Structure and Function of the PiuA and PirA Siderophore-Drug Receptors from *Pseudomonas aeruginosa* and *Acinetobacter baumannii*

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ABSTRACT The outer membrane of Gram-negative bacteria presents an efficient barrier to the permeation of antimicrobial molecules. One strategy pursued to circumvent this obstacle is to hijack transport systems for essential nutrients, such as iron. BAL30072 and MC-1 are two monobactams conjugated to a dihydroxypyridone siderophore that are active against *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Here, we investigated the mechanism of action of these molecules in *A. baumannii*. We identified two novel TonB-dependent receptors, termed *Ab-PiuA* and *Ab-PirA*, that are required for the antimicrobial activity of both agents. Deletion of either *piuA* or *pirA* in *A. baumannii* resulted in 4- to 8-fold-decreased susceptibility, while their overexpression in the heterologous host *P. aeruginosa* increased susceptibility to the two siderophore-drug conjugates by 4- to 32-fold. The crystal structures of PiuA and PirA from *A. baumannii* and their orthologues from *P. aeruginosa* were determined. The structures revealed similar architectures; however, structural differences between PirA and PiuA point to potential differences between their cognate siderophore ligands. Spontaneous mutants, selected upon exposure to BAL30072, harbored frameshift mutations in either the ExbD3 or the TonB3 protein of *A. baumannii*, forming the cytoplasmic-membrane complex providing the energy for the siderophore translocation process. The results of this study provide insight for the rational design of novel siderophore-drug conjugates against problematic Gram-negative pathogens.

KEYWORDS *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, siderophore-drug receptor

The outer membrane of Gram-negative bacteria is an efficient barrier that limits access of antibiotics to their targets. *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are characterized by especially low outer membrane permeability due to slow influx across porin channels and relatively rapid efflux via numerous efflux systems of the resistance-nodulation-division (RND) type (1). There have been several attempts to exploit alternative transport mechanisms to overcome this permeability barrier (2). In this view, the TonB-dependent receptors (TBDRs), able to transport siderophores or vitamin B₁₂ in Gram-negative organisms (3), as well as carbohydrates, cations, and thiamine (4), are particularly attractive. Due to their high-affinity binding sites, substrates are transported even at low substrate concentrations. Furthermore, they can perform concentrative uptake (uphill transport) for enterochelin (5) and vitamin B₁₂ (6) and accommodate large substrate molecules, including cobalamines, heme, or peptidic

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siderophores, such as pyoverdine. In contrast to porin-mediated passive diffusion, the uptake via the outer membrane TBDRs requires energy, derived from the proton motive force, and transmitted via the TonB-ExbB-ExbD complex located in the cytoplasmic membrane.

In order to hijack these uptake systems, conjugates between antibiotics and siderophore molecules have been designed. So far, the most success has been obtained with beta-lactams, since their target is located in the periplasmic space, to which most of the siderophores are delivered (3). The iron-binding moiety of beta-lactam siderophore conjugates is frequently a small catechol-type siderophore, such as dihydroxypyridone or a mixed catechol-hydroxamate (7). Such conjugates have been developed with aminopenicillins (8), cepheems (KP-736 and S-649266) (9–11), and monocyclic beta-lactams (pirazmonam, BAL30072, and MC-1) (12–15). In particular, the monobactam conjugates show potent activity against the Gram-negative nonfermenters *P. aeruginosa* and *A. baumannii* (15–18).

A. baumannii is a nosocomial pathogen associated with severe infections in immunocompromised hosts (19). Besides its chromosomally encoded AmpC-type beta-lactamase (20), *A. baumannii* frequently harbors class B and D carbapenemases (21) and extended-spectrum beta-lactamases (ESBL) of the PER, GES, and VEB types (22). RND-type efflux systems, including AdeABC (23), AdeIJK (24), and AdeFGH (25), further contribute to the multidrug resistance phenotype of clinical isolates. *A. baumannii* harbors between 21 and 27 TonB-dependent receptors compared to 35 in *P. aeruginosa* (26). So far, only FhuE (27) and BauA (28), the receptor for the siderophore acinetobactin (29, 30), have been characterized in *A. baumannii*.

In this study, we identified two novel TonB-dependent receptors of *A. baumannii*, termed PiuA and PirA (*Ab*-PiuA and *Ab*-PirA), required for the activity of two monocyclic beta-lactam siderophore conjugates. When expressed in *P. aeruginosa*, both receptors confer hypersusceptibility to the two drugs. The first crystal structures of PiuA and PirA were obtained, together with their orthologues from *P. aeruginosa*. Although they showed similar overall structures, there were marked differences in their putative substrate binding sites. The data should provide insights for the future rational design of novel siderophore-drug conjugates. (Part of this work was presented at the ECCMID conference, Copenhagen, Denmark, 2015.)

RESULTS

Identification of PiuA and PirA orthologues in *A. baumannii*. We (31) and others (15) previously identified the TBDRs PiuA and PirA as important to the activity of the siderophore-drug conjugates BAL30072 and MC-1 in *P. aeruginosa*. Since BAL30072 also shows potent activity toward *A. baumannii* (14, 18, 32), we attempted to identify its mechanism of action in the organism. We performed a homology search for potential orthologues of PiuA and PirA on the genome of *A. baumannii* reference strain ATCC 19606 (Table 1). The BLAST algorithm identified two uncharacterized open reading frames (ORFs), A1S_0474 and A1S_0980 (ATCC 17978 numbering), as the closest hits, sharing amino acid identities of 44% with PiuA and 51% with PirA of *P. aeruginosa*, respectively. Both genes have been shown to be preceded by putative Fur binding sites (A1S_0474, GCGAATAATAATAATTCTTATTAT, and A1S_0980, GATATTGTTAATAATTATC ATTATT) (33), suggesting regulation by iron availability via the Fur iron uptake regulator. Downstream of the *piuA* gene of *A. baumannii*, we identified an ORF (A1S_0473) showing 52% amino acid identity with *piuC* from *P. aeruginosa* and *ybiX* from *Escherichia coli* (Fig. 1). The corresponding proteins belong to the family of Fe(II)-dependent oxidoreductases (34), and their colocalization with *piuA* might indicate that they act on the natural substrate transported by PiuA/FiuA. In contrast, the genetic environment of the *pirA* region of *A. baumannii* is different from those in *P. aeruginosa* and *E. coli* (Fig. 1). The *pirA* gene in *A. baumannii* is preceded by *nfuA*, coding for an Fe-S-containing protein involved in intracellular iron utilization and oxidative-stress response (35). In *P. aeruginosa*, the gene for the secondary enterobactin transporter PirA is located next to a two-component system comprising the transcriptional regulator PirR (PA0929) and

