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Identification of *rhtX* and *fptX*, Novel Genes Encoding Proteins That Show Homology and Function in the Utilization of the Siderophores Rhizobactin 1021 by *Sinorhizobium meliloti* and Pyochelin by *Pseudomonas aeruginosa*, Respectively

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Rhizobactin 1021 is a hydroxymate siderophore produced by the soil bacterium *Sinorhizobium meliloti* 2011. A regulon comprising *rhtA*, encoding the outer membrane receptor protein for the ferrisiderophore; the biosynthesis operon *rhbABCDEF*; and *rhrA*, the Ara-C-like regulator of the receptor and biosynthesis genes has been previously described. We report the discovery of a gene, located upstream of *rhbA* and named *rhtX* (for "rhizobactin transport"), which is required, in addition to *rhtA*, to confer the ability to utilize rhizobactin 1021 on a strain of *S. meliloti* that does not naturally utilize the siderophore. Rhizobactin 1021 is structurally similar to aerobactin, which is transported in *Escherichia coli* via the IutA outer membrane receptor and the FhuCDB inner membrane transport system. *E. coli* expressing *iutA* and *fhuCDB* was found to also transport rhizobactin 1021. We demonstrated that RhtX alone could substitute for FhuCDB to transport rhizobactin 1021 in *E. coli*. RhtX shows similarity to a number of uncharacterized proteins which are encoded proximal to genes that are either known to be or predicted to be involved in iron acquisition. Among these is PA4218 of *Pseudomonas aeruginosa*, which is located close to the gene cluster that functions in pyochelin biosynthesis and outer membrane transport. PA4218 was mutated by allelic replacement, and the mutant was found to have a pyochelin utilization-defective phenotype. It is proposed that PA4218 be named *fptX* (for "ferripyochelin transport"). RhtX and FptX appear to be members of a novel family of permeases that function as single-subunit transporters of siderophores.

Although iron is abundant, it is not readily available to microorganisms growing aerobically because it is chemically bound in complexes that have a low solubility at neutral pH. Iron is essential for growth, and bacteria have evolved a variety of strategies to overcome its limited availability (40). A common strategy is the production of ferric iron binding compounds, termed siderophores. There is considerable structural variation among siderophores (9), but they are all characterized by a high binding constant for ferric iron and the capacity to acquire iron from the local environment and facilitate its uptake by the bacterial cell. Siderophore receptor proteins that function in the transport of the ferrisiderophore complex into the cell are located in the outer membranes of gram-negative bacteria grown under conditions of iron depletion. The receptors display relatively tight specificity for their cognate siderophores. Translocation of the ferrisiderophore through the receptor and into the periplasm is dependent on the energy-transducing Ton system, comprising two cytoplasmic membrane proteins, ExbB and ExbC, in addition to the TonB protein that transverses the periplasm (27). The receptor proteins commonly possess a conserved sequence, the TonB box, and evidence suggests that the interaction between the siderophore and the receptor leads to a conformational change in the receptor and subsequent association between the TonB protein and the receptor, possibly at the TonB box. In this way TonB provides the energy necessary to transport the siderophore to the periplasm.

Ferrisiderophore transport across the periplasm and cytoplasmic membrane exhibits less specificity than that at the outer membrane. The ferric hydroxamate uptake (Fhu) system of *Escherichia coli* (23) facilitates the transport of different hydroxamate siderophores, including ferrichrome, coprogen, and aerobactin, each of which requires its cognate receptor at the outer membrane. The Fhu system is representative of an ABC transporter, composed of the cytoplasmic membrane integral protein FhuB, which resembles a heterodimer and has each half associated with the ATPase FhuC (16). In addition, the periplasmic protein FhuD binds the ferrisiderophore (24) and chaperones it across the periplasm to the cytoplasmic membrane FhuBC complex.

Infectious pathogens encounter strict iron limitation within an animal host due to the action of iron-withholding proteins such as transferrin and lactoferrin. The production of siderophores has been shown to correlate with bacterial pathogenicity in numerous studies. *Pseudomonas aeruginosa*, an important human opportunistic pathogen, produces two siderophores, pyoverdine and pyochelin (37). Both have been shown to have the capacity to remove iron from transferrin and lactoferrin and to contribute to the virulence of *P. aeruginosa* in animal infection models (47). Plant pathogens do not have to compete with iron binding proteins such as transferrin or lactoferrin. Nevertheless, they must compete with the plant host systems for iron transport and storage, which ensure that iron is accessible as a nutrient to the host while not being

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available to catalyze the formation of reactive free radicals (12). A role for the siderophore chrysobactin in the virulence of plant pathogenic *Erwinia* species has been established (11). In contrast, agrobactin produced by *Agrobacterium tumefaciens* is not necessary for crown gall formation (26). The interaction between root nodule bacteria, which are collectively called the rhizobia, and their leguminous plant hosts results in the formation of a nitrogen-fixing symbiotic interaction that is known to be demanding with regard to iron. However, rhizobactin 1021, produced by *Sinorhizobium meliloti*, and vicibactin, produced by *Rhizobium leguminosarum*, have been shown to be unnecessary for the symbiotic interactions with their respective plant hosts (6, 30). Rhizobia are found free living in soil, and it is likely that the siderophores that they produce function to provide them with an adequate supply of iron in the competitive soil environment.

The siderophore produced by *S. meliloti* 2011, termed rhizobactin 1021, is a citrate-based dihydroxamate that has a core structure identical to that of schizokinen from *Bacillus megaterium* (35). However, unlike shizokinen, rhizobactin 1021 has an unusual lipid moiety appended. The core structures of rhizobactin 1021 and schizokinen are not identical to, but do resemble, the core of aerobactin produced by some members of the *Enterobacteriaciae* and shown to be a virulence factor in *E. coli*. Genetic characterization of rhizobactin 1021 production and utilization by *S. meliloti* 2011 revealed an operon comprising the biosynthesis genes *rhbABCDEF* located upstream from *rhtA*, the outer membrane receptor gene for the siderophore (30). An Ara-C-like regulator encoded between the biosynthesis operon and the receptor gene was shown to positively regulate the production and transport of the siderophore. While some siderophore loci have clearly identifiable inner membrane transport systems encoded close to the biosynthesis and outer membrane receptor genes, there is no obvious transporter in the rhizobactin 1021 region of the sequenced genome of *S. meliloti*.

