Importance of the Ornibactin and Pyochelin Siderophore Transport Systems in *Burkholderia cenocepacia* Lung Infections

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Previously, *orbA*, the gene encoding the outer membrane receptor for ferric-ornibactin, was identified in *Burkholderia cenocepacia* K56-2, a strain which produces ornibactin, salicylic acid, and negligible amounts of pyochelin. A K56-2 *orbA* mutant was less virulent than the parent strain in a rat agar bead infection model. In this study, an *orbA* mutant of *B. cenocepacia* Pc715j which produces pyochelin in addition to ornibactin and salicylic acid was constructed. The gene encoding the outer membrane receptor for ferric-pyochelin (*fptA*) was also identified. An *fptA* mutant was constructed in Pc715j and shown to be deficient in [^{59}Fe]pyochelin uptake. A 75-kDa iron-regulated protein was identified in outer membrane preparations of Pc715j that was absent in outer membrane preparations of Pc715j*fptA*::tp. Pc715j*fptA*::tp and Pc715j*orbA*::tp produced smaller amounts of their corresponding siderophores. Both Pc715j*orbA*::tp and Pc715j*fptA*::tp were able to grow in iron starvation conditions in vitro. In the agar bead model, the Pc715j*orbA*::tp mutant was cleared from the lung, indicating that the pyochelin uptake system does not compensate for the absence of a functional ornibactin system. Pc715j*fptA*::tp persisted in rat lung infections in numbers similar to those of the parent strain, indicating that the ferric-ornibactin uptake system could compensate for the defect in ferric-pyochelin uptake in vivo. These studies suggest that the ornibactin uptake system is the most important siderophore-mediated iron transport system in *B. cenocepacia* lung infections.

Burkholderia cenocepacia (formerly *B. cepacia* genomovar III) is an opportunistic organism which has emerged as a major respiratory pathogen for patients with cystic fibrosis or chronic granulomatous disease (25, 26, 38). Infections with *B. cenocepacia* are a major concern because of the high patient-to-patient transmissibility and the multidrug resistance of the organism. Colonization is often associated with a rapid and fatal pneumonia (25, 26, 38). Recently, it has been determined that the *B. cepacia* complex which infects cystic fibrosis patients consists of nine biochemically similar but genetically distinct species (13, 14, 38). *B. cenocepacia* (70) is the most common *Burkholderia* species in populations of cystic fibrosis patients, causing more than 50% of the infections in these patients (38).

Iron is an essential requirement for bacterial growth because it is required for many biological processes (46). However, little free iron is available in the human host because much of it is bound by proteins such as lactoferrin, transferrin, and heme (17, 45). Under physiological conditions, iron exists in the ferric state and is insoluble, present at not more than 10^{-18} M, while for optimal bacterial growth 10^{-6} to 10^{-8} M is required (27, 46). Pathogenic bacteria have developed specific systems to acquire iron. One mechanism is the production of low-molecular-weight ferric iron chelators known as siderophores (27, 46). Siderophores facilitate the solubilization and transport of ferric iron into the cell by a specific outer membrane receptor-mediated transport system in which TonB,

ExbB, and ExbD provide energy for transport across the inner membrane into the cell (reviewed in references 17, 27, and 45).

The *B. cepacia* complex has been shown to produce four siderophores: ornibactins, salicylic acid (SA), pyochelin, and cepabactin (42, 43, 66, 68, 72). Ornibactins and SA are the predominant siderophores found in cystic fibrosis isolates, but there is species and strain variation (19). Ornibactins are linear hydroxamate/hydroxycarboxylate siderophores similar in structure to the pyoverdines produced by fluorescent pseudomonads, yet they lack a chromophore (43, 68). Pyochelin is a structurally unique siderophore of the phenolate class (16). SA, a biosynthetic precursor of pyochelin, has been shown to possess siderophore activity in *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* (41, 72), and *Burkholderia cepacia* (66, 72).

In previous studies, genes involved in the synthesis (*pvdA* and *pvdD*) (65) and uptake (*orbA*) (63) of ornibactin have been identified. *pvdA* and *orbA* allelic exchange mutants were less virulent than the parent strain in a chronic respiratory infection model. K56*orbA*::tp and K56*pvdA*::tp both showed reduced histopathology as well as reduced persistence, indicated by the fact that they were cleared from the lungs (63, 65). These mutants also demonstrated a reduced ability to grow in iron-restricted conditions in vitro (63, 65). These studies demonstrated a requirement for ornibactin in growth and virulence; however, these studies were performed with a strain that produces negligible amounts of pyochelin.

