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OmpA Is the Principal Nonspecific Slow Porin of *Acinetobacter baumannii*

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***Acinetobacter* species show high levels of intrinsic resistance to many antibiotics. The major protein species in the outer membrane of *Acinetobacter baumannii* does not belong to the high-permeability trimeric porin family, which includes *Escherichia coli* OmpF/OmpC, and instead is a close homolog of *E. coli* OmpA and *Pseudomonas aeruginosa* OprF. We characterized the pore-forming function of this OmpA homolog, $OmpA_{Ab}$, by a reconstitution assay. $OmpA_{Ab}$ produced very low pore-forming activity, about 70-fold lower than that of OmpF and an activity similar to that of *E. coli* OmpA and *P. aeruginosa* OprF. The pore size of the $OmpA_{Ab}$ channel was similar to that of OprF, i.e., about 2 nm in diameter. The low permeability of $OmpA_{Ab}$ is not due to the inactivation of this protein during purification, because the permeability of the whole *A. baumannii* outer membrane was also very low. Furthermore, the outer membrane permeability to cephalothin and cephaloridine, measured in intact cells, was about 100-fold lower than that of *E. coli* K-12. The permeability of cephalothin and cephaloridine in *A. baumannii* was decreased 2- to 3-fold when the $ompA_{Ab}$ gene was deleted. These results show that $OmpA_{Ab}$ is the major nonspecific channel in *A. baumannii*. The low permeability of this porin, together with the presence of constitutive β-lactamases and multidrug efflux pumps, such as AdeABC and AdeIJK, appears to be essential for the high levels of intrinsic resistance to a number of antibiotics.**

Besides *Pseudomonas aeruginosa*, there are other genera of Gram-negative bacteria, such as the *Acinetobacter* species, that often produce multidrug-resistant and even pan-resistant strains (4, 32). Since *P. aeruginosa* produces a major porin of unusually low permeability, or a "slow porin" (40, 43), that plays a major role in its high levels of intrinsic resistance (3, 49), it is suspected that a similar situation may also exist in *Acinetobacter* species. It has been reported that the permeability coefficients of the *Acinetobacter calcoaceticus* outer membrane (OM) to zwitterionic cephalosporins were 2 to 7 times lower than the already very low values for the same β-lactams in the OM of *P. aeruginosa* (37). However, the identity of the major porin species in *Acinetobacter* has not been established so far.

The major protein of the *Acinetobacter baumannii* OM was named OmpAb (18), or HMP-AB (15), and belongs to the OmpA-like family, as sequence comparison revealed a clear homology with the monomeric OM protein A (OmpA) of *Enterobacteriaceae* and OM protein F (OprF) of *Pseudomonas* spp. OmpAb and HMP-AB were reported, however, to produce permeability comparable to or even higher than that of the classical trimeric porins of *Escherichia coli*, such as OmpF and OmpC (15, 18). If this is correct, the presence of such porins does not explain the low permeability of the *Acinetobacter* OM. In contrast, another study found that the OmpA homolog in *A. baumannii* had very low permeability (30). Furthermore, the high permeability of the OmpA homolog is not consistent with our earlier observation that *E. coli* OmpA (41) and *P. aeruginosa* OprF (25, 50) behave as slow porins (43) that produce very slow permeation of solutes yet have pore sizes that are either similar to, or larger than, that of OmpF. Thus, the existing literature is quite confusing, and indeed, one review (47) argues that "one of the limitations of our knowledge of *A. baumannii* is the lack of information concerning its OM proteins and the permeability."

Because the properties of the porin channel in *Acinetobacter* species are crucial for the understanding of their resistance mechanism and also the development of antibiotics for the control of this important pathogen, we reexamined the properties of the OmpA family protein in *A. baumannii* (hereafter it is called $OmpA_{Ab}$ to distinguish it from *E. coli* OmpA). The results show that it produces a low-permeability channel that is similar in both permeability and size to that of *P. aeruginosa* OprF. We then show that an *A. baumannii* mutant that lacks $OmpA_{Ab}$ is defective in the uptake of a model antibiotic, hydrophilic cephalosporins, indicating that this protein is, indeed, the major nonspecific porin of this organism.

MATERIALS AND METHODS

Bacterial strains. *A. baumannii* ATCC 17978 and its derivatives were used for the study of OM permeability. $OmpA_{Ab}$ was purified either from this strain or after its expression in a hexahistidine-tagged form in porinless *E. coli* strain HN705 (*ompC ompF*::Tn*5*) (41). *A. baumannii* strains BM4651 (Δ*adeABC*), BM4679 (Δ*adeIJK*), and BM4652 (Δ*adeABC* Δ*adeIJK*) and its parent strain BM4454 (11, 21) were obtained from P. Courvalin.

Expression plasmid for hexahistidine-tagged $OmpA_{Ab}$. The mature part of the $ompA_{Ab}$ gene (coding for amino acids 23 to 346) was cloned by PCR amplification using the Expand Long Template PCR system (Roche) and inserted between the PstI and NotI sites of the vector plasmid, pKY9790 (40), which was previously modified by inserting the signal sequence of the *E. coli* OmpA protein followed by a hexahistidine tag just in front of the PstI site, generating the plasmid pKY-$OmpA_{Ab}$.

