotic limitations for crop growth (i.e., irrigation, fertilization, genotypes, combination with other beneficial microbes, etc.) could partially explain the inconsistent results in field experiments.

14.5.3 Carriers and Modes of Application Under Field Conditions

Azospirillum sp., among other microbes with recognized beneficial effects on plant growth, has been formulated in inoculants, also named biofertilizers. Generally, the microbes introduced into an inoculant formulation are representative of the bacterial population that is already present in the soil. The inoculation increases the numbers of cells in the rhizosphere, ensuring an early colonization and increasing the probability that the beneficial effects of the selected strains can be exerted on plants (Balandreau 2002).

The inoculation with *Azospirillum* sp. in field crops is generally done treating the seeds with liquid or solid carriers containing the microorganisms or applying them

directly in the sowing furrow during seed sowing. In rice and other intensively grown crops, transplanting seedlings soaked in a solution containing *Azospirillum* sp. has been also described in the bibliography to deliver the microbes to the rhizosphere (Govindarajan et al. 2008; Islam et al. 2012; Khalid et al. 2011). Foliar and soil-sprayed application formulations containing these bacteria were also described in several studies, but with controversial results and not clear recommendations about its management (i.e., growth stage at the moment of application, concentration of living cells delivered per plant, environmental and application conditions for its adequate use, etc.) (El Habbasha et al. 2013; Fukami et al. 2016).

Currently, the seed treatment at the moment of sowing is the preferred mode of inoculation with *Azospirillum* sp., mainly done without the application in slurry with other products (i.e., micronutrients, fungicides, insecticides, polymers, etc.). Most of the reports discussed in this chapter describe the effect of seed treatments mostly applied at the moment of sowing with liquid- or peat-based formulations containing different strains of *Azospirillum* sp. Several other formulations containing *Azospirillum* sp. have been developed for treating the seeds long term before sowing. One of these allows to include the microorganism in the formulation of alfalfa (*Medicago sativa*) coating treatments also in combination with other chemical compounds providing a long-term survival of azospirilla (Díaz-Zorita et al. 2012a). The risk of incompatibility between the microorganisms present in the inoculants and the diverse compounds (i.e., fungicides, insecticides, micronutrients, pigments, etc.) used in seed treatments limits the beneficial contribution of inoculation and has to be analyzed. Thus, alternative methods for seed inoculation as well as the integral management of the seed treatment process are often developed. The studies based on the application of the inoculants in the furrow of sowing are scarce and still under discussion, but there is no doubt that it is a recommended option for reducing the risk of low compatibility or viability of the microbe with other treatments applied to the seeds (Hungria et al. 2013). Fukami et al. (2016) compared diverse methods of inoculation (i.e., in-furrow, soil spray at sowing and foliar spray after seedling emergence) and identified effective alternative methods of inoculation (Fig. 14.1). Foliar spray improved the colonization of leaves, while the soil inoculation favored root and rhizosphere colonization. The authors reported positive effects of inoculation on the volume of corn roots and plant height when the inoculant was sprayed in the soil at the emergence of the seedlings. But, more wheat (*Triticum aestivum* L.) tillers per plant were described when the inoculations were sprayed on the leaves.

Morais et al. (2016), in field trials performed in the Cerrado region of Brazil, showed positive grain yield responses of corn crops when inoculated in the furrow of planting at a dose greater than 200 ml ha^{-1} (Fig. 14.2). These promising results support that, other than seed treatments, this might be applied as an alternative method for the inoculation of crops and soils with *Azospirillum* sp., but still requires several practical adjustments (i.e., dose of inoculant per plant, total volume of application for foliar or soil spraying, etc.).

The excessive manipulation of seeds is discouraged because desiccation, high temperatures, ultraviolet light, or toxic compounds may affect microbial endurance

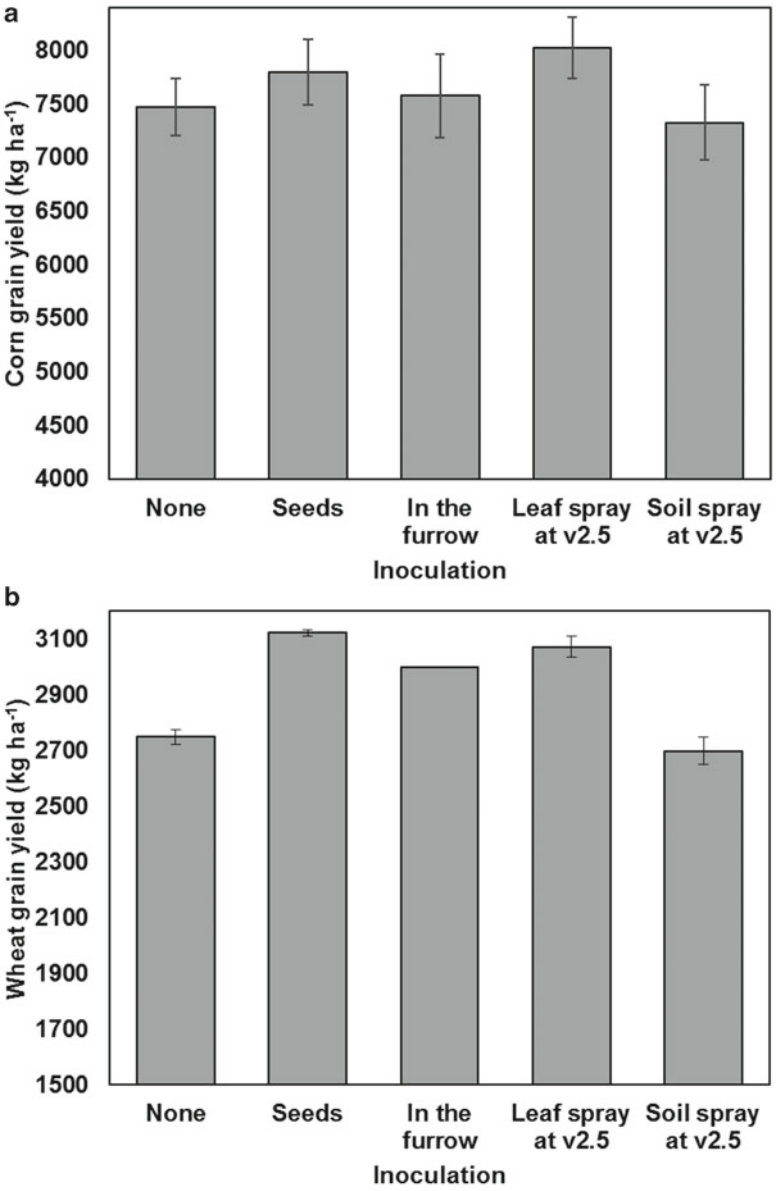

Fig. 14.1 Grain yield of crops inoculated with *A. brasilense* under different systems of inoculation. Mean of two corn sites and one wheat site each under two levels of N fertilization. The vertical bars represent the standard error of the means (Adapted from Fukami et al. 2016)

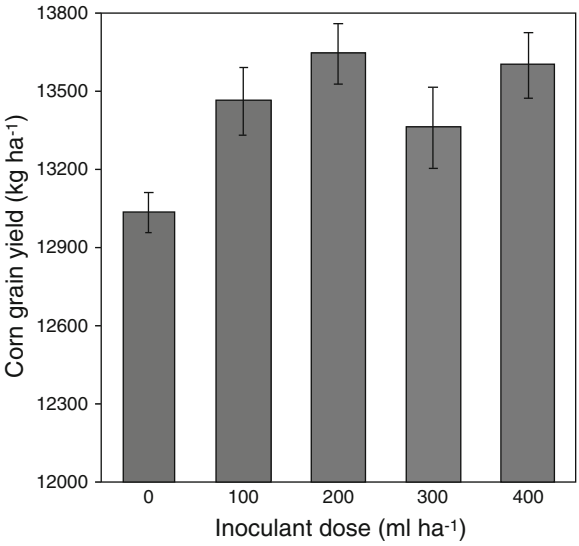

Fig. 14.2 Corn grain yield of crops inoculated with *A. brasilense* in the furrow of planting. Mean of three nitrogen fertilization levels. The vertical bars represent the standard error of the means (Adapted from Morais et al. 2016)

(Díaz-Zorita et al. 2015). Also, sowing as well as the furrow treatment application should be done in a moist seedbed, avoiding the seeds to be exposed to desiccation or direct sunlight.

14.5.4 Changes in Yield Components in Inoculated Crops

Azospirillum sp. inoculation promotes corn productivity under different environmental conditions, usually correlated with the increase of the root surface that leads to an increase in the soil volume exploration. In grasses, the inoculation with *Azospirillum* sp. alters the morphology of the roots increasing the number of lateral roots and root hairs (Steenhoudt and Vanderleyden 2000). More early plant height growth has been frequently reported in *Azospirillum* sp.-inoculated crops; however, the relative contribution in aboveground growth is smaller than in root growth and generally decreased with the development of the crops (Dobbelaere et al. 2001; Naiman et al. 2009). For example, corn crops treated with *A. brasilense* and grown under dryland conditions, among different regions in South America, showed a greater vegetative growth with a greater shoot dry matter accumulation (7.9 %) and also a greater number of grains at harvest (4.3 %). But, no significant differences on single grain weight were described between treatments (Díaz-Zorita et al. 2012b). Similar responses have been described in the case of wheat and barley (*Hordeum vulgare* L.) crops under diverse environmental conditions. The reported benefits in crop grain yield are mostly explained for the increase in the quantity of grain produced in response to the better vegetative growth in inoculated crops with minimal effects on yield components determined during the seed-filling period like single grain weight or its composition (Table 14.2, Fig. 14.3).

Table 14.2 Increase of wheat and barley grain yield parameters due to inoculation with *A. brasilense* Sp246

	Wheat (%)	Barley (%)
Spikes m^{-2}	7.2	6.6
Grains $spike^{-1}$	5.9	8.1
Grains m^{-2}	13.1	14.7
Grain yield m^{-2}	14.7	17.5
Protein content	4.1	5.1

Values represent the percentage of increase compared with non-inoculated plants. Mean of three N fertilization levels and two growing seasons
Adapted from Ozturk et al. (2003)

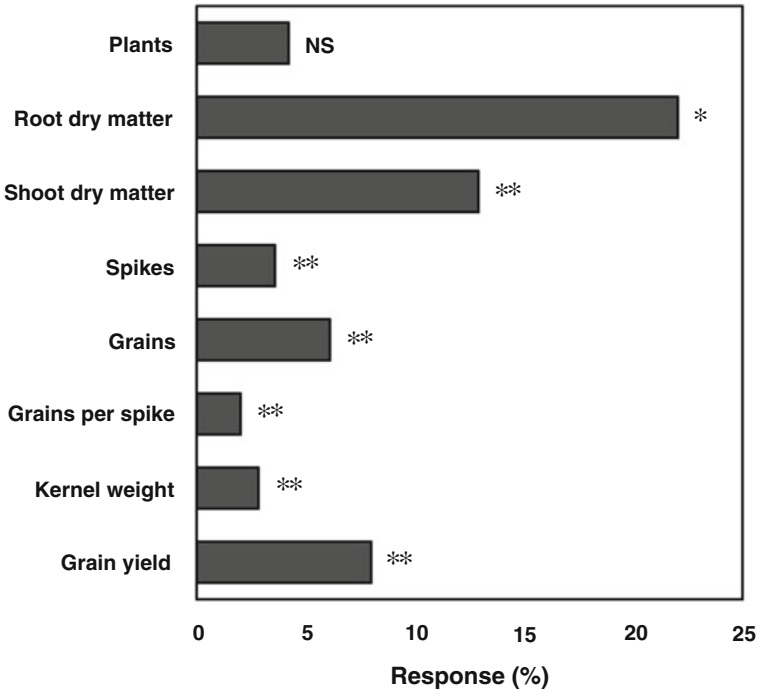

Fig. 14.3 Increase of grain yield parameters and production of *A. brasilense*-inoculated wheat crops in the Pampas, Argentina, compared with non-inoculated plants. Mean of 297 experimental sites. Statistics from LSD (T): *NS* = nonsignificant differences ($p > 0.10$), * = $p < 0.05$; ** = $p < 0.01$ (Adapted from Díaz-Zorita and Fernández-Canigia 2009)

14.5.5 Field Characteristics and Crop Responses to Inoculation

The inoculation of crops with *A. brasilense* has showed the enhancement in plant growth and productivity in diverse environments. But, as crop productivity and grain yield are strongly affected by the environment, the success of the practice lies

Table 14.3 Range of wheat and corn grain yield increase in response to the inoculation with *A. brasilense* in soils from the Buenos Aires province, Argentina, compared to non-inoculated experiments

Soil type	Wheat (%)	Corn (%)
Humic Hapludoll	2–34	6–77
Typic Argiudoll	0–30	0–7
Entic Hapludoll	2–32	3–13
Acuic Argiudoll	0–10	–
Aeric Argiudoll	–	2–94
Vertic Argiudoll	–	5

Adapted from García de Salomone (2012)

on both the inoculation practice and environmental conditions. Unfortunately, most field studies have been performed in single locations or during few production seasons, and the interpretation of the contribution of *Azospirillum* sp. to the crop production under a random temporal and spatial conditions is limited. Only, large agricultural studies performed during multiple growing seasons and experimental sites provide valuable information.

