Protein Secretion in Gram-Positive Bacteria: From Multiple Pathways to Biotechnology

Jozef Anné, Anastassios Economou and Kristel Bernaerts

Abstract A number of Gram-positive bacteria are important players in industry as producers of a diverse array of economically interesting metabolites and proteins. As discussed in this overview, several Gram-positive bacteria are valuable hosts for the production of heterologous proteins. In contrast to Gram-negative bacteria, proteins secreted by Gram-positive bacteria are released into the culture medium where conditions for correct folding are more appropriate, thus facilitating the isolation and purification of active proteins. Although seven different protein secretion pathways have been identified in Gram-positive bacteria, the majority of heterologous proteins are produced via the general secretion or Sec pathway. Not all proteins are equally well secreted, because heterologous protein production often faces bottlenecks including hampered secretion, susceptibility to proteases, secretion stress, and metabolic burden. These bottlenecks are associated with reduced yields leading to non-marketable products. In this chapter, besides a general overview of the different protein secretion pathways, possible hurdles that may hinder efficient protein secretion are described and attempts to improve yield are discussed including modification of components of the Sec pathway. Attention is also paid to omics-based approaches that may offer a more rational approach to optimize production of heterologous proteins.

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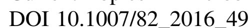

Abbreviations

FEA	Flagella export apparatus
GFP	Green fluorescent protein
GRAS	Generally recognized as safe
LAB	Lactic acid bacteria
mTNFα	Mouse tumor necrosis factor α
PMF	Proton motive force
PSPa	Phage shock protein A
Sec pathway	General secretory pathway
SPase I	Signal peptidase type I
T4SS	Type 4 secretion system
T7SS	Type VII secretion system
Tat	Twin-arginine translocation

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1 Introduction

Biotechnology involves the use of living organisms and their products for the benefit of mankind in different areas. The biotechnological manufacturing of products of biomedical interest such as antibiotics, vaccines, antibodies, and other

biopharmaceuticals are termed red biotechnology. On the other hand, the production of industrial enzymes for the sustainable processing and production of chemicals, materials, and fuels is classified as white biotechnology, while green biotechnology serves agriculture and involves, for example, the development of pest-resistant plants and the control of food and feed. Recently, blue biotechnology gained interest, whereby marine organisms and their products are used for the making of valuable compounds including drugs and bioenergy. Molecules of interest can be synthetized by the original producing organism, but in many cases they are heterologously produced. This means that the necessary genes derived from other organisms are cloned into the host cell of choice with the intention to express the molecules encoded by the cloned DNA so that the host produces the molecules that it normally does not make. The global biotechnology market size is estimated to have a value of more than US$398 billion in 2015 and a growth expectation at a compound annual growth rate of 12.3 %. To be profitable in this vast market, not only the value of the molecules produced counts, but the cost-effectiveness and environmental-friendliness of the production process are at least as important. The main challenge therefore is to have the most effective production process for lowering the cost of the final product to obtain a commercially viable process. Optimization of microbes in production processes has been done over the past decades using, for example, random mutagenesis and selection, identification of metabolic reactions whose activities should be modified to achieve the desired cellular objective, genome-scale modeling of metabolism, and by fermentation optimization. More recently, these approaches are enhanced by synthetic biology tools.

To produce heterologous molecules, a variety of different expression systems has been described, each with its own advantages and disadvantages. The most popular host is *Escherichia coli*, as proven by the fact that nearly half of the approved recombinant biopharmaceuticals are synthesized using this host. Reasons why *E. coli* is so popular in recombinant protein production and as a workhorse in the laboratories of academia and the biopharma industry are obvious: its genetics are far better understood than those of other microorganisms; there are many genetic tools available, and proteins, if expressed, can be obtained in high production yields. As other bacteria, *E. coli* grows quickly on cheap media to high cell density. A drawback, however, is that *E. coli* are Gram-negative bacteria inherently having an outer membrane bilayer (OM) composed of lipopolysaccharides (LPS). The OM acts as an effective permeability barrier hindering secreted proteins from being released into the extracellular medium. LPS contains the endotoxin lipid A, that if released in the blood can cause septic shock, a systemic inflammatory response syndrome. When overexpressed in *E. coli*, many proteins become misfolded and accumulate in the cytoplasm as inclusion bodies. To become active, these inclusion bodies need to be solubilized, and proteins refolded into bioactive molecules, an often cumbersome process, with poor recovery and accounting for the major cost in the production process of recombinant proteins. Therefore, new solubilization techniques have been proposed, as well as genetic approaches to make *E. coli* a better host, for example, engineering *E. coli* strains that possess an

oxidative cytoplasmic environment that favors disulfide bond formation, overexpression of different chaperones or combinations thereof, the fusion of appropriate tags to the N- or C-terminus of the overexpressed protein, secretion in the medium using the alpha-hemolysin secretion system. These improvements can be helpful, but are not used at an industrial scale, and depending on the proteins to be obtained other host cells derived from Gram-positive bacteria might be a better option.

An important asset is that Gram-positive bacteria are monoderm with a cell envelop that surrounds the cytoplasmic membrane with a thick peptidoglycan layer and associated teichoic acids. This structure protects the cell from mechanical or osmolytic lysis and is an anchor place for proteins, glycopolymers, and cations. Notwithstanding its complex structure, the cell envelop of Gram-positive bacteria is permeable to proteins as it does not contain an outer membrane. Consequently, secreted proteins will be released into the culture medium, where they can obtain their native conformation simplifying downstream processing. This is an advantage for the industrial production of heterologous proteins.

In this review, a survey will be presented about the protein secretion pathways in Gram-positive bacteria together with possible applications for specific species. Complementary to the beneficial properties of heterologous expression in Gram-positive bacteria, strategies for enhancing heterologous protein production are developed to acquire commercially acceptable production and yield. This review will further give a brief compilation of approaches tackling the bottlenecks at the level of expression up to metabolic fluxes.

2 Protein Secretion Pathways in Gram-Positive Bacteria

Protein secretion is a vital process for all organisms, since about 35 % of all proteins made in a cell are either membrane-embedded or secreted (Orfanoudaki and Economou 2014). To do so bacteria have different secretion pathways at their disposal. Whereas at least 7 diverse secretion systems (type I–VII) have been identified for Gram-negative bacteria, 6 protein translocation systems were reported for Gram-positive bacteria [for an overview, see Forster and Marquis (2012)] as explained below for Gram-positive bacteria. Only two of them are used for biotechnology purposes (Fig. 1).

2.1 General Secretion (Sec) System

The most important protein secretion pathway is the general secretion (Sec) system, which directs proteins to the cytoplasmic membrane for their insertion into or translocation across the membrane. Proteins destined for secretion are in general synthesized as preproteins with an N-terminally extended sequence, named the signal peptide. The primary sequences of signal peptides are not homologous, although they

Sec pathway	Twin-arginine transport pathway
Secreted folded mature protein Cell wall SPase SecE SecY SecG Cytoplasmic membrane SecA ATP ADP + P_i Cytosol Unfolded preprotein with signal peptide	Secreted folded mature protein SPase TatA TatB TatC Folded preprotein with Tat signal peptide
Used with species such as *B. subtilis, B. licheniformis, B. amyloliquefaciens S. lividans, S. coelicolor, C. sporogenes, C. glutamicum, L. lactis, L. plantarum, C. acetobutylicum, C. sporogenes, C. novyi*-NT, Bacterial enzymes and several eukaryotic proteins	Used with species: *B. subtilis, S. lividans, C. glutamicum* Bacterial enzymes, GFP and other eukaryotic proteins
Non-classically secreted protein pathway	Tested at lab scale with *B. subtilis* for secretion of heterologous bacterial enzymes
Type IV secretion	Transfer of heterologous effector proteins by *Escherichia coli* pKM101-encoded conjugation system (Whitaker et al. 2016)
Type VII secretion system	Not applied
Flagella export apparatus	Secretion of recombinant proteins in *Salmonella* (Vonderviszt et al. 2012)
Holins	Not applied

Fig. 1 Overview of protein secretion pathways of Gram-positive bacteria and their possible use in biotechnology

do have 3 common structural features: a net positively charged N-terminus, a hydrophobic core region (H-region), and a polar C-terminal end containing the signal peptidase recognition site. In bacteria, the signal peptide is between 20 and 30 residues long, but can contain even more than 50 amino acids residues depending on the species and the protein to be secreted. The role of the signal peptide is to guide the protein to the secretion channel following binding to soluble targeting factors. The involvement of chaperones to target newly synthesized proteins to the translocation

pathways, either co- or posttranslationally, is best studied for *E. coli*, as summarized hereafter. The chaperones trigger factor (Tf), DnaK/DnaJ/GrpE (DnaKJE) and GroEL do not only play a major role in the folding of newly synthesized cytosolic proteins, but are also important for posttranslational protein targeting (Grady et al. 2012; Castanie-Cornet et al. 2014). Besides Tf, DnaKJE and GroEL, of prime importance for secretion in proteobacteria, is SecB, which keeps proteins in an unfolded secretion-competent state and delivers them to the Sec translocon SecYEG via its interaction with SecA, the dimeric ATPase subunit of bacterial protein translocase (Karamanou et al. 1999). The peripherally associated motor protein SecA drives then the protein translocation step by repeated cycles of ATP-binding and hydrolysis resulting in SecA membrane insertion/deinsertion and stepwise exportation of the preprotein through the channel (Chatzi et al. 2013). Additional energy promoting translocation, when the preprotein is detached from SecA, comes from the proton motive force [PMF; (Schiebel et al. 1991)]. During or following translocation, the signal peptide is cleaved off by the membrane-embedded signal peptidase I or II (the latter specializing on secreted lipoprotein signal peptides) at the signal peptidase recognition site located in its C-terminal end. This recognition site is often a canonical A-X-A motif, but other residues are permitted as well. It was recently shown by computational analyses of ~1500 genomes that numerous major evolutionary clades have replaced the canonical signal peptide sequence with novel motifs (Payne et al. 2012).

