JOURNAL OF BACTERIOLOGY, Dec. 1980, p. 884-890
0021-9193/80/12-0884/07$02.00/0

Vol. 144, No. 3

# Overproduction of Outer Membrane Protein Suppresses *envA*-Induced Hyperpermeability

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A quantitative study on outer membrane components was performed in a number of envelope mutants of *Escherichia coli* K-12 exhibiting different permeability properties for antimicrobial agents. The *envA1* allele causing an increased influx for both hydrophobic and hydrophilic drugs was found to be associated with a deficiency in the amount of lipopolysaccharides. The *sefA1* allele acts as an extragenic suppressor on the EnvA phenotype. The *sefA1 envA1* double mutant was found to have a higher outer membrane buoyant density, apparently due to an increase in protein content. This double mutant was still low in lipopolysaccharide content.

The outer membrane (OM) of gram-negative enterobacteria constitutes an effective barrier against the penetration of hydrophobic substances of all sizes. Hydrophilic solutes with molecular weights below about 600 (e.g., the bulk of nutrients) pass through the outer membrane via aqueous pores (12). Specific OM proteins, denoted porins, have been shown to be constituents of these pores (10, 13). Molecular weight, charge, and degree of hydrophobicity of the solute greatly affect the rate of penetration through the OM pores (16). Porin-deficient mutants show a reduced rate of diffusion for hydrophilic low-molecular-weight compounds such as β-lactams (17).

Mutants of *Escherichia coli* K-12 and *Salmonella typhimurium* exhibiting an increased sensitivity to hydrophobic antibacterial agents fall into three main groups. One type of mutant lacks most of the carbohydrate portion of its lipopolysaccharide (LPS). Thus, heptose-deficient mutants are characteristically hypersensitive to a number of hydrophobic compounds, such as rifampin, vancomycin, novobiocin, and gentian violet, whereas the response to hydrophilic antibiotics is virtually unaffected (15). It has been suggested that in heptose-deficient mutants exposure of phospholipids on the outer leaflet of the OM enables hydrophobic solutes to penetrate the OM by dissolving into the lipid matrix (8). The second group of mutants is defective in the synthesis of the LPS core (6). These mutants exhibit increased sensitivity predominantly to hydrophobic substances. One of them, NbsA, showed at least a 90% reduction in phosphate content in the lipid A portion of its LPS. The NbsB mutant is defective in racemization of L-glycero-D-mannoheptose. In contrast, no structural defects in LPS have been demonstrated in the third group of mutants. These envelope mutants are in general hypersensitive to both hydrophobic and hydrophilic antibacterial agents (18, 21, 25). Envelope mutants of *E. coli* K-12 carrying the *envA1* allele are chain forming and characteristically hypersensitive to hydrophobic drugs such as rifampin and to hydrophilic antibiotics such as the β-lactams (19). Rifampin-resistant mutants of *envA1*-carrying strains fall into two groups, one of which probably represents true intragenic revertants (19). The mutants in the other group are cold sensitive and defective in septum formation (22). In two independent mutant strains which fall into this class, the mutations were located apart from *envA* and close to *proA* in a locus designated *sefA*.

Except for a relative decrease in phosphatidylglycerol compared with other OM phospholipids, no specific biochemical defect has been found in the OM of *envA1*-carrying strains (20). In this study the OM of *envA1* was compared with the OM of both the wild type and strains carrying the *sefA1* allele. It was concluded that the *envA1* mutation was associated with a decrease in LPS of the OM, and that the *sefA1* mutation increased the amount of protein in the OM.

