3 Siderotyping, a Tool to Characterize, Classify and Identify Fluorescent Pseudomonads

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3.1 Introduction

The genus *Pseudomonas* sensu stricto belongs to the γ subclass of the Proteobacteria (Kersters et al. 1996); it is limited to species of the previous *Pseudomonas* rRNA group I (Palleroni 1984). The revised genus contains about 100 species (http://www.dsmz.de/bactnom/nam2400.htm) found in all the major natural environments and using a wide range of substrates. The fluorescent species produce pyoverdins, a siderophore fluorescent under UV light. Among the fluorescent pseudomonads, pathogenic strains are harmful to human, plants or mushroom; saprophytic ones can be useful in bioremediation, biocontrol or plant growth promotion. Bacterial siderophores can be important determinants of these processes and they can be a characteristic of a species. Therefore, it is useful to determine the siderophores produced or used by a strain in a characterization, classification or identification process, a practice sometimes called siderotyping (Meyer et al. 1997).

In this chapter we will discuss the specificity of siderophores in bacteria. Methods to detect a specific siderophore will then be described. Finally, the presently published siderophores produced by the fluorescent pseudomonads, their biological importance and techniques of detection will be presented. The siderophores corrugatin, cepabactin and norcadamine produced by non-fluorescent pseudomonads (Budzikiewicz 2004) will not be described.

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3.2 Siderophore Specificity

3.2.1 Specificity of Production

Siderophore production is generally specific at the genus level; for example, pyoverdins are produced only by *Pseudomonas* spp., ornibactin by *Burkholderia* spp. and mycobactin by *Mycobacterium* spp. However, there are exceptions: pyochelin and cepabactin are produced by *Pseudomonas* and *Burkholderia* species; enterobactin by *Klebsiella*, *Enterobacter* and *Erwinia* species; and other examples include corynebactin, ferrioxamines and salmochelin (Budzikiewicz 2004; Winkelmann 2004). In the case of yersiniabactin found in the genera *Yersinia*, *Escherichia*, *Citrobacter*, *Klebsiella*, *Salmonella*, *Enterobacter*, *Pseudomonas* and, perhaps, *Photorhabdus*, horizontal gene transfer is responsible for siderophore propagation (Bach et al. 2000; Schubert et al. 2000; Oeschlaeger et al. 2003; Mokracka et al. 2004; Bultreys et al. 2006). Siderophore production can also be specific at the species level. In pseudomonads, corrugatin, pseudomonine and quinolobactin have been found in one species (Budzikiewicz 2004). Also, pyoverdins can be specific at the species level because the peptide part of the molecule varies (Meyer et al. 2002a).

3.2.2 Specificity of Utilization and Heterologous Uptake

The rule is that for each siderophore produced there is a specific receptor to translocate the iron-bound siderophore back to the cell. However, a siderophore can be incorporated by a strain that is unable to produce it. Cross interactions have been observed with pyoverdins of similar structures (Poole and McKay 2003), and the genomes of *P. aeruginosa* PAO1, *P. putida* KT2440, *P. fluorescens* Pf0 and *P. syringae* DC3000 contain 35, 29, 26 and 23 genes, respectively, that encode outer-membrane receptors (Cornelis and Matthijs 2002; Martins dos Santos et al. 2004). *P. aeruginosa* PAO1 produces only pyoverdin and pyochelin, but its pyoverdin receptor binds a structurally different pyoverdin; other receptors are specific for aerobactin, enterobactin, another pyoverdin, cepabactin, deferrioxamines, deferrichrysin, deferrirubin, coprogen, citrate and *myo*-inositol hexakisphosphate (Poole 2004). In these systems, the heterologous siderophore generally activates the production of its receptor. Siderophore uptake experiments using purified siderophore enable the detection of these siderophore-inducible or constitutive iron transport systems (Poole et al. 1990; Champomier-Verges et al. 1996; Ongena et al. 2002). The ability to use heterologous

siderophores is important for competitiveness (Champonier-Verges et al. 1996; Martins dos Santos et al. 2004).

3.3 Siderotyping Methods

In fluorescent pseudomonads, the culture media often used to produce siderophores are succinate (SMM) (Meyer and Abdhalla 1978), CAA (Meyer et al. 1998) and Na-gluconate (Budzikiewicz 1993). The glucose asparagine GASN medium (Bultreys and Gheysen 2000) has also already been used to produce pyoverdin, dihydropyoverdin, yersiniabactin and pyridine-2,6-bis(monothiocarboxylic acid).

The bacteria are generally grown in shaken liquid medium, but they have also been grown in a still Petri dish containing liquid medium and one block of agar medium. This technique enabled a considerable improving in pyoverdin and yersiniabactin productions by *P. syringae*, and in yersiniabactin production by *Escherichia coli*, compared to the technique in shaken Erlenmeyer flasks (Bultreys and Gheysen 2000; Bultreys et al. 2006).