Purification of the hexahistidine-tagged $OmpA_{Ab}$. Purification of hexahistidine-tagged $OmpA_{Ab}$ from HN705 cells with freshly transformed pKY-$OmpA_{Ab}$ was performed essentially as described earlier for His-tagged OprF (39). Briefly, a 1-liter LB culture was grown at 30°C overnight with shaking in the presence of 30 μg/ml chloramphenicol but without IPTG (isopropyl-β-D-thiogalactopyranoside) induction. The

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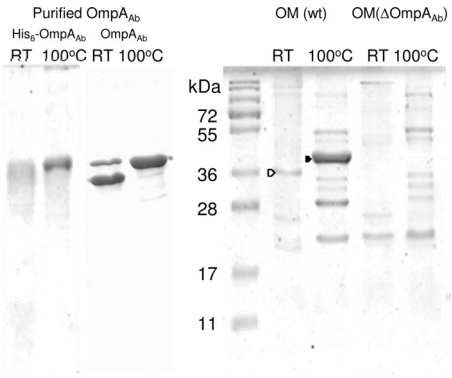

FIG 1 SDS-PAGE of the purified OmpA$_{Ab}$ preparations and the OM proteins from *A. baumannii* ATCC 17978 and its Δ*ompA$_{Ab}$* mutant. Samples containing 5 μg of purified hexahistidine-tagged OmpA$_{Ab}$ or untagged OmpA$_{Ab}$ or 10 μg of total OM proteins from the parent or its ΔOmpA$_{Ab}$ mutant strain were applied to SDS-PAGE either unheated (room temperature [RT]) or after heating at 100°C for 5 min in the sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 5% β-mercaptoethanol, 2% SDS). The gels were analyzed by Coomassie blue staining. The bands containing the partially denatured (heat-modifiable) and completely denatured OmpA$_{Ab}$ are indicated by open and filled arrowheads, respectively.

crude envelope fraction, prepared with a French pressure cell disruption followed by centrifugation, was extracted with 0.5% Sarkosyl to remove inner membrane proteins, and then the OM proteins were solubilized with a buffer containing 1.5% dodecyl-β-D-maltoside and 0.25 mg/ml lysozyme. Hexahistidine-tagged OmpA$_{Ab}$ was isolated with a Ni-NTA Superflow column (Qiagen) as described for His-tagged OprF (39). Imidazole was then removed, and the protein sample was concentrated by using centrifugal filtration with Vivaspin20 (molecular weight cutoff, 10,000; Vivascience, Inc.) (39).

Purification of unmodified OmpA$_{Ab}$. The OmpA$_{Ab}$ protein without a hexahistidine tag was isolated from the outer membrane of *A. baumannii* ATCC 17978. Cells were disrupted with a French pressure cell treatment, and the envelope fraction was extracted with 0.5% Sarkosyl to remove inner membrane proteins. The outer membrane proteins were extracted overnight with 68 mM octyl β-D-glucoside in 20 mM HEPES buffer (pH 7.5) containing 5 mM EDTA and 0.1 mg/ml lysozyme at 0°C. The extract was then mixed with an equal volume of the sample buffer (10% glycerol, 1.5 M aminocaproic acid, 100 mM Bis Tris-HCl [pH 7.0], 0.5% Coomassie brilliant blue G250), and the proteins were separated by blue native gel electrophoresis (38) at 100 V for 4 h at 4°C in a gel system consisting of 4% stacking gel (pH 7.0) and 10% separating gel (pH 7.0). For visualization of protein bands, a narrow lane was cut out and stained with Coomassie brilliant blue R250 staining solution (0.25% Coomassie brilliant blue R250, 50% methanol, 5% acetic acid) for 5 min. The band containing OmpA$_{Ab}$ was cut out from the unstained portion of the gel and was subjected to electroelution in a buffer containing 0.1% octylpolyoxyethylene (octyl-POE), 192 mM glycine, and 25 mM Tris base as described by Dé et al. (12). The sample consisted of an essentially pure preparation of OmpA$_{Ab}$, as seen in Fig. 1.

Determination of pore-forming activity. Pore-forming activity was routinely assayed by determining the osmotic swelling rates of proteoliposomes containing different amounts of OmpA$_{Ab}$ in an isotonic solution of sugars, usually L-arabinose (25). As a measure of pore-forming activity, rates of swelling were expressed in milliunits of optical density (OD)/min/μg protein (mOD/min/μg protein). The pore size was inferred from the dependence of the swelling rates on the sizes of the permeating solutes, as described earlier (25).

