Biometals (2007) 20:431–452
DOI 10.1007/s10534-006-9065-4

Iron acquisition mechanisms of the *Burkholderia cepacia* complex

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Received: 26 August 2006 / Accepted: 28 November 2006 / Published online: 13 February 2007

Abstract The *Burkholderia cepacia* complex (Bcc) is comprised of at least 10 closely related species of Gram-negative proteobacteria that are associated with infections in certain groups of immunocompromised individuals, particularly those with cystic fibrosis. Infections in humans tend to occur in the lungs, which present an iron-restricted environment to a prospective pathogen, and accordingly members of the Bcc appear to possess efficient mechanisms for iron capture. These bacteria specify up to four different types of siderophore (ornibactin, pyochelin, cepabactin and cepaciachelin) that employ the full repertoire of iron-binding groups present in most naturally occurring siderophores. Members of the Bcc are also capable of utilising some exogenous siderophores that they are not able to synthesise. In addition to siderophore-mediated mechanisms of iron uptake, the Bcc possess mechanisms for acquiring iron from haem and from ferritin. The Bcc therefore appear to be well-equipped for life in an iron-poor environment.

Keywords Siderophore biosynthesis · Iron-dependent gene regulation · Pyochelin · Ornibactin · Salicylic acid

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Introduction

The genus *Burkholderia* belongs to the β class within the phylum *Proteobacteriaceae* and comprises approximately 40 species (Coenye and Vandamme 2003, http://www.bacterio.cict.fr/b/burkholderia.html) (Fig. 1). Most species of *Burkholderia* are found in river sediments and soil, particularly in the rhizosphere (Tabacchioni et al. 2002; Berg et al. 2005; Payne et al. 2006). Although many species within the genus are plant pathogens, some exhibit plant growth-promoting activity. In contrast, *B. pseudomallei*, and the subspecies, *B. mallei*, are distinguished by their ability to cause melioidosis and glanders, respectively, in humans and animals. Also residing within this genus are a group of closely related species collectively referred to as the *Burkholderia cepacia* complex (Bcc), which currently comprise ten validly described species (Coenye and Vandamme 2003; Baldwin et al. 2005; Huber et al. 2004) (see Fig. 1). These organisms are characterised by an extraordinary nutritional versatility (Stanier et al. 1966; Alisi et al. 2005), which is reflected in their large genomes, comprising multiple (usually three) chromosomes (Rodley et al. 1995; Lessie et al. 1996; Parke and Gurian-Sherman 2001). For example, *B. cenocepacia* J2315 has a total genome size of 8.06 Mb organised into three circular chromosomes of 3.87, 3.22 and 0.88 Mb, together with a plasmid of 92.7 kb (http://www.sanger.ac.uk/Projects/B_cenocepacia/).

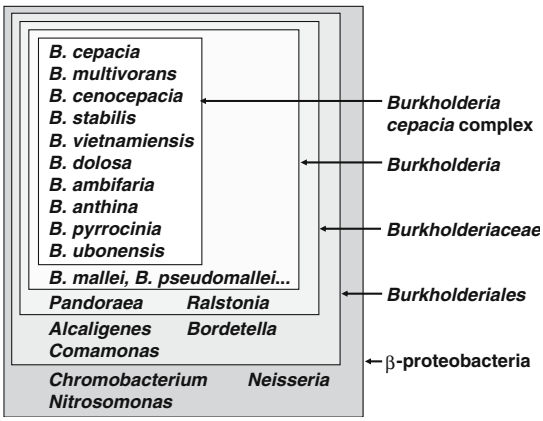

Fig. 1 Taxonomy of the *Burkholderia*. The genus *Burkholderia* currently contains approximately 40 members, of which 12 are shown, including all 10 currently described members of the *B. cepacia* complex (*white box*)

Although they are adapted for life on and around plants, all members of the Bcc can cause opportunistic infections in humans, particularly in patients with cystic fibrosis (CF) or chronic granulomatous disease, as well as patients requiring mechanical ventilation (Isles et al. 1984; Govan and Deretic 1996; Speert 2001). Although all members of the Bcc have been associated with infections in CF patients, the most prevalent are *B. cenocepacia* and *B. multivorans* (LiPuma et al. 2001; Mahenthiralingam et al. 2002; Speert et al. 2002; Reik et al. 2005). Treatment of such infections is extremely difficult due to their high intrinsic resistance to many antibiotics (LiPuma 1998; Speert 2002; Mahenthiralingam et al. 2005). Infection of the lung presents a challenge to colonising bacteria, not only due to the presence of alveolar macrophages, but also because this organ presents an iron restricted environment (Wang et al. 1996). Therefore, the successful establishment of a niche within the lung will require an efficient means of iron capture. This review addresses the mechanisms of iron acquisition utilised by the Bcc and focuses mainly on siderophore-mediated iron uptake, as this is the most intensively studied aspect of iron acquisition in this group of organisms.

Siderophore-mediated iron uptake by the Bcc

Members of the Bcc produce up to four different siderophores, not including salicylic acid (which may not act as a siderophore; see discussion below). The siderophores elaborated by the Bcc contain most of the bidentate ferric iron-chelating groups commonly present in bacterial siderophores, and includes catechols (present in cepaciachelin), linear hydroxamate and α-hydroxycarboxylate groups (both present in ornibactin), a cyclic hydroxamate (hydroxypyridonate) moiety (as in cepabactin), and 2-hydroxyphenyl-thiazoline/-oxazoline and thiazolidine-carboxylate moieties (both present in pyochelin).

Pyochelin

Pyochelin, 2-(2-*o*-hydroxyphenyl-2-thiazolin-4-yl)-3-methylthiazolidine-4-carboxylic acid, is derived from the condensation of salicylic acid with two molecules of cysteine, each of which undergoes cyclisation to thiazoline and thiazolidine ring derivatives following their incorporation into the molecule. Natural pyochelin is present as two spontaneously interconvertible stereoisomers, pyochelins I and II, due to isomerisation at the C-2″ position of the thiazolidine ring (Ankenbauer et al. 1988; Rinehart et al. 1995; Schlegel et al. 2004) (Fig. 2a). The total synthesis of pyochelin from simple precursors has been described (Ankenbauer et al. 1988; Rinehart et al. 1995; Zamri and Abdallah 2000; Ino and Murabayashi 2001; Patel et al. 2003). Iron-free pyochelin exhibits a yellow–green fluorescence under UV light, is highly labile in aqueous solution and unstable to light (Cox and Graham 1979; Cox et al. 1981). The iron-bound form, ferripyochelin, is more stable and more soluble in water than the iron-free form, and it is also non-fluorescent, the resultant complex imparting a wine-red colour to the solution at pH 2.5, changing to orange between pH 4.0 and 7.0 (Cox and Graham 1979; Cox et al. 1981). Although the binding coefficient for ferric iron is low (2.4×10^5 M), this value was determined in ethanol, and the activity of pyochelin as a siderophore suggests that the coefficient might be much higher in aqueous solution (Cox and Graham 1979; Cox 1980; Ankenbauer et al. 1985, 1988; Sriyosachati and Cox 1986). Pyochelin can also form complexes with Zn(II), Cu(II), Co(II), Mo(VI), Ni(II), V(IV) and V(V)

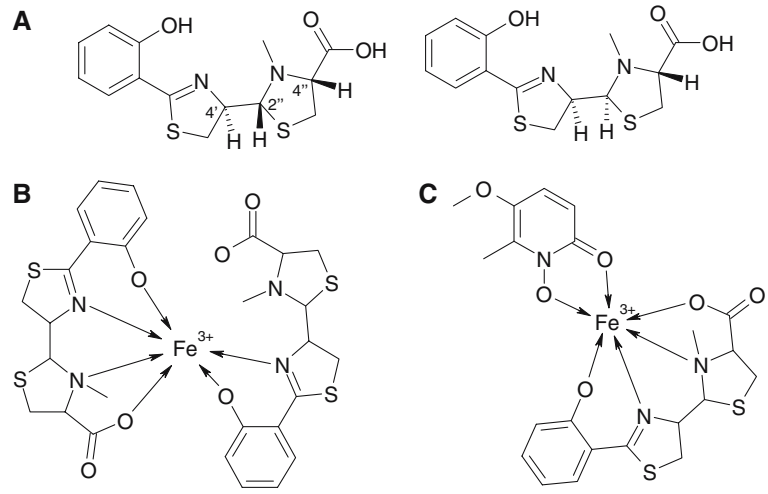

Fig. 2 Structure of pyochelin and its ferric iron complexes. **a** The two naturally occurring stereoisomers of pyochelin, pyochelin I (configuration 4′*R*,2″*R*,4″*R*), on the *left*, and pyochelin II (configuration 4′*R*,2″*S*,4″*R*), on the *right*. **b** Coordination of ferric iron by pyochelin in the 2:1 pyochelin–Fe(III) complex. The most stable geometry is shown (see Tseng et al. 2006). **c** The 1:1:1 cepabactin–pyochelin–Fe(III) complex (see Klumpp et al. 2005)

(Cuppels et al. 1987; Visca et al. 1992; Baysse et al. 2000).