Besides the Sec pathway, there is also the signal recognition pathway (SRP) for cotranslational secretion that in bacteria mainly deals with membrane protein insertion and to a lesser extent with protein secretion. SRP binds to particularly hydrophobic N-terminal signal sequences or hydrophobic transmembrane segments as they emerge from the ribosome. The SRP/RNC (*r*ibosome *n*ascent *c*hain) complex interacts with the membrane-bound *S*RP *R*eceptor (SR) and the delivery of the RNC to the translocation channel SecYEG in the membrane finally leads to the dissociation of the SRP/SR complex, whereupon the preprotein is driven across the translocation channel with the help of continuing translation and/or SecA.

It is generally assumed that the secretion of proteins in Gram-positive bacteria follows similar steps as they occur in *E. coli* since genes involved in the Sec-dependent protein secretion pathway are identified in the genome of Gram-positive bacteria. Nonetheless SecB is absent from the genomes of Gram-positive bacteria, although some SecB-like genes are present. For example, SecB-like protein (Rv1957) is present in the *Mycobacterium tuberculosis* genome where it specifically controls a stress-responsive toxin–antitoxin system. Experiments suggest that Rv1957 could play a role in protein export of *M. tuberculosis* (Sala et al. 2014), but its role is completely different from that of SecB in Gram-negative bacteria. In *Bacillus subtilis*, the SecB chaperone function has been attributed to CsaA (Shapova and Paetzel 2007). How Sec-dependent secretory proteins are kept in a Sec-secretion-competent way, and which chaperones are involved is not clear yet.

2.2 Twin-Arginine Transport Pathway (Tat)

A special characteristic of the Tat pathway is that proteins are transported across the cytoplasmic membrane in a folded state, and the energy for translocation comes from the proton motive force (PMF). Many Tat substrates receive cofactors and fold prior to translocation. Based on genome sequence analysis of prokaryotes, the Tat pathway is present in nearly 80 % of all bacteria, also in *Archaea* (Simone et al. 2013). Results indicate that the Tat pathway is utilized to highly varying extents. It operates in parallel with the Sec pathway. Signal peptides that target proteins to the Tat pathway resemble Sec signal peptides, but with a conserved S/T-R-R-x-FLK consensus motif at the end of the N-region, where the twin-arginines are invariant and normally essential for efficient export by the Tat pathway (Stanley et al. 2000). However, the Tat-specific signal sequence with two arginine residues may not be an absolute prerequisite for the Tat pathway (Watanabe et al. 2009). The Tat translocon comprises two kinds of small membrane proteins: TatC, a highly hydrophobic protein with 6 predicted transmembrane helices, and with its N- and C-termini at the cytoplasmic face of the membrane. The Tat translocon contains also one or two members of the TatA protein family, named TatA and TatB, sequence-related proteins with a common structure, each predicted to comprise a membrane-spanning α-helix at the N-terminus, immediately followed by an amphipathic helix located at the cytoplasmic side of the membrane and a C-terminal region of variable length. TatB and TatC form an oligomeric, multivalent receptor complex that binds Tat substrates, while multiple protomers of TatA assemble at substrate-bound TatBC receptors to facilitate substrate transport (Cleon et al. 2015). Minimal Tat systems contain only one type of TatA and one type of TatC. When the signal peptide of a Tat-dependent protein is recognized, it will be bound by a multi-subunit TatBC complex located in the membrane and Tat secretion is initiated. This binding event triggers the PMF-dependent recruitment and oligomerization of TatA protomers from a pool in the membrane to form the active TatABC-containing translocation site (Berks 2015). Possible cross talk between the Tat- and Sec-dependent protein secretion pathways has been reported (Goosens et al. 2014). This assumed interaction, however, needs to be further investigated.

In actinomycetes including *Streptomyces lividans* and other streptomycetes, *Mycobacterium* (McDonough et al. 2005) and *Corynebacterium* (Oertel et al. 2015), TatA, TatB, and TatC are the components for this pathway, similarly to Gram-negative bacteria. Of the Tat components in *Streptomyces*, TatC is essential whereas TatA and TatB are individually dispensable and are, next to the membrane-embedded localization, also found as active soluble complexes in the cytoplasm (De Keersmaeker et al. 2007). In contrast to the majority of Tat-containing organisms in which the Sec pathway is the major route for protein transport, the Tat pathway seems to be an important protein secretion route in *Streptomyces*. It is estimated that nearly 20 % of proteins of the extracellular proteome is secreted via the Tat pathway (Widdick et al. 2006). Using enhanced green fluorescent protein (eGFP) and mCherry fusions of the proteins of the Tat

machinery, Willemse et al. (2012) tried to determine their subcellular localization in *Streptomyces coelicolor* throughout the complex life cycle of this organism. They showed that TatA, TatB, and TatC dynamically co-localize in the vegetative hyphae, with a strong preference for apical sites in growing hyphae. For *Corynebacterium,* secretion is mainly studied in *Corynebacterium glutamicum* (Kikuchi et al. 2006). A functional *C. glutamicum* Tat system requires TatA and TatC, while the TatB protein seems to be dispensable, but it is important for maximal efficiency, and it was also found to be essential for the secretion of a heterologous Tat-dependent model protein into the *C. glutamicum* culture supernatant (Oertel et al. 2015). It was further shown that TatB (in combination with TatA and TatC) is strictly required for unimpaired aerobic growth (Oertel et al. 2015).

Both *M. tuberculosis* and *Mycobacterium smegmatis* have a functional Tat pathway. As for other actinomycetes, the Tat translocon consists of TatA, TatB, and TatC (McDonough et al. 2005). In *M. tuberculosis,* the Tat pathway is essential for growth (Saint-Joanis et al. 2006) as concluded from the inability to obtain viable deletion mutants. This contrasts with *M. smegmatis* for which viable Tat mutants could be obtained, although these mutants showed growth defects. In addition, increased sensitivity to β-lactam antibiotics was also noticed, this as a consequence of reduced export of β-lactamase BlaS, a protein with a predicted Tat signal peptide (Saint-Joanis et al. 2006).

For staphylococci, only some species contain a functional Tat system, including *Staphylococcus carnosus* (Meissner et al. 2007), *Staphylococcus haemolyticus* (Yamada et al. 2007), and *Staphylococcus aureus* (Biswas et al. 2009). The Tat system is composed of TatA and TatC and was proven to translocate iron-dependent peroxide FepB in *S. aureus* (Biswas et al. 2009). *B. subtilis* secretion system has been studied extensively (Goosens et al. 2014). Genes for two TatC (TatCd and TatCy) and three TatA components (TatAd, TatAy, and TatAc) have been identified in the *B. subtilis* genome. The core Tat complex consists of TatA and TatC, namely TatAy–TatCy, of which the latter is constitutively expressed and exports more substrates, including the Dyp-type peroxidase EfeB (YwbN), the Rieske iron-sulfur protein QcrA, and the alkaline phosphatase YkuE, while TatAdCd is only expressed under phosphate limitation (Pop et al. 2002). A third TatA-like protein TatAc can be combined as TatAcCd and TatAcCy. It is supposed to be an intermediate evolutionary step in TatA–TatB specialization (Goosens et al. 2015).

2.3 Type IV Secretion Systems (T4SSs)

T4SSs transport a diverse array of substrates from DNA to nucleoprotein complexes and effector proteins. They are multi-subunit, membrane-spanning translocation systems found in Gram-positive as well as Gram-negative bacteria and in some archaea (Chandran Darbari and Waksman 2015). They have evolved from a self-transmissible, single-stranded DNA conjugation system with VirB4-like AAA + ATPase to systems with an enormous diversity in their overall structure

and the types of substrates secreted. T4SSs can be divided into 3 groups (Bhatty et al. 2013): (1) a conjugation system to deliver ssDNA and one or more proteins across the membrane to the bacterial or eukaryotic target cell whereby direct cell contact is required; (2) the effector translocation system by a contact-dependent mechanism to deliver proteins to the cytosol of eukaryotic target cells, and (3) a release/uptake system to export/import molecules from/to the extracellular milieu. As a consequence, T4SSs are involved in a variety of functions including type 4 pilus formation, toxin and other protein secretion, gene transfer, and biofilm formation. Secreted substrates are involved in pathogenesis and adaptation to the cellular host environment. T4SSs translocate also proteins that form pilin-like structures (Chen and Dubnau 2004). A typical characteristic of these proteins is the presence of a specialized leader peptide that is cleaved off by a cognate membrane-bound type 4 prepilin peptidase during the process of secretion. Only T4SS conjugation systems are known in Gram-positive bacteria and Archaea to date.