The responses to the inoculation with *Azospirillum* sp. vary among soil types and crops (Table 14.3). It has been proposed that crop stressful conditions like short-term droughts, mainly during early growth stages of the inoculated crops, among others, severely affect the success of the inoculation. It has been described that the wheat grain response to the inoculation with *A. brasilense* in the semiarid and subhumid Pampas region of Argentina decreases when the rainfalls increase relative to the environmental water demand (measured as evapotranspiration) as well as when the soil organic matter content increases (Rodriguez Cáceres et al. 2008).

Díaz-Zorita et al. (2012b), based on 298 experimental trials from Argentina, Bolivia, Brazil, Paraguay, and Uruguay, also concluded that corn responses to the inoculation were greater under stressful conditions during the early growth of the crops or in the absence of nitrogen fertilization practices. When rainfalls are scarce during the early stages of growth of the crops, the positive effect of the inoculation practice on crop yields is evident (Fig. 14.4). Under well-watered conditions, the changes in root development, attributed to *Azospirillum* sp. inoculation, are less evident than under moderate water limitations. During early abiotic stress conditions, *Azospirillum* sp. increases root length, surface, and volume compared with untreated crops (Dobbelaere et al. 1999; Kapulnik et al. 1985). But, under strong stressful growing conditions (i.e., severe droughts, major nutrient limitations, etc.), the responses to the inoculation treatment are barely observed (Lana et al. 2012; Mehnaz et al. 2010; Naiman et al. 2009; Naseri et al. 2013).

The contribution of the environmental factors that affects the growth of the crops and the inoculation practice response normally occurs at random. Under the complexity of interacting random factors, a better interpretation of the results requires the analysis based on the frequency of their occurrence as well as the discrimination among hierarchical productivity factors. For a conclusive conclusion regarding the crop responses to the inoculation with *Azospirillum* sp., a large number of observations are needed to describe significant differences between treatments, being the analysis of distribution response values a more realistic approach to measure the

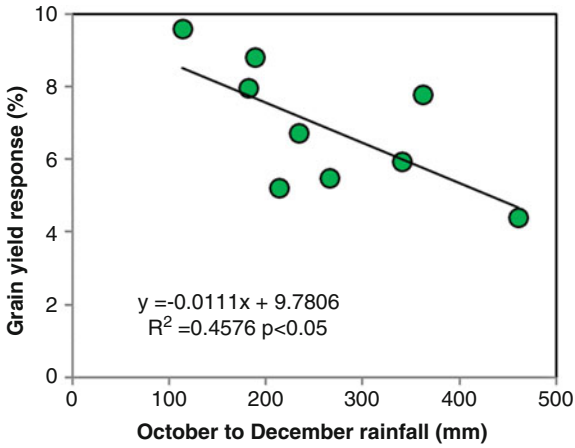

Fig. 14.4 Mean relative seasonal grain yield responses to the application of *A. brasilense* on corn crops of the Pampas region (Argentina) and rainfalls during vegetative (October to December) growing stages (Adapted from Díaz-Zorita 2012)

Table 14.4 Mean values of percentile ranges in the empirical distribution of corn grain yields from control and *A. brasilense*-inoculated crops in a database from 316 field trials performed in the Pampas region (Argentina) under regular production practices

	Range of the empirical distribution				
	0–5	5–25	25–75	75–95	95–100
Treatment	Grain yield (kg ha^{-1})				
Control	3,691	6,118	8,755	11,541	13,838
Inoculated	3,912	6,566	9,216	11,948	14,448
P	ns	<0.001	<0.001	<0.001	ns
n	16	63	157	64	16
SE	209	92	78	82	316

Adapted from Díaz-Zorita et al. (2015)
P statistical significance, *n* number of sites considered in each range, *SE* standard errors of the means, and *ns* nonsignificant differences between means

crop performance (Díaz-Zorita et al. 2015). For example, based on 316 field experiments, corn yields yielded from 2020 kg ha^{-1} to 18654 kg ha^{-1}. Although percentile values differed along the whole distribution, only mean yields of the treatments distribution in the range from 5 to 95 % were significant (Table 14.4). The probability to find differences in both extremes of the data distribution was low; however, in the central 90 % of the distribution, in crops yielding between 6000 and 12000 kg ha^{-1}, crops inoculated with *A. brasilense* had a consistent higher probability of greater yields than control crops (Table 14.4).

14.5.6 Inoculation Responses and Crop Management Practices

Crop management practices influence the growth of crops, including the performance of *Azospirillum* sp.-inoculated crops in the presence of abiotic stresses (i.e., nutrient deficiencies, water stress, etc.). The specificity between *Azospirillum* strains

and plant species and genotypes is not clearly evident; thus, the selection of strains under specific environmental and production (i.e., crops, management practices, etc.) conditions is a key factor for achieving a successful plant response to inoculation (Hungria 2011). For example, the response of rice to *Azospirillum* sp. inoculation is variable and depends on the interaction between plant cultivars and *Azospirillum* species and strains (Okumura et al. 2013). For example, a plant genotype *Azospirillum* sp. has been described in the north part of the corn production region of Argentina (Garcia de Salomone and Dobereiner 1996). But, under regular production conditions, where multiple interactive factors are involved in the response to the inoculation treatment, the type of germplasm in wheat crops seemed to have a minor effect on yield (Fig. 14.5). From the same integral study, among 297

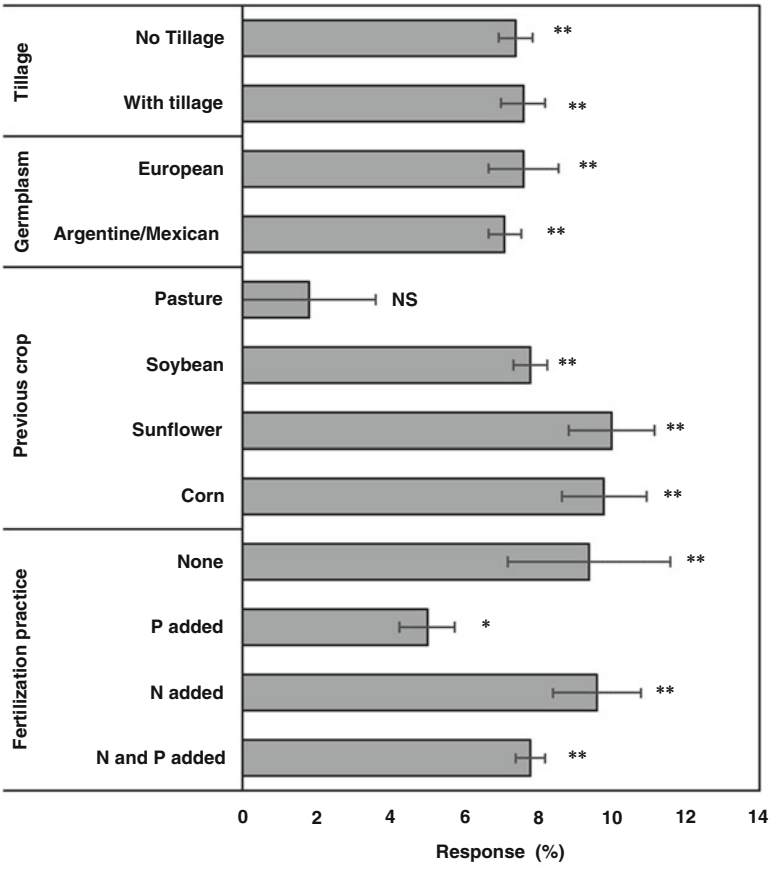

Fig. 14.5 Wheat grain yield response to crop and soil management practices, subjected to seed inoculation with a liquid formulation containing *A. brasilense* across 297 experimental sites in the Pampas region of Argentina. Horizontal bars represent the standard error of the means. Statistics from LSD (T): *NS* = nonsignificant differences ($p > 0.10$), * = $p < 0.05$; ** = $p < 0.01$ (Adapted from Díaz-Zorita and Fernández-Canigia 2009)

experimental sites located in the Pampas region of Argentina, the wheat response to the inoculation with *Azospirillum* sp. showed differences depending on the previous cultivated crop and the fertilization practice.

Best yields of wheat crops were obtained by crop rotation with sunflower (*Helianthus annuus* L.) or corn, suggesting that the soil condition is a limiting factor for the growth of wheat. The sunflower production area in Argentina mostly lies within the semiarid Pampas region, with frequent water stress conditions that limit the normal wheat production. It is well known that the residues from corn crops have a negative effect in wheat establishment, reducing the stands of plants and its productivity. In both situations, sunflower production in Pampas and wheat production in rotation with corn, the inoculation with *Azospirillum* sp. provides beneficial conditions for root growth, enlarging the soil volume exploration and providing more resources for the formation of tillers. In the other hand, the response of wheat to the inoculation was not significant in areas where pastures were frequently grown, but a better soil fertility related with more soil organic matter content was found (Díaz-Zorita and Fernández-Canigia 2009).

Veresoglou and Menexes (2010), among diverse environmental conditions, also reported positive responses in the growth of wheat plants inoculated with *Azospirillum* sp. They concluded that the benefit varied depending on the nitrogen fertilization dose, the genotype of wheat, and also the applied strain of *Azospirillum* sp. The fertilization effect on the response to the inoculation is controversial, and it varies depending on the applied nutrient, the dose, as well as the crop response to this practice itself. It has been described that nitrogen fertilization is beneficial to wheat crop responses to *Azospirillum* sp. inoculation because of the promotion in nitrogen absorption and use (Saubidet et al. 2002). But, the grain yield parameters of wheat and barley crops inoculated with *A. brasilense* diminished when the level of nitrogen fertilization increased. Furthermore, under relatively high nitrogen availability, the plant response to *Azospirillum* sp. inoculation was not observed (Ozturk et al. 2003). In sites where nitrogen availability is a limiting factor for the normal productivity of cereals, the plant response to the inoculation is frequently observed. In a study performed in two representative sites in the semiarid-subhumid Pampas cultivated with rye (*Secale cereale* L.) as a cover crop, it was observed that the mean response in dry matter production of *A. brasilense*-inoculated plants was 534 kg ha^{-1}, equivalent to 13 % of increase (Table 14.5). This response was mainly observed when nitrogen fertilization was performed and suggests the complementary need of both practices (inoculation and fertilization) for achieving fast and abundant above ground biomass.

