

Burkholderia spp. Alter *Pseudomonas aeruginosa* Physiology through Iron Sequestration

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Pseudomonas aeruginosa and members of the *Burkholderia cepacia* complex often coexist in both the soil and the lungs of cystic fibrosis patients. To gain an understanding of how these different species affect each other's physiology when coexisting, we performed a screen to identify *P. aeruginosa* genes that are induced in the presence of *Burkholderia*. A random gene fusion library was constructed in *P. aeruginosa* PA14 by using a transposon containing a promoterless *lacZ* gene. Fusion strains were screened for their ability to be induced in the presence of *Burkholderia* strains in a cross-streak assay. Three fusion strains were induced specifically by *Burkholderia* species; all three had transposon insertions in genes known to be iron regulated. One of these fusion strains, containing a transposon insertion in gene PA4467, was used to characterize the inducing activity from *Burkholderia*. Biochemical and genetic evidence demonstrate that ornibactin, a siderophore produced by nearly all *B. cepacia* strains, can induce *P. aeruginosa* PA4467. Significantly, PA4467 is induced early in coculture with an ornibactin-producing but not an ornibactin-deficient *B. cepacia* strain, indicating that ornibactin can be produced by *B. cepacia* and detected by *P. aeruginosa* when the two species coexist.

Microbial populations in most natural environments consist of a multitude of species, yet laboratory studies of bacterial physiology have focused predominately on pure cultures. Relatively little is known at the molecular level about how different bacteria interact with each other when present in the same environment. Specifically, there is a need to define how the gene expression patterns of a given bacterium are affected by the presence of other microbial species.

To gain insight into ways in which different bacterial species may affect each other, we investigated the interaction between two ubiquitous soil microorganisms, *Pseudomonas aeruginosa* and members of the *Burkholderia cepacia* complex. These bacterial species are phylogenetically distinct yet often coexist in diverse environments. *P. aeruginosa*, a member of the gamma proteobacteria, and *Burkholderia* species, members of the beta proteobacteria, are both catabolically versatile and highly resistant to many antibiotics (1, 23, 38, 47). *Burkholderia* strains are characterized by a high level of genomic diversity, and the *B. cepacia* complex is now comprised of at least nine species (10, 11, 22, 25). These species include *B. cepacia*, initially isolated as an onion pathogen, and *Burkholderia multivorans* and *Burkholderia vietnamiensis*, which have been investigated for use as biocontrol and bioremediation agents (5, 9, 39, 40). *P. aeruginosa* has been found ubiquitously in soil environments (2, 38, 47). Since both genera are common soil isolates and have such similar phenotypic properties, it is likely that they occupy similar niches in many natural environments.

Although they are primarily soil inhabitants, both *P. aeruginosa* and many *Burkholderia* species are also opportunistic human pathogens. *P. aeruginosa* is a major source of nosocomial infections, causing disease in a wide range of immuno-

compromised patients (24). In addition, *P. aeruginosa* colonizes the lungs of almost all cystic fibrosis (CF) patients at an early age. While antibiotic treatment can temporarily decrease bacterial levels in the lungs of CF patients, *Pseudomonas* infections are usually chronic (14).

Subsequent to *P. aeruginosa* infection, CF patients frequently develop secondary infections by a number of other microbial species. Prominent among these are members of the *Burkholderia* genus, which have emerged as particularly problematic pathogens for CF patients (6, 14, 15, 17). Patients with *Burkholderia* infections often succumb to what is termed the "cepacia syndrome," or necrotizing pneumonia, which is usually fatal (7, 49). Currently, it is unclear how the presence of *Burkholderia* leads to such a rapid decline in health.

Because *P. aeruginosa* and *B. cepacia* are found in the same natural environments and can coinfect human hosts, we wanted to investigate how *Burkholderia* affects *P. aeruginosa* gene expression. In order to examine interactions between *P. aeruginosa* and *Burkholderia* in an unbiased manner, we prepared a random gene fusion library in *P. aeruginosa* and performed a screen using a cross-streak assay on solid medium. This screen allowed us to identify *Pseudomonas* genes that are induced in the presence of secreted or cell-associated factors from *Burkholderia*. The gene fusions were used in an assay to identify the inducing activity from *Burkholderia*. We were able to determine that ornibactin, a siderophore (iron-chelating molecule) produced by *Burkholderia*, can strongly affect *P. aeruginosa* gene expression under the conditions tested. This demonstrates that competition for iron can be a factor in *Pseudomonas-Burkholderia* interactions.

MATERIALS AND METHODS

Media, reagents, and bacterial strains. Bacterial strains were grown in Luria-Bertani broth (LB) or succinate minimal medium (27). Tetracycline (TET) was used at concentrations of 150 or 75 $\mu\text{g/ml}$, and carbenicillin was used at a concentration of 750 $\mu\text{g/ml}$. 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside

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TABLE 1. Induction of PA4467::Tn5lacZ with medium conditioned by *Burkholderia* or non-*Burkholderia* species

Lab collection no.	Strain	Miller units \pm SD	Reference or source
ZK 2878	<i>B. cepacia</i> complex, CF isolate 5	295 \pm 11	J. Burns, University of Washington
ZK 2715	<i>B. vietnamiensis</i> G4	284 \pm 28	41
ZK 2875	<i>B. cepacia</i> complex, CF isolate 2	255 \pm 24	J. Burns
ZK 2714	<i>B. cepacia</i> 5424	235 \pm 76	27
ZK 2716	<i>B. multivorans</i> 17616	230 \pm 11	27
ZK 2877	<i>B. cepacia</i> complex, CF isolate 3-2	225 \pm 18	J. Burns
ZK 3331	<i>B. cepacia</i> K56-2	208 \pm 19	27
ZK 2876	<i>B. cepacia</i> complex, CF isolate 3-1	121 \pm 2	J. Burns
ZK 2718	<i>B. cepacia</i> 25416	92 \pm 12	27
ZK 2610	<i>Staphylococcus aureus</i> strain Newman	45 \pm 1	Lab stock
ZK 2757	<i>Bacillus subtilis</i> 3610	38 \pm 11	Lab stock
ZK 2745	<i>Candida albicans</i> SC5314	36 \pm 4	Lab stock
ZK 126	<i>Escherichia coli</i> K-12	32 \pm 1	Lab stock
ZK 2511	<i>Enterococcus</i> sp. clinical isolate	20 \pm 7	Lab stock
ZK 2466	<i>P. aeruginosa</i> PA14	20 \pm 3	Lab stock

(X-Gal) was used at a concentration of 100 μ g/ml. Strains used for conditioned medium experiments are indicated in Table 1.