2.4 Type VII Secretion System (T7SS)

Recent studies have uncovered a T7SS or early secretory antigen 6-KDa (ESX) secretion system. Originally, it was detected via an in silico analysis of the *M. tuberculosis* virulence effectors ESAT-6 (early-secreted antigenic target, 6 kDa) and the associated 10-kDa culture filtrate protein (CFP-10, EsxB) encoded by the *esxA* and *esxB* genes, respectively. They were known to be secreted despite the lack of a recognizable secretion signal (Tekaia et al. 1999). Esx proteins are characterized by their small size ($\sim$100 residues) and a WXG motif in the middle of the protein that forms a hairpin bend (Pallen 2002). Therefore, an alternative name was proposed for T7SS, the WXG100 secretion system (Wss) (Sutcliffe 2011), because distant homologues of ESAT-6/CFP-10 identified in Gram-positive bacteria all share a central WXG motif. T7SSs are widespread in actinomycetes and Gram-positive bacteria and affect a range of bacterial processes including sporulation, conjugation, and cell wall stability (Sysoeva et al. 2014). The T7SS is a complex system with many components and substrates, at least in mycobacteria. *M. tuberculosis* has five T7S systems, designated ESX-1 through ESX-5 (Stoop et al. 2012), which show similarity in gene content and gene order. Of these T7S systems, 3 are important for survival in the host, namely ESX-3, responsible for the uptake of iron and zinc, and ESX-5, responsible for the secretion of immunomodulatory effector proteins, and ESX-1 is most crucial for virulence. First detected in *M. tuberculosis* (Stanley et al. 2003), it was shown afterward also to be present in the non-pathogenic species *M. smegmatis* (Converse and Cox 2005) and the fish pathogen *Mycobacterium marinum* (Abdallah et al. 2009). On the other hand, ESX-1 is absent in *Mycobacterium bovis* BCG, the attenuated vaccine strain. PE/PPE is also secreted by the T7SS. The PE/PPE protein family, which has a conserved signature motif proline–glutamate and proline–proline–glutamate residues near the start of their encoded proteins, affects mycobacterial interactions with

the innate immune system, specifically inhibiting macrophage function (Ahmed et al. 2015). ESX-1 and ESX-5 have been implicated in major roles in the secretion of PE/PPE proteins (Abdallah et al. 2009). ESX secretion seems to be crucial for establishing and maintaining the infection for *M. tuberculosis.* A PSI-BLAST search on sequences retrieved from the NCBI or the ViruloGenome databases further evidenced the presence of ESAT-6 homologues in a number of low-GC Gram-positive bacteria, and also in several actinobacteria other than *Mycobacterium* (Pallen 2002), including all sequenced *Streptomyces* genomes such as *S. coelicolor*, *S. lividans,* and *S. scabies*. The biological importance of this pathway for streptomycetes is, so far, less well-known and begins only just to be revealed. For *S. scabies* no role in virulence for any of the T7SS components in any of the plant infection models tested could be detected, but it was demonstrated that components encoded by the T7SS gene cluster are required for the normal growth and development of *S. scabies* (Fyans et al. 2013). By mutagenesis analysis, it was shown that also proteins encoded by the *esxBA* operon and belonging to the WXG-100 superfamily play a role in morphogenesis in *S. coelicolor* (San Roman et al. 2010). In the sequenced genomes of other Gram-positive bacteria including *B. subtilis, Bacillus anthracis, Clostridium acetobutylicum, Listeria monocytogenes*, and *S. aureus,* ESAT-6 homologues were also discovered (Pallen 2002) and confirmed experimentally, for example, for *S. aureus* (Burts et al. 2005), *B. subtilis* (Sysoeva et al. 2014), *B. anthracis* (Fan et al. 2015).

2.5 Flagella Export Apparatus (FEA)

This specific protein export apparatus serves to secrete proteins that form the flagella hook, filament, and cap (Erhardt et al. 2010). Flagellar T3SS are present both in Gram-positive and Gram-negative bacteria, and it has been proposed that the type III secretion required for pathogenesis evolved from flagellar-specific T3SS (Hueck 1998). To transport proteins that form the flagella hook, filament, and cap to the distal growing end, the FEA utilizes ATP and PMF as the energy source (Paul et al. 2008). The flagellar export apparatus is thought to be the ancestor of all T3SS functions in the export of several components of the flagellum across the cytoplasmic membrane into the channel of the flagellum for assembly. Not much is known, however, about the FEA for Gram-positive bacteria. One report mentions that in *B. subtilis* FlgM is secreted by the flagellar export apparatus, consistent with the model of morphogenetic coupling proposed in *Salmonella enterica* (Calvo and Kearns 2015).

2.6 Holins

Originally holins were used to describe a group of phage-encoded pore forming membrane proteins that control access of phage-encoded endolysins to the

peptidoglycan layer. During the phage lytic cycle, holins insert into the bacterial cell membrane to translocate phage-encoded cell wall hydrolases (Wang et al. 2000). Holins may also be important for a variety of other functions in Gram-positive phage-free bacteria (Saier and Reddy 2015) such as (i) spore morphogenesis and germination in *B. subtilis* (Real et al. 2005); (ii) biofilm formation and DNA release for *S. aureus* (Fischer et al. 2014) (iii) programmed cell death and acetate metabolism in *S. aureus* (Ahn et al. 2012); and (iv) biofilm formation and oxidative stress adaptation in *Streptococcus mutans* (Westbye et al. 2013). A number of practical applications have been described for the holin/lysin systems, for example, aiming to control bacterial or viral infections (Yan et al. 2013; Shi et al. 2012) or to deliver drugs, nucleic acids, and proteins to animal cells (Kuo et al. 2009).

2.7 Non-classically Secreted Proteins

Extracellular proteomic studies revealed that a number of proteins are found in the extracellular medium without any secretion signal (Tjalsma et al. 2004). As their secretion route is not known, they are indicated as "non-classically secreted proteins" (Wang et al. 2016). Although there is a debate if these proteins in the extracellular medium are not a consequence of cell lysis, evidence was given by Yang et al. (2011), who experimentally showed that the *B. subtilis* carboxylesterase Est55, and several other cytoplasmic proteins are secreted through a process in which the protein domain structure plays a contributing role. Furthermore, using enolase to which the heterologous protein GFP was fused it was shown that the intact long N-terminus including the hydrophobic helix domain is required to serve as a non-cleavable signal for the secretion of enolase (Yang et al. 2014). Moreover, signals of "non-classically secreted proteins" could be more generally used for the secretion of heterologous proteins (Chen et al. 2016).

Despite the presence in Gram-positive bacteria of a variety of different export systems, the industrial production of (heterologous) proteins has relied primarily on the Sec-dependent pathway and to a far lesser extent the Tat pathway. Below an overview is given for using these systems in a number of different Gram-positive host cells.

3 Gram-Positive Bacteria as Hosts for Heterologous Protein Production

Gram-positive bacteria are considered interesting hosts for the production of heterologous proteins. An important advantage they have is that secreted proteins are released into the culture medium in which the conditions are favorable for the correct

folding of heterologous proteins. This contrasts to the reducing environment of the cytoplasm, in which secretory proteins that undergo oxidative folding, cannot fold. In addition, secreted proteins have the advantage that the mature protein has no methionine extension, but the authentic N-terminal amino acid sequence because of the cleavage by the signal peptidase. Therefore, several Gram-positive bacteria have been evaluated as hosts for the secretory production of heterologous proteins. Reasons why specific species have been tested are, for example, their industrial importance (or that of their relatives) and known fermentation technology, their proven secretion capacity, the absence of pathogenicity and toxicity and available tools for genetic manipulation. In Table 1, a number of possible advantages/disadvantages are compared for Gram-positive bacteria versus Gram-negative bacteria (*E. coli*).

3.1 Streptomyces

Streptomycetes belong to the phylum *Actinobacteria,* filamentous or rod-shaped bacteria, of which the filamentous forms tend to produce branching filaments. These Gram-positive soil bacteria are widespread in nature, they have a high guanine and cytosine content in their DNA (70–73 % GC) and a remarkably large genome size of up to 11.9 Mbps (*S. bingchenggensis* BCW-1; Accession ID: CP002047) (Wang et al. 2010) and with gene clusters from just a few to more than 30 pathways for the biosynthesis of a diverse range of secondary metabolites (Nett et al. 2009). Various *Actinomycetales* species are the richest source of natural products, they account for about 45 % of all microbial bioactive secondary metabolites with about 80 % of the 7600 compounds being produced by streptomycetes (Berdy 2005). Many of these secondary metabolites are of industrial and pharmaceutical value, including clinically important antibiotics for human and veterinary medicine or applied in agriculture, anticancer, and immunosuppressive agents, other pharmacologically active compounds, antiparasitic agents, and herbicides. Streptomycetes also play an important role in nature. Thanks to the large variety of enzymes they produce, such as cellulases and chitinases, they help to break down decaying vegetation as such playing an important role in the C- and N-cycle and replenishing the soil with nutrients. Typical for streptomycetes is their complex life cycle: Under suitable growth conditions, exospores germinate and subsequently develop into hyphae, which frequently become branched forming the vegetative mycelia that subsequently differentiate to aerial mycelia. Finally, aerial mycelia become divided into long chains of prespore compartments, which eventually mature to thick-walled exospores. In this phase of the life cycle, a large array of secondary metabolites is produced (van Wezel and McDowall 2011).

Strains of the class Actinobacteria include some of the most common soil, freshwater, and marine life. Other Actinobacteria inhabit plants and animals, including some pathogens such as *M. tuberculosis*, several strains of *Corynebacterium, Nocardia, Rhodococcus* spp., and a few *Streptomyce*s species (Goodfellow 2012).

Table 1 Strengths and weaknesses of Gram-positive host cells compared to *E. coli* for heterologous protein production

	Gram-positive bacteria	Gram-negative bacteria (*E. coli*)
Strengths		
Growth and physiology	Fast unicellular growth for a number of strains (e.g., *Bacillus, Lactobacilli*, *Corynebacterium*), facilitating easy fermentation standardization	Fast growth, unicellular facilitating easy fermentation standardization
		Many different expression hosts available
Media	Cheap, no complex production media	Cheap, no complex production media
Genetic tools	DNA sequences of most important strains are available	Extensive knowledge on genetics
		Many genetic tools available, hereby facilitating easy cloning and recombination
		Large variety of promoters (strong and regulated)
Yield		Very high expression levels; high biomass yield in fed-batch fermentation
Safety	GRAS status (Lactobacilli, *B. subtilis, C. glutamicum, S. lividans*)	
Product quality and downstream processing	Correct preprotein processing during secretion creating a native mature protein	
	Extracellular secretion allows easy downstream processing and product recovery	
	Extracellular secretion in the medium promotes correct folding	
	Extracellular secretion minimizes contamination from host protein	
	Hosts with different GC content	
	Extensive knowledge of industrial processes for several hosts	
Weaknesses		
Growth and physiology		Outer membrane with lipopolysaccharides requiring careful downstream processing
	Streptomyces: slower growth, mycelium morphology and clump formation for *Streptomyces* making fermentation more challenging	
Genetic tools	Limited number of genetic tools	
	Less variety of promoters	

(continued)

Table 1 (continued)