Construction of an *ompA$_{Ab}$* null mutant of *A. baumannii*. In order to assess the contribution of OmpA$_{Ab}$ in the nonspecific diffusion across the OM, the *ompA$_{Ab}$* gene was replaced by a kanamycin resistance gene (Kan^r) by using a gene replacement plasmid, pEX18Tc (17). Two approximately 1,000-bp regions located upstream and downstream from the *ompA$_{Ab}$* gene were amplified by PCR using primers (UpFw, 5′-ACGC GTCGACCGAATGCTTCGTCAGTTTGAGGCAACATGGCAAAAACAG CTG; UpRv, 5′-CGGAATTCTTTGAGTTCTTGAACAGTAAAAAAGCG ACTCGTTAGAGTCGC; DownFw, 5′-AACTGCAGGGATATCCTCCA GAGATAACAATTGTTGTTCAAGCTCAGCCT; and DownRv, 5′-CG GGATCCGCAGTATATTGCAAAATGAAGAACGGGTTAGCTTCTGCA TTA). These PCR products, Upstream and Downstream, were digested and ligated between SalI and EcoRI and PstI and BamHI sites, respectively, of pEX18Tc. The *kan* gene (816 bp) of pACYC was amplified by PCR using primers containing PstI and EcoRI restriction sites. This PCR product was digested and ligated between PstI and EcoRI sites of pEX18Tc containing the Upstream and Downstream segments.

For gene replacement, the recombinant plasmid was electroporated into *A. baumannii* ATCC 17978. Integrants of the Kan^r plasmid were selected on LB plates containing 30 μg/ml kanamycin, and these were then plated on LB plates containing 5% sucrose in order to select for strains that have lost the plasmid sequence. Sucrose-resistant (Suc^r) colonies were ascertained to have the tetracycline-sensitive phenotype as the result of plasmid eviction. The absence of the *ompA$_{Ab}$* gene sequence was monitored by PCR and was further confirmed by the absence of the OmpA$_{Ab}$ band in the SDS-PAGE of OMs.

MIC determination. MIC values were determined with 96-well microtiter plates using a standard 2-fold broth microdilution method with LB broth containing 5 mM $MgCl_2$.

Expression of the chromosomally encoded β-lactamases. The strain of *A. baumannii* used is known to contain two β-lactamase genes coding for a class C AmpC enzyme and a class D oxacillinase of the OXA-51 family (1). The standard isoelectric focusing (IEF) procedure was used with a precast gel (Novex pH 3-10, Invitrogen), followed by nitrocefin staining to evaluate the expression of these genes essentially as described by Philippon et al. (34). We used pI 4.45 to 9.6 IEF standards from Bio-Rad as well as the TEM enzyme of pI 5.4 as standards.

Assay of EtBr influx in intact cells. The rate of entry of ethidium bromide (EtBr) into intact cells was measured as an indicator of the permeability of the lipid bilayer domains of OM, because the diffusion across the inner membrane bilayer, composed of the conventional phospholipids, is expected to be orders of magnitude faster, and thus, the flux across the OM becomes the rate-limiting step. Overnight cultures were diluted 20-fold in fresh LB containing 5 mM $MgCl_2$, and the cultures were grown with shaking at 37°C. Ten-milliliter portions were harvested by centrifugation at room temperature, and the cells were washed twice with 50 mM K-phosphate buffer, pH 7, by centrifugation at room temperature. The amount of cells corresponding to 0.4 optical density at 600 nm (OD_{600}) units was added into 2 ml of the same K-phosphate buffer containing a 100 μM concentration of the proton conductor carbonyl cyanide 3-chlorophenylhydrazone (CCCP). After addition of EtBr (6 μM final concentration), the fluorescence of the EtBr-nucleic acid complex generated by the influx of EtBr into the cells was determined at room temperature by using a Shimadzu RF6301 spectrofluorometer with excitation and emission wavelengths of 545 nm and 600 nm, respectively.

Determination of cephalosporin flux. The diffusion rates of cephalosporins through the OM were determined by measuring the rates of their hydrolysis by periplasmic β-lactamase(s) in intact cells (28). Bacteria were grown in 5 ml LB overnight at 37°C, and 1.5 ml of the culture was diluted into 50 ml of LB containing 5 mM $MgCl_2$. The cells were grown at 37°C for 3 h with aeration by shaking and were washed once with 50 mM potassium phosphate buffer, pH 7.0, containing 5 mM $MgCl_2$. Washed cells were resuspended in the same buffer at an OD_{600} of 0.8 (corresponding to 0.24 mg [dry weight]/ml). One portion was sonicated to release all β-lactamase into the solution, and the other portion was used for the intact-cell assay. In order to correct for hydrolysis caused by the enzyme that leaked out into the suspension buffer, one portion of the cell suspension was centrifuged at 13,000 rpm in a microcentrifuge for 2 min and the super-

natant was used for the assay. Hydrolysis was monitored with a Uvikon 860 spectrophotometer, custom designed to minimize the effect of light scattering, at 486 nm for nitrocefin (31). For cephalothin and cephaloridine, 260-nm absorption was used, with cells of 10- and 1-mm light paths, respectively (28). The permeability coefficient *P* was obtained from cells treated with 100 μM CCCP in order to inactivate the efflux pumps.

