ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1991, p. 1203–1207
0066-4804/91/061203-05$02.00/0

Vol. 35, No. 6

Increased Susceptibility to β-Lactam Antibiotics and Decreased Porin Content Caused by *envB* Mutations of *Salmonella typhimurium*

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Received 30 January 1991/Accepted 19 March 1991

Isogenic derivatives carrying *envB6*, *envB9*, or *envB*$^+$ alleles were obtained from a strain of *Salmonella typhimurium* that was partially resistant to mecillinam, a β-lactam antibiotic specific for penicillin-binding protein 2 (PBP 2). Testing of the isogenic strains with several antibacterial agents demonstrated that *envB* mutations either increased resistance (mecillinam) or did not affect the response (imipemen) to β-lactams that act primarily on PBP 2, while susceptibilities to β-lactams that act on PBP 1B, PBP 3, or both were increased. Furthermore, the susceptibilities of *envB* strains to hydrophobic compounds such as rifampin, novobiocin, or chloramphenicol were not modified, even though their susceptibilities to deoxycholate and crystal violet were enhanced. Outer cell membranes of *envB* mutants presented a 50% reduction in protein content compared with that of the isogenic *envB*$^+$ strains, and OmpF and OmpD porins were particularly affected by the reduction. No alteration in the amount or pattern of periplasmic proteins was noticed, and lipopolysaccharides from *envB* mutants appeared to be normal by sodium dodecyl sulfate-urea-polyacrylamide gel electrophoresis. By using derivatives that produced a plasmid-encoded β-lactamase, it was demonstrated that *envB* cells are slightly less permeable to cephalothin than *envB*$^+$ bacteria are. It is concluded that the high susceptibility of *envB* mutants to β-lactams is due to the increased effectiveness of the antibiotics on PBP 1B, PBP 3, or both.

The action of β-lactam antibiotics on enterobacteria is exerted on a group of about seven inner membrane proteins, the penicillin-binding proteins (PBP), that are involved in the synthesis and maturation of murein. The affinity for each of the seven PBPs varies widely among the different β-lactams; however, the bactericidal action of a β-lactam is due to interference with the activity of at least one of three essential PBPs: PBP 1B, which is the main enzyme that performs cell wall elongation; PBP 2, which is involved in rod shape determination; and PBP 3, which acts specifically in cross-wall synthesis (29). To reach their inner membrane targets, β-lactams must traverse the permeability barrier imposed by the outer membrane through hydrophilic channels formed by porins. Much information has accumulated lately on the properties of the different types of pores and also on the effect of size, charge, and hydrophobicity of the β-lactam molecule on permeability (22). In addition, most gram-negative bacteria possess periplasmic β-lactamases, either chromosomal or plasmid encoded, which can destroy the antibiotic before it reaches the target. Therefore, affinity for PBPs, permeability through the outer membrane, and β-lactamase activity are the main factors that determine susceptibility to β-lactam antibiotics (23).

Increased susceptibility of *envB* mutants of *Escherichia coli* and *Salmonella typhimurium* to β-lactams has been reported (3, 25). These cell shape-defective mutants carry an apparently normal PBP 2 (13), yet they form spherical cells and display several pleiotropic alterations such as increased susceptibility to deoxycholate (DOC), increased resistance to UV light, enhanced autolytic activity, abnormal fermentation of some carbohydrates, etc. (3, 25, 34).

In this work, the effect of *envB* mutations on the outer membrane of *S. typhimurium* was studied. The results obtained demonstrate that despite their high susceptibility to most β-lactams, *envB* mutants suffer a drastic reduction in porin content and show decreased permeability to those antibiotics.

MATERIALS AND METHODS

Bacterial strains. All of the bacterial strains used in this study were derivatives of *S. typhimurium* LT2; their origins and relevant genetic properties appear in Table 1. Isogenic transductants were obtained and tested as described by Antón (4). Derivatives carrying plasmid pNK-972 were constructed by transducing pNK972 to strains DA1173 and DA1167 with phage P22 HT105/1 *int-201* grown on strain TT10427. Ampicillin-resistant transductants carrying pNK972 were selected on L agar containing 30 μg of ampicillin per ml. pNK972-carrying derivatives of *envB* strains were not prepared by this procedure because, for unknown reasons, stable ampicillin-resistant transductants could not be obtained with *envB* recipients. Instead, *envB6* and *envB9* alleles were transduced to strain DA1167 (pNK 972) (DA1337) by cotransduction with marker *argR372*::Tn*10* (4).