## MATERIALS AND METHODS

**Bacterial strains.** The origin and properties of the *E. coli* K-12 strains used in this study are listed in Table 1.

**Media and growth conditions.** Bacteria were grown in the defined rich potassium morpholinepropanesulfonate medium of Neidhardt et al. (14). The solid medium used was LB (3) containing 1.5% agar (LA-plate). The bacteria were cultivated on a rotary

TABLE 1. *E. coli K-12 strains used*

| Strain | Relevant markers | Comments and references |
|---|---|---|
| D21 | *ampA1 his proA rpsL trp* | (5) |
| D22 | *envA1*; other markers as in D21 | Hypersensitive to drugs, chain forming (21) |
| D22S1 | *sup-200*; other markers as in D22 | Spontaneous, probably intragenic barrier revertant of strain D22 (19) |
| D2201 | *sefA1*; other markers as in D22 | Rifampin-resistant revertant of strain D22 (22) |
| D2101 | *sefA1 proA*$^+$; other markers as in D21 | *sefA1* transductant of strain D21 (22) |
| D21e7 | *lpsA1*; other markers as in D21 | Lacks rhamnose, galactose, and part of glucose in LPS (5) |
| D21f2 | Markers as in D21 | Heptose deficient (5) |

shaker at 37°C, and growth was assayed by optical density, using a Klett-Summerson colorimeter with a W66 filter. Cells were harvested at 50 Klett units (2 × $10^8$ cells per ml) after six generations of growth. The density of the cultures was kept within the range of 5 to 50 Klett units.

**Resistance determinations.** Resistance levels on plates were defined as the antibiotic concentration in the plates that gave 50% survival of single cells.

**Assay of β-lactamase.** β-Lactamase activity was assayed with the microiodometric method, with the modifications of Zimmerman and Rosselet (28).

**Determination of the penetration rate for cephalosporin C.** The diffusion parameter *C* was calculated according to Zimmerman and Rosselet (28). The strains used carry the *ampA1* mutation and consequently produce high amounts of the *E. coli* chromosomal β-lactamase. The hydrolysis rate for cephalosporin C was determined by keeping the substrate concentration at the $K_m$ for the chromosomal β-lactamase (0.22 mM). The hydrolysis rate in whole cells was determined by adding cephalosporin C directly to the culture. Samples were withdrawn during a period of 8 min for β-lactamase assay. The culture was then filtered through a 0.22-μm filter (Millipore Corp.), and the β-lactamase activity of the medium was determined. With all strains studied more than 90% of the β-lactamase activity was still cell bound at the end of the incubation.

**Preparation of the OM.** The OM was prepared without use of EDTA and lysozyme by a slight modification of the method of Smit et al. (27). Membranes were recovered by centrifugation in a type 65 rotor at 50,000 rpm for 45 min. An outer membrane fraction was obtained after a sucrose gradient centrifugation at 40,000 rpm for 13 h in an SW40 rotor.

**Polyacrylamide gel electrophoresis of proteins.** The gel electrophoresis procedure was as described earlier (T. Edlund, T. Grundström, G. R. Björk, and S. Normark, Mol. Gen. Genet., in press).

**Preparation, chromatography, and electrophoresis of $^{32}$P-labeled LPS.** To 10-ml cultures with a fivefold-reduced content of $K_2HPO_4$ as compared with the morpholinepropanesulfonate medium, 50 μCi of $^{32}P_i$ was added. LPS preparation and paper chromatography were as described by Boman and Monner (5), with their solvent system A. Polyacrylamide gel electrophoresis of $^{32}$P-labeled LPS was performed according to Bailey and Apirion (1). The gels contained 15% acrylamide and 0.2% sodium dodecyl sulfate and were molded with the same component concentrations as those used for electrophoresis of proteins. $^{32}$P-containing spots were visualized by autoradiography overnight.

**OM buoyant density determinations.** From overnight cultures in medium containing 1 mM glycerol, 40-ml cultures supplemented with 0.2 mM glycerol were inoculated. To the D21 culture 0.1 μCi of [*U*-$^{14}$C]glycerol per ml was added, and to the D2201 and D2101 cultures 0.8 μCi of [2(*n*)-$^3$H]glycerol per ml was added. Membranes were prepared as described above and suspended in 0.7 ml of 0.77 M sucrose in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid, pH 7.4. The D21 membranes were distributed equally between the D2201 and D2101 membrane preparations, and the mixtures were layered on 11.9-ml linear 1.0 to 2.0 M sucrose gradients in the same buffer. The gradients were centrifuged for 36 h at 40,000 rpm in a SW40 rotor and fractionated by collecting drops from the bottom of the tube.

