Reduced Expression of Outer-membrane Proteins in β -Lactam-resistant Mutants of *Enterobacter cloacae*

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Two antibiotic-resistant mutants of *Enterobacter cloacae* (AZT-R and AMA-R), obtained by selection with aztreonam and carumonam, were studied. Both mutants were resistant to β -lactam antibiotics. In addition, AMA-R was also resistant to chloramphenicol, trimethoprim and brodimoprim, whereas AZT-R was hypersensitive to these compounds. Cytoplasmic and outer membranes of these bacteria were separated by sucrose density gradient centrifugation. Analysis of the outer membranes using SDS-PAGE showed marked changes in the bands corresponding to the porins (between 35 and 40 kDa). In the two mutants, the 39 kDa band was reduced to approximately 30% of the wild-type and the 36.5 kDa band was absent. Labelling of the outer membranes with the hydrophobic photolabel 3-(trifluoromethyl)-3-(*m*-[¹²⁵I]iodophenyl)diazirine ([¹²⁵I]TID) enabled the above bands as well as a 28.8 kDa band to be identified as integral membrane proteins, thus supporting the suggestion that they correspond to porins and OmpA protein, respectively. Whereas the changes observed in outer-membrane proteins are assumed to be responsible for resistance to β -lactam antibiotics, the basis of hypersensitivity of AZT-R to hydrophobic antibiotics remains to be more clearly defined.

INTRODUCTION

Several mechanisms of resistance to β -lactams have been proposed involving changes of enzyme specificity (Godfrey *et al.*, 1981), overproduction of target enzymes (Sanders & Sanders, 1983) and changes of membrane permeability (Lugtenberg & Peters, 1976; Nikaido, 1985; Nikaido & Vaara, 1985). Mutations leading to changes of membrane permeability are possibly quite common but are still not well understood. We have chosen *Enterobacter cloacae* as a model system to examine changes of membrane structure in Gram-negative bacteria responsible for the development of resistance to β -lactams, because resistant strains are common and are of increasing clinical importance (Sanders & Sanders, 1983; Werner *et al.*, 1985).

Recently we have obtained two β -lactam-resistant mutants (designated AZT-R and AMA-R) from the parent *E. cloacae* 908 S (Then & Angehrn, 1982, 1986) for which changes of membrane permeability probably constitute the principal mechanism of resistance. Both mutants show a broad resistance to various β -lactams like ceftriaxone, ceftazidime, aztreonam, carumonam, cefoxitin, cefotetan and others. Additionally, the AMA-R mutant is also resistant to other kinds of antibiotics, e.g. chloramphenicol, trimethoprim or brodimoprim, whereas the AZT-R mutant shows hypersensitivity to these compounds as compared with the wild-type (Then & Angehrn, 1986). In this study we have characterized in detail the changes in membrane composition observed for the resistant strains.

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Abbreviations: CL, cardiolipin; KDO, 2-keto-3-deoxyoctonate; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; [¹²⁵I]TID, 3-(trifluoromethyl)-3-(*m*-[¹²⁵I]iodophenyl)diazirine.

METHODS

Bacterial strains. The parent strain, *E. cloacae* 908 S_w, as well as the mutants AZT-R and AMA-R have been described recently (Then & Angehrn, 1986). All strains were kept frozen at -80°C in sterile sheep blood and were stable indefinitely.

