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Quinolobactin, a New Siderophore of *Pseudomonas fluorescens* ATCC 17400, the Production of Which Is Repressed by the Cognate Pyoverdine

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Transposon mutant strain 3G6 of *Pseudomonas fluorescens* ATCC 17400 which was deficient in pyoverdine production, was found to produce another iron-chelating molecule; this molecule was identified as 8-hydroxy-4-methoxy-quinaldic acid (designated quinolobactin). The pyoverdine-deficient mutant produced a supplementary 75-kDa iron-repressed outer membrane protein (IROMP) in addition to the 85-kDa IROMP present in the wild type. The mutant was also characterized by substantially increased uptake of ^{59}Fe-quinolobactin. The 75-kDa IROMP was produced by the wild type after induction by quinolobactin-containing culture supernatants obtained from the pyoverdine-negative mutant or by purified quinolobactin. Conversely, adding purified wild-type pyoverdine to the growth medium resulted in suppression of the 75-kDa IROMP in the pyoverdine-deficient mutant; however, suppression was not observed when *Pseudomonas aeruginosa* PAO1 pyoverdine, a siderophore utilized by strain 3G6, was added to the culture. Therefore, we assume that the quinolobactin receptor is the 75-kDa IROMP and that the quinolobactin-mediated iron uptake system is repressed by the cognate pyoverdine.

Several species of rRNA group I pseudomonads (the genus *Pseudomonas* sensu stricto) are characterized, under iron-limiting conditions, by the production of fluorescent, yellow-green, specific iron(III) chelators (siderophores) that are called pyoverdines or pseudobactins (1, 2, 12). Each of these siderophores, which are needed for high-affinity transport of iron(III) to the cell (16), is composed of a dihydroxyquinoline chromophore and a variable peptide chain comprising 6 to 12 amino acids, depending on the producing strain (2). In addition to these high-affinity iron chelators, fluorescent pseudomonads are known to produce other lower-affinity siderophores, such as pyochelin, a derivative of salicylic acid (7), and salicylic acid itself (18, 25). Fluorescent pseudomonads are also characterized by their capacity to take up a variety of structurally unrelated siderophores, including pyoverdines (pseudobactins) produced by other strains (3, 14). When these heterologous siderophores are added to a culture, they induce the production of corresponding siderophore receptors in the outer membrane via a signal cascade relayed by the receptor itself (10, 14). *Pseudomonas fluorescens* ATCC 17400 has been studied in our laboratory as a bacterium that is able to utilize different siderophores, including ferrichrome, deferrioxamine, pseudobactin BN7, and B10 (unpublished results). This bacterium also exhibits iron-repressed in vitro antagonism against the phytopathogen *Pythium debaryanum* (6). It has been demonstrated that iron-deprived *P. fluorescens* ATCC 17400 cells produce not only a specific pyoverdine whose structure is known (8) but also 8-hydroxy-4-methoxy-monothioquinaldic acid, which is readily hydrolyzed in the culture medium to 8-hydroxy-4-methoxy-quinaldic acid (20). A pyoverdine-negative *P. fluorescens* Tn*5* mutant of ATCC 17400 has been isolated in our laboratory (6). In this study, we demonstrated that this mutant produces 8-hydroxy-4-methoxy-quinaldic acid (renamed quinolobactin), which acts as a siderophore and induces a new iron-repressed outer membrane protein, and that the ferrisiderophore uptake system is preferentially induced in the absence of *P. fluorescens* wild-type pyoverdine.

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MATERIALS AND METHODS

***Pseudomonas* strains and culture media.** *P. fluorescens* ATCC 17400 is a wild-type strain and produces pyoverdine in Casamino Acids medium (CAA). Pyoverdine-deficient (nonfluorescent) mutant 3G6 was obtained after Tn*5* mutagenesis as previously described (6). Cultures were grown at 28°C in CAA, which contained (per liter) 5 g of Bacto Casamino Acids (Difco Laboratories), 1.18 g of $K_2HPO_4 \cdot 3H_2O$, and 0.25 g of $MgSO_4 \cdot 7H_2O$. For growth under iron-sufficient conditions, $FeCl_3$ was added at a final concentration of 100 μM. For purification of quinolobactin or pyoverdine, cells were grown in minimal succinate medium (16). Both of the media used contained less than 2 μM iron (11). Unless otherwise indicated, 500-ml cultures were inoculated from an overnight preculture and incubated at 28°C at 200 rpm (New Brunswick Innova shaker). Organisms were grown in the presence of 1 mg of ethylenediaminedihydroxyphenylacetic acid (EDDHA) per ml with and without pyoverdine (150 μM) by using a Bio-Screen apparatus (Lab Systems, Helsinki, Finland). This apparatus allowed 200 liquid microcultures to be incubated and shaken while the growth in each well was measured by determining the absorbance at 600 nm at predetermined time intervals. The following parameters were used: shaking for 30 s every 3 min, absorbance determined every 10 min, and a temperature of 28°C.

