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Roles of Efflux Pumps from Different Superfamilies in the Surface-Associated Motility and Virulence of *Acinetobacter baumannii* ATCC 17978

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ABSTRACT Although the relationship between *Acinetobacter baumannii* efflux pumps and antimicrobial resistance is well documented, less is known about the involvement of these proteins in the pathogenicity of this nosocomial pathogen. In previous work, we identified the AbaQ major facilitator superfamily (MFS) efflux pump and demonstrated its participation in the motility and virulence of *A. baumannii*. In the present study, we examined the role in these processes of *A. baumannii* transporters belonging to different superfamilies of efflux pumps. Genes encoding known or putative permeases belonging to efflux pump superfamilies other than the MFS were selected, and the corresponding knockouts were constructed. The antimicrobial susceptibilities of these mutants were consistent with previously reported data. In mutants of *A. baumannii* strain ATCC 17978 carrying inactivated genes encoding the efflux pumps *A1S_2736* (resistance nodulation division [RND]), *A1S_3371* (multidrug and toxic compound extrusion [MATE]), and *A1S_0710* (small multidrug resistance [SMR]), as well as the newly described ATP-binding cassette (ABC) permeases *A1S_1242* and *A1S_2622*, both surface-associated motility and virulence were reduced compared to the parental strain. However, inactivation of the genes encoding the known ABC permeases *A1S_0536* and *A1S_1535*, the newly identified putative ABC permeases *A1S_*0027 and *A1S_1057*, or the proteobacterial antimicrobial compound efflux (PACE) transporters *A1S_1503* and *A1S_2063* had no effects on bacterial motility or virulence. Our results demonstrate the involvement of antimicrobial transporters belonging at least to five of the six known efflux pump superfamilies in both surface-associated motility and virulence in *A. baumannii* ATCC 17978.

KEYWORDS *Acinetobacter*, efflux pumps, motility, virulence

*A*cinetobacter baumannii is an opportunistic microorganism that causes nosocomial infections of the central nervous system, skin, soft tissues, and bone, as well as pneumonia (1). The health threat posed by this bacterium is related to its enormous potential to develop antimicrobial resistance, its ability to survive in the hospital environment, and its virulence factors (2). Among the resistance mechanisms of *A. baumannii* are enzymatic modification of antimicrobial agents, changes in membrane permeability, alternative metabolic processes, alterations of the site of antimicrobial action, and reduction of the intracellular drug concentration through drug extrusion (3). The last mechanism is carried out by efflux pumps. However, the demonstrated involvement of these antimicrobial determinants in multidrug resistance and thus in the pathogenicity of diverse bacterial species implies their role not only in drug extrusion but also in virulence (4, 5). Efflux pumps can be classified into six superfamilies according to their amino acid similarity, energy source, number of components, number of transmembrane regions, and substrate types (4): major facilitator superfamily (MFS), resistance nodulation division (RND), multidrug and toxic compound extru-

TABLE 1 Efflux pump transporters of *A. baumannii* ATCC 17978 (CP000521.1) analyzed in this study[a]

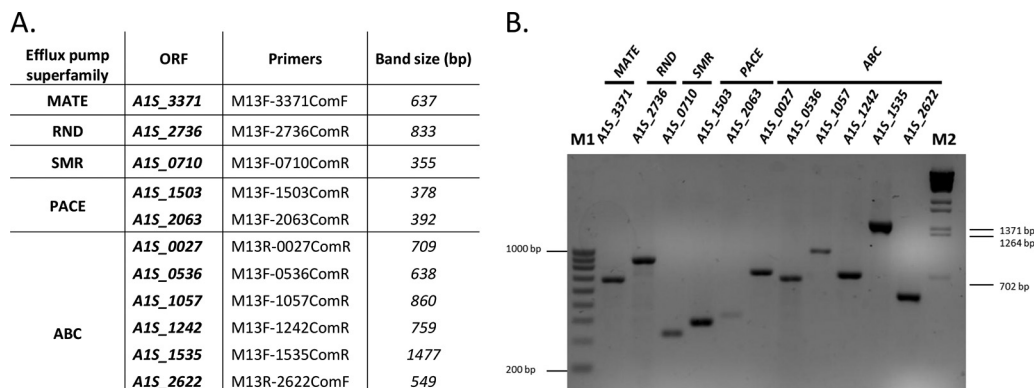| Transporter	Accession no.	Efflux pump superfamily	Participation in motility	Participation in virulence
A1S_2736	WP_000046679.1	RND	+	+
A1S_3371	WP_001135124.1	MATE	+	+
A1S_0710	WP_000312237.1	SMR	+	+
A1S_0536	WP_000165905.1	ABC	–	–
A1S_1535	WP_000922240.1	ABC	–	–
A1S_0027	WP_000031022.1	ABC	–	–
A1S_1057	WP_000536102.1	ABC	–	–
A1S_1242	WP_000988396.1	ABC	+	+
A1S_2622	WP_000657706.1	ABC	+	+
A1S_1503	WP_001161758.1	PACE	–	–
A1S_2063	WP_005135057.1	PACE	–	–

[a]The accession number, superfamily of each protein, and its participation (+) or nonparticipation (–) in motility and virulence are indicated.

sion (MATE), small multidrug resistance (SMR), ATP-binding cassette (ABC), and proteobacterial antimicrobial compound efflux (PACE) (6, 7). In *A. baumannii*, transporters belonging to all six superfamilies have been identified.