**Determination of OM components.** Protein was determined by the method of Lowry et al. (9), using bovine serum albumin as the standard. Phospholipids were extracted by the method of Bligh and Dyer (4) and quantitated by the phosphorus assay of Bartlett (2). The phospholipid content calculations were based on an average molecular weight of 702 calculated from the relative frequency of different phospholipid components in strain D21 and D22 (20). Ketodeoxyoctanate was quantitated by the method of Osborn et al. (23). The ketodeoxyoctanate content of LPS from D21, purified by the method of Galanos et al. (7), was found to be 5.9%. This value was used to calculate the concentrations of LPS. The dry weights of membrane and LPS preparations were measured after drying in vacuum over diphosphoropentoxide. The contamination of OM with inner membrane was estimated by assaying lactate dehydrogenase (23).

**Distribution of phosphate within macromolecules.** Cells were labeled with $^{32}P_i$ as described above. The cell pellets were lysed as described by Bailey and Apirion (1). Polyacrylamide gel electrophoresis of total cell extracts was performed in gels containing 12% acrylamide and 0.2% sodium dodecyl sulfate and molded as described above. The bands containing phospholipids, LPS, and different nucleic acid species were localized by autoradiography overnight. The radioactivity in gel slices was measured after incubation overnight in scintillation fluid supplemented with 0.5 ml of Protosol (New England Nuclear Corp.).

**Aqueous biphasic partitioning.** To 10-ml cultures, 2.5 μCi of L-[4,5-$^3$H]leucine per ml was added. The harvested cells were washed twice in 0.9% NaCl. The phase systems were as described earlier (11), except that 0.1 ml of a suspension of bacteria ($1.5 \times 10^9$/ml) was added. The basal system contained dextran (enriched in the bottom phase) and polyethylene glycol (PEG) (enriched in the top phase). For tests with hydrophobic PEG, 0.13 mmol of palmitic acid was added per gram of PEG polymer by esterification of PEG-palmitate. For the test with PEG with positive charge 12.5% of the PEG had been exchanged with bis-trimethylamino PEG. Partition ratios were calculated as the ratios between the bacterial content (as radioactivity) in the top and bottom phases.

**Materials.** Benzylpenicillin (penicillin G) was kindly provided by A. B. Astra, Södertälje, Sweden. Cephalothin was a gift from Eli Lilly & Co., Indianapolis, Ind. Cephalosporin C and cephaloridine were gifts from Glaxo Laboratories Ltd, Greenford, England. [$^3$H]glycerol (500 mCi/mmol), [$^{14}$C]glycerol (46 mCi/mmol), [$^3$H]leucine (23 Ci/mmol), and $^{32}P_i$ were obtained from Amersham, England. Lysozyme, rifampin, RNase A, and DNase I were from Sigma Chemical Co., St. Louis, Mo. Zulkowsky starch was from E. Merck AG, Darmstadt, West Germany.

## RESULTS

**Barrier properties of the *envA1* mutant and its derivatives.** The chain-forming *envA1* mutant D22 is, when compared with its parental strain D21, hypersensitive to both drugs considered to be hydrophilic (e.g., β-lactams) and drugs considered to be hydrophobic (e.g., rifampin) (Table 2). Thus, strain D22 was 7 to 60 times more sensitive to cephalosporin C, cephalothin, and benzylpenicillin, whereas the cephaloridine sensitivity was virtually the same in the mutant and parental strain. It has been shown for cephaloridine, in contrast to the other listed β-lactams, that a penetration barrier in the OM is practically nonexistent; thus, the rate of penetration is very high even in wild-type *E. coli* cells (24, 28). These results suggested that the diffusion rate for β-lactams other than cephaloridine should be higher in the *envA1* mutant compared with its wild type. This assumption was verified by directly measuring the diffusion rate for cephalosporin C into intact cells by using a modification of the procedure described by Zimmerman and Rosselet (28). By this procedure we recorded a four- to fivefold increase in the diffusion rate of cephalosporin C in the *envA1* mutant D22, as compared with the wild type D21. The *sefA1* mutation suppresses the *envA1*-mediated hypersensitivity to rifampin. In an *envA1 sefA1* double mutant (strain D2201) the diffusion rate for cephalosporin C was 60% lower than for strain D22 (*envA1*). Moreover, the tolerance level to all β-lactams tested except cephaloridine was increased in D2201 to a level intermediate between those of D22 and D21. Strain D2101 (*sefA1*), lacking the *envA1* mutation, did not markedly differ from strain D2201 (*envA1 sefA1*) in its resistance to β-lactams and to rifampin.