The stoichiometry of the ferripyochelin complex in solution is one or two molecules of pyochelin per metal ion, depending on the concentration of pyochelin relative to iron (Cox and Graham 1979; Ankenbauer et al. 1988; Schlegel et al. 2004; Tseng et al. 2006). Mass spectrometric and spectroscopic studies indicate that a single molecule of pyochelin forms a tetradentate ligand with ferric iron in solution (Klumpp et al. 2005; Tseng et al. 2006). In complex with the ferripyochelin receptor of *Pseudomonas aeruginosa* (FptA), a single molecule of pyochelin is observed as a tetradentate molecule that coordinates ferric iron through the single nitrogen atoms of the thiazoline and thiazolidine rings, the phenolate oxygen and one of the carboxylate oxygen atoms (Cobessi et al. 2005; Mislin et al. 2006). The remaining two coordinating groups in the octahedral complex are provided by another molecule not specifically recognised by FptA. In the FptA–ferripyochelin co-crystal, the other two ligands are oxygen atoms present on ethylene glycol (Cobessi et al. 2005). However, in culture supernatants of *B. cepacia*, the bidentate ligand is cepabactin (Klumpp et al. 2005). In pyochelin–ferric iron 2:1 complexes that occur in solution, the octahedral coordination is asymmetrical, with one pyochelin molecule acting as a tetradentate ligand while another behaves as a bidentate ligand (Fig. 2b). The two coordinating atoms of the bidentate pyochelin molecule can be either the single oxygen and nitrogen atoms provided by the hydroxyphenyl-thiazoline moiety, or the thiazolidine nitrogen atom and one of the carboxylate oxygen atoms, with the stablest geometry predicted to be provided by the former pairing (Tseng et al. 2006). It is more likely, however, that the ferric–pyochelin complex occurs as a 1:1 complex in solution, with the remaining two coordination sites occupied by water molecules or hydroxides (Tseng et al. 2006).

Pyochelin was first isolated from culture supernatants of *P. aeruginosa* grown under conditions of iron limitation (Cox and Graham 1979). It was subsequently identified in culture supernatants of some strains of *P. fluorescens*, but not those of *P. putida* or *P. stutzeri* (Sokol 1984; Cuppels et al. 1987; Castignetti 1997; Schmidli-Sacherer et al. 1997). Pyochelin was also shown to be produced by some members of the Bcc (Bukovits et al. 1982; Sokol 1984), including two *B. cepacia* strains, ATCC 25416 and ATCC 17759 (Meyer et al. 1989, 1995), and the clinical isolate 715j, now identified as *B. cenocepacia* (Darling

et al. 1998; Visser et al. 2004; Farmer and Thomas 2004). However, in two surveys of Bcc clinical isolates, ~50% of strains produced little or no pyochelin (Sokol 1986; Darling et al. 1998), including K56-2, which has subsequently been identified as *B. cenocepacia* (Sokol et al. 1999; Mahenthiralingam et al. 2000). Furthermore, the genome sequence of the *B. cenocepacia* epidemic strain, J2315, reveals a frameshift mutation in the middle of the *pchF* gene (see below) which would be expected to block pyochelin biosynthesis (http://www.sanger.ac.uk/Projects/B_cenocepacia/). Among a panel of 14 *B. vietnamiensis* strains, none were shown to produce pyochelin (Meyer et al. 1995). Recently, it has been shown that the *B. pseudomallei* genome encodes the machinery necessary for the biosynthesis and transport of pyochelin (Holden et al. 2004; Tuanyok et al. 2005; Alice et al. 2006).

The pathway for the biosynthesis of pyochelin in *P. aeruginosa* has been the subject of extensive investigation (Quadri 2000). As the gene organisation in *B. cenocepacia* is essentially identical to that of *P. aeruginosa* (see below), a brief discussion of the pathway for pyochelin biosynthesis, its mode of uptake, and its genetic regulation in *P. aeruginosa* follows. Pyochelin is biosynthesised from one molecule of salicylate and two molecules of L-cysteine, and requires the activity of two non-ribosomal peptide synthetases (NRPS), PchE and PchF (Quadri et al. 1999). The three precursors are covalently attached to PchE and PchF through thioester linkages to phosphopantetheine ''arms.'' Correct charging of each precursor is ensured by the activity of the PchC thioesterase (Reimmann et al. 2004). The first step, carried out by PchE, involves a condensation reaction between the activated carboxyl group of salicylate and the amino group of a molecule of cysteine (Fig. 3). PchE subsequently catalyses cyclodehydration of the cysteine moiety, resulting in formation of a thiazoline ring, and random epimerisation at the C-2 position to a mixture of the D- and L-forms (Patel et al. 2003). The resultant D-enantiomer, dihydroaeruginoic acid (Dha), is an antifungal compound which is found in culture supernatants of *P. aeruginosa*, *P. fluorescens* and *B. cenocepacia* (Carmi et al. 1994; Serino et al. 1997; Farmer and Thomas 2004). Dha has Fe(III)-chelating properties, but has no known role as a siderophore (Serino et al. 1997). During pyochelin biosynthesis, the amino group of a second molecule of L-cysteine, bound to PchF, acts as a nucleophile to displace Dha (as a 2-hydroxyphenyl-thiazoline–PchE thioester intermediate) from PchE, followed again by cyclisation of the newly added cysteine moiety (the second cysteine moiety retains the L-configuration). This is followed by reduction of the imine carbon–nitrogen bond in the nascent thiazoline ring, resulting in formation of a thiazolidine ring (Patel and Walsh 2001; Reimmann et al. 2001). After methylation of the thiazolidine ring nitrogen, pyochelin is hydrolysed from the phosphopantetheine arm of PchF by an intrinsic thioesterase activity (Patel and Walsh 2001) (Fig. 3).

Three proteins have been identified as playing a role in ferripyochelin transport. The TonB-dependent pyochelin outer membrane receptor, FptA, and/or its gene, have been identified in *P. aeruginosa*, *P. fluorescens*, *B. cenocepacia* and *B. pseudomallei* (Heinrichs et al. 1991; Ankenbauer 1992; Ankenbauer and Quan 1994; Visser et al. 2004; Paulsen et al. 2005; Alice et al. 2006). Additionally, Southern hybridisation experiments have identified the presence of the *fptA* gene in the genomes of *P. aureofaciens*, *P. putida* and *B. cepacia* 25416, although *P. aureofaciens* and *P. putida* are not known to produce pyochelin (Castignetti 1997). FptA has a predicted molecular mass of 76-kDa and can bind and transport both the pyochelin I and II stereoisomers. Ligand docking experiments predicted that FptA also recognises the pyochelin–Fe(III)–cepabactin complex (Mislin et al. 2006). FptA lacks the additional N-terminal domain present in some TonB-dependent outer membrane transporters (OMT) that are referred to as the OMT_N family or as TonB-dependent transducers (Schalk et al. 2004; Koebnik 2005) (Fig. 4). This is consistent with the absence of a Fec-type ECF σ factor and cognate cytoplasmic membrane regulator for regulating the pyochelin genes in either *P. aeruginosa* or *B. cenocepacia* (Braun and Mahren 2005). Although FptA is not a member of the OMT_N family, it binds the apo-form of its cognate siderophore, i.e. iron-free pyochelin (Hoegy et al.