Although *A. baumannii* lacks flagella, this bacterium is able to move through surface-associated motility (8, 9). In previous reports we identified the MFS efflux pump AbaQ, which in *A. baumannii* is involved not only in quinolone extrusion but also in surface-associated motility and virulence (10, 11). Additional evidence implicating efflux pumps in the pathogenicity of *A. baumannii*, and therefore its virulence has been observed in several studies. For instance, overexpression of the RND efflux pump AdeABC increased bacterial virulence in a murine model (12), while inactivation of the MFS efflux pump AbaF lowered bacterial virulence in a *Caenorhabditis elegans* model (13). However, to our knowledge, whether transporters belonging to the other efflux pump superfamilies participate in the motility and virulence of *A. baumannii* has yet to be determined. Thus, the aim of this work was to investigate the role of transporters belonging to different efflux pumps superfamilies in the surface-associated motility and virulence of *A. baumannii*.

RESULTS

Selection and mutant construction of different *A. baumannii* efflux pumps belonging to the RND, MATE, SMR, ABC, and PACE superfamilies. As noted above, we previously described the role of the MFS AbaQ quinolone-efflux pump in the surface-associated motility and virulence of *A. baumannii* (10, 11). To examine the role of other efflux pumps in these processes, genes encoding transporters belonging to the other efflux pump superfamilies were selected (Table 1). From the RND efflux pump superfamily, the gene *A1S_2736* was chosen. This gene encodes a permease homologous (99% identity) to the AdeJ component corresponding to the AdeIJK RND efflux pump described in *A. baumannii* strain BM4454 (14). The AbeM3 transporter, encoded by the gene *A1S_3371*, and the transporter encoded by the gene *A1S_0710*, both identified in *A. baumannii* ATCC 17978, were chosen as members of the MATE and SMR efflux pump superfamilies, respectively (15, 16). From the ABC efflux pump superfamily, the genes *A1S_0536* and *A1S_1535* were selected. These genes encode permeases from the only two thus-far-identified ABC transporters involved in antimicrobial resistance, and both have been identified in strain ATCC 17978 (17, 18). In addition, by analyzing the annotated genome of the same *A. baumannii* strain, several genes annotated as possible permeases of ABC transporters were identified. Based on their permease-like structure, determined by using the Protter web platform (http://wlab.ethz.ch/protter) to analyze both the amino acid sequence and transmembrane characteristics of the putative proteins, the genes *A1S_0027*, *A1S_1057*, *A1S_1242*, and *A1S_2622* were selected as putative members of the ABC superfamily. Finally, from the PACE superfamily, we selected the only two members of this recently discovered superfamily: the AceI transporter encoded by the *A1S_2063* gene and the *A1S_1503* gene, both identified in *A. baumannii* ATCC 17978 (19). No other PACE members were found in the annotation of the genome of this strain. Mutants of all these genes were obtained using a suicide

A.

Efflux pump superfamily	ORF	Primers	Band size (bp)
MATE	*A1S_3371*	M13F-3371ComF	637
RND	*A1S_2736*	M13F-2736ComR	833
SMR	*A1S_0710*	M13F-0710ComR	355
PACE	*A1S_1503*	M13F-1503ComR	378
	A1S_2063	M13F-2063ComR	392
ABC	*A1S_0027*	M13R-0027ComF	709
	A1S_0536	M13F-0536ComR	638
	A1S_1057	M13F-1057ComR	860
	A1S_1242	M13F-1242ComR	759
	A1S_1535	M13F-1535ComR	1477
	A1S_2622	M13R-2622ComF	549

B.

FIG 1 (A) Primers used to confirm each of the indicated *A. baumannii* mutants. The lengths of the resulting PCR products are shown in base pairs (bp). (B) PCR verification of each *A. baumannii* mutant generated by gene disruption. V Ladder NzyTech (lane M1) and ʎ BstEII-digested DNA (lane M2) were used as molecular size markers.

vector, as described in Materials and Methods. All of the resulting mutants were confirmed by PCR (Fig. 1) and sequencing (Macrogen) using the appropriate primers (Table 2). All of the constructed *A. baumannii* mutants were stable, as confirmed after ten passages without selective pressure (data not shown).

