The *pobA* gene of *Burkholderia cenocepacia* encodes a Group I Sfp-type phosphopantetheinyltransferase required for biosynthesis of the siderophores ornibactin and pyochelin

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INTRODUCTION

The genus *Burkholderia* includes a number of Gram-negative bacterial species that are pathogenic to animals and/or plants (Mahenthiralingam et al., 2005; Compant et al., 2008). One group of closely related species, the *Burkholderia cepacia* complex (BCC), are noted for their ability to cause opportunistic infections in humans, particularly in patients with cystic fibrosis (CF) (LiPuma, 1998; Coenye et al., 2001; Speert, 2002; Mahenthiralingam et al., 2008). Among the members of the BCC, *Burkholderia cenocepacia* is the most prevalent in both CF and non-CF infections (Vandamme et al., 1997; Coenye et al., 2001; Mahenthiralingam et al., 2002, 2005; Reik et al., 2005). The complete genome sequence has recently been determined for a strain of *B. cenocepacia* that caused an epidemic among CF patients in Canada and the UK (Holden et al., 2009).

Colonization of the CF lung by bacterial pathogens requires the expression of high-affinity iron uptake systems due to the decreased availability of iron (Brown et al., 1984; Thompson et al., 1990; Gray-Owen & Schryvers, 1996; Wang et al., 1996). A commonly employed mechanism for
iron acquisition by bacteria is the secretion of low-molecular-weight Fe(III)-binding compounds known as siderophores, which are then transported into the bacterium via specific outer membrane receptors (Braun, 2001; Croas & Walsh, 2002). Most clinical isolates of B. cenocepacia produce the siderophores ornibactin and pyochelin (Sokol, 1986; Visca et al., 1993; Meyer et al., 1995; Darling et al., 1998; Thomas, 2007), and their production has been correlated with morbidity and mortality in CF patients and/or shown to contribute to pathology in animal models of respiratory infection (Sokol, 1986; Sokol & Woods, 1988; Sokol et al., 1999, 2000; Visca et al., 2004; Uehlinger et al., 2009). Ornibactin contains an L-ornithine-D-hydroxyaspartate-L-serine-L-ornithine backbone that requires two non-ribosomal peptide synthetases (NRPSs), OrbI and OrbJ, for its assembly (Stephan et al., 1993; Agnoli et al., 2006). The yellow-green fluorescent siderophore pyochelin is biosynthesized from salicylate by the successive addition and cyclization of two molecules of cysteine (Ankenbauer et al., 1988; Reimmann et al., 1998; Quadri et al., 1999; Quadri, 2000; Croas & Walsh, 2002).

NRPSs have a modular arrangement, consisting mainly of repeated tri-domain elongation units (‘modules’). Each module comprises (i) an amino acid adenylation domain (A), which serves to activate the amino acid building block; (ii) a peptidyl carrier protein (PCP) domain, which is derivatized with a 4’-phosphopantetheine (P-pant) ‘arm’ of ~20 Å in length that is used, in turn, to covalently attach the amino acid to the NRPS via a thioester bond; and (iii) a condensation domain (C), which catalyzes peptide bond formation between the phosphopantetheine-anchored amino acid in one module and a similarly anchored amino acid in the preceding unit (Quadri, 2000; Croas & Walsh, 2002; Challis & Nasmith, 2004; Sieber & Marahiel, 2005). In situations where one of the building blocks is not an amino acid, such as occurs in pyochelin, which has an aryl N-cap derived from salicylate, the PCP domain is substituted by an aryl carrier protein (ArCP) domain, as in the case of PchE. This domain is also a substrate for phosphopantetheinylation. Activation of NRPS PCP and ArCP domains is carried out by a phosphopantetheinyltransferase (PPTase). This enzyme transfers the P-pant moiety from coenzyme A (CoASH) to an invariant serine residue in the conserved phosphopantetheinylation site [FFxLLG(D/H)S(L/I)] of the PCP/ArCP domain (Walsh et al., 1997; Marahiel et al., 1997; Quadri et al., 1998b). A total of four predicted PCP domains are present in the ornibactin biosynthetic machinery (three in OrbI and one in OrbJ), each of which binds one of the four amino acid building blocks, and three PCP/ArCP domains occur in the NRPSs involved in pyochelin biosynthesis (two in PchE and one in PchF) (Quadri, 2000; Agnoli et al., 2006).

A well-characterized PPTase is the Escherichia coli EntD protein, which is required for activating the ArCP domain of EntB, and the PCP domain of EntF during biosynthesis of the catecholate siderophore enterobactin (Lambalot et al., 1996; Gehring et al., 1997). PPTases are also required for the activation of NRPSs and polyketide synthetases (PKSs) that participate in the biosynthesis of compounds other than siderophores (Walsh et al., 1997; Sieber & Marahiel, 2003). For example, the Bacillus subtilis PPTase Spf is required for the biosynthesis of surfactin, a cyclic lipopeptide possessing antibiotic properties, as well as for the catecholate siderophore bacillibactin (Nakano et al., 1992; Grossman et al., 1993; Quadri et al., 1998b; Heerklotz & Seelig, 2001; May et al., 2001). PPTases such as EntD and Spf, which play roles in secondary metabolism, are referred to as Spf-type PPTases, and several different Spf-type PPTases may be encoded by the same bacterium (Lu et al., 2008).