In *S. meliloti* over 400 transporter genes are predicted in the sequenced genome. Of the products of these genes, only the SitABCD metal-type permease, encoded close to the *fur* gene, has been investigated with regard to iron transport (36). It was determined that the SitABCD transporter is not involved in siderophore transport but does function in manganese acquisition. For *P. aeruginosa* there is remarkably little known about inner membrane transport of siderophores. We report the identification of novel genes that function in rhizobactin 1021 and pyochelin utilization. The products of these genes are members of a new family of proteins that appear to function at the inner membrane to facilitate the uptake of ferri-siderophore complexes in a variety of species, including *S. meliloti* and *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used are listed in Table 1. *S. meliloti* was cultured on TY medium (3). *E. coli* and *P. aeruginosa* were cultured on Luria-Bertani medium (43). SOC medium (20) was used in the transformation of *E. coli*. CAS (chrome azurol S) medium for the detection of siderophore production was prepared by the method of Schwyn and Neilands (44) with the modifications described by Reigh and O'Connell (41). Antibiotics were used at the following concentrations: for *S. meliloti*, kanamycin at 100 μg/ml, gentamicin at 30 μg/ml, tetracycline at 10 μg/ml, and streptomycin at 1 mg/ml; for *P. aeruginosa*, kanamycin at 500 μg/ml, gentamicin at 50 μg/ml, tetracycline at 50 μg/ml, and ampicillin at 100 μg/ml; and for *E. coli*, kanamycin at 30 μg/ml, gentamicin at 20 μg/ml, tetracycline at 20 μg/ml, and ampicillin at 100 μg/ml.

Bacterial conjugation and transformation. Bacterial conjugation was carried out as described by O'Connell et al. (33). Transformation was by the method of Inoue et al. (20). *E. coli* XL-1 Blue was used as a host for routine transformation and plasmid preparation.

DNA manipulations. PCRs were undertaken with the temperature gradient block in a Thermo Hybaid PCR Express thermal cycler. DNA restriction and ligation reactions were carried out by standard procedures (43). Plasmid DNA was isolated by the alkaline lysis method (4). Cosmid DNA was prepared by the method of Little (29) but with the modification that the DNA was phenol extracted and was precipitated with 2.5 volumes of ethanol on ice for 30 min. Restriction fragments were cut from ethidium bromide-stained agarose gels as required for subcloning and were purified by using the Perfectprep gel cleanup kit as directed by the manufacturer (Eppendorf). Total genomic DNA was prepared from *S. meliloti* broth cultures by the method of Meade et al. (31) and from *P. aeruginosa* by the method of Chen and Kuo (7). Restricted genomic DNA was separated by agarose gel electrophoresis and transferred to nitrocellulose filters by standard methods (43). Probes for Southern hybridization were labeled and hybridized by using the digoxigenin DNA labeling and detection kit as directed by the manufacturer (Roche).

Strain and plasmid constructions. The *rhtX43* mutation in *S. meliloti* 2011*rhtX43* was constructed by selecting for insertion of the transposon Tn*5lac* in *rhtX* (Sma2337 in the genome sequence) by using the fragment-targeted mutagenesis method described by Lynch et al. (30). *E. coli* A118 was the source of Tn*5lac*.

To construct the mutant *S. meliloti* 2011*rhtX1*, carrying an Ω kanamycin resistance cassette inserted in the *rhtX* gene, a 2-kb region, having the BamHI site within *rhtX* centrally located, was amplified by PCR (forward primer [P_f], 5′-AGATCTGTTGCCGAAGCCCTGCGGGTTC-3′; reverse primer [P_r], 5′-AGATCTGGAAGGCGGAGGCGGAGATGGC-3′). The fragment was ligated to the pCR2.1 vector by TA cloning and then subcloned into the BamHI site of pJQ200ks as a BglII fragment (BglII sites having been incorporated into the primers). This destroyed the BamHI recognition sequence in the vector and left the single BamHI site in the *rhtX* gene available for insertion of the Ω kanamycin resistance cassette, which was cut as a BamHI fragment from plasmid pHP45Ω-Km. The pJQ200ks derivative with *rhtX*::Ω-Km was mobilized into *S. meliloti* 2011 by a triparental mating with pRK600. Integration of the plasmid by a single crossover was selected on gentamicin and streptomycin. After purification, a second crossover and allelic replacement were selected on medium containing 5% sucrose with kanamycin. Gentamicin sensitivity was confirmed, and Southern hybridization was used to confirm the loss of the vector and the correct insertion of the cassette.

S. meliloti 2011 mutant Sma2335*km* was constructed as follows. A region encoding the Sma2335 gene was amplified on a 2-kb fragment from genomic DNA (P_f, 5′-GCGGCCGCCGATGGTCTGCTTCACCCCCTCG-3′; P_r, 5′-CCCGGGCATCCAGGCATTCGGCCGCCGG-3′). The primers introduced XmaI and NotI sites into the fragment, which was ligated to the pCR2.1 vector by TA cloning. The fragment was then subcloned into pJQ200ks that had been cut with XmaI and PspOM1, an enzyme that cuts pJQ200ks such that it can be ligated to the NotI-restricted fragment carrying Sma2335. This ligation destroyed the ApaI site from pJQ200ks, leaving a unique ApaI site in the ligated plasmid within the Sma2335 gene. The kanamycin resistance gene was amplified from plasmid pUC4K with ApaI sites introduced on the PCR primers (P_f, 5′-GGGCCCGACGTTGTAAAACGACGGCCAGTG-3′; P_r, 5′-GGGCCCGGAAACAGCTATGACCATGATTACG-3′). This fragment was initially ligated to the pCR2.1 plasmid by TA cloning and then subcloned into the ApaI site of the Sma2335 gene in pJQ200ks. Allelic replacement and confirmation of the insertion by Southern hybridization were carried out as described above.