Siderophore detection by IEF and CAS. Siderophores were detected by isoelectric focusing (IEF) of CAA culture supernatants, followed by detection of iron-chelating molecules with an overlay consisting of chrome azurol S reagent (CAS) (13, 19, 23).

Siderophore purification. *P. fluorescens* ATCC 17400 pyoverdine was purified as previously described (17).

Quinolobactin was purified from strain 3G6 succinate culture supernatants by

the following procedure. The iron-free lyophilized supernatant was dissolved in water, and the solution (pH 8) was extracted three times with ethyl acetate. The aqueous phase containing quinolobactin was then acidified to pH 3.5 with hydrochloric acid and extracted again, and the ethyl acetate solution was dried with $MgSO_4$. After evaporation, the white-yellow residue was dissolved in slightly alkaline water. Quinolobactin was purified further by absorption on a Sep-Pack/RP 18 cartridge, which was extensively washed with water in order to remove contaminating compounds (mainly the remaining succinic acid from the growth medium). Quinolobactin was then eluted from the cartridge with methanol-water (1:1, vol/vol). Samples were analyzed by electronionization-mass spectrometry (70 eV; direct inlet; Incos 50; Finnigan-MAT, Bremen, Germany), and ^{1}H and ^{13}C nuclear magnetic resonance spectra were determined with a model AMX 500 instrument (Bruker, Karlsruhe, Germany). Pyochelin was purified by the method of Sokol (24).

^{59}Fe-labelled siderophore uptake. Ferric complexes were prepared and uptake experiments with ^{59}Fe-quinolobactin, ^{59}Fe-pyoverdine, or ^{59}Fe-pyochelin were performed as previously described (4).

Outer membrane preparation. Outer membranes were prepared by the Sarkosyl solubilization method (9) as previously described (5). The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide gels) and were stained with Coomassie blue by using standard procedures (22). The following molecular weight markers were used: phosphorylase *b* (molecular weight, 93,000) bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (30,000), trypsin inhibitor (21,000), and lactalbumin (14,000) (Pharmacia, Uppsala, Sweden).

RESULTS

Production of a nonfluorescent iron chelator by pyoverdine-negative mutant 3G6. After Tn*5* mutagenesis, mutant 3G6 was isolated as a nonfluorescent, pyoverdine-negative organism that was not able to grow in CAA containing 1 mg of EDDHA per ml (6). This mutant was found to have two Tn*5* insertions, as determined by Southern hybridization (6). The kanamycin resistance gene and flanking DNA were cloned by using *Sal*I digestion and ligation into vector pUC19. Two different types of clones were obtained, as determined by a restriction pattern analysis. The flanking sequences were analyzed, and the results revealed that one transposon was inserted into a sequence that was similar to the sequence of a *Neisseria meningitidis* lipooligosaccharide biosynthesis gene (accession no. L09189), while the second transposon was inserted into a gene whose product was similar to bacterial peptide synthases (unpublished results), including the product of the *pvdD* gene of *Pseudomonas aeruginosa* known to be involved in the biosynthesis of pyoverdine (15). Pyoverdine production by mutant 3G6 and growth in the presence of EDDHA could be restored by a cosmid containing the complete peptide synthase gene; this gene was also found to hybridize with the flanking region of the second transposon insertion (unpublished results). When a supernatant of a 24- or 32-h CAA culture of strain 3G6 was analyzed by IEF to determine whether siderophores were present, a nonfluorescent, CAS-positive spot was detected (results not shown). This spot was not detected in the wild-type preparation unless the spent medium was concentrated before it was applied to the IEF gel (results not shown).