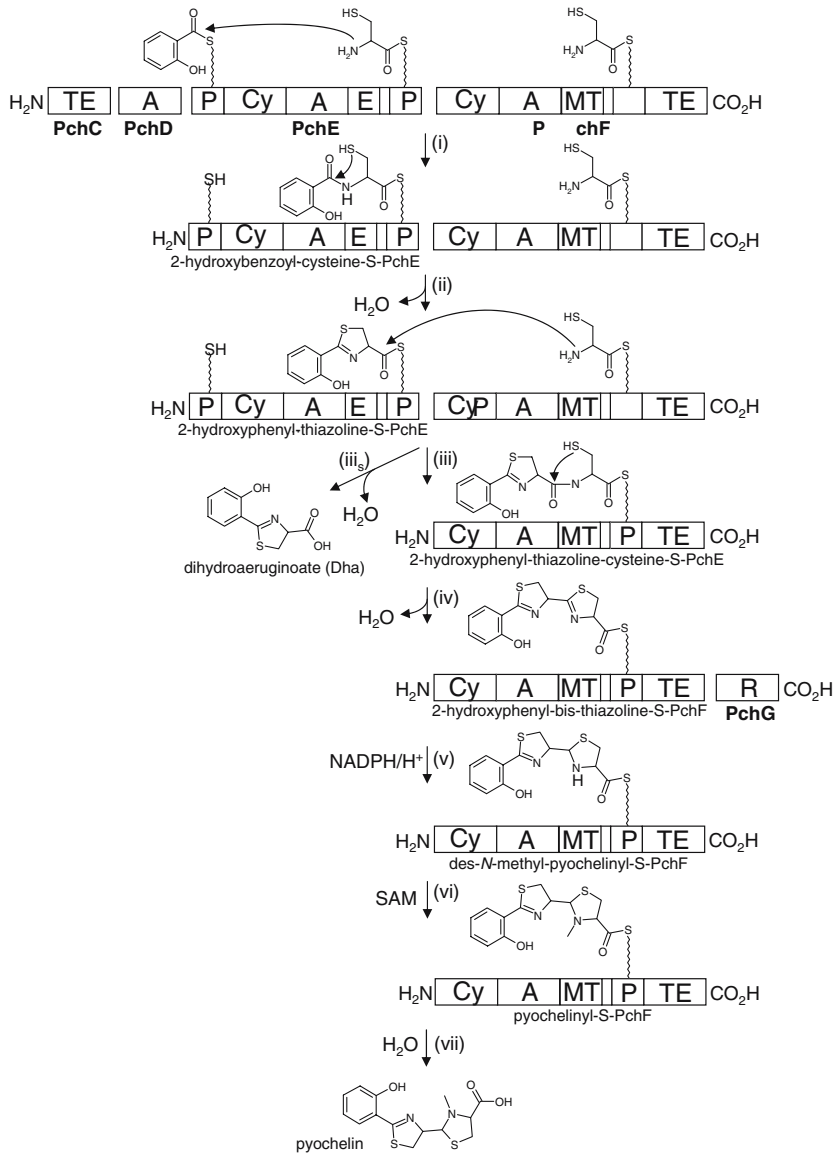

Fig. 3 Biosynthesis of pyochelin. In the first step, salicylic acid and two molecules of cysteine are activated by adenylation, then transferred to the phosphopantetheinyl arms (*zigzag motifs*) attached to the P domains of PchE and PchF. Correct loading of the P domains is ensured by the PchC thioesterase. Synthesis of the first intermediate is catalysed by the PchE Cy domain, and involves nucleophilic attack by the amino group of the PchE-tethered cysteine on the thioester carbon atom of the salicylate group [step (i)], followed by cyclisation of the cysteine moiety and epimerisation to give rise to a mixture of D- and L-forms of the thiazoline ring [step (ii)]. This intermediate can give rise to Dha if it is hydrolysed off PchE [step (iii_s)]. Synthesis of the core pyochelin structure requires the PchF Cy domain, and occurs through nucleophilic attack by the amino group of the PchF-linked cysteine on the thioester carbon atom of the Dha moiety [step (iii)], followed by cyclisation of the cysteine moiety [step (iv)]. The nascent thiazoline ring is reduced to a thiazolidine through the activity of PchG [step (v)] and methylated on the ring nitrogen [step (vi)]. The completed pyochelin molecule is hydrolytically removed from PchF by the C-terminal thioesterase domain [step (vii)]. *A* adenylation (activation) domain, *Cy* condensation and cyclisation domain), *P* phosphopantetheinylation domain [also known as thiolation (*T*), or aryl/peptidyl carrier protein (*ArCP*/*PCP*) domain], *E* epimerase domain, *MT* methyl transferase domain, *R* reductase, *TE* thioesterase domain, *SAM* S-adenosylmethionine. Note that the epimerase and methyl transferase domains are located within the corresponding adenylation domains

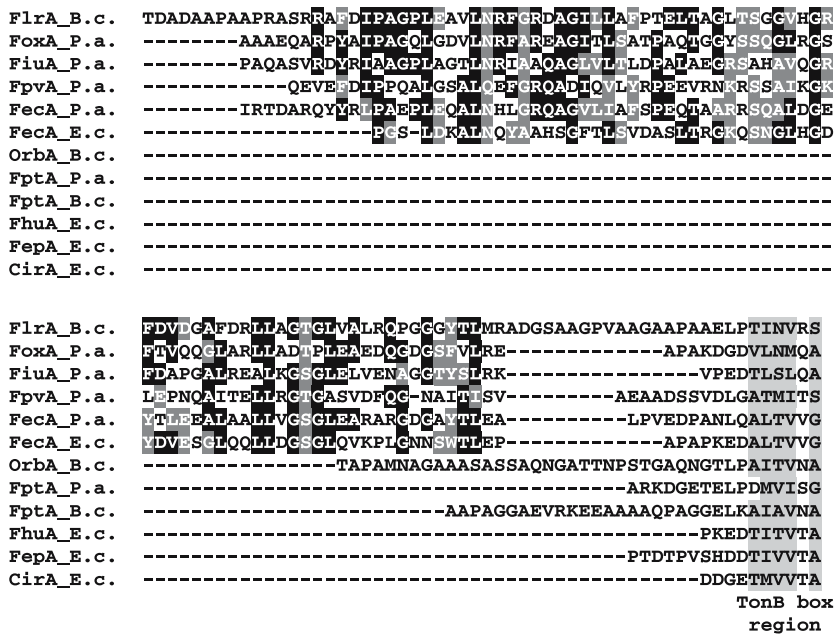

Fig. 4 Alignment of the N-termini of selected TonB-dependent ferric-siderophore transporters. Six representative members of the OMT_N family of receptors from *B. cenocepacia* (B.c.), *P. aeruginosa* (P.a.) and *E. coli* (E.c.) are shown, below which are six representative receptors from the same organisms which are not members of this family. The OMT_N domains were aligned by ClustalW and highlighted using *boxshade*. Amino acids shown in *white font with black shading* are identical at the corresponding position in 50% of the sequences shown, whereas those shown in *white font with grey shading* are similar. Predicted N-termini of the mature (i.e. processed) proteins are shown. The predicted TonB box region is shown in *black font with grey shading*. The corresponding genetic loci are: *B. cenocepacia* FlrA (ferrichrome-like receptor) (BCAL1371), OrbA (BCAL1700) and FptA (BCAM2224); *P. aeruginosa* FoxA (PA2466), FiuA (PA0470), FpvA (PA2398), FecA (PA3901), FptA (PA4221)

2005). The FptA–pyochelin complex can be subsequently loaded with ferric iron (Hoegy et al. 2005). A second putative outer membrane protein of 14 kDa has also been implicated in ferripyochelin uptake in *P. aeruginosa*, but the precise role or nature of the protein is unknown (Sokol and Woods 1983; Sokol 1984, 1987). Recently, a 43.2-kDa polypeptide, FptX, belonging to a novel family of single subunit cytoplasmic membrane transporters, has been proposed as the ferripyochelin permease (O'Cuiv et al. 2004).

The genes required for the biosynthesis of salicylate from chorismate, and for pyochelin from salicylate, have been identified in *P. aeruginosa*, and they form a co-regulated cluster (PA4224–PA4231) (Fig. 5). These genes comprise *pchA* and *pchB*, which are required for conversion of chorismate into salicylate via isochorismate (Serino et al. 1995; Gaille et al. 2002, 2003), *pchD*, which is required for activation of salicylate by ATP (Serino et al. 1997), *pchE* and *pchF*, encoding the Dha and Pch synthetases, respectively (Reimmann et al. 1998), *pchG* encoding an NADPH-dependent reductase that converts the second thiazoline ring into a thiazolidine ring (Patel and Walsh 2001; Reimmann et al. 2001), and *pchC*, specifying a thioesterase that edits incorrectly charged PchE and PchF (Serino et al. 1997; Reimmann et al. 2004). These genes are organised into two divergent operons, *pchDCBA* (PA4228–PA4231) and *pchEFGHI* (PA4226–PA4222). *pchH* and *pchI* encode components of an ABC transporter, although a role in pyochelin transport has not been established (Reimmann et al. 2001). Located adjacent *pchH* and *pchI* are four additional genes that are predicted to encode cell envelope proteins, two of which, *fptA* (PA4221) and *fptX* (PA4218), have been demonstrated to play a role in ferripyochelin transport (see above). The other two genes, *fptB* (PA4220) and *fptC* (PA4219), appear to encode cytoplasmic membrane proteins. It is almost certain that *fptA*

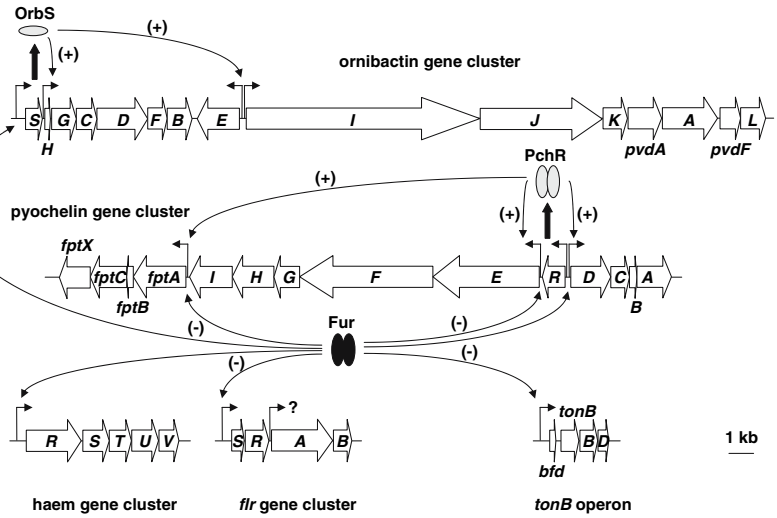

Fig. 5 Putative Fur-regulated operons involved in iron acquisition in *B. cenocepacia*. Five loci are shown: the ornibactin locus (BCAL1688–1702), genes denoted by *single letters* are prefixed by "*orb*"; the pyochelin locus (BCAM2221–2235, corresponding to PA4218–4231 in *P. aeruginosa*), genes denoted by *single letters* are prefixed by "*pch*"; the haem uptake locus (BCAM2626–2630), genes denoted by *single letters* are prefixed by "*bhu*"; the *flr* locus (BCAL1369–1372), genes denoted by *single letters* are prefixed by "*flr*"; the *tonB* locus (BCAL2290–2293), genes denoted by *single letters* are prefixed by "*exb*." Promoters targeted by the indicated regulatory proteins are shown by *arrows*. *Plus symbol* gene activation, *minus symbol* gene repression

and *fptB* constitute an operon under *fptA* promoter control, as they appear to be translationally coupled, and it is possible that *fptC* and *fptX* constitute part of this operon. The organisation of the pyochelin gene cluster in *P. aeruginosa* and *B. cenocepacia* (and *B. pseudomallei*) appears to be identical (Visser et al. 2004; Alice et al. 2006, our unpublished observations). However, the genes flanking this cluster in *P. aeruginosa* (PA4217 and PA4232) are different from those present at the corresponding positions in *Burkholderia* (our own unpublished observations). Interestingly, the genes are organised differently in *P. fluorescens* Pf-5 (Paulsen et al. 2005).