TABLE 1 Bacterial strains and plasmids^a

Strain/plasmid	Relevant characteristics	Reference or source
<i>P. aeruginosa</i>		
PAO1	PAO1; <i>mexT</i> nonfunctional	Laboratory collection
PAO1 Δ <i>piuA</i>	Unmarked deletion of <i>piuA</i> gene	This study
<i>A. baumannii</i>		
ATCC 19606	<i>A. baumannii</i> reference clinical isolate	HUG ^b collection
307-0274	<i>A. baumannii</i> clinical isolate	T. A. Russo; SUNY, Buffalo, NY
ATCC 19606 Δ <i>piuA</i>	<i>piuA</i> (A1S_0474) deletion mutant	This study
ATCC 19606 Δ <i>pirA</i>	<i>pirA</i> (A1S_0980) deletion mutant	This study
ATCC 19606 Δ <i>piuA</i> Δ <i>pirA</i>	<i>piuA</i> - <i>pirA</i> double-deletion mutant	This study
<i>E. coli</i>		
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17(r_K⁻ m_K⁺) supE44 relA1 lac [F' <i>proAB lacI^q</i> ΔM15Tn10(Tc)]</i>	Stratagene
ST18	<i>pro thi hsdR</i> ⁺ Tmp ^r Sm ^r ; chromosome::RP4-2 Tc::Mu-Kan::Tn7/ λ <i>pir</i> Δ <i>hemA</i>	68
Plasmids		
pMKm	Contains a 1.7-kb Km cassette carrying a <i>neo</i> gene; Km ^r	69
pEX18Ap	Gene replacement vector; Ap ^r	53
pEX18Gm	Gene replacement vector; Gm ^r	53
pEX18Km	pEX18Ap derivative; 0.4-kb fragment of the <i>bla</i> gene replaced with 1-kb <i>neo</i> gene from pMKm via AatII/Scal; Km ^r	This study
pEX18Gm- <i>pirA</i> -ko	Contains a 1.4-kb fusion fragment of the up- and downstream regions of <i>pirA</i> ; Gm ^r	This study
pEX18Km- <i>piuA</i> -ko	1.1-kb fusion fragment of the 5' and 3' regions of <i>piuA</i> ; Km ^r	This study
pEX1.8	Inducible expression vector; Ap ^r	70
pApX2	Broad-host-range expression vector; Ap ^r	I. Attree (Grenoble, France)
p <i>piuA</i> 1.1	<i>piuA</i> from PAO1 cloned into pApX2; Ap ^r	This study
p <i>pirA</i> 1.1	<i>pirA</i> from PAO1 cloned into pApX2; Ap ^r	This study
p <i>piuA</i> -Ab6.3	<i>piuA</i> -Ab cloned into pEX1.8; Ap ^r	This study
p <i>pirA</i> -Ab4.1	<i>pirA</i> -Ab cloned into pApX2; Ap ^r	This study

^aAp, ampicillin; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Tc, tetracycline; Tmp, trimethoprim.

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the sensor kinase PirS (PA0930) (36). The closest orthologue of PirA in *E. coli* is the ferric enterobactin transporter FepA, whose gene is embedded within the enterobactin biosynthesis region (not shown in Fig. 1). Based on this evidence, we adopted the nomenclature from *P. aeruginosa* and termed these proteins PiuA (A1S_0474) and PirA (A1S_0980), designated below Ab-PiuA and Ab-PirA.

PiuA and PirA contribute similarly to siderophore–beta-lactam activity in *A. baumannii*. To test the involvement of PiuA and PirA in the activity of BAL30072 and MC-1 against *A. baumannii*, we constructed *piuA* (A1S_0474) and *pirA* (A1S_0980) gene deletion mutants in *A. baumannii* strain ATCC 19606. Both the *piuA* and the *pirA* mutants showed 2- to 8-fold increases in the MICs for both drug conjugates, in agreement with decreased transport in the individual mutants. The *piuA*-*pirA* double mutant showed a further increase in MICs, which was more pronounced for MC-1 than for BAL30072 (Table 2). This suggests that in the wild-type strain and under the conditions of the MIC assay, both receptors have a basal expression level and may contribute similarly to the uptake of BAL30072 and MC-1. This is in contrast to the

FIG 1 Chromosomal loci of *piuA* and *pirA* genes in *A. baumannii*, *P. aeruginosa*, and *E. coli*. The TBDR gene *piuA* is colocalized with the Fe(II)-dependent oxidoreductase gene *piuC*, while the *pirA* loci differ among the three organisms. Only genes relevant to the study context are shown.

TABLE 2 Susceptibilities of *piuA* and *pirA* deletion mutants of *A. baumannii*

Strain	MIC (mg/liter)		
	BAL30072	MC-1	ATM
ATCC 19606	0.25	1	32
ATCC 19606 Δ <i>piuA</i>	1	2	32
ATCC 19606 Δ <i>pirA</i>	1–2	4	32
ATCC 19606 Δ <i>pirA</i> Δ <i>piuA</i>	2–4	32	32

situation in *P. aeruginosa*, since deletion of *pirA* in PAO1 has no effect on susceptibility to BAL30072 and MC-1, and the contribution of this TBDR is seen only in a *piuA* deletion background (15).