Purification and identification of quinolobactin from strain 3G6 spent medium. Mutant 3G6 was grown in succinate medium for 48 h, and the lyophilized culture supernatant was extracted with ethyl acetate, from which quinolobactin was purified as described above. The molecular mass of this compound, as determined by electron ionization-mass spectrometry, was 219 Da, and its fragmentation pattern corresponded to that of 8-hydroxy-4-methoxy-quinaldic acid as described by Neuenhaus et al. (20). Since this compound is a quinolone, we decided to name it quinolobactin. Its structure was confirmed by its nuclear magnetic resonance spectra (CD_3OD), as follows: ^{1}H (δ ppm, multiplicity): 4.16, s, 3H: OCH_3: 7.67, s, 1H: H-3; 7.63, dd, 1H: H-5; 7.45, t, 1H: H-6; 7.12, dd, 1H: C-7; ^{13}C (ppm): 57.1: OCH_3; 152.2: C-2; 101.1: C-3; 166.2: C-4; 113.1: C-5; 129.6: C-6; 113.6: C-7; 154.3: C-8; 137.3: C-9; 123.7: C-10; 169.2: CO. The structure of quinolobactin is shown in Fig. 1. Purified quinolobactin decolorizes CAS (23), indicating that it could form a complex with Fe(III). While the free compound in aqueous solution (pH 9.5) is yellow, addition of Fe(III) results in the appearance of a dark green color, indicating that an iron complex is formed, probably at a 3:1 ratio as with other quinoline derivatives (21). When free quinolobactin was examined, two absorbance peaks were observed, at 216 and 249 nm; when the iron complex was examined, peaks were observed at 212 and 250 nm. The extinction coefficients (ε) were 1.1×10^4 and 0.94×10^4 for the free ligand at 216 and 249 nm, respectively, and 6.3×10^4 and 4.78×10^4 for the ferric complex at 212 and 250 nm, respectively. It is not yet clear how quinolobactin forms complexes with iron(III) since both carboxyl and hydroxyl groups are present in the molecule (Fig. 1). We found that after 40 h of growth at 28°C in CAA, the wild type produced 3 to 5 mg of quinolobactin per liter, while the mutant produced 12 to 29 mg of quinolobactin per liter (measured after extraction and purification by weighing the purified compound).

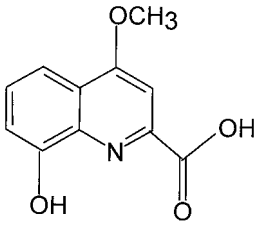

FIG. 1. Structure of 8-hydroxy-4-methoxy-quinaldic acid.

Growth stimulation of pyoverdine-negative mutant 3G6 by quinolobactin and pyoverdines. Figure 2 shows that quinolobactine stimulated the growth of pyoverdine-negative mutant 3G6 when it was added to CAA. The same siderophore, however, did not restore the growth of this mutant in the presence of EDDHA (Fig. 2). Both the homologous pyoverdine and a heterologous pyoverdine (from *P. aeruginosa* PAO1) restored the growth of mutant 3G6 in the presence of EDDHA. These results indicate that quinolobactin probably has a lower affinity for iron(III) than EDDHA has, while pyoverdines, with their high affinity for Fe(III), strip EDDHA from iron. We found that quinolobactin did not stimulate the growth of the wild type in CAA (results not shown).

Induction of a 75-kDa IROMP in mutant 3G6. When outer membranes from the wild type and strain 3G6 grown for 40 h in CAA were analyzed, it was evident that strain 3G6 outer membranes contained a major 75-kDa iron-repressed outer membrane protein (IROMP) in addition to the 85-kDa IROMP present in the wild type (Fig. 3). Adding filter-sterilized culture supernatant from strain 3G6 or pure quinolobactin to the wild type just after inoculation resulted in the appearance of the 75-kDa IROMP (Fig. 3, lane 7). Both the 85-kDa protein and the 75-kDa protein were IROMPs, since they disappeared when iron was added (Fig. 3, lanes 3 and 5). Although a fuzzy band around 75 kDa was still visible in outer membrane preparations obtained from the wild type and the mutant grown in the presence of iron, this band always migrated slightly above the 75-kDa IROMP band in SDS-PAGE gels. Therefore, we did not believe that this band could result from incomplete repression by iron of the production of the quinolobactin receptor. This assumption was confirmed by the absence of ^{59}Fe-quinolobactin uptake by cells grown in the presence of iron (see below).