3.3.1 Siderophore Uptake Experiments

A positive response in these tests is not always indicative of the ability to produce the siderophore because heterologous uptake can occur (Fuchs et al. 2001). Growth stimulation tests: a solid culture medium containing the strong iron chelator ethylenediaminedihydroxyphenyl-acetic acid (Meyer et al. 1997) or dipyridyl (Bultreys et al. 2001) is used. Plates are inoculated and a paper disc impregnated with a siderophore is placed on the agar. Growth stimulation around the paper disc is indicative of the uptake of the tested siderophore.

Siderophore-mediated ^{59}Fe uptake: a ^{59}Fe-siderophore complex is incubated in the presence of iron-depleted bacteria and the suspension is filtered. After washing, the radioactivity of the cells on the filter is determined (Munsch et al. 2000).

3.3.2 Electrophoretic Methods

Pyoverdin and yersiniabactin receptors and peptide synthetases can be detected after *SDS-PAGE*, either by immunoblotting or by radiography after

growth with ^{35}S-labeled amino acids (Meyer et al. 1997; Schubert et al. 1998). *Isoelectric Focusing Electrophoresis (IEF)* followed by an overlay with an iron-containing blue chrome azurol S (CAS) agarose gel (Schwyn and Neilands 1987) is a method used to detect siderophores (Koedam et al. 1994). The siderophores are separated according to their isoelectric point (pI). Pyoverdins are detected after IEF under UV light. Other siderophores are detected after overlay; in the presence of a siderophore, the iron is locally removed from the blue CAS gel, which becomes orange. Fe(III)-chelates of the pyoverdin and dihydropyoverdin of *P. syringae* can be detected after IEF by their natural color (Bultreys et al. 2001).

3.3.3 Chromatographic Methods

Pyochelin can be detected by thin layer chromatography (TLC) (Sokol 1984). Siderophores are detected by high performance liquid chromatography (HPLC), either after extraction, as for pyochelin and pseudomonine (Serino et al. 1997; Kilz et al. 1999; Mercado-Blanco et al. 2001), or in the culture medium, as for pyoverdins and yersiniabactin (Bultreys et al. 2003, 2006). They are identified by their retention times and UV spectra analyzed with a photodiode array detector.

3.3.4 Mass Spectrometry (MS)

One method couples HPLC with electrospray ionisation (ESI)-MS; it enables the determination of the molecular ion of pyoverdins (Kilz et al. 1999). Also, free pyoverdin extracts analyzed by ESI-MS and collision activation can provide information on pyoverdin structures (Fuchs and Budzikiewics 2001).

3.3.5 Use of Modified Indicator Strains

In uptake tests, a strain unable to produce a siderophore but able to use it detects this siderophore in the culture supernatant of tested strains (Mokracka et al. 2004). In another test, the up-regulation of *fyuA* in the presence of yersiniabactin, monitored by a *fyuA-gfp* (green fluorescent protein) reporter fusion, indicates the presence of yersiniabactin in the culture supernatant of tested strains (Schubert et al. 2000).

3.3.6
Genetic Tests

DNA hybridization and/or PCR were used for aerobactin, a novel catecholate siderophore, yersiniabactin, pyridine-2,6-bis(monothiocarboxylic acid) and pseudomonine (Johnson et al. 2001; Mercado-Blanco et al. 2001; Sepúlveda-Torres et al. 2002; Bultreys et al. 2006). Repressed siderophores can be detected. However, the possession of a gene does not always correlate with the ability to produce the siderophore. Also, sequence variations can induce false PCR negatives.

3.4
Siderophores of Fluorescent Pseudomonads

3.4.1
Pyochelin and its By-Product Salicylic Acid

3.4.1.1
Description and Biological Importance

Pyochelin is a salicylic acid-derived siderophore with the formula $C_{14}H_{16}N_2O_3S_2$ (molecular mass 324) produced by strains of *P. aeruginosa*, *P. fluorescens*, *Burkholderia cepacia* and *Burkholderia multivorans* (Cox and Graham 1979; Cox et al. 1981; Sokol 1984). Pyochelin exists in nature as two interconvertible stereoisomers: pyochelin I and II (Rinehart et al. 1995). Fe(III)-dipyochelin has a low stability constant of 5×10^5 (Visca et al. 1992). Pyochelin complexes with Zn(II), Cu(II), Co(II), Mo(VI), and Ni(II) might deliver these metal ions to the cell (Visca et al. 1992). Complexes of pyochelin with vanadium have antibacterial effects (Baysse et al. 2000).