In the majority of bacterial species, a second type of PPTase, known as holo-ACP synthase (ACPS) or AcpS-type PPTase, is required for activation of acyl carrier protein (ACP), a versatile protein that participates in the biosynthesis of fatty acids, phospholipids, lipid A, lipoic acid, RTX toxins, acylhomoserine lactones and the aldehyde substrate of bacterial luciferase (Lambalot & Walsh, 1995; Flugel et al., 2000). The polypeptide chain length of AcpS-type PPTases (~120 amino acids) is approximately half that of Spf-type PPTases and, unlike the monomeric Spf-type PPTases, they form homotrimers. AcpS-type PPTases also activate the ACP domain(s) of PKSs but not those of NRPSs (Gehring et al., 1997). The Spf-type PPTases produced by some bacteria (for example, EntD) cannot substitute for AcpS-type PPTases, and therefore in these bacteria the AcpS-type PPTase is essential for growth (Flugel et al., 2000). However, in some cases the Spf-type PPTase can activate both NRPSs and ACP. For example, a Bacillus subtilis acpS mutant supports fatty acid biosynthesis, and Bacillus subtilis Spf will modify E. coli or Bacillus subtilis apo-ACP in vitro (Quadri et al., 1998b; Mootz et al., 2001). In the case of Pseudomonas aeruginosa, the cross-reactivity of the Spf-type PPTase is obligatory, as only the Spf-type PPTase (PcpS) is present (Finking et al., 2002; Barekzi et al., 2004).

As part of our investigation into iron homeostasis in B. cenocepacia, we have used a novel transposon to isolate mutants that are unable to produce ornibactin and pyochelin. Characterization of the mutants has allowed us to infer that a single Spf-type PPTase is required to activate the four NRPSs involved in biosynthesis of the two siderophores.

**METHODS**

**Bacterial strains, media and growth conditions.** All bacterial strains used are shown in Table 1. B. cenocepacia strains were cultured at 37°C on M9 minimal salts agar (Clowes & Hayes, 1968) containing glucose (0.5%) as the carbon source, and subsequently maintained on this medium at room temperature (~20°C). E. coli strains were cultured at 37°C on MacConkey agar or on LB agar containing appropriate antibiotics for plasmid selection [IST agar (Oxoid) was used when the antibiotic was trimethoprim], and were...
Table 1. Bacterial strains, plasmids and transposons used in this study