Isolation of inner and outer membranes. Cells grown to the stationary phase in tryptic soy broth (Difco) were harvested by centrifugation at 4000 *g* for 10 min at 4°C , washed twice with 10 mM-sodium phosphate buffer (pH 7.0) and resuspended with 50 mM-Tris/HCl (pH 8.0), 1 mM-MgCl₂, 0.2 mM-DTT. After addition of deoxyribonuclease, ribonuclease (50 μg each ml⁻¹) and phenylmethylsulphonyl fluoride (final concentration 0.1 mM), the cells were broken by passing three times through a French pressure cell at 1100 atm (111 MPa). The outer and the cytoplasmic membranes were separated essentially according to Burnell *et al.* (1980). Unlysed cells were removed by centrifugation at 3000 *g* for 20 min at 4°C . The supernatant was centrifuged at 17000 *g* for 1 h at 4°C . At this stage most of the inner membrane should be in the supernatant and the pellet should consist mainly of outer membrane. After resuspension of the outer membrane with 50 mM-Tris/HCl (pH 8.0), 1 mM-MgCl₂, 0.2 mM-DTT, 0.2 M-KCl, the membranes were obtained by centrifugation at 200000 *g* for 90 min at 4°C . The pellet was resuspended with a syringe in buffer containing no KCl and layered on top of a sucrose step gradient of 35–55% (w/w). After 17 h centrifugation at 21000 r.p.m. in a SW 27 rotor (Beckman) at 4°C , the main band was isolated and diluted with 50 mM-Tris/HCl (pH 8.0), 1 mM-MgCl₂, 0.2 mM-DTT. KCl was again added to 0.2 M final concentration and the pellet, obtained after centrifugation at 200000 *g* for 60 min at 4°C , was resuspended in KCl-free buffer. Membranes which were not used the same day were frozen with liquid nitrogen and stored at -70°C . The inner membranes were isolated from the supernatant of the 17000 *g* centrifugation by the same treatment. The quality of the separation of the two membranes was determined by measuring the NADH-oxidase activity as described by Osborn *et al.* (1972), by the LPS/protein ratio and by the patterns of the proteins in SDS-polyacrylamide gels.

Hydrophobic photolabelling of outer membranes. To 2 ml outer-membrane suspension [0.5 mg protein (ml buffer)⁻¹], 5 μl of an ethanolic solution of 3-(trifluoromethyl)-3-(*m*-[¹²⁵I]iodophenyl)diazirine ([¹²⁵I]TID; 5 mCi ml⁻¹, 185 GBq ml⁻¹, Amersham) (Brunner & Semenza, 1981) was added. After incubation for 10 min at room temperature, the samples were irradiated by using a Philips TL40 W/08 black-light lamp in quartz cuvettes for 10 min at the same temperature. Nonphotolysed [¹²⁵I]TID and noncovalently bound photolysis products were removed by washing the membranes twice with buffer containing 0.2 M-KCl. After the second ultracentrifugation at 200000 *g* for 90 min at 4°C , the pellet was resuspended in 0.3 ml KCl-free buffer. Samples were frozen in liquid nitrogen and stored at -70°C . For the analysis of the labelling of outer-membrane components, samples containing 3.5×10^5 c.p.m. were electrophoresed on an SDS-polyacrylamide gel and autoradiographed.

Analytical procedures. Protein was determined according to a modified Lowry procedure (Peterson, 1977), omitting the precipitation step with TCA. To determine the LPS content, the amount of 2-keto-3-deoxyoctonate (KDO) after TCA precipitation of membrane samples (Osborn *et al.*, 1972) containing 0.2–1 mg protein was determined (Karkhanis *et al.*, 1978). As a standard the ammonium salt of KDO from Sigma was used. The lipid content was determined (Chen *et al.*, 1956) after extraction of the isolated membranes (samples containing 1–4 mg protein in 1 ml aqueous solution) according to Bligh & Dyer (1959), using methylene chloride instead of chloroform. The lipid composition was determined by phosphate analysis of thin-layer chromatography plate scrapings (elution in chloroform/methanol/water, 85:30:4, by vol., on silica gel plates 60 F-254 from Merck). SDS-PAGE was done essentially as described by Laemmli (1970). The protein samples, however, were solubilized in 58 mM-Tris/HCl (pH 6.8), 5% (v/v) glycerol, 50 mM-DTT, 2% (w/v) SDS, 0.02% bromophenol blue at 95°C for 90 s and applied onto slab gels (1.5 mm thick, 20 cm long). The stacking and the running gel contained 3.75 and 12% (w/v) acrylamide, respectively, 0.16% *N,N'*-methylene-bisacrylamide and 0.1% SDS. For autoradiography the running gels were dried and exposed on X-ray films from Kodak (X-Omat AR) at -70°C .