Media and antibiotics. Unless otherwise stated, growth medium was L broth containing the following per liter: 10 g of tryptone (Difco Laboratories), 5 g of yeast extract (Difco), and 5 g of NaCl. In some experiments, nutrient broth (Difco) containing 0.3 M NaCl or with no NaCl added was used. Solid medium was obtained by adding 15 g of agar (Difco) per liter to liquid medium. Semisolid agar for motility tests was L broth with 3.5 g of agar per liter.

The following antibiotics were kindly provided by the companies given in parentheses: mecillinam (Productos Roche, Buenos Aires, Argentina), imipenem (Parke Davis,

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TABLE 1. Bacterial strains used in this study

Strain	Relevant properties or derivation	Source or reference
LT2	Wild type	P. E. Hartman
SB2298	*hisF3031 trpA3*	P. E. Hartman
SB2299	*hisF3031 trpA3 hisO1242*	P. E. Hartman
TT10427	LT2(pNK972)	J. R. Roth
DA692	Same as SB2299 but *envB6*	5
DA743	Same as SB2298 but *envB9*	5
DA947	Same as DA692 but *argR372*::Tn*10*	4
DA1041	Same as DA743 but *argR372*::Tn*10*	4
DA1167	Spontaneous Mec^r derivative of LT2	This study
DA1168	*argR372*::Tn*10 envB6* transductant of DA1167[a]	This study
DA1172	Same as DA1168 but *envB9*	This study
DA1173	Same as DA1168 but *envB*$^+$	This study
DA1336	Same as DA1173 but carrying pNK972[b]	This study
DA1337	Same as DA1167 but carrying pNK972[b]	This study
DA1338	*argR372*::Tn*10 envB6* transductant of DA1337[a]	This study
DA1339	Same as DA1338 but *envB9*	This study

[a] Transduction was mediated by phage P22 HT105/1 *int-201* with either DA947 or DA1041 as the donor; selection was for resistance to tetracycline (4).

[b] Transduction was mediated by phage P22 HT105/1 *int-201* with TT10427 as the donor; selection was for resistance to ampicillin.

Raleigh, N.C.), piperacillin (Laboratorios Lederle, Buenos Aires, Argentina), and cephalothin (Laboratorios Glaxo, Buenos Aires, Argentina). The following antibiotics were purchased from the indicated companies: penicillin G, carbenicillin, and rifampin (Sigma Chemical Co., St. Louis, Mo.), ampicillin (Laboratorios Bagó, Buenos Aires, Argentina), cefotaxime (Argentia S.A., Buenos Aires, Argentina), streptomycin (Lepetit, Buenos Aires, Argentina), and chloramphenicol (Parke Davis, Buenos Aires, Argentina).

Susceptibility to antibiotics and chemicals. MICs were determined by placing 20-μl samples of diluted stationary-phase cultures containing about 1,000 cells on L-agar plates containing twofold serial dilutions of the substance tested. Appropriate dilutions spread onto L agar served to assess the number of cells plated. Growth was scored after incubation for 2 days at 37°C. Colony formation by less than 5% of the cells plated was recorded as inhibition. Only differences between MICs of at least fourfold were considered significant.

Permeability. Permeability to β-lactam antibiotics was assayed by the procedure of Zimmermann and Rosselet (35), as modified by Nikaido et al. (24), by using derivatives of *envB* and *envB*$^+$ strains carrying plasmid pNK972. This plasmid encodes the β-lactamase from plasmid R_1 (30) that has been reported not to affect outer cell membrane characteristics (22).