RESULTS

Characterization of the *A. baumannii* $OmpA_{Ab}$ channel. As described in the introduction, there is no consensus on the rate of solute influx through the $OmpA_{Ab}$ channel. We therefore reexamined the properties of the $OmpA_{Ab}$ channel by producing a recombinant $OmpA_{Ab}$ containing the *E. coli* OmpA signal peptide and a hexahistidine tag at the N terminus. This protein was expressed in *E. coli* strain HN705, which lacks both OmpF and OmpC, so that the final product would not be contaminated by the high-permeability porins of *E. coli*. Under our condition of expression utilizing the basal transcription level of the pTac promoter, most of $OmpA_{Ab}$ was inserted into the *E. coli* OM (not shown) and could be extracted under the conditions which led to extraction of OM proteins (39, 44).

Figure 1 shows the Coomassie-stained SDS-PAGE of the purified hexahistidine-tagged $OmpA_{Ab}$ and the OM protein profile of *A. baumannii* grown at 37°C in L broth. $OmpA_{Ab}$ is by far the most abundant protein in the OM of *A. baumannii*, as described earlier (18), and this protein showed a characteristic mobility shift, sometimes called "heat modifiability," so that it travels more slowly after denaturation at 100°C, similar to *E. coli* OmpA and *P. aeruginosa* OprF (44). The purified hexahistidine-tagged OmpA protein (expected size, 38.8 kDa) appeared as a broad band with an apparent molecular mass of around 40 kDa, and this protein migrated more slowly after the heat denaturation. The SDS-PAGE profile in Fig. 1 suggests that the majority of $OmpA_{Ab}$ is monomeric, especially after heating at 100°C, although it is possible that some of it is loosely associated in an oligomeric state before heating.

For the functional assay, the purified hexahistidine-tagged $OmpA_{Ab}$ was reconstituted into proteoliposomes, and the rates of their osmotic swelling in isotonic L-arabinose were determined (Fig. 2A). The specific pore-forming activity of $OmpA_{Ab}$ was 7.2 ± 0.9 mOD/min/μg, which was similar to the activities of *E. coli* OmpA (6 mOD/min/μg [41]) and *P. aeruginosa* OprF (4.5 mOD/min/μg [23]) and which was nearly 100-fold lower than the value obtained with *E. coli* OmpF (400 mOD/min/μg [27]). This low permeability is not caused by the hexahistidine tag sequence, because tag-free $OmpA_{Ab}$, purified from the outer membrane of *A. baumannii* ATCC 17978 as described in Materials and Methods, showed a similarly low permeability (7.9 ± 0.4 mOD/min/μg) (data not shown). Furthermore, it is not due to its inactivation during purification or to improper folding during expression in *E. coli*, as the proteoliposomes reconstituted with fragments of the intact OM of *A. baumannii* also showed about 200-fold lower permeability (that was similar to the results with the OM of *P. aeruginosa*) than the vesicles reconstituted with the OM of wild-type *E. coli* (Fig. 1B). The OM permeability of *E. coli* HN705 was quite low, as the high-permeability porins, OmpF and OmpC, were absent (Fig. 1B). These data strongly suggest that the OM of *A. baumannii* does not contain significant amounts of high-permeability porins, a situation similar to the OM of *P. aeruginosa*.

The pore size of $OmpA_{Ab}$ was estimated by the osmotic swelling of proteoliposomes in sugars of different sizes (Fig. 3). The

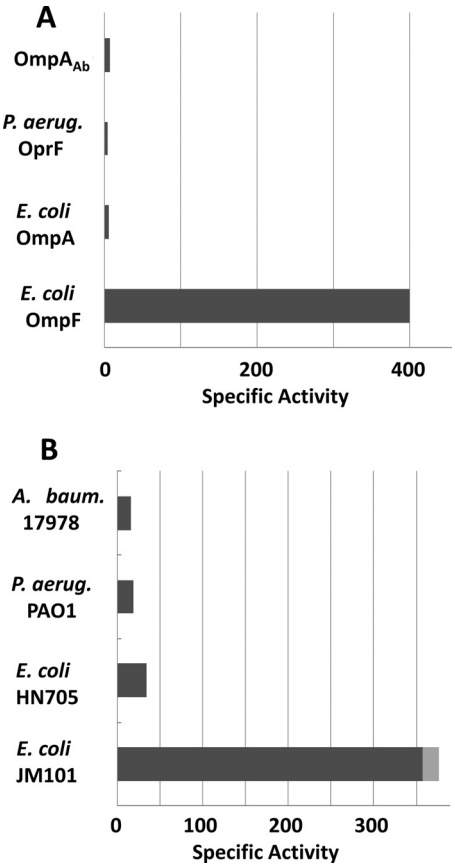

FIG 2 Comparison of specific pore-forming activities of OmpA homolog porins from different origins, including *A. baumannii* (*A. baum.*), *P. aeruginosa* (*P. aerug.*), and *E. coli*. (A) Specific pore-forming activity for L-arabinose diffusion through various purified porins in mOD min^{-1} mg^{-1}. (B) Specific pore-forming activity measured for L-arabinose diffusion through the proteoliposomes reconstituted from crude OM fragments of different strains (0.5 μg of OM from *E. coli* JM101, 5 μg of OM from *E. coli* HN705 [$OmpF^-$, $OmpC^-$], *P. aeruginosa* PAO1, and *A. baumannii* ATCC 17978). With JM101, the standard deviation is shown as a gray box. The unit is mOD min^{-1} μg^{-1}.

hexahistidine tag-free $OmpA_{Ab}$ behaved in a nearly identical manner (data not shown). The behavior of $OmpA_{Ab}$ appeared to be very similar to that of *P. aeruginosa* OprF (25), and thus, the size of the $OmpA_{Ab}$ channel is larger than those of the classical high-permeability porins, such as *E. coli* OmpF. This combination of large channel size and a slow permeation rate, which may appear paradoxical, is common among OmpA family "slow porins" and is caused by the fact that only a minority of these protein molecules folds into an open-channel conformation (23, 39, 40, 43).