Pyochelin is synthesized from chorismate and two moles of cysteine. Salicylic acid and the iron-chelator and antibiotic dihydroaeruginoic acid (Carmi et al. 1994) are by-products (Crosa and Walsh 2002). PchA and PchB transform chorismate into salicylate (Gaille et al. 2002, 2003). Salicylic acid plays a role in plant defense by inducing systemic acquired resistance (SAR) (Durrant and Dong 2004), and bacteria secreting salicylic acid can induce SAR in plants (De Meyer and Höfte 1997; Maurhofer et al. 1998; De Meyer et al. 1999).

Pyochelin contributes to the virulence of *P. aeruginosa* in mice and humans (Cox 1982; Britigan et al. 1997; Takase et al. 2000), possibly because of siderophore activity (Ankenbauer et al. 1985), but ferripyochelin also enhances hydroxyl radical formation and pulmonary epithelial and artery endothelial cell injury in presence of pyocianin (Britigan et al. 1992, 1997).

Pyochelin and especially ferripyochelin have the capacity of degrading toxic organotins found in the environment, like triphenyltin chloride, by a mechanism involving hydroxyl radical formation (Sun et al. 2006; Sun and Zhong 2006).

Pseudomonads producing pyochelin can play a role in biocontrol. Pyochelin and pyocianin induce resistance against *Botrytis cinerea* in tomato, probably resulting from the formation of reactive oxygen species which play a role in plant defense (Audenaert et al. 2002). Also, pyochelin- and pyoverdin-mediated iron competition protects tomato against *Pythium* (Buysens et al. 1996).

3.4.1.2
Detection Methods

The media used to produce pyochelin are CAA, SMMCA and GGP (Cox and Graham 1979; Visca et al. 1992; Serino et al. 1997; Darling et al. 1998; Reimmann et al. 1998; Takase et al. 2000). The use of 1/10-strength nutrient broth-yeast extract amended with glucose or glycerol increases pyochelin and salicylic acid production (Duffy and Défago 1999).

Pyochelin is a light yellow siderophore with a yellowish-green fluorescence, which can be masked by the pyoverdin. In methanol, it forms a wine-red (pH 2.5) to orange (pH 7.0) non-fluorescent complex with iron. Iron free pyochelin displays absorption maxima at 218, 248 and 310 nm and iron-saturated pyochelin at 237, 255, 325, 425 and 520 (pH 2.5) or 488 (pH 7.0) nm (Cox and Graham 1979). The most widely used methods of detection are TLC and HPLC (Sokol 1984; Ankenbauer et al. 1988; Serino et al. 1997; Darling et al. 1998; Reimmann et al. 1998; Duffy and Défago 1999; Takase et al. 2000; Visser et al. 2004). In TLC, salicylic acid and pyochelin are detected in concentrated chloroform or dichloromethane extracts of acidified culture supernatants. In HPLC, pyochelin isomerizes spontaneously to pyochelin I and II (3:1 ratio); ethyl acetate extracts of acidified culture supernatants are concentrated before injection. Salicylic acid, dihydroaeruginoic acid and pyochelin I and II are identified by their retention times and UV spectra. Pyochelin can be detected by IEF and CAS overlay (Meyer and Geoffroy 2004). The PCR primers 5'-AGATGGACAAAGCGCCCTGC-3' and 5'-GATGGGCGGAGACGAACAGG-3' amplify (Tm 60 °C) 2139 bp of *pchD* of *P. aeruginosa* PAO1 encoding the salicyl-AMP ligase used in pyochelin synthesis (Serino et al. 1997; Takase et al. 2000).

3.4.2
Pseudomonine

Pseudomonine is a salicylic acid-based siderophore with the formula $C_{16}H_{18}N_4O_4$ (molecular mass 330). It is produced with salicylic acid by *P. fluorescens* AH2

isolated from spoiled Nile perch from Lake Victoria (Anthoni et al. 1995). It is also produced with pyoverdin and salicylic acid by the plant growth-promoting *P. fluorescens* WCS374 (Mercado-Blanco et al. 2001). Iron-regulated metabolites produced by WCS374 induce systemic resistance and the suppression of *Fusarium* wilt in radish, but the role of pseudomonine is not clarified.

The genes *pmsCEAB* are involved in pseudomonine synthesis (Mercado-Blanco et al. 2001). PmsB and PmsC show similarities with PchB and PchA of *P. aeruginosa*, involved in salicylate synthesis.

Pseudomonine was produced in asparagine sucrose broth (Anthoni et al. 1995) or in SMM using a pyoverdin-defective mutant (Mercado-Blanco et al. 2001). Pseudomonine emits a blue fluorescence under UV light, detectable when the pyoverdin is repressed. It shows absorbance maxima at 298, 237 and 203 nm in water (Anthoni et al. 1995), and it is detectable by HPLC (Mercado-Blanco et al. 2001). The PCR primers SAL01 5'-GAACCTCAATGACATTCGAG-3' and SAL02 5'-GTAGAGCTTCTCGACGAAAG-3' amplify (Tm 56 °C) 214 bp of *pmsB* of *P. fluorescens* WCS374. Pseudomonine production was detected by RT-PCR (Mercado-Blanco et al. 2001).