The objectives of the present study were to construct mutants defective in either pyochelin-mediated iron uptake or ornibactin-mediated iron uptake in a strain that produced both siderophores to determine if a single functional siderophore system was sufficient for growth in iron-restricted conditions and for survival in a chronic respiratory infection model.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference(s)
E. coli		
DH5α	F^- φ80 *lacZ*ΔM15 Δ(*lacZYA-argF*) *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 deoRU169*	Invitrogen
HB101	F^- Δ(*gpt-proA*)62 *leuB6 glnV44 ara-14 galK2 lacY1* Δ(*mcrC-mrr*) *rpsL20* (Str^r) *xyl-5 mtl-1 recA13*	57
TOP10	F^- *mcrA* Δ (*mrr-hsdRMS-mcrBC*) φ80 *lacZ*ΔM15 Δ *lacX74 deoR recA1 araD139* Δ (*ara-leu*)7697 *galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
SM10	Mobilizing strain, RP4 genes integrated in chromosome, Km^r	62
B. cenocepacia		
Pc715j	CF respiratory isolate; Orn, Pch, SA	19, 40
Pc715j*orbA*::tp	*orbA*::Tp^r derivative of Pc715j; Tp^r	This study
Pc715j*fptA*::tp	*fptA*::Tp^r derivative of Pc715j; Tp^r	This study
K56-2	CF respiratory isolate; Orn, SA	19, 39
K56*orbA*::tp	*orbA*::Tp^r derivative of K56-2; Tp^r	63
Plasmids		
pRK2013	ColE1 *tra*(RK2)$^+$; Km^r	23
pCC524T	pEX18Tc with a 3-kb EcoRI-BglII *orbA* fragment from K56-2 containing a 0.6-kb Tp^r cassette inserted into the *orbA* gene; Tc^r Tp^r	63
pCR^{R}2.1TOPO	Cloning vector for PCR products; Km^r Ap^r	Invitrogen
pEX18Tc	Suicide vector; *sacB* Tc^r	31
p34E-Tp	Source of trimethoprim resistance cassette; Tp^r	20
pEKH103	pCR^{R}2.1TOPO containing a 2.35-kb *fptA* PCR product; Ap^r Km^r	This study
pEKH107	pEKH103 with a 0.67-kb SalI fragment containing the Tp^r cassette from p34E-Tp inserted into the SalI site in the *fptA* gene; Ap^r Km^r Tp^r	This study
pEKH113	pEX18Tc with a 2.2-kb HindIII-PstI *fptA*::tp fragment from pEKH107; Tc^r Tp^r	This study

[a] Tc, tetracycline; Tp, trimethoprim; Km, kanamycin; Ap, ampicillin; Orn, ornibactin; Pch, pyochelin; SA, salicylic acid; Str, streptomycin; CF, cystic fibrosis.

MATERIALS AND METHODS

Strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. K56-2 produces ornibactins, SA, and negligible amounts of pyochelin, while Pc715j produces ornibactins, SA, and pyochelin (19). When appropriate, antibiotics were added at the following concentrations: 100 μg of ampicillin, 15 μg of tetracycline, 50 μg of kanamycin, and 1.2 mg of trimethoprim per ml for *Escherichia coli* and 300 μg of tetracycline and 100 μg of trimethoprim per ml for *B. cenocepacia*. A 100-mg/ml stock of trimethoprim was prepared in *N,N*-dimethylacetamide.

DNA manipulations. For genetic manipulations, cultures were grown in Luria-Bertani (LB) broth (Invitrogen, Burlington, Canada) or on 1.5% LB agar at 37°C. Molecular biology techniques were generally performed as described by Sambrook et al. (57). Genomic DNA was isolated from Pc715j as described by Ausubel et al. (5). Restriction enzymes and oligonucleotide primers were purchased from Invitrogen (Burlington, Canada), while T4 DNA ligase was purchased from Promega (Madison, Wis.). PCR products were cloned with the pCR^{R}2.1TOPO kit according to the manufacturer's directions (Invitrogen). Recombinant plasmids were electroporated into *E. coli* DH5α or TOP10 with a Gene Pulser (Bio-Rad, Richmond, Calif.) as recommended by the manufacturer.

To construct an *orbA* allelic exchange mutant of strain Pc715j, pCC524T (63), containing an insertionally inactivated *orbA* gene, was used. This plasmid was transferred to *B. cenocepacia* Pc715j by triparental mating with pRK2013 (23) as previously described (63). Insertional inactivation was confirmed by PCR and Southern hybridization.

To amplify the *fptA* gene from Pc715j, the *fptA* gene sequence from *Pseudomonas aeruginosa* PAO1 (4) was used to search the *B. cepacia* genome sequence of *B. cenocepacia* strain J2315 produced by the sequencing group at the Sanger Institute (http://www.sanger.ac.uk/Projects/B_cenocepacia). Primers fptAup (5′-GTGACGAGCTCAATACGGGCCG-3′) and fptAdo (5′-CCCCAAGCTTGA CGCCATCAGA-3′) were used to amplify a 2.3-kb product containing the *fptA* gene from Pc715j. This product was cloned into pCR^{R}2.1TOPO to create pEKH103.

For the construction of an *fptA* allelic-exchange mutant of Pc715j, a 0.92-kb internal SalI fragment was removed from the *fptA* gene and replaced with a 0.67-kb SalI fragment from p34E-Tp (20) containing the trimethoprim resistance cassette to create pEKH107. A 2.2-kb HindIII-PstI fragment from pEKH107 consisting of the insertionally inactivated *fptA* gene was inserted into the HindIII and PstI sites in pEX18Tc (31) to form pEKH113. This plasmid was transferred to Pc715j by triparental mating with pRK2013 (23) as described above. Insertional inactivation of *fptA* was confirmed by both Southern hybridization and PCR.