To obtain the *fptX* (PA4218) gene of *P. aeruginosa* for mutagenesis, a 3.6-kb XhoI/XmaI fragment containing the coding region was subcloned from the cosmid pMO 012405 and inserted in pJQ200ks. Insertion in the multiple cloning site of the vector with these enzymes removed the SalI site from the vector and allowed the kanamycin resistance gene from pUC4K to be inserted into the SalI site within *fptX*. Allelic exchange to introduce the mutated gene into the *P. aeruginosa* genome, constructing strain PA4218-km1, was carried out. However, sucrose sensitivity as a result of the *sacB* gene on pJQ200ks was not as effective as in *S. meliloti*, and it was necessary to screen a large number of colonies by replica plating on medium containing kanamycin only and medium containing kanamycin and gentamicin to detect clones having the gentamicin-sensitive, kanamycin-resistant phenotype resulting from recombination events that excised

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
Strains		
E. coli		
XL-1Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F′ *proAB lacI*^{q} *Z*Δ*M15*Tn*10*(Tet^r)]	43
DH5α	*endA1 gyrA96 thi-1 hsdR17 supE44 relA1 recA1* Δ*lacU169*(ϕ80Δ*lacZ*Δ*M15*)	43
A118	Chromosomally located Tn*5lac*	45
H1443	K-12 strain, *aroB*	17
RK4375	*fhuC*::Tn*10 fepA9 aroE24* (previously *fhuB478*::Tn*10*)[b]	*E. coli* Genetic Stock Center
S. meliloti		
2011	Wild type[c]; Sm^r Nod$^+$ Fix$^+$	
2011*rhtX*1	2011 with Ω-Km cassette in *rhtX* (*rhtX1*)	This study
2011*rhtX*43	2011 with Tn*5lac* insertion in *rhtX* (*rhtX43*)	This study
2011Sma2335*km*	2011 with Km^r cassette in Sma2335	This study
2011*rhtA45*	Tn*5lac* insertion in *rhtA*	30
2011*rhrA26*	Tn*5lac* insertion in *rhrA*	30
102F34	Does not produce or utilize rhizobactin 1021; Nod$^+$ Fix$^+$	41
P. aeruginosa		
PA01	Wild type; produces pyoverdine and pyochelin	
CDC5	*pvd-2*; produces pyochelin, does not produce pyoverdine	2
DH143	*pvd-2 fptA*	19
PA4218-km1	CDC5 with a Km^r cassette in *fptX* (PA4218)	This study
B. megaterium ATCC 19213 (91-02)	Schizokinen producer	NCIMB[d] culture collection
Plasmids		
pJQ200ks	Gm^r *mob sacB*	39
pRK600	Tra Cm^r	15
pCR2.1	Ap^r	Invitrogen
pMO 012405	Cosmid carrying genomic region of PAO1 including *fptX* coding sequence	*Pseudomonas* Genetic Stock Center
pSUP104	Tc^r *mob*	38
pBBR1MCS-5	Gm^r *mob*	25
pPOC4	pBBR1MCS-5::*rhtX*	This study
pPOC5	pSUP104 with the region carrying *rhrA* and *rhtA*	This study
pEN7	*iutA*	18
pRG13	*iucABCD*	18
pHP45Ω-Km	Source of Ω-Km	13
pUC4K	Source of Km^r cassette	Amersham
pPC35	pCR2.1 *fhuCDB*	This study
pPC29	pCR2.1 *fhuCD*	This study
pPC20	pCR2.1 *fhuDB*	This study

[a] Ap^r, Cm^r, Gm^r, Km^r, Sm^r, and Tc^r, ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, and tetracycline resistance, respectively; Tra, transfer.
[b] The strain carries the mutation previously described as *fhuB478*::Tn*10* by Kadner et al. (22), which was determined to be *fhuC*::Tn*10* in this work. See text for details.
[c] *S. meliloti* 2011 is the parent strain of 1021 (31). 1021 was used to sequence the genome of the species and for the purification and chemical characterization of rhizobactin 1021 (35).
[d] NCIMB, National Collections of Industrial, Food and Marine Bacteria.

the vector from the chromosome. Correct insertion of the cassette in the chromosome was confirmed by Southern hybridization.

Plasmid pPOC4, carrying *rhtX* expressed from a *lac* promoter, was constructed by amplifying the gene with its ribosome binding site by using primers that introduced XhoI and PstI restriction sites (P_f, 5′-CTCGAGGCCGGGCAGTG GCAGTTTTCGATGC-3′; P_r, 5′-CTGCAGTCATCGTGATCTTGAAGGAC GCGCTTTC-3′) and inserting the gene in the pCR2.1 vector by TA cloning. The fragment was then subcloned into the mobilizable broad-host-range vector pBBR1MCS-5 that had been cut with XhoI and PstI, placing the gene in the correct orientation for expression from the *lac* promoter of the plasmid.

The plasmid pPOC5 carrying *rhrA* and *rhtA* was constructed by a two-step amplification as follows. First, the *rhtA* open reading frame was amplified (P_f, 5′-GAATTCCCTGTTGACGTTCGCATGC-3′; P_r, 5′-TCTAGATTAAAAAA CCTTTCTCAGCGA-3′) and cloned by TA cloning in the pCR2.1 vector. Second, a fragment containing the *rhrA* gene and a section of *rhtA* including the XhoI site was amplified (P_f, 5′-GAATTCTCAAGCGGCGGCTGCCAGCC-3′; P_r, 5′-CTCGAGCGCGGAATCGCCCACG-3′) and cloned in the pCR2.1 vector. The first fragment was subsequently cloned in the broad-host-range vector pSUP104. The *rhrA* open reading frame was completed in this construct by inserting the region extending from the XhoI site to the end of the *rhrA* open reading frame, obtained from the second amplification described above. This construct then contained the *rhrA* and *rhtA* genes complete with their promoter regions cloned in pSUP104.

The plasmids pPC35, pPC29, and pPC20 were constructed by amplifying *fhuCDB* (P_f, 5′-GAATTCGTGCCCATTTCACAAGTTGGCTGTTATGC-3′; P_r, 5′-GGATCCTTAACGGCTCTGCTTTCTCAACAAATAGATAA-3′), *fhuCD* (P_f, as for *fhuCDB*; P_r, 5′-GGATCCTCACGCTTTACCTCCGATGGCGTTAT CC-3′), and *fhuDB* (P_f, 5′-GAATTCCTGCACCTGTGAGTTTTGTTTATTGA

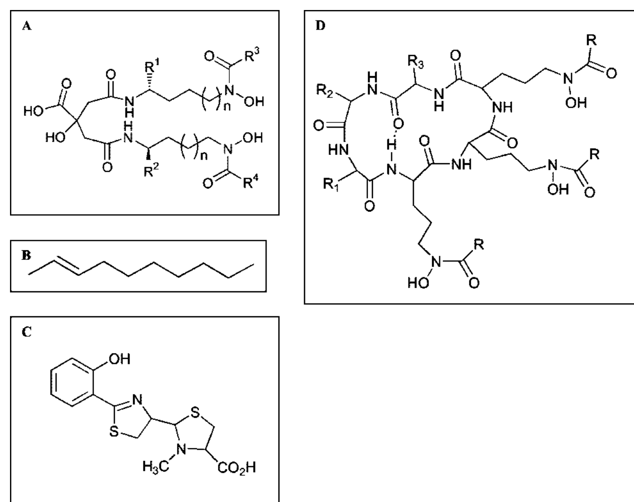

FIG. 1. Chemical structures of the siderophores used in this study. (A) Aerobactin ($R^1 = R^2 = COOH$; $R^3 = R^4 = H$; $n = 2$), schizokinen ($R^1 = R^2 = H$; $R^3 = R^4 = H$; $n = 0$), rhizobactin 1021 ($R^1 = R^2 = R^3 = H$; R^4 = the structure in panel B; $n = 0$). (C) Pyochelin. (D) Ferrichrome ($R = CH_3$; $R^1 = R^2 = R^3 = H$).