Abbreviations: ApR, encodes ampicillin resistance; CmR, encodes chloramphenicol resistance; KmR, encodes kanamycin resistance; TcR, encodes tetracycline resistance; RifR, rifampicin-resistant; SmR, streptomycin-resistant; TpR, trimethoprim-resistant or encodes trimethoprim resistance; Pch−, pyochelin-negative phenotype; Orb−, orbacinin-negative phenotype; BHR, broad host range.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>B. cenocepacia strains</strong></td>
<td></td>
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</tr>
<tr>
<td>715j</td>
<td>CF isolate, prototroph (Orb+ Pch−)</td>
<td>Darling et al. (1998)</td>
</tr>
<tr>
<td>AHA1</td>
<td>Pyochelin-negative spontaneous mutant of 715j (Orb+ Pch−)</td>
<td>This study</td>
</tr>
<tr>
<td>AHA27</td>
<td>715j-pobA::mini-Tn5Cm262YA (Orb− Pch−)</td>
<td>This study</td>
</tr>
<tr>
<td>IW10</td>
<td>715j-pobA::mini-Tn5Cm262YA (Orb+ Pch−)</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC1061</td>
<td>hsdR araD139 Δ(ara-leu)7697 ΔlacX74 galIU galK rpsL (SmR)</td>
<td>Casadaban &amp; Cohen (1980)</td>
</tr>
<tr>
<td>JM83</td>
<td>F− ara D(lac-proAB) rpsL ᵃ800lacZΔM15 (SmR)</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>CC118(pir)</td>
<td>araD139 Δ(ara-leu)7697 ΔlacX74 galIU galK pBAD24 thi-1 rpsL argE3 (am) recA1 pir (R'R)</td>
<td>Herrero et al. (1990)</td>
</tr>
<tr>
<td>SM10(pir)</td>
<td>thi-1 thr leu tonA lacY supE recA1</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>S17-1</td>
<td>thi proA hsdR recA RP4-2-Tc::Mu (KmR) (pir)</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>BW19851</td>
<td>ΔuidA::pir+ recA1 hsdR17 creBS10 endA1 zfi-5 thi RP4-2-Tc::Mu-1 kan::Tn7 integrant (TpR, SmR)</td>
<td>Metcalf et al. (1994)</td>
</tr>
<tr>
<td>AN90</td>
<td>thi leuB proC trpE lacY mtl xyl rpsL azi flaA tsx supA recA1endD</td>
<td>Cox et al. (1970)</td>
</tr>
<tr>
<td><strong>Plasmids and transposons</strong></td>
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</tr>
<tr>
<td>pBRR1MC5</td>
<td>Mobilizable BHR cloning vector, IncP- and ColE1-compatible (CmR)</td>
<td>Kovach et al. (1994)</td>
</tr>
<tr>
<td>pAHA14</td>
<td>pBRR1MC5 with the dfrB2 gene inserted in the EcoRI site of cat (TpR)</td>
<td>This study</td>
</tr>
<tr>
<td>pAHA14-pobA</td>
<td>pAHA14 containing the B. cenocepacia 715j pobA gene under the control of the lacZ promoter (TpR)</td>
<td>This study</td>
</tr>
<tr>
<td>pUC8-sfp</td>
<td>pUC8 containing the Bacillus subtilis sfp gene (ApR)</td>
<td>Nakano et al. (1992)</td>
</tr>
<tr>
<td>pBRR1MC5-sfp</td>
<td>pBRR1MC5 containing the Bacillus subtilis sfp gene (ApR)</td>
<td>This study</td>
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<tr>
<td>pET28a-entD</td>
<td>pET28a containing the E. coli entD gene (KmR)</td>
<td>Lambalot et al. (1996)</td>
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<tr>
<td>pAHA14-sfp</td>
<td>pAHA14 containing the Bacillus subtilis sfp gene (TpR)</td>
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<tr>
<td>pAHA14-entD</td>
<td>pAHA14 containing the E. coli entD gene (TpR)</td>
<td>This study</td>
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<tr>
<td>pRW500</td>
<td>RK2-derived reporter vector harbouring lacZYA genes (TcR)</td>
<td>This study</td>
</tr>
<tr>
<td>pUC19</td>
<td>E. coli-specific cloning vector (ApR)</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pUC18NotlacZYA</td>
<td>pUC18Not containing 5.89 kb promoterless lacZYA cassette (ApR)</td>
<td>This study</td>
</tr>
<tr>
<td>pUTmini-Tn5Cm5</td>
<td>Mobilisble suicide vector, pUT, containing mini-Tn5Cm5 (CmR, ApR)</td>
<td>de Lorenzo et al. (1990)</td>
</tr>
<tr>
<td>pUTmini-Tn5StplacZYA</td>
<td>pUTmini-Tn5Cm5 containing lacZYA cassette in NotI site</td>
<td>This study</td>
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<tr>
<td>pUTmini-Tn5StplacZYA</td>
<td>pUTmini-Tn5Cm5 containing lacZYA cassette in NotI site</td>
<td>This study</td>
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Maintained at 4 °C. Where indicated, casamino acids (CAA; Difco) were added to M9-glucone medium to a final concentration of 0.1%, and is referred to as M9-CAA medium. Antibiotics were used at the following concentrations: ampicillin, 100 μg ml−1 (E. coli); trimethoprim, 25–50 μg ml−1 (E. coli) and 50 μg ml−1 for B. cenocepacia growing on LB medium and in M9-CAA medium, 100 μg ml−1 on M9-glucose agar; tetracycline (E. coli), 20 μg ml−1; kanamycin (E. coli), 25 μg ml−1. For strains carrying lacZ fusions, X-Gal was included in solid medium at 40 μg ml−1. CAS plates used to screen B. cenocepacia mutants for siderophore production were made as described by Agnoli et al. (2006). For assaying enterobactin production by E. coli strains, CAS agar was made by adding 10 ml CAS reagent (Schwyn & Neilands, 1987) to 90 ml M9 salts agar supplemented with glucose (0.2%), casamino acids (0.2%), leucine (20 μg ml−1), proline (20 μg ml−1), tryptoarin (10 μg ml−1) and thiamine (5 μg ml−1). EDDHA plates were made by adding ethylenediaminedi(o-hydroxyphenylacetic) acid (EDDHA) to M9-CAA agar at a final concentration of 35 μg ml−1.

For measurement of the effect of iron availability on the bacterial growth rate, B. cenocepacia strains were grown to stationary phase at 37 °C (i.e. ~20 h) in M9-CAA medium (25 μg ml−1 trimethoprim was included when pAHA14 derivatives were employed). Cells from these cultures were inoculated at 100-fold dilution into 50 ml fresh medium containing either 2,2'-dipyridyl (100 μM) to generate iron-starvation conditions or 50 μM ferric chloride for iron-replete conditions, and were grown in flasks at 37 °C with vigorous shaking. For β-galactosidase assays, B. cenocepacia strains were grown to stationary phase (~20 h) at 37 °C in the same medium used to culture the cells for the assay, and were diluted 100-fold into 5 ml of this medium. To determine the effect of iron availability on gene expression, this medium was used as the same that used for growth rate determination.

