7 Pyoverdine Synthesis and its Regulation in Fluorescent Pseudomonads

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7.1 Introduction

7.1.1 Historical Perspective

The genus *Pseudomonas* (γ-subclass of *Proteobacteria*) comprises a number of species belonging to the rRNA homology group I of *Pseudomonas sensu lato* (De Vos et al. 1989). These species are also referred to as the fluorescent pseudomonads group, which include *Pseudomonas aeruginosa, Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas syringae*, and some minor species. Fluorescent pseudomonads are Gram-negative motile rods that usually grow aerobically and show remarkable nutritional and ecological versatility (Palleroni 1992). They are typical inhabitants of water and soil that can very commonly be isolated from the rhizosphere of many plants and have been well studied as biocontrol agents (Haas and Defago 2005). The type species of the genus, *P. aeruginosa*, is a widespread bacterium that has gained increasing medical significance as an opportunistic pathogen, though it normally occupies soil and water habitats (Hofte et al. 1990; Lyczak et al. 2000). The fluorescent pseudomonads are usually distinguished by the production of diffusible yellow-green fluorescent pigments, although some of the so-called fluorescent pseudomonads (e.g., *Pseudomonas stutzeri*, *Pseudomonas mendocina* and *Pseudomonas alcaligenes*) do not actually produce such pigments (Palleroni and Moore 2004). A number of terms have been used for the fluorescent compounds released by *Pseudomo-*

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nas spp., including fluorescein, pseudobactin and pyoverdine (sometimes spelt pyoverdin). The last of these is now in general use and will be used here.

Pyoverdines were first studied in the late nineteenth century and attracted considerable interest in the first part of the last century, in particular with regard to the growth media and conditions that promoted their production (reviewed in Budzikiewicz 2004); however, their biological function was not known. An important advance was the development of a minimal medium and methodology for the purification of pyoverdines which permitted subsequent chemical characterization (Meyer and Abdallah 1978). In the same paper, the authors recognized that the biosynthesis of pyoverdine by *P. fluorescens* is suppressed by the presence of free iron in the growth medium and that the pyoverdine they were studying had a very high affinity for iron Fe^{3+} ions. It was proposed that pyoverdines act as siderophores (iron transporters) for the pseudomonads and this was demonstrated in an accompanying paper (Meyer and Hornspreger 1978).

A large number of pyoverdines have now been purified from different strains and species of *Pseudomonas*, with each strain making a single (and characteristic) form of pyoverdine although they can utilize many different pyoverdines (see chapters by Meyer and by Cornelis, this volume). *P. aeruginosa* strains, for example, secrete one of three distinct pyoverdines (Types I–III). Pyoverdines are composed of three parts: (i) a fluorescent dihydroxyquinoline chromophore that is common to all pyoverdines; (ii) an acyl side chain (either dicarboxylic acid or amide) bound to the amino group of the chromophore; and (iii) a strain-specific peptide chain linked *via* an amide bound to the C1 (rarely C3) carboxyl group

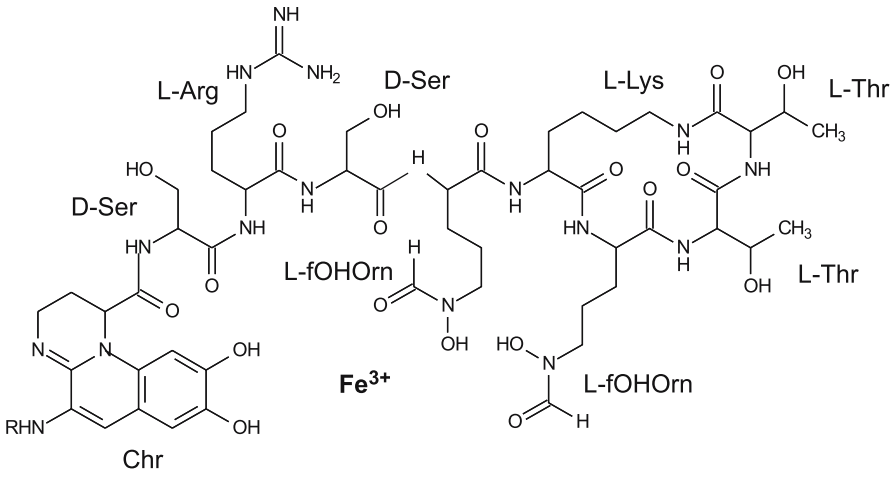


Fig. 7.1. Pyoverdine made by *P. aeruginosa* strain PAO1. The chemical structure of this pyoverdine was determined by Briskot et al. (1989). R is a variable acyl side chain (dicarboxylic acid or amide; usually α-ketoglutarate, succinate or glutamate). The catecholate group on the chromophore (Chr) and the hydroxamate groups on the L-N^5-formyl-N^5-hydroxyornithine (L-fOHOrn) residues constitute a high-affinity binding site for an Fe^{3+} ion. Amino acids are abbreviated with the conventional three-letter code. The structures of pyoverdines synthesised by other fluorescent pseudomonads are described elsewhere in this book (Meyer, this volume)

of the chromophore (Fig. 7.1). The catecholate and the hydroxamate (or sometimes β-hydroxy acid) groups provide a high-affinity binding site ($K_f \sim 10^{24}\ M^{-1}$) for Fe^{3+} ions. Pyoverdines are of considerable scientific interest because of their contribution to pathogenicity of *P. aeruginosa* (Meyer et al. 1996; Takase et al. 2000) and because of their role in the ecology of soil pseudomonads and their possible involvement in biological control of plant pathogens (O'Sullivan and O'Gara 1992; Loper and Henkels 1999; Weller et al. 2002; Haas and Défago 2005). Consequently, there has been considerable research into the genetics and biochemistry of pyoverdine synthesis and into the regulation of pyoverdine production. Most research has been carried out with a strain of *P. aeruginosa* (strain PAO1, ATCC 15692) and this strain will be the main focus of this chapter. Studies with other pseudomonads are also reviewed and indicate that *P. aeruginosa* PAO1 is a suitable model for understanding pyoverdine synthesis and regulation.

7.2 Pyoverdine Genes in *Pseudomonas aeruginosa* PAO1

The structure of pyoverdine synthesized by the PAO1 strain of *P. aeruginosa* is shown in Fig. 7.1. Initial genetic studies of mutations causing pyoverdine deficiency showed that most of the genes specific to this process clustered at a single chromosomal locus, although a few mutations were mapped to a second minor locus (Ankenbauer et al. 1986; Hohnadel et al. 1986; Visca et al. 1992). A subsequent study of 24 transposon-insertion mutations, all of which mapped to the major pyoverdine locus, showed that pyoverdine synthesis genes (*pvd* genes) were present over a chromosomal region of about 90 kbp (Tsuda et al. 1995). However, some mutations in this region did not affect pyoverdine synthesis, showing that genes with other functions were also located in this part of the genome.

These gene mapping studies were followed by DNA cloning approaches that allowed the molecular characterization of genes required for synthesis of pyoverdine (Visca et al. 1994; Cunliffe et al. 1995; Merriman et al. 1995; Miyazaki et al. 1995; McMorran et al. 1996, 2001; Stintzi et al. 1996, 1999; Lehoux et al. 2000; Mossialos et al. 2002; Vandenende et al. 2004). The functions of individual genes are described below. A major breakthrough in this field was the publication of the complete genome sequence of *P. aeruginosa* strain PAO1 (Stover et al. 2000) and the availability of a web-based genome database (www.pseudomonas.com). Further genes that are required for pyoverdine synthesis were identified in the genome using micro array and candidate gene approaches (Ochsner et al. 2002; Lamont and Martin 2003). A full-list of genes required for synthesis of pyoverdine by strain PAO1 is given in Table 7.1. The organization of these genes in the genome is shown in Fig. 7.2. It is likely that most if not all of the genes required for synthesis of pyoverdine by strain PAO1 have now been identified. Different genes or groups of genes are considered below.