TGAGC-3′; P_r, as for *fhuCDB*), respectively, and inserting them in the pCR2.1 vector. The cloned genes were expressed with their own ribosome binding sites from the *lac* promoter of the vector.

Siderophore production and utilization assays. Siderophore production was detected by the CAS assay. A 10-μl aliquot of culture adjusted to an optical density at 600 nm of 1.0 was delivered onto the surface of a CAS plate and the plate was incubated at 30°C for 3 days, after which the appearance of an orange halo indicated siderophore production.

The siderophore utilization bioassay was carried out as described by Lynch et al. (30) with 2,2′-dipyridyl incorporated in the medium at concentrations of 300 μM for *S. meliloti*, 2 mM for *P. aeruginosa*, and 400 μM for *E. coli*. For the siderophore utilization bioassays, the supernatants containing the siderophores were prepared from strains as follows: rhizobactin 1021 from *S. meliloti* 2011, schizokinen from *B. megaterium* ATCC 19213 (91-02), pyochelin from *P. aeruginosa* CDC5, and aerobactin from *E. coli* H1443 carrying pRG13. The *aroB* mutation in *E. coli* H1443 abolishes the production of enterobactin, while aerobactin biosynthesis is encoded by *iucABCD* on pRG13. The strains were grown in medium containing 2,2′-dipyridyl at the concentrations described above and at 300 μM for *B. megaterium*. Ferrichrome was purchased from Sigma. Figure 1 shows the chemical structures of the siderophores used in this study. As negative controls, supernatants were prepared from strains grown under iron-replete conditions.

Plant nodulation and nitrogen fixation assays. Alfalfa (*Medicago sativa*) seeds were surface sterilized in ethanol and domestic bleach and examined for the absence of contaminants by germination on TY medium. Germinated seedlings were transferred to Jensen medium, as described by Ogawa et al. (34), in test tubes and inoculated with approximately 10^5 *S. meliloti* cells per tube. Nitrogen fixation by whole plants was determined after 30 days by the acetylene reduction assay (49).

DNA sequencing and sequence analysis. DNA was sequenced by MWG Biotech, Milton Keynes, United Kingdom. Database searches were undertaken with BLASTX and BLASTP (1). Multiple-sequence alignments were performed with Multalin (8) and Genedoc. The Tmpred program was used to predict protein structure and folding. Access to genome sequence data was obtained at http://sequence.toulouse.inra.fr/meliloti.html for *S. meliloti* and at http://www.pseudomonas.com for *P. aeruginosa*.

RESULTS

Identification and mutagenesis of *rhtX*. To fully delineate the rhizobactin regulon, the region upstream from the biosynthesis operon *rhbABCDEF* (Fig. 2) was mutagenized. A Tn*5lac* insertion in the open reading frame upstream of *rhbA* was isolated by the fragment-targeted transposon mutagenesis method described by Lynch et al. (30). The strain carrying the insertion, *S. meliloti* 2011*rhtX43*, was tested for its ability to produce rhizobactin 1021 and to utilize the siderophore. The plate bioassay revealed that the mutant was totally defective in rhizobactin 1021 utilization. In the CAS assay the mutant produced a diminished halo, which initially suggested that the *rhbABCDEF* operon located downstream from the transposon insertion site may be transcribed from two promoters, one located between the transposon insertion site and the operon and the second upstream from the transposon. However, it came to our attention that the Tn*5lac* transposon can have outward promoter activity which could be responsible for the low-level expression of the *rhbABCDEF* operon in the mutant. We therefore constructed a mutation in the same gene with the

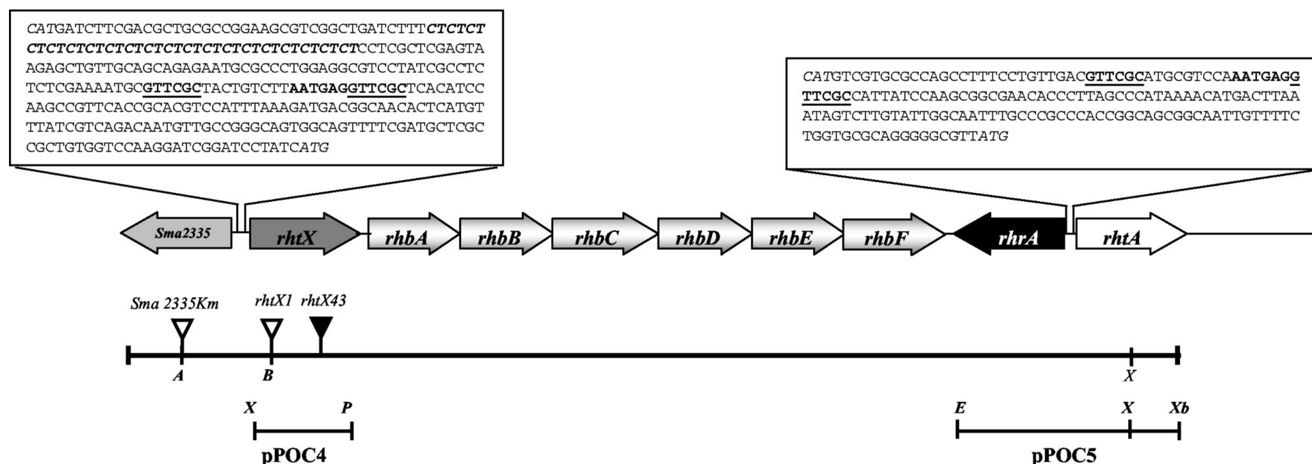

FIG. 2. Genetic organization of the region encoding rhizobactin biosynthesis (*rhb*), regulation (*rhr*), and transport (*rht*) genes in *S. meliloti*. The DNA sequences upstream of *rhtX* and between *rhrA* and *rhtA* are shown, having the six-base repeat (underlined) interspersed by 15 bases. Identical sequences in the two regions are shown in boldface. The locations of the kanamycin cassette insertions in the mutants 2011Sma2335*km* and 2011*rhtX1* are shown (▽). The location of the Tn*5lac* insertion in mutant 2011*rhtX43* is also shown (▼). Start codons are in italic. The CT repeats are in boldface italic. Restriction sites: *A*, ApaI; *B*, BamHI; *E*, EcoRI; *P*, PstI; *X*, XhoI; *Xb*, XbaI.