Regulation of the *pch* genes requires the Fur protein, a global repressor of iron-regulated genes, and PchR, an AraC family transcriptional regulatory protein (Heinrichs and Poole 1993, 1996; Ochsner et al. 1995; Martin and Rosner 2001; Lowe et al. 2001; Andrews et al. 2003) (Fig. 5). The *pchR* gene (PA4227) is located within the pyochelin biosynthetic gene cluster, and its protein product activates the *pchDCBA*, *pchEFGHI* and *fptABCX* operons (Heinrichs and Poole 1996; Serino et al. 1997; Reimmann et al. 1998, 2001; Michel et al. 2005). PchR, in complex with its cofactor, pyochelin (probably in the iron-loaded form), recognises a 32-bp DNA sequence motif, known as the PchR-box, located upstream of the *pchD*, *pchE* and *fptA* promoters. Binding of holo-PchR to the PchR-box located at the *pchD* promoter also represses transcription of *pchR*, as this protein binding site is located downstream from the *pchR* promoter (Heinrichs and Poole 1996; Michel et al. 2005). The requirement for intracellular pyochelin for induction of pyochelin gene expression may explain the previous observations that *fptA* mutants show a marked decrease in *fptA* gene expression (in *P. aeruginosa*) and produce significantly less pyochelin than the wild type strain (in *B. cenocepacia*) (Heinrichs et al. 1991; Gensberg et al. 1992; Heinrichs and Poole 1996; Visser et al. 2004).

The *pchD*, *pchE*, *pchR* and *fptA* promoters are also repressed by Fur in the presence of iron, and for the *pchD*, *pchE* and *fptA* promoters this repression is dominant over activation by PchR (Ankenbauer and Quan 1994; Ochsner et al. 1995; Serino et al. 1997; Reimmann et al. 1998; Michel et al. 2005). The organisation of Fur- and PchR-

boxes in the *B. cenocepacia* pyochelin gene cluster appears to be the same as described for *P. aeruginosa* (our own unpublished observations). It has also been shown that other transition metal ions [Co(II) at 10 μM, Cu(II), Mo(VI), Ni(II) and Zn(II) at 100 μM, and Al(III) at 2 mM] repress synthesis of pyochelin, and [apart from Al(III)] they have also been shown to result in decreased abundance of a 75-kDa outer membrane protein (probably FptA) in *P. aeruginosa* (Visca et al. 1992; Koedam et al. 1994).

Pyochelin biosynthesis also appears to be regulated by sulphur availability in *B. cenocepacia* and *P. aeruginosa*, although it is not clear whether this regulatory input acts at the level of transcription. First, it was observed that the presence of cysteine in the growth medium enhanced the production of pyochelin by *P. aeruginosa* (Audenaert et al. 2002; Gaille et al. 2003). Subsequently, it was shown that sulphate transport mutants of *B. cenocepacia*, or wild type *B. cenocepacia* growing under conditions of sulphate limitation, were restricted in their ability to produce pyochelin (Farmer and Thomas 2004). Supplementation with cysteine, but not methionine, restored pyochelin production, suggesting that pyochelin synthesis was responsive to the intracellular cysteine pool. Interestingly, under certain conditions of sulphur limitation, *B. cenocepacia* produced salicylate but not pyochelin (Farmer and Thomas 2004). As salicylate biosynthesis requires expression of the *pchBA* genes, and as these genes are co-regulated with the genes specifying pyochelin biosynthesis, it would appear that the mechanism for inhibition of pyochelin production in response to a depleted cysteine pool is post-transcriptional.

Ornibactin

Ornibactin, L-Orn^1(N^5-OH, N^5-acyl)-D-*threo*-Asp(β-OH)-L-Ser-L-Orn^4(N^5-OH, N^5-formyl)-1,4-diaminobutane, is a linear tetrapeptide derivative that chelates iron by providing three bidentate metal chelation groups (Stephan et al. 1993). These groups (two hydroxamates and an α-hydroxycarboxylate) are generated by modification of the sidechains of three of the amino acids in the peptide (the N- and C-terminal ornithines, and the D-aspartate), with the serine residue serving only as a spacer (Fig. 6). The δ-amino group nitrogen of both ornithine residues is hydroxylated and acylated. For the N-terminal ornithine, the acyl group is provided by a β-hydroxy acid with a carbon chain length of 4, 6 or 8, which gives rise to the three species of ornibactin: ornibactin-C4 (ornibactin B), ornibactin-C6 (ornibactin D) and ornibactin C8 (ornibactin F), respectively. In contrast, the C-terminal ornithine is acylated with formic acid. Hydroxylation and acylation of the ornithine sidechain nitrogen atom results in generation of the two hydroxamate groups. The α-hydroxycarboxylate group of ornibactin is produced by hydroxylation of the β-carbon in the aspartate side chain. The tetrapeptide is embellished by amidation of the C-terminal carboxyl group with putrescine (1,4-diaminobutane). This final modification plays no role in metal chelation but may be important for the mechanism of ornibactin biosynthesis (see below). Ornibactin appears to be the only siderophore produced by the *Burkholderia* which can form a hexadentate complex with ferric iron with a stoichiometry of 1:1, and although the stability constant for the ferric–ornibactin complex has not been determined, it is likely to be much higher than that of complexes formed between other *Burkholderia*-derived siderophores and ferric iron.

Ornibactin was first isolated from culture supernatants obtained from an environmental strain of *B. vietnamiensis*, but was subsequently identified in a further 13 strains, including two clinical isolates (Stephan et al. 1993; Meyer et al. 1995). It was also identified in two environmental strains of *B. cepacia* (ATCC 17759 and ATCC 25416), *B. ambifaria* strain PHP7, and in a survey of 63 Bcc strains, most of which were clinical isolates that included *B. cenocepacia*, ornibactin was identified in culture supernatants of 60 of them (Meyer et al. 1995; Barelmann et al. 1996; Darling et al. 1998). The nature of the species that did not produce ornibactin in the Darling et al. study are not known (P. Sokol, personal communication). More recently, strong evidence has been provided that the siderophore malleobactin, produced by *B. pseudomallei*, is, in fact, ornibactin (Alice et al. 2006). Indeed, ornibactin appears

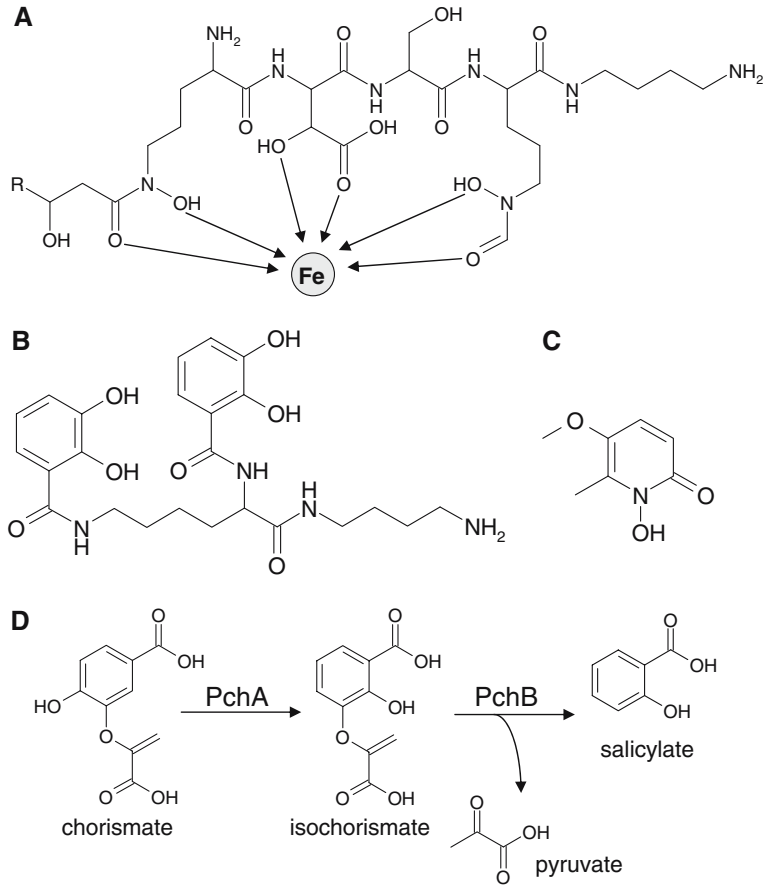

Fig. 6 Structure of ornibactin, cepabactin, cepaciachelin and salicylate. **a** Ornibactin shown in complex with ferric iron. $R = CH_3–$, $CH_3–CH_2–CH_2–$ or $CH_3–CH_2–CH_2–CH_2–CH_2–$ for ornibactin C-4, C-6 and C-8, respectively. **b** Structure of cepaciachelin. **c** Structure of cepabactin. **d** Biosynthesis of salicylic acid from chorismic acid. The gene products required for carrying out this transformation in *B. cenocepacia* and *P. aeruginosa* are shown

to be the most prevalent siderophore among the *Burkholderia*, and is produced by a much greater proportion of clinical isolates than is pyochelin (Darling et al. 1998). Furthermore, individual strains within the Bcc can produce all three ornibactin derivatives. Ornibactin is not made by fluorescent pseudomonads such as *P. aeruginosa* and cannot be utilised by them (Meyer et al. 1995).