Table 7.1. Genes associated with pyoverdine synthesis in *P. aeruginosa* PAO1

Gene	Function/homologous genes	Phenotype of mutant strain [a]
pvcABCD	Enzymes associated with synthesis of the pyoverdine chromophore (Stintzi et al. 1996, 1999)	Pvd^{-} [b]
ptxR	Transcriptional regulator required for expression of *pvc* genes (Stintzi et al. 1999)	Pvd^{-} [b]
pvdQ	38% identity with Aculeacin A acylase from *Actinoplanes utahensis* (Inokoshi et al. 1992)	Pvd^{-}
pvdA	L-Ornithine hydroxylase (Visca et al. 1994)	Pvd^{-}
fpvI	ECF sigma factor required for expression of *fpvA* (Beare et al. 2003)	Pvd^{+}
fpvR	Antisigma factor for PvdS and FpvI (Lamont et al. 2002, Beare et al. 2003)	Pvd^{+}
PA2389	Over 30% identity with periplasmic membrane fusion proteins (MFP) of RND/MFP/OMF-type efflux systems (Poole 2001; Zgurskaya and Nikaido 2000)	Pvd$^{\pm}$
PA2390	Over 40% identity with resistance-nodulation-division (RND)-type transporter components of RND/MFP/OMF-type efflux systems (Poole 2001; Zgurskaya and Nikaido 2000)	Pvd$^{\pm}$
PA2391	Over 30% identity with outer membrane factor (OMF) proteins of RND/MFP/OMF-type efflux systems (Poole 2001; Zgurskaya and Nikaido 2000)	Pvd$^{\pm}$
pvdP	No known function	Pvd^{-}
pvdM	23% identity with porcine dipeptidase (Rached et al. 1990)	Pvd^{-}
pvdN	26% identity with isopenicillin N epimerase from *Streptomyces clavuligerus* (Kovacevic et al. 1990)	Pvd^{-}
pvdO	No known function	Pvd^{-}
pvdF	N^5-hydroxyornithine transformylase (McMorran et al. 2001)	Pvd^{-}
pvdE	ABC transporter (secretion) (McMorran et al. 1996)	Pvd^{-}
fpvA	Ferripyoverdine receptor protein (Poole et al. 1993b)	Pvd^{+}
pvdD	Pyoverdine peptide synthetase (Merriman et al. 1995)	Pvd^{-}
pvdJ	Pyoverdine peptide synthetase (Lehoux et al. 2000)	Pvd^{-}
pvdI	Pyoverdine peptide synthetase (Lehoux et al. 2000)	Pvd^{-}
PA2403–PA2410	Expression of these genes is co-regulated with pyoverdine synthesis genes; their roles in pyoverdine synthesis (if any) are not known. Mutations in PA2403 and PA2407 do not prevent pyoverdine synthesis (Ochsner et al. 2002)	Pvd^{+}
PA2411	36% identity with thioesterase GrsT from *Bacillus brevis* (Kratzschmar et al. 1989)	Pvd^{+}
PA2412	No known function	Pvd^{-}
pvdH	Aminotransferase (Vandenende et al. 2004)	Pvd^{-}
pvdL	33% identity with TycB peptide synthetase from *Bacillus brevis* (Mootz and Marahiel 1997)	Pvd^{-}
pvdG	34% identity with GrsT thioesterase from *Bacillus brevis* (Kratzschmar et al. 1989)	Pvd^{-}

[a] For *pvd* genes, as determined by Ochsner et al. (2002) and Lamont and Martin (2003) unless otherwise indicated

[b] The effects of mutations in the *pvc* genes on pyoverdine synthesis is dependent on the growth medium (see text)

Table 7.1. *(continued)* Genes associated with pyoverdine synthesis in *P. aeruginosa* PAO1

Gene	Function/homologous genes	Phenotype of mutant strain [a]
pvdS	ECF iron sigma factor (Cunliffe et al. 1995; Miyazaki et al. 1995)	Pvd^-
pvdY	No known function (Vasil and Ochsner 1999)	$Pvd^{\pm}$
pvdX	No known function (Vasil and Ochsner 1999)	Pvd^+

[a] For *pvd* genes, as determined by Ochsner et al. (2002) and Lamont and Martin (2003) unless otherwise indicate

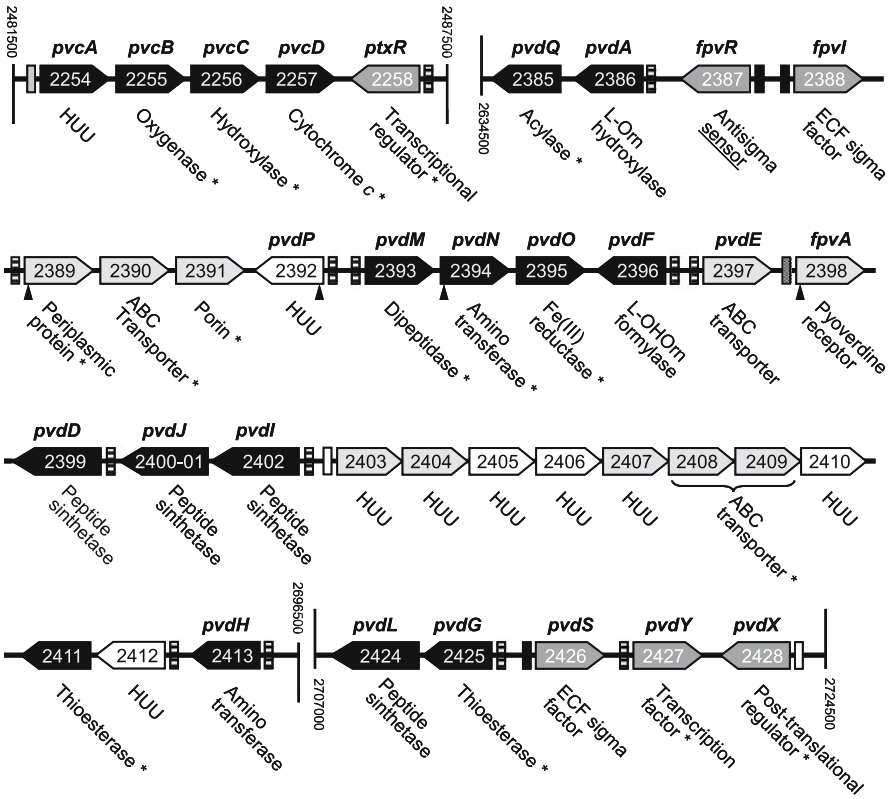


Fig. 7.2. Pyoverdine synthesis genes in *P. aeruginosa* PAO1. Genes (not drawn to scale) are oriented according to the direction of transcription. Gene numbers and map positions are according to the Pseudomonas Genome Project (www.pseudomonas.com). Gene names and function (if known) are also shown; *asterisks* denote protein functions inferred from in silico prediction. Uncharacterised gene products are indicated as HUU (hypothetical, unclassified, unknown). The *grey scale* differentiates predicted or confirmed protein functions, as follows: *black*, biosynthetic enzymes; *dark grey*, regulatory proteins; *grey*, membrane or transport proteins; *white*, HUU. Binding sites for Fur repressor protein are shown as *black boxes*, PvdS-dependent promoters as *dashed boxes*, the PtxR-dependent promoter as a *grey box*, the FpvI-dependent promoter as a *dotted box*, and uncharacterised promoters as *white boxes*. Note that PA2403–PA2410 is transcribed from an iron-regulated promoter lacking an obvious IS box. For further details and references, see Table 7.1 and the text

7.2.1
Peptide Synthetase Genes *pvdD*, *pvdI*, *pvdJ* and *pvdL*

One of the first pyoverdine synthesis genes to be characterized was *pvdD* (Merriman et al. 1995; Ackerley et al. 2003) with a mutation in this gene preventing pyoverdine synthesis. DNA sequencing showed that this gene encodes an enzyme that is part of a large family of enzymes, the non-ribosomal peptide synthetases (NRPSs). Enzymes in this class catalyze the formation of peptide bonds between substrate amino acids, and are responsible for the synthesis of a very wide range of peptides and peptide-like secondary metabolites including siderophores (reviewed in Crosa and Walsh 2002; Finking and Marahiel 2004). Although enzyme-catalyzed peptide bond formation is energetically inefficient compared to ribosomal peptide synthesis (due to the need to have a specific enzyme for each product) it enables peptide formation from amino acids that cannot be joined ribosomally, such as non-proteinogenic, D- and methylated amino acid residues.

NRPSs are very large enzymes that have a modular architecture, with each module (~1000 amino acid residues) catalyzing the incorporation of one substrate amino acid into the peptide product through a carrier thiotemplate mechanism (Kleinkauf and von Dohren 1996; Lautru and Challis 2004; Finking and Marahiel 2004). Different modules can be part of the same or different polypeptides. Each module contains a domain that is responsible for recognition and adenylation (activation) of the substrate amino acid (the A domain); a thiolation (T) domain that is the covalent attachment site for a phosphopantetheine cofactor; and, with the exception of the module responsible for incorporation of the first amino acid, a condensation (C) domain that catalyses peptide bond formation. Modules may also contain auxiliary domains that catalyze epimerization or methylation of the substrate amino acids. Each module adenylates the cognate amino acid which is then transferred to the phosphopantetheine cofactor; the C domains catalyze sequential peptide bond formation between the carboxyl group of the nascent peptide and the amino acid carried by the flanking module. The peptide is synthesized in an N-terminal to C-terminal direction. The last module is followed by a thioesterase domain that releases the assembled peptide from the phosphopantetheine cofactor. This reaction is often accompanied by cyclisation of the peptide. In most NRPSs, the organisation and the order of the modules corresponds to the amino acid sequence of the peptide product (the so-called *co-linearity* rule). The different peptides present in different pyoverdines are due to the different substrate specificities of pyoverdine-synthesising NRPSs in different strains and species (Ravel and Cornelis 2003).