	Gram-positive bacteria	Gram-negative bacteria (*E. coli*)
Product quality and downstream processing	Product degradation likely for several *Bacillus* strains due to an excess of proteases	N-terminal methionine of non-secreted proteins; extracellular protein secretion not easily achieved
		Proteins are not secreted and inclusion bodies can be formed
		Inclusion bodies make downstream processing more expensive and environmentally unfriendly
	Minimal posttranslational modification of proteins	Minimal posttranslational modification of proteins
	Multi-domain eukaryotic proteins expressed are difficult to express as functional proteins	Multi-domain eukaryotic proteins expressed are difficult to express as functional proteins

Over the last 20 years, some of these high-GC Gram-positive bacteria have been studied extensively as an alternative expression system [reviewed in Anné et al. (2014)]. *Streptomyces* is extremely well suited for the expression of DNA from other actinomycetes and genomes of high-GC content. Furthermore, due to its high innate secretion capacity, *Streptomyces* can be a better system than *E. coli* for the production of many extracellular proteins. The host of choice for secretory protein production of heterologous proteins using Streptomyces is *S. lividans*. The main reasons are its limited restriction–modification system as such avoiding the requirement to use non-methylated DNA for transformation or conjugation, and its low endogenous protease activity, when compared to many other streptomycetes (Butler et al. 1996). Based on whole-genome sequence analysis of *S. lividans* TK24 (Ruckert et al. 2015) and RNAseq analysis, *S. lividans* transcribes only a limited number of genes encoding proteases under standard growth conditions in minimal media. Only one-third of the genes encoding secreted proteases are transcribed at medium to high level (Tobias Busche and Jörn Kalinowski, personal communication). In case of cytoplasmic or membrane-bound proteases, 75 % of the encoding genes are transcribed at medium to high level.

A wide variety of host–vector systems have been developed, many of which are based on plasmid pIJ101, such as pIJ702 and pIJ486 (Kieser et al. 2000), but in addition, a large array of new vectors has been developed including replicative plasmid vectors, integrative plasmid and phage vectors, and special vectors for integrating DNA into the *Streptomyces* chromosome [for an overview see Rebets et al. (2016)]. As protoplast transformation with *Streptomyces* is time-consuming, conjugative plasmids are most often used for cloning purposes.

A number of heterologous prokaryotic and eukaryotic proteins have been successfully produced to economically interesting yields. For example, the L-Lysine α-oxidase (LysOX) gene from *Trichoderma viride,* a homodimeric 112-kDa flavoenzyme LysOX, was cloned and heterologously expressed in *S. lividans* TK24

with an enzyme activity up to 9.8 U/mL. Cel6A-(His)6 was secreted in *S. lividans* supernatant after 84 h of cultivation amounted to 5.56 U/mL. The maximum expression level of Cel6A-(His)6 in *S. lividans* supernatant reached up to 173 mg/L after 84 h of cultivation (Li et al. 2013). Using the promoter and signal sequence of subtilisin inhibitor of *S. venezuelae* CBS762.70 (Van Mellaert et al. 1998) yields of up to 300 mg/mL biologically active mouse TNFα could be obtained and monomeric red fluorescent protein yielded up to 500 mg/mL. In some cases, proteins which could hardly or not be produced in *B. subtilis* or *E. coli* such as e.g., xyloglucanase from *Jonesia* sp. (Sianidis et al. 2006) and CelA from *Rhodothermus marinus* (Halldórsdóttir et al. 1998) were successfully produced as secreted proteins with *S. lividans*. *Mycobacterium* Ag85A produced in *S. lividans* used in combination with rCFP-10, rESAT-6, rAPA, rPstS-1 obtained via *E. coli* heterologous production in an ELISA multi-antigen was shown to be an efficient, complementary tool for the diagnosis of active pulmonary tuberculosis (Ayala et al. 2015). In other cases, however, only low yields could be obtained, a phenomenon also experienced with other expression systems. For the examples mentioned above, the Sec-dependent secretion pathway was used. For a more complete overview of heterologous proteins secreted using recombinant *S. lividans*, see Anné et al. (2012). After the detection of the Tat-dependent pathway in bacteria, one was convinced that the latter pathway could be a solution for the production of heterologous proteins not or hardly produced via the Sec pathway. However, so far this hope has not been materialized. This does not mean, of course, that the Tat pathway cannot be used for the production of heterologous proteins, but it still has to be investigated at a larger scale. Surprisingly, Sec-dependent translocation in *tat* deletions mutants and especially in Δ*tatB* mutants showed an increase (Schaerlaekens et al. 2004). No real explanation for this phenomenon could be given up to now.

3.2 Corynebacterium

Other Gram-positive bacteria with high-GC content are corynebacteria. Some species such as *C. diphtheria* are important pathogens, while the majority are not pathogenic and some are industrially very important as major producers of amino acids including glutamic acid, lysine, threonine and valine (Mitsuhashi 2014), nucleotides, and vitamins. In particular, *C. glutamicum* is of major industrial importance. Corynebacteria are also able to produce large amounts of extracellular proteins despite their diderm-mycolate cell wall. *C. glutamicum* has several attractive features, making it a potentially interesting host for the production of heterologous proteins at an industrial scale: it secretes few endogenous proteins, and no proteases in the culture filtrate are detected, although a proteome analysis revealed the presence of more than 40 proteins in the culture supernatant (Hermann et al. 2001). As a consequence, *C. glutamicum* has been shown to be a valuable host for the production of heterologous proteins including functionally active human

epidermal growth factor (Date et al. 2006), thus demonstrating its potential for industrial-scale production of human proteins. Expressed proteins can be secreted through the Sec or the Tat pathway. For example, isomaltodextranase (IMD) of *Arthrobacter globiformis* and *Streptomyces mobaraensis* pro-transglutaminase (MTG) was produced via the *C. glutamicum* Tat pathway and yields could reach approximately 100 mg/L in flask cultures. This achievement implies a great potential for the industrial-scale production of proteins that are not efficiently secreted via other systems (Kikuchi et al. 2006).

3.3 Bacillus

Bacillus species are aerobic, endospore-forming, rod-shaped cells that are ubiquitously present in nature. Various *Bacillus* species including *B. subtilis, Bacillus licheniformis,* and *Bacillus amyloliquefaciens* can produce various enzymes including proteases, amylases, and lipases in amounts up to 25 g/L. These proteins are used in different industrial and household applications such as in the cleaning, paper, textile, food, and feed industry and also for bioremediation. Because of the efficient protein secretion of these bacilli, their secretion process is intensively investigated, in particular for *B. subtilis* which for this purpose is considered the model organism among Gram-positive bacteria. More insight in the fundamentals of the secretion process is meaningful to develop strains with superior secretion capacity. Whereas improvement of protein secretion was in general quite successful for homologous proteins, production of heterologous proteins was more cumbersome. The most important reasons therefore are a combination of the properties of the secretion pathway, the bacterial cell envelope, and the presence of a number of membrane-bound, cell-wall-bound, and secreted proteases (Westers et al. 2008). For example, the quality control proteases, WprA, HtrA, and HtrB, and feeding proteases, NprB, AprE, Epr, Bpr, NprE, Mpr, and VprA, quickly degrade slow-folding, or wrongly folded proteins (Pohl and Harwood 2010). Nevertheless, some heterologous prokaryotic and eukaryotic proteins could be well expressed, as quantities ranging from less than 10 μg up to more than 200 mg/L could be obtained [for an overview, see Schumann (2007), Kang et al. (2014)] Other reports mention the overproduction of α-amylase from *B. licheniformis* in a recombinant *B. subtilis* strain (Chen et al. 2015a); and the accumulation of biologically active hIL-3 in the growth medium in amounts of up to 100 mg/L (Westers et al. 2006).

3.4 Lactobacilli

Lactic acid bacteria (LAB) are a phylogenetically diverse group of Gram-positive, aerotolerant, non-spore-forming rods or cocci with a low-GC genome. They ferment carbohydrates with lactic acid as the major end-product. They are commonly

used to ferment food and as probiotics. *Lactobacilli* are part of the normal flora of humans and animals. The reason why strains of LAB, mainly lactococci and lactobacilli, are chosen as cell factories are plentiful: (1) many species are generally recognized as safe (GRAS) organisms because they are traditionally used in food products; *Lactobacillus* infections occur very rarely; if so, they are opportunistic infections, especially in immunocompromised individuals (Schlegel et al. 1998); (2) genetic tools for manipulation of LAB are well-developed; (3) strains of *Lactococcus lactis* secrete relatively few proteins and express very few membrane-bound or secreted proteases. In such strains, HtrA is the only protease that has been characterized on the extracellular surface (Poquet et al. 2000). (4) A variety of constitutive and inducible vector systems have been developed including the well-known 2-component NIsin-Controlled gene Expression system (NICE). This system derives from the auto-induced expression of nisin, an antibacterial polycyclic peptide produced by some strains of *L. lactis* (Kuipers et al. 1998). When nisin binds to the receptor NisK, a membrane-associated protein kinase, NisR becomes phosphorylated. The activated NisR then induces the nisin promoter (Mierau and Kleerebezem 2005). Small amounts of nisin are sufficient to activate the promoter. The NICE system is widely used for the expression of heterologous proteins in *L. lactis* (Mierau and Kleerebezem 2005).

Several proteins could be efficiently secreted using *L. lactis* and *Lactobacillus plantarum* as hosts as illustrated hereafter with a few examples. *S. aureus* nuclease NucA was secreted in amounts of more than 200 mg/L culture medium (Tremillon et al. 2010; Karlskas et al. 2014); the C-terminal region of staphylococcal HtrA transmembrane proteins could efficiently be produced and secreted in *L. lactis* as correctly folded proteins (Samazan et al. 2015); *L. lactis* was shown to be a suitable host to express a variety of structurally different glycoside hydrolases of LAB in their native, multi-meric form (Schwab et al. 2010); *B. subtilis* oxalate decarboxylase (Anbazhagan et al. 2013); *Thermobifida fusca* cellulases and xylanases to convert biomass to biofuels using *Lactobacillus plantarum* as a host (Morais et al. 2013); recombinant *L. lactis* was able to secrete biologically active human interferon-γ inducible protein-10 (Villatoro-Hernandez et al. 2008). Chitosanase (CsnA) and a β-mannanase (ManB) from *B. licheniformis* and *B. subtilis*, respectively, were efficiently produced in *L. plantarum* (Sak-Ubol et al. 2016). More examples can be found in an overview given by Le Loir et al. (2005). For the expression and secretion of the heterologous proteins, mentioned in these examples, different plasmids (inducible), promoters, and signal peptides have been used.