The response of the crops to the inoculation under severe availability of nutrients is also limited or negligible. For example, from the evaluation of ten wheat production sites located in the Pampas region with limitations in the soil extractable phosphorus levels, it was observed that the grain yield response to the seed inoculation with *A. brasilense* was only significant in treatments with phosphorus fertilization (Fig. 14.6). In these sites, the mean response to the fertilization was almost 55 % greater in the inoculated crops (819 kg ha^{-1}) than in the absence of this practice (529 kg ha^{-1}). The mean response to the inoculation in the fertilized crops was

Table 14.5 Shoot dry matter production of rye cover crops inoculated with *A. brasilense*. Parameters were measured after 150 days of growth in two sites (A and B) from the semiarid-subhumid Pampas

Seed inoculation	N fertilization (kg ha^{-1})	Sites		
		A	B	Mean
		Dry matter (kg ha^{-1})		
None	0	4,270	2,893	**3,961**
	40	5,087	3,593	
Azospirillum brasilense	0	5,227	3,348	**4,495**
	40	5,775	3,628	

Adapted from Cristian Alvarez, pers.com

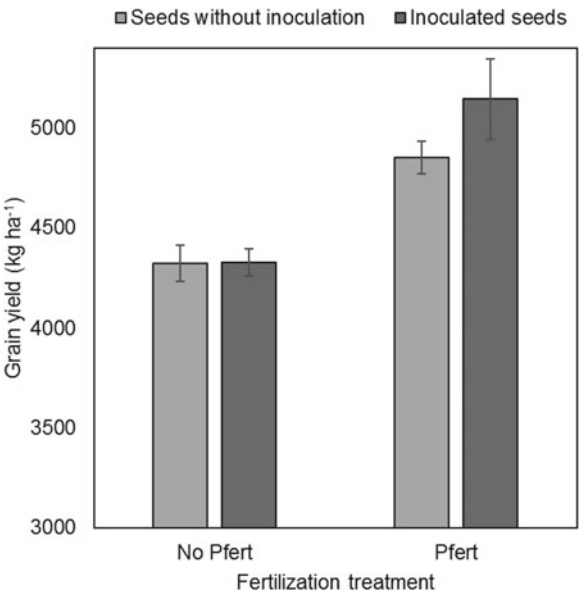

Fig. 14.6 Wheat grain yield response seed inoculation with *A. brasilense* and phosphorus fertilization (Pfert) in the Pampas region of Argentina. Mean of ten sites performed during the 2015/2016 growing season. Vertical bars represent the standard error of the means (Adapted from Díaz-Zorita *unpublished data*)

294 kg ha^{-1}, equivalent to the increment in 6 % the grain yields compared with the control without the use of the biological seed treatment.

The combined inoculation of legumes with rhizobia and azospirilla, among other beneficial soil microorganisms, because of their complementary biological activities in plants, could improve the performance of the plants. Although the contribution of the co-inoculation with rhizobia and azospirilla on the productivity of diverse legume crops and pastures is promising, the available information under large production conditions is limited. The results from 21 field trials with alfalfa pastures performed in the Pampas region of Argentina showed that the application of biological seed treatments combining *Sinorhizobium meliloti* and *A. brasilense* enhanced both the establishment of plants and the forage production (Díaz-Zorita et al. 2012a). The inoculation with *S. meliloti* showed 14.7 % more

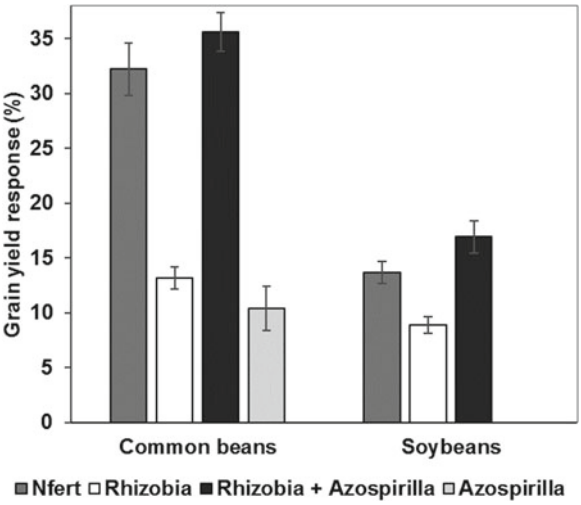

Fig. 14.7 Grain yield response of common beans and soybeans crops to rhizobia and azospirilla inoculation. Mean of four sites from Brazil. Nfert = nitrogen fertilization, 80 kg of N ha^{-1} in common beans and 200 kg ha^{-1} in soybeans. The vertical bars represent the standard error of the means (Adapted from Hungria et al. 2013)

dry matter production than the control without biological seed treatment. When *A. brasilense* was combined in the biological treatment, the dry matter response was doubled (28.5 %). Between both biological seed treatments, the dry matter production per plant after 150 days of growth was greater when *A. brasilense* was combined with *Sinorhizobium meliloti* (1.69 kg $plant^{-1}$) than when only *S. meliloti* was applied (1.65 kg $plant^{-1}$). Hungria et al. (2013) also showed more grain yields of soybean and common bean (*Phaseolus vulgaris*) when combining rhizobia seed inoculation with in-furrow application of *A. brasilense* in four sites from Brazil (Fig. 14.7).

14.6 Registration and Production of Biological Products with *Azospirillum* sp. in South America

In the south cone of South America, there are 104 biological commercial products containing *Azospirillum* sp., produced for more than 50 companies and mainly formulated in liquid carriers (Tables 14.6, 14.7, 14.8 and 14.9). Most of them are produced in Argentina (85 products) and only 16 in Brazil, 1 in Paraguay, and 2 in Uruguay. All the available products for commercialization in the Argentine market are produced in Argentina (Table 14.6). But in Brazil (Table 14.7) and Uruguay (Table 14.8), the products are locally produced or imported from Argentina, while the Paraguayan market (Table 14.9) produces and imports, from both Argentina and Brazil, these inoculants.

Azospirillum brasilense strain Az39 is the active principle in 65 % (68 products) of these inoculants. In addition, the Az39 strain is also used in combination with other *A. brasilense* strains (or with *Pseudomonas fluorescens* strains (5 products)). Even a significant number of formulations are produced by the combination of *A. brasilense* Abv5 and Abv6 strains (11 products) or *A. brasilense* Az78 and Az70

Table 14.6 Biological products formulated with *Azospirillum* sp. registered in Argentina at 2015

Origin	Brand	Active principle	Company	Carrier	Crop
Argentina	Axion Plus	*A. brasilense* Az39	Agro Franquicias SA	Liquid	W
Argentina	Full Bacter	*A. brasilense* Az39	Agro Invest SRL	Liquid	W, C
Argentina	Alter Promaz Tls	*A. brasilense* Az39	Alterbio SA	Liquid	W, B, O
Argentina	Facyt Az	*A. brasilense* Az39	Ayui SRL	Liquid	W, C, Sun, B, O
Argentina	Nitrafix	*A. brasilense* 11005	BASF Argentina SA	Liquid	W, C, S, Soy
Argentina	Nitro Fix Az	*A. brasilense* Az39	Bilab SA	Liquid	W, C, Sun, Cot
Argentina	Axion Plus Az	*A. brasilense* Az39	Bilab SA	Liquid	C
Argentina	Axion Plus Az	*A. brasilense* Az78 + Az79	Bilab SA	Liquid	W, C
Argentina	Hober Soy	*A. brasilense* Az78 + Az79	Bilab SA	Liquid	Soy
Argentina	Azomix	*A. brasilense* Az39	Bionet SRL	Liquid	W
Argentina	Azomax	*A. brasilense* Az39	Campomax SH	Liquid	W
Argentina	Beterseed Azospirillum	*A. brasilense* Az39	Cao Rocio	Liquid	W, C
Argentina	Grow Azp	*A. brasilense* Az39	Ceres Demeter SRL	Liquid	W, C
Argentina	Azosnitro	*A. brasilense* Az39	Cergen SRL	Liquid	W, C
Argentina	Gramibac	*A. brasilense* Az39	Chemical-Bio SA	Liquid	W
Argentina	Radixius	*A. brasilense* Az39	Chemical-Bio SA	Liquid	C
Argentina	Crinigan Maíz	*A. brasilense* Az39	Crinigan SA	Peat	C
Argentina	Ray Green	*A. brasilense* Az39	Ecofertil SRL	Peat	Soy
Argentina	Ray Green	*A. brasilense* 1003	Ecofertil SRL	Liquid	W, C, Sun
Argentina	G3 Azubac	*A. brasilense* Az39	Emfag SA	Liquid	W
Argentina	Sower	*A. brasilense* Az38	Farmchem SA	Liquid	W
Argentina	Azos B	*A. brasilense* Az39	Fitogenia SRL	Liquid	W, C
Argentina	Forte	*A. brasilense* Az39	Fitoquimica SA	Liquid	W, C
Argentina	Azp 2000	*A. brasilense* Az39	FPC SA	Liquid	W, C, B

(continued)

Table 14.6 (continued)

Origin	Brand	Active principle	Company	Carrier	Crop
Argentina	Trigalazo	*A. brasilense* Az39	Fragaria SRL	Liquid	W
Argentina	Graminazo Plus	*A. brasilense* Az39	Fragaria SRL	Liquid	W, C, S
Argentina	Cazo	*A. brasilense* Az39	Fragaria SRL	Liquid	C
Argentina	FosW N	*A. brasilense* Az39	Green Quality SA	Liquid	W, C, S
Argentina	Graminante Maíz	*A. brasilense* AzM3	Lab. Alquimia SA	Liquid	C
Argentina	Graminante Trigo	*A. brasilense* AzT5	Lab. Alquimia SA	Liquid	W
Argentina	N Azospirrillum	*A. brasilense* V5 + V6	Laboratorio Biotech SA	Liquid	W, Sun, Sug, S, Cot
Argentina	BioNitrosem Azo	*A. brasilense* Az39	Laboratorio Biotech SA	Liquid	W, C, Sun
Argentina	Azollum H	*A. brasilense* Tuc 27/85	Laboratorio San Pablo SRL	Liquid	W
Argentina	Azollum	*A. brasilense* Az39	Laboratorio San Pablo SRL	Liquid	W
Argentina	Azollum Maíz	*A. brasilense* Tuc 27/85	Laboratorio San Pablo SRL	Liquid	W, C
Argentina	Tabazoll Duo Plus	*A. brasilense* Az39	Laboratorio San Pablo SRL	Liquid	T
Argentina	Macromix	*A. brasilense* Tuc10/1	Laboratorio San Pablo SRL	Liquid	Sun
Argentina	Ene-2	*A. brasilense* Az39	Laboratorios Arbo SRL	Liquid	W, C, G
Argentina	Rhizoflo Premium	*A. brasilense* Az39	Laboratorios CKC SA	Liquid	W, C, Sun, S, Soy
Argentina	Rhizoflo Liquid Trigo	*A. brasilense* Az39	Laboratorios CKC SA	Liquid	W
Argentina	Rhizoflo Liquid Maiz	*A. brasilense* Az39	Laboratorios CKC SA	Liquid	C
Argentina	Azo LQ	*A. brasilense* Az39	Lanther Quimica SA	Liquid	W, C
Argentina	Marketing Agricola	*A. brasilense* Az39	Marketing Agricola SRL	Liquid	W, C, Sun
Argentina	Nitragin Maiz	*A. brasilense* Az39	Monsanto Argentina SAIC	Liquid	C
Argentina	Nitragin Wave	*A. brasilense* Az39	Monsanto Argentina SAIC	Liquid	W, Wg
Argentina	Azotrap	*A. brasilense* Az39	Nitrap SRL	Liquid	W
Argentina	Azotrap Plus	*A. brasilense* Az39	Nitrap SRL	Liquid	C, Sun, L

Argentina	Graminasoil	*A. brasilense* Az39	Nitrasoil Argentina SA	Liquid	C
Argentina	Graminosoil L	*A. brasilense* Az39	Nitrasoil Argentina SA	Liquid	W
Argentina	Bio-Enhance	*A. brasilense* Az39	Nitrasoil Argentina SA	Liquid	Soy
Argentina	Nivel Azo	*A. brasilense* Az39	Nivelagro SA	Liquid	W, C
Argentina	Promozion	*A. brasilense* Az39	Nova SA	Liquid	W, C
Argentina	Nitragin Semillero	*A. brasilense* Az39	Novozymes BioAg SA	Solid	W, C, Sun, Soy
Argentina	Palaversich Biopower	*A. brasilense* Az39	Palaversich y Cia. SAC	Liquid	W, C, S
Argentina	Buscador N	–	Raparo, Angel Ruben	Peat	W, C
Argentina	Azogrowth	*A. brasilense* Az39	Red Surcos SA	Liquid	Cot, Sun,C, Soy, S, W
Argentina	Rizospirilum	*A. brasilense* Az39	Rizobacter SA	Liquid	W, C
Argentina	Zaden Gramineas	*A. brasilense* Az39	Semillera Guasch SRL	Liquid	W
Argentina	Nitrofull G	*A. brasilense* Az39	Serv-Quim SA	Liquid	W
Argentina	Noctin Azo	*A. brasilense* Az39	Sintesis Quimica SAIC	Liquid	W, C, Sun
Argentina	Vigor Part B	*A. brasilense* Az39	Sintesis Quimica SAIC	Liquid	–
Argentina	Masterfix L	*A. brasilense* Az39	Stoller Biociencias SRL	Liquid	W, C
Argentina	Azzea Uno	*A. brasilense* Tuc27/85	Tres E	Liquid	C