Sequence analysis showed that PvdD contained two almost-identical peptide synthetase modules and the second of these was followed by a thioesterase domain (Merriman et al. 1995). This led to the hypothesis that PvdD is responsible for incorporating two L-threonine (L-Thr) residues at the carboxyl terminus of the pyoverdine peptide with the thioesterase domain catalysing release of the

nascent peptide from the enzyme, accompanied by partial cyclisation (Fig. 7.1). The first part of this hypothesis was tested in subsequent biochemical studies with recombinant protein containing the first module of PvdD. The enzyme showed high substrate specificity, having high activity with L-Thr but not with D-Thr, L-allo-Thr or L-serine (L-Ser) in enzyme assays (Ackerley et al. 2003).

Genes encoding other NRPSs required for pyoverdine synthesis were identified immediately upstream of *pvdD* (Lehoux et al. 2000). Initially, three such genes were thought to be present but this was due to an error in the draft *P. aeruginosa* genome sequence, and when this error was corrected two genes (*pvdI* and *pvdJ*) were present. Mutations in either of these genes prevent pyoverdine synthesis (Lehoux et al. 2000; Ochsner et al. 2002; Lamont and Martin 2003). Sequence analysis showed that the PvdI and PvdJ proteins contain four and two peptide synthetase modules, respectively. The substrate amino acids for different peptide synthetase modules can be predicted through the *co-linearity* rule and through bioinformatic methods (Stachelhaus et al. 1999; Challis et al. 2000). PvdI is predicted to direct incorporation of (in order) D-Ser, L-arginine (L-Arg), D-Ser, and L-N^5-formyl-N^5-hydroxyornithine into the pyoverdine peptide, with PvdJ predicted to incorporate L-lysine (L-Lys) and the second L-N^5-formyl-N^5-hydroxyornithine residue (Ravel and Cornelis 2003).

Another NRPS, called PvdL, is also required for pyoverdine synthesis and is involved in synthesis of the chromophore group (Mossialos et al. 2002). The *pvdL* gene is located in the pyoverdine gene cluster though it is not adjacent to the other NRPS genes (Fig. 7.2). PvdL is composed of four modules. The first module is predicted to be an acyl CoA ligase, as it has sequence similarities with likely acyl CoA ligase domains in the first modules of NRPSs involved in synthesis of bleomycin (Du et al. 2000) and saframycin (Pospiech et al. 1996). The predicted substrates of the remaining modules are L-glutamate (L-Glu), L-tyrosine (L-Tyr) and/or L/D-tri-hydroxyphenylalanine, and L-2,4-diaminobutyric acid (L-Dab) (Mossialos et al. 2002).

Prior to the availability of the *P. aeruginosa* PAO1 genome sequence, Georges and Meyer (1995) identified high-molecular weight proteins in a number of *Pseudomonas* species that were synthesized under conditions of iron limitation and that they termed iron-regulated cytoplasmic proteins (IRCPs). Mutant strains unable to synthesize pyoverdine had altered IRCPs. This led the authors to propose that the IRCPs are NRPSs required for pyoverdine synthesis. The estimated sizes of the four largest IRCPs for *P. aeruginosa* PAO1 (approximately 550, 480, 290 and 250 kDa) correlate very well with the sizes of PvdL, PvdI, PvdD and PvdJ predicted from the genome sequence (Table 7.1), validating the proposal that the IRCPs are indeed NRPSs. These are among the largest proteins encoded by the *P. aeruginosa* PAO1 genome (www.pseudomonas.com).

As indicated above, NRPSs require a phosphopantetheine cofactor and covalent attachment of the cofactor is catalyzed by a phosphopantetheine transferase. A phosphopantetheine transferase, PcpS, has been identified in *P. aeruginosa* and shown to be required for pyoverdine synthesis (Finking et al. 2002; Barekzi et al. 2004). Other bacterial species have at least two phosphopantetheine trans-

ferases, one for primary metabolism (fatty acid synthesis) and one for secondary metabolism, but PcpS is apparently the only phosphopantetheine transferase in *P. aeruginosa*.

7.2.2 The *pvdA* and *pvdF* Genes

The *pvdA* gene was first identified via a mutation *pvd-1* that was characterized during analysis of pyoverdine-deficient mutants of *P. aeruginosa* PAO1 (Visca et al. 1992). Assays of cell-free extracts of the *pvd-1* (synonym of *pvdA*) mutant showed that it was unable to produce L-N^5-hydroxyornithine and its L-N^5-formyl derivative. Addition of L-N^5-hydroxyornithine to the growth medium restored the ability of the mutant strain to make pyoverdine and to grow under iron-limiting conditions. These findings provided strong evidence that the *pvd-1* mutation prevents synthesis of the L-N^5-formyl-N^5-hydroxyornithine residues that are present in pyoverdine. The *pvdA* gene was subsequently cloned through complementation of the *pvd-1* mutation (Visca et al. 1994). A mutation was engineered in the chromosomal *pvdA* gene and resulted in the same phenotype as that of the *pvd-1* mutant, confirming identification of the correct gene. Analysis of the deduced PvdA sequence predicted a high degree of similarity to two enzymes that catalyze similar reactions during siderophore synthesis, an L-lysine-N^6-hydroxylase from *Escherichia coli* and an L-ornithine-N^5-oxygenase from the fungus *Ustilago maydis*. A *pvdA* homologue, called *psbA*, was later identified in the rhizobacterium *Pseudomonas* B10 and found to be essential for pyoverdine synthesis (Ambrosi et al. 2000). Recently, PvdA was successfully purified and its biochemical characterization confirmed that PvdA is an L-ornithine-N^5-oxygenase that is required for conversion of L-ornithine to L-N^5-hydroxyornithine (Ge and Seah 2006).

The *pvdF* gene was identified through sequencing of cloned DNA that originated from the pyoverdine locus (McMorran et al. 2001), with a mutation that was engineered in this gene preventing pyoverdine synthesis. The sequence showed that the PvdF enzyme had some similarities to glycinamide ribonucleotide transformylases, a group of enzymes that catalyze a formylation reaction as part of the purine synthesis pathway. This led to the hypothesis that PvdF catalyses formylation of L-N^5-hydroxyornithine to form L-N^5-formyl-N^5-hydroxyornithine that is then incorporated into pyoverdine. Chemical analyses of extracts from wild-type and PvdF mutant bacteria were consistent with this hypothesis (McMorran et al. 2001).

These data indicate that PvdA and PvdF catalyse sequential reactions to synthesize L-N^5-formyl-N^5-hydroxyornithine from L-ornithine. The L-N^5-formyl-N^5-hydroxyornithine is then available for incorporation into pyoverdine by the PvdI and PvdJ NRPSs (see above).

7.2.3 The *pvdH* Gene

The *pvdH* gene was identified through bioinformatic and gene-expression approaches and mutations that were engineered into this gene showed that it is required for pyoverdine synthesis (Ochsner et al. 2002; Lamont and Martin 2003). Sequence analysis suggested that it was an aminotransferase. This was confirmed by Vandenende and co-workers (Vandenende et al. 2004) who carried out an extensive characterization of the PvdH enzyme. It was shown to catalyze an aminotransferase reaction interconverting aspartate β-semialdehyde and L-Dab. The latter amino acid is predicted to be one of the substrates for the PvdL NRPS that is almost certainly required for synthesis of the chromophore component, as described above.

7.2.4 Other *pvd* Genes

Mutations in several other genes have been shown to prevent pyoverdine synthesis (Table 7.1). Biochemical studies have not yet been carried out on the corresponding proteins but bioinformatics analyses can give insights into the possible roles of some of these proteins/enzymes in pyoverdine production.

The first gene to fall into this category is *pvdE* (McMorran et al. 1996). The predicted sequence of the PvdE protein has all the characteristics of ATP-binding-cassette (ABC) membrane transporter proteins. Such proteins are found in many species (from microbes to humans) and are responsible for transport of a very wide range of substrates including proteins, polysaccharides, peptides, drugs, sugars, amino acids, and metal ions (Higgins 2001). ABC transporters can catalyze either import or export of their substrates and PvdE has most similarity to family members that are required for export (Saurin et al. 1999). The substrate that is transported by PvdE has not been determined; it may be pyoverdine or a pyoverdine precursor, or a protein or other factor that is required extra-cytoplasmically for pyoverdine synthesis.