TABLE 2 Oligonucleotides used in this study

| Oligonucleotide	Sequence (5′–3′)	Application
MUT0027F	AGTCGAATTCGTGGAACGAAGCAAGTCG	Mutant construction
MUT0027R	AGTCGAATTCTTTCATCAATACCGAACC	Mutant construction
COM0027R	GCTTGTGCTGAAATAGTCTCAGC	Mutant verification
MUT0710F	AGTCGAATTCGCAGGTGTATTTGAAGTG	Mutant construction
MUT0710R	AGTCGAATTCCAATGCCTGTCCAAACCG	Mutant construction
COM0710R	CAGTCCTGATACAATCAGTACAGCG	Mutant verification
MUT0536F	AGTCGAATTCTGAAGGTGAGCTGGTTGC	Mutant construction
MUT0536R	AGTCGAATTCATCGTATGACCAGCCGCG	Mutant construction
COM0536R	TTCGATAATACGCGTTGC	Mutant verification
MUT1057F	AGTCGAATTCGTGGTCGTGCAAATTGCG	Mutant construction
MUT1057R	AGTCGAATTCCACTAGGTTTTGTAATGG	Mutant construction
COM1057R	GGATAGTCTCGCTGAACAATTGC	Mutant verification
MUT1242F	AGTCGAATTCCAGCCTTGCGTACAGGCG	Mutant construction
MUT1242R	AGTCGAATTCAACCACGCGGTCATACAC	Mutant construction
COM1242R	CACGTAAATCAGCACGTTTACC	Mutant verification
MUT1535F	AGTCGAATTCCCTGAAAGTTTCTTGAGC	Mutant construction
MUT1535R	AGTCGAATTCGTAGGCGTCAGTTACACGG	Mutant construction
COM1535R	GCAGTAGTAGCAGCAATTACGC	Mutant verification
MUT2063F	AGTCGAATTCGGTCATCATTGCGATTGC	Mutant construction
MUT2063R	AGTCGAATTCGCAATCAGCAAACCACCC	Mutant construction
COM2063R	GGTATAAACCAAAATACATAAGG	Mutant verification
MUT2622F	AGTCGAATTCCCAAGTAATTTAACGC	Mutant construction
MUT2622R	AGTCGAATTCCTGCAAAACGAATAGG	Mutant construction
COM2622F	GTCATTGGCCAAACAGCCAATAGC	Mutant verification
MUT2736F	AGTCGAATTCCGTCTTTCAATTGAAAGTG	Mutant construction
MUT2736R	AGTCGAATTCGTAGCTATGCGACATGCG	Mutant construction
COM2736R	TCACGATTTATGCTCCTGAG	Mutant verification
MUT3371F	AGTCGAATTCGTCCGGTACCATTAACAC	Mutant construction
MUT3371R	AGTCGAATTCTGATGCTATAGCCGGTGC	Mutant construction
COM3371R	ACGGTTCGGCCGAAGTGG	Mutant verification
CMPL0710F	ATCGTCTAGAATGAAAATGTCTGAAGG	Mutant complementation
CMPL0710R	ATCGCCATGGTTAGGATGGAGACGAA	Mutant complementation
CMPL1242F	ATCGTCTAGAATGATGACAAAAATAAATTAC	Mutant complementation
CMPL1242R	ATCGTCTAGATCATGAATTGCCCCCTTGG	Mutant complementation
CMPL2736F	ATCGTCTAGAATGCCTATTGCACAATATC	Mutant complementation
CMPL2736R	ATCGTCTAGATCACGATTTATGCTCCTGAG	Mutant complementation
CMPL3371F	ATCGTCTAGAATGCCACCAAGACAGTC	Mutant complementation
CMPL3371R	ATCGTCTAGACTAAATAAGAACTTTG	Mutant complementation
M13FpUC	GTTTTCCCAGTCACGAC	Sequencing primer for pCR-BluntII-TOPO and pVRL Gmʳ vectors
M13RpUC	CAGGAAACAGCTATGAC	Sequencing primer for pCR-BluntII-TOPO and pVRL Gmʳ vectors

TABLE 3 MICs of the tested antimicrobials for wild-type *A. baumannii* ATCC 17978 (WT) and the derivatives carrying mutant efflux pumps[a]

Antimicrobial agent	WT	MIC (mg/liter)								
		Superfamily								
		RND (A1S_2736)	MATE (A1S_3371)	SMR (A1S_0710)	PACE		ABC			
					A1S_1503	A1S_2063	A1S_0536	A1S_1535	A1S_1242	A1S_2622
Ciprofloxacin	0.25	**0.031**	0.25	**0.125**	0.25	0.25	0.25	0.25	0.25	0.25
Levofloxacin	0.125	**0.0156**	0.125	**0.062**	0.125	0.125	0.125	0.125	**0.062**	0.125
Nalidixic acid	8	**2**	8	**4**	8	8	8	8	**4**	8
Trimethoprim	16	**4**	16	**8**	16	16	16	16	**8**	16
Novobiocin	8	**0.062**	8	8	8	8	**2**	8	**4**	8
Meropenem	2	**0.5**	2	2	**1**	2	2	2	2	2
Ampicillin	16	16	16	16	16	16	16	16	16	16
Amikacin	1	**0.5**	1	1	**0.5**	1	**0.5**	1	1	1
Gentamicin	0.5	**0.25**	0.5	0.5	0.5	0.5	0.5	**0.25**	0.5	**0.25**
Erythromycin	4	**2**	4	4	4	4	**2**	4	4	4
Colistin	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Chloramphenicol	16	**8**	16	16	16	16	16	**8**	16	16
Tetracycline	4	**1**	**2**	**2**	4	4	4	4	**2**	4
Minocycline	0.062	**0.0038**	0.062	**0.031**	**0.031**	0.062	0.062	0.062	**0.031**	**0.031**
Rifampin	4	**1**	4	4	4	4	4	4	4	4
DC	5,000	**62.5**	5,000	**1,250**	5,000	5,000	5,000	**2,500**	5,000	5,000
SDS	2,500	**156**	2,500	**1,250**	2,500	2,500	2,500	2,500	2,500	2,500
Acriflavin	12.5	12.5	12.5	12.5	**6.25**	12.5	12.5	12.5	12.5	12.5
Chlorhexidine	6	6	6	6	6	**3**	6	6	6	6

[a]Mutants that did not at all differ from the wild type are not included (*A1S_0027* and *A1S_1057*). Differences compared to the WT strain are indicated in boldface.