Source: SENASA and INTA (unpublished data)
References: *C* corn, *W* wheat, *S* sorghum, *Soy* soybean, *Sun* sunflower, *O* oats, *B* barley, *Wg* winter grasses, *G* grasses in pastures, *Cot* cotton, *L* lettuce, *Sug* sugarcane, *T* tobacco

Table 14.7 Biological products formulated with *Azospirillum* sp. registered in Brazil at 2015

Origin	Brand	Active principle	Company	Carrier	Crop
Argentina	Rizospirillum	–	Rizobacter	Liquid	C, W
Brazil	Grap Nod A	*A. brasilense* AbV5 + Abv6	Agrocete	Liquid	C, W
Brazil	Gelfix Gramíneas	*A. brasilense* BR11005 (Sp45)	BASF	Liquid	C
Brazil	Biomax Milho	*A. brasilense* AbV5	Bio Soy	Liquid	C
Brazil	Azzofix	*A. brasilense* AbV5 + Abv6	Microquímica	Liquid	C
Brazil	Nitro1000 Gramíneas	*A. brasilense* AbV5 + Abv6	Nitro1000	Liquid	C, W
Brazil	Nodugran L	*A. brasilense* AbV5 + Abv6	NoduSoy	Liquid	C
Brazil	Azomax	*A. brasilense* AbV5 + Abv6	Novozymes BioAg	Liquid	C
Brazil	Masterfix Gramíneas	*A. brasilense* AbV5 + Abv6	Stoller	Liquid	C, W, R
Brazil	Masterfix Gramíneas	*A. brasilense* AbV5 + Abv6	Stoller	Peat	C, W, R
Brazil	Azototal	*A. brasilense* AbV5 + Abv6	Total Biotecnologia	Liquid	C, W
Brazil	Azototal	*A. brasilense* AbV5 + Abv6	Total Biotecnologia	Peat	C, W

Source: ANPII and EMBRAPA (unpublished data)
References: *C* corn, *W* wheat, *R* rice

Table 14.8 Biological products formulated with *Azospirillum* sp. registered in Uruguay at 2015

Origin	Brand	Active principle	Company	Carrier	Crop
Argentina	Nitragin Maíz	*A. brasilense* Az39	Novozymes BioAg SA	Liquid	C
Uruguay	Bioprom	*A. brasilense* Az39	Calister SA	Liquid	C, W
Uruguay	Graminosoil	*A. brasilense* Az39 + CFN535	Lage y Cia. SA	Liquid	C, S

Source: MAGyP (unpublished data)
References: *C* corn, *W* wheat, *S*, sorghum

strains (2 products). Other azospirilla strains used in single inoculants are Abv5, AzM3, AzT5, 1003, Tuc 27/85, Tuc 10/1, and 11005. There are no other species of *Azospirillum* used for the formulation of azospirilla-containing inoculants in the region.

Liquid carriers are the most commonly used for the formulation of these biological products (82 %), but a few products are formulated on solid carriers like peat or bentonite (8 %). The more frequent shelf life of the registered products is 6 months

Table 14.9 Biological products formulated with *Azospirillum* sp. registered in Paraguay at 2015. SENAVE (Paraguay), ANPII (Brazil), and SENASA (Argentina) (unpublished data)

Origin	Brand	Active principle	Company	Carrier	Crop
Argentina	Azp 2000	*A. brasilense* Az39	FPC SA	Liquid	C, W
Argentina	Graminazo	*A. brasilense* Az39	Fragaria SA	Liquid	C
Argentina	Graminante Maíz	*A. brasilense* AzM3	Lab. Alquimia SA	Solid	C
Argentina	Graminante Trigo	*A. brasilense* AzT5	Lab. Alquimia SA	Solid	W
Argentina	Rhizoflo Liquid Maíz	*A. brasilense* Az39	Laboratorios CKC	Liquid	C
Argentina	RhizoFlo Premiun	*A. brasilense* Az39	Laboratorios CKC	Liquid	C
Argentina	Nitragin Maíz	*A. brasilense* Az39	Monsanto	Liquid	C
Argentina	Nitragin Semillero	*A. brasilense* Az39	Monsanto	Liquid	C
Brazil	Gelfix Gramineas	*A. brasilense* BR11005 (Sp45)	BASF	Liquid	C
Brazil	Biomax Premiun	*A. brasilense* AbV5	Bio Soy	Liquid	C
Brazil	Grammy Crop	*A. brasilense* BR11005 (Sp45)	Forquímica	Liquid	C, W, R
Brazil	Masterfix Gramíneas	*A. brasilense* AbV5 + Abv6	Stoller	Liquid	C
Brazil	Azototal	–	Total biotecnologia	Liquid	C, W
Paraguay	Nutrichem	*A. brasilense* Az40	Chemtec	Liquid	C

References: *C* corn, *W* wheat, *R* rice

(data not shown), but there is at least one product showing longer survival of the azospirilla life and has been recommended for its use in industrial seed treatments (i.e., pelletized alfalfa).

Although the inoculation with these biological products has been recommended for 13 crops, the registration of the products has been performed mainly for corn (73 products) and wheat (64 products) crops. The rest of the crops recommended for the application of commercial products containing *A. brasilense* are sorghum (*Sorghum bicolor*) (29), sunflower (13), soybean (7), grasses and winter cereals for grazing (4), rice (3), barley (3), cotton (*Gossypium hirsutum*) (3), oats (*Avena sativa*) (2), sugarcane (*Saccharum officinarum*) (1), tobacco (*Nicotiana tabacum*) (1), and lettuce (*Lactuca sativa*) (1).

In Argentina, during 2014, most of the products containing *A. brasilense* were used in wheat, winter grasses, soybean, and corn covering almost 500.000 has. In Brazil, most of the commercialized products were allocated in the corn grain production market. Based on 2014 data, approximately 2.9 million doses of azospirilla inoculants were commercialized covering almost 2.8 million has. Almost 2000 has cultivated with corn have been treated with azospirilla inoculants during the 2014 growing season in Uruguay.

14.7 Concluding Remarks

Azospirillum sp. is probably the most studied genus of associative plant growth-promoting rhizobacteria due to its capacity to colonize more than hundreds of plant species. Multiple and complex mechanisms have been described in the microbe-plant interaction (i.e., nitrogen fixation and phytohormone biosynthesis), but none of them individually has been identified to support the changes in plant growth. Currently, it has been agreed that the combination or the sum of these mechanisms operate together providing variable benefits to the growth of the plants, according to the diverse stressful environmental conditions that the azospirilla plant has to face. During the last two decades, the inoculation of selected *Azospirillum* sp. strains on diverse crops under field conditions has been intensively studied. It has been widely shown that these microorganisms consistently benefit the plant, improving growth, development, and productivity under controlled and field conditions. The use of azospirilla inoculants for crop production is a promising and increasing practice mostly in crop management conditions (i.e., dryland, limited fertilization, etc.), where the occurrence of abiotic growth stresses is frequent. The benefit of azospirilla uses complements the application of other good practices, like nitrogen or other nutrient fertilization, or inoculation with other beneficial microbes, among others. In general, the yield of grain yield production by the applications of azospirilla inoculants has been showing a better result in summer cereals rather than in other crops like legumes or winter cereals. The benefits of azospirilla on plant (and crop) growth have been mainly explained by their effect during the early growth stages and, in particular, by the enhancing root growth. The variability of results in grain production in the field is explained for changes in the late-season growth conditions.

Part of the current challenges in the promising use of azospirilla inoculants is the development of successful formulations for seed treatments at diverse application strategies, storage handling, and environmental conditions. For example, it has been recommended the combination of azospirilla inoculation with synthetic seed treatments, showing thus a long-term survival of the microbes that allows its use in industrial processes. However, their formulations also show several manufacture limitations due to factors like the need of applying a large volume of liquids or the handling and attachment of dry powders. On the other hand, the development of alternative application systems like the delivery of azospirilla in the seeding furrow simultaneously with the planting operation is seen as a solution to surpass the limitations for on-seed inoculation.

Azospirillum sp. supports the natural growth of diverse plant species mitigating multiple abiotic stresses and providing a direct contribution, not only increasing crop yields but also enhancing the efficiency of diverse production resources (i.e., fertilizers, land, etc.), with extended benefits to the environment. However, a strong and coordinated communication program informing the beneficial effects of the inoculation with *Azospirillum* sp. strains in current extensive (i.e., cereals and legumes) and also intensive (i.e., nursery and transplanting for forestry, vegetables, etc.) crop practices has to be encouraged. This communication network should

include not only direct users of these products (i.e., growers, consultants and extension agents, public and private researchers and developers, inoculant producers, etc.) but also other actors from rural and urban environments and local regulatory agencies. The area of *Azospirillum* sp. research needs also to be intensified, focusing the studies in the mechanisms of action and the convenience of their use with other beneficial microorganisms as well as the genomic identification of markers that will facilitate strain selection and improvement.

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Fluorescent *Pseudomonas:* A Natural Resource from Soil to Enhance Crop Growth and Health

15

María Lis Yanes and Natalia Bajsa

Abstract

Fluorescent *Pseudomonas* had always played an important role in the development of biopesticides and biofertilizers since the concern for more sustainable agricultural production systems exists. They produce a distinctive soluble yellowish-green siderophore called pyoverdin, show an excellent root-colonizing ability, and exert a wide battery of mechanisms to promote plant growth, either directly by facilitating nutrient acquisition or synthesizing phytohormones or indirectly by biological control of plant pathogens. Fluorescent *Pseudomonas* have been applied successfully to control plant pathogens on different pathosystems due to their ability of producing secondary metabolites such as antibiotics, induction of systemic resistance in the host plant, and/or competition for niches and nutrients. They are very suitable for developing market inoculants, as they are abundant in soil and roots, can use a variety of carbon sources, have a high growth rate, can be introduced into the rhizosphere by seed bacterization, and are amenable to genetic manipulation. However, compared to the volume of research that has been performed with these bacteria, few strains have been successfully developed into commercial products for plant biocontrol and biostimulation. Some drawbacks for their field application need to be overcome, as variations observed in field performance, and the constraints found during registration of market products, due to some opportunistic human pathogenic *Pseudomonas* that have been reported. The development of suitable formulations for bacterial delivery, genetic modification of promising strains, and coinoculation with other plant growth-promoting microorganisms are discussed as potential ways of strengthening the use of *Pseudomonas* spp. in agricultural systems.

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15.1 Introduction

Fluorescent *Pseudomonas* had always played an important role in the development of biopesticides and biofertilizers since the concern for more sustainable agricultural production systems exists. A huge background regarding the application of these microorganisms for plant fertility purposes can be traced to the first patent obtained in the 1910s (Coates 1910). Since that, fluorescent *Pseudomonas* have gained more and more relevance, and nowadays they can be compared to rhizobia in terms of scientific knowledge and applied research.

Fluorescent *Pseudomonas* are a heterogeneous collection of non-enteric strains, Gram-negative chemoheterotrophs, and generally aerobic, non-fermentative, and mobile bacteria which have a polar flagellum (Dwivedi and Johri 2003). The genus is comprised of the species *P. aeruginosa*, *P. aureofaciens*, *P. chlororaphis*, *P. fluorescens*, *P. putida*, and more recently *P. protegens* and also plant-pathogenic species such as *P. cichorii* and *P. syringae*. Fluorescent *Pseudomonas* differ from other *Pseudomonas* spp. for their distinctive soluble yellowish-green pigment, which corresponds to the siderophore called pyoverdin, which emits light when exposed to ultraviolet radiation (Meyer and Abdallah 1978). What makes pseudomonads so interesting? It is because of the fact that they have excellent root-colonizing ability and a wide battery of mechanisms to promote plant growth (i) directly by facilitating nutrient acquisition or synthesizing phytohormones and (ii) indirectly by biological control of plant pathogens. Besides, they are very suitable for being applied as inoculants; for instance, they can use a variety of carbon sources, are amenable to genetic manipulation, are abundant in soil and roots, have a high growth rate, and can be introduced into the rhizosphere by seed bacterization (Whipps 2001).