Mutations in *pvdG* prevent pyoverdine synthesis (Lamont and Martin 2003) although it is possible that this is because of the effect of the mutation on expression of the downstream *pvdL* gene that is predicted to be operonic with *pvdG* (Fig. 7.2). PvdG is predicted to be a thioesterase. A second predicted thioesterase in *P. aeruginosa* PAO1 is PA2411, but a mutation in this ORF does not prevent pyoverdine synthesis (Ochsner et al. 2002). Thus, three predicted thioesterases (including the thioesterase domain present in PvdD) could be involved in pyoverdine synthesis. Other NRPS systems also have more than one thioesterase. There is evidence that the additional thioesterases may act as proofreaders, removing

incorrect substrates or aberrant intermediates that have been covalently linked to an NRPS enzyme (Heathcote et al. 2001; Schwarzer et al. 2002; Reimmann et al. 2004). Alternatively, PvdG and PA2411 may assist with release of the pre-pyoverdine peptide from PvdD, accompanied by partial cyclisation of the peptide.

The *pvdM*, *pvdN* and *pvdO* genes are likely to form an operon (Lamont and Martin 2003; Ochsner et al. 2002). Mutations in all three of these genes prevent pyoverdine synthesis. While it cannot be ruled out that the effects of the *pvdM* and *pvdN* mutations are polar on *pvdO*, it seems most likely that all three genes are required for pyoverdine synthesis. Amongst biochemically-characterised proteins, PvdM has highest sequence similarity with mammalian dipeptidases (Table 7.1). It is possible that PvdM catalyses processing of a pyoverdine precursor. Intriguingly, a gene encoding a putative dipeptidase with sequence homology to PvdM is also clustered with NRPS genes involved in synthesis of the fungal secondary metabolite sirodesmin (Gardiner et al. 2004), although for many other NRPS-generated peptides there is no evidence of a requirement for a (di)peptidase. Amongst characterized proteins, PvdN has highest sequence similarity to an isopenicillin N epimerase from *Streptomyces clavuligerus* (Kovacevic et al. 1990). However, the exact role of PvdN in pyoverdine synthesis has yet to be determined. PvdO is suggested to be an Fe^{3+} reductase (Ochsner et al. 2002) and its exact role in pyoverdine synthesis is also unclear.

The *pvdP* gene is expressed divergently from *pvdMNO*. Homologues of PvdP protein are restricted to the fluorescent pseudomonads; the exact role of this protein in pyoverdine synthesis is not known. The *pvdQ* gene is downstream from *pvdA*. PvdQ has sequence similarity to aculeacin A acylase from *Actinoplanes utahensis* as well as other penicillin amidases. The PvdQ enzyme has been shown to hydrolyse the amide bond in an acyl homoserine lactone that acts as a quorum-sensing signaling molecule in *P. aeruginosa* (Huang et al. 2003). This provides evidence that the enzyme is indeed an acylase although it presumably has a different substrate during pyoverdine synthesis; the physiological relevance of PvdQ-mediated hydrolysis of acyl homoserine lactone is not clear (Roche et al. 2004).

7.2.5 The *pvc* Genes

A cluster of four genes, the *pvc* genes, that is located about 150 kb away from the *pvd* genes have been implicated in the maturation of the pyoverdine chromophore as mutations in *pvc* genes prevented synthesis of pyoverdine (Stintzi et al. 1996, 1999). These genes are likely to be expressed as an operon, with regulation of expression having similarities to that of the *pvd* genes. However, under some conditions, *pvc* mutants are able to make pyoverdine (P. Cornelis, personal communication) and homologues of the *pvc* genes are not present in other *Pseudomonas* species that make pyoverdines (Baysse et al. 2002; Mossialos et al. 2002). The exact role of the *pvc* genes in pyoverdine synthesis is therefore not clear.

7.2.6 Intermediate-Based Studies of Pyoverdine Synthesis

Studies on the biosynthesis of pyoverdine have been carried out using classical biochemical approaches based on the identification and characterization of intermediate compounds in the biosynthetic pathway, in particular in the laboratory of H. Budzikiewicz (reviewed in Budzikiewicz 2004). A detailed description of these studies is beyond the scope of this book. Instead, we describe two particularly important intermediates that were purified and characterized and shed light on synthesis of the pyoverdine chromophore. These are ferribactin and 5,6-dihydropyoverdine. Ferribactin is pyoverdine in which, instead of the dihydroxyquinoline chromophoric group, there is the tripeptide γ–L-Glu – D-Tyr – L-Dab (Hohlneicher et al. 2001). Dihydropyoverdine is identical to pyoverdine except that two carbons in the chromophore are not saturated; consequently this form of pyoverdine does not fluoresce. The characterisation of these compounds, in conjunction with other studies, has led to a proposed pathway for chromophore biosynthesis that is described below.

7.2.7 The Biosynthetic Pathway of Type I Pyoverdine in *P. aeruginosa* PAO1

The biochemical pathway of pyoverdine synthesis is understood only in part. Genetic and biochemical studies described above show that a variety of different enzymes are involved in this process. There is good evidence that both the chromophore and the peptide moiety of pyoverdine are synthesized in a multistep reaction by the NRPSs, namely, PvdL, PvdI, PvdJ and PvdD. The pyoverdine chromophore backbone is the condensation product of L-Glu, D-Tyr and L-Dab (Bockman et al. 1997), while the pyoverdine PAO1 (Type I) peptide chain results from the condensation and partial cyclisation of eight amino acids (Fig. 7.1) (Briskot et al. 1989). The composition of pyoverdine PAO1 corresponds very well with the predicted substrates of the peptide synthetases (see above) with each NRPS module catalysing incorporation of one amino acid into the pyoverdine precursor. Furthermore, because PvdL is the only NRPS lacking an initial C-domain, its role in the first step of pyoverdine biogenesis has presumptively been assigned (Mossialos et al. 2002). The architecture of this enzyme consists of two basic modules (II and IV), a third central module with an additional epimerization domain and a first, unusual short module with a T-domain preceded by an acyl-CoA ligase-like domain, whose role has not yet been elucidated in non-ribosomal peptide synthesis (Mossialos et al. 2002). On the other hand, PvdD is the only NRPS which ends with a thioesterase domain, arguing for a function in siderophore release at the end of the assembly step (Ackerley et al. 2003). The other two synthetases, PvdI and PvdJ, are composed

of four and two basic modules respectively, with two supplementary epimerization domains in the first and third modules of PvdI, almost certainly involved in epimerization of two substrate L-Ser residues (Ravel and Cornelis 2003).

Except for PvdD, which was found to activate two L-Thr residues (Ackerley et al. 2003), the biochemistry of these peptide synthetases has not yet been addressed. However, their established modular organisation, together with in silico prediction of the substrate specificity of each A domain (Stachelhaus et al. 1999; Challis et al. 2000), makes possible the prediction of a nearly complete NRPS pathway for pyoverdine biogenesis (Fig. 7.3). Briefly, PvdL catalyzes the formation of the chromophore backbone by condensation of L-Glu, D-Tyr and