PiuA and PirA from *A. baumannii* are functional in *P. aeruginosa*. To test whether the *A. baumannii* proteins are functional in *P. aeruginosa*, plasmids ppirA-Ab4.1 and ppiuA-Ab6.3, expressing, respectively, the *pirA* and *piuA* genes of *A. baumannii* from a constitutive or an inducible promoter, were transferred into *P. aeruginosa* strain PAO1 and into a *piuA* deletion mutant. While expression of the vector plasmids alone had no significant effect on the BAL30072 or MC-1 MIC, the expression of both *piuA* and *pirA* from *A. baumannii* conferred increased susceptibility to these conjugates in *P. aeruginosa*. As expected, susceptibility to aztreonam (ATM) was not affected by overexpression of the TBDR genes (Table 3). Similar results were obtained in a *piuA* deletion strain showing a 32- to 64-fold increase in susceptibility to BAL30072 and MC-1 when the *pirA* gene of either *P. aeruginosa* or *A. baumannii* was overexpressed. However, Ab-PiuA expression from plasmid ppiuA-Ab6.3 did not seem to confer increased susceptibility on the *piuA* deletion strain (Table 3). A possible explanation is that the induced expression level of Ab-PiuA in the *piuA* deletion strain might be insufficient to restore wild-type susceptibility.

Structure comparison of PiuA and PirA from *P. aeruginosa* and *A. baumannii*.

The sequence alignment of the PiuA and PirA orthologues revealed high conservation within the putative substrate binding domains NL1 (PiuA and PirA) and NL2 and NL3 (PirA) (Fig. 2). The structures of PiuA and PirA from *P. aeruginosa* and *A. baumannii* have been solved. As expected, these structures were similar to those of other TBDRs, sharing a two-domain organization consisting of a 22-stranded transmembrane β -barrel (Fig. 3, green ribbons) and an N-terminal plug domain (blue ribbons) folded inside the barrel.

TABLE 3 Effects of heterologous expression of Ab-PiuA and Ab-PirA in *P. aeruginosa* PAO1 and PAO1 Δ *piuA* on susceptibility to monocyclic beta-lactam antibiotics

Strain/plasmid	MIC (mg/liter) ^a		
	BAL30072	MC-1	ATM
PAO1	1–2	0.5	4
pEX1.8	2	0.25	4
pApX2	1	0.5	8
ppiuA1.1 (<i>P. aeruginosa</i>)	0.125	ND	4
ppiuA Δ L1.1 (<i>P. aeruginosa</i>)	2	ND	4
ppiuA-Ab6.3	0.25	0.06	4
ppirA1.1 (<i>P. aeruginosa</i>)	0.25	ND	8
ppirA-Ab4.1	0.06	0.03	4
PAO1 Δ <i>piuA</i>	8–16	2	4
pApX2	16	4	8
pEX1.8	16	4	4
ppiuA1.1 (<i>P. aeruginosa</i>)	0.125	0.03	8
ppiuA-Ab6.3	8	2	2
ppirA1.1 (<i>P. aeruginosa</i>)	0.25	0.06	8
ppirA-Ab4.1	0.25	0.125	4

^aDetermined in MH medium supplemented with carbenicillin at 100 mg/liter and with 1 mM IPTG for strains harboring pEX1.8 and ppiuA-Ab6.3. ND, not done.

FIG 2 Structure-based sequence alignment of PiuA and PirA proteins from *P. aeruginosa* and *A. baumannii*. Secondary-structure elements of Ab-PiuA are illustrated above the alignment. Regions predicted to be involved in the binding of the siderophores are boxed in red. Residues spatially equivalent to those involved in substrate binding in FhuA, FptA, and FpvA are highlighted in green. Key residues of the secondary binding site of FepA and conserved in the PirA structure are shown in yellow. Positions of residues of the nonconserved initial binding site are indicated by orange triangles. The plug domain is boxed in cyan. The alignment was drawn using Aline (71).

FIG 3 Crystal structures of PiuA and PirA from *P. aeruginosa* and *A. baumannii*. β -Barrels are colored green, and N-terminal plug domains are blue. Loops NL1, NL2, and NL3 of the plug domains are shown in red. Residues likely to be part of the siderophore substrate binding site are shown as yellow sticks. The last solved residues of the flexible loops are represented as spheres.

As often observed with TBDRs, several extracellular loops were missing in the *Pa*-PiuA structure. Loops NL1 (83 to 90), L7 (505 to 526), and L8 (560 to 567) could not be modeled due to the high flexibility of these regions. In contrast, the equivalent extracellular loops were present in the structure of *Ab*-PiuA, perhaps due to the presence of a phosphate ion that coordinated with Glu73 of NL1, Asp303 of L4, Arg316 of β 8, and His697 of L11. Structural comparison of *Pa*-PiuA and *Ab*-PiuA (root mean square deviations [RMSD] of 1.3 Å for 616 aligned C- α atoms) showed differences in the lengths and positions of several extracellular loops (Fig. 4; see Fig. S1 in the supplemental material). *Ab*-PiuA contained additional amino acids in loops L7 and L9, but fewer in L6 and L8 than *Pa*-PiuA. Strikingly, loop L7 of *Ab*-PiuA folded inside the barrel close to the top of the plug domain (Fig. 4; see Fig. S1 in the supplemental material). A mutant protein with five amino acids (EGGNP; positions 73 to 76) deleted from loop NL1 was generated in PiuA from *P. aeruginosa*. When expressed in strain PAO1, the NL1 deletion mutant was unable to confer hypersusceptibility to BAL30072, as observed with the PiuA wild-type protein (Table 3). We conclude that loop NL-1 is likely involved in the initial binding of BAL30072 in PiuA.