Transposon library construction and insertion mapping. A random library of Tn5-B21 transposon insertion mutants (*lacZ* fusion strains) was generated in *P. aeruginosa* PA14 as described previously (36, 43). The transposon library consists of mutants derived from 20 independent conjugations. Transposon insertion sites were identified by arbitrary PCR amplification as described previously (8, 37). PCR products were sequenced, and results were compared to the *P. aeruginosa* genome database (<http://www.pseudomonas.com>).

Cross-streak assay. Cultures of *Burkholderia* strains were grown in LB at 37°C overnight, and 100 μ l was streaked down the center of square petri dishes containing LB agar with X-Gal. These plates were incubated for 24 h at 30°C. Fusion strains were taken from LB-TET (75 μ g/ml) plates, individually resuspended in 100 μ l of LB, and allowed to incubate at 37°C for 4 h. A toothpick was used to streak each fusion strain suspension from the edge of the plate towards the *Burkholderia* streak, with a small area of overlap. Plates were incubated at 30°C for 42 h and observed for the appearance of blue pigment (indicative of β -galactosidase activity) in the *P. aeruginosa* fusion strains.

Liquid induction assay. Conditioned medium was obtained from cultures grown in LB or succinate minimal medium with or without 10 mM ornithine at 30° for 40 h. Cells were pelleted by centrifugation, and the supernatant was filtered through a 0.2 μ m-pore-size filter. Conditioned medium was added 1:1 with fresh medium (LB or succinate minimal) to the assay.

PA4467::Tn5lacZ (fusion I) cells were prepared for the β -galactosidase assay as follows: liquid overnight cultures were grown in LB-TET (75 μ g/ml) at 37°C, diluted 1:200 in LB with TET, allowed to grow to an optical density at 600 nm (OD₆₀₀) of 0.3, and diluted again 1:200 in LB with TET. The cultures were centrifuged when they reached an OD₆₀₀ of 0.1, and the pellets were resuspended in 1/20 of the original volume of LB. Concentrated cells were added to the assay medium such that the final cell density was an OD₆₀₀ of 0.1. Fusion cells were incubated in the indicated medium for 45 min at 37°C with shaking. After incubation, cells were placed immediately on ice, β -galactosidase assays were performed, and Miller units were calculated as described previously (31). PAO1 *lacZ* fusion strains (*pvdJ*, *pvdA*, *regA*, and *pvdS*) were grown with carbenicillin and assayed for induction as described above.

RNA isolation and microarray analysis. Total RNA was isolated from *P. aeruginosa* PAO1 cells that were treated as follows. PAO1 cultures were harvested at an OD₆₀₀ of 0.3, centrifuged, and resuspended in LB, an equal volume of *Burkholderia* Bc2-conditioned medium and LB, or an equal volume of PAO1-conditioned medium and LB. For conditioned media, *Burkholderia* Bc2 and PAO1 were grown in LB at 37°C for 24 h and filter sterilized. *P. aeruginosa* was incubated in these conditions at 37°C for 30 min with shaking. RNA was isolated with RNeasy columns (QIAGEN) after treating the cells with 3 mg of lysozyme/ml. The GeneChip probes were prepared according to the protocol supplied by Affymetrix, and data were analyzed by using MICROARRAY SUITE software (Affymetrix).

Biochemical techniques. Ferric chloride was added to various media to test for the presence of iron-binding compounds. One microliter of a FeCl₃ · 6H₂O 25% (wt/vol) solution was added to 500 μ l of conditioned medium. A change in color

relative to fresh medium indicated the presence of an iron-chelating compound (29). The chrome azurol S (CAS) assay with or without shuttle solution was used as described previously (42). Absorbance at 630 nm was measured relative to water alone. The ratio of absorbance with the medium of interest mixed with the CAS solution to that of water or fresh medium with the CAS solution was designated the relative CAS activity and is a measure of the iron-chelating activity.

Ornibactin purification and quantitation. Ornibactin was purified as described previously (13, 30, 48) with the following modifications. *Burkholderia* strain K56-2 was grown in 150 ml of succinate medium with 10 mM ornithine at 30°C for 40 h. Cells were pelleted by centrifugation, and the supernatant was removed and filtered through a 0.2 μ m-pore-size filter. Conditioned medium was applied to a C₁₈ column (15 by 1.5 cm), rinsed with water, and eluted with 100% methanol. This eluant was concentrated by evaporation, added to a Sephadex LH-20 column (30 by 1.5 cm), and eluted with 100% methanol. After testing for CAS activity, every fifth CAS-active fraction was pooled, and the solution was concentrated by evaporation. Fifty microliters was applied to a silica gel G thin-layer chromatography (TLC) plate. The TLC plate was developed in butanol-acetic acid-water (3:1:1). After drying, the plate was sprayed with 0.1 M FeCl₃ in 0.1 N HCl.

CAS-active fractions were dried to completeness through evaporation, resuspended in water, and pooled. This was used as the ornibactin stock solution because the TLC plate indicated that no other iron-binding compounds were present and this is the preparation used in other studies involving ornibactin (30). The concentration of ornibactin in the stock solution was quantitated by using the molar extinction coefficient of ornibactin in water (1,267 M⁻¹ cm⁻¹) as described by Meyer et al. (30). The CAS assay was used to correlate purified ornibactin and ornibactin present in K56-2-conditioned medium.