An additionally interesting aspect of recombinant lactococci is that they can be used as live vectors for the delivery of antigenic or therapeutic proteins to mucosal surfaces in the framework of the treatment of allergic, infectious, and gastrointestinal diseases. This use has the potential to elicit antigen-specific secretory immunoglobin A responses at mucosal surfaces (Pontes et al. 2011; Bermudez-Humaran et al. 2011). *L. lactis* engineered to secrete bioactive molecules such as Interleukin-10 (IL-10), an anti-inflammatory cytokine, was shown to be beneficial in the treatment of inflammatory bowel disease (IBD). *L. lactis* producing IL-10 markedly reduced the pathology of colitis in several mouse models. Another

strain expressing a Fab against TNF-α was also effective in the treatment of IBD (Vandenbroucke et al. 2010). A truncated version of the A2 antigen from *Leishmania donovani* expressed in *L. lactis* as cell wall anchored protein effectively gave induced high levels of antigen-specific serum antibodies (Yam et al. 2011). Subcutaneous immunization with live *L. lactis* expressing the LACK antigen anchored to the cell wall and *L. lactis* secreting IL-12 significantly delayed footpad swelling in *Leishmania major* infected BALB/c mice (Hugentobler et al. 2012). For a more extensive overview of protection studies with LAB vaccines, see among others in Wells and Mercenier (2008).

In addition, lactobacilli can be used for the delivery of DNA at the mucosal membrane. To improve the delivery, so-called invasive *L. lactis* strains were developed. These recombinant strains expressed *S. aureus* fibronectin-binding protein A or internalin A of *Listeria monocytogenes* (de Azevedo et al. 2015) or a mutated form thereof (Pontes et al. 2014) to increase the invasiveness of the strain and subsequent DNA delivery. Several examples showed the feasibility of this approach to elicit an immune response using DNA vaccination with *L. lactis* as a vector.

3.5 Clostridium *and* Bifidobacterium

These genera have in common that they are both anaerobic and Gram-positive. Clostridia are rod-shaped, endospore-forming bacteria with a low-GC content. *Clostridium* is mainly known for its pathogens like *Clostridium tetani, Clostridium botulinum,* and *Clostridium perfringens*, which secrete potent toxins leading to the life-threatening diseases tetanus, botulinum, and gangrene, respectively. *Clostridium difficile* is mainly a nosocomial pathogen and the causative agent of antibiotic-associated pseudomembranous enterocolitis. From the biotechnology point of view, *C. acetobutylicum* is an important producer of butanol and acetone. Pasteur was the first to report the fermentation process of butanol already in 1861 (Jones and Woods 1986). Stimulated by the First World War, the acetone and butanol fermentation gradually became a most important industrial fermentation processes until the 1950s. Then, the interest for the fermentative production of butanol and acetone wasted away because of cheap crude oil prices as raw material for their chemical synthesis. However, the acetone and butanol fermentation recently regained importance in the framework of renewable resources for biobutanol production. For this reason, several individual cellulosomal components and mini-cellulosomes from *C. thermocellum* and *Clostridium cellulolyticum* have been cloned and expressed in *C. acetobutylicum* and their gene products such as Cel5A, Cel8C, and Cel9M were successfully secreted into the medium. On the other hand, other cellulosomal component proteins such as Cel48F, Cel9G, and Cel9E could

not be recombinantly obtained (Mingardon et al. 2011). The development of allele-coupled exchange (ACE) (Heap et al. 2012) for *Clostridium* allowed the generation of stable and iterative integrations within a relatively short period of time. As such three genes of *C. thermocellum*-derived cellulosome components inserted into the genome of *C. acetobutylicum* could be efficiently expressed, with subsequent secretion and complex formation (Kovacs et al. 2013).

Clostridium spp. came also in the focus of research for a totally different application, notably in the framework of anticancer therapy. As anaerobic bacteria survive and multiply only under anaerobic conditions, after intravenous administration they selectively colonize, if present, in the hypoxic/necrotic areas of solid tumor tissue, a consequence of inconsistent and insufficient blood flow within regions of the tumor. When administered to a tumor-bearing body, the hypoxic/necrotic zones in solid tumors are ideal niches for the growth of anaerobic bacteria, as other tissues in a body are well oxygenized. This selectivity is repeatedly demonstrated with experimental animals (Umer et al. 2012; Roberts et al. 2014). Strains tested during these experiments belong to the following species: *C. acetobutylicum*, *C. sporogenes*, an attenuated *C. novyi*-NT or *C. beijrinckii* and more recently also *C. ghonii* (Wei 2013). When multiplying in these tumor tissues, they destroy (part of) the tumor by the hydrolytic enzymes they produce. Besides, by combinatorial treatment antitumor activity can be increased by using recombinant strains in which genes for prodrug converting enzymes are cloned. Examples of such genes are nitroreductase that converts the CB1954 prodrug to an active antitumor drug (Theys et al. 2006; Heap et al. 2014) or cytosine deaminase (CDase) which converts 5-fluorocytosine to the cytotoxic drug 5-fluorouracil. Using appropriate signal peptides, CDase can be secreted in sufficient amounts to be of biological relevance as is also the case for cloned TNF-α or IL-10, cytokines with an antitumoral but also with an immune stimulating activity to combat the tumors [for an overview see Umer et al. (2012)].

Bifidobacteria are non-spore-forming, non-motile, often branched rod-shaped bacteria with a % GC value of circa 60. They are ubiquitously found in the intestines, and because they have a probiotic function they are often used in yogurt. Engineered *Bifidobacterium adolescentis* expressing endostatin (specifically inhibiting the proliferation of vessel endothelial cells stimulated by basic fibroblast growth factor, and hence also inhibiting tumor growth), when intravenously administered to tumor-bearing mice were found only in the tumor. They inhibited angiogenesis and tumor growth (Li et al. 2003). It must be mentioned that in this case endostatin was not secreted into the medium, but was expressed intracellularly. The above-mentioned results show the potential of using (recombinant) anaerobic bacteria as tumor-specific vectors to transport anticancer genes/proteins to tumor tissues.

4 Bottlenecks in Protein Secretion and Possible Remediation

To be economically interesting, recombinant strains should produce sufficient amounts of the protein of interest. However, in many cases concentrations are low or too low. Various reasons could be at the root of the problem (see Table 2). As a consequence, several approaches can be followed in an attempt to increase the yield: from strain engineering at several levels up to fermentation optimization and this using either rather empirical approaches up to more sophisticated ones, applying state-of-the-art technologies (Fig. 2). In the following paragraphs, several examples will be used to illustrate these possibilities.

4.1 Modulation of Components of the Protein Secretion Pathway

It is evident that the promoter is of utmost importance for high expression levels. Looking into the literature, a wide variety of promoters are available for different bacteria, either constitutive or inducible. Sources of promoters vary from native to

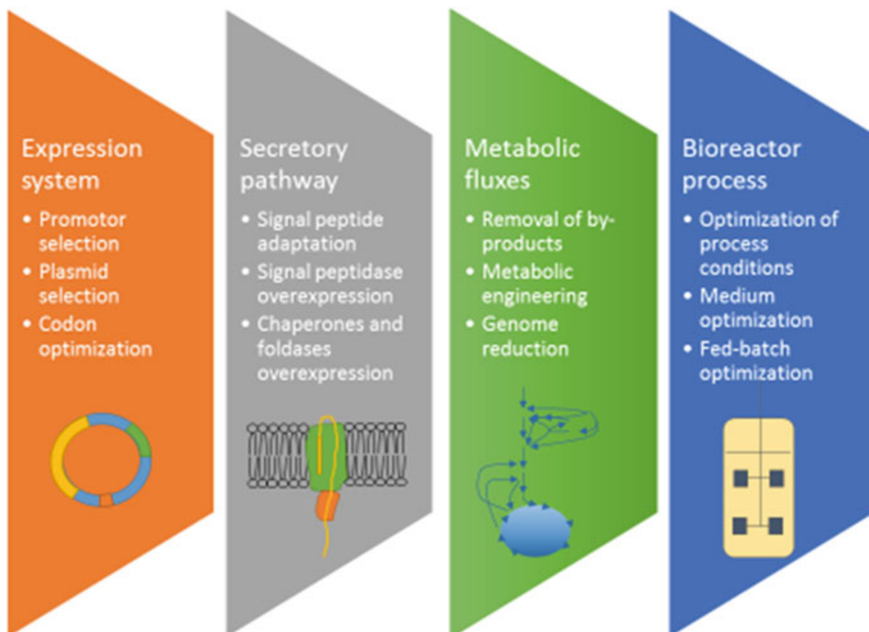

Fig. 2 Schematic overview of possible strategies for increased protein production

Table 2 Potential bottlenecks for the secretory production of recombinant proteins by Gram-positive bacteria and possible solutions

Bottleneck	Possible solutions
Unsatisfactory expression	Change promoter
	Change host strain
	Change expression vector
Translation	Optimize Ribosome Binding Site
	Use codon-optimized genes
Secretion	Modify signal peptide
	Use Tat instead of Sec secretion (or vice versa)
	Overexpress chaperones/foldases
	Overexpress signal peptidase(s)
	Overexpress Sec or Tat components
	Mutate or make hybrid SecA
	Use fusion proteins
Incorrect folding	Overexpress foldases and chaperones
Breakdown of protein of interest	Delete protease(s)
Metabolic burden	Identify problem via—omics analysis
	Bypass or solve problem by strain modification (overexpression or deletion of target gene)
	Reduce genome
Yield	Optimize fermentation
	Change carbon source and/or medium

synthetic promoter libraries. Promoter strength can be compared using reporter proteins such as β-glucuronidase (GusA) (Siegl et al. 2013), mCherry (Heiss et al. 2016) and others. To have a recent overview of different promoters used for Streptomycetes, we refer to Rebets et al. (2016).