3.4.3 Yersiniabactin

3.4.3.1 Description and Biological Importance

Yersiniabactin is salicylic acid based siderophore of formula $C_{21}H_{27}N_3O_4S_3$ (molecular mass 481) produced by *P. syringae* (Bultreys et al. 2006). It was initially characterized in *Yersinia pestis*, the causal agent of bubonic plague (Haag et al. 1993; Drechsel et al. 1995; Chambers et al. 1996). Yersiniabactin is widespread among human and animal pathogenic enterobacteria, such as *Yersinia* spp., *Escherichia coli*, *Citrobacter* spp., *Klebsiella* spp., *Salmonella enterica* and *Enterobacter* spp. (Bach et al. 2000; Schubert et al. 2000; Carniel 2001; Oelschlaeger et al. 2003; Mokracka et al. 2004). The complete yersiniabactin iron uptake system, called the yersiniabactin locus, is located in a genomic high-pathogenicity island transmissible by horizontal gene transfer (Carniel 2001; Perry 2004; Schmidt and Hensel 2004; Antonenka et al. 2005). Genes that are similar to yersiniabactin genes have been detected in the insect pathogen *Photorhabdus luminescens* (Duchaud et al. 2003) and in the plant pathogen *P. syringae* (Buell et al. 2003), which is divided into many pathovars and nine genospecies (Gardan et al. 1999). A yersiniabactin locus was recently detected in three genospecies of *P. syringae*, but most generally not in the other genospecies and in representatives of other pseudomonads (Bultreys et al. 2006). However, the locus organization and gene sequences are different compared to the enterobacteria, and

the locus is not located in a high-pathogenicity island as usual; also, the gene organizations of *P. luminescens* and *P. syringae* are closer to each other than to the *Y. pestis* group. Interestingly, a *Pseudomonas* strain produces the Zn-, Cu- or Fe-containing antimycoplasma agent micacocidin A, B and C, which strongly resembles yersiniabactin (Kobayashi et al. 1998).

Yersiniabactin is synthesized from chorismate by the salicylate synthase Irp9/YbtS, the salicyl-AMP ligase Irp5/YbtE, the peptide synthetase high-molecular-weight protein (HMWP) 2, the polyketide synthase/peptide synthetase HMWP1 and the thiazoline reductase Irp3/YbtU (Crosa and Walsh 2002). Only one protein usually converts chorismate in salicylate in yersiniabactin synthesis (Kerbarh et al. 2005), but two genes homologous to *pchA* and *pchB* of *P. aeruginosa* seem involved in this conversion in *P. syringae* (Bultreys et al. 2006).

In *Yersinia* spp., yersiniabactin is a virulence factor: it is indispensable in the early stage of infection of *Y. pestis*, and defective mutants of *Y. pseudotuberculosis* and *Y. enterocolitica* show a loss of virulence (Perry 2004). In *E. coli*, the involvement of the HPI in virulence is not as clear, and two in vivo studies drew different conclusions (Schubert et al. 2002; Lefranc Nègre et al. 2004).

In *P. syringae*, the existence of the genospecies 1, 2, 4 and 6 within which the strains do not produce yersiniabactin indicates that pathogenicity is possible without producing yersiniabactin. Strains defective in pyoverdin production belonging to the pathovars *tomato* and *persicae* produce yersiniabactin, but the advantage of producing both yersiniabactin and pyoverdin is unclear; the very high stability constant (4×10^{36}; Perry et al. 1999) of ferriyersiniabactin (compared with 10^{25} for the pyoverdin) could carry an adaptive advantage (Bultreys et al. 2006).

3.4.3.2
Yersiniabactin and Taxonomy

Yersiniabactin is informative on the evolution within *P. syringae* and in classification. The different GC contents in the yersiniabactin locus and in the chromosome indicate a yersiniabactin locus acquisition by horizontal gene transfer, either by an ancestor of the producing pathovars followed by stabilization in the chromosome, or by an ancestor of *P. syringae* followed by a locus deletion in an ancestor of the non producing pathovars (Bultreys et al. 2006). This is confirmed by a DNA hybridization study (Gardan et al. 1999): only the pathovars of the genospecies 3, 7 and 8 have a yersiniabactin locus, except for two pathovars belonging to the genospecies 2. Only two exceptions in the genospecies 3 (Bultreys et al. 2006) are an error and a possible misidentification: the negative strain *P. syringae* pv. *ribicola* LMG 2276 (CFBP 2348) actually belongs to the genospecies 6 (Gardan et al. 1999); *P. syringae* pv. *maculicola* CFBP 1657 used in the DNA hybridization study has a yersiniabactin locus (Bultreys and Gheysen, unpublished), whereas the genospecies of the negative strain LMG 5295 is unknown. This correlation renders yersiniabactin detection

a strong information in early classification of a strain, and in the study of the *P. syringae* evolution.