Mixed cultures. *P. aeruginosa* and *Burkholderia* strains were grown overnight in succinate medium with 20 μ M ferrous sulfate. Cultures were diluted into fresh succinate medium at 1:100 of the desired final OD₆₀₀ and incubated at 30°C until cultures reached the desired optical densities. When fusion I cultures were at an OD₆₀₀ of 0.1 and *Burkholderia* cultures were at an OD₆₀₀ of either 0.1 or 1.0, aliquots were centrifuged, rinsed, and resuspended in warm succinate medium. Cells were added to 5 ml of warm succinate medium at the following densities: fusion I to an OD₆₀₀ of 0.1 and *Burkholderia* K56-2 or K56pvdA::tp to an OD₆₀₀ of either 0.1 or 1.0. Cultures were incubated with shaking at 30°C. Aliquots were taken at the indicated time points, and 10⁻⁵ and 10⁻⁶ dilutions were plated in duplicate on LB plates. Plates were incubated for 48 h at 37°C. The remaining samples were placed on ice and used for β -galactosidase assays. Cell counts and β -galactosidase activity normalized to cell number were obtained by counting *P. aeruginosa* and *Burkholderia* colonies. The colonies were readily distinguishable on the basis of morphology. It was previously determined that, at the dilutions used, the presence of one species did not affect the ability of the other species to grow. To determine the β -galactosidase activity normalized to fusion I cell number, calculations were performed as described for Miller units (see above), with the OD₆₀₀ value replaced with CFU/ml and multiplied by a factor of 10⁸. This value was designated β -galactosidase activity/CFU.

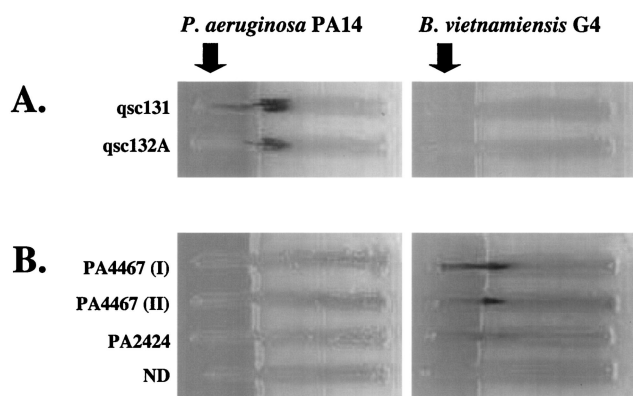


FIG. 1. *P. aeruginosa* PA14 and *B. vietnamiensis* G4 were tested for their ability to induce either *P. aeruginosa* PAO1 (*lasI rhII*) quorum-sensing-controlled fusions (qsc131 and qsc132A) (A) or *P. aeruginosa* PA14 Tn5*lacZ* fusions (B). ND indicates that the transposon insertion site of the fusion was not determined. *B. vietnamiensis* and *P. aeruginosa* PA14 strains were incubated on LB-X-Gal plates for 24 h at 30°C prior to inoculation of the fusion strains. Plates were photographed after 40 h of coincubation at 30°C.

RESULTS

Isolation of *Burkholderia*-responsive *P. aeruginosa* gene fusions. A screen was designed to identify *P. aeruginosa* gene fusions induced by either secreted or cell-associated factors from *Burkholderia* species. The screen was based on an assay in which random *P. aeruginosa lacZ* gene fusions are cross-streaked against a *Burkholderia* strain. For fusions that are induced by *Burkholderia*, *lacZ* expression should be high proximal and low distal to the cross-streak. To validate the screen, we tested gene fusions that are induced by secreted *P. aeruginosa* quorum-sensing molecules, the homoserine lactones PAI-1 and PAI-2 (41). These gene fusions were in a strain background unable to synthesize either PAI-1 or PAI-2 (*lasI rhII*) (52). When cross-streaked with wild-type *P. aeruginosa*, *lacZ* expression was evident proximal to the cross-streak (Fig. 1A).

A library of approximately 6,000 transposon insertion strains was screened for fusions that are specifically induced by *Burkholderia*. A Tn5*lacZ* insertion library was constructed in *P. aeruginosa* strain PA14 as described previously (35, 36). The transposon contains a promoterless *lacZ* gene, creating a transcriptional fusion upon insertion. Isolated fusion strains were tested in the cross-streak assay for their ability to become induced in the presence of *Burkholderia* strains. Separate LB agar plates containing the chromogenic galactoside X-Gal were inoculated with vertical streaks of two different *Burkholderia* strains, *B. vietnamiensis* G4 (an environmental isolate) and *B. cepacia* 5424 (a CF clinical isolate). The *Burkholderia* strains were allowed to grow for 24 h before the *P. aeruginosa* fusion strains were cross-streaked with them. The *P. aeruginosa* fusions were observed over a period of 42 h for the appearance of blue pigment proximal to the *Burkholderia* streak. Of the fusion strains screened, 36 initial candidates were apparently inducible by *Burkholderia*. These fusions were rescreened against a number of other microbial species to rule out fusions that were not induced specifically by *Burkholderia*. Candidate