RESULTS

Resistance profile

The antibiotic susceptibilities of the parent *E. cloacae* 908 S_w and the two mutants were obtained by the agar dilution assay (Then & Angehrn, 1986) (Table 1). Both mutants showed resistance to a variety of β -lactams but clearly differed from one another in sensitivity to other classes of antibiotics. For instance, AMA-R was also resistant to erythromycin, chloramphenicol, trimethoprim and brodimoprim, whereas AZT-R was hypersensitive to these compounds. The mechanism of resistance against β -lactam antibiotics in the mutants was due neither to an overproduction of β -lactamase nor to a decrease in affinity of the penicillin-binding proteins (Then & Angehrn, 1986). We therefore suspected that the changes in antibiotic susceptibility were connected to alterations in the barrier properties of the cell envelope, probably at the level of the outer membrane.

Table 1. *Minimal inhibitory concentration of various antibiotics for E. cloacae* 908 *S*_{wi} and the two mutants, AZT-R and AMA-R, respectively

Antibiotic	Minimal inhibitory concn ($\mu\text{g ml}^{-1}$)		
	<i>S</i> _{wi}	AZT-R	AMA-R
Ampicillin	16.0	8.0	256.0
Aztreonam	0.2	16.0	32.0
Carumonam	0.1	16.0	16.0
Cefotetan	0.4	102.0	25.6
Ceftazidime	0.2	6.4	6.4
Ceftriaxone	0.1	3.2	1.6
Lamoxactan	0.5	51.0	25.6
Ticarcillin	1.6	204.0	409.0
Brodinoprim	0.8	0.1	25.6
Ciprofloxacin	0.06	0.06	0.5
Chloramphenicol	6.4	1.6	25.6
Erythromycin	204.0	25.6	204.0
Nalidixic acid	3.2	1.6	25.6
Trimethoprim	0.2	0.05	3.2

Table 2. *Characterization of the inner- and outer-membrane fractions of E. cloacae* 908

Strain	Membrane	NADH-oxidase activity*	Phospholipid/protein ratio†	KDO/protein ratio‡
		(%)	[nmol P (mg protein) ⁻¹]	[nmol (mg protein) ⁻¹]
<i>S</i> _{wi}	CM	98, 99	415	14
	OM	2, 1	265	205
AZT-R	CM	90, 95	480	20
	OM	10, 5	295	142
AMA-R	CM	96, 98	535	23
	OM	4, 2	380	154

CM, cytoplasmic membrane (inner membrane); OM, outer membrane.

* Total activity (mg protein)⁻¹ in inner- and outer-membrane fractions is 100% [= 340 and 6 nmol NADH oxidized min⁻¹ (mg protein)⁻¹ for strain *S*_{wi}]; results for two separate preparations are shown.

† Mean of determinations on two separate extractions.

‡ KDO was measured five times.

Separation of cytoplasmic and outer membranes

The cytoplasmic and outer membranes were separated using sucrose density gradient centrifugation. Initial attempts at membrane separation using the EDTA/lysozyme method of Osborn *et al.* (1972) as modified by Witholt *et al.* (1976) were successful with the wild-type only. Although spheroplasts could be obtained with all three strains, the membranes of the mutants could not be separated by subsequent sucrose density centrifugation.