**Plasmid and transposon construction.** All plasmids and transposons used are shown in Table 1. Recombinant DNA techniques were performed essentially as described in Sambrook et al. (1989). Construction of pUC18NotlacZYA and its progenitor, pRW500, is
described in the Supplementary Material and in Supplementary Figs S1 and S2). To construct mini-Tn5CmlacZYA and mini-Tn5TpacZYA, the 5.89 kb NotI lacZYA cassette of pUC18NotlacZYA was inserted into the unique NotI site located between the ω-Cm interposon and the O end of mini-Tn5Cm, and between the dfr (Tp) cassette and the O end of mini-Tn5Tp, and derivatives in which the N-terminal coding region of the lacZ gene was positioned adjacent to the O end of the mini-transposon were selected.

pAHA14 is identical to pBBRTP (DeShazer & Woods, 1996), and was constructed by transferring the dfrB2 (Tp9 trimethoprim-resistance) cassette (which includes the dfrB2 promoter) from p34E-Tp into the EcoRI site located within the cat gene of pBBR1MCS. dfrB2 is inserted in the opposite orientation to the cat gene. pAHA14-pobA was constructed by amplifying the pobA gene from B. cenocepacia 715j genomic DNA with Pfu Turbo DNA polymerase (Stratagene) in the presence of DMSO (5%) using primers pobAfor (5′-GGCG-AAGCTTGTTAAACGATGCATGATCGAACGTT-3′) and pobArev (5′-GGCGGAGTACCAAATGGTTCATGAAGCCG-3′) restriction sites underlined, and cloning the resultant product between the HindIII and BamHI sites of pAHA14. The pobAfor primer contained a stop codon (bold type) to ensure no translational readthrough of the 5.89 kb mini-Tn5Cm with mini-Tn5Tp, and derivatives in which the N-terminal coding region of the lacZ gene was positioned adjacent to the O end of the mini-transposon were selected.

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**RESULTS**

**Construction of new reporter transposons for use in Burkholderia**

Tn5 derivatives have been shown to be useful for genetic analysis of members of the genus *Burkholderia* (Dennis & Zylstra, 1998; Sokol et al., 1999; Farmer & Thomas, 2004; Agnoli et al., 2006). To increase the versatility of these transposons, we inserted a promoterless lacZ cassette into the unique NotI site of mini-Tn5Cm and mini-Tn5Tp, thereby allowing lacZ to serve as a reporter of transcription originating in genomic DNA flanking the O end of the integrated transposon. The resultant transposons were named mini-Tn5CmlacZYA and mini-Tn5TpacZYA (Fig. 1), and are contained in the mobilizable suicide plasmid pUT (de Lorenzo & Timmis, 1994). The transferred lacZ gene is devoid of the segments of the *E. coli* trpB and trpA genes that are fused to the 5′ end of lacZ in many transcriptional reporter plasmids and transposons (see Supplementary Material). To prevent the formation of translational fusions, translation termination codons are present in all three reading frames upstream of the lacZ Shine–Dalgarno sequence. The nucleotide sequence of the region upstream of the lacZ translation initiation codon is shown in Supplementary Fig. S2.

**Isolation of B. cenocepacia mutants in which production of both ornibactin and pyochelin is decreased or abolished**

*B. cenocepacia* strain 715j produces the siderophores ornibactin and pyochelin (Darling et al., 1998; Visser et al., 2004; Farmer & Thomas, 2004; Agnoli et al., 2006). To test the effectiveness of mini-Tn5CmlacZYA, it was used to generate mutants of 715j that were deficient in production of both siderophores (Orb\(^\text{−}\) Pch\(^\text{−}\) phenotype).
In two separate mutagenesis experiments, siderophore-deficient mutants were identified on CAS agar by the absence of an orange halo surrounding the colony or the presence of a halo of decreased size. Only one mutant was identified (AHA27) in which the production of a halo was completely abolished, although several mutants were identified which generated a very small halo. As expected, analysis of individual siderophore production showed that AHA27 failed to produce either ornibactin or pyochelin when growing in liquid culture under iron-limiting conditions (Fig. 2a, b). Production of the pyochelin precursor salicylic acid by this mutant was reduced although not abolished. Of the mutants that produced a very small orange zone around the growth, IW10 produced trace amounts of pyochelin in liquid culture, whereas production of salicylate and ornibactin was not significantly affected (Fig. 2c, d). The phenotypes of AHA27 and IW10 in the CAS plate assay are shown in Fig. 2(e). The absence of an orange zone around the growth of AHA27 on CAS plates confirms a previous observation that salicylate is not active as a siderophore in the CAS assay, although it has been proposed as a siderophore for \textit{B. cenocepacia} (Sokol et al., 1992, 1999; Visca et al., 1993).