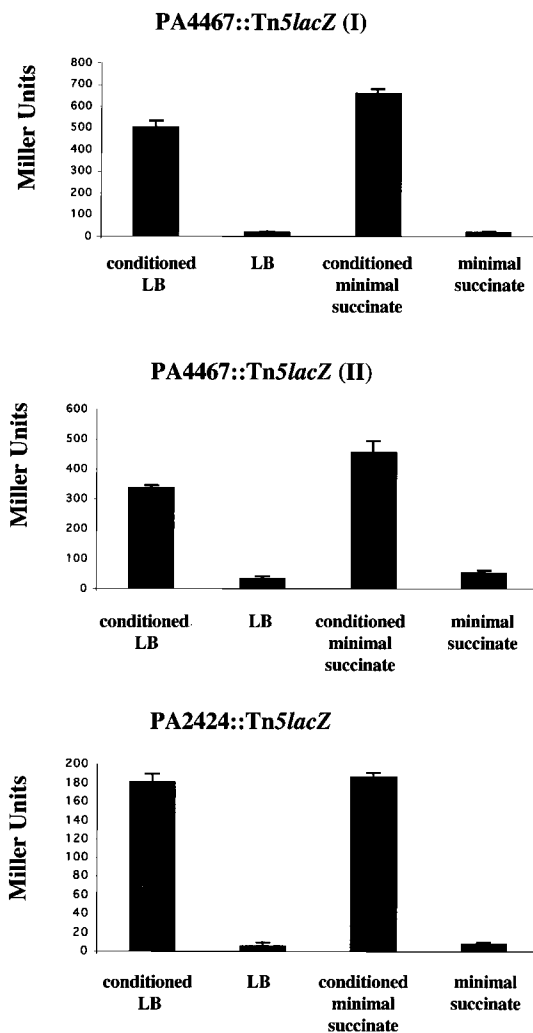


FIG. 2. Fusions were tested for their ability to be induced by *Burkholderia*-conditioned medium in a liquid β -galactosidase assay. The assay was performed and Miller units were calculated as described in Materials and Methods. All three fusion strains were induced by both rich (LB) and minimal (succinate) media conditioned by *B. vietnamiensis* G4. The results shown are the averages of data from three separate experiments (error bars indicate standard deviations [SD]).

fusions were also screened against wild-type *P. aeruginosa* to rule out fusions that were induced by general nutrient depletion. Of the 36 initial candidates, three fusions were identified as being induced specifically by *Burkholderia* (Fig. 1B).

Induction of *P. aeruginosa* gene fusions by *Burkholderia*-conditioned medium. To quantitate the level of induction of the fusions by *Burkholderia*, we utilized a liquid β -galactosidase assay. *Burkholderia* strains were grown in either rich or succinate minimal medium to stationary phase, and cell-free supernatants of these cultures were collected and referred to as conditioned medium. Early-exponential-phase cultures (OD₆₀₀ of 0.1) of the *P. aeruginosa* fusion strains were resuspended in a 1:1 mixture of *Burkholderia*-conditioned medium and fresh medium. Fusion cells were then incubated for 45 min before the β -galactosidase assays were performed (Fig. 2). All three *P. aeruginosa* fusion strains were induced in the presence

of *Burkholderia*-conditioned medium relative to medium alone, regardless of whether rich or minimal medium was used. In both types of conditioned media, the fusions were induced between 10- and 20-fold. For all of our subsequent experiments, we focused on a single fusion strain, PA4467::Tn5lacZ (I), or fusion I for short.

In addition to the initial *Burkholderia* strains tested in the cross-streak assay, we found that numerous strains, representing multiple species in the *B. cepacia* complex, strongly induced fusion I. Microbial strains were inoculated into LB and allowed to grow for 40 h at 30°C, and the resulting conditioned media were tested for their ability to induce the fusion. All nine of the *Burkholderia* strains tested strongly induced the fusion, while conditioned media from other microbial species, including *P. aeruginosa* itself, did not induce the fusion (Table 1).

Transposon insertions into iron-regulated genes. The transposon insertion sites of the three *Burkholderia*-responsive fusions were identified by using arbitrary PCR and DNA sequence analysis. Two of the fusion strains contain transposon insertions in or directly following the same open reading frame (ORF), PA4467. We called these fusion I (insertion site 4,997,281) and fusion II (insertion site 4,996,465), respectively. The ORF of unknown function is located at the end of an operon containing the manganese-cofactored superoxide dismutase and a fumarate hydratase. The operon has been shown to be induced under low-iron conditions, and biochemical studies show that Fur, the ferric uptake regulator, binds in the promoter region (18, 19). While this operon has also been shown to be regulated by the PAI-1 quorum-sensing system, regulation by iron has been shown to be dominant (3, 20). The third fusion strain, fusion III (insertion site 2,717,251), contains a transposon insertion in PA2424, a gene recently named *pvdL* for its role in pyoverdine biosynthesis. The *pvdL* gene encodes a nonribosomal peptide synthetase and is thought to be in a bicistronic operon with a gene, PA2425, encoding a putative thioesterase. The operon is upregulated in low-iron conditions and is under the control of the alternative sigma factor, PvdS (33).

Microarray analysis of *P. aeruginosa* genes induced by *Burkholderia*. Microarray experiments were performed to complement the screen to identify *P. aeruginosa* genes induced in the presence of *Burkholderia*-conditioned medium. A mid-exponential-phase culture of *P. aeruginosa* (strain PAO1) was incubated for 30 min in (i) LB, (ii) an equal volume of *Burkholderia* Bc2-conditioned medium and LB, and (iii) an equal volume of *P. aeruginosa* PAO1-conditioned medium and LB. RNA was isolated, and Affymetrix GeneChips were used to identify *P. aeruginosa* genes induced in the presence of *Burkholderia*-conditioned medium. Table 2 shows all of the genes whose levels of expression increased 20-fold or greater in the presence of *Burkholderia*-conditioned medium relative to LB alone. Among the most highly induced genes in the microarray experiment were those identified in the genetic screen, PA2424 and the operon containing PA4467 (PA4467-PA4471). In addition, other genes known to be iron regulated, such as those involved in pyoverdine and pyochelin biosynthesis, were highly induced. While PA2424 was also induced in *P. aeruginosa*-conditioned medium relative to LB, the level of induction was much lower than that with *Burkholderia*-conditioned medium. In addition, other known iron-regulated genes were not in-

TABLE 2. *P. aeruginosa* PAO1 genes with increased expression in *Burkholderia*-conditioned medium relative to LB^a