Influx of antibiotics and dyes into an *A. baumannii* Δ$ompA_{Ab}$ mutant. In order to confirm that $OmpA_{Ab}$ plays a major role in the penetration of solutes across the OM, we made a mutant lacking $OmpA_{Ab}$ by replacing the chromosomal $ompA_{Ab}$ gene with the Kan^r cassette. The OM from the Δ$ompA_{Ab}$ strain indeed showed a complete absence of $OmpA_{Ab}$ (Fig. 1).

The Δ$ompA_{Ab}$ mutant grew relatively well in L broth with a doubling time of 29 min, only slightly slower than that of the parent strain (26 min). However, we noted that the Δ$ompA_{Ab}$ mutant had a significantly increased permeability for hydrophobic dyes, as seen by the uptake rates of EtBr in the presence of the

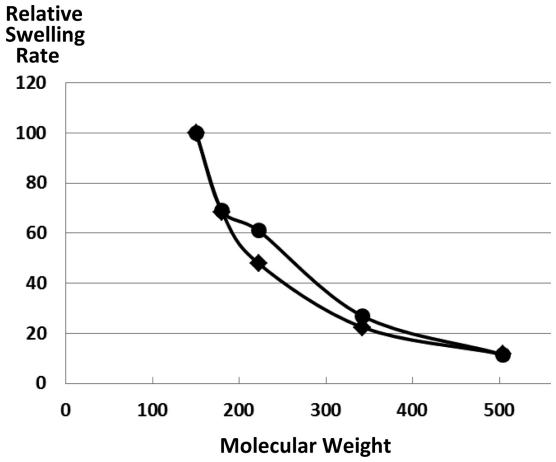

FIG 3 Diffusion rates of solutes of various sizes through the *A. baumannii* $OmpA_{Ab}$ porin. A total of 20 μg of hexahistidine-tagged $OmpA_{Ab}$ was reconstituted into proteoliposomes as described in Materials and Methods. They were diluted in isosmotic solutions of L-arabinose (molecular weight, 150), D-glucose (molecular weight, 180), *N*-acetyl-D-glucosamine (molecular weight, 221), sucrose (molecular weight, 342), and raffinose (molecular weight, 504), and the initial rates of swelling of the liposomes are shown relative to that in L-arabinose. The data with hexahistidine-tagged OmpA (circles) were similar to previous data with OprF purified from *P. aeruginosa* PAO1 (squares) (26, 28).

TABLE 1 Antibiotic susceptibilities of the wild type and the Δ$ompA_{Ab}$ mutant of *A. baumannii*

Agent	MIC (μg/ml)	
	Wild type	Δ$ompA_{Ab}$ mutant
Ampicillin	250	125
Benzylpenicillin	125	62
Carbenicillin	31	31
Nitrocefin	500	125
Cephaloridine	250	250
Cephalothin	500	500
Ceftriaxone	31	31
Cefotaxime	31	31
Aztreonam	31	31
Imipenem	0.3	0.3
Tetracycline	6	6
Chloramphenicol	250	250
Novobiocin	10	5
Gentamicin	6	6
Polymyxin B	20	2.5

proton conductor CCCP (Fig. 4). Since EtBr is large, lipophilic, and rather rigid, it is believed to diffuse mainly across the bilayer region of OM rather than through the narrow porin channel. Thus, it seems likely that the absence of this major protein, $OmpA_{Ab}$, produces empty spaces in the OM, which becomes filled by phospholipids, producing phospholipid bilayer domains in the OM, as seen in the "deep rough" mutants of enteric bacteria (2). A similar phenotypic change was observed in *P. aeruginosa* when the *oprF* gene was deleted, as shown by the severalfold increases in the entry of the hydrophobic fluorescent probe 1-*N*-phenylnaphthylamine (48).