Determination of the substrate specificity of the different efflux pumps. The antimicrobial susceptibility of the *A. baumannii* ATCC 17978 wild-type (WT) strain and its derivative mutants was determined by the microdilution method (Table 3). The results confirmed the susceptibility of the RND efflux pump mutant *A1S_2736* to a wide range of antimicrobial agents (such as β-lactams and quinolones) and to other compounds (such as sodium dodecyl sulfate [SDS]), as previously described (14). Moreover, this mutant was highly susceptible to previously untested compounds, including deoxycholate (DC). The *A1S_3371* MATE efflux pump mutant only showed a slight difference in tetracycline even though no antimicrobial resistances were previously associated with this transporter (15). The *A1S_0710* SMR efflux pump mutant exhibited greater susceptibility than the wild type to compounds such as SDS and DC. Accordingly, overexpression of this transporter in *Escherichia coli* increased its resistance to both compounds (16). In addition, this same mutant was slightly more susceptible than the wild-type to several antimicrobials, including quinolones, tetracycline, and trimethoprim. Among the ABC efflux pump mutants, the genes *A1S_0536* and *A1S_1535* exhibited resistance to erythromycin and to gentamicin and chloramphenicol, respectively, as previously described (17, 18). Mutants of two newly described ABC permeases, encoded by the genes *A1S_1242* and *A1S_2622*, showed modest differences in their resistance profiles compared to the wild-type strain (Table 3). However, inactivation of the putative ABC permeases encoded by the genes *A1S_0027* and *A1S_1057* did not result in differences in susceptibility to any of the compounds tested. Finally, among members of the PACE superfamily, the AceI (*A1S_2063*) mutant was more susceptible to chlorhexidine and the *A1S_1503* mutant to acriflavin, as previously reported (7, 19). The latter mutant also showed a slight susceptibility to meropenem, amikacin, and minocycline (Table 3).

Surface-associated motility of the *A. baumannii* efflux pump mutants. To examine whether the selected efflux pumps play important roles in the surface-associated motility of *A. baumannii*, the motility of the knockout mutants on agar motility plates was investigated. A loss of motility was determined in mutants of the RND (*A1S_2736*), MATE (*A1S_3371*), and SMR (*A1S_0710*) efflux pumps (Fig. 2A). Introduction of plasmid pVRL1 Gm[r], carrying the corresponding wild-type gene, restored the parental surface-
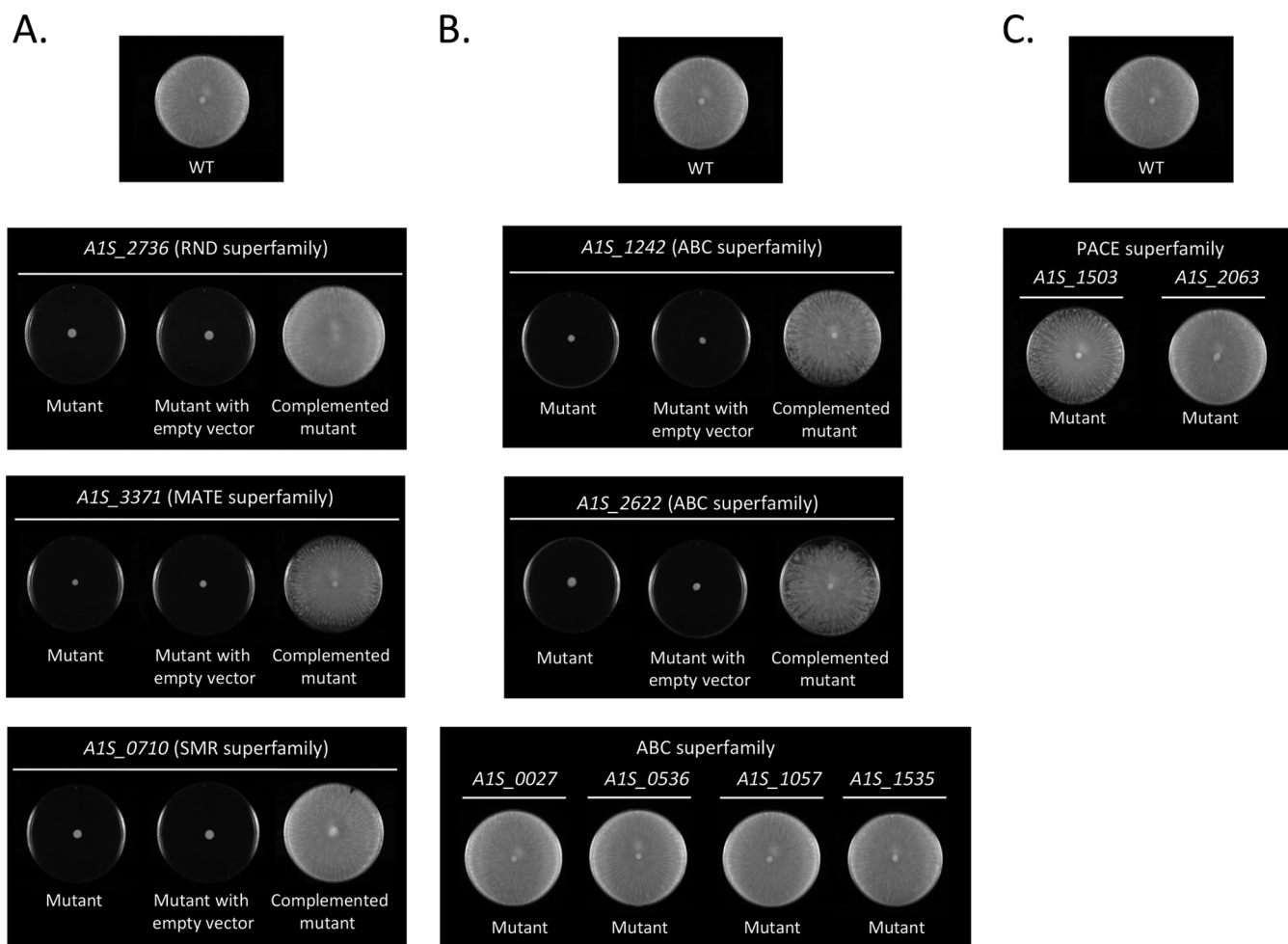
FIG 2 Surface-associated motility assays of wild-type *A. baumannii* strain ATCC 17978 (WT) and the indicated derivative mutants belonging to the RND, MATE, and SMR (A), ABC (B), and PACE (C) superfamilies. The surface-associated motility of the indicated mutants carrying either the empty expression vector pVRL1 Gm^r (mutant with empty vector) or the same plasmid containing the corresponding wild-type (WT) gene (complemented mutants) is also shown.