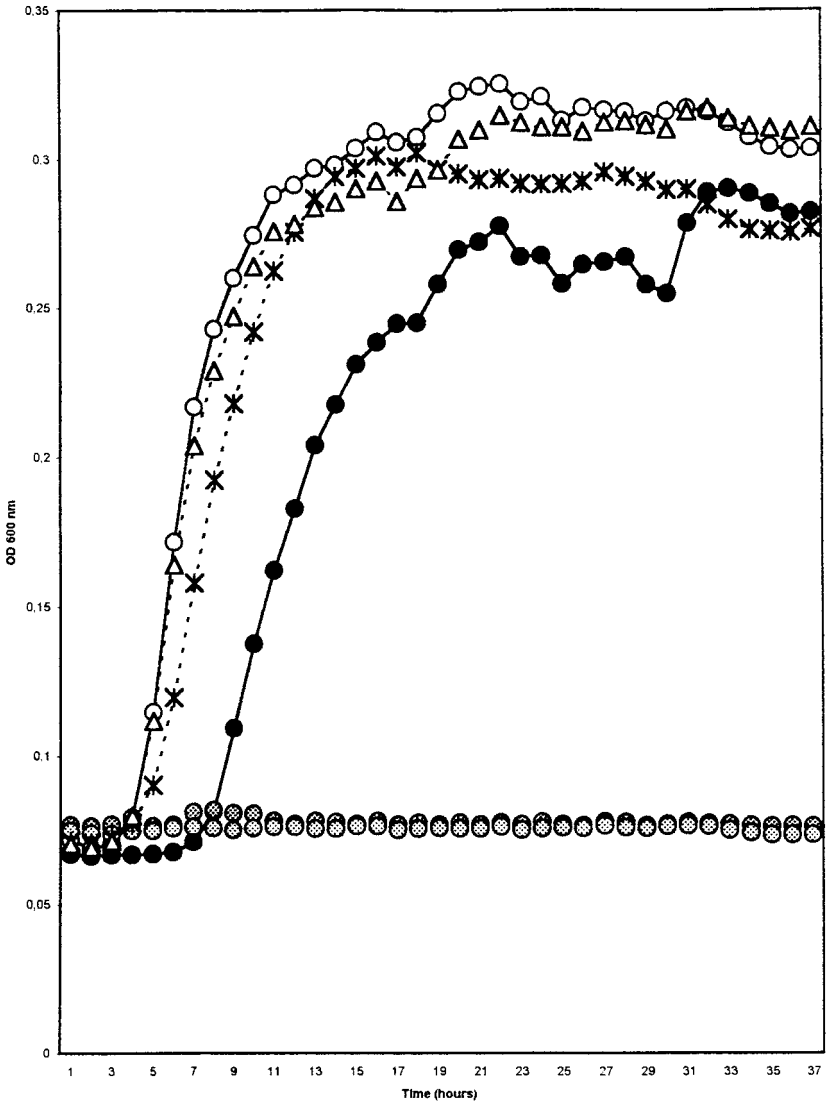

FIG. 2. Growth curves for pyoverdine-negative mutant 3G6 in CAA (●), CAA containing quinolobactin (50 μM) (○), CAA containing EDDHA (1 mg/ml) (◍), CAA containing EDDHA and quinolobactin (◍), CAA containing EDDHA and homologous pyoverdine (150 μM) (△), and CAA containing EDDHA and *P. aeruginosa* PAO1 pyoverdine (*). Growth was monitored with a Bio-Screen apparatus as described in the text. OD 600 nm, optical density at 600 nm.

Effects of pyoverdines from *P. fluorescens* ATCC 17400 and *P. aeruginosa* PAO1 on the production of the 75-kDa IROMP. As shown above, purified pyoverdine from wild-type *P. fluorescens* ATCC 17400 or from *P. aeruginosa* PAO1 could restore the growth of mutant 3G6 in CAA containing 1 mg of EDDHA per liter (Fig. 2). When purified *P. fluorescens* ATCC 17400 pyoverdine was added to a strain 3G6 culture, the 75-kDa IROMP disappeared (Fig. 4A). Conversely, addition of pure PAO1 pyoverdine had no effect on the outer membrane protein profile of mutant 3G6 (Fig. 4A, lane 3) except for induction of a new IROMP at 82 kDa, which could be separated from the 85-kDa IROMP only after prolonged migration (Fig. 4B).