Some of this increased EtBr permeability was suppressed by the addition of 5 mM $MgCl_2$ to the assay mixture (Fig. 4). Therefore, we used LB broth supplemented with 5 mM $MgCl_2$ for the measurement of antibiotic susceptibility. The broth 2-fold dilution assay of MICs (Table 1) showed that instead of the expected increase in MICs, in the Δ$ompA_{Ab}$ mutant, there was a small decrease in the MICs of lipophilic compounds, such as benzylpenicillin, nitrocefin, and novobiocin, and a large decrease for the

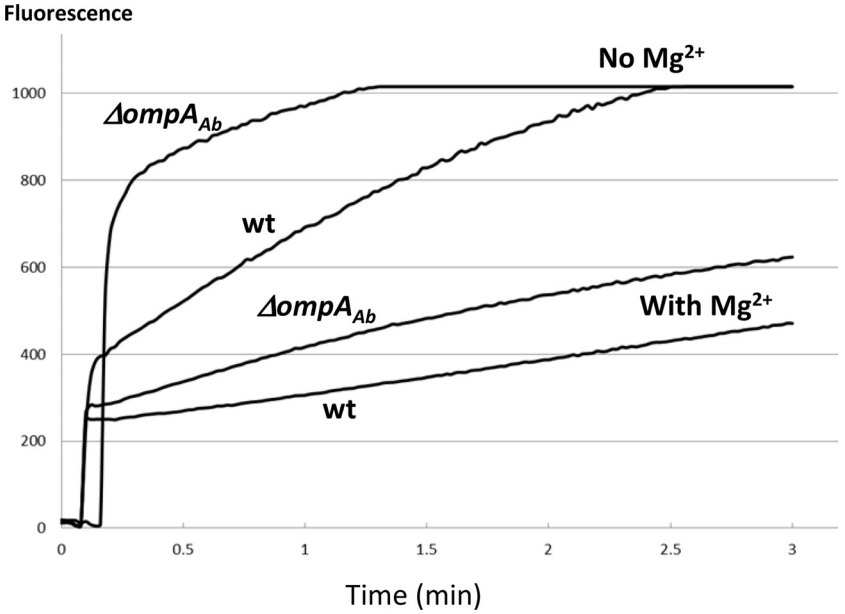

FIG 4 Accumulation of ethidium bromide (EtBr) in intact cells of the Δ$ompA_{Ab}$ mutant and its parent strain. The amount of cells corresponding to 0.4 OD_{600} units was added into 2 ml of 50 mM K-phosphate buffer, pH 7.0, containing 100 μM CCCP with or without 5 mM $MgCl_2$. Ten seconds later, EtBr (final concentration, 6 μM) was added to the mixture, and the fluorescence of the EtBr-nucleic acid complex generated by the influx of EtBr into cells was determined as described in Materials and Methods. Intensity of the fluorescence emission (ordinate) is in arbitrary units.

TABLE 2 OM permeability to cephalosporins

Substrate (concn [mM])	Strain	Hydrolysis rate (nmol/s/mg)[b]		Permeability coefficient (cm/s)
		Intact cells	Extract	
Nitrocefin (0.1)	Parent	0.006 ± 0.002	0.253 ± 0.013	0.4×10^{-6}
	Δ*ompA*$_{Ab}$ mutant	0.018 ± 0.006	0.250 ± 0.013	1.4×10^{-6}
Cephalothin (0.1)	Parent	0.022 ± 0.007	0.295 ± 0.045	1.7×10^{-6}
	Δ*ompA*$_{Ab}$ mutant	0.011 ± 0.008	0.248 ± 0.020	0.9×10^{-6}
Cephaloridine (1)	Parent	0.071 ± 0.020	0.434 ± 0.048	0.57×10^{-6}
	Parent (no CCCP)	0.027 ± 0.013	0.434 ± 0.048	0.21×10^{-6}
	Δ*ompA*$_{Ab}$ mutant[a]	0.033 ± 0.014	0.539 ± 0.066	0.26×10^{-6}

[a] Although we also attempted to determine the cephaloridine permeability without CCCP in this mutant, the net influx was so low that it was impossible to obtain reproducible values.
[b] Values are means and standard deviations.

agent that attacks the lipopolysaccharide (LPS)-phospholipid asymmetric bilayer of the OM, polymyxin B. Although many common antibiotics are normally thought to traverse the OM barrier mainly through the water-filled channels of porins (24, 29), in the Δ*ompA*$_{Ab}$ mutant, the increased diffusion across the modified lipid bilayer domain (described above) now presumably plays a predominant role, causing this increased susceptibility to lipophilic agents.

Expression of endogenous β-lactamases in *A. baumannii* ATCC 17978. Since we used the endogenous β-lactamase(s) in the OM permeability assay described below, it was important to assess the expression levels of the two endogenous enzymes, AmpC (pI > 9.0) (33) and OXA-51 (pI = 7.0) (7). When about 0.1 mg of the extract of ATCC 17978, grown in LB, was analyzed by isoelectric focusing (see Materials and Methods), the only activity seen had a pI close to 9 and no activity was seen in the area close to a pI of 7 (results not shown). We therefore assume that under our growth conditions, only the AmpC enzyme is expressed to a significant degree.

When the kinetics of hydrolysis of cephalosporins were examined by using crude extracts of ATCC 17978 cells, Kan values estimated by curve fitting were 63, 72, and 550 μM for nitrocefin, cephalothin, and cephaloridine, respectively, quite similar to the values reported earlier for two clinical strains of *A. baumannii* (33) and for a strain of *A. calcoaceticus* (presumably *A. baumannii*) (37). The V_{max} values were 0.40, 0.48, and 0.70 nmol/s/mg (dry weight) cells, respectively, and again the relative rates among the three agents were similar to those reported earlier (33).