associated motility phenotype in all cases (Fig. 2A). In the only two *A. baumannii* ABC efflux pumps described so far (*A1S_0536* and *A1S_1535*), there were no changes in the motility profile compared to the respective wild-type strain (Fig. 2B). However, mutants of the ABC permeases identified here and encoded by the genes *A1S_1242* and *A1S_2622* showed a loss of surface-associated motility, while the motility of mutants *A1S_0027* and *A1S_1057* was similar to that of the wild-type strain (Fig. 2B). Introduction of the plasmid pVRL1 Gm^r, carrying the corresponding wild-type gene, restored motility in both *A1S_1242* and *A1S_2622* mutants (Fig. 2B). Finally, the motility of both mutants of the PACE superfamily transporters (*A1S_1503* and *A1S_2063*) was similar to that of the wild-type strain (Fig. 2C). Since the growth curves of all mutants grown in liquid medium (Luria-Bertani [LB]) were comparable to that of the respective wild-type strain (data not shown), the observed defects in surface-associated motility cannot be attributed to reductions in the bacterial growth rate.

Effect of the inactivation of genes encoding efflux pumps on the virulence of *A. baumannii.* The nematode *C. elegans* has been used as a model organism to study the virulence of *A. baumannii* by counting the progeny of worms fed with the strain of interest (20). In the present study, the virulence of mutants of the efflux pumps encoded by the genes *A1S_2736* (RND), *A1S_3371* (MATE), and *A1S_0710* (SMR), as well as that of mutants of the newly described ABC permeases (*A1S_1242* and *A1S_2662*), was attenuated compared to the wild-type strain (Fig. 3). However, in the mutants
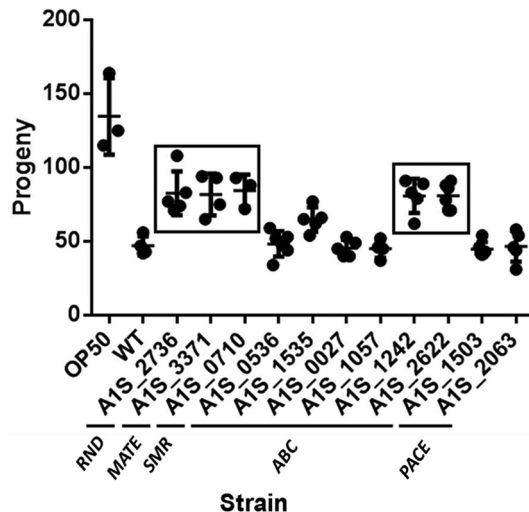
FIG 3 Representative *C. elegans* fertility assay results for wild-type *A. baumannii* strain ATCC 17978 (WT) and derivative mutants. Boxes indicate strains that differed significantly ($P < 0.05$) compared to the WT strain. *E. coli* OP50 was included as a low-virulence control.

A1S_0536, *A1S_1535*, *A1S_0027*, and *A1S_1057* (ABC), as well as mutants *A1S_1503* and *A1S_2063* (PACE), the level of virulence was similar to that of the wild-type strain (Fig. 3). The validity of the *Galleria mellonella* killing in studies of *in vivo* host-pathogen interactions in *A. baumannii* has been reported (21). Accordingly, we used this animal model to verify the results obtained in the nematode fertility assay. As expected, the virulence of the same *A. baumannii* mutants, lacking the genes encoding the permeases A1S_2736 (RND), A1S_3371 (MATE), A1S_0710 (SMR), A1S_1242 (ABC), and A1S_2662 (ABC), was significantly decreased compared to the wild-type strain (Fig. 4).

DISCUSSION

Bacterial efflux pumps actively prevent the intracellular accumulation of toxic compounds and thus, play an important role in antimicrobial resistance (6). In previous studies, we identified a quinolone efflux pump, AbaQ, belonging to the MFS superfamily and involved in the motility and virulence of *A. baumannii* (10, 11). This led us to examine the participation of other transporters, belonging to different efflux pump superfamilies, in the motility and virulence of *A. baumannii*. Mutants of genes encoding the selected transporters of each of the other five remaining superfamilies (Table 1) were therefore constructed using *A. baumannii* ATCC 17978 (Fig. 1). Their MICs to different compounds were determined using the microdilution method (Table 3).