ORF	Gene	Induction (fold) in <i>Burkholderia</i> -conditioned LB versus LB ^b	Induction (fold) in <i>Pseudomonas</i> -conditioned LB versus LB ^{b,c}
PA2411		158 ± 5	7 ± 2
PA4471		152 ± 2	NC
PA2424	<i>pvdL</i>	124 ± 2	6 ± 1
PA2452		124 ± 3	NC
PA3407	<i>hasA</i>	115 ± 2	NC
PA4470	<i>fumC</i>	111 ± 2	NC
PA2413		91 ± 2	NC
PA4469		82 ± 1	NC
PA2412		71 ± 2	NC
PA4468	<i>sodM</i>	66 ± 1	NC
PA2400		60 ± 2	4 ± 1
PA2399	<i>pvdD</i>	58 ± 4	NC
PA2393		52 ± 2	NC
PA2034		52 ± 2	NC
PA4710		45 ± 1	NC
PA2386	<i>pvdA</i>	42 ± 2	NC
PA2402		38 ± 1	NC
PA4467		38 ± 1	NC
PA2396	<i>pvdF</i>	36 ± 2	NC
PA2426	<i>pvdS</i>	36 ± 3	NC
PA2392		32 ± 3	NC
PA4228	<i>pchD</i>	32 ± 2	NC
PA4896		28 ± 1	NC
PA2394		28 ± 2	NC
PA2033		26 ± 2	NC
PA2444	<i>glyA</i>	24 ± 5	13 ± 4
PA3530	<i>bfd</i>	24 ± 3	NC
PA4168		23 ± 4	NC
PA2397	<i>pvdE</i>	22 ± 1	NC
PA1999		20 ± 3	15 ± 3
PA2401		20 ± 1	NC
PA0672		20 ± 3	NC

^a Results are shown for genes induced more than 20-fold.

^b Data shown are the averages of two experiments ± SD.

^c NC, no change above background.

duced in *P. aeruginosa*-conditioned medium relative to LB. This indicates that the pattern of gene induction in the presence of *Burkholderia* is not a generic response to conditioned media from stationary-phase cultures. The results further indicate that there is an overlap between genes induced in low-iron conditions and those induced by *Burkholderia*-conditioned medium.

Iron regulation of fusion I expression. Because both PA4467 and *pvdL* are known to be iron regulated, we confirmed that fusion I could be induced under iron-limiting conditions. We monitored fusion I β-galactosidase activity in medium with increasing concentrations of diethylenetriaminepentaacetic acid (DTPA), an iron-chelating agent (21). As predicted, iron limitation was sufficient to induce fusion I (Fig. 3A), as the fusion was induced in the presence of DTPA at concentrations greater than 4 μM. To test whether excess iron could suppress the inducing capability of *Burkholderia*-conditioned medium, we added increasing concentrations of ferrous sulfate to *B. vietnamiensis* G4-conditioned medium. As shown in Fig. 3B, the addition of iron incrementally decreased the induction of fusion I by conditioned medium, suggesting that iron limitation was required for the ability of *Burkholderia*-conditioned medium to induce the fusion.

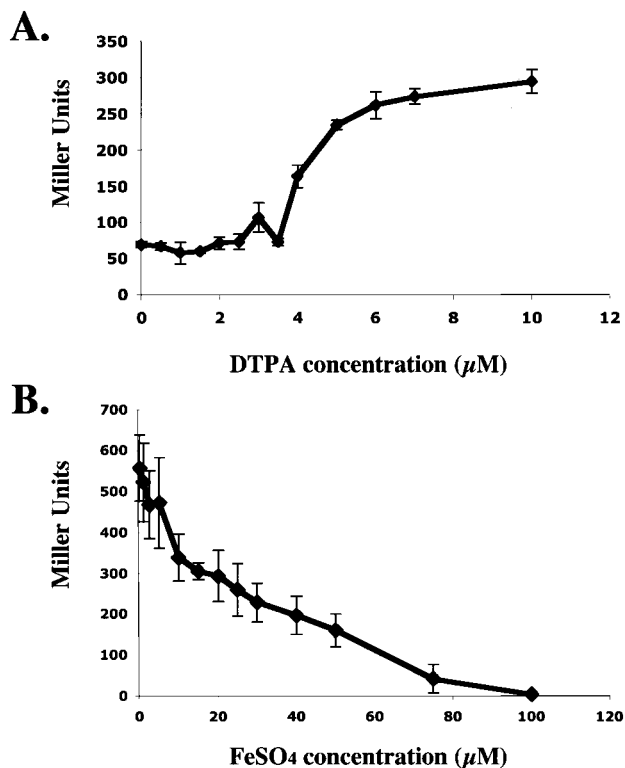


FIG. 3. (A) DTPA, a synthetic iron chelator, was used to test whether iron depletion of fresh medium was able to induce fusion I. DTPA was added to succinate minimal medium at the concentrations shown. Values are the averages of data from three separate experiments (error bars indicate SD). (B) The ability of ferrous sulfate to suppress induction by *B. vietnamiensis* G4-conditioned medium was tested. The indicated concentrations of ferrous sulfate were added to conditioned succinate minimal medium. The induction level was determined as described in Materials and Methods. Values are the averages of data from three separate experiments (error bars indicate SD).

Iron-chelating activity in *Burkholderia*-conditioned medium.

From the above results, we hypothesized that an iron-chelating activity from *Burkholderia* was sequestering iron away from *P. aeruginosa* in these experiments. To test this hypothesis, we used several assays to detect the presence of iron chelators in *Burkholderia*-conditioned medium. Both the ferric chloride and CAS assays (see Materials and Methods) can detect the presence of iron-chelating compounds (29, 42). With both assays, *Burkholderia*-conditioned medium tested positive for iron-chelating activity (data not shown). We wanted to investigate whether this iron-chelating activity corresponded to the activity that induced fusion I.

Previous studies had identified four siderophores made in various combinations by *Burkholderia* strains: pyochelin, salicylic acid, cepabactin, and ornibactin (28, 30, 44, 46, 48). Darling et al. (13) showed that roughly 90% of all *B. cepacia* complex strains tested produced ornibactin and salicylic acid, whereas pyochelin and cepabactin were produced by far fewer strains. In addition, Meyer et al. (30) found that one well-characterized strain, ATCC 25416, produced ornibactin at a level of 109 mg/liter, while cepabactin and pyochelin were produced at levels of 46 mg/liter and 11 mg/liter, respectively.