Permeability of cephalosporins through the OM of *A. baumannii* in an intact-cell assay. Because the OM integrity is compromised as described above in the Δ*ompA*$_{Ab}$ mutant, we turned to the direct quantitative determination of OM permeability by using cephalosporins as test solutes, partly because cephalosporins, owing to their strong acidic groups, are likely to traverse the OM mainly through aqueous porin channels (as shown experimentally in *E. coli* [28]). In this assay, we measured the periplasmic concentration of cephalosporins from the rates of hydrolysis of cephalosporins by the periplasmic β-lactamase(s) in intact cells and calculated the permeability coefficient *P* from the hydrolysis rate and the concentration gradient of the drugs across the OM (28, 51). We used the endogenous chromosomally coded AmpC enzyme, which apparently plays a predominant role in the hydrolysis of β-lactams in the uninduced cells of ATCC 17978, as described above.

The hydrolysis rates of three cephalosporins, including nitrocefin, cephalothin, and cephaloridine, were determined in intact cells treated with 100 μM CCCP in order to inactivate multidrug efflux pumps (Table 2). For nitrocefin and cephalothin, the rates were corrected for hydrolysis due to enzyme that had leaked out into the suspension buffer. Even after correction for the enzyme leakage, the apparent permeability coefficient for nitrocefin was surprisingly high (Table 2). This most likely means that most of this quite lipophilic compound is crossing the OM through the lipid bilayer region that has become more permeable, as described above (rather than through the porin channel).

With the more-hydrophilic cephalothin, we observed a 2-fold decrease in permeability in the Δ*ompA*$_{Ab}$ mutant (Table 2). We expected a somewhat larger difference in the permeability coefficients of cephaloridine, since the diffusion rate of this zwitterionic compound across phospholipid bilayers is very low (19). The mutant lacking OmpA$_{Ab}$ showed a significant decrease in the permeability coefficient of this compound, although the difference was only 2.2-fold. We note also that the permeability coefficient of cephaloridine in the *A. baumannii* wild-type OM was 0.57×10^{-6} cm/s: this value is about 100-fold lower than the value obtained for the *E. coli* OM (28) and similar to that for *P. aeruginosa* (around 1×10^{-6} cm/s) (49). Still, these values were probably overestimates as a measure of porin permeability because of the contributions from leaked-out enzymes and the penetration across the bilayer region of OM.

In this intact-cell assay, measurements were carried out with the active efflux inactivated by de-energization of the cytoplasmic membrane with a proton conductor, 100 μM CCCP. We examined, in two ways, if the efflux pumps in *A. baumannii* affected the measurement of OM permeability. First, carrying out assays in the absence of CCCP strongly decreased the apparent permeability coefficient of cephaloridine (Table 2). Second, in the mutant BM4652, lacking two known efflux pumps, AdeABC and AdeIJK, we found that there was a 2- to 4-fold decrease in MIC for nitrocefin, cephalothin, and cephaloridine compared to that of the parent strain BM4454 (data not shown), as was found earlier for more-recently introduced cephalosporins (10).

DISCUSSION

Clinical isolates of *A. baumannii* often show elevated resistance levels to a large number of antibiotics (4, 32). The strain we used (ATCC 17978) was isolated back in 1951, but it is already resistant to penicillins and cephalosporins introduced early, presumably

owing to the intrinsic resistance of this species (Table 1). There are two β-lactamase genes in the *A. baumannii* ATCC 17978 genome (1) which code for class C AmpC and class D Bla_{oxa-51} enzymes. We found that the AmpC enzyme that hydrolyzes early cephalosporins rapidly was expressed at a fairly high level under our conditions of growth. However, β-lactamases alone cannot produce significant resistance levels, and we need the collaboration between the periplasmic β-lactamase and the low OM permeability (26). Indeed, the OM of a related species, *A. calcoaceticus*, was reported to show very low permeability (37).

In this work, we purified the major OM protein $OmpA_{Ab}$ from *A. baumannii* and showed that it produced permeability channels that allowed only a very slow penetration of solutes (Fig. 2). Since the permeability of the unfractionated OM was similarly low (Fig. 2), it appears that $OmpA_{Ab}$ is the major porin in this species. In order to confirm this conclusion, we measured the OM permeability for cephalosporins in intact cells with the classical Zimmermann-Rosselet method (51) using an Δ*ompA*$_{Ab}$ mutant. However, measurement of permeability in intact cells of *A. baumannii* was difficult because, owing to the extremely slow permeation through the porin channels, the spontaneous diffusion across the asymmetric lipid bilayer region became relatively significant, especially when the composition of the lipid domain became altered due to the absence of the major OM protein (2). Since β-lactams contain strongly acidic carboxylate groups, this may seem surprising. However, we have shown experimentally that lipophilic β-lactams, such as benzylpenicillin, can traverse the usual phospholipid bilayers with measurable rates most probably in their rare protonated forms (19). Thus, when the lipophilic nitrocefin was used as the probe, the permeation rate was unexpectedly high already in the wild type and became even higher in the Δ*ompA*$_{Ab}$ mutant (Table 2). The situation was somewhat better with the more hydrophilic cephalothin and cephaloridine, which do not easily diffuse across bilayers (19): here the *ompA*$_{Ab}$ deletion decreased permeability to about one-half (Table 2). Nevertheless, these data do not necessarily mean that one-half of cephalothin and cephaloridine molecules permeate through a porin(s) other than $OmpA_{Ab}$, because they may be crossing the OM through the bilayer or the hydrolysis could have occurred by leaked-out enzyme that was not fully corrected for. It should also be noted that the permeability coefficient for cephaloridine in the wild-type strain (0.57×10^{-6} cm/s) (Table 2) was more than two orders of magnitude smaller than those found for OmpF- and OmpC-producing *E. coli* strains (5.3×10^{-4} and 0.45×10^{-4} cm/s, respectively [28]).