Comparison with other TBDRs showed that the closest structural relatives of PiuA are the pyochelin (FptA) and pyoverdine (FpvA) receptors of *P. aeruginosa* (37, 38), with an RMSD of 1.84 Å (567 aligned C- α atoms) and 1.99 Å (555 aligned C- α atoms),

FIG 4 Putative binding sites in PiuA of *P. aeruginosa* and *A. baumannii* and PirA of *A. baumannii*. The color scheme is the same as that in Fig. 3. The phosphate ion present in the *Ab*-PiuA structure is colored orange and red, and residues Asp303 and Glu73, which coordinate it, are represented as green and white sticks.

TABLE 4 Susceptibilities of *A. baumannii* mutants selected on BAL30072

Strain	MIC (mg/liter)			Mutation ^a
	BAL30072	MC-1	ATM	
307-0294	0.25	1	32	WT
307-0294-R4	>8	>8	16	ExbD3 (Q4fs)
307-0294-R5	>8	>8	16	TonB3 (K106fs)
307-0294-R6	>8	>8	16	TonB3 (K80fs)

^afs, frameshift; WT, wild type.

respectively, and the *E. coli* ferrichrome iron receptor FhuA, with an RMSD of 1.83 Å (600 aligned C-α atoms) (39, 40) (see Fig. S1 in the supplemental material).

As for PiuA, the extracellular loops of PirA from *P. aeruginosa* were highly flexible, and the structural analysis of *Pa*-PirA was focused on the structure of PirA from *A. baumannii*, where these loops were experimentally located. PirA was similar to the ferric enterobactin receptor FepA of *E. coli* (41), with an RMSD of 1.09 Å (618 aligned C-α atoms), as expected from the elevated sequence similarity (51% amino acid identity) (see Fig. S1 in the supplemental material). This is consistent with a previous report identifying PirA of *P. aeruginosa* as a secondary transporter of ferric enterobactin (36).

Selection of *A. baumannii* mutants with decreased susceptibility to BAL30072.

We investigated the possibility of resistance emergence in *A. baumannii*. Colonies of strains 307-0294 and ATCC 19606 able to grow in the presence of BAL30072 concentrations of 4 or 8 μg/ml were obtained at frequencies of 7×10^{-8} to 7×10^{-9} . After determining their susceptibility profiles, we selected colonies that presented increased MICs of BAL30072 and MC-1 but unchanged MICs of ATM. Mutations in such colonies are expected to affect mechanisms specific to the siderophore-drug conjugate. Three out of 6 clones (R4, R5, and R6) obtained with strain 307-0294 and none of the 8 clones tested from strain ATCC 19606 fulfilled this criterion (Table 4). Therefore, clones R4, R5, and R6 were selected for further analysis and submitted to whole-genome sequencing. Mapping of the reads on the genome of strain 307-0294 revealed a single but distinct mutation in each of the three strains. Mutant R4 had acquired a frameshift mutation (insertion of A at position 9) in the *exbD3* gene, while mutations in clones R5 (deletion of A at position 319) and R6 (insertion of A at position 243) resulted in frameshift mutations in the *tonB3* gene (Table 4). Interestingly, the *tonB3* gene, but not the *tonB1* and *tonB2* genes (see Fig. S2 in the supplemental material), has recently been shown to be induced under iron-limiting conditions (42). The TonB3-ExbB3-ExbD3 system could therefore be a likely candidate for providing energy to PiuA and PirA siderophore receptors in *A. baumannii*.

Decreased growth rates of spontaneous *exbD3* and *tonB3* mutants of *A. baumannii*. We observed that the spontaneous mutants R4, R5, and R6 formed slightly smaller colonies than the wild type when grown on lysogeny broth (LB) agar plates. To assess whether the mutations in the energy-transducing TonB3 system impose a fitness cost, we compared the growth of the mutants to that of the wild-type strain in Mueller-Hinton (MH) broth. As shown in Fig. 5, the *tonB3* (R5 and R6) and *exbD3* (R4) mutants were characterized by a longer lag phase and a lower final cell density after 20 h growth than the wild type. When the growth medium was supplemented with Fe(II)Cl₂ at a final concentration of 20 μM, the growth phenotype of mutants R4 to R6 could be restored to reach nearly wild-type levels. The data suggest that TonB3-ExbB3-ExbD3 is important for siderophore-mediated Fe³⁺ acquisition and that the fitness of such mutants is likely to be severely decreased *in vivo*, where iron availability is limited.

DISCUSSION

In this study, we have identified two siderophore receptors, termed *Ab*-PiuA and *Ab*-PirA, that are very likely involved in transport of the siderophore-monosulfactam conjugates BAL30072 and MC-1 into *A. baumannii*. The numbers of TBDRs found in different strains of *A. baumannii* range from 21 to 27 (compared to 35 in *P. aeruginosa*)

FIG 5 Growth of spontaneous mutants with decreased susceptibility to BAL30072. The strains were grown in MH broth (MHB) with or without Fe(II)Cl₂ supplementation (20 μM final concentration). Growth was followed in a plate reader with regular OD₆₀₀ readings. The data represent the average OD₆₀₀ value for duplicate cultures. Experiments were repeated on three different occasions, yielding similar results. Data from one representative experiment are shown. The OD₆₀₀ values, measured after 18 h for each of the three mutants, were statistically different from that of the wild type (Student *t* test; *P* < 0.05) in the absence, but not in the presence, of iron, as indicated by asterisks (top).

(26). The close phylogenetic relationship between *Acinetobacter* and *Pseudomonas* prompted us to search in *A. baumannii* for orthologues of the previously identified TonB-dependent receptors PiuA and PirA from *P. aeruginosa* (15, 31). For each of these proteins, we identified one orthologue (>40% amino acid identity). The highest similarity was found within the putative substrate binding sites located in the N-terminal regions of the TBDRs (Fig. 2).