TABLE 2. Siderophore utilization by *S. meliloti*

Strain	Relevant genotype	Utilization of:			
		Rhizobactin 1021	Schizokinen	Aerobactin	Ferrichrome
2011	*rhrA*$^+$ *rhtA*$^+$ *rhtX*$^+$	+	+	−	+
2011*rhtX43*	*rhrA*$^+$ *rhtA*$^+$ *rhtX43*	−	−	−	+
2011*rhtA45*	*rhrA*$^+$ *rhtA45 rhtX*$^+$	−	−	−	+
102F34	Lacks *rhrA, rhtA*, and *rhtX*	−	−	−	+
102F34(pPOC4)[a]	*rhtX*$^+$	−	−	−	+
102F34(pPOC5)[b]	*rhrA*$^+$ *rhtA*$^+$	−	−	−	+
102F34(pPOC4, pPOC5)	*rhrA*$^+$ *rhtA*$^+$ *rhtX*$^+$	+	+	−	+

[a] *rhtX* is expressed constitutively.
[b] *rhtA* expression is activated by RhrA.

Ω kanamycin resistance cassette, to ensure complete termination of transcription by the cassette insertion. Mutant *S. meliloti* 2011*rhtX1* carrying the cassette was found to be defective in rhizobactin 1021 utilization. However, unlike *S. meliloti* 2011*rhtX43*, it was totally defective in the production of the siderophore as tested by the CAS assay, indicating that there was a polar effect on siderophore production in *S. meliloti* 2011*rhtX1*. It can be concluded, therefore, that the diminished halo which had been observed in *S. meliloti* 2011*rhtX43* was due to the polar effect of the Tn*5lac* insertion, which was partially relieved by the outward promoter activity from the Tn*5lac* transposon transcribing the *rhbABCDEF* genes at a low level. The gene carrying the mutations, called Sma2337 in the published genome sequence, was named *rhtX* (for "rhizobactin transport") in recognition of the observed phenotype and the homology to YbtX from *Yersinia pestis* (discussed below). The evidence implies that *rhtX* is the first gene in an operon that extends through *rhbABCDEF*.

To determine whether the gene further upstream of *rhtX* (Fig. 2) also functioned in the production or utilization of the siderophore, a mutation was constructed in the neighboring open reading frame. By allelic replacement a kanamycin resistance cassette was inserted, as described in Materials and Methods, in the small open reading frame annotated in the *S. meliloti* genome as Sma2335, to give strain *S. meliloti* 2011Sma2335*km*. The phenotype of the strain was determined by using the CAS assay to detect siderophore production and the plate bioassay to examine siderophore utilization. A phenotype identical to that of the wild type was observed for the strain, indicating that Sma2335 does not function in production or utilization of rhizobactin 1021. Further upstream from *rhtX* and next to Sma2335 there is an operon showing very good homology to the *kdp* operon present in many bacterial species and determined to function in potassium transport. This operon is unlikely to function in transport of the siderophore.

Complementation of the mutations in *rhtX* was undertaken with plasmid pPOC4, carrying the *rhtX* gene in the broad-host-range plasmid pBBR1MCS-5, under the control of the *lac* promoter of the plasmid. The presence of plasmid pPOC4 in *S. meliloti* 2011*rhtX1* restored the wild-type phenotype for siderophore transport in the bioassay but did not restore the wild-type phenotype for siderophore production on CAS plates. This further confirmed the conclusion that *rhtX* does not have a role in siderophore biosynthesis and that the reason for the low-level siderophore production in the *S. meliloti* 2011*rhtX43* mutant was the effect of the outward gene expression from the Tn*5lac* transposon on the downstream biosynthesis genes.

Analysis of the sequence upstream of *rhtX* revealed direct repeats of the sequence GTTCGC separated by 15 bases (Fig. 2). Moreover, 20 repeats of the CT dinucleotide are found consecutively in the published genome, upstream as shown in Fig. 2. Interestingly, we sequenced this region independently and found 19 repeats of the dinucleotide at the same location.

Mutagenesis of *rhtX* did not affect the ability of *S. meliloti* to nodulate alfalfa and fix nitrogen. Nitrogenase activity, as measured by the acetylene reduction assay, of plants nodulated by *S. meliloti* 2011*rhtX43* was comparable to that of the wild type (data not shown).

Introduction of *rhtX* and *rhtA* confers the ability to utilize rhizobactin 1021 in *S. meliloti* 102F34, a strain that otherwise cannot utilize the siderophore. It has been shown that *rhtA* encodes the outer membrane receptor for rhizobactin 1021 (30, 41). The RhrA activator positively regulates transcription of the gene. Plasmid pPOC5, carrying *rhtA* and *rhrA* in the broad-host-range vector pSUP104, was shown to complement mutations in *rhtA* and *rhrA* when introduced into *S. meliloti* 2011*rhtA45* and *S. meliloti* 2011*rhrA26*, respectively. *S. meliloti* 102F34 is a strain that does not produce or utilize rhizobactin 1021. When pPOC5 was introduced into *S. meliloti* 102F34, it did not confer the ability to utilize the siderophore. However, when pPOC4, carrying *rhtX*, was introduced into *S. meliloti* 102F34 along with pPOC5, the strain was able to utilize rhizobactin 1021 in the plate bioassay (Table 2; Fig. 3). The presence of both plasmids also conferred the ability to utilize shizokinen but not aerobactin. The introduction of pPOC4 alone did not confer the ability to utilize rhizobactin 1021 on *S. meliloti* 102F34.

***E. coli* can utilize rhizobactin 1021 via either the Fhu transport system or RhtX.** We investigated the utilization of rhizobactin 1021 and the structurally related siderophores schizokinen and aerobactin in *E. coli*. The outer membrane receptor IutA and the FhuCDB transport system are required for utilization of aerobactin by *E. coli* (23). We found that rhizobactin 1021 was utilized in the presence of *iutA* and the *fhu* operon (Table 3). The strain also utilized schizokinen, a hydroxamate siderophore produced by *B. megaterium*.