**Genomic loci of transposon insertions in pyochelin-deficient mutants**

Genomic DNA isolated from the two pyochelin-deficient mutants was digested with the restriction enzyme \textsc{SacI} and probed with mini-Tn5Cm\textit{lacz}YA DNA following Southern blotting. The probed digestions revealed that a single transposon insertion had occurred in each case (results not shown). Nucleotide sequence analysis of the DNA flanking the transposons in AHA27 and IW10 revealed that in both mutants, the transposon had integrated into a 251 codon ORF, which we have named \textit{pobA} (pyochelin and ornibactin biosynthesis) (Fig. 3). This corresponds to BCAL2248 in the recently published genome sequence of \textit{B. cenocepacia} strain J2315 (Holden et al., 2009), and the protein product of the 715\textit{pobA} gene differed at only one amino acid position from the predicted product of BCAL2248 (the substitution of alanine for valine at amino acid position 215). In IW10, the \textit{lacz} gene was inserted in the same orientation as \textit{pobA}, whereas in AHA27 the \textit{lacz} was inserted in the reverse orientation (Fig. 3).

The \textit{pobA} gene encodes a polypeptide that exhibits strong similarity to bacterial PPTases required for activating ArCP and PCP domains of NRPSs. The strongest matches (38\% identity over the matching region) to proteins outside the genus \textit{Burkholderia} were to a putative Sfp-type PPTase proposed to be involved in the biosynthesis of the lipopeptide antibiotic iturin A by \textit{Synechocystis} spp. and to the Hetl protein, a PPTase which has been proposed to be required for biosynthesis of a secondary metabolite involved in heterocyst formation by \textit{Nostoc} spp. (Black & Wolk, 1994; Kaneko et al., 1996; Lambalot et al., 1996). Both organisms are members of the Cyanobacteria. The amino acid sequence of the predicted \textit{PobA} polypeptide was also similar to those of characterized Sfp-type PPTases, including the \textit{Streptomyces venezuelae} \textit{jadM} gene product, required for biosynthesis of the antibiotic jadomycin, the \textit{Mycobacterium tuberculosis} \textit{pptT} gene product, required for biosynthesis of the siderophore mycobactin, the \textit{E. coli} PPTase \textit{EntD}, and Sfp itself (Lambalot et al., 1996; Quadri et al., 1998a, b; Wang et al., 2001). The amino acid sequence alignment showed that \textit{PobA} contains the signature sequence that characterizes both Sfp- and AcpS-type PPTases, and also revealed an additional motif, ‘ppt-S’, that is conserved in all Sfp-type PPTases (see Discussion). Furthermore, the alignment also showed that the Sfp subclass of PPTases falls into two conserved subgroups, I and II.

Consistent with the inability of AHA27 to produce siderophores, mini-Tn5Cm\textit{lacz}YA had inserted between codons 98 and 99 of \textit{pobA} in this mutant. However, in IW10, the transposon had inserted between the first and second bases of the predicted initiation codon for \textit{pobA}.

**Fig. 1.** Schematic representation of mini-Tn5T\textit{placZYA} and mini-Tn5Cm\textit{lacz}YA. The \textit{NotI} \textit{lacz}YA cassette introduced into mini-Tn5T and mini-Tn5Cm contains the \textit{lacz}, \textit{lacY} and \textit{lacA} genes (shown with grey shading). Preceding the \textit{lacz} translation initiation codon is a 200 bp leader region containing an RNase III processing site and translation termination codons in all three reading frames. Also present on the cassette are 600 bp of DNA that is located downstream of the \textit{lacA} translation stop codon on the \textit{E. coli} chromosome. The origin of the \textit{lacz}YA cassette is described in the Supplementary Material. Transcription termination signals located at each end of the \textit{o-Cm} interposon used to construct mini-Tn5Cm are represented by lollipop motifs. Note that the orientation of the \textit{cat} gene in mini-Tn5Cm has been confirmed by DNA sequencing, and is different from that shown elsewhere (Fellay et al., 1987; de Lorenzo et al., 1990; de Lorenzo & Timmis, 1994). The 19 bp \textit{O} and \textit{I} end repeat sequences flanking each transposon are indicated by vertical black bars. Sites for commonly used restriction enzymes are shown (note that single \textsc{SacI} sites are also located in \textit{lacz} and \textit{dfr}).
translation. As the terminal base of the transposon I end is a G residue, transposon insertion resulted in replacement of the native ATG codon by an alternative initiation codon (GTG), which may give rise to low levels of PobA synthesis in this mutant. A decreased level of PobA may have led to the observed negative effect on pyochelin biosynthesis if the enzyme possesses an intrinsically low phosphopantetheinylation activity towards one or more of the ArCP or PCP domains in PchE or PchF.