Iron uptake assays. For pyochelin uptake assays, cultures were grown overnight at 32°C with maximum aeration in 20 ml of Casamino Acids-phosphate-sulfate (CPS) medium (2) supplemented with 5 μg of pyochelin per ml. For SA uptake assays, cultures were grown as above in tryptic soy broth medium treated with Chelex 100 (Bio-Rad, Richmond, Calif.) and subsequently dialyzed (TSB-DC) (48). Cultures were washed with 10 ml of medium and resuspended to an A_{600} of 0.3. The iron binding efficiency of the pyochelin stock was determined with the Chrome Azurol S (CAS) assay (60). $^{59}FeCl_3$ (3.6 nmol) was mixed with 4.8 nmol of actively binding pyochelin in a volume of 100 μl and equilibrated for 60 min. Salicylic acid (7.2 nmol) was mixed with 3.6 nmol of $^{59}FeCl_3$ in a volume of 100 μl and equilibrated as above. Uptake reactions were initiated by the addition of 100 μl of ^{59}Fe-siderophore mixture to 10 ml of cells. Samples (1 ml) were then removed at selected time points, filtered through cellulose acetate filters (0.45 μm), and washed with 3 ml of 10 mM Tris (pH 7.5)–0.9%NaCl. The amount of accumulated ^{59}Fe on the filters was measured with an LKB gamma counter (66).

Growth determination. To determine the effects of siderophores on growth, cultures were grown overnight in 10 ml of iron-deficient TSB-DC at 32°C with maximum aeration and subcultured into 15 ml of medium to an A_{600} of 0.02. Pyochelin and ornibactins were added to a final concentration of 10 μg/ml. To determine the effects of nonsiderophore iron chelators on growth, 20 μM ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDHA) (Sigma, Oakville, Canada) was added to the medium in one experiment. Growth experiments were performed in triplicate, and growth was measured by determining A_{600}.

Siderophore assays. For the determination of siderophore activity via CAS assays (60) and ornibactin production assays, cultures were grown at 32°C for 40 h in succinate medium (44) supplemented with 10 mM ornithine (43). Ornibactins were purified by Sephadex LH-20 chromatography and quantitated in CAS assays as previously described (35, 36). To determine the amount of SA and pyochelin produced, cultures were grown in deferrated Casamino Acids medium (64) at 32°C for 24 h. SA and pyochelin were isolated by thin-layer chromatography and quantitated as previously described (36, 65).

Outer membrane protein isolation. For outer membrane protein isolation, Pc715j, Pc715j*orbA*::tp, and Pc715j*fptA*::tp were grown overnight in CPS medium (2) at 32°C, subcultured into 500 ml of fresh medium, and grown to an A_{600} of approximately 1.0. Outer membranes were isolated as described previously. Some cultures were supplemented with 10 μg of pyochelin per ml and 50 μM $FeCl_3$ as required.
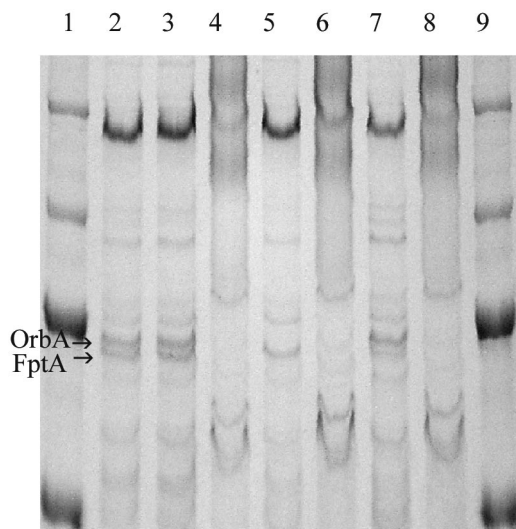
FIG. 1. Outer membrane protein profiles of Pc715j, Pc715j*fptA*::tp, and Pc715j*orbA*::tp. Ten micrograms of outer membrane preparations from cultures grown in CPS medium was electrophoresed on an SDS–10% PAGE gel and stained with Coomassie brilliant blue. Lanes 1 and 9, 150-, 100-, 75-, and 50-kDa molecular mass markers; lane 2, Pc715j in medium with no additions; lane 3, Pc715j in medium plus 10 μg of pyochelin per ml; lane 4, Pc715j in medium plus 50 μM FeCl₃; lane 5, Pc715j*orbA*::tp in medium with no additions; lane 6, Pc715j*orbA*::tp in medium plus 50 μM FeCl₃; lane 7, Pc715j*fptA*::tp in medium with no additions; lane 8, Pc715j*fptA*::tp in medium plus 50 μM FeCl₃.