Knowledge of the role of efflux pumps in the motility and virulence of *A. baumannii* is limited. We therefore examined the motility and virulence of mutants lacking representative transporters belonging to all superfamilies of efflux pumps using motility plates and two *in vivo* animal models, respectively. The mutant lacking the RND efflux pump *A1S_2736* had an altered motility pattern and an attenuated virulence compared to the wild-type strain (Fig. 2A, 3, and 4). In *A. baumannii*, some members of the RND superfamily have been implicated in the pathogenicity of the bacterium. For example, in *A. baumannii* strain AB5075, the attenuated virulence of two mutants (*arpA* and *arpB*) lacking components of an RND system was demonstrated in the *G. mellonella* model (22). Furthermore, inactivation of the gene *A1S_0114*, involved in the synthesis of a lipopeptide, reduces the motility and virulence of *A. baumannii*. *A1S_0114* is located on the same operon as *A1S_0116*, which encodes an RND transporter possibly involved in the secretion of this lipopeptide (23).

During the preparation of the manuscript, Knight et al. reported that the protein AdeJ, the permease component of an RND system, is involved in the motility of *Acinetobacter nosocomialis* whereas the permease AdeB, which also belongs to the
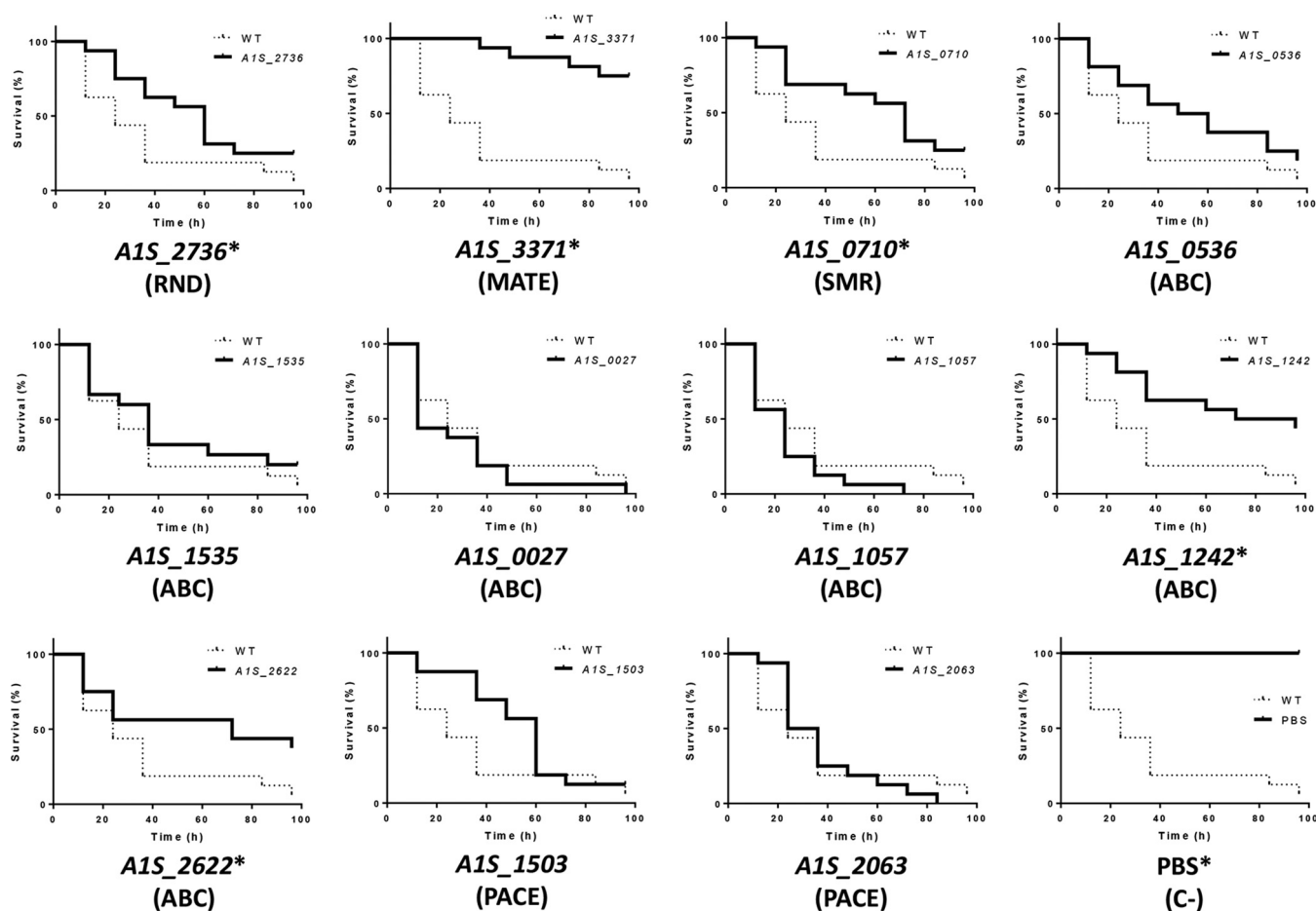
FIG 4 Survival of *G. mellonella* larvae ($n = 16$ per group) infected with approximately 10^6 CFU of the indicated *A. baumannii* strains or PBS. The concentrations of all mutant strains differed by <0.5 log CFU/larva with respect to the wild-type (WT; strain ATCC 17978). In the negative-control group (C–) the caterpillars were inoculated with PBS to take into account killing due to physical trauma. All assays were carried out simultaneously. For clarity, the results obtained from cultures inoculated with the different mutants or PBS are presented separately and compared to the group inoculated with the WT strain. In those comparisons, a *P* value of <0.05, indicated by an asterisk (*), was considered to indicate significant decreased virulence relative to the WT strain. The results of a representative assay are shown.

same efflux pump superfamily, is not (24). Although our study does not include an analysis of the AdeB protein, we analyzed the permease encoded by the gene *A1S_2736*, which has 99% identity with AdeJ of *A. nosocomialis*, and obtained the same results. Previous studies showed that the absence of the gene encoding the AdeRS two-component system, which regulates the RND efflux pump AdeABC in *A. baumannii*, causes a loss of motility but not of virulence (25). Further alterations in the motility and virulence of *A. nosocomialis* resulting from the deregulation of efflux pumps have also been demonstrated. Specifically, deletion of the repressor of the RND efflux pump, AcrAB (AcrR), increased both the motility and the virulence of the mutant (26).