Effect of pobA disruption on growth under iron-limiting conditions

To examine the effect of disruption of pobA on growth of B. cenocepacia, the ability of both mutants to grow under conditions of iron limitation was examined. On agar containing the iron(III) chelator EDDHA, IW10 exhibited the same colony-forming ability as its parent strain, 715j, whereas growth of AHA27 was inhibited on this medium. In contrast, on standard minimal salts agar (without addition of iron) the two mutant strains formed colonies with equal efficiency (results not shown). Growth of the mutants was also monitored in liquid culture under iron-replete and iron-limiting conditions. Under iron-replete conditions, both mutants grew at a similar rate to the parent strain (Fig. 4a). In the presence of the iron chelator 2,2'-dipyridyl, the growth of the wild-type strain and IW10 was similar to that of iron-fed cells during early to mid exponential growth, but their growth rates became retarded relative to iron-fed cells during the mid to late exponential phase (i.e. at OD~0.5). In contrast, growth of the AHA27 mutant was almost completely abolished under iron-limiting conditions (Fig. 4a).

Complementation of the pobA mutant by characterized PPTase genes

To confirm that the observed phenotypes of AHA27 and IW10 are a direct result of disruption of the pobA reading frame, a complementation test was performed using pAHA14-pobA, in which the pobA gene is placed under the control of the lac promoter. Introduction of this plasmid into the pobA mutants resulted in a wild-type siderophore phenotype in the CAS plate assay (Fig. 5a; result for IW10 not shown). Analysis of individual siderophores produced by the complemented mutants showed that the wild-type copy of pobA restores normal production of pyochelin to mutant IW10, and of both pyochelin and ornibactin to AHA27 (Fig. 5b and results not shown). The results also showed that pAHA14-pobA, but not pAHA14, conferred on AHA27 the ability to grow...
at the wild-type rate under iron-limiting conditions (Fig. 4b).

To test the hypothesis that \(pobA\) encodes a PPTase, we carried out a complementation test with two well-studied PPTase genes: the \(E.\ coli\ entD\) gene, required for biosynthesis of the siderophore enterobactin, and the \(B.\ subtilis\ sfp\) gene, required for biosynthesis of surfactin. Both \(entD\) and \(sfp\) (without their cognate promoters) were subcloned into pAHA14 under the control of the \(lac\) promoter, and the resultant plasmids were introduced into AHA27 and IW10. Fig. 5(a, b) shows that both PPTase genes were able to restore the wild-type phenotype to AHA27 in the CAS plate assay as well as normal production of pyochelin.

**Complementation of an \(E.\ coli\ entD\) mutant by \(pobA\)**

The CAS plate assay can be used to distinguish between enterobactin-producing and non-producing strains (Schwyn & Neilands, 1987). Thus, the \(E.\ coli\ entD\) mutant AN90, which fails to biosynthesize enterobactin, gave a negative result in this assay, whereas the \(entD^+\) \(E.\ coli\) strain JM83 gave a positive result (Fig. 5c). As expected, expression of \(entD\) in AN90 in trans resulted in the restoration of siderophore production, yielding a phenotype identical to that of JM83. The same result was observed for the heterologous PPTase gene \(sfp\), as reported elsewhere (Grossman et al., 1993). Moreover, \(pobA\) was also able to substitute for \(entD\) in the complementation assay (Fig. 5c). This observation is consistent with the results of the \(pobA\) mutant complementation assay, and confirms that \(pobA\) encodes an Sfp-type PPTase.

**Effect of iron limitation on expression of the \(pobA\) gene**

As \(pobA\) is required for growth under iron-starvation conditions, mutant IW10 was used to monitor the regulation of \(pobA\) in response to iron. Due to the requirement for a fully functional \(pobA\) gene to ensure efficient siderophore production under iron-starvation conditions, \(\beta\)-galactosidase assays were carried out using a strain harbouring pAHA14-pobA. The results showed
that in cells containing multicopy pobA, iron had very little effect on pobA transcription. In the absence of the pobA plasmid, transcription of the pobA gene in iron-starved cells increased significantly relative to that in the pobA⁺ strain growing under the same conditions (~57% increase), and this was repressed by iron to a level similar to that measured in pobA⁻ cells growing under iron-replete conditions (Fig. 6).

**DISCUSSION**

In this study we have demonstrated that a single Sfp-type PPTase, PobA, is required for ornibactin and pyochelin biosynthesis in *B. cenocepacia*. The function of PobA is almost certainly to activate the OrbI, OrbJ, PchE and PchF NRPSs that participate in the biosynthesis of these siderophores. In contrast to the Sfp-type PPTase of *P. aeruginosa* (PcpS), which activates NRPSs involved in pyochelin and pyoverdine biosynthesis and also serves as the ACPS, PobA is not essential for growth of *B. cenocepacia* under iron-sufficient conditions. This suggests that an ACPS, or an additional Sfp-type PPTase with broad substrate specificity, is encoded by the *B. cenocepacia* genome. TBLASTN analysis of the genomes of *B. cenocepacia* strains HI2424 and J2315 reveals that they encode an ACPS [in fact, chromosome 1 of strain J2315 contains a duplication of 57 kb which gives rise to two identical copies of an acpS orthologue: BCAL1009 and BCAL2861 (Holden et al., 2009; Sousa et al., 2008)]. Furthermore, there are no additional Sfp-type PPTases encoded by the genome of strain J2315. Therefore, the non-lethality of the pobA null mutation is almost certainly due to the presence of an ACPS in *B. cenocepacia*. Although it is possible that PobA also serves as an ACPS, we have not explored whether, in the absence of an ACPS, PobA is able to support the growth of *B. cenocepacia*.