In contrast, well-separated membrane preparations could be obtained by the method of Schnaitman (1970) (optimized by Burnell *et al.*, 1980), which employs the French pressure cell for initial cell breakage followed by differential and finally sucrose density gradient centrifugation (see Methods). The resuspended pellet from the 17000 *g* centrifugation was obtained as a broad white band near the bottom of the centrifuge tube in the final gradient centrifugation step and was designated the H-band (high density). Similarly the supernatant of the 17000 *g* step appeared in the upper third of the final gradient and was designated the L-band (low density). For the wild-type, the separation of inner and outer membranes as judged by the specific activity of NADH-oxidase in H- and L-fractions was very good (L/H = 100), and acceptable (though somewhat poorer than for the wild-type) for AMA-R (L/H = 50) and AZT-R (L/H = 20) (Table 2). The distribution of KDO (which occurs exclusively in the LPS of outer membranes) was the reverse of that of NADH-oxidase (i.e. present almost exclusively in the outer membrane), thus confirming the assignment of L- and H-bands to inner and outer membranes, respectively. Finally, the lipid/protein ratio of the cytoplasmic membrane was consistently higher than that of the outer membrane (Table 2). Similar results have been reported previously for *Salmonella typhimurium* (Osborn *et al.*, 1972) and *Escherichia coli* (Burnell *et al.*, 1980; Overath *et al.*, 1975; Schnaitman, 1970).

Table 3. *Phospholipid composition of inner- and outer-membrane fractions of E. cloacae 908*

Phospholipids were extracted from membrane fractions after gradient centrifugation as described in the text. The amount of individual phospholipids was determined by phosphate analysis of thin-layer chromatography scrapings. Numerical values are given as a percentage of the total and represent the mean of two separate extractions.

Strain	Membrane	PE	lyso-PE	Total (PE + lyso-PE)	PG	CL	Total (PG + CL)
<i>S</i> _{wi}	CM	73	0.4	73	23	3.5	27
	OM	77	9	86	6	8	14
AZT-R	CM	80	1	81	17	2	19
	OM	76	10	86	5	9	14
AMA-R	CM	79	1	80	17	3	20
	OM	82	6	88	5	6	11

CM, Cytoplasmic membrane (inner membrane); OM, outer membrane.

The phospholipid composition was determined for each of the separated membrane fractions (Table 3). In all three strains phosphatidylethanolamine (PE) was the major phospholipid present in both cytoplasmic and outer membranes. Phosphatidylglycerol (PG) and cardiolipin (CL) were present in both membranes of all strains although cytoplasmic and outer membranes were consistently enriched in PG and CL, respectively. Similar overall phospholipid composition has been reported for *S. typhimurium* (Osborn *et al.*, 1972) and *E. coli* (Lugtenberg & van Alphen, 1983) although the specific enrichment of the charged phospholipids in inner and outer membranes, respectively, was somewhat different to that reported here. Significant quantities of lysophosphatidylethanolamine, probably derived from hydrolysis of PE by an endogenous phospholipase (Lugtenberg & van Alphen, 1983; White *et al.*, 1972), were observed for the outer membranes.

Protein composition of membranes of E. cloacae 908 S_{wi} and its mutants

SDS-PAGE analysis of H- and L-fractions showed the two membrane populations to be significantly different (Fig. 1). Cytoplasmic membranes contained a broad distribution of proteins (Fig. 1*a-c*) whereas the outer membranes contained only four or five major proteins (Fig. 1*d-f*). In all three strains the most intense band (probably OmpA protein) had a molecular mass of 28.8 kDa (Fig. 1*d-f*). This band was increased by 10 and 40% (by densitometry, data not shown) relative to that of the wild-type in the AMA-R and AZT-R mutant, respectively. In our experiments, OmpA protein migrated somewhat faster than other OmpA proteins from different Gram-negative bacteria (Behr *et al.*, 1980; Hancock & Carey, 1979; Lugtenberg & Peters, 1976). The fast migration of this protein was probably due to the presence of DTT as the reducing agent in the sample buffer (Fig. 2*a*). When DTT was replaced by 2-mercaptoethanol (Laemmli, 1970), the 28.8 kDa band could not be detected. Instead, the 37.5 kDa band became much more intense (Fig. 2*b*) and the molecular mass was very similar to those described for the heat-modified OmpA proteins (33–35 kDa). We therefore concluded that the 28.8 kDa and the 37.5 kDa band represented two conformational forms of the OmpA protein.