Strains were grown in L broth containing 30 μg of ampicillin per ml, to exclude plasmid loss, and 5 mM $MgCl_2$, to prevent cell breakage. Cells were harvested in the mid-exponential phase, washed, resuspended, and assayed in 50 mM sodium phosphate buffer–5 mM $MgCl_2$ (pH 7.0). Hydrolysis of 1 mM cephalothin by intact and sonically disrupted cells was followed spectrophotometrically at 260 nm in a 1-mm-light-path cuvette (24). The K_m for cephalothin (270 μM) was determined with supernatants of *envB*$^+$ disrupted cells. Hydrolysis data obtained with intact cells were corrected for the extracellular activity released from damaged cells and also for cell sedimentation, since this was significant with exponential-phase *envB* cells, which are large, nonmotile spheres.

Isolation of cell fractions. Cell envelopes were obtained as described by Ames (1), with some modifications. Cells were disrupted either by sonication or by passage through a French pressure cell. Disrupted cells were centrifuged at 14,000 × *g* for 15 min, and total protein in the supernatant was assessed. The envelope fraction was sedimented by centrifugation of the supernatant at 120,000 × *g* for 45 min. Treatment of cell envelopes with 1% Sarkosyl solubilized the inner membrane proteins (9); the outer membrane was pelleted by centrifugation at 120,000 × *g* for 90 min and resuspended, and the protein content was assayed. The amount of protein in this fraction was referred to the amount of protein in the supernatant of disrupted cells.

Cell envelopes and outer membranes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (16), except for the composition of the separating gel, which was modified as described by Lugtenberg et al. (19). Identification of porin bands was effected by comparison of the outer membrane protein of wild-type LT2 grown in nutrient broth either without NaCl or with 3 M NaCl. Reduction of OmpF in the latter with the corresponding OmpC increase and the reciprocal change in nutrient broth without NaCl allowed identification of those protein bands (32).

Periplasmic proteins were obtained by the procedure of Neu and Heppel (21) and analyzed by SDS-PAGE in 12.5% polyacrylamide gels (16). Lipopolysaccharide (LPS) was extracted by the aqueous butanol method of Leive and Morrison (17), and samples containing similar amounts of 3-deoxy-sugars (2-keto-3-deoxyoctonate and abequose) were submitted to electrophoresis in 16% polyacrylamide gels containing 4 M urea (SDS-urea-PAGE). 3-Deoxy-sugars were estimated as described by Dröge et al. (8). Silver staining of LPS gels was performed by the procedure of Tsai and Frasch (31).

RESULTS

To test the effect of *envB* mutations on the susceptibilities to several antibacterial agents, isogenic strains carrying the *envB6*, *envB9*, or *envB*$^+$ allele were prepared by cotransduction with the selective marker *argR372*::Tn*10* (4). It has been demonstrated that *envB9* and some other *envB* alleles are not viable in wild-type recipients but are tolerated by strains displaying resistance to mecillinam, a β-lactam antibiotic that elicits a round cell shape (4). On this account, DA1167, a spontaneous mecillinam-resistant (Mec^r) derivative of the wild-type strain LT2, was used as the recipient in the construction of the isogenic strains.

Susceptibilities to several β-lactam antibiotics, antibiotics belonging to other families, and DOC and crystal violet were scored. The results (Table 2) demonstrated that the strain used as a tolerant recipient for *envB* mutations acquired, simultaneously with mecillinam resistance, partial resistance to imipenem (another β-lactam that confers a round cell shape) and streptomycin and increased susceptibility to chloramphenicol. As reported before (4), introduction of *envB* mutations into strain DA1167 increased its level of resistance to mecillinam, while the response to imipenem was not modified (Table 2). Contrary to the MICs of mecillinam and imipenem, the MICs of all the other β-lactams tested decreased in both *envB* mutants, but more so in the

TABLE 2. MICs of several antibacterial compounds for *envB* and *envB*$^+$ strains