To determine whether the utilization of rhizobactin 1021 in *E. coli* was dependent on the Fhu system, we sought mutants with mutations in the *fhuCDB* genes. *E. coli* RK4375 carries the mutation described as *fhuB478*::Tn*10* by Kadner et al. (22). This mutation was constructed prior to the discovery of the

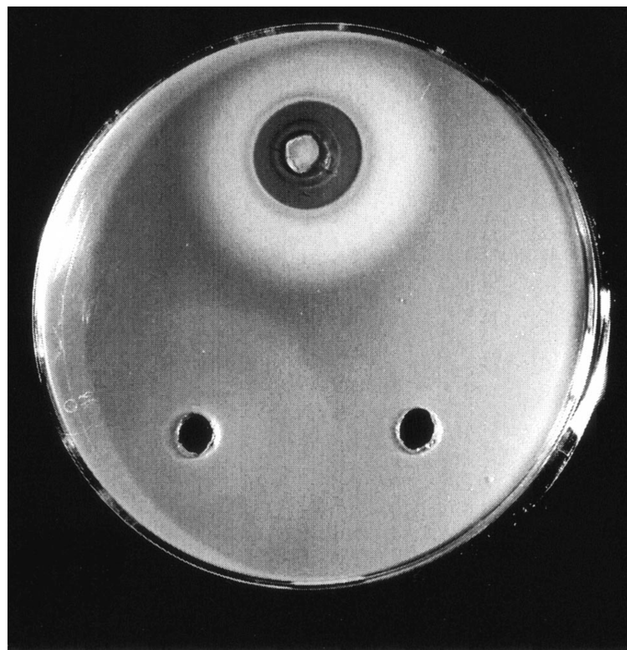

FIG. 3. Iron nutrition bioassay. The medium was seeded with *S. meliloti* 102F34, carrying plasmids pPOC4 and pPOC5. Ferric chloride (100 mM) was added to the top well, a test solution containing rhizobactin 1021 was added to the well on the left, and a test solution lacking rhizobactin 1021 was added to the well on the right.

fhuC and *fhuD* genes within the same operon, and therefore it was necessary to determine the precise gene within the *fhu* operon into which Tn*10* had inserted. Plasmids pPC35, pPC29, and pPC20 were constructed to express the *fhuCDB*, *fhuCD*, and *fhuDB* genes, respectively, and complementation of the *fhuB478*::Tn*10* mutation was undertaken with each of the plasmids. Plasmid pPC35 complemented the mutation, resulting in restoration of the ability to transport ferrichrome as determined by bioassay. Neither pPC29 nor pPC20 restored the phenotype. On this basis it was concluded that the Tn*10* insertion in *E. coli* RK4375 is in the *fhuC* gene, from which it exerts a polar effect on the downstream *fhuDB* genes.

Plasmid pEN7 was introduced into *E. coli* RK4375 to express *iutA*, and the strain was tested for the ability to utilize rhizobactin 1021, schizokinen, aerobactin, and also ferrichrome (the FhuA receptor is intact in this strain). The negative result that was observed (Table 3) indicated that the Fhu system is necessary for the transport of both rhizobactin 1021 and schizokinen in *E. coli*. As expected, aerobactin utilization and ferrichrome utilization were also abolished by the mutation in *fhuC*. However, when pPOC4, expressing *rhtX*, was introduced into this mutant, in the presence of pEN7, it restored the ability to utilize rhizobactin 1021 and schizokinen, although aerobactin and ferrichrome were still not utilized (Table 3). The results indicate that RhtX can act as an alternative to the inner membrane FhuCDB functions to facilitate the utilization of rhizobactin 1021 and schizokinen in *E. coli*.

A homologue of RhtX functions in the utilization of pyochelin by *P. aeruginosa*. Homologues of RhtX identified by a BLAST search included PA4218, the product of a gene located close to the regulon for pyochelin production in the genome of *P. aeruginosa*. A mutation in the PA4218 gene was constructed by inserting a kanamycin resistance cassette in the genomic copy of the gene in *P. aeruginosa* PA4218-km1. This mutant is a derivative of *P. aeruginosa* CDC5, a pyochelin-producing strain that does not produce pyoverdine. The mutant was tested for pyochelin utilization in the plate bioassay and was found to have lost the ability to utilize the siderophore. The elimination of pyochelin utilization was similar to that observed for the *fptA* mutant *P. aeruginosa* DH143. We propose that the gene annotated as PA4218 in the *P. aeruginosa* genome be named *fptX* (for "ferripyochelin transport") in recognition of its role in pyochelin utilization and the sequence homology to RhtX that the encoded protein displays.

DISCUSSION

The transport of siderophores across the periplasm and cytoplasmic membrane of gram-negative bacteria has been found to be facilitated by periplasmic binding protein-dependent transport (PBT) systems of the ABC superfamily. Here we report that rhizobactin 1021 can be utilized by *E. coli* expressing the aerobactin outer membrane receptor IutA and a functional Fhu PBT system. The recognition of rhizobactin 1021 by IutA is not surprising given the structural similarity between aerobactin and rhizobactin 1021 and the high identity (40%) and similarity (61%) between IutA and RhtA, the receptor for rhizobactin 1021 in *S. meliloti*. It is also not surprising that utilization is dependent on the Fhu system, as it is known that a number of citrate hydroxamate siderophores such as aerobactin are transported by the Fhu PBT system in *E. coli*. Many gram-negative bacteria possess transport systems that show homology to the Fhu system of *E. coli*. Among the rhizobia, *R. leguminosarum* biovar viciae has been shown to have a Fhu system that is required for the uptake of the trihydroxamate siderophore vicibactin (46). However, analysis of the

TABLE 3. Siderophore utilization by *E. coli*

Strain	Relevant genotype	Utilization of:			
		Rhizobactin 1021	Schizokinen	Aerobactin	Ferrichrome
H1443	*fhuC*$^+$ *fhuD*$^+$ *fhuB*$^+$	−	−	−	+
H1443(pEN7)	*fhuC*$^+$ *fhuD*$^+$ *fhuB*$^+$ *iutA*$^+$	+	+	+	+
RK4375[a]	*fhuC*::Tn*10*	−	−	−	−
RK4375(pEN7)	*fhuC*::Tn*10* *iutA*$^+$	−	−	−	−
RK4375(pPOC4)	*fhuC*::Tn*10* *rhtX*$^+$	−	−	−	−
RK4375(pPOC4, pEN7)	*fhuC*::Tn*10* *iutA*$^+$ *rhtX*$^+$	+	+	−	−