The genes required for ornibactin biosynthesis and utilisation in *B. cenocepacia* have been identified through a combination of molecular genetic and in silico approaches. Using *B. cenocepacia* K56-2, a strain which produces ornibactin and salicylate but only low levels of pyochelin, two transposon mutants were isolated which failed to produce ornibactin (Sokol et al. 1999). In one mutant, the transposon was inserted in the *pvdA* gene, encoding L-ornithine N^5-oxygenase, an enzyme that is required for hydroxylation of the δ-amino group nitrogen of ornithine as the first step in the formation of a hydroxamate group (Visca et al. 1994). In the other mutant, the transposon had inserted into a gene that was predicted to encode a NRPS that bore similarity to the PvdD component of pyoverdine synthetase. Both mutants were also defective in uptake of exogenously supplied ferric–ornibactin. Downstream from *pvdA*, Sokol and colleagues identified the *orbA* gene, encoding the 78-kDa ferric–ornibactin outer membrane receptor. Mutation of this gene has been shown to inhibit the uptake of

ferric–ornibactin (Sokol et al. 2000). The *orbA* gene is followed by *pvdF*, encoding N^5-hydroxy-ornithine transformylase (Sokol et al. 2000; McMorran et al. 2001) (Fig. 5).

In a separate transposon mutagenesis experiment, eight ornibactin-deficient mutants of *B. cenocepacia* KLF1, a pyochelin-negative derrivative of strain 715j, were obtained, in which the transposon had integrated into one or other of two NRPS genes, *orbI* and *orbJ* (Agnoli et al. 2006). As the partial sequence obtained for *pvdD* was not deposited in the database, it is not possible to say with certainty which of these two genes corresponds to *pvdD*. Mutants in which *orbA*, *orbI*, *orbJ* and *pvdA* are inactivated also failed to grow in the presence of concentrations of the iron chelators EDDHA and/or dipyridyl that do not restrict growth of the parent strains (Sokol et al. 1999, 2000; Agnoli et al. 2006). Upstream of *orbI*, and transcribed in the opposite direction, a gene (*orbE*) was identified encoding a predicted transport protein exhibiting a high similarity to PvdE of *P. aeruginosa* (McMorran et al. 1996; Agnoli et al. 2006). The subsequent availability of the complete genome sequence of *B. cenocepacia* J2315 (http://www.sanger.ac.uk/Projects/B_cenocepacia/) showed that the *pvdA–orbA–pvdF* and *orbE–orbI–orbJ* units were components of a cluster of 14 genes (BCAL1689-BCAL1702) required for biosynthesis and utilisation of ornibactin (Fig. 5). This analysis suggested that all of the genes required for biosynthesis and export of ornibactin, together with those required for the uptake of ferric–ornibactin and removal of iron from the siderophore are present at the same genomic locus.

Analysis of these genes resulted in proposal of the following pathway for ornibactin biosynthesis and utilisation (Agnoli et al. 2006). According to this model, synthesis of the modified amino acids required as precursors for the ornibactin tetrapeptide requires the products of the *orbG* (BCAL1690), *orbK* (BCAL1698), *pvdA* (BCAL1699), *pvdF* (BCAL1701) and *orbL* (BCAL1702) genes (Fig. 7). As outlined above, PvdA and PvdF act sequentially to hydroxylate and formylate the sidechain nitrogen of ornithine, as occurs in *P. aeruginosa* during pyoverdine biosynthesis. As *orbK* and *orbL* appear to encode acetylases, it is likely that PvdA, and either OrbK or OrbL, sequentially hydroxylate and acylate the sidechain nitrogen of a second ornithine with a β-hydroxy acid. Aspartic acid is likely to be hydroxylated on the β-carbon by the product of *orbG*, a protein exhibiting a high degree of similarity to α-ketoglutarate-dependent hydroxylases (Hausinger 2004). Genes similar to *orbG* are present in the *pvd* loci of *Pseudomonas* strains such as *P. putida* and *P. syringae* that incorporate β-hydroxy aspartate into their pyoverdines, but are not present in the corresponding loci of *P. aeruginosa*, a species which does not utilise this amino acid as a building block for pyoverdine biosynthesis (Meyer 2000; Ravel and Cornelis 2003). The three modified amino acids, together with L-serine, are assembled into ornibactin by ornibactin synthetase, comprising two NRPSs, OrbI and OrbJ (BCAL1696 and BCAL1697) (Fig. 8). OrbI is predicted to be responsible for formation of an L-Orn(N^5-OH, N^5-acyl)-D-Asp(β-OH)-L-Ser intermediate, with isomerisation of L-aspartate to the D-enantiomer being catalysed by an epimerase domain present on OrbI (Agnoli et al. 2006). The final step in synthesis of the tetrapeptide involves peptide bond formation between the carboxyl group of the tripeptide and the amino group of N^5-hydroxy-N^5-formyl-ornithine tethered to OrbJ. This results in displacement of the tripeptide from the phosphopantetheine arm anchored to the C-terminal peptidyl carrier protein domain of OrbI. The tetrapeptide is then released from the phosphopantetheine group on OrbJ as a result of nucleophilic attack by an amine group of putrescine. This step obviates the requirement for a C-terminal thioesterase domain in OrbJ or for a free-standing thioesterase, and results in derivatisation of the tetrapeptide C-terminus with a primary amine (Fig. 8).

The presence of genes in this cluster that encode predicted ferric-siderophore transport proteins permits some informed speculation regarding the trafficking of ornibactin and processing of the iron-loaded siderophore. OrbE (BCAL1695) is very similar to the predicted PvdE polypeptide of *P. aeruginosa*, which is required for production of pyoverdine (McMorran et al. 1996). Both proteins possess the

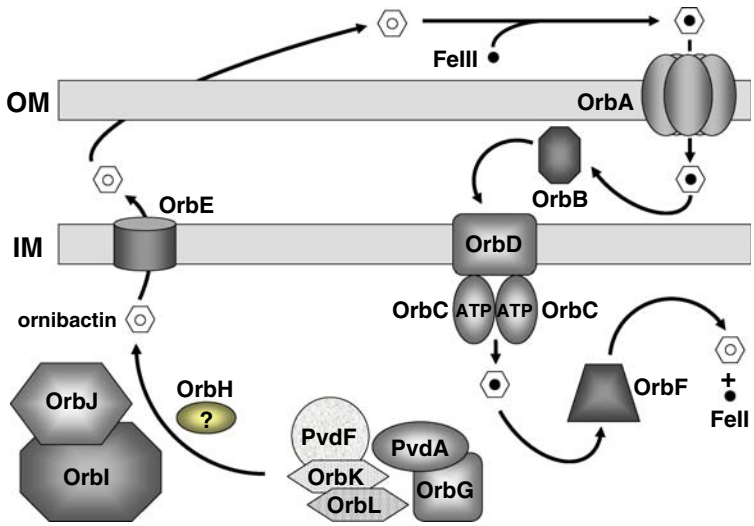

Fig. 7 Model for the biosynthesis and transport of ornibactin in *B. cenocepacia*. According to the model, amino acid precursors are modified by PvdA, PvdF, OrbG, OrbK and OrbL and assembled into ornibactin by the two NRPSs, OrbI and OrbJ. The role of OrbH in ornibactin biosynthesis is unknown. Ornibactin is then exported across the cytoplasmic membrane by OrbE. The extracellular ferric–ornibactin complex is transported across the outer membrane by OrbA, using the TonB–ExbB–ExbD complex to transduce energy derived from the protonmotive force generated at the cytoplasmic membrane. Transport of ferric–ornibactin across the cytoplasmic membrane requires a permease consisting of OrbB, OrbC and OrbD. Ferrous iron is probably released from the internalised ferric–ornibactin complex by the action of OrbF. See text for details

characteristics of an ABC transporter in which both the transmembrane and ATPase components reside within the same polypeptide, and it is probable that they constitute part or all of the export systems for these siderophores (Agnoli et al. 2006).