Fluorescent *Pseudomonas* have been applied successfully to control plant pathogens on different pathosystems, and as the interest of reducing chemical pesticide inputs on different crops increases, the number of host plant increases too. Biocontrol mechanisms are diverse and can be simultaneously exerted by a single strain or a combination of strains. The main mechanisms involve the synthesis of secondary metabolites like antibiotics, the induction of systemic resistance in the host plant, and the competition for niches and nutrients.

Direct promotion of plant growth mediated by fluorescent *Pseudomonas* is another approach to reduce the use of chemical fertilizers. Fluorescent *Pseudomonas* exert this effect by improving plant nutritional status and/or by synthesizing plant hormone-like compounds.

15.2 Mechanisms that Promote Plant Health by Fluorescent *Pseudomonas* spp.

15.2.1 Antibiotics: Diversity of Antimicrobial Metabolites Produced by *Pseudomonas* spp.

Early studies on suppression of plant pathogens by antagonistic bacteria were focused on their ability to produce siderophores which efficiently compete for iron

acquisition, so that pathogens remain devoid of this nutrient (Kloepper et al. 1980). However, in the last 30 years, it was demonstrated that other secondary metabolites such as antibiotics, enzymes and volatile compounds have an important role in controlling pathogen development (Weller 2007; De La Fuente et al. 2004).

Most fluorescent *Pseudomonas* strains are capable of synthesizing one or more antibiotics (Weller 2007). The structure, biosynthetic pathways and regulation of the main antibiotics produced by *Pseudomonas* spp. have been fully characterized. Among them are the phenazine derivatives, 2,4-diacetylphloroglucinol, pyrrolnitrin, pyoluteorin and cyclic lipopeptides.

Phenazines are heterocyclic nitrogen-containing secondary metabolites, produced not only by *Pseudomonas* but also by various microbial genera like *Brevibacterium*, *Burkholderia* and *Streptomyces*, among others (Mavrodi et al. 2006). It has been described over 100 phenazine derivatives of microbial origin, and a single microorganism can produce up to ten of these ones (Dwivedi and Johri 2003; Mavrodi et al. 2013). Phenazine compounds have a broad spectrum of activity against bacteria, fungi, and parasites. Their mode of action relies on its redox properties and the capacity to promote the production of toxic reactive oxygen species (ROS) in the target organism (Mavrodi et al. 2012). However, their biocontrol activity has also been attributed to the induction of systemic resistance (ISR) in the host plant and the reduction in toxin production by the pathogen. For instance, phenazine 1-carboxylic acid (PCA)-producing *Pseudomonas fluorescens* LBUM223 controls the development of common scab in potato caused by *Streptomyces scabies*. But the antagonistic effect was demonstrated not to be mediated by antibiosis but, instead, by a reduction in the expression of thaxtomin A by *S. scabies* which is necessary to produce the necrotic lesions or scabs. In field trials, an increase of 46 % in tuber weight was achieved by inoculation with LBUM223, which did not reduce pathogen soil populations, but altered the expression of a key pathogenesis gene, leading to reduced virulence (Arseneault et al. 2013, 2015).

Five enzymes are required to transform the chorismate in pyocyanin (PYO) or phenazine-1,6-dicarboxylic acid (PDC), which are encoded by a gene cluster that also contains genes involved in regulation, transport, resistance and transformation of PYO/PDC to strain-specific phenazine derivatives (Mavrodi et al. 2013). Several studies have demonstrated that phenazines are involved in the control of soilborne fungal pathogens. It was shown that some indigenous PCA-producing *Pseudomonas* strains could locally reach high concentrations of the antibiotic around the roots, estimated over 100 mM, enough to inhibit *Rhizoctonia solani* AG-8, an important pathogen of wheat (Mavrodi et al. 2012). The phenazines produced by *Pseudomonas* spp. are also able to inhibit pathogens such as *F. oxysporum*, *Pythium* spp., *Gibberella avenacea*, *Alternaria* spp. and *Drechslera graminea* (Mavrodi et al. 2006).

The antimicrobial metabolite 2,4-diacetylphloroglucinol (DAPG) is another example of a widely characterized antibiotic produced by fluorescent *Pseudomonas* spp. This polyketide compound is responsible for suppressiveness of plant diseases such as tobacco black root rot (Keel et al. 1996) and wheat take-all (Raaijmakers and Weller 1998). DAPG spectrum of action is quite broad and covers bacteria, fungi, oomycetes and nematodes. Recent studies in *Saccharomyces cerevisiae*

provided evidence of the mode of action of DAPG. This compound acts as a proton ionophore interrupting the proton gradient in the mitochondrial membrane, which explains their broad spectrum of action in various eukaryotes (Gleeson et al. 2010; Troppens et al. 2013). The biosynthetic locus involved in the antibiotic production includes *phlA*, *phlC*, *phlB* and *phlD*, responsible for the production of monoacetylphloroglucinol (MAPG) and its conversion to 2,4-DAPG, together with genes encoding efflux and repressor proteins (Bangera and Thomashow 1996; Bangera and Thomashow 1999).

Pyrrolnitrin and pyoluteorin are also extensively characterized antibiotics. The first is a chlorinated phenylpyrrole which is synthesized from L-tryptophan and whose mode of action involves the inhibition of the respiratory chain in fungus. Given its strong antifungal activity, it has been used to develop topical antimycotics for human use or even fungicides for agricultural application (Gross and Loper 2009). The biosynthetic gene cluster involved in pyrrolnitrin synthesis consists of four highly conserved genes, *prnABCD*, that encode enzymes responsible for the conversion of L-tryptophan in pyrrolnitrin in *P. fluorescens*. On the other hand, pyoluteorin is an aromatic polyketide antibiotic that consists in a resorcinol ring attached to a dichlorinated pyrrole moiety. A hybrid nonribosomal peptide synthetase/polyketide synthase (NRPS/PKS) is responsible for pyoluteorin synthesis: the resorcinol ring is synthesized by PKS, while the dichloropyrrol is synthesized from L-proline by NRPS. The biosynthetic operon contains 17 genes, including transport and regulatory genes (Nowak-Thompson et al. 1999). Pyoluteorin is especially toxic to oomycetes and some fungi and bacteria (Howell and Stipanovic 1980), but its mode of action has not yet been clarified.

Fluorescent *Pseudomonas* spp. can also synthesize volatile antimicrobial compounds whose actions are exerted at greater distances than diffusible antibiotics. Within volatile compounds, hydrogen cyanide (HCN) has been the most studied and it is commonly produced by pseudomonads inhabiting soils (Gross and Loper 2009). HCN is a strong inhibitor of metalloenzymes such as cytochrome oxidase; therefore, it is toxic for most organisms (Blumer and Haas 2000). Its production in the rhizosphere environment has shown to contribute to the control of pathogens. The compound is synthesized from glycine by an HCN synthase complex, which is encoded by three structural genes *hcnABC*. The operon is highly conserved among cyanogenic *Pseudomonas* (Loper and Gross 2007), but the genomic context is not conserved, differing among species. The HCN biosynthetic locus had also been reported in *Chromobacterium violaceum* and several species of *Burkholderia*. An example of biological control of a plant pathogen mediated by HCN production is by the use of *P. fluorescens* CHA0 whose ability to produce HCN contributes to the biocontrol of black root rot caused by *Thielaviopsis basicola*, in tobacco (Voisard et al. 1989). There are reports of *Pseudomonas* that inhibit the growth of pathogenic fungi by the production of volatile compounds other than HCN. In many cases these compounds were not identified (Fernando et al. 2005; Trivedi et al. 2008; Weisskopf 2013). But, in other cases, the presence of undecene, undecadiene, (benzyloxy)benzonitrile (Kai et al. 2007), nonanal, benzothiazole and 2-ethyl-1-hexanol (Athukorala et al. 2010) was found.

Another group of secondary metabolites widely distributed among the fluorescent *Pseudomonas* spp. is cyclic lipopeptide (CLP) biosurfactants. These compounds are also produced by *Bacillus* spp. They are amphipathic compounds with an enormous structural diversity that reflects a broad variety of natural roles, some of which may be unique to the producing microorganism (Raaijmakers et al. 2010). CLP are basically composed of a lipid chain attached to a short oligopeptide (8–25 amino acids) that can be linear or cyclic (Raaijmakers et al. 2006). The peptide moiety is synthesized by NRPS which are able to incorporate non-proteinogenic amino acids such as D-amino acids, β-amino acids and hydroxy- or N-methylated residues (Mootz et al. 2002). These non-proteinogenic amino acids protect the synthesized peptide against the action of ubiquitin-dependent proteases (Hashizume and Nishimura 2008). An initial condensation domain is responsible for the attachment of the lipid moiety to the growing peptide chain, which can be of different length even for the same CLP. These compounds usually show strong lytic and growth-inhibitory activities against a variety of microorganisms including viruses, mycoplasma, bacteria, fungi and oomycetes, giving to the producing microorganisms an important advantage in the competition for niches (Raaijmakers et al. 2010). The proposed mode of action is the disruption of cell membranes by pore formation which triggers an imbalance in transmembrane ion fluxes and cell death (van de Mortel et al. 2009). CLP also have a role in bacterial motility and solubilization and diffusion of substrates. Mutants unable to produce CLP usually show little or no motility, which can be recovered by exogenously addition of CLP (de Bruijn et al. 2007). This function is important for microbial dispersal and colonization of ecological niches. Various strains of *Pseudomonas* spp. have proven to be more effective in colonization of roots than their mutant strains (Braun et al. 2001; Nielsen et al. 2005; Tran et al. 2007). CLP might also have an important role in adhesion to surfaces and biofilm formation that depends on the structure of the CPL. Some mutant strains of *Pseudomonas* spp., unable to produce CLP, generate unstable biofilm as compared with the wild strain (Roongsawang et al. 2003; Kuiper et al. 2004). In other cases, the absence of CLP caused a significant reduction in biofilm formation (de Bruijn et al. 2007; de Bruijn et al. 2008). Finally, CLP may have a role in the induction of systemic resistance (ISR) in plants. For example, inoculation of tomato plants with a massetolide (CLP)-producing strain or the pure compound increased the plant leaves' resistance against *Phytophthora infestans* infection (Tran et al. 2007). It is unknown if specific receptors are needed in host plants, but it is thought that CLP can generate distortions or transient channels in plant membranes, initiating a cascade of responses that leads to an increased expression of the plant defense system (Jourdan et al. 2009).