Animal studies. Animal infection experiments were performed in the chronic respiratory infection model in rats as described by Cash et al. (10). For the preparation of agar beads, cultures were grown overnight in 20 ml of TSB-DC at 32°C with maximum aeration. Groups of 10 male Sprague-Dawley rats weighing 150 to 170 g (Charles River Canada, Inc.) were tracheotomized under anesthesia and inoculated with approximately 10^5 CFU of the appropriate strain embedded in agar beads as previously described. On days 7 and 14 postinfection, the lungs from four or five animals in each group were removed aseptically and homogenized (Polytron homogenizer; Brinkmann Instruments, Westbury, N.Y.) in 3 ml of phosphate-buffered saline (0.05 M, pH 7.2, containing 0.9% saline). Serial dilutions were plated on Trypticase soy agar (TSA) (Difco, Detroit, Mich.) and TSA plus the appropriate antibiotic or *B. cepacia* selection agar (30), and bacterial counts were performed.

RESULTS

Construction of *orbA* and *fptA* mutants. An *orbA* mutant of Pc715j was constructed as previously described (63) and designated Pc715j*orbA*::tp. Outer membrane profiles were prepared for Pc715j and Pc715j*orbA*::tp grown in both low- and high-iron conditions. Two iron-regulated proteins migrated near the 75-kDa molecular mass marker and are marked with arrows in Fig. 1. The upper band, believed to be the ornibactin receptor, is present in Pc715j but absent in Pc715j*orbA*::tp (Fig. 1, compare lanes 2 and 5) and absent in the presence of 50 μM FeCl₃ in both Pc715j and Pc715j*orbA*::tp (Fig. 1, compare lanes 2 and 4 and lanes 5 and 6). These results are similar to those reported for K56-2 and K56*orbA*::tp (63).

The ferric-pyochelin outer membrane receptor gene *fptA* has been identified in *P. aeruginosa* PAO1 (4). The *P. aeruginosa* PAO1 FptA amino acid sequence was used to search the *B. cenocepacia* J2315 sequence (http://www.sanger.ac.uk /Projects/*B.cenocepacia*). The deduced amino acid sequence of

B. cenocepacia J2315 FptA was determined to be 64% identical to that of *P. aeruginosa* FptA (4). This amino acid sequence was also used to search the GenBank database with the BLASTP algorithm (1, 37) for similar matches. Identity ranged from between 34 and 35% for various hydroxamate-type siderophore receptors, including the PupB (32) and PupA (6) pseudobactin receptors of *P. putida*; FpvA, the pyoverdine receptor of *P. aeruginosa* (50); and coprogen-, rhodotorulic acid-, and ferrioxamine-type receptors such as *E. coli* FhuE (58). FptA was also 35% identical to a *Bordetella* sp. FauA (7), the receptor for the siderophore alcaligin. Alcaligin is a macrocyclic dihydroxamate-type siderophore that requires ornithine as a precursor (8).

B. cenocepacia fptA is predicted to encode a 737-amino-acid polypeptide with a molecular mass of 80,766 Da and a pI of 8.67. A probable signal sequence cleavage site was detected between amino acids 41 and 42 with the SignalP program (47). The mature form of FptA is predicted to have a molecular mass of 76,040 Da and a pI of 6.95. A TonB-dependent signature sequence 2 was identified at the C-terminal end from residues 721 to 738 by a Prosite search. Ten of 18 residues in this region matched the consensus sequence.

The sequence surrounding *fptA* was searched for open reading frames with Artemis (56; www.sanger.ac.uk/Software /Artemis), and BLASTP was used to search for similarities to the amino acid sequences encoded by selected open reading frames. It appears that upstream of *fptA*, gene organization is similar to that in *P. aeruginosa*. Open reading frames that showed identity to genes involved in pyochelin synthesis (*pchG*) and ABC transporter proteins with export functions (*pchH* and *pchI*) (54) were identified. Downstream of *fptA* was an open reading frame with approximately 35% homology to a gene encoding iron-regulated membrane proteins of unknown function, as reported for *P. aeruginosa* (4).

An *fptA* mutant of Pc715j was constructed and designated Pc715j*fptA*::tp. Outer membrane preparations isolated from Pc715j and Pc715j*fptA*::tp in both low-iron and high-iron conditions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The smaller of the two iron-regulated proteins in the 75-kDa range was absent in Pc715j*fptA*::tp (Fig. 1, compare lanes 2 and 7) and migrated close to the expected mass for mature FptA. Although a faint protein with a molecular mass similar to that of FptA was detectable in lane 7, this was probably another protein of similar size, since it was present in much smaller amounts than the FptA band observed in Pc715 or Pc715j*orbA*::tp (lanes 2, 3, and 5). Efforts to clearly separate these two proteins by SDS-PAGE with different percentages of acrylamide were not successful.