**Surface charge and hydrophobicity of *envA1*, *sefA1*, and LPS mutants.** The effects of mutations on surface charge and liability to hydrophobic interaction were studied by partitioning the bacteria in aqueous biphasic systems of dextran and PEG containing positively charged bis-trimethylamino-PEG or hydrophobic PEG-palmitate. It was observed that all strains were transferred toward the top phases by bis-trimethylamino-PEG, suggesting that they all possessed a net negative surface charge (Table 3). The LPS mutant D21f2 was apparently less negatively charged, as reflected by a smaller change of the partition ratio on the addition of trimethylamino-PEG. No such effect was found for the *envA1* mutant D22. The effect of PEG-palmitate on the partition was such that the wild type D21 showed almost no liability to

TABLE 2. *Resistance to antibiotics and penetration rate for cephalosporin C in D21 and its envelope mutant derivatives*

| Strain | Resistance[a] (μg/ml) | | | | | Penetration rate for cephalosporin C[b] |
|---|---|---|---|---|---|---|
| | Rifampin | Benzylpenicillin | Cephaloridine | Cephalothin | Cephalosporin C | |
| D21 (wild type) | 4 | 160 | 2.4 | 21 | 210 | 1 |
| D22 (*envA1*) | 0.003 | 2.5 | 1.3 | 1 | 30 | 4.5 |
| D22S1 (*envA1 sup-200*) | 1 | 130 | 1.7 | 19 | 160 | 1.3 |
| D2201 (*envA1 sefA1*) | 1 | 22 | 1.0 | 11 | 85 | 1.8 |
| D2101 (*sefA1*) | 1 | 39 | 1.0 | 12 | 63 | 2.1 |
| Partition coefficient[c] | 65 | 0.6[d] | 0.12[d] | 0.26[d] | 0.002 | |

[a] Resistance was determined as the level of a drug permitting 50% of single cells to form colonies.
[b] Diffusion parameter *C* relative to D21, measured as described in the text.
[c] Partition coefficient in isobutanol–0.02 M phosphate buffer (pH 7.4)–0.9% (wt/vol) NaCl at 37°C. A high value indicates a hydrophobic substance.
[d] Data taken from Zimmerman and Rosselet (28).

TABLE 3. *Partition coefficient[a] in a dextran-PEG two-phase system of D21 (wild type) and the mutants*

| Strain | Partition coefficient | | |
|---|---|---|---|
| | Basal | TMA-PEG[b] | P-PEG[b] |
| D21 (wild type) | 0.087 (0.025) | 21.5 (1.5) | 0.094 (0.001) |
| D22 (*envA1*) | 0.056 (0.005) | 24.0 (6.3) | 0.166 (0.009) |
| D22S1 (*envA1 sup-200*) | 0.075 (0.001) | 32.3 (0.3) | 0.100 (0.008) |
| D2201 (*envA1 sefA1*) | 0.064 (0.017) | 32.3 (11.1) | 0.054 (0.030) |
| D2101 (*sefA1*) | 0.048 (0.017) | 24.0 (0.3) | 0.119 (0.002) |
| D21f2[c] | 0.104 (0.018) | 10.4 (3.4) | 0.415 (0.019) |

[a] The partition coefficient was calculated as the ratio between the bacteria content (as radioactivity) in the top phase enriched in PEG and the bottom phase enriched in dextran.