The role of members of the RND superfamily in motility and virulence has also been studied in other pathogens. Thus, in *Pseudomonas aeruginosa*, the RND transporter MexEF-OprN was shown to be involved in the transport of a signal molecule that mediates quorum sensing and therefore swarming motility, biofilm formation, and the production of virulence factors (27). In the opportunistic pathogen *Stenotrophomonas maltophilia*, mutants lacking the RND transporter SmeYZ have an increased susceptibility to human serum and neutrophils, exhibit a decreased swimming motility, and are less virulent (28). The absence of the RND AcrAB efflux system in the respiratory pathogen *Klebsiella pneumoniae* results in a lower capacity of infection (29), while in *Enterobacter cloacae* a transporter homologous to the AcrAB-TolC system contributes to environmental adaptation, fitness, and virulence (30). There are also several examples

linking other RND efflux pumps to virulence in the pathogens *Borrelia burgdorferi* (31), *Salmonella enterica* (32), *Vibrio cholerae* (33), and *Erwinia amylovora* (34).

Mutants in the MATE efflux pump *A1S_3371* and the SMR efflux pump *A1S_0710* also show a reduced motility and an attenuated virulence in *A. baumannii* (Fig. 2A, 3, and 4). In *Ralstonia solanacearum* the absence of the MATE DinF efflux pump causes a decreased virulence of the bacterium in tomato plants (35), and in *K. pneumoniae* the SMR KpnEF efflux pump participates in capsule synthesis and therefore, presumably, virulence as well (36).

While the involvement of ABC efflux pumps in the virulence of *A. baumannii* has not been demonstrated, we previously reported a gene encoding a putative taurine ABC transporter component involved in both motility and virulence of this bacterial species (10). Inactivation of the ABC transporters encoded by the previously described genes *A1S_0536* and *A1S_1535* had no effects on the motility or virulence of *A. baumannii*. However, in this work we newly identified two genes encoding ABC permeases (*A1S_1242* and *A1S_2622*) that seem to be involved in both functions (Fig. 2B, 3, and 4). In contrast, mutants of other genes encoding putative components of ABC transporters (*A1S_0027* and *A1S_1057*) did not show changes in motility or virulence (Fig. 2B, 3, and 4). Genes encoding ABC efflux pump components in other pathogens have also been implicated in virulence, such as the ABC efflux pumps MacAB in *S. enterica* (37), MrtAB in *Yersinia pseudotuberculosis* (38), YejABEF in *Brucella melitensis* (39), and Ssu in *Xanthomonas citri* (40).

The motility and virulence profiles of *A. baumannii* mutants belonging to the PACE superfamily (*A1S_1503* and *A1S_2063*) were very similar to those of the wild-type strain (Fig. 2C, 3, and 4). To our knowledge, the role of the recently discovered PACE superfamily in virulence has not been studied in any other microorganism. However, since these protein are highly conserved in other pathogenic bacteria, while their physiological role remains to be determined, their participation in drug resistance seems to be accidental (41).

The mechanisms by which efflux pumps participate in bacterial pathogenicity have yet to be fully elucidated. However, it is clear that the loss of an efflux pump may in itself, have secondary consequences, by altering the composition and structure of the cell envelope. Furthermore, efflux pumps also may contribute to the secretion of virulence factors, as observed for the RND-type transporter VexB of *V. cholerae* and the ABC-type transporter MacAB of *E. coli* (42, 43). In addition, efflux pumps may translocate host-derived antimicrobial molecules, allowing survival of the pathogen. This is the case of the MFS efflux pump FarAB of *Neisseria gonorrhoeae*, which protects the bacterium against long-chained antibacterial fatty acids produced by the host (44). Other examples are the RND efflux pumps AcrAB and CmeABC (belonging to *E. coli* and *Campylobacter jejuni*, respectively), both of which translocate the bactericidal bile encountered in the intestinal tracts of their host animals (45, 46). Likewise, efflux pumps may affect motility and virulence, by translocating molecules that modulate processes such as quorum sensing, as shown for the RND efflux pump MexEF-OprN of *P. aeruginosa* (27). In *A. baumannii*, the function of these efflux systems might be the translocation of surfactants or molecules related to surface-associated motility or quorum sensing, since alterations in molecules mediating bacterial population responses have been related to the inhibition of motility (47).