The protein corresponding to FptA was not expressed in the presence of 50 μM FeCl₃ in either Pc715j or Pc715j*fptA*::tp (Fig. 1, compare lanes 2 and 4 and lanes 7 and 8). Expression of the pyochelin receptor in *P. aeruginosa* has been shown to be inducible by the presence of pyochelin (24). The effect of the addition of pyochelin on FptA expression in Pc715j was examined. In low-iron conditions, the addition of pyochelin resulted in no observable differences in protein profiles (Fig. 1, compare lanes 2 and 3). Since Pc715j produces pyochelin, it is not surprising that an increase in the amount of pyochelin in the medium did not result in a further increase in expression of the receptor. Addition of 50 μM FeCl₃ to the medium resulted in
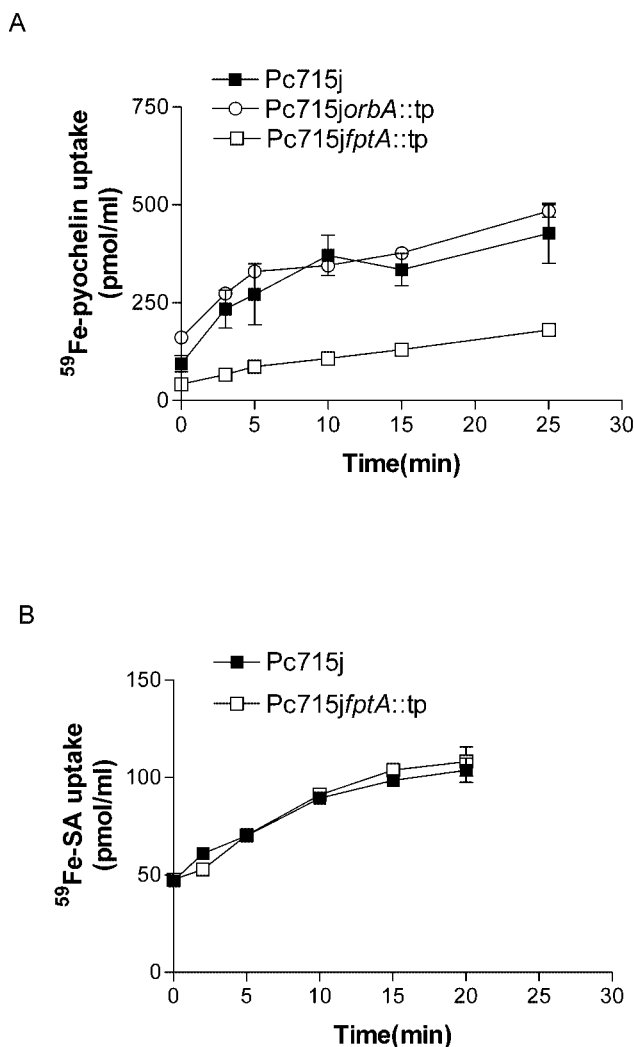
A

B

FIG. 2. Uptake of ^{59}Fe-labeled siderophore complexes by Pc715j, Pc715j*fptA*::tp, and Pc715j*orbA*::tp. (A) [^{59}Fe]pyochelin; (B) [^{59}Fe]SA. Reactions were initiated by the addition of the ^{59}Fe-labeled siderophore complex. Samples were removed at selected intervals, and the amount of ^{59}Fe accumulated was determined. Values are the means ± standard deviations for triplicate assays. The cultures in panel A were grown in CPS medium, and the cultures in panel B were grown in TSB-DC medium.

altered expression of several proteins in addition to FptA and OrbA and appeared to slightly retard the migration of others (Fig. 1. lanes 4, 6, and 8). These proteins have not yet been identified.

Role of *fptA* and *orbA* in iron transport and siderophore production. OrbA has previously been confirmed to be required for ferric ornibactin uptake (63). In order to confirm that *fptA* was required for pyochelin uptake in *B. cenocepacia*, the ability of Pc715j*fptA*::tp to take up [^{59}Fe]pyochelin was compared to that of Pc715j. Pc715j*fptA*::tp was unable to accumulate [^{59}Fe]pyochelin in the assay period (Fig. 2A). The ability of Pc715j*orbA*::tp to take up [^{59}Fe]pyochelin was also determined. Pc715j*orbA*::tp was able to take up [^{59}Fe]pyochelin at a rate similar to that of Pc715j, indicating that pyochelin uptake is not affected by an *orbA* mutation (Fig. 2A).

SA is a biosynthetic precursor to pyochelin (3) in addition to functioning as a siderophore in *P. aeruginosa* and *B. cepacia* species in vitro (66, 72). It is not known whether a specific receptor protein is involved in the uptake of SA (55). To determine the effects of an *fptA* mutation on SA uptake, the ability of Pc715*fptA*::tp to take up [^{59}Fe]SA was examined. Pc715j and Pc715j*fptA*::tp were able to take up [^{59}Fe]SA at similar rates (Fig. 2B), indicating that an *fptA* mutation does not affect the ability to acquire SA. These data suggest the presence of a specific outer membrane receptor for SA uptake.

The effects of *orbA* and *fptA* mutations on siderophore production were also determined (Table 2). Pc715j*orbA*::tp produced significantly less ornibactin than Pc715 ($P < 0.05$). These results were similar to those reported previously for strain K56-2 (63). Pyochelin production by Pc715j*fptA*::tp was significantly less than that by Pc715j ($P < 0.05$), whereas the amount of pyochelin produced by Pc715j*orbA*::tp was similar to that by Pc715j. Interestingly, SA production by Pc715j*fptA*::tp was increased fivefold compared to that by Pc715j ($P < 0.05$).