3.4.3.3
Methods and Yersiniabactin Use in Identification

As evoked in 'Siderotyping methods', yersiniabactin is detected among enterobacteria using growth stimulation tests, SDS-PAGE, HPLC of the culture medium, modified indicator strains and genetic tests.

Among pseudomonads (Bultreys et al. 2006), yersiniabactin was produced in solid-liquid GASN or King B media in still Petri dishes. Yersiniabactin is nearly colorless and orange when chelated to iron. Ferriyersiniabactin was detected in the culture medium by HPLC and identified by its spectral characteristics: absorbance maxima near 227, 255, 305, and 386 nm at pH 5.3 and pH 7.0. The PCR primers PSYE2 5'-GGCACCTGGAACAGG-3' and PSYE2R 5'-GCCAGATCGTCCATCAT-3' amplify (Tm 64 °C) a fragment of the *irp1* gene (encoding HMWP1) of 943 bp in *P. syringae* and 925 bp in *Escherichia coli*, but they are ineffective for the *P. syringae* pathovars *glycinea* and *phaseolicola* of genospecies 2, which also have a yersiniabactin locus. Dot blot using several washing conditions enabled a general detection of *irp1* in both *P. syringae* and enterobacteria. A PCR test using the primers PT3 and PT3R is specific for *P. syringae* and is proposed to identify all the yersiniabactin producing pathovars on their respective hosts (Bultreys and Gheysen 2006).

3.4.4
Pyridine-2,6-bis(monothiocarboxylic acid) (PDTC)

PDTC (molecular mass 198) was purified from strains of *P. putida* (Ockels et al. 1978) and *P. stutzeri* KC (Lee et al. 1999). It converts the pollutant CCl_4 to CO_2 in iron-limiting conditions (Lee et al. 1999; Lewis et al. 2001). In *P. putida*, PDTC is a siderophore repressed by the pyoverdin (Lewis et al. 2004). It forms 2:1 complexes of comparable stability (~10^{33}) with iron, nickel and cobalt (Stolworthy et al. 2001). PDTC forms complexes with 14 metals and can protect bacteria from mercury, cadmium, as well as selenium and tellurium oxyanions; it is involved in an initial line of defense of bacteria against toxicity from various metals and metalloids (Cortese et al. 2002a; Zawadzka et al. 2006). Cu(II) and PDTC render strains able to reduce amorphous Fe(III) oxyhydroxide (Cortese et al. 2002a).

At least five genes are involved in the PDTC system in *P. stutzeri* KC (Lewis et al. 2000; Sepúlveda-Torres et al. 2002). Two of them, *orfF* and *orfI*, were not detected in seven *P. stutzeri*, one *P. balearica*, or a *P. putida* producing PDTC. This suggests that *P. stutzeri* KC may possess a distinct biosynthetic pathway

(Sepúlveda-Torres et al. 2002), which may have been acquired from mycobacteria and cyanobacteria (Cortese et al. 2002b).

PDTC is produced in DRM (Lee et al. 1999) or GASN (Bultreys and Gheysen 2000) shaken liquid media (Zawadzka et al. 2006). It forms a blue Fe(II)-complex and a brown Fe(III)-complex; the absorbance maxima of Fe(III)-$(PDTC)_2$ are 345, 468, 604 and 740 nm and of Fe(II)-$(PDTC)_2$ are 314 and 687 nm (Cortese et al. 2002a). PDTC concentration is usually determined by measuring the absorbance of Fe(II)-$(PDTC)_2$ at 687 nm (Budzikiewicz et al. 1983). CCl_4 degradation can indicate PDTC production: cultures in medium D supplemented with CCl_4 are incubated under denitrifying conditions and CCl_4 is measured by gas chromatography (Tatara et al. 1993). The PCR primers CC109f 5'-GTTACAGCCGCCACCTACTGAT-3' and CC110r 5'-GCTAGGCAGAGAAGAGTCCACG-3' amplify 1112 bp of *orfF* and the primers CC111f 5'-GGCTGCTCAGTATCGGCAGTAT-3' and CC112r 5'-GGGGCGTTGACAGAGAAGTAAG-3' 1385 bp of *orfI* of *P. stutzeri* KC, and a Southern hybridization method is described (Sepúlveda-Torres et al. 2002).

3.4.5 Quinolobactin

Quinolobactin (8-hydroxy-4-methoxy-2-quinoline carboxylic acid) is a secondary siderophore with a low affinity constant for Fe(III) produced by *P. fluorescens* (Neuenhaus et al. 1980; Mossialos et al. 2000). Quinolobactin is produced in the first 16 h of iron stress before it is suppressed by the pyoverdin; this could be the first way of producing strains dealing with iron limitation (Mossialos et al. 2000; Cornelis and Matthijs 2002). A pathway for quinolobactin synthesis from xanthurenic acid has been proposed (Matthijs et al. 2004).