The availability of strong promoters does not guarantee that the protein will be produced at sufficient levels, because bottlenecks are mainly at the secretion level and more downstream. Therefore, several approaches have been attempted related to the protein secretion pathway itself (Fig. 1).

4.1.1 Signal Peptide Adaptation

It is not clear what determines the sequence of an "efficient" signal peptide; therefore, several approaches are being investigated including single amino acid replacements in the N-terminal region of the signal peptide (Lammertyn and Anné 1998) or testing large Sec-type signal peptide libraries (Mathiesen et al. 2009; Degering et al. 2010). Considerable differences exist between different signal peptides but also for different mature proteins for the same set of signal peptides and this independently of the host tested. Amino acid extension by which the amino

acids in the neighborhood of the signal peptidase cleavage site are conserved could also be helpful (Sevillano et al. 2016).

4.1.2 Signal Peptidase Overexpression

As explained above, several proteins constitute the protein secretion pathway with proteins different for the Sec- and Tat-dependent pathway. What they have in common is the signal peptidase type I, an enzyme needed to release the signal peptide from the mature protein upon translocation. Most Gram-negative bacteria have only 1 chromosomally encoded signal peptidase I, but some have more. For example, *Pseudomonas aeruginosa* has two (LepB and PA1303), each with a different role in virulence and physiology (Waite et al. 2012). On the other hand, with the exception of i.a. *Streptococcus pneumoniae*, *M. tuberculosis,* and *S. aureus,* many Gram-positive bacteria have more than 1 SPase I with a maximum for *B. subtilis* which contains 5 chromosomally encoded SPase I genes (*sip*); namely *sipT*, *sipS*, *sipU*, *sipV,* and *sipW*. In addition, various *B. subtilis* strains contain in addition 2 plasmid encoded (*sipP*) SPase I genes (van Roosmalen et al. 2004). SipS and SipT are key to preprotein processing, while SipU, SipV, and SipW appear to play minor roles in protein secretion (Tjalsma et al. 1998). Overexpression of signal peptidases resulted in an increased level of secretion (Pummi et al. 2002). Similarly, in *Bacillus megaterium* MS941, co-overexpression of its unique signal peptidase SipM increased the heterologously expressed *Leuconostoc mesenteroides* dex-transucrase (Malten et al. 2005).

S. lividans has 4 chromosomally encoded SPases I (SipW, SipX, SipY, and SipZ) (Parro et al. 1999). None of the individual SPases I was found to be essential for cell viability, indicating they have an overlapping substrate specificity. Nevertheless, SipY was shown to be the major SPase as the secretome of a SipY-deficient strain is severely affected (Palacin et al. 2002) on growth as well as on morphology (Gullón et al. 2012). Moreover, in particular cases the SipY mutant was shown to have some interesting advantages compared to the wild-type *S. lividans* for the overproduction of extracellular agarose probably as a consequence of the diminished extracellular proteolytic activity (Gabarró et al. 2016). Alternatively, co-overexpression of all 4 *sip* genes led to the highest increase in total preprotein processing capacity of the cell, and also to a higher amount of extracellular human CC16. It can thus be concluded that for *S. lividans* both overexpression and inactivation of individual Sip proteins can be advantageous for yield improvement of secretory proteins (Geukens 2002).

4.1.3 Overexpression of Chaperones and Foldases

When heterologous proteins are expressed, they ought to obtain their correct conformation, both for activity and stability, as incorrectly folded proteins are more prone to proteases and aggregation. Correct conformation is obtained with the help

of chaperones. Folding facilitators are, for example, the DnaK chaperone (DnaK, DnaJ, and the nucleotide-exchange factor GrpE) and GroEL/ES (mainly studied in *E. coli*) which assist the folding of newly synthesized proteins and prevent protein aggregation. Following secretion, peptidyl-prolyl cis/trans isomerases (PPIases) and disulfide bond formation proteins (Dsb) are needed for formation and rearrangement of disulfide bonds. Six Dsb (A-G) have been identified in *E. coli*. In Gram-positive bacteria, this folding process is hardly investigated except for *Bacillus*. The main components responsible for secretory protein folding and quality control in *B. subtilis* are summarized in Sarvas et al. (2004). The lipoprotein PrsA, a putative peptidyl-prolyl cis/trans isomerase, plays a major role in protein secretion by helping the posttranslocational extracellular folding of several secreted proteins. The presence of the extracytoplasmic enzymes thiol-disulfide oxidoreductases (TDOR) in *B. subtilis* were identified based on data searches. They were named BdbA (YolI), BdbB (YolK), BdbC (YvgU), and BdbD (YvgV) (Kouwen and van Dijl 2009). It was shown that BdbB and BdbC are involved in the folding of tested proteins including PhoA and A13i-Bla (Bolhuis et al. 1999). Overexpression of chaperones is considered an attractive approach to increase yield of heterologous proteins (Mogk et al. 2002). Overexpression of *prsA* in *Bacillus,* for example, increased the secretion of α-amylases, recombinant protective antigen, and a protease (Williams et al. 2003; Vitikainen et al. 2005; Chen et al. 2015b). Overexpression of the *B. subtilis* TDOR genes, however, did not improve the folding of the secreted heterologous proteins as investigated with PhoA. On the other hand, overexpression of the DsbA from *S. aureus* or the *S. carnosus* DsbA allowed the secretion of active PhoA at elevated levels (Kouwen and van Dijl 2009). Folding modulators in other Gram-positive bacteria and their impact on heterologous protein production have not yet been investigated. Based on homology searches in the genome of the *S. lividans* TK24, chaperones and peptidyl-prolyl cis/trans isomerases have been identified (Tobias Busche and Jörn Kalinowski, personal communication), but their effect on (heterologous) protein secretion has still to be investigated.

4.1.4 Sec Components

The Sec translocase consists of the integral membrane complex SecYEG, the ATPase SecA, and two additional membrane proteins that promote the release of the mature peptide across the cytoplasmic membrane (SecD and SecF). In *E. coli,* SecD and SecF are two separate membrane proteins, whereas in *B. subtilis* they are present as one polypeptide, named SecDF (Bolhuis et al. 1998). It is required to maintain a high capacity for secretion. It is not essential, but its deletion results in low-temperature sensitivity, aberrant cell division, and impaired protein secretion. The *secDF* deletion mutant exhibits a reduced level of secreted proteins (Vorös et al. 2014). Few attempts have been made to modulate specific Sec proteins to improve protein secretion. One example is the co-expression in *B. subtilis* of the *E. coli* SecB and a hybrid SecA of *B. subtilis* in which the 32 C-terminal amino

acids end was replaced by the corresponding SecA fragment of *E. coli* (Diao et al. 2012). This artificial protein targeting pathway led to a significant increase in the secretion of 2 model proteins tested, mutant maltose binding protein (MalE11) and alkaline phosphatase (PhoA), which *B. subtilis* could hardly export using the native secretion pathway. Kakeshita et al. (2010) deleted 61 amino acids of the C-terminus of SecA, a region, known to bind SecB in *E. coli.* In Gram-positive bacteria; however, SecB is absent, and the C-terminal region is not essential for protein secretion nor for growth. Moreover, the 61 amino acid deletion dramatically increased the extracellular production of the heterologous proteins alkaliphilic *Bacillus* sp. thermostable alkaline cellulase (Egl-237) and human interferon a (hIFN-a2b) in *B. subtilis*. Differential expression of SecA demonstrates that various precursors may exhibit major differences in their dependency on the amount of functional SecA in the cell (Leloup et al. 1999). In some cases, therefore, SecA overexpression or mutation might be beneficial for improved protein secretion as shown for cutinase in *B. subtilis* (Brockmeier 2006).

4.1.5 Tat Translocon Overexpression

The Tat pathway represents an alternative pathway for the production of secreted recombinant proteins, in particular for proteins prefolded in the cytoplasm. Notwithstanding this particular property, this pathway is so far not much explored for the industrial production of (heterologous) proteins. Several reasons account for this: Protein yield of Tat-exported proteins is in general substantially lower than of Sec-secreted proteins, and much of the synthesized proteins is retained in the cytoplasm (DeLisa et al. 2004). This might be a consequence of the fact that the export machinery becomes easily saturated not only by overexpressed target proteins, but even for native Tat-exported proteins (Barrett et al. 2003). The saturation of the export machinery can partially be relieved by co-expression of proteins of the Tat translocon. The stoichiometry of the TatABC components seems, however, critical for export function. For example, in *E. coli* overexpression of *tatB* resulted in complete loss of Tat transport, overexpression of *tatA* has a less severe but nonetheless significant effect on translocation (Sargent et al. 1999), while high expression of *tatC* can relieve saturation of the Tat pathway (DeLisa et al. 2004). Therefore, most attempts to relieve the saturation problem of the Tat translocon have been done by the coordinated overexpression of TatABC. This can certainly have a positive effect on the secretion of Tat-dependent proteins as illustrated for different organisms both Gram-positive and Gram-negative bacteria. When TatABC were overproduced in *S. lividans*, a fivefold increased xylanase C secretion was noticed. Surprisingly, the overproduction of TatABC in *S. lividans* caused a strong reduction in the secretion of the monitored Sec-dependent substrates (De Keersmaeker et al. 2006), suggesting a possible cross talk between the Tat- and Sec-dependent protein secretion pathway. Also for *C. glutamicum* overexpression of Tat components dramatically increased the secretion of *Chryseobacterium proteolyticum* pro-protein glutaminase (pro-PG) and *Streptomyces mobaraensis*

pro-transglutaminase (pro-TG). The amounts of secreted pro-PG were more than threefold higher when TatC or TatAC was overexpressed, and there was a further threefold increase when TatABC were overexpressed (Kikuchi et al. 2009). More recently, Albiniak et al. (2013) investigated the ability of an *E. coli tat* null mutant containing *B. subtilis* TatAdCd system to export the Tat-dependent model protein GFP. These cells do indeed export GFP to the periplasm with high efficiency; moreover, the protein was subsequently released into the extracellular medium during batch fermentation. The latter property was a consequence of the fact that the *E. coli tat* null mutant strain has impaired outer membrane integrity (Ize et al. 2003). Such an example shows that within Gram-positive bacteria, similar approaches can be tested to optimize secretion yield of (heterologous) proteins and to broaden the spectrum of proteins that can be produced by Gram-positive bacteria.