Effect of *fptA* and *orbA* mutations on growth in the presence of siderophores or EDDHA. The ability of Pc715j*orbA*::tp and K56*orbA*::tp to utilize ornibactins and pyochelin for growth in vitro in iron-deficient medium was examined. Wild-type and mutant strains grew at similar rates in the absence of added siderophores (compare Fig. 3A and B). The addition of pyochelin had no effect on the growth of any of the strains. When ornibactins were added, Pc715j*orbA*::tp had a slight decrease in growth rate between 6 and 12 h (Fig. 3A). This was markedly different from that observed for K56*orbA*::tp, which grew very poorly with the addition of ornibactins to the medium (Fig. 3B). This indicates that Pc715j*orbA*::tp can acquire iron effectively with the pyochelin transport system. To determine the effects of other iron chelators on growth, 20 μM EDDHA was added to the growth medium instead of siderophores. Pc715j and Pc715j*orbA*::tp grew at similar rates in the presence and absence of EDDHA (data not shown), which also suggests that pyochelin is an effective siderophore in vitro. This is in contrast to the previously reported effects of EDDHA on the growth of the non-pyochelin-producing strain K56*orbA*::tp (63).

The ability of Pc715j*fptA*::tp to utilize pyochelin and ornibactins for growth in iron-deficient medium was also examined. Pc715j and Pc715j*fptA*::tp grew at similar rates in the absence of siderophores (Fig. 3C). The addition of ornibactins had no effect on the growth of either strain. When pyochelin was added to the growth medium, a slight decrease in growth was observed for Pc715j*fptA*::tp between 5 and 12 h. Pc715j and Pc715*fptA*::tp grew at similar rates in both the absence and

TABLE 2. Effect of *fptA* and *orbA* mutations on siderophore production in *B. cenocepacia* Pc715j

Strain	Amt[a] (μg/ml of supernatant)		
	Salicylic acid	Ornibactin	Pyochelin
Pc715j	0.1 ± 0.01	23.5 ± 8.2	4.8 ± 2.1
Pc715j *fptA*::tp	0.5 ± 0.1[b]	26.0 ± 1.3	0.1 ± 0.2[b]
Pc715j*orbA*::tp	0.3 ± 0.3	5.5 ± 2.5[b]	3.3 ± 2.2

[a] Values are the means ± standard deviations for triplicate assays. Assays were performed three times with similar results.
[b] Significantly different from results for Pc715j ($P < 0.05$) by analysis of variance, Dunnett multiple-comparisons test.
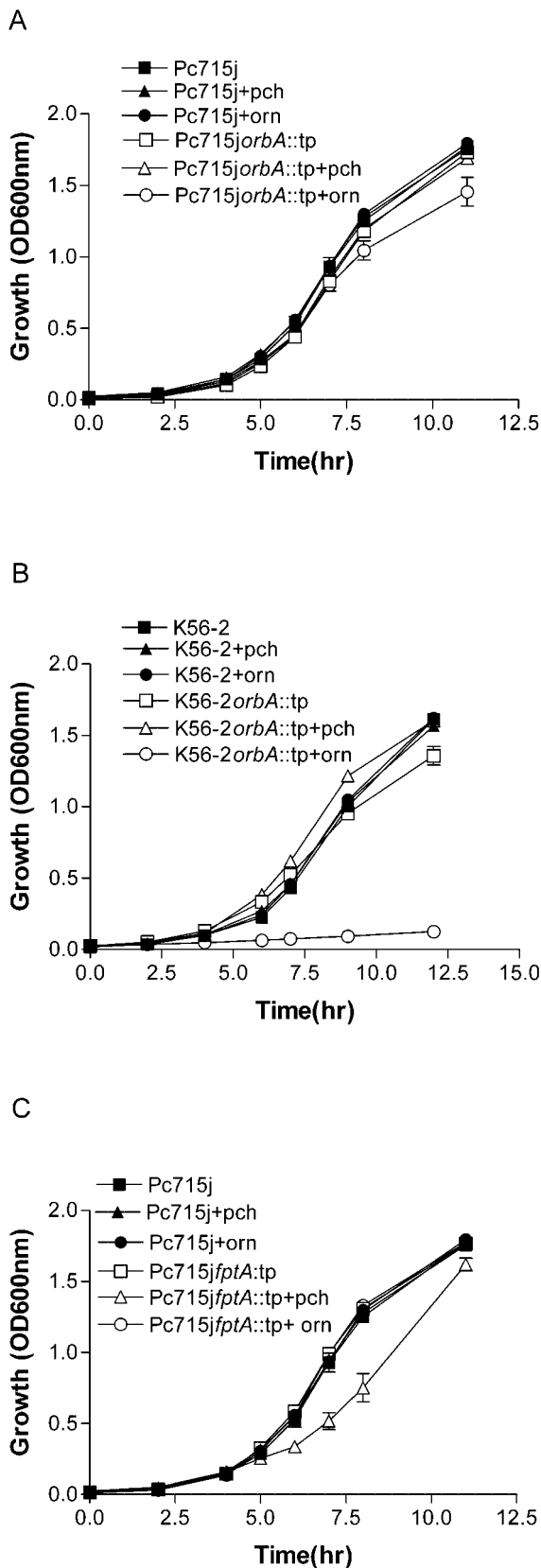
A