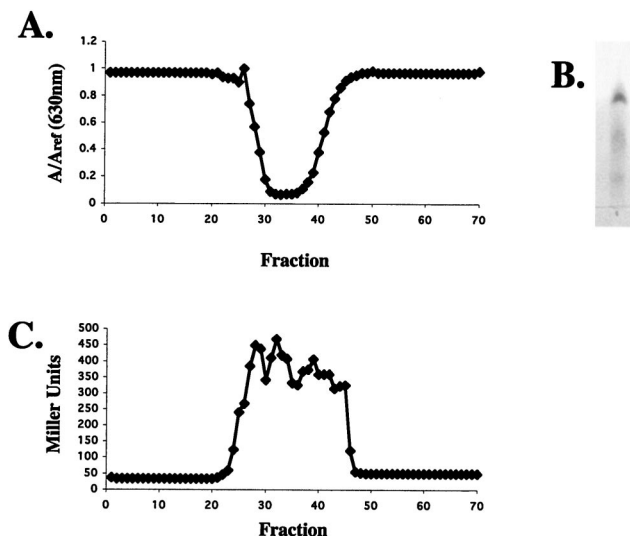


FIG. 4. (A) Fractions from a Sephadex LH-20 column were tested for iron-chelating activity. Two hundred microliters of each 1-ml fraction was concentrated, resuspended in water, and tested in the CAS assay. (B) The CAS-active fractions were pooled and run on a silica gel G TLC plate, which was then sprayed with ferric chloride. The only iron-reactive spots were the three bands indicative of the three forms of ornibactin (containing acyl side chains of different lengths; R_f values, 0.25, 0.18, and 0.09). (C) One hundred microliters of each 1-ml fraction was dried by lyophilization, resuspended in buffered medium, mixed 1:1 with fresh succinate medium, and tested for its ability to induce fusion I.

Several studies have examined the ability of *P. aeruginosa* to utilize *Burkholderia* siderophores. Both pyochelin and salicylic acid are known to be made and used by *P. aeruginosa* to scavenge iron (12, 26, 51). In a study examining the use of heterologous siderophores, Meyer (26) demonstrated that cepabactin could be taken up and used for iron acquisition by *P. aeruginosa*. However, studies with radiolabeled ornibactin showed that *P. aeruginosa* could not take it up over a 15-min assay period, suggesting that ornibactin cannot be used as an iron source (30).

We hypothesized that ornibactin may make iron unavailable to *P. aeruginosa*, causing iron-regulated operons to be induced. To test this hypothesis, we purified ornibactin using the published protocol (13, 30, 48). In the final step of the purification process, we tested fractions obtained from a Sephadex LH-20 column for iron-chelating activity using the CAS assay (Fig. 4A). CAS-active fractions were pooled, run on a TLC plate, and sprayed with ferric chloride. Three bands with R_f values of 0.25, 0.18, and 0.09 were detected, indicating the presence of ornibactin (Fig. 4B). The column fractions were then tested for their ability to induce fusion I. As seen in Fig. 4C, fractions containing purified ornibactin were able to induce fusion I.

Ornibactin requirement for fusion I induction by *Burkholderia*-conditioned medium. To test whether ornibactin production was necessary for the inducing capability of *Burkholderia*-conditioned medium, we obtained a strain defective in ornibactin biosynthesis from P. Sokol (University of Calgary, Calgary, Alberta, Canada). The parental strain, *B. cepacia* K56-2 is an isolate from a CF patient and is a member of the genomovar III group of the *B. cepacia* complex. It was shown

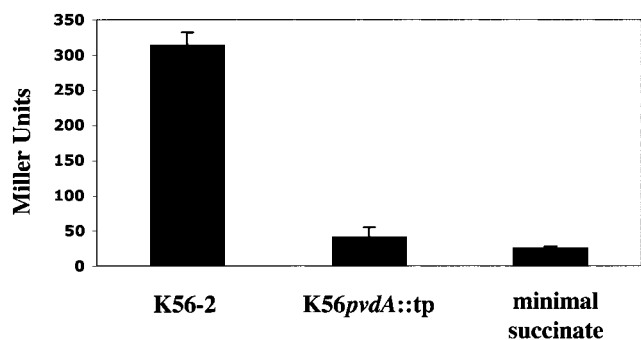


FIG. 5. Conditioned succinate minimal media from ornibactin-producing strain *B. cepacia* K56-2 and the ornibactin-deficient mutant *K56pvdA::tp* were tested for their ability to induce fusion I. *Burkholderia* strains were grown in succinate minimal medium with 10 mM ornithine for 40 h at 30°C. Values are the averages of data from four separate experiments (error bars indicate SD).

to produce ornibactin, salicylic acid, and negligible amounts of pyochelin. A nonpolar replacement mutant in *pvdA*, a gene encoding an L-ornithine *N*⁵-oxygenase, is defective in ornibactin production (45). We tested the ability of conditioned medium from the wild-type strain and from the mutant strain to induce fusion I. While the wild-type strain induced the fusion, the mutant strain did not (Fig. 5). These data indicate that ornibactin is necessary for the ability of *Burkholderia* K56-2-conditioned medium to induce fusion I.

Conditioned media from K56-2 and *K56pvdA::tp* were also used to test whether ornibactin biosynthesis is necessary for the ability of *Burkholderia* to induce other *Pseudomonas* iron-regulated genes. As shown in Table 3, conditioned medium from the wild-type strain but not the ornibactin-deficient mutant is able to induce the iron-regulated pyoverdine biosynthesis genes *pvdJ* and *pvdA*, as well as iron-regulated transcriptional regulators *pvdS* and *regA* (34). This finding indicates that the induction of numerous iron-regulated genes in the presence of *Burkholderia* is due to ornibactin production.