Tat-dependent secretion could be further increased if the phage shock protein (PspA) was overexpressed as shown for *S. lividans* (Vrancken et al. 2007), which was also true for the Gram-negative *E. coli* (DeLisa et al. 2004). The beneficial effect of PspA overproduction could be a consequence of its effector role in the maintenance of the integrity of the cytoplasmic membrane and proton motive force (Darwin 2005), the latter providing the energy in Tat-dependent protein translocation.

4.2 Omics Approaches for Enhanced Protein Secretion

Thanks to the new and fast techniques of DNA sequencing for genome analysis and RNAseq for transcriptome analysis, the availability of new methods for proteome analyses and the massive amounts of data and intelligent bioinformatics tools, it has now become more easy to have an insight at the systems-level burden caused by the overproduction of proteins, by the presence of plasmids and the biosynthesis and secretion of heterologous proteins. Stepping closer to the observed phenotype, metabolites and metabolic fluxes matter most and can be investigated using metabolomics and fluxomics techniques. Omics approaches are new drivers for rational engineering of host strains for improved fitness and increased productivity. Despite the availability of these new resources and their potentialities, so far not much research has been done in this field.

4.2.1 Transcriptomics and Proteomics

One of the first studies in which transcriptomics and proteomics studies were combined to understand the physiological and metabolic changes that occurred in high cell density cultivation (in order to obtain higher yield) was done with *E. coli* (Yoon et al. 2003). A recent study with *B. licheniformis* investigated the early responses to physical stress and nutrient starvation using integrated transcriptomics and proteomics (Voigt et al. 2014). With this approach, they were able to identify

general and specific marker proteins for different stress and starvation conditions including high protein secretion. Such markers might be interesting to follow the production process, and when needed to adapt it accordingly.

Because of its improved Tat-dependent protein secretion, the transcriptional profile of the *S. lividans pspA* mutant (see Sect. 4.1.5) was compared with the wild-type strain to see whether genes were differentially expressed in the *pspA* mutant. A number of genes were shown to be up- or downregulated in the mutant strain using a microarray screen containing all genes of *S. lividans* (Anné et al. 2014). Sixty-seven genes were twofold or more upregulated in the *pspA* mutant, while 117 genes were down-regulated. Among the genes encoding proteins for which a function is known or predicted, there are several which are linked to stress regulation (cold shock proteins, sigma factors), while others are involved in metabolic processes such as energy production and conversion and general metabolism. Among others, an increased expression of *sco6996* in the *pspA* mutant was identified. The corresponding protein SCO6996 shows some homology to the RNA polymerase sigma factor RpoE and experiments in *Salmonella* Typhimurium previously showed that RpoE can (at least partially) compensate for the lack of PspA (Becker et al. 2005). Loss of either *pspA* or *rpoE* leads to a depolarization of the membrane potential, indicating that both can affect the PMF. Moreover, PspA overproduction could partially compensate for the loss of RpoE in a *Salmonella* Typhimurium ΔrpoE strain. Furthermore, Gordon et al. (2008) recently showed that overexpression of one particular sigma factor (SigU) in *S. coelicolor* could lead to a significant alteration in the secretome. The SigU-overproducing strain secreted a much greater quantity and diversity of proteins than the wild-type strain, revealing that modification of the sigma factor expression in *S. lividans* might also affect protein secretion. Overexpression of *sco6996* led to an increased secretion of the tested proteins (XylC, eGFP) through the Tat pathway. This increase was far less pronounced than in the case of PspA overexpression, but still yielded a 20 % increase in final protein yield, which is still highly interesting. In another study, transcriptomics expression profiles of *S. lividans* TK24 strains producing the heterologous proteins human/mouse tumor necrosis factor alpha (hTNFα/mTNFα), monomeric red fluorescent protein, and xyloglucanase were compared to the corresponding control strain containing the empty vector only. Based on these analyses, a number of genes showed a significant twofold change in the recombinant strains overproducing the heterologous proteins. One gene, encoding a phosphoenolpyruvate carboxykinase (PEP carboxykinase) involved in the tricarboxylic acid (TCA) cycle and gluconeogenesis, was selected for further investigation. Overexpression of this gene in *S. lividans* TK24 hTNFα and xyloglucanase C production strains increased almost twofold the yield of recombinant hTNFα (Lule et al. 2012) and XylC in comparison with the initial production strains. Overall, these results show that a transcriptomics-based approach represents a useful tool for a rational optimization of heterologous protein secretion in *S. lividans*.

4.2.2 Metabolomics and Fluxomics

Metabolomics refers to the comprehensive analysis of small molecules produced by cellular metabolism. Metabolites inside as well as outside the cell (referred to as the endo- and exometabolome) can help to understand phenotypic behavior of recombinant strains, can be used for metabolic flux estimation, and can assist in strain development when combined with other omics data. Analysis is mostly done with mass spectrometry (MS) preceded by chromatographic separation, for which the technique of choice depends on the depth of analysis, the targeted metabolites, and the type of application [e.g., Garcia-Ochoa and Gomez (2009)]. Metabolomics, however, does not reach the same high resolution as RNAseq- or MS-based proteome analysis [e.g., Goodacre et al. (2004)]. From the vast pool of small molecules, some hundreds of metabolites can be detected in untargeted analysis but less than a hundred metabolites can usually be identified and quantified in a targeted metabolome analysis. When analyzing for intracellular metabolites, rapid quenching is required (e.g., in cold methanol, in liquid nitrogen) since their metabolite levels can quickly change (e.g., order of seconds in the central carbon metabolism) upon sampling. Quenched cells are then separated from the culture broth (e.g., centrifugation), and metabolites are extracted from the cell pellet (e.g., freeze-thawing cycles, ethanol boiling). Final derivatization follows when using GC-MS analysis. All stages need to be carefully evaluated and optimized to avoid leakage during quenching, to ensure complete extraction of metabolites, and to minimize loss of metabolites. Protocols can be found in literature but require validation prior to their application. Some examples of exo- and endometabolome analysis for Gram-positive hosts for heterologous protein expression are given in the next paragraph.

D'Huys et al. (2011) performed a comprehensive exometabolome profiling of wild-type, empty plasmid-containing and mTNFα-producing *S. lividans*. Metabolite profiles revealed that glutamate and aspartate are two important growth-determining amino acids. Cometabolization with glucose results in a high growth rate, although this fast biomass accumulation did not coincide with the highest mTNFα to biomass yield. Overflow of alanine and organic acids was typical for the fast growth phase and pointed out the imbalance in carbon and nitrogen metabolism. After depletion of aspartate and glutamate, growth slows down and mTNFα yield increases. Entering the stationary growth phase after glucose depletion, a diauxic shift toward consumption of overflow metabolites can be observed and mTNFα yield was maximal. Fed-batch processing is proposed as a strategy for tackling overflow metabolism. Based on the protocol for endometabolome analysis developed in Kassama et al. (2010), Muhamadali et al. (2015) performed a complementary endometabolome analysis which confirmed the intracellular metabolic shifts and observed organic acids and sugar overflow inside mTNFα-producing *S. lividans*.

A first example of using metabolome profiling for debottlenecking heterologous protein production is described in Korneli et al. (2012). Green fluorescent protein (GFP) production by *B. megaterium* was investigated. Large-scale bioreactor conditions are mimicked in small-scale bioreactors by intermitted feeding of

substrate, hereby inducing periods of feast and famine and resulting in a reduced process performance and product yield. Detailed time course of intracellular metabolites uncovered limitations in particular amino acids which could be resolved by supplementation of these amino acids during fermentation.

To fully understand the nature of metabolic bottlenecks and associated metabolome profiles, however, one needs to investigate metabolic fluxes in metabolic reaction networks. Fluxomics refers here to any technique applicable for this metabolic flux analysis. A genome-wide analysis of metabolic fluxes uses constrained-based metabolic modeling approaches [e.g., Lewis et al. (2012) and cited references therein], in which flux balance analysis (FBA) forms a central methodology. This FBA method is based on measured exchange rates of substrates and products, a genome-scale stoichiometric network model, steady-state assumption for intracellular metabolites, reaction flux constraints, and the optimization of a cellular objective function such as biomass growth or redox potential. This technique is tractable because of its genome-wide scope and commonly used for testing metabolic capacity of strains and for development of in silico strain engineering programs [e.g., Kim and Reed (2010), Schellenberger et al. (2011) and Wiechert (2001)]. However, exact knowledge of parallel reactions, bidirectional reactions, cycles, and flux split ratios requires ^{13}C-based metabolic flux analysis [e.g., Wiechert (2001); Zamboni et al. (2009)]. ^{13}C-based fluxomics is particularly suited for accurate flux calculations in the central carbon metabolism. Fluxes are estimated from intracellular mass isotopomer distributions in free intracellular metabolites or proteinogenic amino acids observed after feeding a ^{13}C-labeled carbon source. ^{13}C-labeling distributions will be determined by the actual reaction rates. Published flux maps are usually snapshots taken during a specific growth phase adopting a pseudo steady-state condition, but transient profiles of metabolic fluxes can also be modeled using dynamic FBA [e.g., Hjersted and Henson (2009)] or in stationary ^{13}C-based flux analysis [e.g., Wiechert and Nöh (2013)].