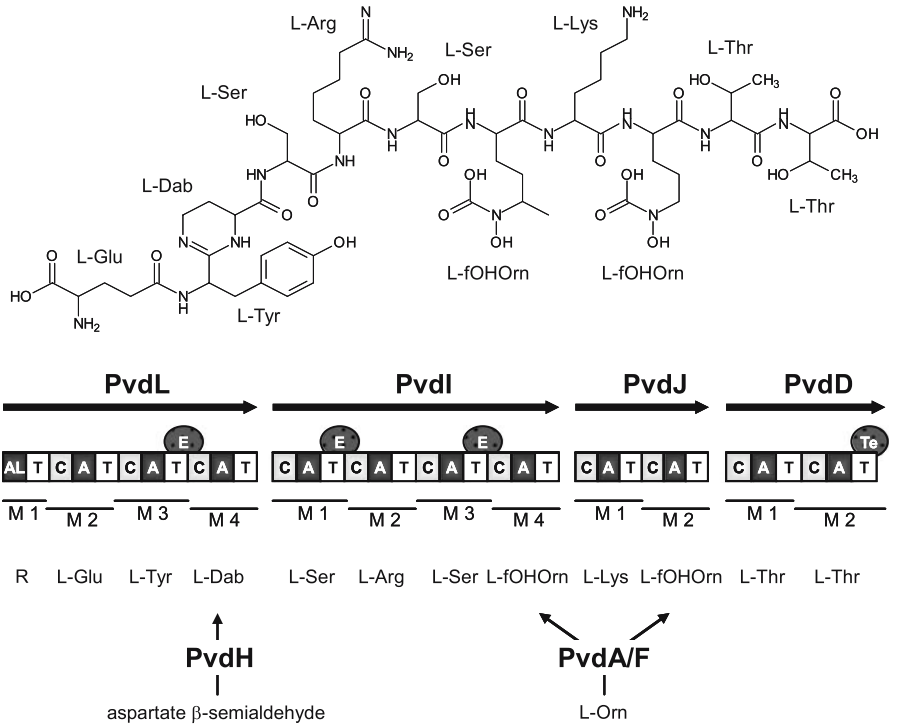


Fig.7.3. Pyoverdine biosynthesis in *P. aeruginosa* PAO1: predicted amino acid specificity of non-ribosomal peptide synthetases. NRPS enzymes are shown as *black arrows* with the respective modular domains as *adjacent squares*. Protein domains are: AL, acyl CoA ligase; A, adenylation; T, thiolation; C, condensation. Each enzyme module is indicated as M with progressive numbering. *Circles* indicate auxiliary modules: E, epimerisation; Te, thioesterase. The immature pyoverdine backbone (ferribactin) resulting from the activity of NRPSs modules is shown on the *upper part*. Amino acid substrates recognized by each module are indicated: L-Dab, L-2,4-diaminobutyrate; L-fOHOrn, L-N^5-formyl-N^5-hydroxyornithine. Other amino acids are abbreviated with conventional three-letter code. Note that the existence of E domains in PvdL (M3) and PvdI (M1 and M3) results in the incorporation of D-isomers in the pyoverdine precursor peptide. The substrate R of the putative AL domain (M1) is not known. Precursor-generating enzymes PvdH, PvdA and PvdF are shown in the *lower part*

L-Dab (Mossialos et al. 2002); the C-domain of module one of PvdI then promotes the attachment of this precursor to the first amino acid residue (D-Ser) of the growing chain. Further elongation of the peptide is provided by the linear progression of the PvdI, PvdJ and PvdD modules, while release of the peptide arises from the PvdD thioesterase domain activity. According to this model, the order of the peptide chain assembly would exactly match the physical order and orientation of NRPS gene modules responsible for peptide moiety biosynthesis, from the N-terminus of PvdI to the C-terminus of PvdD. The structure of ferribactin (see above and Fig. 7.3) exactly matches the predicted structure of this proposed precursor peptide.

In support of the proposed pathway, the first A domain of the NRPSs cluster is always specific for the first amino acid of the peptide moiety, i.e. the one joined to the chromophore, as also documented for other *P. aeruginosa* pyoverdine types (Smith et al. 2005). Moreover, PvdL is the only pyoverdine-related NRPS that is encoded by the genomes of all fluorescent *Pseudomonas* (Ravel and Cornelis 2003). Conversely, NRPSs responsible for peptide chain elongation are highly variable among different pseudomonads, consistent with the amino acid variability of the peptide moiety (Ravel and Cornelis 2003).

Although the specific modular organization of the pyoverdine NRPSs corresponds well with the amino acid sequence of the pyoverdine peptide backbone, there is no evidence for a putative cyclisation domain (Ravel and Cornelis 2003) that would be expected from the partially cyclic peptide chain structure (Fig. 7.1). Given that isolated thioesterase domains have been involved in peptide cyclisation (Kohli et al. 2001), this enzymatic activity could be provided either *in cis* by the thioesterase domain of PvdD or *in trans* by PA2411 and/or PvdG.

Genetic and biochemical characterization of PvdA and PvdF, described above, shows that they are necessary for synthesis of L-N^5-formyl-N^5-hydroxyornithine that is present in the pyoverdine peptide (Fig. 7.1). Whether these modification events occur before or during the amino acid incorporation into the peptide chain has still to be fully determined. However, both *pvdA* and *pvdF* mutants are non-fluorescent (Visca et al. 1992, 1994; McMorran et al. 2001), suggesting that N^5-formyl-N^5-hydroxyornithine formation is a pre-requisite for chromophore assembly or maturation.

The events leading to maturation of the pyoverdine chromophore are not so fully understood. PvdH catalyses the formation of L-Dab, one of the predicted substrates of PvdL. The isolation of ferribactins (described above) is consistent with the proposal that PvdL catalyses condensation of L-Dab, L-Glu and D-Tyr (although a subsequent reaction must take place so that L-Glu is joined through the γ- rather than the α-carbon). Transformation of ferribactins to pyoverdines requires a series of redox reactions followed by tautomerization leading to dihydroxyquinoline ring formation (Dorrestein et al. 2003). Furthermore, the L-Glu attached to the original D-Tyr by its γ-carboxyl group can be transformed to α-ketoglutaric acid, succinamide, malamide or hydroxylated to free acid (Schafer et al. 1991). It has been proposed that some modifications

of the chromophore moiety could take place in the periplasm by the action of hemoproteins (Baysse et al. 2002), since heme has been identified as a necessary component for pyoverdine biosynthesis (Baysse et al. 2001). It may be that the *pvc* genes contribute to this step. Additionally, *pvd* genes that have not yet been assigned biochemical functions (see above; Table 7.1) may catalyse reactions involved in chromophore maturation.

Following (or concomitant with) synthesis, pyoverdine must be secreted into the extra cellular milieu. The mechanism of secretion is not known although one obvious candidate protein that may be involved is PvdE (see above). PA2389–PA2391 are co-regulated with pyoverdine genes (Ochsner et al. 2002), encode proteins with the characteristics of a secretion system and are located at the pyoverdine locus (Fig. 7.2) so that it is tempting to speculate that they are involved in pyoverdine synthesis. However, mutations in these genes do not prevent secretion of pyoverdine (Ochsner et al. 2002; Lamont and Martin 2003). A different secretary system has also been implicated in pyoverdine secretion (Poole et al. 1993a) but the possible contribution of this system has not been further studied.

7.3 Iron Regulation of Pyoverdine Synthesis: The Master Roles of Fur and PvdS

As for all siderophores, pyoverdines are produced in response to nutritional iron deficiency. This is inferred by the evidence that pyoverdine synthesis is repressed in suspension cultures grown in chemically-defined laboratory media containing more than 5 μM $FeCl_3$ (Meyer and Abdallah 1978). Above this concentration, Fe(III) is likely to diffuse passively into the cell where it is reduced to Fe(II) in the cytoplasm, thereby acting as a co-repressor for the global regulatory protein Fur (ferric uptake regulator) (Escolar et al. 1999). Iron-dependent transcriptional repression is primarily dependent on the binding of the Fur-Fe(II) holorepressor complex to the promoters of iron-regulated genes. In the case of pyoverdine genes Fur is not the only regulatory protein. Positive control of siderophore synthesis also occurs through a membrane-spanning signalling mechanism (Sect. 7.4).

Fur is an essential protein in *P. aeruginosa* PAO1 and in other fluorescent pseudomonads which tolerate only partial loss of Fur function through amino acid substitutions resulting from point mutations in the *fur* gene (reviewed in Vasil and Ochsner 1999; Venturi et al. 1995b). *P. aeruginosa fur* mutants show pleiotropic phenotypes and deregulation of siderophore synthesis (Vasil and Ochsner 1999). The iron regulon has been extensively investigated in *P. aeruginosa* PAO1, confirming the role of Fur as the master regulatory protein in iron metabolism. The expression of more than 100 genes shows strict dependence on iron deficiency (Ochsner et al. 2002; Palma et al. 2003), but only a minority of these genes contain a Fur-binding sequence (the Fur box, GATAATGATAAT-

CATTATC) (Lavrrar and McIntosh 2003) in their promoter. This apparent discrepancy is due to the occurrence of Fur-controlled transcriptional regulators that regulate subsets of genes, most of which are involved in the uptake of endogenous or exogenous iron chelates (Visca et al. 2002). The crystal structure of *P. aeruginosa* Fur has been solved (Pohl et al. 2003). The protein consists of two domains: the dimerisation domain containing the regulatory Fe(II)-binding site and the DNA-binding domain containing a structural Zn(II) atom. Fur-Fe(II) associates as a dimer, and two dimers have been proposed to recognise 5-bp spaced operator sites located on opposite sides along the DNA sequence, thereby hindering access of vegetative (RpoD-dependent) RNA polymerase (RNAP) to the promoter. Within the pyoverdine region of the *P. aeruginosa* PAO1 chromosome, Fur-binding sequences were identified in the promoter regions of the *pvdS*, *fpvI* and *fpvR* genes (Beare et al. 2003; Cunliffe et al. 1995; Lamont et al. 2002; Miyazaki et al. 1995; Ochsner and Vasil 1996).