[b] Increase in partition coefficient compared with the basal system is indicating negative charge in the bis-trimethylamino-PEG (TMA-PEG) system and hydrophobicity in the PEG-palmitate (P-PEG) system. The figures within parentheses represent the range (two experiments performed).

[c] The LPS of D21f2 is heptose deficient (5).

hydrophobic interaction, whereas the degree of hydrophobicity was very pronounced in the heptose-deficient mutant D21f2. The effect of PEG-palmitate on the partition of the *envA1* mutant D22 was not as drastic as for D21f2, but was clearly greater than for the wild type, suggesting increased exposure of hydrophobic surface entities in D22. The *sefA1* allele suppressed *envA1*-induced effects on the physical properties of the cell surface. These biophysical data suggested that measurable biochemical differences should be observed in both the *envA1* and the *sefA1* mutants when compared with the wild type.

**Qualitative study of the OM components of *envA1* and *sefA1* strains.** Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis of OM preparations did not reveal any significant differences in the OM protein profiles of wild-type and *envA1* cells. Likewise, strains D22S1 (*envA1 sup-200*), D2201 (*envA1 sefA1*), and D2101 (*sefA1*) showed the same OM protein profile as did strain D21 (data not shown). The LPS moiety of strains D21 and D22 (*envA1*) has previously been demonstrated to contain the same molar ratios of carbohydrates and lipid A fatty acids (20). To examine the possibility of less drastic differences in the structure of the LPS of the studied strains, $^{32}$P-labeled LPS was prepared and separated by either paper chromatography or by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. In both cases the LPS of the mutants D22 (*envA1*), D22S1 (*envA1 sup-200*), D2201 (*envA1 sefA1*), and D2101 (*sefA1*) displayed the same $R_f$ values as did that of their parental strain D21. The *lpsA1* mutant D21e7, on the other hand, which lacks rhamnose in its LPS, showed an $R_f$ value of 0.60 in the paper chromatography system and an $R_f$ value of 0.61 in the polyacrylamide gel electrophoresis system, as compared with 0.20 and 0.67, respectively, for the other strains. These results strongly argued against qualitative LPS differences in the *envA1* strain and in its barrier revertants.

A modest relative reduction in OM phosphatidylglycerol of *envA1*-carrying strains has been reported. However, no marked differences in the relative distribution of the phospholipid classes in the OM of D21, D22 (*envA1*), and D2201 (*envA1 sefA1*) were found (19; unpublished data).

**OM buoyant density of *envA1* and *sefA1* strains.** We were intrigued by the possibility that quantitative rather than qualitative changes might exist in the OM of the mutants studied. In *sefA1*-carrying strains this idea was supported by the observation that the OM of D2201 (*envA1 sefA1*) and of D2101 (*sefA1*) banded slightly lower in the sucrose gradient as compared with the OM of D21. In the experiment shown in Fig. 1 a $^3$H-labeled membrane

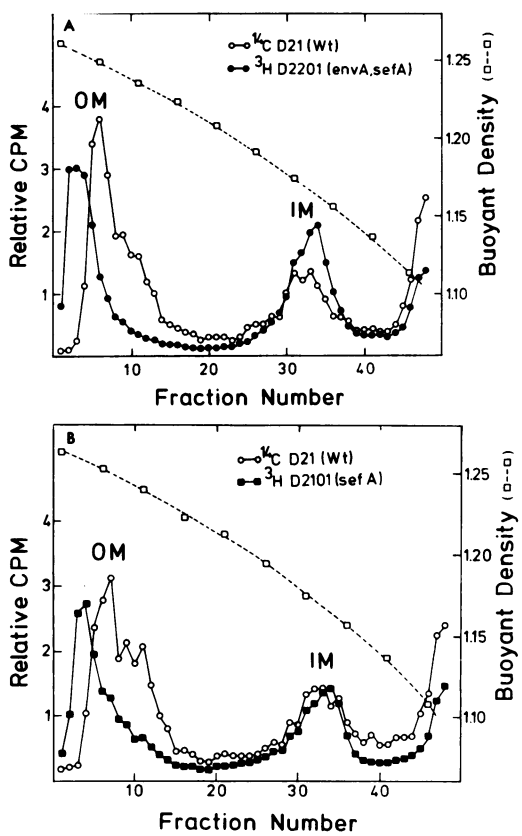


FIG. 1. *Isopycnic buoyant density gradient centrifugation of membrane material prepared from [$^{14}$C]-glycerol-labeled D21 cells and [$^3$H]glycerol-labeled D2201 (envA1 sefA1) and D2101 (sefA1) cells as described in the text.*