Genome-scale FBA was applied by D'Huys et al. (2012) to get understanding in the metabolome profiles and growth of *S. lividans* in a complex medium. In contrast to the maximum biomass formation capacity predicted from the complex medium, *S. lividans* shows suboptimal growth illustrating that rich media do not necessarily support maximum biomass growth. Overflow metabolism could not be predicted but needed to be imposed by constraints. Uptake of amino acids clearly contributed to biomass growth by augmenting the pool of available amino acids and by increasing the fluxes in the tricarboxylic (TCA) cycle. Genome-scale analysis of metabolic fluxes during human growth hormone production with *B subtilis* also showed metabolic shifts during batch fermentation on a minimal medium as well as shifts in the number of reactions that carried fluxes (Özdamar et al. 2010). A ^{13}C-based fluxomics was performed by Umakoshi et al. (2011) on batch cultures of *C. glutamicum* secreting heterologous transglutaminase (TGase). An increased flux through the pentose phosphate pathway for NADPH generation and also an increased flux through the TCA cycle augmenting the NADH/NAD and ATP/ADP ratios could be observed. This inspired the authors to increase the NADH/NAD

ratio by promoting lactate production. Elevation of the pH from 6.2 to 7.0 gave a small yet notably 1.4-fold increase in product yield.

Fluxomics can also form the foundation for rational strain engineering, i.e., for the identification of interesting gene knockouts redirecting fluxes and leading to higher yields of the desired product. Advantage of fluxomics-based strain design is that the interconnected nature of the cellular metabolic reactions is taken into account. In the broader context of LAB-based vaccine production (Oddone et al. 2009), for example, applied dynamic genome-scale FBA to identify targets for enhanced heterologous protein production in *L. lactis*. Green fluorescent protein (GFP) expression (as a model protein) could be increased with 15 % by implementing predicted gene targets.

A recent trend to increase production performance of microbial host cells is genome reduction where large segments of the genomic DNA are removed with the intension of removing metabolic ballast and increasing resources of product formation. Genome reduction efforts often focus on production of secondary metabolites [e.g., Gomez-Escribano and Bibb (2011)], but their application to heterologous protein production has also been reported by Toya et al. (2014) and Lieder et al. (2015). The genome-reduced *Pseudomonas putida* strain created exhibits a 40 % increased GFP production (Lieder et al. 2015). Toya et al. (2014) transformed a genome-reduced *B. subtilis* (Morimoto et al. 2008) to produce heterologous cellulase and investigated fluxes in the central carbon metabolism using ^{13}C fluxomics. A 1.7-fold increase in specific cellulase production rate as compared to the parental strain with empty plasmid was attained and flux maps reflects higher pentose phosphate pathway flux and thus NADPH generation, which seems to be a general requirement for enhanced recombinant protein production.

5 Fermentation

Development of a recombinant protein production process starts under laboratory conditions in small volume shake flasks. Many screening experiments for strain selection, testing of vectors, promoters and signal peptidases, and medium optimization are required. Laboratory-scale bioreactor experiments are performed for defining optimal culture conditions such as pH, dissolved oxygen, stirrer speed, and process operation (mostly batch or fed-batch). Screening can be greatly speed up by using high-throughput microbioreactor platforms. A first proof of principle for filamentous bacteria is reported for *S. coelicolor* by Sohoni et al. (2012). Rohe et al. (2012) developed a milliliter bioreactor screening platform and validated this setup for heterologous protein secretion of *Fusarium solani pisi* cutinase by *C. glutamicum*. Multiple cultures are run in parallel in a microtiter plate cultivation system (Biolector®) in which each well is stirred and dissolved oxygen, pH and biomass are monitored online. A liquid-handling robot enables swift media preparation, online dosing (e.g., for optimization of inducer concentration and time) and sampling. Auxiliary devices can be added for sample handling and online assaying

(Unthan et al. 2015). Scalability for *C. glutamicum* was proven excellent to 1-L bioreactors and even up to 20-L bioreactors. This advanced automated platform of Rohe et al. (2012) proved also be applicable for *Streptomyces*, a species with a more complex growth physiology including clump formation (J. Koepff and M. Oldiges, Forschungszentrum Jülich GmbH, personal communication).

Yield of secreted heterologous proteins and productivity are affected by the medium constitution, growth phase and fermentation time. Optimization of the medium composition can be done randomly but a more rational approach is guided by design of experiments techniques [e.g., Mandenius and Brundin (2008)]. A satisfactory recombinant protein production typically requires nutrient-rich media containing amino acids. Pozidis et al. (2001), for example, illustrate fermentation upscaling and medium selection for murine tumor necrosis factors alfa (mTNF-α) production with *S. lividans* TK24 and tested different amino acid rich media. Final biomass and heterologous protein concentration show no consistency and protein yields are better in a less efficient growth medium, even with the use of a constitutive promotor. D'Huys et al. (2011) further demonstrated that the yield of mTNF-α increases after glutamate and aspartate depletion from the nutrient-rich medium and when the biomass growth rate slows down. Secreted protein yield becomes highest in the stationary phase but the fermentation must be stopped when degradation by extracellular protease activity is observed. Although complex media are commonly used in *Streptomyces* cultivations, cells do not exploit their nutritional resources optimally toward biomass formation and by-product formation is usually observed (D'Huys et al. 2012). Media can also be defined and identification of the most essential amino acids can be a tedious job. Nowruzi et al. (2008), for example, screened for the impact of different amino acids and defined mixtures of them on the heterologous expression of recombinant human interleukin 3 (rHuIL-3) in *S. lividans* 66.

Batch and fed-batch cultivation are both industrially relevant *modi operandi* in heterologous protein production. Batch operation is simple and flexible, but controlled substrate addition in fed-batch fermentations enables metabolic control and high density growth by avoiding by-product formation related to overflow metabolism. Minimization of acetate overflow by restricting the specific biomass growth rate is a common practice in *E. coli* recombinant protein production [e.g., Eiteman and Altman (2006)]. After reaching the high cell density at the end of the substrate feeding phase, heterologous protein expression is started by inducer addition. Heterologous protein production by Gram-positive bacteria can also be favored by fed-batch operation, although the fed-batch control strategy depends on the expressed protein, the promotor used and other factors. No standardized approaches as those established for *E. coli* seem to exist. A good illustration of the diversity and complexity in fed-batch operation strategies is given in Oztürk et al. (2016). Fed-batch processes for homologous and heterologous expression by *Bacillus* are reviewed and associated fed-batch operation strategies are derived. No consensus fed-batch operation strategy could be found and feeding strategies largely depended on promoter choice.

From a practical and financial point of view, microorganisms growing as single cells are more favorable for easy fermentation. A filamentous growth morphology increases the power input requirements in aerated bioreactors, increases cooling requirements and results into clump formation which introduces diffusion limitations and biomass heterogeneity. Not all cells in pellets and clumps of *Streptomyces* are biologically active and metabolically equal (Manteca et al. 2008; Rioseras et al. 2014). Large and dense clump formation can be partially counteracted by addition of hydrophilic polymers like polyethylene glycol [e.g., Kieser et al. (2000)]. Clump formation, mycelial differentiation and programmed cell death have been linked to the production of antibiotics and are important factors for secondary metabolite production by *Streptomyces* [Rioseras et al. (2014) and cited references therein]. Studies linking heterologous protein secretion and cellular morphology are limited, but van Wezel and coworkers found that overexpression of *ssgA* in *Streptomyces* leads to more fragmented growth without substantial clump formation and increases heterologous protein yields in *S. lividans* 1326 (van Wezel et al. 2006; Sevillano et al. 2016). Morphology engineering could be a strategy for enhanced protein secretion.

Upscaling from laboratory-scale to industrial-scale bioreactors generally reduces the final product yields due to spatial gradients, oxygen transfer limitations, shear stress, medium differences, etc. Guidelines for upscaling of bioreactor processes are described in many handbooks on bioreactor process engineering or fermentation technology [e.g., Doran (2013) and McNeil (2008)]. Many empirical relations have been established to estimate important parameters like oxygen transfer coefficients in large-scale fermenters, but upscaling generally remains a trial and error process based on some generally accepted rules of thumbs [e.g., Garcia-Ochoa and Gomez (2009)]. Keeping a constant dissolved oxygen concentration for aerobic processes with non-filamentous organisms or keeping a constant impeller tip speed for filamentous organisms are good starting points for upscaling. Oxygen supply to the production biomass is of key importance, and spatial differences are common in large bioreactors. Oscillations in oxygen availability can lead to temporary metabolic shifts, by-product formation, and eventually multi-substrate growth. Kass et al. (2014) characterize these effects for *C. glutamicum* and strain robustness can be guaranteed when temporary gradients are limited to the scale of a few minutes. Metabolic robustness toward spatial gradients in large bioreactors is a desired property of a robust production strain, but effects on heterologous protein secretion are not yet characterized.

6 Conclusion

Bacteria are fast-growing organisms able to be replicate in cheap culture media. This property makes them potentially attractive cell factories in biotechnological processes such as for the production of heterologous proteins. For well-known reasons, *E. coli* remains the host of choice but cannot meet all expectations as not

all proteins are equally well produced in this Gram-negative host. Therefore, several Gram-positive bacteria are being explored and used as alternative bacterial hosts for the production of heterologous proteins. An important motivation therefore is that Gram-positive bacteria secrete proteins in the extracellular medium allowing correct folding, a problem encountered with *E. coli* in which case proteins often are precipitated in inclusion bodies impeding the purification process of the proteins to correctly folded active compounds.

The use of Gram-positive bacteria for heterologous proteins in secreted form shows mixed successes. While some proteins are produced in industrially viable amounts as secreted proteins, others give only small or disappointingly low yields. The new techniques that in recent years became available, including next-generation sequencing (NGS), RNA-seq, proteomics, metabolomics, and fluxomics combined with more advanced bioinformatics, and the improved understanding of the protein secretion pathways help to understand the cellular background that underlies production yield. Using this understanding allows rational strain engineering, possibly in combination with synthetic biology tools, and will undoubtedly broaden the applicability of Gram-positive bacteria for efficient use in protein secretion biotechnology.

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