A quinolobactin spot was detected by IEF and CAS overlay of CAA culture supernatants for a pyoverdin-defective mutant; the spot was detected in the wild-type preparation only after concentration (Mossialos et al. 2000).

3.4.6 Pyoverdin (Pseudobactin)

3.4.6.1 Description and Biological Importance

A pyoverdin is made up of (i) a quinoline chromophore, (ii) a peptide chain of 6 to 12 amino acids containing about half *d*-amino acids and (iii) an acid (amide) side chain consisting of a dicarboxylic acid (amide) (Budzikiewicz

1993, 1997, 2004). The peptide chain is strain specific, except in one case (Barelmann et al. 2003), and is variable among strains and species. About 50 peptide chains are known, but 106 are predicted (Meyer and Geoffroy 2004). The catechol of the chromophore and two amino acids are involved in iron chelation; the amino acids are either β-hydroxy aspartic acid (in one case, β-hydroxy histidine) or hydroxamic acids derived from ornithine. Several pyoverdins varying according to the presence of a cycle in the peptide chain and by the side chain can be found in the culture medium; some are degradation products of the secreted forms (Schäfer et al. 1991; Bultreys et al. 2004). Pyoverdin precursors such as ferribactin and dihydropyoverdin, as well as isopyoverdins, vary in the nature of the chromophore; with rare exceptions (Jacques et al. 1995; Bultreys et al. 2001) they are produced at a much lower concentration than the pyoverdin. Azotobactin produced by pseudomonads and by *Azotobacter vinelandii* differs in the structure of the chromophore and the absence of the acid side chain (Demange et al. 1988).

The uptake of the Fe(III)-chelated pyoverdin is carried out by a specific receptor located in the outer membrane, which recognizes the peptide chain of its cognate pyoverdin. Pyoverdins with small differences (Ruangviriyachai et al. 2001; Barelmann et al. 2002, 2003; Fernández et al. 2003; Bultreys et al. 2004) or a common motif (Meyer et al. 1999, 2002b; Weber et al. 2000; Schlegel et al. 2001) in their peptide chain are incorporated at a reduced or high rate by a same receptor. Heterologous pyoverdins can stimulate the production of specific additional receptors and be incorporated (Morris et al. 1992; Koster et al. 1993, 1995; Leoni et al. 2000).

Pyoverdins are the principal siderophore of the fluorescent pseudomonads. The pyoverdin of *P. aeruginosa* is involved in virulence in animal models (Meyer et al. 1996; Handfield et al. 2000; Takase et al. 2000). It is able to acquire iron from transferrin and lactoferrin (Xiao and Kisaalita 1997) and it regulates the production of three virulence factors: exotoxine A, an endoprotease and pyoverdin itself (Lamont et al. 2002; Beare et al. 2003). On the other hand, the pyoverdin of *P. syringae* is not involved in virulence in cherry fruits (Cody and Gross 1987), but its production is stimulated in conditions found on plant surface when *P. syringae* has to use amino acids as carbon sources (Bultreys and Gheysen 2000).

Because of their high affinity constants for iron, between 10^{24} and 10^{27} (Budzikiewicz 2004), pyoverdins can be involved in competitiveness, growth promotion and biocontrol (Kloepper et al. 1980; Loper and Buyer 1991; Lemanceau et al. 1992; O'Sullivan and O'Gara 1992; Raaijmakers et al. 1995a; Buysens et al. 1996; Ambrosi et al. 2000; Mirleau et al. 2001). Strains producing a specific pyoverdin and using heterologous siderophores are favored (Buyer and Leong 1986; Jurkevitch et al. 1992; Raaijmakers et al. 1995b; Mirleau et al. 2000; Martins dos Santos et al. 2004). Pyoverdins can also play a role in bioremediation by degrading triphenyltin, an aquatic pollutant armful to plankton, gastropods and fish (Inoue et al. 2000, 2003). Pyoverdins bind and oxidize Fe(II) (Xiao and Kisaalita 1998).

3.4.6.2
The Peptide Chain of Pyoverdins and its Evolution

In *P. aeruginosa*, the pyoverdin genes are located in the *pvd* locus, or in a distant place for several chromophore-related genes (Poole 2004). The peptide synthetases PvdD, PvdI and PvdJ are responsible for the non-ribosomal synthesis of the peptide chain (Merriman et al. 1995; Lehoux et al. 2000). They contain as many enzymatic modules as there are amino acids in the pyoverdin; each module is specific for one amino acid (Kleinkauf and von Döhren 1996; von Döhren et al. 1999). The synthesis occurs by transfer of the intermediate from one module to the next without releasing it into the cytoplasm. One domain in each module is selective for one amino acid. A modification in this domain can induce the replacement of one amino acid by another. This is probably one way that pyoverdins evolve, as noted between the pyoverdins of *P. syringae* and *P. cichorii* differing by the replacement of one serine by glycine (Bultreys et al. 2004). Also, a deletion in a peptide synthetase can give a shorter peptide, as noted for the rare fourth type pyoverdin of *P. aeruginosa* which differs from the third type in that there is a missing glutamine (Smith et al. 2005).