To test whether ornibactin accounted for all of the inducing activity present in *Burkholderia*-conditioned medium, we determined the concentration of ornibactin in *B. cepacia* K56-2-conditioned medium. Using a published molar extinction coefficient, we determined the concentration of ornibactin in our purified preparation. This allowed us to correlate ornibactin concentration with CAS activity (Fig. 6A). Using dilutions of K56-2-conditioned medium in the CAS assay, we made a sec-

TABLE 3. Induction of *P. aeruginosa* PAO1 iron-regulated genes by conditioned media from wild-type and ornibactin-deficient *Burkholderia* strains

lacZ fusion	Conditioned media (Miller units ± SD) ^b		
	SMM ^a	SMM + K56-2	SMM + <i>K56pvdA::tp</i>
<i>pvdA</i>	18 ± 6	81 ± 29	11 ± 4
<i>regA</i>	31 ± 26	172 ± 69	23 ± 7
<i>pvdJ</i>	17 ± 7	289 ± 160	20 ± 5
<i>pvdS</i>	31 ± 10	412 ± 266	29 ± 1

^a SMM, succinate minimal medium.

^b Values are the averages of results from three separate experiments.

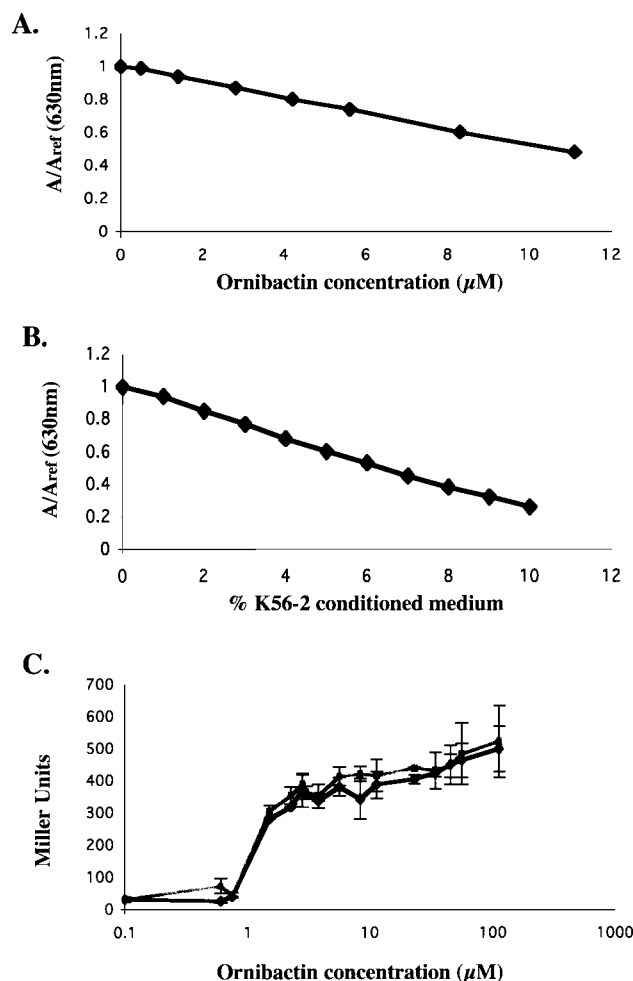


FIG. 6. The concentration of ornibactin in K56-2-conditioned succinate medium was determined. (A) Dilutions of the ornibactin stock solution were assigned activity in the CAS assay. (B) Dilutions of K56-2-conditioned medium, shown as the percentage of conditioned medium in water added to the CAS assay, were assigned CAS activity. (C) Known ornibactin concentrations (squares) and the volumes of conditioned medium that corresponded to the known ornibactin concentrations (diamonds) were tested for induction of fusion I. Values are the averages of data from three separate experiments (error bars indicate SD). The volume differences were made up with buffered medium (minimal medium lacking succinate) and were mixed 1:1 with fresh succinate medium.

ond curve correlating conditioned medium with CAS activity (Fig. 6B). Equating CAS activities from both curves, we could assign values of ornibactin concentration to the *Burkholderia*-conditioned medium. After growth of *Burkholderia* K56-2 in succinate minimal medium supplemented with 10 mM ornithine, the conditioned medium contained 166 μM ornibactin. A dose-response experiment was performed in which known amounts of ornibactin were added to cultures of the fusion I strain. The induction levels were compared to those of fusion cells incubated with conditioned medium corresponding to equivalent amounts of ornibactin. As shown in Fig. 6C, purified ornibactin and K56-2-conditioned medium with equivalent ornibactin concentrations induced fusion I to the same level. This result indicates that ornibactin is the major activity in

Burkholderia K56-2 conditioned medium responsible for the induction of this fusion.

Induction of fusion I in mixed cultures of *P. aeruginosa* and *Burkholderia*. To address the role of ornibactin in *P. aeruginosa*-*Burkholderia* interactions, we examined the effect of ornibactin production on *P. aeruginosa* in mixed cultures. Specifically, we compared the expression level of fusion I in the presence of an ornibactin-producing *B. cepacia* strain relative to an ornibactin-deficient *B. cepacia* strain. *B. cepacia* cultures at an OD₆₀₀ of 1.0 were pelleted by centrifugation and rinsed to remove any residual ornibactin. These cells were mixed with fusion I cells at an OD₆₀₀ of 0.1, such that the majority of the cells were *Burkholderia* cells. The cultures were incubated at 30°C, and aliquots were taken for CFU counts and β-galactosidase assays at various time points. As shown in Fig. 7A, fusion I cells were induced immediately in the presence of an ornibactin-producing but not an ornibactin-deficient *B. cepacia* strain even though the growth rates of the two *B. cepacia* strains were roughly equivalent.

To examine whether ornibactin could induce fusion I when the two species are mixed at comparable numbers and physiological states, we added both species in the early exponential phase at an OD₆₀₀ of 0.1. As shown in Fig. 7B, ornibactin production by *Burkholderia* species is able to induce fusion I even when both strains are inoculated at a low density. While PA4467 is known to become induced in stationary phase, it is reproducibly induced earlier in the presence of an ornibactin-producing *B. cepacia* strain. Importantly, this occurs even though the cultures are not initially iron limited and overall CFU counts and growth rates in the different cultures are equivalent. This demonstrates that in conditions where *P. aeruginosa* and *Burkholderia* are found in similar physiological states and numbers, ornibactin can play a role in the response of *Pseudomonas* to the presence of *Burkholderia*.