Quinolobactin and pyoverdine-mediated iron uptake. The kinetics of iron incorporation into iron-starved cells of *P. fluorescens* ATCC 17400 and mutant 3G6 (grown in succinate minimal medium) showed that both the cognate ferripyoverdine and ferriquinolobactin were actively taken up, whereas pyochelin was not taken up (Fig. 5). For wild-type cells, pyoverdine was the most efficient siderophore since ferriquinolobactin mediated the uptake of iron at a lower level, which did not change during 15 min of incubation. On the other hand, strain 3G6 cells exhibited increased ^{59}Fe-quinolobactin uptake efficiency compared to wild-type cells. Although the level of uptake of PAO1 ferripyoverdine was low with wild-type *P. fluorescens* cells, it was reproducible, and the level of uptake was greater in 3G6 cells (Fig. 5). No incorporation of ferrisiderophores was observed when cells were grown in iron-supplemented medium, even in the presence of ^{59}Fe-quinolobactin, which demonstrated that the ca. 75-kDa outer membrane protein observed in iron-sufficient cells was not the quinolobactin receptor.

DISCUSSION

Pyoverdines and pseudobactins, which can be detected easily by their yellow-green color and by their fluorescence under UV light, are not the only siderophores produced by fluorescent

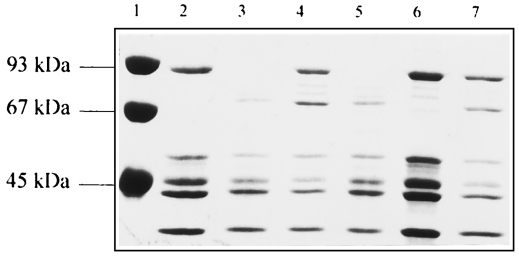

FIG. 3. SDS-PAGE of outer membrane proteins from wild-type cells grown in CAA (lanes 2 and 6), wild-type cells grown in CAA containing iron (50 μM) (lane 3), mutant 3G6 cells grown in CAA (lane 4), mutant 3G6 cells grown in CAA containing iron (lane 5), and wild-type cells grown in CAA containing purified quinolobactin (50 μM) (lane 7). Lane 1 contained the molecular weight standards (Pharmacia-LKB).

pseudomonads. Other siderophores produced by these bacteria include pyochelin of *P. aeruginosa* (7) and salicylic acid of *P. fluorescens* CHA0 and *P. aeruginosa* (18, 25). These compounds can be considered secondary siderophores since they are usually produced in much lower amounts than pyoverdines (a few milligrams per liter versus 100 to 200 mg liter^{-1}) and are less efficient in iron binding and uptake. However, they can be considered compounds that provide rescue iron uptake systems, and it has been shown that their production (in the case of salicylic acid) and their role in iron uptake (in the case of pyochelin) are greater in pyoverdine-deficient mutants than in wild-type cells (10, 17, 18). Production of 8-hydroxy-4-methoxyquinoline-2-carboxylic acid (quinolobactin) by *P. fluorescens* has been described previously (1, 20), but the role of this molecule as a siderophore was not described. We demonstrate here that quinolobactin is a new siderophore, based on the following evidence: it is produced under iron-limiting conditions, as previously demonstrated by other workers (20); it is actively taken up by *P. fluorescens* ATCC 17400 and is more efficiently taken up by a pyoverdine-negative mutant; and it induces the production of a new 75-kDa IROMP. Quinolobactin can be considered a low-affinity siderophore since the pyoverdine-negative mutant 3G6 which produces quinolobactin is not able to grow in the presence of the strong iron chelator EDDHA unless the homologous pyoverdine or a heterologous pyoverdine (from *P. aeruginosa* PAO1) is added. Quinolobactin is a quinaldic acid derivative, and its biosynthesis probably involves the shikimic acid track, anthranilic acid branch.