[a] RK4375 carries a Tn*10* insertion in *fhuC* that is polar on *fhuD* and *fhuB* (see text).

sequenced genome of *S. meliloti* reveals no gene products with significant homology to the components of the Fhu transport system, apart from two homologues of FhuA. No homologue of FhuD has been identified, while any homology to FhuB and FhuC is of negligible significance (our unpublished observations). The absence of recognizable Fhu genes in *S. meliloti* raises a question regarding the transport system that facilitates the utilization of rhizobactin 1021 and ferrichrome. The identification of *rhtX* in the region encoding rhizobactin 1021 biosynthesis and the observation that mutants with mutations in this gene are defective in utilization of the siderophore suggested that RhtX may be a novel permease for rhizobactin 1021. This was supported by the observation that the introduction of *rhtX* and *rhtA* into *S. meliloti* 102F34, a strain that does not utilize rhizobactin 1021, was sufficient to confer the ability to utilize the siderophore. Further evidence for the role of RhtX as a transporter is provided by the restoration of rhizobactin 1021 utilization in an *E. coli fhuC* polar mutant by the introduction of *rhtX*, in the presence of the outer membrane receptor IutA. This result supports a role for RhtX as a transporter of either intact ferric rhizobactin 1021 or a derivative of the siderophore. RhtX does not function in the transport of ferrichrome in *S. meliloti* (Table 2) or *E. coli* (Table 3), and it is not clear how ferrichrome is transported in *S. meliloti*, given the absence of a recognizable FhuBCD transport system. The absence of FhuBCD in *S. meliloti* and the inability of RhtX to facilitate ferrichrome transport imply that another novel transport system for a hydroxamate siderophore must exist in the organism.

The Tmpred program predicts that RhtX has 12 transmembrane domains. Comparison of the protein sequence by BLASTP (1) analysis identified homology to a number of proteins, two of which have been investigated in detail, namely, AmpG from *E. coli* and YbtX from *Y. pestis* (which is 99% similar to Irp8 from *Yersinia enterocolitica*). AmpG is a permease for muropeptides that functions in cell wall recycling and generates signal molecules for the induction of β-lactamase (10, 21, 28). While the similarity to AmpG was low over the entire length of RhtX, the 155 amino acids extending from position 38 to 193 in RhtX show 51% similarity to the aligned region of AmpG. The genes encoding YbtX and Irp8 are located proximal to the genes that encode biosynthesis and uptake of yersiniabactin in their respective hosts. They have 52% similarity in the region extending from position 38 to 193 in RhtX. Both *ybtX* and *irp8* have been mutagenized in the analysis of yersiniabactin transport, and in each case it was found that the mutations did not affect the transport of yersiniabactin (5, 14). Yersiniabactin transport is mediated by proteins encoded by *ybtP* and *ybtQ* in *Y. pestis* and the corresponding genes *irp6* and *irp7* in *Y. enterocolitica*. Both sets of proteins show homology to ABC transporters with an export function. The ABC transporter genes are within the same operon as *ybtX* and *irp8* in the respective species. It is possible that YbtX and Irp8 represent redundant or inefficient permeases that have been functionally replaced by more efficient ABC transporters for yersiniabactin transport. In *S. meliloti*, however, there is no obvious ABC transporter located proximal to the rhizobactin 1021 regulon. The effect of the mutations in *rhtX* on siderophore utilization, the homology of RhtX to AmpG, and the capacity of the gene to effect utilization of the siderophore in the nonutilizing strain of *S. meliloti* and in the *E. coli fhuC* mutant combine to suggest that it encodes a dedicated permease for rhizobactin 1021.

A number of the proteins showing highest homology to RhtX are encoded within clusters of genes that are predicted to be involved in iron transport. A homologue of interest in regard to *S. meliloti* is Smc02889. This gene is located beside a putative outer membrane receptor that shows homology to the yersiniabactin receptor and may confer on *S. meliloti* the ability to utilize yersiniabactin encountered in the soil environment. Furthermore, located 3′ to Smc02889 and transcribed divergently from it is an Ara-C-like regulator. A number of the homologues of RhtX are found in gene clusters that appear to be regulated by Ara-C-like regulators. Another homologue of particular interest is the product of the gene named PA4218 in the *P. aeruginosa* genome, which has been assigned the name *fptX* in this work. It is located adjacent to the region encoding biosynthesis and transport of pyochelin. The bioassay result that we observed for *P. aeruginosa* PA4218-km1, carrying a mutation in the *fptX* gene, indicates that the gene functions in pyochelin utilization. Microarray analysis of *P. aeruginosa* has shown that *fptX* (PA4218) is upregulated under low-iron growth conditions (32).

There is no confirmed cytoplasmic membrane transport system encoded within the pyochelin region. While the amino acid sequences of proteins encoded by *pchH* and *pchI* show similarity to those of ABC transporter proteins (including YbtP and YbtQ of *Y. pestis* and Irp6 and Irp7 of *Y. enterocolitica*), no function could be assigned to these proteins in either export or utilization of pyochelin (42). The situation with regard to pyochelin, whereby PchI and PchH, the predicted transporters, have no apparent role in utilization while FptX has a function, is in contrast to that observed for yersiniabactin, where the predicted transporters YbtPQ in *Y. pestis* (Irp6 and Irp7 in *Y. enterocolitica*) function in yersiniabactin utilization but YbtX/Irp8 appear to be redundant or of minor importance.

The discovery that *rhtX* is the first gene in an operon that extends through *rhbABCDEF* implies that it is under the control of RhrA and iron, both of which were previously demonstrated to regulate this operon at the transcriptional level (30). This led us to examine the sequence between the operon and the upstream open reading frame Sma2335, which is transcribed in the opposite direction. The putative RhrA binding site GTTCGC$(N)_{15}$GTTCGC that is observed exactly matches a sequence found upstream of *rhtA* (Fig. 2). RhrA has also been shown to regulate *rhtA* (30). It is likely that the regulation of iron response is also effected in this region. An iron response regulator of multiple operons, RirA, has been identified in *R. leguminosarum* (48), and it would be of interest to examine the role of the *rirA* homologue that exists in *S. meliloti* in regard to iron regulation of the rhizobactin 1021 regulon. Interestingly, further upstream of the region shown in Fig. 2 and before the start codon of Sma2335 there are 20 CT dinucleotide repeats. Phase variation is known to be mediated by nucleotide repeats that may be lost or gained by strand slippage and repair, leading to frameshifting when the repeat is within an open reading frame or to an effect on RNA polymerase binding when the repeat occurs in a promoter. Variation in the number of repeats was actually confirmed in this case, as we sequenced this region independently and found 19