A transcriptome analysis performed in *A. baumannii* strain ATCC 17978 recently showed that expression of both *piuA* and *pirA* is induced under iron-limiting conditions (33). *A. baumannii* strains harbor at least three distinct siderophore clusters, one of them being the acinetobactin biosynthesis operon. However, neither PiuA nor PirA is part of these clusters. This suggests that the two TBDRs are likely involved in the uptake of xenosiderophores, although the substrate(s) of PiuA has not been identified. The closest orthologue of PiuA in *E. coli* is Fiu (35% amino acid identity), a TBDR also involved in the uptake of cephalosporins conjugated to catechol siderophores (12, 13).

PirA is an orthologue of the enterobactin receptor FepA in *E. coli* (51% amino acid identity). Enterobactin is a tricatechol siderophore produced by several enterobacterial species. However, the FepA receptor does not seem to play a role in the transport of catechol-based siderophore-drug conjugates in *E. coli* (12, 13), but it can transport beta-lactams linked to enterobactin (43, 44).

It is assumed in the design philosophy that BAL30072 interacts with the siderophore receptors PiuA and PirA in a manner similar to that of their natural siderophores.

However, the structures of the loops differ between the orthologous proteins from the two organisms. There are even larger differences between the loop structures of PirA and PiuA (see Fig. S1 in the supplemental material). Since the natural siderophores of PiuA and PirA are not known, we rely on comparisons with known complexes to inform us as to the recognition elements. The binding site of known siderophore receptors is in general formed by loops NL1, NL2, and NL3 of the plug domain and loops L3, L7, and L11 and strands $\beta 7$, $\beta 8$, and $\beta 9$ of the β -barrel (Fig. 2) (37, 39, 45).

The closest structural homologues of PiuA are FptA, FpvA, and FhuA, and their complexes with cognate siderophores show a cluster of aromatic and positively charged residues (Fig. 4). The side chains of Tyr239 on loop L3 of *Pa*-PiuA and Trp298 in $\beta 7$ of *Ab*-PiuA have positions similar to those of Trp362 in FpvA and Met271 in FptA, both involved in siderophore binding. Tyrosines 307 and 296 on loop $\beta 7$ of *Pa*-PiuA and *Ab*-PiuA occupy positions equivalent to those of FpvA Trp391 and FhuA Tyr116. A positively charged residue on $\beta 8$ (Lys329 for *Pa*-PiuA and Arg316 for *Ab*-PiuA) is in the same position as the side chain of Lys344 in FhuA. Trp498, present in loop L7 of *Ab*-PiuA, occupies the same volume as Tyr231 and Phe115 in NL3 of FpvA and FhuA, respectively. A tyrosine-histidine couple (residues 694 and 697 in *Ab*-PiuA and 697 and 700 in *Pa*-PiuA) is superimposed with Phe795 and Tyr796 in FpvA and Phe704 and Tyr707 in FhuA. We note that a phosphate ion is found in the *Ab*-PiuA structure, where it is coordinated by Arg316 and His697. There are important differences, however, between *Pa*-PiuA and *Ab*-PiuA. In particular, loops NL2 and NL3 are shorter in *Pa*-PiuA, while NL1 extends above the plug domain of *Ab*-PiuA (Fig. 3). Consequently, NL1 in *Ab*-PiuA would clash with the siderophore location seen in FpvA, FptA, and FhuA (see Fig. S1 in the supplemental material). This may reflect a genuine difference with a functional consequence or simply an inactive conformation.

Although there is no structure for the siderophore complex of FepA from *E. coli*, several binding and mutation studies have been reported (46–48). These studies suggest a biphasic binding kinetic with an initial binding site in the loop extremities and a secondary site deeper inside the barrel leading to the translocation. Loops L2, L4, L7, L8, L10, and L11 were found to be involved in the binding of ferric enterobactin (41, 49). These loops are different in structure and sequence from those found in PirA, consistent with the observation that FepA does not transport BAL30072. Interestingly, it also indicates that PirA, which can recognize enterobactin, may use a different constellation of residues than FepA. The presumed second binding site, on top of the plug domain, is highly conserved between the two PirAs and FepA. Deletion of loop NL1 or NL3 in FepA or mutation of the conserved Trp101 in NL3 (corresponding to Trp111 and Trp110 in *Ab*-PirA and *Pa*-PirA) drastically decreased the uptake of ferric enterobactin (48, 49). Other key residues known to be important in FepA are Tyr260 of L3 (262 in *Ab*-PirA and 264 in *Pa*-PirA), Arg316 on $\beta 7$ (*Ab*-PirA R320 and *Pa*-PirA R321), and Tyr478 of L7 (*Ab*-PirA Tyr478 and *Pa*-PirA Tyr479), which are conserved in PirA. As in FepA, loops L3, L4, L7, and L10 cover the top of the barrel, making the plug domain less accessible than for the other TBDRs.

TBDRs interact with the cytoplasmic-membrane protein TonB, which transmits energy derived from the proton gradient to the receptor via interaction with the N-terminal TonB box, to drive substrate transport (see Fig. S2 in the supplemental material). *A. baumannii* harbors three distinct chromosomally encoded TonB systems, among which TonB3-ExbB3-ExbD3 was recently shown to be iron regulated (42). Spontaneous resistant clones able to grow on >4 mg/liter of BAL30072 carried frame-shift mutations in *tonB3* or *exbD3* (see Fig. S2 in the supplemental material). It is therefore highly likely that the TonB3-ExbB3-ExbD3 system interacts with PiuA and PirA to provide the energy for the uptake of BAL30072 and MC-1. Of note, the MICs of BAL30072 for the *tonB3* and *exbD3* mutants were higher than those for the individual TBDR *piuA* and *pirA* mutants or the *pirA-piuA* double mutant (compare the values in Tables 2 and 4). Although the strain backgrounds are different (ATCC 19606 versus 307-0294), it suggests that additional TBDRs could be involved in transport of siderophore-drug conjugates in *A. baumannii* strain 307-0294. This assumption would

also explain the selection of *exbD3* and *tonB3* mutants at 4 mg/liter of BAL30072, which display higher MICs than the individual *pirA* and *piuA* mutants in strain ATCC 19606.