Fur can also act as a positive regulator of the expression of some genes involved in oxidative stress response (superoxide dismutase, *sodB*), Krebs cycle (succinate dehydrogenase, *sdh*) and iron storage (bacterioferritin, *bfrB*). Positive iron regulation of gene expression is indirect and involves two small RNAs, PrrF1 and PrrF2, tandemly arranged on the *P. aeruginosa* PAO1 chromosome (Wilderman et al. 2004). PrrF RNAs act at the post-transcriptional level to reduce the expression of target genes. They reduce mRNA stability by increasing the rate of its decay through complementary base pairing at the 5´-end of target transcript (Wilderman et al. 2004). Transcription of PrrF1 and Prrf2 only occurs in low-iron conditions as expression of these RNAs is repressed by Fur-Fe(II) binding to their promoters. This system enhances expression of target genes (including *sodB*, *sdh* and *bfrB*) in response to iron proficiency.

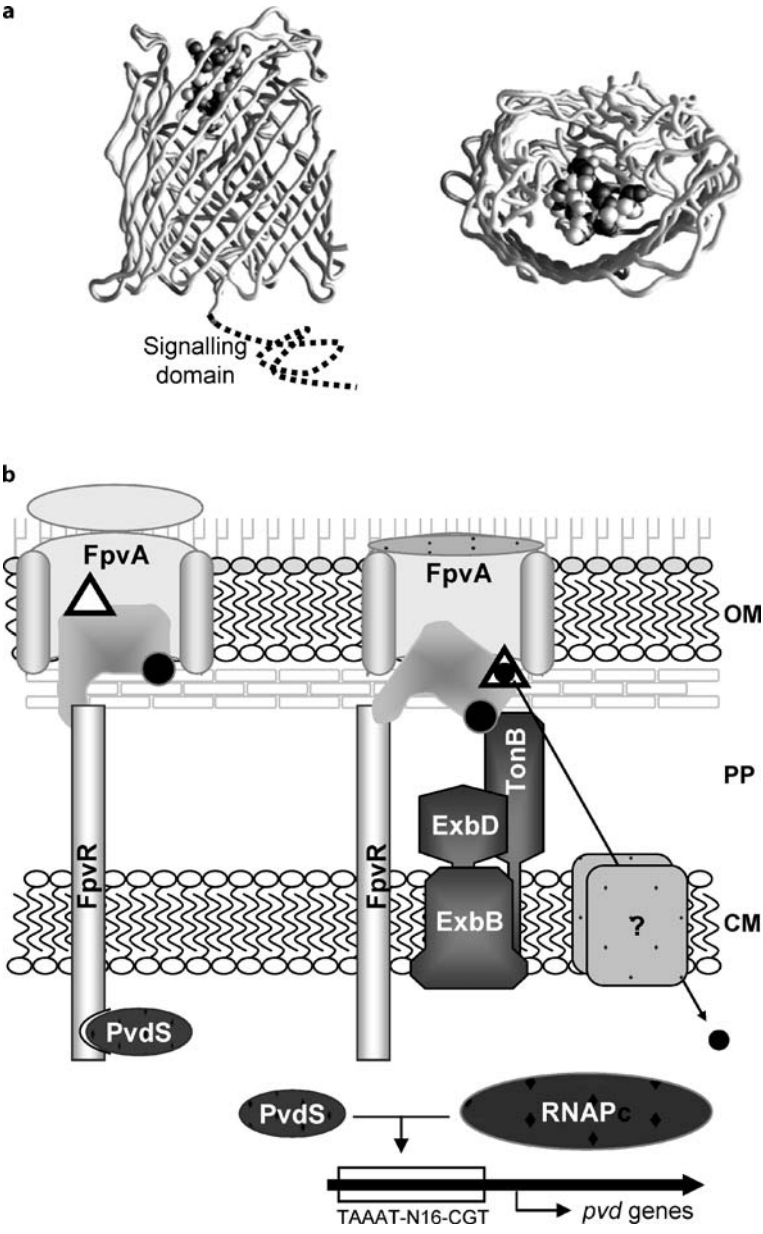
Despite their iron-regulated expression profile, all pyoverdine biosynthesis and uptake genes or operons lack Fur-binding sequence in their promoter region but contain the iron starvation (IS) box (originally described as [G/C]CTAAATCCC, but currently reconsidered as TAAAT), a typical sequence signature involved in the recognition and binding of the alternative sigma factor PvdS (Rombel et al. 1995; Wilson and Lamont 2000). PvdS is the prototypic member of IS sigma factors, an iron-responsive subgroup of the extracytoplasmic function (ECF) family of RpoD-like sigmas (Leoni et al. 2000). The *pvdS* promoter region contains three overlapping Fur boxes and binds Fur in vitro (Ochsner et al. 1995), thus explaining the tight iron-regulated expression of pyoverdine genes. PvdS is a 187-amino acid protein that spontaneously associates with the core fraction of RNAP to form a transcriptionally active RNAP holoenzyme (Leoni et al. 2000; Wilson and Lamont 2000). As a typical ECF sigma, PvdS is very much smaller than the primary sigma factor RpoD and has only a low amount of sequence similarity. This is consistent with its promoter recognition sequence, which is markedly different from the RpoD consensus. PvdS-dependent promoters are all characterised by an IS box at about position –33 relative to the transcription start point, followed by the CGT triplet 16/17 nt downstream (Ochsner et al. 2002; Wilson and Lamont 2000). The TAAAT-$N_{16/17}$-CGT element has

been recognised with minor variation in the promoter region of 19 out of the 26 PvdS-regulated genes of *P. aeruginosa* PAO1 (Ochsner et al. 2002).

7.4 Receptor-dependent Autoregulation of Pyoverdine Synthesis

Positive control of siderophore transport has been extensively investigated in *P. aeruginosa*. In this bacterium, siderophores account for at least three levels of regulation and signalling. First of all, iron supply via siderophores enables *P. aeruginosa* to switch-on the activity of Fur repressor thereby silencing the Fur regulon. Second, receptor binding by both endogenous and exogenous siderophores communicates the real efficacy of a given siderophore in iron supply, acting as a stimulus for co-ordinate expression of the cognate uptake and, eventually, biosynthesis genes. Third, and apparently unique to the pyoverdine system, binding of the siderophore to its receptor acts as an extracytoplasmic stimulus for a signal transduction cascade leading to the overexpression of several exoproducts, including a protease, an exotoxin, and pyoverdine itself. Four protein partners located in distinct cellular compartments enable the pyoverdine signal to be transmitted from the cell surface to the cytoplasm. These are the pyoverdine receptor FpvA, the antisigma factor FpvR and two alternative sigma factors, PvdS and FpvI. An overall view of transport-coupled pyoverdine signalling is shown in Fig. 7.4.

► **Fig. 7.4 a,b.** Receptor-dependent pyoverdine signaling in *P. aeruginosa* PAO1: **a** crystal structure of the pyoverdine-loaded FpvA receptor (Cobessi et al. 2005). *Side* (*left*) and *top* (*right*) view of FpvA showing 22 antiparallel β strands filled by the plug domain. The pyoverdine molecule, depicted in space filling representation, is shown in the ligand binding pocket of the receptor. The structure of the periplasmic N-terminal extension implicated in signalling is not known and this is represented by a dotted line; **b** model for the mechanism of pyoverdine signalling. The *left part* of the figure shows the resting state of the signalling complex. Under low-iron conditions, the FpvA receptor is permanently engaged with iron-free pyoverdine (*white triangle*) and interaction between the membrane-spanning antisigma factor FpvR and the PvdS sigma reduces transcription of *pvd* genes. Within the FpvA schematic structure, the cork-bound signalling domain and the TonB box are depicted as a *grey plug* and a *black sphere*, respectively. The *right part* of the figure shows the model for the activated state of the signalling complex. Displacement of apo pyoverdine by ferric pyoverdine (*black circle embedded in white triangle*) causes a conformational transition of the FpvA receptor that results in TonB-dependent ferric pyoverdine transport into the periplasm followed by transport of iron into the cytoplasm (*black arrow*) via an unknown mechanism. Concomitantly, release of PvdS by the FpvR antisigma causes PvdS to bind the core enzyme of RNA polymerase (RNAPc) and direct transcription of pyoverdine biosynthetic genes (*pvd*). These are characterised by the typical TAAAT-$N_{16–17}$-CGT promoter motif. A similar pattern of interaction can be predicted between FpvR and the *fpvA*-specific IS sigma factor FpvI (not shown). OM, outer membrane; PP, periplasm; CM, cytoplasmic membrane. For further details see the text


a
Signalling
domain
b
FpvA
FpvA
OM
TonB
PP
ExbD
FpvR
FpvR
ExbB
?
CM
PvdS
PvdS
RNAPσ
TAAAT-N16-CGT
pvd genes