Figure 5 shows that mutant 3G6 takes up quinolobactin more efficiently than the wild type takes up quinolobactin. This effect was observed repeatedly and can be explained by the presence of larger amounts of the receptor in the mutant, which in turn is induced by the siderophore. Indeed, we demonstrated that the pyoverdine-negative mutant produces about four to six times more quinolobactin than the wild type produces. This result is consistent with previous observations made with pyoverdine-negative mutants of *P. aeruginosa*, which showed that there was increased production of pyochelin and its receptor (11). The induction of cognate receptors was demonstrated previously when fluorescent pseudomonads were grown in the presence of an endogenous or exogenous siderophore (3, 14). Interestingly, the 75-kDa IROMP was not detected after the homologous *P. fluorescens* pyoverdine was added to a culture of strain 3G6. When the experiment was repeated with purified *P. aeruginosa* PAO1 pyoverdine, the amount of the 75-kDa outer membrane protein remained unchanged, although PAO1 pyoverdine clearly restored the growth of 3G6 in the presence of EDDHA and induced the production of a new ca. 82-kDa IROMP. In this case the mutant also took up more PAO1 ferripyoverdine than the wild type took up, which is consistent with induction of the 82-kDa receptor. The uptake of the cognate pyoverdine by the pyoverdine-negative mutant was unchanged compared to the wild type (Fig. 5), as was the presence of the 85-kDa pyoverdine receptor (Fig. 2). This could mean that the cognate pyoverdine system is not loop regulated by the siderophore. This is not unusual since it was also found to be the case for *Pseudomonas putida* WCS 358, in which the cognate siderophore receptor PupA is not loop regulated (14). Cells grown in the presence of PAO1 pyoverdine are probably less iron deficient than cells grown without an externally added siderophore. This suggests that the disappearance of the 75-kDa IROMP resulting from the addition of the homologous pyoverdine from *P. fluorescens* ATCC 17400 is not the result of repression due to the iron present in the cell. To our knowledge, repression of a siderophore receptor by another siderophore, which seems to occur with the putative 75-kDa quinolobactin receptor in the presence of the homologous pyoverdine, has not been described previously. At this stage, we do not know whether quinolobactin is a siderophore used by other fluorescent

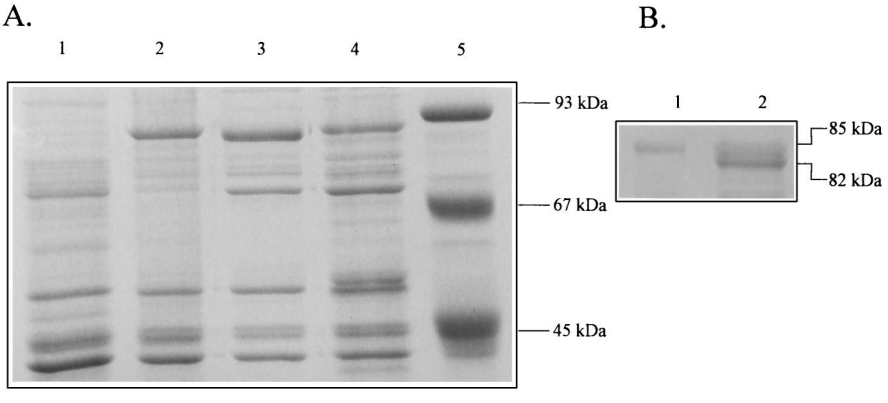

FIG. 4. (A) SDS-PAGE of the pyoverdine-negative mutant 3G6 outer membrane proteins after growth in CAA containing iron (lane 1), CAA containing purified *P. fluorescens* pyoverdine (150 μM) (lane 2), CAA containing purified *P. aeruginosa* PAO1 pyoverdine (150 μM) (lane 3), and CAA (lane 4). Lane 5 contained the molecular weight standards (Pharmacia-LKB). (B) Close-up of IROMPs from mutant 3G6 grown in CAA (lane 1) and in CAA containing purified *P. aeruginosa* PAO1 pyoverdine (150 μM) (lane 2) after 10 h of migration on a 10% acrylamide gel.