The central part of the *pvd* locus is the most divergent locus between strains of the three principal pyoverdin types in the *P. aeruginosa* genome; a high variation is localized in the genes encoding the membrane receptor FpvA, the ABC transporter PvdE and the peptide synthetases PvdD, PvdJ and PvdI (Spencer et al. 2003; Smith et al. 2005). Horizontal gene transfers probably explain these differences because there are unusual codon and tetranucleotide usages. The pyoverdin and its receptor co-evolve and the changes in the receptor, resulting from horizontal gene transfers probably from other pseudomonads, *Agrobacterium tumefaciens* and *Azotobacter vinelandii*, appear to lead to further changes in the pyoverdin (Smith et al. 2005).

3.4.6.3
Pyoverdins and Phylogeny

In phytopathogenic fluorescent pseudomonads, an evolution is apparent in the peptide chains of pyoverdins (Bultreys et al. 2003, 2004): *P. syringae*, *P. viridiflava* and *P. ficuserectae* produce the same pyoverdin; the related species *P. cichorii* produces a pyoverdin differing in the replacement of one serine by glycine; and the distant species *P. fuscovaginae* and *P. asplenii* produce a clearly different, but related, pyoverdin. All these pyoverdins contain two Asp-based iron ligands and, interestingly, the producing species, apart from *P. fuscovaginae* and *P. asplenii*, are arginine dihydrolase-negative. In the arginine dihydrolase-positive species, the 4 pyoverdins of *P. aeruginosa* contain

2 Orn-based ligands, 19 pyoverdins of *P. fluorescens* contain either 2 Orn- or 1 Orn- and 1 Asp-based ligands, and 13 pyoverdins of *P. putida* always contain 1 Orn- and 1 Asp-based ligands; the rest of the peptide chains of these pyoverdins, however, are highly variable. Then, it appears that the amino acids involved in iron chelation evolve slowly because of their necessity for the pyoverdin activity and they could therefore be useful markers in phylogeny (Bultreys et al. 2003). This is confirmed because *pvdA* and *pvdF* necessary for the synthesis of the iron ligand formyl-hydroxyornithine from Orn (Visca et al. 1994; Wilson et al. 2001), and the Orn-based ligands, are conserved in the 4 *P. aeruginosa* pyoverdin types (Smith et al. 2005), although the conserved *pvdF* is positioned just beside the highly variable region of the *pvd* locus.

It is difficult to find filiations between pyoverdins of the arginine dihydrolase-positive species, except when a pyoverdin is produced by different species (Fuchs and Budzikiewicz 2001; Meyer and Geoffroy 2004). The diversifying selection observed in the *pvd* locus in *P. aeruginosa* (Smith et al. 2005) indicates that the heterogeneity apparent in pyoverdins can be higher than the general heterogeneity in a species. Horizontal gene transfers could explain the numerous pyoverdin structures found in *P. fluorescens* and *P. putida* and restrict the phylogenic information available from pyoverdins. It is also a sign of a high selection pressure for new specific iron-chelating systems in the rhizosphere (Smith et al. 2005).

3.4.6.4
Pyoverdins and Taxonomy

Siderotyping of pyoverdins by IEF and siderophore uptake is used to revise the genus *Pseudomonas*, alongside polyphasic taxonomic approach, and the general rules are defined: (i) all strains belonging to a given species produce an identical pyoverdin; and (ii) each species is characterized by an original pyoverdin. Indeed, complex taxonomic studies were elegantly and rapidly confirmed by the description of one corresponding siderotype for at least 11 species: *P. mandelii*, *P. monteilii*, *P. rhodesiae*, *P. tolaasii*, *P. costantinii*, *P. brassicacearum*, *P. thivervalensis*, *P. salomonii*, *P. mosselii*, *P. libanensis* and *P. kilonensis* (Meyer et al. 2002a; Meyer and Geoffroy 2004). However, the same pyoverdin can be produced by related species: *P. syringae*, *P. viridiflava* and *P. ficuserectae*; *P. asplenii* and *P. fuscovaginae*; *P. fluorescens*, *P. cedrella*, *P. orientalis*, *P. palleroniana* and *P. veronii*; *P. brenneri* and *P. gessardii*; and *P. jessenii* and *P. migulae* (Bultreys et al. 2003; Meyer and Geoffroy 2004). Also, several pyoverdins can be produced in one species: *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. grimontii* and *P. lini* (Fuchs and Budzikiewicz 2001; Meyer and Geoffroy 2004). The recent observation that the heterogeneity in pyoverdins can be higher than in the species (Smith et al. 2005) should be noted in future work.