External iron-loaded ornibactin is transported across the outer membrane by the ferric–ornibactin receptor, OrbA (BCAL1700) (Fig. 7). The predicted amino acid sequence of OrbA shows that it contains a TonB box near the N-terminus of the mature protein (our unpublished observations) (Fig. 4). Like the ferripyochelin receptor, FptA, OrbA is not a member of the OMT_N family and does not harbour the N-terminal signalling domain characteristic of such receptors (Schalk et al. 2004; Koebnik 2005, our unpublished observations). However, in contrast to the pyochelin receptor, synthesis of which is inducible by its cognate iron-loaded siderophore (Heinrichs and Poole 1996; Michel et al. 2005), *orbA* gene expression is not stimulated by the presence of ferric–ornibactin, and the ability to utilise ornibactin is not a prerequisite for production of ornibactin (Sokol et al. 2000). Similar observations have been made regarding the malleobactin receptor of *B. pseudomallei* (Alice et al. 2006). Transport of ornibactin across the cytoplasmic membrane is likely to be specified by the *orbB*, *orbC* and *orbD* genes (BCAL1694, BCAL1691 and BCAL1692, respectively). The OrbB, OrbC and OrbD polypeptides are predicted to constitute an ABC transporter specific for ferric–ornibactin (Agnoli et al. 2006) (Fig. 7). The *orbF* gene (BCAL1693) is predicted to encode a 28.5-kDa polypeptide containing a $CC(X)_{11}C(X)_2C$ motif. OrbF is similar to the *E. coli* FhuF protein, that possesses a similar cysteine-rich motif required for formation of a novel [2Fe–2S] centre (Muller et al. 1998). As FhuF has been suggested to be involved in reduction of ferric iron bound to internalised ferrioxamine B, OrbF may play a similar role in the release of iron from ferric–ornibactin in the cytoplasm of *B. cenocepacia*.

One of the genes in the cluster, *orbH* (BCAL1689), encodes an 80 amino acid polypeptide of unkown function. OrbH is highly homologous to MbtH, encoded by the mycobactin locus

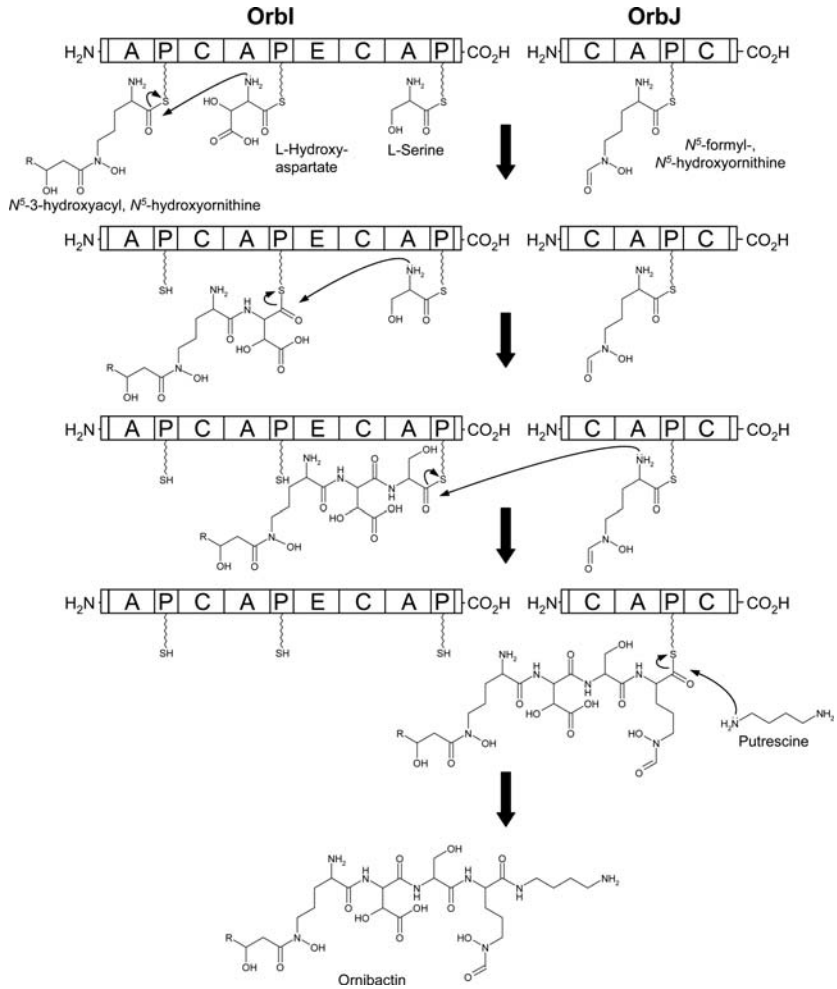

Fig. 8 Predicted mechanism of the non-ribosomal assembly of the ornibactin tetrapeptide. Each NRPS, OrbI and OrbJ (represented by the *two large rectangles*), is composed of adenylation (*A*), peptidyl carrier (*P*) and condensation (*C*) domains. OrbI also contains a predicted epimerase domain (*E*) to convert L-hydroxyaspartate to the *D* form. Phosphopantetheine (*P-pant*) ''arms,'' to which activated amino acids are linked by a thioester bond, are shown as *vertical zigzag motifs*. *Curved arrows* represent peptide bond formation by nucleophilic displacement of one amino acid from the P-pant arm by the amino group of the next amino acid in the peptide chain. The amino acid precursors are shown in their modified forms, although it is not clear at what step the N^5-amino group of the N-terminal ornithine is derivatised with a β-hydroxy acid. See text for details

of *Mycobacterium tuberculosis*, and the product of PA2412, a gene located within the pyoverdine gene cluster of *P. aeruginosa* (Quadri et al. 1998; Ravel and Cornelis 2003). OrbH-like polypeptides are also encoded by gene clusters that specify non-ribosomally synthesised peptides that are not siderophores.

As with the majority of siderophores, ornibactin is produced in response to iron deprivation (Meyer et al. 1995). Consistent with this, it was shown that transcription of the *B. cenocepacia pvdA* gene is strongly repressed by the presence of iron in the medium (Sokol et al. 1999; Lewenza and Sokol 2001). Subsequently, RT-PCR analysis demonstrated that all the genes within the ornibactin locus are repressed by iron (Agnoli et al. 2006). More recently, a gene encoding an ECF σ factor was identified adjacent to the ornibactin gene cluster in *B. cenocepacia* (BCAL1688). Inactivation of this gene resulted in inhibition of

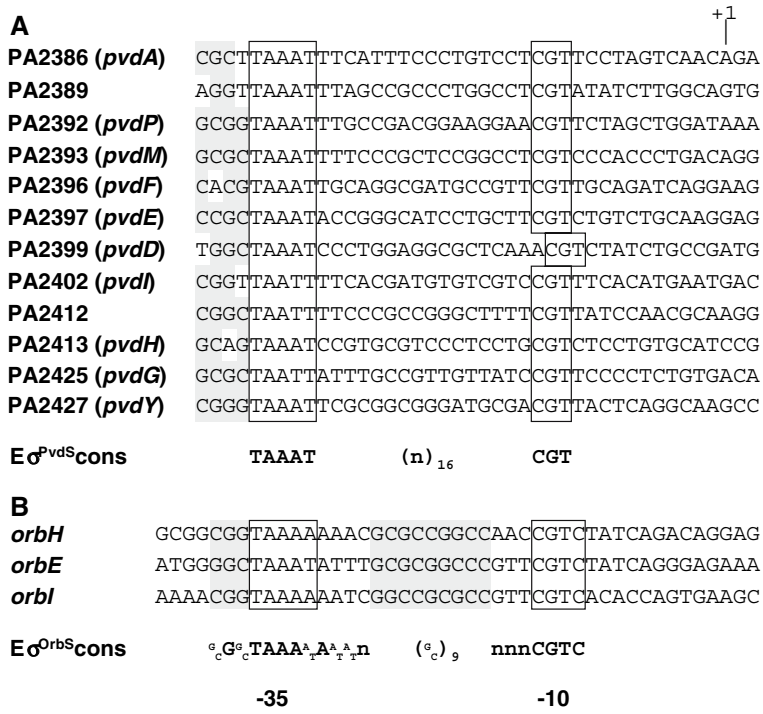

Fig. 9 DNA sequences of PvdS- and OrbS-dependent promoters. **a** PvdS-dependent promoters for the indicated genes from the pyoverdine loci of the *P. aeruginosa* genome are shown, with the consensus sequence given below. **b** OrbS-dependent promoters for the indicated genes from the ornibactin locus of the *B. cenocepacia* genome, with the consensus sequence given below. For (**a**) and (**b**), proposed –35 and –10 regions are enclosed in *boxes*. G + C-rich sequences flanking the –35 region are highlighted with *grey shading* (*n* = any base)

ornibactin production, but had no effect on pyochelin biosynthesis (Agnoli et al. 2006). *orbS* mutants were also growth restricted under iron starvation conditions. The protein product of this gene, OrbS, exhibits 36.5% identity to the pyoverdine gene regulator, PvdS, an iron-starvation ECF sigma factor (Leoni et al. 2000). One notable difference between the two proteins is that OrbS possesses an N-terminal extension of 29 amino acids that is not present in PvdS (Agnoli et al. 2006).

The activity of PvdS is modulated directly by a membrane-anchored anti-σ factor, FpvR, in response to the binding of iron-loaded pyoverdine to its cognate receptor, FpvA, a member of the OMT_N family of ferric-siderophore receptors (Lamont et al. 2002; Visca et al. 2002; Schalk et al. 2004; Koebnik 2005). However, there does not appear to be a candidate anti-OrbS sigma factor encoded by the *B. cenocepacia* genome, and the absence of an N-terminal signalling domain on OrbA lends further support to the idea that such a transmembrane signalling pathway is not employed for regulating OrbS activity. How, then, is OrbS activity regulated? It has been demonstrated that *orbS*, like *pvdS*, is transcribed from a σ^{70}-type promoter that is subject to regulation by the Fur repressor (Ochsner et al. 1995; Agnoli et al. 2006). Thus, when cells are growing under iron replete conditions, the *orbS* promoter is repressed (Fig. 5). Whether this regulatory system alone is enough to keep ornibactin synthesis in check when the siderophore is not required is not yet clear. In this regard, the system for regulating ornibactin gene expression appears to be distinct from those controlling pyochelin (and pyoverdine) synthesis, where two overlapping regulatory systems operate: one involving Fur, and the other requiring the presence of iron-loaded siderophore and its cognate

receptor (although the precise mechanism by which this signal is communicated varies) (Visca et al. 2002; Michel et al. 2005).