Strain	MIC[a]													
	MEC	IPM	PCG	APC	PIPC	CPC	CET	CTX	SM	CM	NOV	RIF	DOC	CV
LT2	0.08	0.08	10	1.25	5	5	2.5	50	25	5	250	10	10	50
DA1167	10	0.64	10	0.64	5	5	2.5	50	100	1.25	250	10	10	50
DA1168	40	0.64	0.64	0.16	0.64	1.25	0.64	6.4	100	0.64	250	10	1.25	NT[b]
DA1172	40	0.64	0.16	0.04	0.32	0.32	0.16	3.2	100	1.25	125	10	1.25	3.2
DA1173	10	0.32	10	1.25	5	5	2.5	50	100	1.25	250	10	10	50

[a] All MICs are expressed in micrograms per milliliter except for the MICs of penicillin G (international units), cefotaxime (nanograms per milliliter), and DOC (milligrams per milliliter). MEC, mecillinam; IPM, imipenem; PCG, penicillin G; APC, ampicillin; PIPC, piperacillin; CPC, carbenicillin; CET, cephalothin; CTX, cefotaxime; SM, streptomycin; CM, chloramphenicol; NOV, novobiocin; RIF, rifampin; DOC, deoxycholate; CV, crystal violet.
[b] NT, not tested.

envB9 than in the *envB6* derivative. On the other hand, no important changes were observed with antibiotics belonging to other groups with either hydrophobic (rifampin, novobiocin, chloramphenicol) or hydrophilic (streptomycin) characteristics (Table 2). Furthermore, *envB* mutations were found to increase susceptibility to DOC and crystal violet (Table 2).

Since porins are the major pathway for entrance of β-lactams (22), outer membranes of the original *envB* mutants, their isogenic derivatives, and related control strains were analyzed for protein content (Table 3). The amount of protein was strongly decreased in the outer membrane of *envB* strains DA692 (*envB6*) and DA743 (*envB9*); the decrease was much more in the former than it was in the latter. When isogenic *envB6*, *envB9*, and *envB*$^+$ derivatives of strain DA1167 were compared, both *envB* mutations were found to produce a 50% decrease in the amount of outer membrane proteins. It was also observed that strain DA1167 itself possessed only half the amount of protein shown by wild-type LT2. This alteration seems to be common in Mec^r derivatives of strain LT2, since it was observed in five independent Mec^r isolates of that strain but did not appear in Mec^r derivatives of other strains (unpublished data).

The effect of *envB* mutations on the level of membrane proteins was investigated by SDS-PAGE of isolated envelopes (Fig. 1). The main difference observed between *envB* and *envB*$^+$ strains grown in L broth was a strong reduction in the flagellin band (molecular weight, about 50,000 [1]) shown by the former. As expected from this result, *envB* mutants behaved as nonmotile strains when tested on semisolid medium, whereas related *envB*$^+$ strains, DA1167 and DA1173, proved to be as motile as the wild-type strain LT2.

TABLE 3. Protein content in outer membranes of *envB* and *envB*$^+$ strains

Strain	*envB* allele	Protein content (μg/mg of total protein)[a]
SB2298	*envB*$^+$	30.2
DA743[b]	*envB9*	14.6
SB2299	*envB*$^+$	39.5
DA692[b]	*envB6*	4.9
LT2	*envB*$^+$	45.1
DA1167	*envB*$^+$	24.1
DA1168[c]	*envB6*	11.2
DA1172[c]	*envB9*	10.6
DA1173[c]	*envB*$^+$	23.1

[a] Micrograms of protein in the outer cell membrane fraction per milligram of protein in the supernatant of disrupted cells (see text).
[b] Original *envB* mutants (see Table 1 for their origins and properties).
[c] Isogenic derivatives of strain DA1167.

Differences in the group of bands formed by OmpA and the porins were also noticeable (Fig. 1); therefore, because the high osmolarity of L broth reduces the OmpF level (32), outer membrane proteins of strains grown in a medium that allows full expression of OmpF (nutrient broth without NaCl) were examined. As shown in Fig. 2, neither OmpF nor OmpD porins were observed in *envB* strains grown under those conditions.

The same amounts of periplasmic proteins were observed in *envB6*, *envB9*, and *envB*$^+$ isogenic strains (9.5, 9.6, and 9.9% of the total cell protein, respectively), and their electrophoretic patterns were also similar (data not shown); therefore, protein export does not appear to be altered in *envB* mutants.