ACPS and Sfp-type PPTases share a common signature sequence (Lambalot et al., 1996; Walsh et al., 1997; Quadri...
et al., 1998b). With the huge increase in the number of bacterial genomic sequences now available, search and alignment tools have allowed us to make comparisons between a large number of Sfp-type PPTases and ACPSs, representing most of the major bacterial taxa. As outlined below, this analysis supports the idea that the subfamilies of bacterial Sfp-type PPTases should be divided into two distinct groups (Copp & Neilan, 2006), one of which shares a more extended region of homology with the ACPSs.

(i) Our analysis confirms that the originally proposed signature sequence for PPTases, composed of two conserved motifs \([(V/I)G(V/I)D(x)_{40-45}(F/W)(S/C/T)xKE(A/S)hK, where x is any amino acid and h is an amino acid with a hydrophobic sidechain\)], generally holds true (Lambalot et al., 1996; Walsh et al., 1997). However, we have extended and refined the signature sequence to \[(I/V/L/G)(V/I/L/T)(D/I/V/L/A)(x)_{n}(F/W)(A/S/T/C)xKE(S/A)h(h/S)k(A/G)\] for bacterial PPTases (where \(n\) is 42–48 for ACPSs and 38–41 for the Sfp subfamily of PPTases). We refer to the two conserved motifs as ppt-1 and ppt-3 (Fig. 7). The previously identified invariant glutamate, corresponding to E127 in Sfp (Reuter et al., 1999), forms part of a less well-conserved \[(L/I/V/M)(S/T)H(S/T/C/A)\], which is conserved among all the bacterial Sfp-type PPTases (Fig. 7). As this motif (ppt-S) resides in a region of the Sfp-type PPTases that is not present in ACPSs, it can be considered diagnostic for this class of PPTase. Sanchez et al. (2001) identified a motif they termed P1 \[PxWPxG(x)_{2}GS(M/L)THCxGY\] in a subset of Sfp-type PPTases encoded by members of the actinomycetes, within which ppt-S is present (underlined). However, P1 is not conserved among all the bacterial Sfp-type PPTases [see (iii) below]. The P1 motif has been referred to as ‘Motif 1’ by Copp & Neilan (2006), who also recognized that it is not conserved in all Sfp-type PPTases. Bacterial Sfp-type PPTases are also characterized by a

(ii) Located 12–23 amino acids N-terminal to the PPTase signature sequence is a tetrapeptide motif \[(L/I/V/M)(S/T)H(S/T/C/A)\], which is conserved among all the bacterial Sfp-type PPTases (Fig. 7). This motif (ppt-S) resides in a region of the Sfp-type PPTases that is not present in ACPSs, it can be considered diagnostic for this class of PPTase. Sanchez et al. (2001) identified a motif they termed P1 \[PxWPxG(x)_{2}GS(M/L)THCxGY\] in a subset of Sfp-type PPTases encoded by members of the actinomycetes, within which ppt-S is present (underlined). However, P1 is not conserved among all the bacterial Sfp-type PPTases [see (iii) below]. The P1 motif has been referred to as ‘Motif 1’ by Copp & Neilan (2006), who also recognized that it is not conserved in all Sfp-type PPTases. Bacterial Sfp-type PPTases are also characterized by a

![Fig. 7. Alignment of the central conserved region of bacterial Sfp-type PPTases with the N-terminal region of AcpS-type PPTases. A selection of both types of PPTase was aligned using the CLUSTAL W program (Thompson et al., 1994) and highlighted using the Boxshade program accessed through the Swiss Institute of Bioinformatics website (http://www.ch.embnet.org/index.html). Amino acid residues that are identical at the corresponding position in ≥50% of sequences are shown in white type with a black background. Similar amino acids are shown in white type with a grey background. The alignment was then gapped manually in order to highlight the motifs characteristic of the Group I and Group II Sfp-type PPTases. Only a representative number of the total residues aligned is shown. Consensus sequences of the conserved motifs described in the text were derived from a more comprehensive alignment than is shown here. The PPTases shown, in descending order, are B. cenocepacia PobA (ABD60228), Nostoc sp. HetI (AA220003), Stigmatella aurantica MtaA (AAF19809), S. venezuelae JadM (AAF34678), Bacillus subtilis Sfp (CAA44858), M. tuberculosis PptT (CAA15589), Yersinia pestis YbtD (NP_669888), Vibrio cholerae VipD (NP_230429), P. aeruginosa PcpS (NP_249856) and E. coli EntD (NP_415115), and the AcpS-type PPTases of Bacillus subtilis (B.s.; NP_388343), E. coli (E.c.; NP_417058) and B. cenocepacia (B.c.; CAR51316).](http://mic.sgmjournals.org)
highly conserved glutamate or glutamine residue located immediately C-terminal to ppt-1. In the crystal structure of Sfp, the carboxyl group of the glutamate residue at this position provides a ligand for a metal ion that is probably magnesium (Reuter et al., 1999).