7.4.1 The FpvA Receptor

The ferric pyoverdine receptor is an iron-regulated outer membrane protein of ca. 86 kDa, encoded by the *fpvA* gene (Poole et al. 1993b). Its crystal structure has recently been solved (Cobessi et al. 2005) (see Fig. 7.4a). FpvA has a periplasmic N-terminal domain that is responsible for signal transduction and transcriptional regulation of *pvd* genes, a typical feature of TonB-dependent receptors involved in surface signalling (Schalk et al. 2004). Consistent with its periplasmic location, this domain is sensitive to proteolysis (James et al. 2005). At the structural level, the membrane-associated portion of FpvA is folded into two domains: a transmembrane 22-stranded *beta*-barrel domain occluded by an N-terminal plug domain containing a mixed four-stranded *beta*-sheet. The *beta*-strands of the barrel are connected by long extracellular loops and short periplasmic turns. The iron-free pyoverdine is bound at the surface of the receptor in a pocket lined with aromatic residues while the extracellular loops do not completely cover the pyoverdine binding site. The N-terminal domain of ca. 70 amino acids that is implicated in signal transduction is not present in this structure. The TonB box that is involved in intermolecular contacts with the TonB protein(s) is poorly defined but could be modelled in an extended flexible conformation (Cobessi et al. 2005).

FpvA-bound ferric pyoverdine is located on the extracellular side of the outer membrane. Translocation of the siderophore into the periplasm requires the proton motive force and energy coupling in the inner membrane through the TonB complex. Three TonB homologues (*tonB1*, *tonB2* and PA0695) have been identified in the *P. aeruginosa* genome (Poole et al. 1996; Zhao and Poole 2000). The *tonB1* gene is iron-regulated, Fur-repressible, and essential for siderophore-mediated iron uptake (Poole et al. 1996). Homologues of *exbB* and *exbD* that are required for ferrisiderophore transport in *E. coli* were identified downstream of the *tonB2* gene, but the whole *tonB2* gene system seems to be dispensable for iron acquisition (Zhao and Poole 2000). Therefore, TonB1 could act in concert with the *exb* homologues encoded by PA0693 and PA0694, or with other proteins, or it could be capable of working autonomously.

Functional studies highlighted that FpvA is capable of binding both unsaturated and iron-saturated pyoverdine (Schalk et al. 1999, 2001). This indicates that pyoverdine-loaded FpvA can be the normal state of the receptor. FpvA-bound apo pyoverdine is not transported by *P. aeruginosa*, while the iron-loaded siderophore efficiently enters the cell (Schalk et al. 1999, 2001). The TonB system is essential for the efficient displacement of FpvA-bound apo pyoverdine by ferric pyoverdine, but dispensable for siderophore binding (Schalk et al. 2001). Iron-saturated pyoverdine does not exchange the metal with the receptor-bound apo siderophore in vivo but replaces it in the receptor pocket, showing fast displacement kinetics that parallel the rate of iron uptake. Thus, FpvA appears to adopt different conformations depending on its loading status, the transport

(and likely signalling) competent state being achieved upon binding of the ferrisiderophore (Cobessi et al. 2005; Schalk et al. 2004).

The involvement of the flexible N-terminal extension of FpvA in pyoverdine signalling was documented by genetic studies (James et al. 2005; Shen et al. 2002). Deletion of *fpvA* curtails both pyoverdine synthesis and transport, while complementation with a truncated version of *fpvA* lacking the 5′ (N-terminal) coding region restores transport of exogenously supplied pyoverdine, but not pyoverdine synthesis (Shen et al. 2002). Mutations within the N-terminal domain do not affect pyoverdine transport but reduce pyoverdine production, consistent with a regulatory function for this region (James et al. 2005). Mutational analysis also provided evidence that FpvA-dependent transport and signalling are not mutually required processes, and that either function can be independently inactivated by insertion mutagenesis (James et al. 2005). Thus, the periplasmic extension of FpvA is definitively involved in the regulation of expression of pyoverdine biosynthesis genes.

P. aeruginosa PAO1 contains an additional receptor FpvB that enables uptake of Type I pyoverdine (Ghysels et al. 2004). FpvB can substitute for pyoverdine transport in an *fpvA*-deficient background. The *fpvB* gene is iron-regulated, but is located outside of the *pvd* locus and is differentially expressed with respect to *fpvA*, depending on the carbon source. Its role in pyoverdine signalling has not yet been addressed.

7.4.2 The FpvR Antisigma

How does binding of (ferri) pyoverdine to the FpvA outer membrane receptor trigger the activity of sigma factor PvdS in the cytoplasm of *P. aeruginosa*? Signal transduction requires the *fpvR* gene product. FpvR is a 331 amino acid protein with a predicted single transmembrane helix (residues 93–115) and a cytoplasmic N-terminus. The overall topology of FpvR is very similar to that observed for two other transmembrane sensors, namely, FecR of *E.coli* and PupR of *P. putide*, and the similarity extends to the functional level (Lamont et al. 2002). The site of interaction between FpvA and FpvR is predicted in the periplasmic N-terminal part of the receptor, in a region (residues 50–59) called the FecR-box (sequence I-LL-GTGA in FpvA) (Visca 2004). There is a high conservation of Trp residues in the N-terminal region of sensor proteins implicated in siderophore signalling, including FpvR (Visca 2004), and the corresponding residues of FecR are known to be crucial for signal transduction to the sigma factor FecI (Stiefel et al. 2001). FpvR has typical antisigma functions (Fig. 7.4b); when overexpressed it causes transcriptional repression of *pvd* genes, while its deletion has only minor effects (Lamont et al. 2002). Genetic evidence indicates that the N-terminal part of FpvR is located in the cytoplasm and interacts with the alternative sigma factors PvdS and FpvI (Lamont et al. 2002; Beare et al. 2003; Rédly

and Poole 2005). *fpvR* is transcribed from a Fur-regulated promoter (Ochsner and Vasil 1996) and is located in opposite orientation to the *fpvI* gene that encodes a sigma factor responsible for transcription of *fpvA* (Fig. 7.2) (Beare et al. 2003; Rédly and Poole 2003).

7.4.3 A Divergent Signalling Pathway from FpvR to PvdS and FpvI Sigma Factors

Transcription of *pvd* genes is primarily dependent upon the expression of the Fur-repressible *pvdS* gene (Cunliffe et al. 1995; Miyazaki et al. 1995). As an alternative sigma factor, PvdS forms a transcriptionally active RNAP holoenzyme complex and shows fair conservation of regions implicated in promoter binding and recognition of core RNAP (reviewed in Visca et al. 2002). As anticipated, the main determinant of promoter recognition by PvdS is the IS box (see Sect. 7.3), a sequence signature also present in the promoters of genes regulated by the PvdS homologues PfrI, PbrA and PsbS in other fluorescent pseudomonads (Rombel et al. 1995).

An additional IS ECF sigma factor has been identified that is encoded by the *fpvI* gene, adjacent to *fpvR* (Fig. 7.2) (Beare et al. 2003; Rédly and Poole 2003). Transcription of both *fpvI* and *fpvR* is co-regulated by iron due to the presence of Fur boxes in their divergently oriented promoters (Ochsner and Vasil 1996; Ochsner et al. 2002). FpvI is implicated in the regulation of FpvA expression, as inferred by the reduced *fpvA* transcription in the *fpvI* mutant and by the increased *fpvA* transcription resulting from *fpvI* overdosage (Beare et al. 2003; Rédly and Poole 2003). Since FpvA is also implicated in the control of *pvd* genes through the FpvR-PvdS signalling cascade, both pyoverdine production and transcription of *pvd* genes are reduced in either *fpvI* or *fpvA* mutants (Beare et al. 2003; Shen et al. 2002).

The available evidence is consistent with a model whereby the N-terminal extension of FpvA interacts with the C-terminal domain of FpvR in the periplasm and the N-terminal portion of FpvR interacts with PvdS and FpvI in the cytoplasm. On this model, binding of (ferri) pyoverdine to FpvA on the outer membrane enables FpvR to transmit a signal through the cytoplasmic membrane to the two sigma factors PvdS and FpvI, leading to transcription of pyoverdine biosynthesis (*pvd*) and uptake (*fpvA*) genes, respectively (Fig. 7.4b). The proposed dual activity of FpvR on both PvdS and FpvI, and the response of *P. aeruginosa* PAO1 to the endogenous pyoverdine are features that differentiate the FpvA/FpvR/FpvI-PvdS system from other surface signalling devices such as the Fec and the Pup systems (Beare et al. 2003; Lamont et al. 2002).