3.4.6.5
Pyoverdins and Identification

The strains producing the same pyoverdin are grouped into siderovars. Once the siderovars are defined in a species, pyoverdins are accurate tools of identification; this can be achieved by comparison with a reference (Bultreys et al 2001, 2003), or a general database regrouping the characteristics of all the pyoverdins can be consulted (Meyer et al. 2002a; Meyer and Geoffroy 2004).

Identification is often required for phytopathogenic species. The presence of two Asp-based iron ligands in the peptide chain of the pyoverdins of *P. syringae*, *P. cichorii* and *P. fuscovaginae* influences the color and spectral characteristics of the Fe(III)-chelates between pH 3 and 7. This is easily detected using visual and spectrophotometric tests differentiating phytopathogenic and saprophytic species; the pathogens are identified by HPLC (Bultreys et al. 2001, 2003).

3.4.6.6
Methods

Yellowish-green pyoverdins are detected in King B medium (King et al. 1954) under UV light (365 nm) by their bluish-green fluorescence. Absorbance maxima of free pyoverdins in the visible are 365 and 380 nm (pH<5), 402 nm (pH 7) and 410 nm (pH 10) (Meyer and Abdallah 1978). The absorbance maximum of Fe(III)-chelated typical pyoverdins is near 400 nm (pH 3–8), with broad charge transfer bands at 470 and 550 nm (Budzikiewicz 1993). The Fe(III)-chelated atypical pyoverdins containing two Asp-based iron ligands of *P. syringae* and *P. cichorii* behave as a typical pyoverdin at pH<3.5 (brown), but the maximum shifts at 408 nm at pH>5.5, without charge transfer bands (orange); the pyoverdin of *P. fuscovaginae* behaves identically at neutral pH but the maximum shifts to only 402.5 nm at pH 3, without marked charge transfer bands (dark orange). This can be observed visually and by spectrophotometry in GASN medium, which enables the detection of phytopathogenic species (Bultreys et al. 2001, 2003).

Three principal methods are used to analyse pyoverdin diversity. *MS*-related methods are the most powerful but they are expensive (Kilz et al. 1999; Fuchs et al. 2001). *IEF* of iron-free pyoverdins coupled with ^{59}Fe uptake experiments have become the methods of choice (Meyer et al. 2002a). The pI of pyoverdins detected under UV light from concentrated CAA pyoverdin extracts (generally 2 or 3 isoforms differing in the side chain) are determined. Each strain is defined by its IEF pattern. This method enables a database to be constituted. The problems encountered are the migration of the pyoverdins at the cathode or the anode because the limits of analysis are between pH 4 and 9 (Achouak et al. 2000; Fuchs et al. 2001), the observation of the same pI for pyoverdins varying by a neutral amino acid (Bultreys et al. 2003; Fernández et al. 2003) and the ob-

servation of different patterns for strains producing the same pyoverdin (Fuchs et al. 2001; Meyer and Geoffroy 2004). Free or Fe(III)-chelated pyoverdins of *P. syringae* and *P. cichorii* are visually detected but not differentiated by IEF (Bultreys et al. 2003). *HPLC* analysis of GASN culture medium is used to identify phytopathogenic fluorescent pseudomonads (Bultreys et al. 2003). Atypical pyoverdins of *P. syringae*, *P. cichorii* and *P. fuscovaginae* containing two Asp-based iron ligands are differentiated and identified by their retention time and their absorbance maximum being near 408 nm at pH 5.3. The technique is easy to use and more accurate than IEF, but less suited to developing a general database.

3.5 Conclusions

The diversifying evolution detected in pyoverdin genes in *P. aeruginosa* and the abundance of outer membrane receptors in pseudomonads indicate that iron competition is an important selection pressure in the rhizosphere. The existence of secondary siderophores and the production of outer membrane receptors in the presence of heterologous siderophores imply sophisticated regulatory mechanisms in iron-deficient environments. Therefore, the importance of siderophores in fitness and competitiveness seems clear and it can explain the observed involvement of siderophores in virulence, plant growth promotion and biocontrol. Also, siderophores can have useful or toxic secondary effects, and the information from siderophores produced by a pseudomonad is important in understanding, controlling and using pseudomonad behavior. Recent findings on the horizontal gene transfers of complete or partial siderophore systems indicate that gene exchange has been a creative force during evolution, alongside clonal divergence and periodic selection. This probably restricts the phylogenic information available from siderophores, although this information is sometimes clear, and can occasionally be linked to the transfer event itself. Some siderophores currently appear to be specific to certain species or to a group of closely related species and they are therefore of interest in taxonomy and identification. Existing methods enable researchers to determine rapidly the siderophores produced by a pseudomonad, and they are of interest for medicine, agronomy, environmental science and systematic bacteriology.

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