DISCUSSION

We describe here a genetic approach to examine the interaction of two microbial species. We show that the production of the siderophore ornibactin by members of the *B. cepacia* complex can significantly alter the expression of *P. aeruginosa* gene PA4467. It has been shown previously that the operon containing PA4467 is controlled by Fur, a regulatory factor that derepresses the operon under iron-limiting conditions (18). Since ornibactin was shown to not be taken up by *P. aeruginosa* (30), it is likely that the mechanism by which ornibactin causes PA4467 induction is by limiting the amount of iron available to *Pseudomonas*. Iron availability is a global regulatory signal for *P. aeruginosa*; numerous genes, including many virulence factors, are induced under low-iron conditions (50). Therefore, ornibactin, as a nonutilizable siderophore, likely causes a global response, inducing a large number of *P. aeruginosa* genes. The induction of many iron-regulated genes by *Burkholderia*-conditioned medium in our microarray experiment supports this idea. Furthermore, we show that a range of iron-regulated genes are induced by conditioned medium from ornibactin-producing but not ornibactin-deficient *Burkholderia* strains.

We wanted to determine whether the observed effect of ornibactin on *P. aeruginosa* occurred in *Burkholderia*-*Pseudo-*

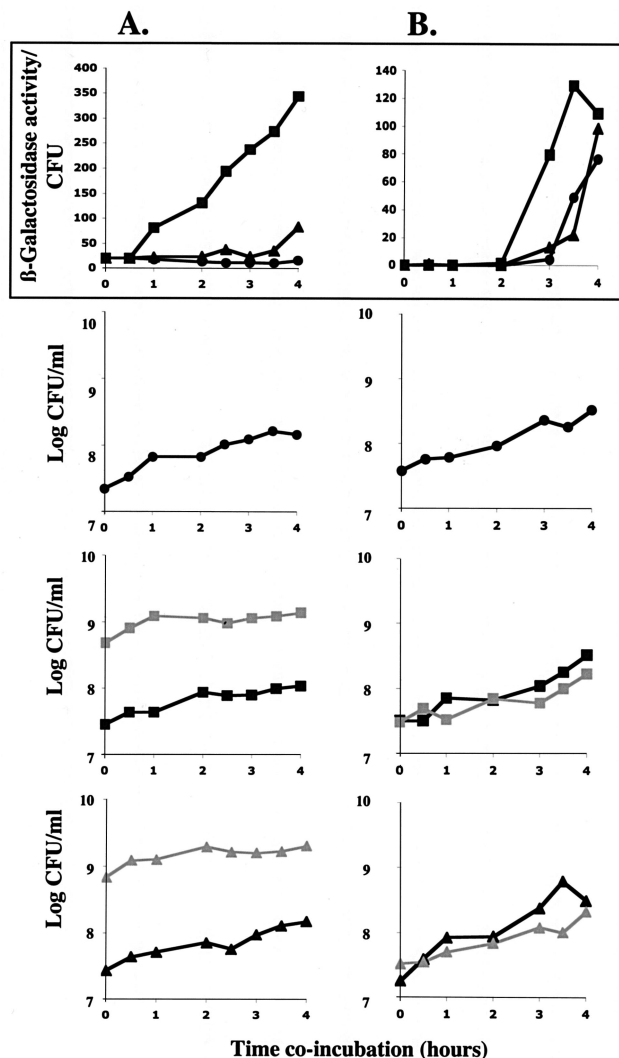


FIG. 7. The ability of *B. cepacia* K56-2 or K56pvdA::tp to induce fusion I in mixed cultures was determined under different conditions. (A) *Burkholderia* cells were in the majority and in stationary phase (added at an OD₆₀₀ of roughly 1.0 to fusion I cells at an OD₆₀₀ of 0.1). (B) There were equal numbers of *Burkholderia* cells and fusion I cells and *Burkholderia* cells were in early logarithmic growth (added at an OD₆₀₀ of 0.1 to fusion I cells at an OD₆₀₀ of 0.1). The top panels indicate the expression levels of fusion I cells incubated alone (black circles), with *B. cepacia* K56-2 (black squares), or with K56pvdA::tp (black triangles). The lower three sets of panels show growth (log CFU/ml) of the strains in the different mixed cultures; fusion I cells (black circles, black squares, and black triangles) are in culture alone, with *B. cepacia* K56-2 (gray squares), or with *B. cepacia* K56pvdA::tp (gray triangles). The experiments shown are representative of the results obtained; the experiments were repeated four times.

monas mixed cultures. To address this, we tested whether ornibactin could induce PA4467 when the two species were mixed at comparable numbers and in comparable physiological states. We show that even when the two species are mixed during early exponential phase, an ornibactin-producing *Burkholderia* strain, but not an ornibactin-deficient strain, induces *P. aeruginosa* PA4467. This occurs despite equivalent growth rates and cell numbers in the different cultures. This suggests that, in some environments where the two species are in similar

physiological states, ornibactin may be produced by *Burkholderia* and detected by *P. aeruginosa*.

It is well established that both soil and the lungs of CF patients are environments with low concentrations of bioavailable iron (50). Siderophores are important for microbial growth in the soil and have been isolated directly from soil in several studies (4, 32). In addition, *P. aeruginosa* siderophores have been isolated from the sputa of CF patients (16). Similar studies have not yet been done with ornibactin, but it is likely that ornibactin, produced in vitro by the vast majority of clinical *Burkholderia* strains, is also produced in the lungs of CF patients. Supporting this, studies by Sokol et al. have shown that a strain deficient in ornibactin production is less virulent than its ornibactin-producing parent in a mouse model (45). Thus, it appears likely that, like *P. aeruginosa* siderophores, ornibactin is synthesized by *Burkholderia* in both the soil and the lungs of CF patients. Further studies are required to address the significance of ornibactin production in *Burkholderia-Pseudomonas* interactions in both the CF lung and soil environments.

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