When analyzed by SDS-urea-PAGE, most LPS preparations from *envB* strains were not found to differ from *envB*$^+$ LPS except for a slight reduction in the bands produced by LPS molecules carrying medium-sized O side chains (Fig. 3). This alteration was not observed when *envB* strains were grown in minimal medium instead of L broth. Yet, some LPS preparations, usually from *envB* strains extracted soon after construction, displayed an anomalous pattern characterized by the almost exclusive appearance of the bands corresponding to the two fastest-migrating LPS molecules (core and core plus one unit side chain) (27). Upon storage and/or subculturing of the strains, the LPS pattern became normalized.

Increased susceptibility to β-lactams accompanied by diminished amounts of porins could indicate that β-lactams

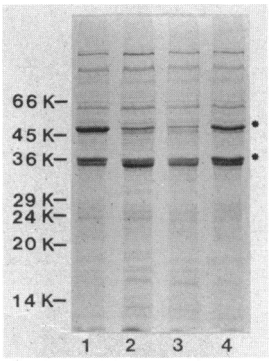

FIG. 1. SDS-PAGE of cell envelopes. Lane 1, strain DA1167 (*envB*$^+$); lane 2, DA1168 (*envB6*); lane 3, DA1172 (*envB9*); lane 4, DA1173 (*envB*$^+$). Separation was performed on a 12.5% slab gel. The positions of molecular weight standards are shown, and their corresponding molecular weights (in thousands [K]) are indicated. Asterisks indicate differences between *envB* and *envB*$^+$ strains.

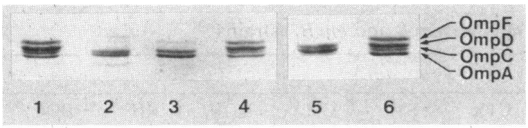

FIG. 2. SDS-PAGE of outer membrane proteins on a 12.5% slab gel. Cells were grown in nutrient broth without NaCl. Lane 1, strain DA1167 (*envB*$^+$); lane 2, DA1168 (*envB6*); lane 3, DA1172 (*envB9*), lane 4, DA1173 (*envB*$^+$). Identification of porin bands was as follows: lane 5, wild-type LT2 grown in nutrient broth with 0.3 M NaCl; lane 6, LT2 grown in nutrient broth without NaCl. Reduction of the OmpF band with the corresponding increase in the OmpC band at high osmolality (lane 5) and the reciprocal change at low osmolarity (lane 6) served to identify those porin bands (30). Only the significant part of the gel is shown.

enter the cell via a pathway other than porins. To test this possibility, permeability to cephalothin was measured in β-lactamase-producing derivatives of *envB* and *envB*$^+$ strains by the procedure devised by Zimmermann and Rosselet (35). The results are shown in Table 4. The permeability parameter (35) was lower for both *envB* mutants than it was for the *envB*$^+$ derivative, thus demonstrating that enhanced entrance was not occurring.

A stronger decrease in permeability could be expected for *envB* cells grown in nutrient broth without NaCl since, in this case, the low osmolarity of the medium would aggravate the effect of *envB* mutations on porins. However, attempts to measure permeability in such cultures were impeded by the fact that cells grown under those conditions were very fragile and, even in the permanent presence of Mg^{2+}, liberated large amounts of β-lactamase into the medium.

DISCUSSION

A strong decrease in the protein content of the outer membrane of *envB* mutants was observed in this study, and although quantitation of the loss suffered by each protein was not performed, it seems probable that most of the reduction was caused by the fall in flagellins and OmpF and OmpD porins.

Genetic evidence suggests that the *envB* gene plays a vital role in wild-type *S. typhimurium* (4), and a recent report showed that the *envB* product exerts negative control on the PBP 3 structural gene (33); notwithstanding this, precise information on *envB* function is still lacking. It also is not known in what manner *envB* mutations influence outer membrane proteins, but protein export does not seem to be involved since periplasmic proteins are not affected.