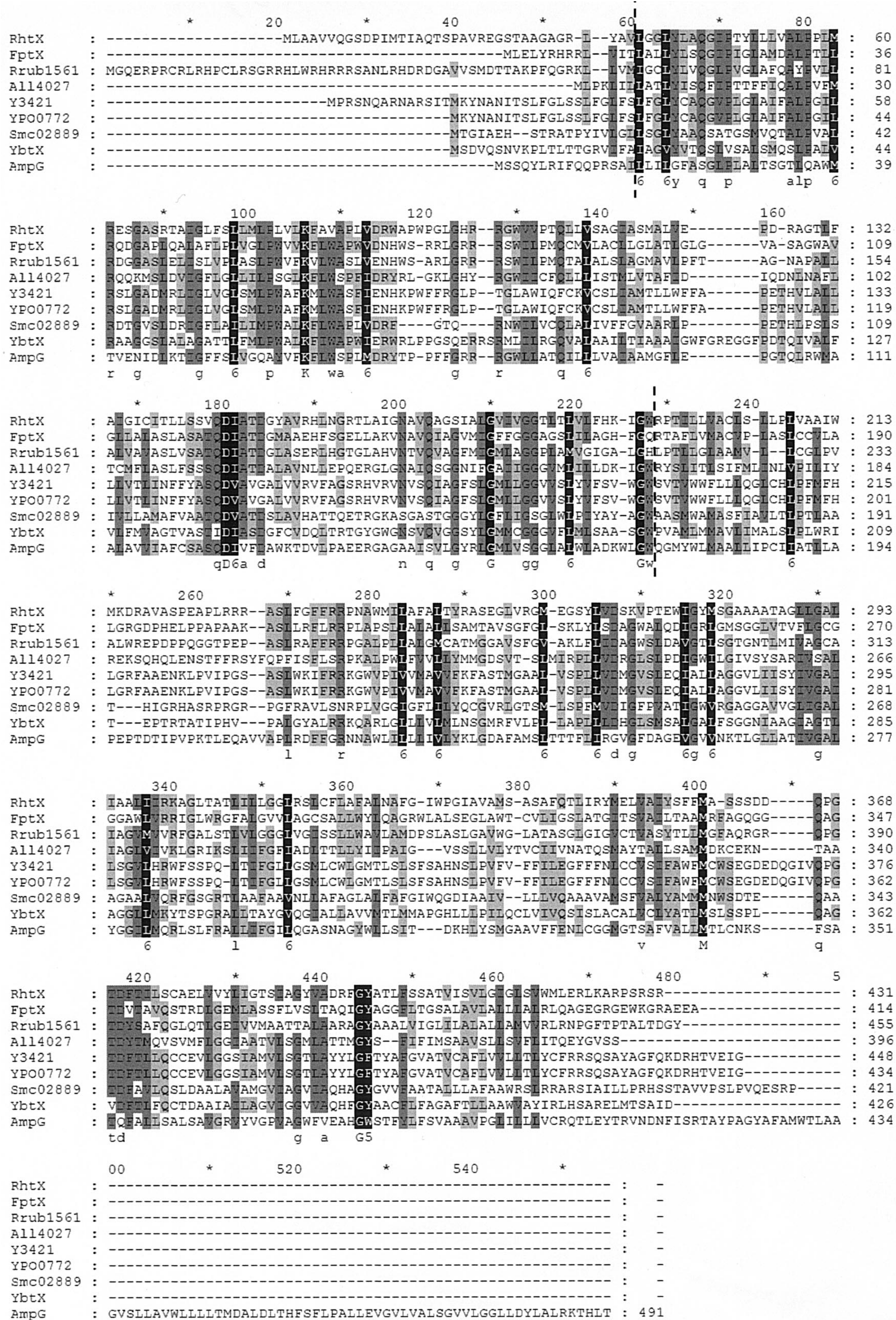

FIG. 4. Alignment of RhtX, the five proteins showing highest homology, and FptX. Similar residues are shaded. Deletions (dashes) are indicated. The accession numbers of the compared proteins are as follows: RhtX (AAK65915), FptX (NP_252908), Rrub1561 (ZP_00014546), All4027 (NP_488065), Y3421 (AAM86970), YPO0772 (CAC89621), Smc02889 (NP_384320), YbtX (CAC90731), AmpG (P36670).

repeats, in contrast to the 20 repeats in the published genome sequence. It is not clear if the variation affects expression of the operon containing *rhtX* or expression of neighboring genes in the upstream region and what effect, if any, this may have.

Schizokinen from *B. megaterium* is structurally identical to rhizobactin 1021 without the lipid moiety and shows a utilization pattern similar to that of rhizobactin 1021 (Fig. 1; Tables 2 and 3). On that basis we can conclude that the unusual lipid of rhizobactin 1021 is not required for its utilization via RhtX. In contrast, aerobactin, which has minor structural differences in comparison to the other two siderphores, is not utilized by strains relying on RhtX to facilitate utilization, even in *E. coli*, where the outer membrane receptor IutA is present.

It is striking that neither RhtX nor FptX, nor any of the other homologues in what appears to be a novel family of permeases, has associated proteins that function in energy coupling or as chaperones of the siderophores across the periplasm. FptX is encoded beside a gene of unknown function (PA4219) with which it would appear to be translationally coupled. However, PA4219 does not have any obvious ATPase or periplasmic binding protein motifs. There is no homologue of PA4219 associated with RhtX or encoded within the *S. meliloti* genome.

Alignment of the sequences of RhtX homologues revealed that homology is greatest in the N-terminal regions of the proteins (Fig. 4), and there is strong conservation of a motif of four amino acids, QD(V/I)A (at position 180 in the sequence as annotated in Fig. 4), which is predicted to be located in a cytoplasmic loop in RhtX. In the absence of an energizing protein, it is interesting to speculate that this represents a conserved domain for such an interaction. Members of the RhtX family of proteins may function by transporting unaltered siderophores, or they may chemically modify the siderophores while releasing iron. They may function in recycling the siderophores. Also, it may be significant that members of the RhtX family of homologues are found, in most cases, located in clusters of genes regulated by Ara-C-like transcriptional activators. The mechanism by which this family of proteins functions may provide signal molecules for their respective Ara-C-like activators of transcription.

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