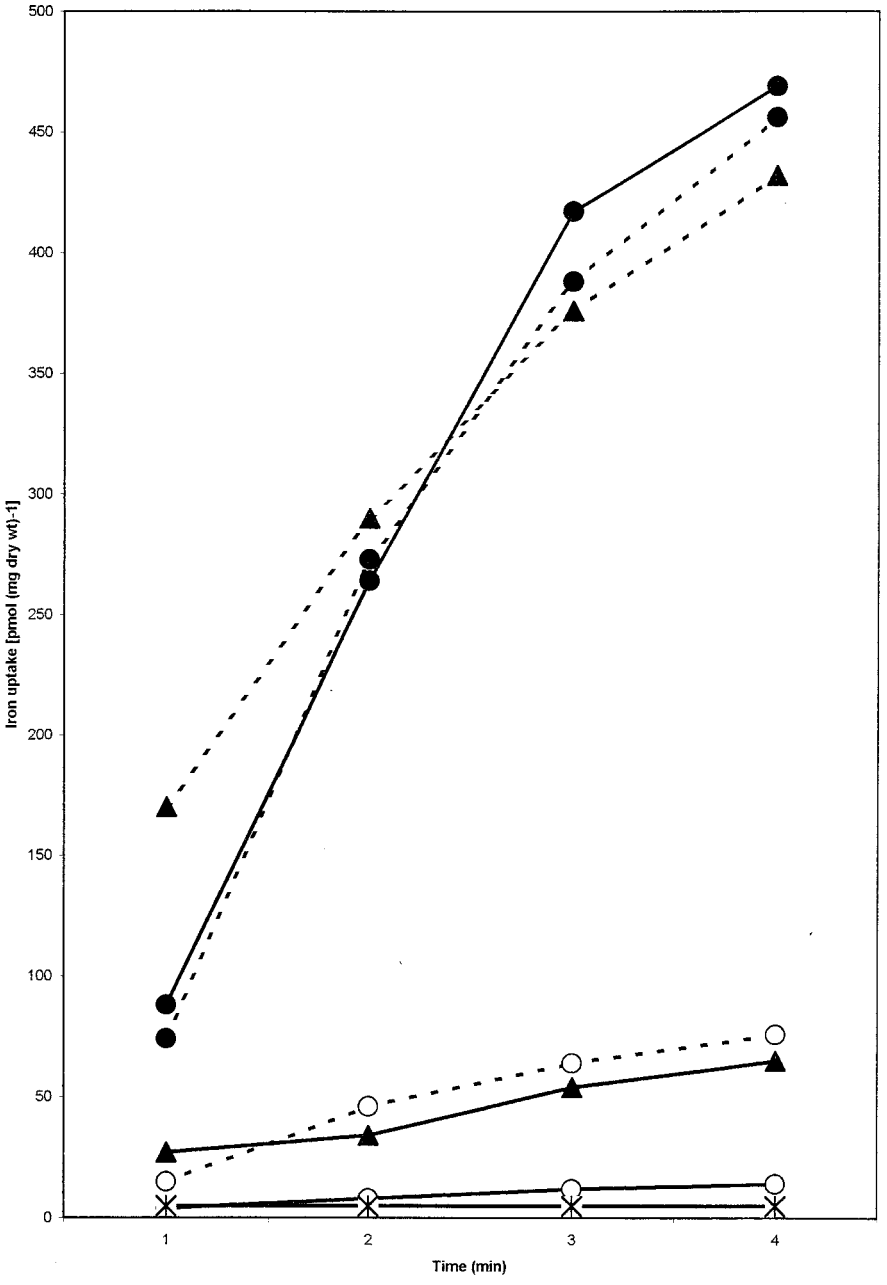

FIG. 5. Uptake of *P. fluorescens* ATCC 17400 ^{59}Fe-pyoverdine (●), *P. aeruginosa* PAO1 ^{59}Fe-pyoverdine (○), and ^{59}Fe-quinolobactin (▲) by wild-type *P. fluorescens* ATCC 17400 (solid lines) and by mutant 3G6 (dashed lines). The incorporation of ^{59}Fe-quinolobactin and ^{59}Fe-labelled *P. fluorescens* pyoverdine obtained with wild-type and mutant 3G6 cells grown in the presence of iron or with iron-depleted cells with pyochelin is also shown (∗).

pseudomonads; this question will be answered only after the genes for the biosynthesis and uptake of quinolobactin are cloned and can be used as a probe. In conclusion, we found that quinolobactin is a new siderophore produced by a fluorescent pseudomonad and that the diversity of siderophores produced by fluorescent pseudomonads may be greater than previously thought.

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