(iii) Amino acid sequences located N-terminal to ppt-S and C-terminal to ppt-3 highlight a clear division within the bacterial Sfp-type PPTases. Highly conserved amino acid sequence motifs within these regions allow the assignment of an Sfp-type PPTase to one or other ‘group’. For example, Group I Sfp-type PPTases, which include PobA and Sfp, are characterized by the sequence G(R/K)Px(L/I/V)-(x)7–13-FN (termed ppt-S1N) immediately preceding ppt-S, and GxG(L/I/F)(x)3(L/I/M/V) (ppt-S1C) immediately following ppt-3. The latter motif is also present at the corresponding position in ACPSs, therefore effectively extending the PPTase signature region for ACPSs and Group I Sfp-type PPTases (Fig. 7). On the other hand, Group II Sfp-type PPTases, which include EntD and PcpS, are characterized by the presence of the sequence PxWPxxhGS (ppt-S2N) immediately preceding ppt-S, and the tripeptide x(Y/F)P (ppt-S2C) is generally present C-terminal to ppt-3 (although the latter motif is not conserved strongly enough to be diagnostic of Group II Sfp-type PPTases). The closely related Sfp-type PPTases analysed by Sanchez et al. (2001) fall within Group II, and hence all contain ppt-S2N fused to S-ppt, giving rise to the proposal of the P1 motif. However, the P1 motif is not present in Group I Sfp-type PPTases.

The Sfp-type PPTase dichotomy may also be discerned by reference to amino acid residues within and adjacent to the ppt-3 motif. Thus, the first residue of ppt-3 is always tryptophan (W) for Group I Sfp-type PPTases, whereas a phenylalanine (F) is always present at this position in Group II members (and ACPSs). This difference has been employed to similarly subdivide the Sfp-type PPTases based on whether they contain the WxxKEA or FxxKES motif in ppt-3 (Copp & Neilan, 2006). However, a serine residue may be found at the position occupied by an alanine in most members of the W/KEA subfamily (Group I), such as in Sfp, and the opposite is also true for PptT. We also note that position 8 of ppt-3 is always a phenylalanine or a tyrosine (Y) in the Group II Sfp-type PPTases, whereas amino acids with aromatic side chains are not present at this position in members of Group I (and ACPSs). A phenylalanine is usually located at the fourth position N-terminal to ppt-3 in Group I members (Fig. 7).

The dichotomy within the Sfp-type PPTases is highlighted in the phylogenetic tree shown in Fig. 8. Note that Group I and Group II Sfp-type PPTases are not restricted to particular bacterial phyla or classes. As pointed out before, members of the same genera may encode phylogenetically distant PPTases (Copp & Neilan, 2006). Specifically, JadM, a Group I PPTase, is found in S. venezuelae, while NysF and PptA are Group II PPTases produced by Streptomyces noursei and Streptomyces verticillus, respectively. One possibility is that the division within the Sfp-type PPTases reflects a functional difference. However, although EntD and PcpS both fall within the Group II Sfp-type PPTases, only one of them (PcpS) can substitute for AcpS (Flugel et al., 2000; Finking et al., 2002; Barekzi et al., 2004). Similarly, Sfp and PcpS are members of different PPTase groups, but both can substitute for AcpS (Fig. 7; Mootz et al., 2001).
We have described a reporter transposon, the utility of which was tested by investigating the regulation of the \( poBA \) gene. Our results imply that in wild-type cells, the \( poBA \) gene is not subject to strong regulation by iron. This is consistent with a role for \( poBA \) in the biosynthesis of other non-ribosomally synthesized peptides that are not involved in iron acquisition. However, in a mutant lacking a fully functional Sfp-type PPTase growing in iron-poor media, \( poBA \) transcription is enhanced. The mechanism for this is not clear but there are no obvious Fur box-like sequences present upstream of \( poBA \) or BCAL2249. Moreover, the degree of iron-dependent regulation is modest compared with many other iron- or Fur-regulated genes.

In a recent assessment of the role of different potential virulence determinants of \( B. \) cenocepacia in a variety of animal model systems, it was shown that the ability to produce the siderophores ornibactin and pyochelin was consistently required for virulence in all the model systems tested (Uehlinger et al., 2009). Thus, \( poBA \) represents a potential target for the development of therapeutic agents to control infections by \( Burkholderia \) spp.

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