Autogenous control of siderophore synthesis has broad biological implications. In species like *P. aeruginosa* that produce and acquire multiple siderophores, coupling between siderophore uptake and biosynthesis ensures that each siderophore is produced only when it is effective in delivering iron to the cell, namely after binding to the cognate receptor.

7.4.4 Additional Regulatory Proteins Involved in Modulation of Pyoverdine Gene Expression

Other regulators of the pyoverdine system have been identified in addition to PvdS, FpvI and FpvR. The effect of AlgQ (or AlgR2) on pyoverdine production by *P. aeruginosa* PAO1 has been elucidated (Ambrosi et al. 2005). AlgQ is a global regulatory protein that activates alginate, ppGpp, and polyP synthesis through a cascade involving nucleoside diphosphate kinase (Ndk). At the sequence level, AlgQ is similar to the *E. coli* Rsd protein that is an antisigma factor for RpoD and, like Rsd, AlgQ is capable of interacting with region 4 of RpoD (Dove and Hochschild 2001). In *P. aeruginosa* PAO1, deletion of *algQ* results in moderate but reproducible reduction in pyoverdine production as the result of diminished transcription of *pvd* genes. Complementation with wild-type *algQ* fully restores pyoverdine production and expression of *pvd* genes, while *ndk* does not. Thus, AlgQ is a functional homologue of Rsd, operating as an antisigma factor for RpoD and so assisting recruitment of core RNAP by PvdS and hence transcription of *pvd* genes. Expression of pyoverdine synthesis genes in *P. putida* requires the presence of the PfrA protein that is similar to AlgQ (Venturi et al. 1993) and could enable the *P. putida* PfrI sigma factor to compete with RpoD for RNAP core enzyme. However, AlgQ has a subtle effect on transcription of *pvd* genes in *P. aeruginosa* (Ambrosi et al. 2005), while PfrA is absolutely essential for the expression of homologous *P. putida* genes (Venturi et al. 1993).

Expression of the *pvc* genes that are associated with synthesis of the pyoverdine chromophore (see Sect. 7.2.5) is controlled by the LysR-type regulator PtxR, which in turn is controlled by PvdS (Stintzi et al. 1999). The *ptxR* gene is absent in fluorescent pseudomonads other than *P. aeruginosa* (Ravel and Cornelis 2003; Stintzi et al. 1999), consistent with the absence of the *pvc* genes from those species. Additional regulatory proteins that could affect PvdS expression or activity are the PA2427 and PA2428 gene products (PvdY and PvdX, respectively; see Table 7.1 and Fig. 7.2), though their functions are merely speculative at the moment (Vasil and Ochsner 1999). Thus, while the role of sigma factors in directing the expression of genes required for the synthesis of pyoverdines is conserved among fluorescent pseudomonads, other regulatory factors involved in modulation of siderophore gene expression can differ between *P. aeruginosa* and other fluorescent *Pseudomonas* species.

7.4.5 Strain- and Species-dependent Variability of Pyoverdine Synthesis and Regulation

The diversity of pyoverdine structures and receptors is mirrored by a huge variability of the pyoverdine genomic region. A whole-genome diversity study com-

paring one environmental and two clinical *P. aeruginosa* strains with the PAO1 type strain recognised the pyoverdine region as the most variable alignable locus (Spencer et al. 2003), coherent with the pyoverdine type diversity (Meyer, this volume). Comparing the pyoverdine locus from nine *P. aeruginosa* strains producing different types of pyoverdine, the highest divergence was observed in the genomic region encompassing the *pvdE*, *fpvA*, *pvdD*, *pvdI* and *pvdJ* genes (Smith et al. 2005). Three divergent sequence types were identified, corresponding to the three pyoverdine structures. In each case, NRPSs for the three pyoverdine types were co-linear and consistent with the variable peptide sequence. Moreover, strong evidence of co-evolution between the *fpvA* and *pvd* genes was observed, indicating that the receptor may have driven diversity at the *pvd* locus. Emergence of receptor variants is likely to occur in natural populations exposed to bacteriocins and phages (Baysse et al. 1999), and these must be compensated by reorganisation of biosynthesis genes through re-arrangement of NRPS modules. Most divergence arose from recombination between types leading to generation of novel pyoverdine structures (Smith et al. 2005). Such an evolutionary dynamic would be advantageous to fluorescent pseudomonads in natural communities, as it would avoid siderophore parasitism by competing microorganisms (Tummler and Cornelis 2005). Conversely, comparison of *pvd* clusters from different *P. aeruginosa* strains revealed a significant conservation of pyoverdine signalling genes *pvdS*, *fpvI* and *fpvR* (Smith et al. 2005), suggesting a positive selection for this regulatory network in *P. aeruginosa*.

Pyoverdine biosynthesis genes have also been identified in the genomes of other fluorescent pseudomonads, both experimentally (Adams et al. 1994; Devescovi et al. 2001; Mossialos et al. 2002; Lamont and Martin 2003; Putignani et al. 2004) and through genomic analysis (Lamont and Martin 2003; Ravel and Cornelis 2003). The available information indicates that the pathway of pyoverdine synthesis will be generally the same as in *P. aeruginosa*, with substrate amino acids being assembled into a precursor peptide by NRPSs and subsequent modifications resulting in formation of pyoverdine. Differences between species are likely to reflect differences in the pyoverdines that are made.

Siderophore-dependent induction of gene expression has been documented in *P. fluorescens* M114, *Pseudomonas* strain B10 and in *P. putida* WCS358 (Callanan et al. 1996; Ambrosi et al. 2002; Venturi et al. 1995a, b), raising the possibility that similar signalling pathways exist in other fluorescent pseudomonads. However, analysis of the *P. putida* KT2440, *P. syringae* DC3000 and *P. fluorescens* Pf0-1 *pvd* locus for the presence of putative PvdS, FpvR and FpvI homologues revealed significant variability. Although the *pvdS* homologue is present in a conserved genetic context for all four genomes analysed, an *fpvR*-like gene is missing in species other than *P. aeruginosa*. In fact, the gene showing highest homology with *P. aeruginosa fpvR* maps far away from the *pvd* locus of *P. putida* (PT3555) and *P. syringae* (PSPTO2358), in a genomic region apparently unrelated to iron uptake (www.tigr.org). It has been proposed that homologues of the *P. syringe* phosphorelay sensor SyrP could compensate for the absence of FpvR to drive positive control of pyoverdine synthesis in such species, and

a *syrP* homologue has indeed been identified in the pyoverdine region of *P. putida*, *P. syringae* and *P. fluorescens* (Ravel and Cornelis 2003). An *fpvI* homologue is present in *P. putida* KT2440 and *P. fluorescens* Pf0-1 but apparently absent in *P. syringae* DC3000, suggestive of a different mechanism of regulation of pyoverdine uptake in this species. Intriguingly, the presence of an IS box upstream of the tandemly-arranged *fpvA*-like receptor genes of *P. syringae* (Ravel and Cornelis 2003) suggests a role for the *pvdS* homologue (PSPTO2133) in biosynthesis-coupled control of pyoverdine receptor expression in this species. Given the heterogeneity of the pyoverdine genomic region in fluorescent pseudomonads, functional studies are needed to clarify the molecular partners involved in autogenous control of pyoverdine synthesis in species other than *P. aeruginosa.*

Putative AlgQ homologues were also identified in several Pseudomonadaceae (Ambrosi et al. 2005). The whole *algQ* genomic locus appeared remarkably conserved in *P. aeruginosa* PAO1, *P. putida* KT2440, *P. syringae* DC3000 and *P. fluorescens* PfO-1, suggesting that AlgQ homologues may play similar regulatory functions in Pseudomonadaceae. However, the different impact of the *algQ* (*pfrA*) mutation on pyoverdine biosynthesis in different fluorescent species deserves further investigation.

7.5 Conclusions

The synthesis of pyoverdines, and its regulation, has been a subject of research for many years. A combination of genetic and biochemical approaches has led to a good, though still incomplete, understanding of these processes in *P. aeruginosa*. For example, further research will be needed to determine the complete biosynthetic pathway and the roles of genes with as-yet-unassigned function. Similarly, the molecular interactions that control expression of pyoverdine synthesis genes are not yet fully understood. Other species have been less intensively studied. The data so far available show that in other fluorescent pseudomonads there are clear parallels but also significant differences in pyoverdine synthesis and its regulation when compared to *P. aeruginosa*. A major challenge will be to relate differences in biosynthesis and regulation seen in the laboratory to the processes that occur during growth and survival of these bacteria in complex soil ecosystems.

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