The wild-type strain contained three discrete bands in the porin region (39, 37.5 and 36.5 kDa). In the mutants, AZT-R and AMA-R, the relative intensity of the 39 kDa protein was reduced to approximately 25 and 35% relative to that of the wild-type, respectively. Neither mutant contained the wild-type 36.5 kDa band. The 37.5 kDa band was slightly increased for the AZT-R strain and decreased to approximately 60% of the wild-type intensity for the AMA-R strain. Significant changes were also observed in the 22.7 and 19.6 kDa bands (AZT-R: 22.7 kDa very weak, 19.6 kDa decreased with respect to wild-type; AMA-R: 22.7 kDa very weak, 19.6 kDa increased with respect to wild-type).

Fig. 1. SDS-PAGE of cytoplasmic (CM) and outer membranes (OM) of *E. cloacae* 908. Proteins were analysed on 12% polyacrylamide gels as described in Methods. Samples (50 μ l) were applied containing either 200 μ g cytoplasmic membrane protein (CM) or 100 μ g outer-membrane protein (OM). The gel was stained in 0.1% Coomassie blue R-250 (Serva): CM proteins of wild-type *E. cloacae* 908 S_{wi} (lane a), of mutant AZT-R (lane b) and mutant AMA-R (lane c); OM proteins of strain S_{wi} (lane d), AZT-R (lane e) and AMA-R (lane f). Lane (g), standard proteins (Pharmacia) containing phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa).

Fig. 2. SDS-PAGE of outer membranes (OM) of wild-type *E. cloacae* 908 S_{wi} using two different reducing agents. Proteins were analysed in 12% gels by Coomassie blue staining. The reducing agent in the sample buffer was 50 mM-DTT (lane a) and 10% (w/v) 2-mercaptoethanol (lane b). Samples (50 μ l) containing 100 μ g protein were applied. Molecular mass standards were the same as in Fig. 1.

Hydrophobic photolabelling of outer membranes

In order to establish whether the above proteins of interest were indeed integral membrane proteins, the isolated outer membranes were labelled with the reagent [125 I]TID. This recently developed carbene precursor has the advantage of being extremely hydrophobic (the partition

Fig. 3. SDS-PAGE of [125 I]TID-labelled outer membranes from *E. cloacae* 908. Proteins were analysed on 12% polyacrylamide by Coomassie blue staining (lanes *a-c*) and by autoradiography (lanes *d-f*). Samples (63 μ l) containing approximately the same amount of radioactivity were applied. Outer-membrane proteins of the wild-type (3.6×10^5 c.p.m., 115 μ g protein, lanes *a* and *d*), of the mutants AZT-R (3.6×10^5 c.p.m., 150 μ g protein, lanes *b* and *e*) and AMA-R (3.6×10^5 c.p.m., 160 μ g protein, lanes *c* and *f*). Molecular mass standards were the same as in Fig. 1.

coefficient between egg phosphatidylcholine or erythrocyte membranes and aqueous buffer at 25 °C is approximately 10^4 (Brunner & Semenza, 1981) and its photogenerated carbene reacts quite nonspecifically. With the relatively weak light source employed, approximately 15% of the starting radioactivity was incorporated covalently into the membranes within 10 min. Most of the label migrated with the tracking dye or slightly ahead of it (Fig. 3) as a result of reaction with the lipids. Similar observations have been made with erythrocytes (Brunner & Semenza, 1981) and brush border membranes (Spiess *et al.*, 1982). The autoradiograph (Fig. 3*a-c*) shows the