The *A. baumannii* *tonB3* and *exbD3* mutants selected in strain 307-0294 on BAL30072 showed decreased growth rates under iron-limiting conditions. A similar growth impairment under iron deficiency was reported by Zimbler et al. for *tonB1* and *tonB2* deletion mutants of strain ATCC 19606 (42). Since the authors of the study could not generate a *tonB3* deletion mutant by homologous recombination, the growth phenotype could not be assessed, and the authors concluded the TonB3-ExbB3-ExbD3 system was essential in *A. baumannii*. Whether this was due to the experimental conditions or to differences in strain backgrounds is unclear. Supplementation with iron could restore the growth defects in their *tonB1* and *tonB2* mutants (42), as well as in our spontaneous *tonB3* and *exbD3* mutants of strain 307-0294 (Fig. 5). It remains to be determined if *tonB1* and *tonB2* mutants also show altered susceptibilities to siderophore-drug conjugates. Altogether, the data suggest that *tonB* mutants, if selected *in vivo*, would be impaired in their fitness and probably also be less virulent in the patient, where iron availability is limited.

Both endogenous and heterologous overexpression of *Ab-PiuA* and *Ab-PirA* conferred increased susceptibility to both BAL30072 and MC-1 on *P. aeruginosa* strain PAO1 (Table 3). This establishes the ability of these two proteins to transport the two antibiotics; moreover, it indicates that the amounts of PiuA and PirA expressed in PAO1 are probably the bottleneck for the transport of siderophore-drug conjugates, rather than the availability of the TonB-ExbB/ExbD systems. There must be mutual recognition between the C-terminal part of the cytoplasmic-membrane protein TonB in *P. aeruginosa* and the TonB box located in the N-terminal domains of PirA and PiuA from *A. baumannii*. In *P. aeruginosa*, the energy coupling to iron-transporting TBDRs is mediated by TonB1 (50), while our data suggest that TonB3 is the equivalent protein in *A. baumannii*. However, TonB1 (*P. aeruginosa*) and TonB3 (*A. baumannii*) show only limited amino acid sequence identity. The TonB3 domain of *A. baumannii* displayed the highest sequence similarity to the C-terminal regions of TolA (amino acids [aa] 277 to 322; 48% identity) and TonB2 (aa 178 to 223; 39% identity) of *P. aeruginosa*. These domains are located in the periplasm and are thought to interact with the corresponding TBDR in the outer membrane (51). Which TonB protein of *P. aeruginosa* is interacting with PiuA and PirA from *A. baumannii* remains to be determined. To our knowledge, this is the first report on heterologous complementation between TonB-dependent receptors in Gram-negative nonfermenters.

In summary, the previously uncharacterized TBDRs PiuA and PirA are the major uptake systems for the siderophore-drug conjugates BAL30072 and MC-1 in *A. baumannii*. Despite their overall similar crystal structures, differences in the external loops of the plug domain could be important for substrate selectivity. We obtained evidence for heterologous recognition between a TonB protein(s) of *P. aeruginosa* and TBDRs of *A. baumannii*. Our study highlights the potential of siderophore-drug conjugates as an efficient strategy to increase antimicrobial transport into Gram-negative bacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in this study are listed in Table 1, and the primers used are shown in Table S1 in the supplemental material. *A. baumannii* strains were grown in double-strength yeast tryptone (dYT) broth and *E. coli* and *P. aeruginosa* in LB at 37°C with shaking (220 rpm). *E. coli* XL-1 Blue and DH10B were used as cloning hosts. *E. coli* ST18 was the donor strain for biparental matings, requiring supplementation with 50 µg/ml 5-aminolevulinic acid. Gentamicin (25 µg/ml) or kanamycin (25 µg/ml) was added for plasmid-carrying strains. MIC determinations were performed in MH broth according to CLSI guidelines (52) and were repeated on at least three different occasions.

Mutant selection and growth assay. Spontaneous mutants of the fully sequenced *A. baumannii* strain 307-0294 were selected on LB agar plates supplemented with BAL30072. Individual colonies were picked and grown in the absence of antibiotic. MICs were determined, and strains with decreased susceptibility to BAL30072 were retained for further analysis. The growth of mutant strains was performed in microtiter plates in a total volume of 200 µl. Strains were grown for 7 h in LB with shaking (250 rpm) at 37°C. Cultures were diluted to an optical density at 600 nm (OD₆₀₀) of 0.015, and 4 µl was inoculated into 196 µl of MH broth. Growth was followed in a BioTek Synergy 1 plate reader at 37°C with

shaking for 1 min before hourly measurement of the optical density at 600 nm. The growth curves represent the average OD₆₀₀ values for duplicate cultures.

PCR amplifications and DNA modifications. PCR primers are listed in Table S1 in the supplemental material. All primer sequences were based on the genome of *A. baumannii* ATCC 19606. Screening PCRs were carried out using the DreamTaq DNA polymerase (Thermo Scientific), in accordance with the manufacturer's instructions and the optimized annealing temperature for each primer set. For screening PCRs, bacterial cells were boiled at 95°C for 5 min and subsequently pelleted at 13,000 rpm for 1 min. Phusion DNA polymerase (Thermo Scientific) was used for high-fidelity PCRs (supplemented with 5% dimethyl sulfoxide [DMSO]). Restriction digestions were performed using Thermo Scientific restriction enzymes, according to the manufacturer's instructions, at the appropriate temperature. All ligation reactions were carried out at room temperature using Thermo Scientific T4 DNA ligase. DNA purifications were performed using either the GeneJet PCR purification or the GeneJet gel extraction kit (Thermo Scientific), following the manufacturer's instructions.