B

C

FIG. 3. Effect of *orbA* and *fptA* mutations on growth in the presence of siderophores. Cultures were grown in iron-deficient TSB-DC medium containing either 10 μg of pyochelin (pch) or ornibactin (orn)

TABLE 3. Effect of *orbA* and *fptA* mutations on persistence of *B. cenocepacia* Pc715j in the rat lung chronic-infection model

Expt	Strain	Inoculum (log CFU)	Log CFU/ml of lung homogenate[a]	
			Day 7 p.i.	Day 14 p.i.
1	Pc715j	4.7	5.5 ± 0.82	3.8 ± 1.6
	Pc715j*orbA*::tp	4.6	2.0 ± 2.5^{b}	0.4 ± 0.7^{b}
2	Pc715j	5.8	5.0 ± 0.3	$3.5 \pm 1.5*$
	Pc715j*fptA*::tp	6.0	5.2 ± 0.5	$4.9 \pm 0.7*$

[a] Values are means ± standard deviations for four animals per group or, if marked with an asterisk, five animals per group at 7 and 14 days postinfection (p.i.).
[b] Significantly different from results for Pc715j ($P < 0.05$) by analysis of variance, Bonferroni multiple-comparisons test.

presence of EDDHA (data not shown). These studies suggest that although pyochelin can function as a siderophore in vitro, it is not required for in vitro growth in iron-limited conditions.

Effects of *orbA* and *fptA* mutations on virulence. Previous studies have indicated that ornibactin biosynthesis and uptake are important factors in the ability of at least some strains of *B. cenocepacia* to persist in experimental lung infections (63). To determine the relative importance of pyochelin uptake in virulence, the persistence of Pc715j*orbA*::tp and Pc715j*fptA*::tp was compared to that of Pc715j in a chronic respiratory infection model. Rats were infected with Pc715j, Pc715j*orbA*::tp, and Pc715j*fptA*::tp, and on days 7 and 14 postinfection, quantitative bacteriological analysis was performed on the lungs. On days 7 and 14 postinfection, approximately 3 logs fewer Pc715j*orbA*::tp than parent strain organisms were recovered from the lungs, while there was no significant difference in the number of Pc715j*fptA*::tp organisms recovered from the lungs (Table 3). These results demonstrate that although a functional ferric-ornibactin uptake system is sufficient for persistence in the lung, a functional ferric-pyochelin uptake system is unable to compensate for a nonfunctional ferric-ornibactin uptake system in vivo.

DISCUSSION

In this study, we compared the importance of the ferric-ornibactin and ferric-pyochelin siderophore-mediated transport systems in *B. cenocepacia* in vitro and in a chronic lung infection model. Pc715j*orbA*::tp was able to overcome iron limitation in vitro and grew at rates similar to those of Pc715j. The addition of ornibactin to Pc715j*orbA*::tp cultures resulted in only a slight decrease in growth rate compared to the parent (Fig. 3A). This is markedly different than the result in K56-2, where an *orbA* mutation severely hindered growth under both of these conditions (Fig. 3B) (63). Pc715j*orbA*::tp is likely able to overcome these limitations by producing sufficient pyochelin to effectively acquire iron in vitro, even though pyochelin has a

per ml or no additions. (A) Comparison of growth rates of Pc715j and Pc715j*orbA*::tp. (B) Comparison of growth rates of K56-2 and K56*orbA*::tp. (C) Comparison of growth rates of Pc715j and Pc715j*fptA*::tp. Optical density (OD) was determined at various intervals. Values are the means ± standard deviations for triplicate assays.

lower binding affinity for iron (16, 72) than ornibactin. Although an affinity constant for iron has not been reported for ornibactin, based on its structural similarity to pyoverdine, which has a binding coefficient for iron of 10^{33} (44), ornibactin likely has a higher affinity for iron than pyochelin (5×10^5) (16). Although ornibactin would bind much of the iron, some could be bound by pyochelin and still be available for iron accumulation by Pc715j*orbA*::tp, which was not inhibited by the addition of ornibactin to the medium to the same extent that K56*orbA*::tp was (Fig. 3A and B).

Pc715j*orbA*::tp was less able to persist in the lung (Table 3,) and the number of bacteria surviving in the lung was similar to the number observed previously for K56*orbA*::tp (65). Even though the amount of pyochelin produced in vitro by Pc715j*orbA*::tp was similar to that produced by Pc715j, this mutant does not appear to compete effectively for iron and survive in the lungs. Although we have not measured pyochelin production directly in vivo, these data suggest that a functional ferric-pyochelin system is unable to compensate for a nonfunctional ferric-ornibactin system. In an immunosuppressed mouse infection model, a *P. aeruginosa* PAO1 pyoverdine-deficient mutant grew poorly in the lung and was recovered in lower numbers in the blood, even though pyochelin production was similar to that of strain PAO1. These data suggest that pyoverdine is more important than pyochelin in *P. aeruginosa* infections in vivo (69).