Successful examples of biological control mediated by antibiotic-producing fluorescent *Pseudomonas* were described for forage legumes damping-off seedling diseases (De La Fuente et al. 2004; Quagliotto et al. 2009; Yanes et al. 2012). For instance, *P. protegens* UP61 strain, which can produce HCN, 2,4-diacetylphloroglucinol, pyrrolnitrin and pyoluteorin, can densely colonize the roots of lotus (*Lotus corniculatus*) and alfalfa (*Medicago sativa* L.) and is capable of controlling damping-off caused by *Pythium* spp. on both forage legumes (De La

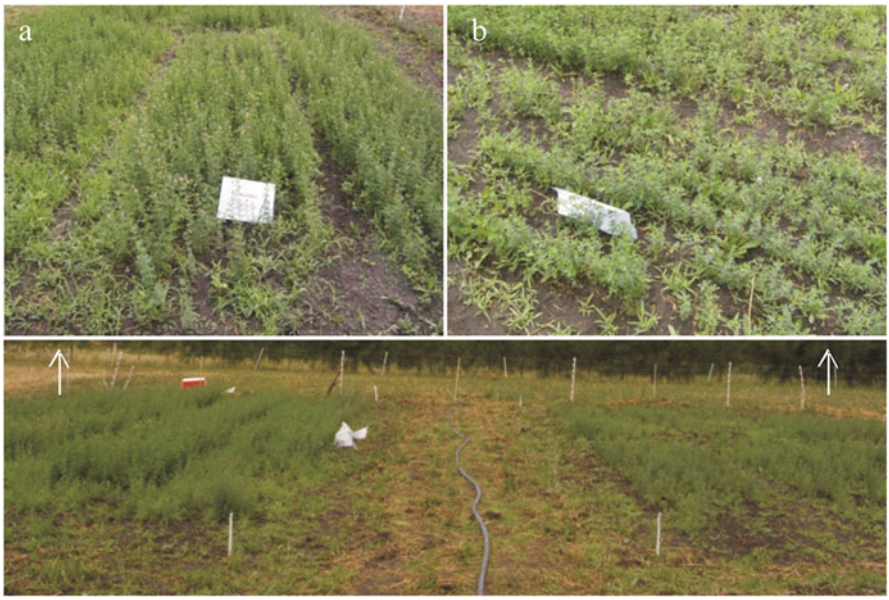

Fig. 15.1 Field assay conducted to determine the biocontrol activity and plant growth-promoting effect of *P. fluorescens* C119 strain. Alfalfa seeds were coinoculated with the pseudomonad strain and *Sinorhizobium meliloti* using a peat-based formulation. Coinoculated plants (**a**) showed a significant increase in alfalfa yield in comparison to the control plants (**b**) which were inoculated only with *S. meliloti*. The picture in the *bottom* shows a partial view of the field assay

Fuente et al., 2004; Quagliotto et al. 2009). Several strains isolated from alfalfa rhizosphere also demonstrated a significant ability to control seedling diseases caused by *Pythium debaryanum* and promoted alfalfa growth (Yanes et al. 2012). Among these strains *P. fluorescens* C119, which produces a CLP with antimicrobial activity, notably promoted the growth of alfalfa (Fig. 15.1).

15.2.2 Biological Control Mediated by Niche and Nutrient Competition

Successful root colonization depends on the ability to compete for nutrients in the root. Competition between beneficial and pathogenic microorganisms by niches in the root surface can result in a decrease in the severity of crop diseases (Kamilova et al. 2005). It is important that beneficial microorganisms colonize efficiently the site of the root that the pathogen uses to get into the plant. Pliego and coworkers (2008) isolated the strains *Pseudomonas pseudoalcaligenes* AVO110 and *P. alcaligenes* AVO73, which were selected by their excellent root-colonizing ability in avocado and antagonism against *Rosellinia necatrix.* Both strains efficiently colonized

different sites of the root, but only AVO110, which was located on the same site as the pathogen, demonstrated a significant protection effect against avocado white root rot.

Competition for iron as biological control mechanism has been widely documented. In the soil, iron availability in the soluble form Fe^{3+} is generally limited. To get access to this nutrient, many microorganisms produce siderophores which are chelant molecules with high affinity for iron. Competition for iron was first reported by Kloepper and colleagues in 1980. Siderophores are structurally diverse and are classified according to their functional group as catechols (enterobactin, vibriobactin, yersiniabactin and pyochelin), hydroxamates (alcaligin and deferoxamine B), and carboxylates (staphyloferrin A and achromobactin) (Miethke and Marahiel 2007). Some *Pseudomonas* are able to internalize the ferric ion using heterologous siderophores synthesized by cohabiting rhizosphere microorganisms, producing a nutrient deficiency that affects the development of competing microorganisms (Loper and Henkels 1999; Saharan and Nehra 2011). An example of an iron competitor strain is the pseudobactin producer *P. putida* WCS358 that is capable to control diseases caused by *Fusarium* spp. in carnation and radish (Weisbeek and Gerrits 1999). The effectiveness of this mechanism was demonstrated by an increase in potato yield when seeds were treated with WCS358 wild-type strain, whereas a siderophore-negative mutant had no effect on tuber yield in field assays (Duijff et al. 1994).

15.2.3 Induction of Defense Responses in Plants

Some nonpathogenic rhizobacteria interact with host plants stimulating an alertness state that would protect plants against subsequent pathogen attack, a phenomenon known as induction of systemic resistance (ISR). This state of preparation for a possible attack enables a fast triggering of defense responses which spread to every organ of the plant, in the presence of a pathogen. While several molecules synthesized by inducing defense bacteria are known to stimulate ISR, there is little information about the molecular mechanisms involved in plant cell recognition of such elicitors. Bacteria-produced molecules involved in the ISR phenotype are lipopolysaccharide (Leeman et al. 1995), siderophores (Maurhofer et al. 1994), flagella (Meziane et al. 2005), and some antibiotics such as those produced by fluorescent *Pseudomonas* spp.: 2,4-diacetylphloroglucinol (Weller et al. 2012), pyocyanin (Audenaert et al. 2002), biosurfactants (Ongena and Jacques 2008), and volatile organic compounds (Ryu et al. 2004). Given this wide variety of elicitors and the enormous diversity of microorganisms that inhabit the rhizosphere, we would expect that plants stand in a continuous state of alert. However, field studies have determined that an effective ISR requires a population density of at least 10^7 CFU of the inducing bacteria per gram of root. For *Pseudomonas* spp., a dose-response study has shown that there is a threshold of 10^5 CFU per gram of root to trigger ISR responses (Raaijmakers et al. 1995). As it is very unlikely to find such a population

density of a specific bacterial genotype on a rhizospheric soil, thus it is very unlikely to find a natural phenomenon of ISR in a soil, except in a suppressive soil (Bakker et al. 2013).

15.3 Mechanisms Involved in Plant Growth Improvement by Rhizospheric *Pseudomonas* spp.

15.3.1 Nutrient Contribution to the Host Plant

Some bacteria including fluorescent *Pseudomonas* spp. can promote plant growth by two major mechanisms: assisting plant nutrition by solubilization of soil nutrients and/or contributing to the pool of the host plant's growth hormones.

Microorganisms that produce metabolites or enzymes that increase the bioavailability of essential plant nutrients such as nitrogen, phosphorus and iron are called biofertilizers (Glick et al. 2007; Berg 2009). The supply of nitrogen by atmospheric N_2-fixing bacteria is the most widely studied example of biofertilization. These bacteria can be endosymbionts, as rhizobia, or free-living diazotrophs that colonize plant rhizosphere, as *Azospirillum*. They synthesize an enzyme complex called nitrogenase which catalyzes, by an energetically costly process, the reduction of N_2 to ammonia; then, the ammonia is taken up by the plant for the biosynthesis of N compounds. Both plants and microorganisms are benefited, because the plants in turn provide carbonated compounds (photosynthates) to the symbiont (Berg 2009). Until a few years ago, it was thought that the genus *Pseudomonas* did not perform atmospheric nitrogen fixation. However, after sequencing the entire genome of *Pseudomonas stutzeri* A1501, it was found that the strain has all the genes necessary to fix N_2, organized in a genomic island possibly obtained by horizontal gene transfer (Yan et al. 2008). This genomic region of *P. stutzeri* A1501 was used to transform *P. protegens* Pf-5 strain, an excellent biocontrol agent of plant diseases. The transformed strain was able to promote the growth of *Arabidopsis thaliana* and other plants of agronomic interest such as alfalfa, fescue and corn under conditions of nitrogen deficiency, opening a new perspective for the production of genetically modified inoculants (Setten et al. 2013).

Phosphorus is an important nutrient and the second limiting element of plant growth, after nitrogen (Gyaneshwar et al. 2002). Phosphate-solubilizing bacteria are another example of biofertilizers, which are taking great relevance due to the high agronomic demand for this nutrient. Most agricultural soils have large reserves of phosphorus due to the repeated application of chemical fertilizers. However, phosphates react with numerous ionic components of the soil, being rapidly immobilized in organic forms by metabolic reactions or in soil mineral particles by adsorption and precipitation. These forms of phosphate are not bioavailable to plants, and only a small fraction of these phosphate forms are available for root absorption (Gyaneshwar et al. 2002). Between 30 and 70 % of the phosphorus in the soil is in organic forms (Shang et al. 1996). Soil microorganisms play an important role in the phosphorus cycle as they produce molecules that assist phosphorus

assimilation by plants (Richardson and Simpson 2011). Two processes are involved in the mobilization of phosphates by soil microorganisms: mineralization of organic phosphorus and solubilization of phosphorus from inorganic sources. Various enzymes such as nonspecific phosphatases, phytases, phosphonatases and C-P lyases release the phosphorus from the organic fraction, while the inorganic phosphorous is mainly released by organic acids such as gluconic acid (Berg 2009; Werra et al. 2009). In summary, phosphate-solubilizing bacteria (PSB) can increase the phosphorus content in plant tissues (Awasthi et al. 2011). Among the best-known PSB are species belonging to the genera *Pseudomonas*, *Bacillus*, *Paenibacillus*, *Escherichia*, *Acinetobacter*, *Enterobacter* and *Burkholderia* (Collavino et al. 2010).

15.3.2 Plant Growth Promotion Mediated by Microbial Phytohormone Production

Plant hormones govern plant growth by spatial and temporal control of division, growth and differentiation of cells. Phytohormones also play an essential role in responses to biotic and abiotic stresses (Peleg and Blumwald 2011). Microorganisms that produce phytohormone-like compounds contribute to the pool of hormones produced by the host plant, intervening in the physiology and promoting plant survival (Dodd et al. 2010; Morel and Castro-Sowinski 2013). Phytohormones act at low concentrations and can be classified in three types: auxins, cytokinins and gibberellins. Indole-3-acetic acid (IAA) is the most commonly found auxin among rhizobacteria. There are at least five biosynthetic pathways for the synthesis of IAA, which mostly use tryptophan (Trp) as precursor. The presence of IAA in the rhizosphere stimulates the formation of lateral roots and root hairs, which greatly increases the absorption surface of roots (Duca et al. 2014). Plants that produce root exudates rich in tryptophan are more prone to be affected by IAA-producing bacteria than those that do not excrete this amino acid precursor. For example, a growth-promoting effect was observed in radish plants, which excrete large amounts of Trp, when inoculated with the IAA-producing strain *P. fluorescens* WCS365, while no effect was observed in plants that excrete tenfold less Trp like tomato, cucumber and pepper (Kamilova et al. 2006).

Cytokinins are involved in cell division and have effects on roots, leaves, flowers, fruits and seeds. The apex of the roots and germinating seeds contains high concentration of cytokinins (Pliego et al. 2011). Members of the genera *Pseudomonas*, *Agrobacterium*, *Erwinia*, *Paenibacillus*, *Azotobacter*, *Azospirillum*, *Bacillus* and *Rhizobium* are cytokinin-producing bacteria, which are also capable of promoting plant growth (García de Salamone et al. 2001).

Gibberellins are involved in various physiological processes of higher plants, in especial root elongation (Pliego et al. 2011). There are more than 130 known gibberellins, which are diterpenoids synthesized by fungi and bacteria (Dodd et al. 2010). The first report of a bacterial gibberellin biosynthetic pathway was for a strain of *B. japonicum* (Morrone et al. 2009). Kang and coworkers (2014)

demonstrated that the gibberellin-producing strain *P. putida* H-2-3 provides the hormone to gibberellin biosynthesis-deficient mutant Waito-C rice plants and also enhances the growth of soybean under saline or drought stresses.