Fig. 4. Distribution of radioactivity in $[^{125}\text{I}]\text{TID}$ -labelled outer membranes. After electrophoresis of $[^{125}\text{I}]\text{TID}$ -labelled membranes on a 12% SDS-polyacrylamide gel (cp. legend of Fig. 3), the running gel was dried and the lanes sliced in 2 mm pieces, which were counted on a γ -counter (Kontron). Outer membranes of *E. cloacae* 908 S_{wi} (a), AZT-R (b) and AMA-R (c). The originally applied amount of radioactivity was 1.82×10^5 , 1.78×10^5 and 1.65×10^5 c.p.m., respectively. 1, 39 kDa band; 2, 37.5 kDa band; 3, 36.5 kDa band; 4, 28.8 kDa band. The inserts show the densitometric scans of the gels stained with Coomassie blue. TD, tracking dye.

Table 4. *Percentage distribution of 125 I-label in the main components of outer membranes*

After SDS-PAGE of [125 I]TID-labelled outer membranes of the three *E. cloacae* 908 S strains S_{wi}, AZT-R and AMA-R as described in the legend of Fig. 3, lanes were cut into 2 mm pieces, the radioactivity was counted and the relative amounts present in distinct gel regions were calculated.

Gel region (kDa)	S _{wi}	AZT-R	AMA-R
39	1.52	0.49	0.49
37.5	0.81	0.80	0.41
36.5	0.99	0	0
28.8	6.73	6.83	5.22
Slice nos 80–103*	81.12	88.57	91.59

* Slice no. 1 is at the top of the running gel, 97 at the tracking dye front (cp. Fig. 3).

28.8 kDa protein and the proteins in the 35–40 kDa region to be the most extensively labelled. After slicing the lanes the 39 kDa band was shown to be specifically reduced in radioactivity to approximately 30% with respect to that of the wild-type for the AZT-R and AMA-R mutants (Fig. 4, Table 4). This compared well with the decrease in protein observed for this band as determined by Coomassie blue staining. For both mutants the radioactivity in the 36.5 kDa region was only slightly above background and was thus consistent with the results obtained by Coomassie blue staining.

Membrane separation using the detergent Sarkosyl

SDS-polyacrylamide gels of membranes of the mutants prepared according to the modified (Burnell *et al.*, 1980) procedure of Schnaitman (1970) clearly showed a decrease of the 39 and 36.5 kDa bands. These bands stained very heavily in the wild-type. In contrast, with outer membranes prepared by the often-used Sarkosyl extraction procedure (Filip *et al.*, 1973; Gutmann & Chabbert, 1984; Sawai *et al.*, 1982; Werner *et al.*, 1985) (data not shown) the wild-type 39 kDa band was weak and not visible at all in the two mutants.

DISCUSSION

The development of β -lactam resistance in bacteria has been suggested to arise from a variety of molecular mechanisms: (1) overproduction of β -lactamases; (2) a reduction in affinity of the penicillin-binding proteins; (3) changes in membrane permeability (see Nikaido, 1985; Nikaido & Vaara, 1985; Sanders & Sanders, 1983). In the mutant strains of *E. cloacae* examined we have excluded (1) and (2) (Then & Angehrn, 1986), so presumably only the mechanism (3) plays a significant role in the development of resistance. Since the β -lactam target sites are in the periplasmic space, only changes in permeability of the outer membrane are of importance. We therefore have restricted our studies to the isolation and biochemical characterization of this membrane. Inner and outer membranes of all three strains (wild-type and two mutants) of *E. cloacae* were separated easily using the method of Schnaitman (1970). This method is rapid and allows the isolation of membranes in a native, functional form, thus allowing the determination of important physical and physiological parameters (e.g. enzyme activity, lipid/protein ratio) not obtainable by other techniques such as 'selective' detergent extraction of total membranes for instance with Sarkosyl.