In contrast to Pc715j*orbA*::tp, Pc715j*fptA*::tp appeared to be able to effectively acquire iron both in vitro and in vivo. Infection with Pc715j*fptA*::tp in a chronic lung infection model resulted in the same level of persistence as infection with Pc715j (Table 3), suggesting that a functional ferric-ornibactin uptake system is able to compensate for a nonfunctional ferric-pyochelin uptake system in vivo. No difference in the ability of a *P. aeruginosa* PAO1 pyochelin-deficient strain and PAO1 to persist in the lungs and blood of immunosuppressed mice was observed, which also provides evidence that a pyochelin uptake system is less important in vivo (69). Although pyochelin is not sufficient for *B. cenocepacia* to acquire iron in vivo, it can enhance virulence. In the agar bead model, exogenously supplied pyochelin was shown to increase the virulence of pyochelin-negative *B. cepacia* strains (67). In addition to its role in iron acquisition, pyochelin also plays a role in tissue injury. Iron bound to pyochelin has been shown to be an efficient catalyst for hydroxyl radical formation and to increase injury to pulmonary artery endothelial cells and epithelial cells resulting from exposure to superoxide and hydrogen peroxide (9, 15).

Previously we demonstrated that a strain with a functional SA uptake system but lacking the ability to synthesize or take up ornibactin was unable to survive in the lung (63, 65). The effect of mutations in the SA uptake system has not been examined directly.

Interestingly, SA production was increased approximately fivefold and pyochelin production was decreased by a similar amount in Pc715j*fptA*::tp. A decrease in pyochelin production might be expected because, in the absence of the pyochelin receptor protein, pyochelin is not needed. Although we have not ruled out the possibility of polar effects due to the *fptA* mutation, we have confirmed that the decrease in ornibactin synthesis observed in the K56*orbA*::tp mutant (65) can be complemented by the *orbA* gene (data not shown), suggesting that

the *orbA* mutation is not polar on the downstream biosynthetic genes. Unfortunately, complementation studies with Pc715j are problematic due to the lack of useful antibiotic resistance markers. Pyochelin biosynthesis has been shown to be positively regulated in *P. aeruginosa* PAO through the action of a transcriptional regulator, PchR (28, 29). PvdS, an alternative sigma factor, has been identified as a positive regulatory protein of pyoverdine biosynthesis in *P. aeruginosa* PAO (18). Iron-responsive regulators that affect the production of pyoverdine-like siderophores have also been identified in *P. putida* WS358 (33, 71) and *P. fluorescens* M114 (61). Additional factors or regulatory proteins may also be involved in the PvdS regulatory network (34). The mechanisms of regulation of pyochelin and ornibactin biosynthesis in *B. cenocepacia* are not yet known, but our studies suggest that there may be a complex network of regulation similar to that of the pseudomonads.

Hyperproduction of SA has also been observed in a K56-2 *pvdA* mutant (65). The rate of [^{59}Fe]SA uptake was also increased in K56*orbA*::tp (63), suggesting that the increase in SA production is a compensation mechanism for the decreased amount of pyochelin and ornibactin produced. Mutants of *P. fluorescens* CHAO that were unable to synthesize pyoverdines produced SA in much larger amounts (41).

SA has also been shown to act as an endogenous siderophore under iron-limiting conditions in vitro in organisms such as *Mycobacterium* spp. (51) and *Azospirillum lipoferum* (59) in addition to *P. aeruginosa* (72), *P. fluorescens* (41), and *B. cenocepacia* (66, 72). SA has been shown to be an intermediate in the biosynthesis of pyochelin as well as being present in other siderophores (11, 22, 49, 52). Our data suggest that even though SA is a precursor in pyochelin synthesis, there is a specific receptor for SA. The ability of Pc715j*fptA*::tp to take up [^{59}Fe]SA as effectively as the parent strain (Fig. 2B) indicates that Fe-SA complexes are not taken up by the pyochelin receptor.

It is interesting that SA is effective at binding ferric iron only in the absence of other ions such as phosphate. However, phosphate ions are abundant in culture media as well as in host tissues and fluids (53). Recently, a theoretical study to calculate the concentration and speciation of ions was used to conclude that SA is not a bacterial siderophore (12). In the presence of 10^{-4} M SA, the concentration of free ferric iron increased only slightly, by approximately 8.4×10^{-9} M, compared to the value in the absence of SA. SA also had little effect on the solubilization of iron in the presence of 40 mM phosphate.

A requirement for mycobactin T, a salicylate-derived siderophore, for growth of *M. tuberculosis* in macrophages has been determined (21). Mutation of the *mbtB* gene, involved in mycobactin biosynthesis, interrupted all salicylate-derived siderophore production. This mutant was deficient in growth in low-iron medium as well as in a macrophage cell line, providing evidence for the ability of SA to act as a siderophore.

B. cenocepacia produces three siderophores, ornibactin, pyochelin, and SA. In this study, we identified the siderophore receptor for pyochelin and provided additional evidence that the ferric-ornibactin uptake system is the most important iron acquisition system in chronic *B. cenocepacia* lung infections.

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