Some bacteria, including *Pseudomonas* spp., are able to decrease the formation of ethylene by plants through the production of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase. The enzyme hydrolyzes ACC, a precursor of ethylene synthesis, with the formation of α-ketobutyrate and ammonium. Plants produce ethylene in response to environmental stresses (Penrose et al. 2001). In these situations plants produce two pulses of ethylene: a small pulse a few hours after the sustained stress is imposed, which is likely to activate the defense genes, and a second pulse of production, a more intense one a few days later. This second pulse of ethylene production generates negative effects on the plant such as senescence, abscission and chlorosis (Glick et al. 2007). It has been proposed a model that explains the promoting effect of ACC deaminase-producing bacteria (Glick et al. 2007) as follows: IAA-producing bacteria can stimulate the synthesis of ACC synthase in plants, which converts S-adenosyl methionine (SAM) in ACC. The ACC is partly exuded by the roots where it is hydrolyzed by ACC deaminase-producing bacteria. To maintain the balance between the concentrations of ACC in and out of the roots, the plant secretes more ACC, decreasing the amount of available ACC necessary to produce ethylene and thus reducing the inhibition effect imposed by ethylene in a stressful situation. The repression of IAA synthesis mediated by ethylene also decreases which results in an increased production of this hormone, increasing the promoting effect. Genes for synthesis of ACC deaminase have been characterized in several strains of *Pseudomonas* spp. (Klee et al. 1991; Cheng et al. 2007), and many of them had proved to be involved in the phenotype of plant growth promotion under stress conditions. For instance, Zahir and colleagues (2011) reported a significant improvement of root elongation, nodulation, and yield of lentil when plants were coinoculated with ACC deaminase-producing *Pseudomonas jessenii* and *Rhizobium leguminosarum* in pot assays and field trial. They showed the implication of ACC deaminase-producing bacteria in the recovery of stressed lentil plants by a bioassay that involved the addition of 6 mM ACC to etiolated lentil seedlings. This triggered an ethylene-specific triple response that consisted in a decrease in root length and shoot length and an increase in shoot diameter. Such effect was significantly reduced when seedlings were coinoculated with both rhizobacteria, which were even more effective than the addition of $CoCl_2$, an inhibitor of ethylene production.

15.4 Fluorescent *Pseudomonas* into the Market

Fluorescent *Pseudomonas* have been widely explored as biocontrol agents and biofertilizers due to the several characteristics mentioned above. However, compared to the volume of research that has been performed with these bacteria, few strains have been successfully developed into commercial products. In Table 15.1, a list of

Table 15.1 *Pseudomonas* spp.-based commercial products for biocontrol or biofertilization, formulated alone or in combination with other PGP microorganisms

Species and strain	Product (name/ quantity of products)	Activity or pathogen controlled	Plant culture	Company/country	References
P. fluorescens EG1053	(2)	*Pythium ultimum*, *Rhizoctonia solani*	Cotton	USA	Cook et al. (1996)
P. fluorescens 1629RS	Frostban A y D (2)	Ice nucleation by *Pseudomonas* spp.		Frost Technology Corporation (USA)	Cook et al. (1996)
P. fluorescens NCIB 12089	(1)	Bacterial blight	Edible fungi	USA	Cook et al. (1996)
P. fluorescens A506	BlightBan A506; Frostban A y B (3)	*Erwinia amylovora*, *Pseudomonas* spp. causing blight and freeze damage	Fruits, tomato, potato	Plant Health Technologies Nufarm Inc. Frost Technology Corporation (EEUU)	Cook et al. (1996), Mark et al. (2006), and Zeller (2006) www.nufarm.com
P. aureofaciens Tx-1	BioJect Spot-Less	*Pythium aphanidermatum*, *Sclerotinia homeocarpa*, *Colletotrichum graminicola*, *Microdochium nivale*	Grass	Eco Soil Systems Inc. Turf Science Laboratories (EEUU)	Mark et al. (2006) www.ecosoil.com
P. chlororaphis 63-28	AtEze	*Pythium* spp., *R. solani*, *Fusarium oxysporum*	Garden and greenhouse plants	Eco Soil Systems Inc. Turf Science Laboratories (USA)	Mark et al. (2006) www.ecosoil.com
P. fluorescens CL0145A	Zequanox	*Dreissena polymorpha*, *D. bugensis* (aquatic animals)		Marrone Bio Innovations (USA)	www.marronebioinnovations.com

(continued)

Table 15.1 (continued)

Species and strain	Product (name/ quantity of products)	Activity or pathogen controlled	Plant culture	Company/country	References
P. fluorescens	Rizofos Liq Maíz Rizofos Liq Trigo (2)	Phosphorous solubilization and mineralization; production of phytohormones, siderophores, and antibiotics	Maize, wheat	Rizobacter (Argentina)	Naiman et al. (2009) www.rizobacter.com.ar
P. fluorescens	Fosforiz	Phosphorous solubilization	Beet	FUNDASES (Colombia)	www.fundases.org
P. fluorescens mogo5	Bioprotection Fosforin	*Pythium* sp., *Phytophthora* sp., phosphorous solubilization and mineralization		Dr. Obregón Laboratories (Costa Rica)	www.doctor-obregon.com
P. fluorescens RA56	AgroBac (4)	Several, e.g., *Streptomyces scabies*	Potato, legumes, cereals	Phytobacter (Germany)	Behn (2008)
Pseudomonas sp. DSMZ13134	Proradix (6)	*R. solani*, *Phytophthora* sp., *Erwinia* sp., *Fusarium* sp., *Microdochium nivale*, *P. ultimum*	Potato, tomato, cucumber, paprika, zucchini, grass	Sourcon-Padena (Germany)	Buddrus-Schiemann et al. (2010) http://www.sourcon-padena.com
P. chlororaphis MA 342	Cedomon, Cerall, Cedress	Seed-borne fungi and leave diseases	Cereals (barley, oat, wheat, rye)	BioAgri (Austria, Finland, Norway, Sweden)	Johnsson et al. (1998) and Mark et al. (2006) www.bioagri.se
P. fluorescens 2P24		*Ralstonia solanacearum*, *F. oxysporum*, *R. solani*		China	Gao et al. (2012)

P. fluorescens	Ecomonas	*R. solani*	Rice	India	Vijay Krishna Kumar et al. (2009)
P. fluorescens	Florezen P	*R. solani*	Rice	India	Vijay Krishna Kumar et al. (2009)
P. fluorescens	Bio Protector	Damping-off, wilt, blight	Rice	Manidharma Biotech (India)	www.manidharmabiotech.com
P. fluorescens				Zen Cropcare (India)	www.zencropcare.com
P. fluorescens	Deepa Bio Plus – Pseudo	Wilt, blight		Deepa Farm Inputs (India)	www.indiamart.com/deepa-farminputs-pvtltd
P. fluorescens	Basmonas	Damping-off, wilt, blight	Several	Basarass Biocon (India)	www.indiamart.com/basarass-bioconpvtltd
P. fluorescens		Bactericide, fungicide		Bharat Biocon (India)	www.indiamart.com/bharat-biocon
P. fluorescens	TNAU – Pf1	Soil fungi	Several	Tamil Nadu Agricultural University (India)	www.indiamart.com/tnau
P. fluorescens		Pathogen fungi		Rajathi Group (India)	www.indiamart.com/rajathi-group
P. fluorescens	Yash Pseudomonas			Yash Krishi (India)	www.indiamart.com/yashkrishi-takniki-ewam
P. fluorescens	Conquer/Victus	*Pseudomonas tolaasii*	Edible fungi	Mauri Foods/Sylvan Spawn (Australia)	Nakkeeran et al. (2006) and Fernandéz and Juncosa (2002)
P. fluorescens	Biomonas	*Schlerotinia*, *Rhizoctonia*, *Pythium*, *Alternaria*, *Ascochyta*, *Cercospora*, *Macrophomina*, *Myrothecium*, *Ramularia*, *Xanthomonas*, *Erwinia*, *Fusarium*, *Verticillium*, *downy mildews*, *powdery mildews*	Cotton, cereals, pulses, vegetables, oilseeds, fruit, and floriculture	Biotech International (India)	www.biotech-int.com Bettiol et al. (2012)

(continued)

Table 15.1 (continued)

Species and strain	Product (name/ quantity of products)	Activity or pathogen controlled	Plant culture	Company/country	References
P. syringae ESC-10 and ESC-11	Bio-save 10 and 11	Postharvest diseases (*Penicillium*, *Botrytis*, *Mucor*, *Helminthosporium*, *Rhizopus*)	Apple, pear, cherry, potato, sweet potato	Jet Harvest Solutions (USA)	Bettiol et al. (2012)
P. fluorescens (yeast and *two Bacillus* spp.*)*	BioGro	Nitrogen fixation, phosphorous solubilization, organic matter mineralization	Rice	Hanoi University of Science (Vietnam)	Adesemoye and Egamberdieva (2013)
P. fluorescens, *Trichoderma viride*	ANOKA	Control of damping-off, seed and root, nematode wilt		Bio Sciences (India)	Woo et al. (2014)
P. fluorescens, *Trichoderma viride*, *Trichoderma harzianum*, *Bacillus subtilis*	Bio Protector	Root-/soilborne and air borne diseases		Bacto Agro Culture Care	Woo et al. (2014)
Pseudomonas, *Trichoderma harzianum*, *Glomus intraradices*	Micover Gold & Plus	Soilborne pathogens		Agrifutur (European Union)	Woo et al. (2014)
Pseudomonas	Micosat Fito	Induced resistance to bacteria, soilborne fungi, pathogens, insects, and nematodes		CCS Aosta (Italy)	Woo et al. (2014)
T. harzianum					
Glomus spp., *Pichia*					

Pseudomonas-based products for plant biocontrol and biostimulation has been reviewed.

A successful example is the biocontrol agent *Pseudomonas chlororaphis* strain MA342, which has been formulated in several commercial products. Initially, MA342 was tested in 105 field experiments in different zones of Sweden for 5 years to control seed-borne diseases of barley, oats, wheat and rye. After consistent results, comparable to chemical fungicide application, an inoculant formulation was developed for the protection of cereals and then for peas. However, the performance of these microorganisms may not always be consistent, as showed by their high effectiveness against *Alternaria* spp. on cabbage and carrots and against *Colletotrichum lindemuthianum* on beans, but a poor performance in seed-borne *Ascochyta* spp. on peas (Amein et al. 2011; Johnsson et al. 1998; Tinivella et al. 2009). Thus, all plant-pathogen interactions can not be overcome by the inoculation with a single *Pseudomonas* strain.

15.5 Benefits and Challenges of Using Fluorescent *Pseudomonas* for Plant Growth Promotion

The application of beneficial microorganisms to improve plant health of economically important crops has many advantages over chemically synthesized pesticides and fertilizers as they are safer with the environment, have a specific targeted activity, do not favor the development of resistant pathogens due to the diversity of mechanisms involved in their biocontrol phenotype, are effective in small doses, decay faster, and can be applied either in conventional or integrated management systems (Berg 2009). Fluorescent *Pseudomonas* are a good example of the sustainable use of microbes, but the commercial adoption of agricultural products based on these bacteria is not so widespread, due to some drawbacks that their application still present and that need to be overcome.

15.5.1 Consistency in Field Performance

As also happens with other plant growth-promoting rhizobacteria (PGPR) (except from rhizobia and some free-living nitrogen fixers), the lack of consistency in field performance of *Pseudomonas*-based bioproducts, in different crops and conditions, is a limitation for its commercial application (Barreto et al. 2010). In some cases, there is a gap between results obtained in controlled laboratory conditions and those achieved in the field. Different host-plant genotypes produce root exudates with different chemical compositions. These variations may play a role in the bacterial responses, which are needed to support inoculum presence and activity (Khalid et al. 2004; Dalmastri et al. 1999). Usually, the process of plant breeding for the selection of cultivars does not take into account the association of plant with beneficial microorganisms. Considering this aspect, it would be a valuable tool to coordinate the selection of host plant varieties and biofertilizers; this strategy could

enhance the ability of the microorganism to interact with the plant and to express promotion traits, e.g., production and responsiveness to hormones (Remans et al. 2008).

The failure of microorganism-based protectant formulations under field conditions has been explained by their low stability, with limited persistence and resistance, but not by a lack of effectiveness (Dorn et al. 2007). This phenomenon can be sometimes explained by the complex regulation of biocontrol-related traits as colonization and production of active metabolites, which are highly dependent on environmental conditions. In some cases, the need for the inoculation with a high population of the biocontrol agent, in order to achieve effective control of the pathogen, is also a limiting factor.

Usually, successful field inoculation experiments have been difficult to establish due to the low disease incidence of plant pathogens during the assays. The strain *P. chlororaphis* R47 was highly active in the protection of potato against *Phytophthora infestans* in vitro and in greenhouse, but its activity could not be verified in the field due to unfavorable infection conditions (Guyer et al. 2015). In another study, from 20 field trials installed in two different regions of Uruguay, and during a 4-year evaluation of effectiveness of three *P. fluorescens* strains to protect alfalfa from emergence diseases, only 11 trials were conducive for damping-off (Quagliotto et al. 2009).