The biochemical characterization of the outer membranes of the three strains used in this study allows exclusion of several potential causes of changes in membrane permeability to β -lactams. The phospholipid compositions are essentially identical in all three strains (Table 3) as are the KDO contents (the KDO content of the wild-type is marginally higher by a factor of 1.3 which is probably not significant), suggesting that these do not constitute the major causal factors of the differing β -lactam permeabilities observed. We note, however, that in the mutants the total phospholipid (mg protein)⁻¹ of the outer membrane is increased, raising the possibility that the distribution of phospholipids between the inner and outer leaflets of the outer

membrane (since KDO is always confined to the outer leaflet) may play a role in modulating the permeability to antibiotics other than β -lactams. This idea has been discussed in more detail by Nikaido (1985).

By far the most striking changes are observed in the membrane protein composition in the outer membrane, by the criteria both of Coomassie blue staining and of labelling with the nonspecific photolabel [125 I]TID (Figs 3 and 4). Changes are observed in the 35–40 kDa porin region, the 28.8 kDa region [OmpA protein (Behr *et al.*, 1980; Hancock & Carey, 1979; Heller, 1978; Lugtenberg & Peters, 1976)] and also in the low-molecular-mass range. However, only the porins are responsible for building nonspecific membrane channels in the outer membrane (Nikaido *et al.*, 1983; Nikaido & Rosenberg, 1983; Yoshimura & Nikaido, 1985) and it seems reasonable to suggest that the changes in this region are responsible almost exclusively for the observed changes in β -lactam permeability. In the following discussion we restrict ourselves to the 39 and 36.5 kDa proteins as these are the proteins which show the largest changes in the mutants. These two proteins are in fact those that Kaneko *et al.* (1984) and others (Gutmann & Chabbert, 1984; Werner *et al.*, 1985; Sawai *et al.*, 1982) have assigned to the porins. The 37.5 kDa band probably corresponds to a nondenatured OmpA protein conformation (see Results). In both of the mutants examined, one of the two porin bands (36.5 kDa) was completely absent and the other (39 kDa) was reduced with respect to the wild-type. A similar phenomenon has also been observed for different enterobacteria (Gutmann & Chabbert, 1984; Sawai *et al.*, 1982; Werner *et al.*, 1985). This therefore suggests that porin deletion may be a general mechanism for reducing membrane permeability to β -lactams. A further mechanism involving a change in porin specificity cannot be excluded but is probably of minor importance in this case. We have confirmed this porin deletion by hydrophobic labelling using the nonspecific photolabel [125 I]TID. This label, which partitions to greater than 99.9% within the hydrocarbon interior of the membrane, should randomly label all membrane proteins with which it comes into contact. The final specific radioactivity observed reflects the fraction of membrane-embedded proteins and should more accurately reflect changes in protein content than staining by Coomassie blue alone. In the event, the Coomassie blue and [125 I]TID profiles were very similar in the porin region (see Figs 3 and 4) and somewhat different in the higher-molecular-mass region. Quantification of the specific radioactivity shows that both mutant strains contained only one-third of the 39 kDa porin as compared to the wild-type (Table 4). This suggests that not only deletion of a porin but also reduction in the number of porin molecules may play a role in determining the level of resistance.

Finally, the mutant strains of *E. cloacae* differ dramatically in their susceptibility to antibiotics not related to β -lactams (Table 1). At least some of these antibiotics are hydrophobic in character, which may be a prerequisite to their ability to penetrate the inner membrane in order to reach the target site (e.g. trimethoprim, chloramphenicol). In these cases the mechanisms responsible for antibiotic resistance or hypersensitivity are probably more complex than those described above, possibly involving changes in lipid permeability (see Nikaido, 1985). In this context, the changes in the KDO/phospholipid ratio described above may be of significance but at present we have no satisfactory explanation for the unusual susceptibility pattern of strain AZT-R.

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