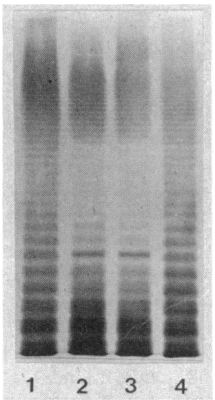

FIG. 3. SDS-urea-PAGE of LPS. Cells were grown in L broth. Lane 1, strain DA1167 (*envB*$^+$); lane 2, DA1168 (*envB6*), lane 3, DA1172 (*envB9*); lane 4, DA1173 (*envB*$^+$).

TABLE 4. Permeabilities of β-lactamase-producing derivatives of *envB* and *envB*$^+$ strains to cephalothin[a]

Strain	*envB* allele	Hydrolysis rate (nmol/min/mg [dry weight])		Periplasmic concn (μM)	Permeability parameter (ml/s/mg [dry weight])
		Disrupted cells	Intact cells		
DA1336	*envB*$^+$	388	63	39	1.1×10^{-3}
DA1338	*envB6*	368	41	26	7.0×10^{-4}
DA1339	*envB9*	207	33	39	5.7×10^{-4}

[a] Tests were performed with 1 mM cephalothin.

Alterations in the pattern of outer membrane proteins are not uncommon and have been observed in normal cells under special conditions (12, 20, 28, 32) and also in several mutants. Thus, deep-rough mutants of *S. typhimurium* that synthesize grossly defective LPSs suffer a pronounced decrease in flagellins and OmpF and OmpD porins (2), and a similar finding has been reported for heptoseless LPS mutants of *E. coli* (15) and *E. coli rfaD* mutants that, owing to a failure to synthesize L-glycero-D-mannoheptose, produce core-defective LPS (6). No core alterations were observed in the LPS of *envB* mutants by SDS-urea-PAGE. Therefore, the diminished content of outer membrane proteins in these strains does not seem to be related to the effects of *envB* mutations on LPS.

It is well established that under normal conditions β-lactam antibiotics traverse the outer membrane through hydrophilic channels formed by porins (22), and decreased porin content associated with enhanced resistance to β-lactams has been documented (10, 14). Contrary to those cases, OmpF and OmpD were drastically decreased in *envB* mutants, and yet, susceptibilities to all β-lactams tested except mecillinam and imipinem were increased.

Mecillinam binds almost specifically to PBP 2, and imipinem also binds strongly to PBP 2 and, with a lower affinity, to PBP 1B (11), whereas most of the other β-lactams act primarily on PBP 1B, PBP 3, or both (7). It has been suggested lately that in addition to its role in cell shape determination, PBP 2 performs a still unidentified function which is essential in wild-type cells but dispensable in *lov* and *cya* strains (26). It could be assumed that this function is also dispensable in *envB* mutants. Therefore, β-lactams that act primarily on PBP 2 would affect *envB* strains only when their periplasmic concentration reached the level required for inactivation of the other PBPs. This concentration should be quite high for mecillinam, because of its low specificity for PBPs other than PBP 2, and much lower for imipinem, which binds readily to PBP 1B.

Increased susceptibilities of *envB* strains to other β-lactams could not be attributed to indiscriminate entrance through phospholipid bilayer regions because, despite their high susceptibilities to DOC and crystal violet, *envB* mutants show a normal response to hydrophobic drugs such as chloramphenicol, novobocin, and rifampin. Moreover, the demonstration that permeability to cephalothin was not increased in *envB* strains provides conclusive evidence that there is not enhanced penetration of β-lactam antibiotics. Furthermore, susceptibility could not be caused by chromosomal β-lactamase failure because *S. typhimurium* does not possess this enzyme (O. Olsson, quoted in reference 18).

It is then concluded that PBP 1B, PBP 3, or both are more susceptible to β-lactam action in *envB* mutants. Because *envB* strains contain an apparently normal set of PBPs (26a), the question is whether the action of β-lactams on PBPs is facilitated or the effects of PBP inhibition are aggravated by the modification in cell shape brought about by *envB* mutations.

ACKNOWLEDGMENTS

The excellent technical assistance of Ana María Flores is gratefully acknowledged.

D.N.A. is a Career Investigator of Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina).

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