Construction of *pirA* and *piuA* knockout mutants in *A. baumannii*. The generation of unmarked knockout mutants was based on the protocol described by Hoang et al. (53). Briefly, DNA fragments of 500 to 700 bp were PCR amplified using primer pairs A1/A2 and B1/B2, respectively (see Table S1 in the supplemental material). For deletion of *pirA*, the up- and downstream regions flanking the gene were PCR amplified. For knockout of *piuA*, the amplified fragments were located in the 5' and 3' regions of the genes. After amplification, the obtained A and B fragments were gel purified, and approximately 40 ng of each fragment was used in a PCR fusion with primers A1 and B2, which share an 18-bp homologous region. The resulting fusion products were gel purified and further cloned into the suicide vectors pEX18Gm, via HindIII/EcoRI restriction sites (*pirA*), and pEX18Km, via Sall/EcoRI (*piuA*). The cloned knockout fragments were verified by sequencing. The replacement vectors were mobilized into *A. baumannii* via biparental conjugation, and the generation of the unmarked mutants was carried out as previously described (54). In order to confirm a gene deletion, locus-specific primers (out1 and out2) that bind up- and downstream of the gene were designed and used for amplification of the knockout region. Sequencing of the obtained PCR fragments confirmed successful deletion of the genes.

Construction of expression plasmids. The *piuA* and *pirA* coding regions were amplified by PCR from genomic DNA of *P. aeruginosa* reference strain PAO1. The *piuA* coding region was amplified with primers *piuA*-Xba and *piuA*-Hind and cloned as a 2,540-bp XbaI-HindIII DNA fragment into the expression vector pApX2, yielding plasmid ppiuA1.1. Deletions of residues 73 to 76 from loop NL1 (ppiAΔL1.1) were constructed with a modified protocol of the QuikChange method (55) using primers *piuA*-Δ1 and *piuA*-Δ2. The *pirA* coding region was amplified as a 2,498-bp DNA fragment obtained with primers *pirA*-Bam and *pirA*-Hind2 (see Table S1 in the supplemental material). The PCR product was digested by BamHI and HindIII and cloned into pApX2, yielding plasmid ppirA1.1. The coding regions of *piuA* (A15_0474) and *pirA* (A15_0980) were amplified by PCR from genomic DNA of *A. baumannii* strain 307-0294 (provided by T. A. Russo, SUNY, Buffalo, NY, USA), using primers listed in Table S1 in the supplemental material. The 2,436-bp-long *piuA* fragment was digested with EcoRI and PstI and ligated to the EcoRI- and PstI-cleaved vector pEX1.8, yielding plasmid ppiuA-Ab6.3. The 2,557-bp-long *pirA* fragment was cleaved with XbaI and PstI and ligated to plasmid pApX2 cleaved with the same restriction enzymes, yielding plasmid ppirA-Ab4.1. The plasmids were transferred into *P. aeruginosa* by electroporation. The *piuA* gene of *A. baumannii* (*piuA*-Ab) was further subcloned by BamHI-PstI digestion into the *A. baumannii* expression vector pBAV1K-5T-gfp (56) (kindly provided by G. Wilharm, Robert Koch Institut, Werningerode, Germany), yielding plasmid ppiuA-Ab7.1. The *piuA*-Ab gene was further subcloned by XbaI-PstI digestion into pBAV1K-5T-gfp, yielding plasmid ppirA-Ab3.1. The PCR conditions were as follows: denaturation at 95°C for 2 min, followed by 27 cycles of 95°C for 20 s, 57°C for 30 s, and 72°C for 2 min and a final extension at 72°C for 4 min. Plasmids were introduced into *P. aeruginosa* and *A. baumannii* by electroporation. Plasmid-containing colonies were selected on LB agar supplemented with carbenicillin at 200 mg/liter (*P. aeruginosa*) or kanamycin at 50 mg/liter (*A. baumannii*).

Cloning, overexpression, and purification of PiuA and PirA from *P. aeruginosa* and *A. baumannii*. The positions of the signal peptide cleavage sites of the proteins were predicted with Signal P4.0 (57). The coding sequence of the mature protein *Pa*-PiuA (PA4514) was amplified from PAO1 genomic DNA using primer pairs *Pa*-PiuA-F/*Pa*-PiuA-R (see Table S1 in the supplemental material) and *Pfu* DNA polymerase. The PCR product was digested by BspHI and EagI restriction enzymes and cloned into vector pET20b using restriction enzymes NcoI and EagI so that the protein was expressed with a PelB signal sequence for the outer membrane localization and a noncleavable C-terminal His₆ tag. Codon-optimized *Pa*-PirA (PA0931) with a C-terminal, noncleavable His₆ tag was purchased from DNA2.0 and inserted into vector pET20b using HindIII and EcoRV restriction sites.

The coding sequences of the mature proteins *Ab*-PiuA and *Ab*-PirA were amplified by PCR from *A. baumannii* strain ATCC 19606 using KOD DNA polymerase (Novagen) and the primer pairs *Ab*-PiuA-F/*Ab*-PiuA-R and *Ab*-PirA-F/*Ab*-PirA-R (see Table S1 in the supplemental material). Both genes were cloned with an N-terminal tobacco etch virus (TEV) protease-cleavable His₆ tag in vector pTAMAHISTEV using NcoI and BamHI restriction sites. The pTAMAHISTEV expression vector was obtained by replacing the PelB signal peptide of plasmid pPELBHISTEV (courtesy of Huanting Liu, University of St Andrews, St Andrews, United Kingdom) with the TamA signal peptide, as described by Liu et al. (58).

Proteins were overexpressed in *E. coli* strain C43(DE3) or BL21(DE3). Cells were grown at 37°C in LB medium containing 100 mg/liter ampicillin to an OD₆₀₀ of 0.7 and then induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 25°C overnight.