RT-PCR analysis allowed identification of three of the transcriptional units within the ornibactin gene cluster, comprising *orbS* to *orbB*, *orbE*, and *orbI* to *orbL* (Agnoli et al. 2006). The *orbE* transcript was shown to include antisense RNA corresponding to at least a part of *orbB*, but it has not been determined where this transcript terminates. The similarity between OrbS and PvdS facilitated the identification of candidate OrbS-dependent promoters. PvdS recognises the consensus sequence TA-AAT(N)$_{16}$CGT (Wilson et al. 2001; Ochsner et al 2002; Visca et al. 2002) (Fig. 9). Consistent with the transcriptional organisation of the ornibactin cluster, sequences identical or nearly identical to the PvdS consensus were identified upstream of *orbH*, *orbE* and *orbI*, but were not present anywhere else at this locus (Fig. 9). As well as containing the consensus –35 and –10 sequences for PvdS-dependent promoters, the candidate OrbS-dependent promoters also possess G + C tracts upstream of the predicted –35 region and in the spacer region separating the –35 and –10 motifs (Fig. 9). DNA fragments containing these sequences were shown to direct iron-regulated transcription of a reporter gene in wild type *B. cenocepacia*, but were silent in an *orbS* mutant (Agnoli et al. 2006). The presence of an OrbS-dependent promoter upstream of *orbH* indicated that an additional transcript was generated within the ornibactin gene cluster, which overlapped with the *orbS–orbB* transcript. Thus, iron-regulated transcription of the *orbE* and *orbI–orbL* operons is indirectly regulated by Fur through its influence on OrbS abundance. However, as transcripts corresponding to *orbH* through to *orbB* appear to be generated from both the *orbS* and *orbH* promoters, regulation of these genes will be subject to direct and indirect regulation by Fur (Fig. 5).

It has also been shown that ornibactin biosynthesis is influenced by quorum sensing. Mutations in the *cepR* or *cepI* genes, encoding a LuxR/LuxI-type quorum sensing regulatory system, result in an approximately 65% increase in ornibactin biosynthesis in *B. cenocepacia* K56-2, but exert no effect on salicylate or pyochelin production (Lewenza et al. 1999; Lewenza and Sokol 2001; Mallot et al. 2005). This increase in ornibactin production was accompanied by a similar increase in *pvdA* transcription during the stationary phase of growth in the presence or absence of iron (Lewenza and Sokol 2001). The DNA sequence recognised by CepR is CTGTnAanntTnACAG, but the location of the binding site(s) for CepR within the ornibactin locus have not been determined (Weingart et al. 2005). Ornibactin synthesis was not affected by mutation in the *cciIR* quorum sensing system (Mallot et al. 2005). Synthesis of ornibactin is also induced approximately twofold by ornithine and some other amino acids, but is not affected by putrescine (Meyer et al. 1995).

Cepabactin

Cepabactin, 1-hydroxy-5-methoxy-6-methyl-2(1H)-pyridinone, is a cyclic hydroxamate (i.e. a hydroxypyridonate) and for that reason can also be considered as a heterocyclic catecholate (Meyer et al. 1989) (Fig. 6a). It was first identified as a metal-binding antibiotic that is secreted into the culture medium by *P. alcaligenes* strain NCIB 11492, and was termed G1549 (Barker et al. 1979). Independently, it was isolated from another pseudomonad (strain BN-227) and named BN-227 (Itoh et al. 1979, 1980). BN-227 was also shown to have antimicrobial properties and formed a complex with ferric iron. A compound with the same structure was subsequently isolated from the *B. cepacia* type strain, ATCC 25416, and *B. cepacia* ATCC 17759 (Meyer et al. 1989, 1995). This compound, named cepabactin, was shown to have all the characteristics of a siderophore: biosynthesis of cepabactin occurred only under iron starvation conditions (maximal production occurred in the presence of 2 μM $FeCl_3$; complete abrogation of cepabactin production occurred at 10 μM $FeCl_3$), and exogenous addition of cepabactin caused growth stimulation under iron-limiting conditions and facilitated iron uptake (Meyer et al. 1989). However, it was found that among 12 environmental and 2 clinical isolates of *B. vietnamiensis*, not one produced cepabactin (Meyer et al. 1995). Furthermore, in a survey of over 60

Bcc clinical isolates, only 6 (comprising a single RAPD type) were demonstrated to produce cepabactin, and in no case was it the sole siderophore produced (Darling et al. 1998). Some of the strains which did not produce cepabactin (i.e. K56-2 and 715j) have now been identified as *B. cenocepacia*. Nevertheless, it is possible that members of the Bcc that do not make cepabactin may still have the ability to utilise this siderophore. A precedent has been set by the fluorescent pseudomonads, as exogenous addition of cepabactin to *P. aeruginosa* strain PAO1 promotes iron uptake, suggesting that a cepabactin-specific receptor exists in this strain (Meyer 1992; Mislin et al. 2006).

The total synthesis of cepabactin has been described (Ohta et al. 1990; Klumpp et al. 2005). Cepabactin forms an orange complex with ferric iron possessing a stoichiometry of three bidentate molecules of cepabactin per metal ion (Itoh et al. 1979; Klumpp et al. 2005). Interestingly, in the presence of pyochelin, a purple-coloured complex is formed, comprised of ferric iron, cepabactin and pyochelin with a 1:1:1 stoichiometry, in which cepabactin provides two of the six dentates and pyochelin provides the other four (Fig. 2c). This complex appears to be less stable than the ferric–cepabactin complex but is more stable than the ferripyochelin complex (Klumpp et al. 2005). It has yet to be determined whether cepabactin serves as an effective siderophore in vivo. The genes responsible for the production and utilisation of cepabactin have not been described.

Cepaciachelin

Cepaciachelin, 1-*N*-[2-*N*′,6-*N*′-di(2,3-dihydroxybenzoyl)-L-lysyl]-1,4-diaminobutane, is a catecholate siderophore first isolated from the culture supernatant of *B. ambifaria* strain PHP7 (LMG 11351), a rhizosphere isolate, grown under iron-limiting conditions (Barelmann et al. 1996). It is comprised of a single molecule of lysine derivatised with 2,3-dihydroxybenzoic acid (DHBA) on the α and ε amino groups, and with diaminobutane (putrescine) on the carboxyl group (Fig. 6b). Thus, cepaciachelin is similar to protochelin, a linear tri-catecholate siderophore produced by a methanolotrophic bacterium and by *Azotobacter vinelandii*, but lacks one of the three 2,3-dihydroxybenzoate moieties (Taraz et al. 1990; Cornish and Page 1995). Biosynthesis of cepaciachelin is likely to involve azotochelin as an intermediate, the probable precursor of protochelin. The effect of iron supplementation on cepaciachelin production has not been reported and its ability to promote iron uptake or growth of *B. ambifaria* has not been examined. However, its status as a siderophore was inferrred from its structural relationship to known siderophores. The genes responsible for the biosynthesis and utilisation of cepaciachelin and the regulation of its production have not been described.

Salicylic acid

Salicylic acid, 2-hydroxybenzoic acid, is biosynthesised by members of several bacterial genera under iron-limiting conditions, where it serves as a precursor for the production of a variety of siderophores. Examples of salicylate-derived siderophores include pyochelin, produced by some pseudomonads and *Burkholderia*, including members of the Bcc (Ankenbauer and Cox 1988), the mycobacterial siderophores, mycobactin and carboxymycobactin (Ratledge and Hall 1972; Adilakshmi et al. 2000), vulnibactin, a siderophore of *V. vulnificus* (Okujo et al. 1994), yersiniabactin, produced by *Yersinia* spp. (Gehring et al. 1998), parabactin of *Paracoccus denitrificans* (Peterson and Neilands 1979), the salicyloyl (i.e. 2-hydroxybenzoyl) peptide derivative, maduraferrin, manufactured by the actinomycete *Actinomadura madurae* (Keller-Schierlein et al. 1988), and pseudomonine, a blue fluorescent compound produced by *P. fluorescens* (Anthoni et al. 1995; Mercado-Blanco et al. 2001). However, in such bacteria, salicylate also accumulates in the culture medium (Ratledge and Winder 1962; Sokol et al. 1992; Meyer et al. 1992; Visca et al. 1993). Additionally, salicylate is produced by some bacteria that are not known to make salicylate-derived siderophores, such as *Azospirillum lipoferum* (Saxena et al. 1986) and some pyochelin-negative Bcc isolates (Sokol et al. 1992; Darling et al. 1998), and it has been proposed as a siderophore in its own right (Meyer et al. 1992; Visca et al. 1993).