The challenges to overcome this limitation for a wider adoption of *Pseudomonas* spp. as biofertilizers include the characterization of the ecology and colonization behavior of these bacteria in the rhizosphere at different situations, as well as their mechanisms of plant promotion and regulation. The development of more stable formulations will enable the strains to be established at adequate cell densities in order to behave as plant promoters under field conditions. A comprehensive consideration of the biology and ecology of the crop system, and the agricultural practices, will help to reduce the variation of results in field performance of these inoculant bioformulations (Barreto et al. 2010; Guyer et al. 2015).

15.5.2 Is Genetic Modification an Option?

Some researchers have proposed the development of genetically modified (GM) microorganisms aimed to combine different plant growth-promoting abilities in a single microbe. For example, the introduction of a plasmid carrying the genomic information for IAA production into *P. fluorescens* BSP53a, a strain that has the ability to block the development of some phytopathogenic fungi due to siderophore synthesis, was seen as an interesting inoculant strain, which combines plant stimulation and protection effects. The recombinant strain produced an increase in root weight and changes in root morphology, as compared to the wild-type strain in black currant, but not in cherry plants (Dubeikovsky et al. 1993).

Several studies have incorporated antimicrobial traits in *Pseudomonas* spp strains. When their impact on resident microbial communities was analyzed, the effect caused by inoculation was transient, suggesting a rapid inactivation of the antibiotic

in the soil (Bajsa et al. 2013). However, a long-term effect in wheat rhizosphere was caused by *P. putida* strains that constitutively express PCA or DAPG, which changed the resident bacterial communities in a 4-year experiment (Viebahn et al. 2006).

This approach, the use of GM microorganisms, is a matter of serious controversy in terms of the spread of a GM bacterium in nature, especially if the microbe has better colonization, competence, or persistent abilities in the natural environment. The constraints for the registration of natural microbes for their application in the environment are numerous, but even more are with GM strains that would involve a more detailed risk assessment.

15.5.3 Let's Cooperate: Coinoculation

Another strategy to enhance effectiveness and reduce variation in performance is the use of bioproducts with a *Pseudomonas* strain in combination with another microorganism, presenting additive or synergistic effects, such as nitrogen-fixing bacteria, mycorrhizal fungi, or other plant biocontrol agents. The rhizosphere is a complex ecosystem, as are the interactions involved in plant growth stimulation or disease, so the use of bioformulations containing several PGPR can improve their effectiveness, as compared with single bacterial inoculation (Barreto et al. 2010; Morel and Castro-Sowinski 2013).

Nitrogen-fixing bacteria are widespread PGPR used to improve crop yield, and the nitrogen fertilization using chemicals could be completely prevented if the right strain and adequate management practices are employed. The combination of diazotrophs with other PGPR strain has been widely reported. The coinoculation practice, using *Pseudomonas* spp. and *Rhizobium* spp., was demonstrated to enhance nodulation, nitrogen fixation, plant biomass and grain yield in various leguminous species including alfalfa, soybean, green gram, and chickpea. For example, the nodule occupancy by a *Rhizobium* strain in pigeon pea increased from 50 % to 85 % in the presence of *Pseudomonas putida*, improving plant growth and nitrogen fixation (Tilak et al. 2006; Remans et al. 2007). In addition, *in vitro* and in-field experiments carried on in lotus and alfalfa plants showed that the coinoculation with rhizobia and fluorescent *Pseudomonas* decreases damping-off incidence and fixes nitrogen, compared with single inoculation (Quagliotto et al. 2009; De La Fuente et al. 2002).

The success of this mixed formulation depends also on abiotic factors as nutrient availability, as showed by the following examples. ACC deaminase-producing *P. putida* UW4 is able to enhance nodulation by *Rhizobium* in *Phaseolus vulgaris* mainly under P-deficiency conditions. In this stress situation, the production of ethylene is increased and bacterial ACC deaminase activity can reduce this stress response and revert nodulation inhibition (Remans et al. 2007). ACC deaminase-producing *Pseudomonas* sp. also promotes groundnut (*Arachis hypogea*) nodulation by *Bradyrhizobium* under saline stress condition (Saravanakumar and Samiyappan 2007).

Some bacteria, including strains of *Pseudomonas* spp., can act as mycorrhiza helper bacteria (MHB), assisting the establishment or promoting the functioning of

arbuscular or ectomycorrhizal symbiosis. They can mobilize nutrients from soil, fix nitrogen and protect plant from pathogens, which can lead to enhance fungal germination and root colonization (Frey-Klett et al. 2007; Velivelli et al. 2015). A few examples include the increased infection by ectomycorrhizal fungi as *Laccaria* spp. and *Pisolithus alba* in Douglas fir, *Eucalyptus* and *Acacia* trees, higher colonization by endomycorrhizal fungi *Glomus* spp. in diverse plant species (maize, potato, tomato, wheat, barley, clover and *Acacia* sp.), and protection of papaya from *Fusarium oxysporum* infection by coinoculation with *Pseudomonas* spp. and *Glomus* spp. (Frey-Klett et al. 2007; Hernández-Montiel et al. 2013). The molecular mechanisms involved in these tripartite interactions are poorly understood. For the establishment of the bacteria/mycorrhizal fungus/plant network, the release of active biomolecules and physical contact among the partners seem important, where quorum sensing, biofilm formation and secretion systems seem to be involved (Bonfante and Anca 2009). For instance, ACC deaminase and ethylene production by *P. putida* is involved in the promotion of endomycorrization by *Gigaspora rosea* in cucumber (Gamalero et al. 2008). Carbon sources may also be involved in the selection of bacterial communities associated with the mycorrhizosphere. The secretion of trehalose, the main carbohydrate used as carbon sink by the fungus, acts in facilitating the colonization of MH *Pseudomonas* and formation of biofilms on hyphae (Duponnois and Kisa 2006; Uroz et al. 2007).

Several *Pseudomonas* strains showed positive effects on the growth of the ectomycorrhizal fungus *Laccaria bicolor* and the effectiveness of the symbiosis with *Populus deltoides* roots. Results suggest that these effects are due to the induction of the presymbiotic status of the fungus (Labbé et al. 2014). In other types of interactions, the production of antibiotics by the MHB is stimulated by the mycorrhiza, but this mechanism has not been described yet in interactions with *Pseudomonas* spp. (Riedlinger et al. 2006).

Plant protection can also be enhanced by the combination of *Pseudomonas* strains with other protective microorganisms. The coinoculation of faba bean (*Vicia faba*) with *Pseudomonas fluorescens* and *Rhizobium leguminosarum* reduced the symptoms produced by yellow mosaic virus, probably by the induction of the systemic resistance of the plant. Among other examples, the coinoculation of cucumber with *Pseudomonas putida* and *Serratia marcescens* and the coinoculation of cucumber and *Arabidopsis thaliana* with *Trichoderma harzianum* and *Pseudomonas* sp. showed additive and positive effects on plants infected with *Fusarium* wilt (Mohr et al. 2008; Liu et al. 1995; Elbadry et al. 2006; Alizadeh et al. 2013).

15.5.4 Suitable Formulations for *Pseudomonas* Delivery

For the successful application of a bioproduct, the formulation (a mixture of ingredients prepared for the direct application on the field or seeds) must deliver the microbial agent in a physiologically active state and with the potential to express all the microbial capabilities (Barreto et al. 2010). Moreover, the product has to be long

lasting for a certain period of time to ensure the proper microbial state for effective field application. As *Pseudomonas* spp. are bacteria that do not produce resistant structures as spores, refrigeration for preservation of the inoculants may be necessary. Temperatures of 4–10 °C enable the conservation of some formulations up to 2–6 months, at room temperature about 3 weeks, but freeze conditions are needed for longer periods. Certain products are marketed as powder-soluble formulas containing lyophilized bacteria and compounds (e.g., milk or canola oil) that improve cell survival and/or effectiveness (Bettiol et al. 2012).

On the other hand, the bacterization of seeds (pre-inoculation of seeds) is also an adequate option of product marketing due to the long-lasting useful life of inoculated seeds that can be stored for long periods (1–2 years) without losing the promoting activity, as it has been shown for *P. chlororaphis*-based products (Johnsson et al. 1998; Bettiol et al. 2012).

Sterile peat is a carrier that has been extensively used for rhizobial formulations, because it allows bacterial survival during storage and commercialization of the final product. It was demonstrated that this carrier is also suitable for fluorescent *Pseudomonas*, maintaining constant populations of $10x10^9$ UFC per gram of peat at 4 °C during 6 months (Bagnasco et al. 1998; Date 2001).

15.5.5 The Tougher Step: Registration

During the process of registration of market products containing microorganisms, and especially when they are based on *Pseudomonas* spp., a large number of constraints are found (Mathre et al. 1999). A risk assessment to human health and to the environment is needed before releasing a product (Fravel 2005). Bacteria of the genus *Pseudomonas* include a few species and strains that can be opportunistic human pathogens, so the massive use of formulations containing this microbe is always under debate. Bacteria unable to grow at human body temperature could circumvent this concern, but forward studies are needed to clearly differentiate truly health threat bacteria and safe ones (Bodilis et al. 2004).

As more information regarding safety of microbial formulation is generated, the big challenge may be the simplification of registration procedures, as it occurs with chemical products whose registration is much more standardized despite its potential but known toxicity.

The ecological impact of some *Pseudomonas* strains is expected to be low, as their populations rapidly decrease after been inoculated, in 1–2 weeks or months in field or greenhouse conditions. This could be a favorable trait for environmental safety but not for its performance in agricultural systems, except if the action that the microbes exert in the rhizosphere is quick enough to induce the positive effect on plants, e.g., during the pathogen control of seedling diseases (Guyer et al. 2015; Quagliotto et al. 2009). Further studies are required to better characterize the ecological behavior of bacteria before their release to the soil.

15.6 Final Remarks

Plant-associated *Pseudomonas* are multifaceted bacteria, with a high metabolic diversity and interesting plant growth traits (Fig. 15.2). Even if there is no doubt regarding the benefits of *Pseudomonas* spp. inoculation on plant growth promotion, there is also a public concern due to their potential as opportunistic pathogenic agents. Thus, further knowledge and experience about their use in agroecosystems, in single or mixed formulation, will give insight to the potential use of *Pseudomonas* spp. in agricultural systems.

The adoption of sustainable agronomical practices for preservation of the native, active and adapted *Pseudomonas* populations in every location would reduce the need for reintroducing them as inoculants (Agaras et al. 2012).

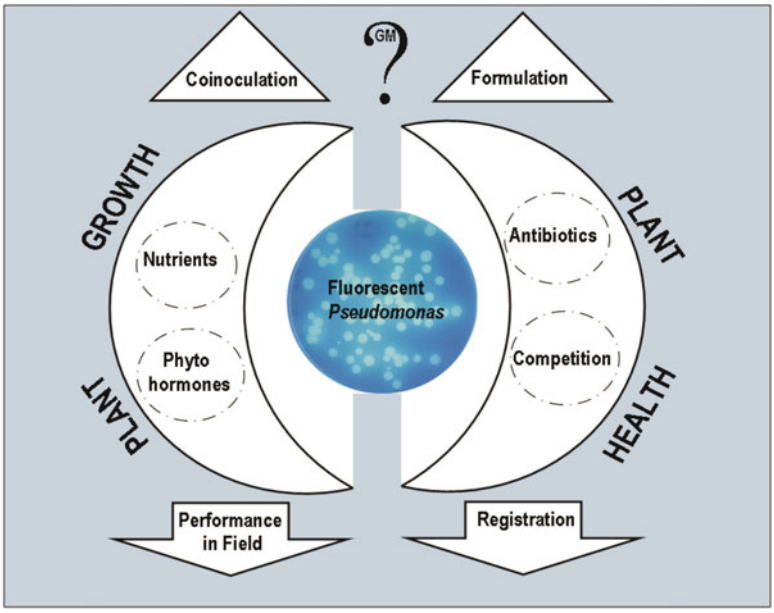

Fig. 15.2 Fluorescent *Pseudomonas*: plant health and plant growth-promoting activities. Limitations in field performance and in registration process have delayed the wider commercial adoption of *Pseudomonas*-based inoculants. The improvement of commercial formulations and/or the coinoculation with other PGPR may overcome those drawbacks. Genetic modification (GM) is a more questionable alternative

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