Our results thus show that $OmpA_{Ab}$ is (a) a slow porin (defined in reference 43) and (b) the porin largely responsible for the nonspecific diffusion across the OM of *A. baumannii*. On the first point, we are confirming the results of Nitzan and coworkers (30) as well as those of Sato and Nakae (37). Jyothisri et al. (18) concluded that their $OmpA_{Ab}$ preparation had a pore-forming specific activity about equal to that of *E. coli* OmpF; however, the data in their paper show a specific activity about 100-fold lower than that of OmpF, and their conclusion was apparently due to a mistake in calculation. Gribun et al. (15) concluded that their $OmpA_{Ab}$ preparation had an activity three to four times lower than that of *E. coli* OmF. This is still quite high, but we note that the same investigator in this team, Y. Nitzan, had earlier published data showing that $OmpA_{Ab}$ had specific activity 35- to 50-fold lower than that of *E. coli* OmpF (30).

Thus, the major porin of *Acinetobacter* is the OmpA homolog, $OmpA_{Ab}$, a situation similar to that found in *P. aeruginosa*, in which another OmpA homolog, OprF, is the major porin (25). This is not surprising, because these two species belong to the same order, *Pseudomonadales* (6). OmpA has long been believed to fold exclusively as a two-domain protein, with its N-terminal half inserted into the OM and the C-terminal half folded into a globular domain in the periplasm (36). However, this conformation belongs only to the majority conformer within the population of OmpA (and OprF) (42, 43). With OprF, we have shown that the low permeability is due to the fact that only a minority of the protein folds into a different, one-domain, open-channel conformation (40), and we found several factors that affect the divergent folding pathway of this protein (39). It is reasonable to assume that a similar mechanism applies to $OmpA_{Ab}$, although this has not yet been experimentally proven.

We can examine if the measured low permeability and the properties of the β-lactamase can explain the high MIC values for the cephalosporins (Table 1). We can calculate, from the permeability coefficient and the kinetic properties of the β-lactamase, the periplasmic concentration (*Cp*) of the β-lactam by solving equation 3 of reference 26. Thus, at the MIC of cephaloridine (250 μg/ml or 0.6 mM), its periplasmic concentration is predicted to be 18 μg/ml. This is somewhat on the high side, as the minimal periplasmic concentration inhibiting cell growth for these β-lactams is usually expected to be in the range of 1 to 5 μg/ml (26). The most likely explanation of this discrepancy is the significant contribution from the constitutive multidrug efflux pumps (10) that would lower the periplasmic concentration of the drugs. Indeed, if we use the apparent permeability coefficient obtained without the inactivation of pumps by CCCP (Table 2), the predicted periplasmic concentration becomes 10 μg/ml, close to the expected range. On the other hand, if the *A. baumannii* OM had the high permeability of the *E. coli* OM for cephaloridine, we can calculate that the MIC will be essentially identical to its periplasmic concentration, and thus, the constitutive AmpC enzyme alone cannot produce any resistance, underscoring the importance of the OM permeation barrier.

P. aeruginosa also shows high intrinsic resistance levels to earlier cephalosporins, but it used to be quite susceptible to imipenem. It was discovered that imipenem bypasses the inefficient nonspecific porin OprF and uses a basic amino acid channel, OprD (14, 45, 46). In the organisms with slow major porins, we expect to see numerous specific channels for the uptake of various nutrients (16), and imipenem "hijacks" one of them. Interestingly, *A. baumannii* appears to use a similar strategy, as numerous studies implicated minor proteins other than $OmpA_{Ab}$ for the influx of carbapenems. Thus, a decreased expression of 22- and 33-kDa proteins was reported in an imipenem-resistant isolate (5). A 29-kDa protein called CarO is apparently important in the permeation of carbapenems (8, 20, 22). Imipenem-resistant *Acinetobacter* isolates were also reported to have diminished levels of 47-, 44-, and 37-kDa OM proteins (35). Yet another investigator reported that an imipenem-resistant *A. baumannii* isolate had decreased levels of a 33- to 36-kDa protein (9). Yet another candidate for a carbapenem channel is the homolog of *P. aeruginosa* OprD, identified through proteomics (13). Possibly there is more than one channel that can serve for the penetration of carbapenems in *Acinetobacter* spp.

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