Overall, our study provides new insights into the efflux pumps of *A. baumannii*, especially their involvement in the motility and virulence of the bacterium. Although not all efflux pumps contribute to motility and virulence, our results demonstrate that in *A. baumannii* ATCC 17978, all superfamilies of efflux pumps, except the recently discovered PACE superfamily, include transporters involved in surface-associated motility. Moreover, all mutants lacking an efflux transporter involved in this kind of motility were also less virulent. While the mechanisms underlying the participation of these efflux pumps in motility and virulence remain unclear, further studies of these proteins will broaden current understanding of the pathogenicity of *A. baumannii*. In addition, the results reported here suggest that inhibitors against these transporters will be

valuable tools with which to fight multiresistant pathogens, by targeting not only antimicrobial resistance but also bacterial motility, thereby reducing the capacity for host colonization.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *A. baumannii* ATCC 17978 and *Escherichia coli* (strains DH5α and OP50) were grown at 37°C in LB agar or in LB broth with shaking at 180 rpm. Plasmids were maintained and selected in *E. coli* and *A. baumannii* hosts using the same medium but supplemented as needed with kanamycin (50 mg/liter) or gentamicin (20 mg/liter for *E. coli* or 40 mg/liter for *A. baumannii*). Bacterial growth was monitored as follows. A bacterial culture in LB broth was inoculated with a 1:100 dilution of an overnight culture and incubated with shaking (180 rpm) at 37°C. The optical density at 600 nm (OD_{600}) was measured hourly. Each test was performed in triplicate. The plasmids pCR-BluntII-TOPO (Invitrogen) and pVRL1 Gm^r (48) were used for mutant construction and complementation, respectively.

Construction of *A. baumannii* knockouts and complementation. Gene inactivation was carried out as previously described (49). Briefly, an internal fragment of the target gene was PCR amplified with the corresponding primers (Table 2) using genomic DNA from *A. baumannii* ATCC 17978 as the template. This fragment was cloned into the kanamycin and zeocin resistance plasmid pCR-BluntII-TOPO, which is unable to replicate in *A. baumannii*, and then propagated in *E. coli* DH5α. The recombinant plasmids (0.1 μg) were electroporated into *A. baumannii* ATCC 17978. Candidate transformant clones were selected on kanamycin-containing plates. Mutants were confirmed by sequencing (Macrogen) of the PCR products. To determine the stability of the obtained mutants, the cultures were passaged 10 times (every 24 h) without selective pressure and the colonies that developed on LB agar plates with or without kanamycin were counted. For complementation, all genes were cloned into the XbaI-NcoI site or, when necessary, into the XbaI site of the pVRL1 Gm^r vector. The recombinant plasmids were propagated in *E. coli* DH5α and then electroporated into the corresponding *A. baumannii* mutant. The complemented mutant was selected on plates containing gentamicin. The resulting recombinant plasmids were verified by PCR and sequencing. The primers used in this study are shown in Table 2.

***In vitro* antimicrobial susceptibility assay.** MICs for all antimicrobials and disinfectants (Sigma) were determined on 96-well plates by the broth microdilution method, using Mueller-Hinton medium (Merck), according to CLSI guidelines (50). All MIC assays were repeated a minimum of three times.

Motility assays. Motility assays were conducted on fresh LB agar motility plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.5% glucose, and 0.5% Difco agar) used on the day of their preparation. To test the motility of the different *A. baumannii* strains, a 5-μl drop of an exponential-phase culture of each strain was placed in the center of the plate. The inoculated plates were incubated at 30°C, usually for 16 to 20 h, until growth of the wild-type strain reached to the plate border. All assays were carried out in triplicate in a minimum of three independent experiments. The representative images shown in the figures were obtained using the ChemiDoc XRS+ System (Bio-Rad).

***In vivo* nematode model of virulence.** The virulence of the different *A. baumannii* strains was determined in a nematode fertility assay performed as previously described (20). Briefly, *C. elegans* strain N2 was fed with low-virulence *E. coli* strain OP50 grown as a lawn on nematode growth medium (NGM) plates. To physiologically synchronize the worms, the nematode eggs were hatched in M9 medium (0.02 M KH_2PO_4, 0.04 M Na_2HPO_4, 0.08 M NaCl, and 0.001 M $MgSO_4$) and worms in the first larval (L1) stage were growth arrested overnight at 16°C. The L1 worms were then grown to the L4 stage on NGM plates seeded with the bacterial strains investigated in this study. One nematode in the L4 state was placed on a peptone-glucose-sorbitol plate with the same strain and incubated at 25°C. During the next 3 days, adult nematodes were transferred to new plates seeded with the same bacterial strain. To determine the fertility of the worms, their progeny was counted daily, 48 h after removal of the parent, using a stereomicroscope (Olympus SZ51). Six independent replicates were established for each strain and each fertility assay was performed in triplicate.

***G. mellonella* killing assay.** The wax worm model of virulence was used following the methodology described by Peleg et al. (21) with some modifications. Briefly, 16 caterpillars (approximately 300 mg in weight) were used for each *A. baumannii* strain. Cells from bacterial inocula prepared from exponential cultures (OD_{600} = 0.5, approximately 10^8 CFU/ml) were washed with phosphate-buffered saline (PBS) and adjusted again to the same OD_{600}. The concentrations of the inocula were confirmed by bacterial colony counts on LB agar. A 10-μl inoculum of the corresponding strain or 10 μl of PBS (as a negative control) was injected into the hemocoel of each worm through the last left proleg. The caterpillars were incubated at 37°C in the dark, and their survival was checked every 12 h for a total of 96 h. All *G. mellonella* killing experiments were performed at least twice.

Statistics. Data for the nematode model of virulence are presented as means ± the standard deviations and were analyzed using two-tailed, one-way analysis of variance, followed by the Tukey test for *post hoc* multiple group comparisons. In the *G. mellonella* killing assay, survival curves were plotted using the Kaplan-Meier method. Differences in survival were calculated using the log-rank test. In all analyses, a *P* value of <0.05 was considered to indicate statistical significance.

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