In bacteria, salicylic acid is biosynthesised from chorismic acid (Fig. 6d), the product of the shikimic acid pathway that serves as the precursor of aromatic amino acids, folic acid, ubiquinone, phenazines and 2,3-DHBA. In *P. aeruginosa*, the conversion of chorismate to salicylate occurs via isochorismate, and requires the successive action of isochorismate synthase (ICS) and isochorismate pyruvate-lyase (IPL) (Fig. 6d) (Serino et al. 1995; Gaille et al. 2002, 2003). In *P. aeruginosa*, ICS is encoded by *pchA* (PA4231). As the isomerisation of chorismate to isochorismate is also required for the biosynthesis of DHBA (a precursor of many catecholate siderophores, including the *E. coli* siderophore enterochelin), orthologues of *pchA* are found in bacteria that produce such siderophores but do not produce salicylate-based iron chelators (i.e. *entC* encodes ICS in *E. coli*, but *E. coli* does not produce salicylate). The second step, involving elimination of pyruvate from isochorismate, is encoded by *pchB* (PA4230). The PchB enzyme (IPL) also possesses a less efficient chorismate mutase activity that results in formation of prephenate, an intermediate in phenylalanine and tyrosine biosynthesis (Gaille et al. 2002).

In *Y. pestis* and *M. tuberculosis*, it has been conjectured that salicylate may be produced in a one-step pathway, as only a single gene (*ybtS* and *mbtI*, respectively) that exhibits homology to genes involved in salicylate biosynthesis has been identified in the yersiniabactin and mycobactin gene clusters (Gehring et al. 1998; Quadri et al. 1998). Subsequently, it was shown that the *Y. enterocolitica irp9* gene encodes a salicylate synthase which converts chorismate to salicylate through the intermediate isochorismate (Kerbarh et al. 2005). It has also been shown that isochorismate can serve as a precursor for salicylate synthesis in *M. smegmatis*, suggesting that if a single enzyme is required for salicylate biosynthesis in this organism, it also involves isochorismate as an intermediate (Marshall and Ratledge 1971, 1972).

pchA and *pchB* are present as part of a cluster of genes required for the biosynthesis and transport of pyochelin in *P. aeruginosa* and *B. cenocepacia* (Fig. 5) (Serino et al. 1995, 1997). In the genome of *B. cenocepacia*, orthologues of *pchA* (BCAM2235) and *pchB* (BCAM2234) are located in a similarly organised cluster on chromosome 2 (see Sect. ''Pyochelin'' and Fig. 5), and the protein products exhibit 47.4 and 74.3% identity, respectively, with *P. aeruginosa* PchA and PchB (our unpublished observations). This suggests that the regulation of salicylate biosynthesis in *Burkholderia* is identical to that in the fluorescent pseudomonads (see Sect. ''Pyochelin'').

The role of salicylate as a bacterial siderophore is controversial. Various investigators have provided evidence that suggests salicylate may be a siderophore. First, salicylate has efficient iron-binding properties, forming a violet-coloured complex with iron in aqueous solution due to coordination of a single Fe(III) ion by three molecules of salicylate (Ogawa and Tobe 1966; Ratledge et al. 1974; Martell and Smith 1977; Meyer et al. 1992; Visca et al. 1993). Secondly, salicylate appears to promote iron uptake in *P. aeruginosa*, *P. fluorescens* and *B. cenocepacia* (Meyer 1992; Meyer et al. 1992; Sokol et al. 1999; Visser et al. 2004), and promotes growth of *P. aeruginosa* and members of the Bcc under iron-limiting conditions (Sokol et al. 1992; Visca et al. 1993). Finally, the production of salicylate by bacteria, and/or expression of the salicylate biosynthesis genes, is iron-regulated in *P. aeruginosa*, *P. fluorescens*, *M. tuberculosis* and the Bcc (Meyer et al. 1992; Sokol et al. 1992; Visca et al. 1993; Serino et al. 1997; Mercado-Blanco et al. 2001; Rodriguez et al. 2002).

Other experimental data provide less support for salicylate playing a role as a siderophore in vivo (Ratledge and Dover 2000). For example, it has been shown that salicylate is ineffective at sequestering ferric ions at neutral pH in the presence of inorganic phosphate (Ratledge et al. 1974; Chipperfield and Ratledge 2000). Bacteria that secrete salicylate but do not elaborate other siderophores are negative in the CAS plate assay (Schwyn and Neilands 1987; Sokol et al. 1999; Agnoli et al. 2006). Only when salicylate is applied at high concentrations to CAS agar is there a colour shift, and this is only a transitory change (Mercado-Blanco et al. 2001). Culture supernatants of *B. cenocepacia* mutants that hyperproduce salicylate are also not active in the CAS assay, even in the presence of the

shuttle, 5-sulfosalicylate (Sokol et al. 1999). Mutants that produce only salicylate as a potential siderophore have been shown to be growth limited relative to the wild type strain under iron-restrictive conditions (De Voss et al. 2000; Agnoli et al. 2006; A.M. Cook and M.S. Thomas, unpublished results), and such strains are much less virulent than the siderophore-producing parent (De Voss et al. 2000; Sokol et al. 2000). Furthermore, there are no reports of the characterisation of a ferric salicylate-specific outer membrane receptor which would presumably be required for transport of the ferric tri-salicylate complex.

The clustering of genes responsible for the biosynthesis of salicylate from chorismic acid with those required for the synthesis of salicylate-based siderophores is suggestive that the primary function of salicylate biosynthesis is as a precursor for more complex siderophores. Thus, in *P. fluorescens* strain WCS373, these genes (*pmsC* and *pmsB*) are linked to the pseudomonine biosynthesis genes (Mercado-Blanco et al. 2001), while in *P. aeruginosa*, *P. fluorescens* Pf-5, *B. cenocepacia* and *B. pseudomallei*, the *pchA* and *pchB* genes comprise part of a gene cluster encoding pyochelin biosynthesis and transport functions (Serino et al. 1997; Holden et al. 2004; Paulsen et al. 2005, http://www.sanger.ac.uk/Projects/B_cenocepacia/). In *M. tuberculosis*, the *mbtI* gene is similarly clustered with the genes specifying mycobactin synthesis (Quadri et al. 1998). This observation also explains the iron-responsive regulation of salicylate production. Furthermore, in strains producing pyochelin, expression of *pchA* and *pchB* will require the presence of pyochelin. This suggests that transcription of *pchA* and *pchB* cannot be uncoupled from that of the other pyochelin biosynthetic genes. The stimulatory effect of salicylate on growth of some bacteria could also be explained by the incorporation of salicylate into a salicylate-derived siderophore, which then serves to promote growth. More difficult to explain using this argument, is the fact that salicylate facilitates uptake of ferric iron. However, it is noteworthy that in one set of experiments which suggested that salicylate stimulated iron uptake by wild type *B. cenocepacia*, ornibactin-deficient mutants exhibited impaired salicylate-mediated iron uptake (Sokol et al. 1999). One possible explanation for this is that the iron uptake measured for the wild type strain was actually due to the action of ornibactin.

Xenosiderophores

Very little information is available in the literature regarding the nature of xenosiderophores that can be utilised by members of the Bcc. It has been shown that the *B. cepacia* type strain, ATCC 25416 cannot utilise pyoverdines obtained from *P. aeruginosa* PAO1 (ATCC 15692) and two different *P. fluorescens* strains (Meyer et al. 1989). Plate bioassays have revealed that *B. cenocepacia* can utilise ferrichrome and ferrioxamine B (desferal) but not citrate (our unpublished observations).

Other mechanisms of iron acquisition by the Bcc

Haem uptake

Burkholderia cenocepacia can use haemin (oxidised haem) as an iron source (Whitby et al. 2006; our unpublished observations). The haem uptake system of *B. cenocepacia* is organised similarly to that of *B. pseudomallei* and is encoded by the *bhu* operon (*Burkholderia* haem uptake) (G. Shalom, J. Shaw and M. Thomas, in preparation). This operon is comprised of five genes, *bhuRSTUV*, and appears to be under the control of a σ^{70}-dependent promoter (Fig. 5). Overlapping this promoter is a sequence possessing a good match (15/19) to the Fur box consensus sequence, suggesting that it is subject to Fur-mediated repression (our unpublished observations). Orthologues of the *bhuRSTUV* genes encode haem uptake systems in other Gram-negative bacteria (Wandersman and Stojiljkovic 2000; Genco and Dixon 2001; Stojiljkovic and Perkins-Balding 2002). We predict that *bhuR* encodes the specific TonB-dependent outer membrane receptor for haem, while *bhuT*, *-U* and *-V* encode the periplasmic binding protein, cytoplasmic membrane transporter, and ATPase components, respectively, of the haem permease. BhuS, is similar to HemS, HmuS and ShuS. Recent evidence

suggests that ShuS acts as a type of shuttle, transferring haem from the haem-specific transport system to haem proteins or haem-degrading proteins (Wyckoff et al. 2005).

Utilisation of ferritin

Recently, it has been shown that *B. cenocepacia* can utilise ferritin as an iron source (Whitby et al. 2006). The mechanism of ferritin utilisation appears to require degradation of ferritin by a secreted serine protease. Ferritin concentrations are particularly high in the lungs of CF patients, and it is tempting to speculate that *B. cenocepacia* takes advantage of its ability to utilise ferritin under these conditions.

Acknowledgements The author would like to thank Cornelia Reimmann and Colin Ratledge for critical reading of the manuscript, and Kirsty Agnoli for help with the MDL ISIS Draw Program.

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