For *Pa*-PiuA and *Pa*-PirA, harvested cells were resuspended in lysis buffer (50 mM sodium phosphate, pH 7.5, 250 mM NaCl, 10% glycerol, 100 μ g/ml lysozyme, and 20 μ g/ml DNase). The cells were lysed by two passages through a cell disruptor at 30,000 lb/in² (Constant Systems). Unbroken cells were removed by centrifugation (20 min at 10,000 $\times$ g), and membrane fractions were isolated by ultracentrifugation (100,000 $\times$ g; 1 h). The supernatant was discarded, and inner membrane proteins were solubilized in buffer 1 (50 mM sodium phosphate, pH 7.5, 250 mM NaCl, 10% glycerol, and 1% [wt/vol] *N*-lauroylsarkosine [Sigma]). The insoluble outer membrane fraction was isolated by ultracentrifugation (100,000 $\times$ g; 1 h), and outer membrane proteins were solubilized in buffer 2 (50 mM sodium phosphate, pH 8, 250 mM NaCl, and 7% [vol/vol] octylpolyoxyethylene [octylPOE] [Bachem]). Insoluble material was removed by ultracentrifugation (100,000 $\times$ g; 1 h). The supernatant was applied to a charged Histrap nickel-sepharose high-performance column (GE Healthcare) equilibrated with 1% (vol/vol) octylPOE, 250 mM NaCl, 10 mM imidazole, and 50 mM sodium phosphate, pH 8, and washed with the same buffer containing 15 mM imidazole. Protein was eluted with a linear gradient of imidazole (5 to 150 mM). Fractions containing the protein were pooled; dialyzed against 50 mM Tris, pH 8.4, and 1% octylPOE; loaded on an anion exchange column (Resource Q [GE Healthcare]) equilibrated with the dialysis buffer; and eluted with a linear gradient of 0 to 600 mM NaCl over 12 column volumes. Fractions were pooled and loaded on a Superdex S200 gel filtration column (GE Healthcare) equilibrated with 10 mM Tris, pH 8, 150 mM NaCl, and 0.45% (vol/vol) *n*-octyltetraoxyethylene (C₈E₄) (Bachem). Protein fractions were pooled and concentrated to 8 mg/ml.

The expression and initial purification steps for *Ab*-PiuA and *Ab*-PirA were the same as for *Pa*-PiuA and *Pa*-PirA. After applying them to a first immobilized-metal affinity chromatography (IMAC) column, imidazole was removed by dialysis (50 mM Tris, pH 8, 250 mM NaCl, 10% glycerol, 2 mM mercaptoethanol, and 1% octylPOE) before adding TEV protease to cleave the His₆ tag. The protein was applied to a second IMAC column, followed by Superdex S200 gel filtration chromatography (GE Healthcare) (10 mM Tris, pH 8, 150 mM NaCl, 0.45% C₈E₄). The protein fractions were pooled and concentrated to 10 mg/ml.

Crystallization and structure determination. Crystals of *Pa*-PiuA appeared at 20°C after several days by mixing 1 μ l of protein solution (8 mg/ml) with 1 μ l of reservoir solution containing 32% polyethylene glycol (PEG) 400, 0.05 M Tris, pH 8.5, 0.05 M sodium sulfate, and 0.05 M lithium chloride. *Pa*-PirA crystals were obtained at 20°C by mixing 1.5 μ l of the protein solution (7.5 mg/ml) containing 2.5 mM *n*-dodecylphosphocholine (Anatrace) with 1 μ l of reservoir solution containing 17% PEG 3350, 0.2 M magnesium chloride, 0.1 M Tris, pH 7. The crystals were frozen with the same solution containing 25% PEG 400.

Crystals of *Ab*-PiuA appeared at 20°C after several days by mixing 1 μ l of protein solution (10 mg/ml) with 1 μ l of reservoir solution containing 1 M diammonium hydrogen phosphate, 0.1 M sodium acetate, pH 4.6. The crystals were cryoprotected by supplementing the mother liquor with 30% sucrose. Prior to crystallization, Fe³⁺-enterobactin was added to *Ab*-PirA at a final concentration of 1 mM before loading on a Superdex S200. *Ab*-PirA crystals appeared a week after mixing at 20°C 1 μ l of protein solution (7 mg/ml) with 1 μ l of reservoir solution containing 16% PEG 8000, 0.15 M magnesium sulfate, and 0.1 M bicine, pH 9.

Data were collected at beamlines IO4 and IO3 at the Diamond Light Source, Oxfordshire, United Kingdom, and ID14-4 at European Synchrotron Radiation Facility (ESRF). Data were processed with XIA2 (59–63). The structure of *Pa*-PiuA was solved by molecular replacement using the coordinates of FhuA (Protein Data Bank [PDB] code 2FCP) (39) as a model with the program PHASER (64). The structure of *Ab*-PiuA was solved using *Pa*-PiuA coordinates, and the structures of *Pa*-PirA and *Ab*-PirA were solved using FepA coordinates (PDB code 1FEP) (41). Models were adjusted with COOT (65), and refinement was carried out using REFMAC in the CCP4 program suite with TLS parameters (66). The final refinement statistics are given in Table S2 in the supplemental material. The quality of all structures was checked with MOLPROBITY (67). Figures were drawn using PyMOL (version 1.8; Schrödinger, LLC).

Whole-genome sequencing. Samples were sequenced on an Illumina MiSeq V2 instrument. Assembly and analysis were performed off instrument using CLCBio Genomics Workbench v 6.5 (Cambridge, MA). Detection of single nucleotide polymorphisms (SNPs)/indels was accomplished through mapping to a parent reference assembly using the same parameters. Quality-based SNPs were detected at a minimum frequency of 80% using default criteria and verified by examination of *de novo* assembly. Annotation of parental strains was accomplished through ORF calling using Prodigal software and BLAST analysis at NCBI for regions of interest. Sequence analyses were performed using the BLAST algorithm on genomic data provided by the MIST2.2 database (mistdb.com).

Accession number(s). Atomic coordinates and structure factors have been deposited in the Protein Data Bank (accession no. 5FOK, 5FP2, 5FP1, and 5FR8).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.02531-16>.

TEXT S1, PDF file, 0.9 MB.

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