fraction from D2201 and a $^{14}C$-labeled membrane fraction from D21 were mixed, and the outer and inner membranes were separated by buoyant sucrose density gradient centrifugation. The radioactivity peaks corresponding to the cytoplasmic membrane showed the same density in D2201 and D21, whereas the OM fractions clearly differed in this respect. The higher density observed for the D2201 OM must be due to the *sefA1* allele, as the *sefA1* transductant D2101 also showed a similar increased outer membrane density (Fig. 1). The *envA1* mutation might also affect the buoyant density of the OM since the *envA1* OM in double-labeling experiments was usually found one fraction closer to the top of the gradient than OM prepared from the wild-type strain D21 (data not shown).

**Quantitative analysis of prepared OM from D21 and the envelope mutants.** The membrane fractions were washed three times with double-distilled water and then analyzed for various components (Table 4). Lactate dehydrogenase was chosen as a marker for inner membrane. Activity measurements of this enzyme indicated that the outer membrane preparations were 5 to 10% contaminated with inner membrane. The recovery of protein, phospholipid, and LPS was 92 to 101% of the determined dry weight of all preparations (Table 4). As the preparation method did not include lysozyme, the prepared OM might contain peptidoglycan (27). However, the amount of attached peptidoglycan was not determined.

The main quantitative OM difference between the wild type and the *envA1* mutant was a decrease in the LPS content of the latter which was not associated with an increased phospholipid content. In the *envA1* mutant the LPS/protein ratio was decreased by 30% when compared with the wild type. In strain D22S1 (*envA1 sup-200*), carrying an apparent intragenic suppressor mutation, the OM composition was very similar to that of the wild type.

TABLE 4. *Outer membrane composition in D21 (wild type) and the cell division mutants*[a]

| Strain | % Protein | % Lipopolysaccharide | % Phospholipid |
|---|---|---|---|
| D21 (wild type) | 42 (0.8) | 43 (1.6) | 16 (4.0) |
| D22 (*envA1*) | 46 (0.6) | 33 (1.4) | 13 (0.7) |
| D22S1 (*envA1 sup-200*) | 40 (0.7) | 40 (1.2) | 16 (2.0) |
| D2201 (*envA1 sefA1*) | 56 (6.0) | 31 (3.6) | 12 (1.5) |
| D2101 (*sefA1*) | 51 (1.4) | 36 (2.2) | 13 (1.7) |

[a] Expressed as percentage (wt/wt) of OM dry weight. The figures are the mean of at least five estimations. The figures within parentheses are the standard deviations.

In the *sefA1* mutant D2101 a 24% increase in protein content of the OM was found, combined with a 13 and 19% decrease of LPS and phospholipid, respectively. In the *envA1 sefA1* mutant D2201 a combination of the *envA1* and *sefA1* effects on OM composition was recorded. A 35% increase in protein content was found compared with D21 and D2101, and when compared with D2101, a decrease of LPS was found which was more pronounced than was the reduction of phospholipid (14 and 8%, respectively).

**Relative content of nucleic acid, LPS, and phospholipid in the envelope mutants.** The distribution of $^{32}P$ within macromolecules was determined in the envelope mutants and their parental strain D21 by polyacrylamide gel electrophoresis. The percentage distribution of nucleic acid, LPS, and phospholipid was measured. The result confirmed the quantitative analyses of OM components described above; that is, the *envA1* mutant D22 when compared with the wild type showed 29 and 38% less $^{32}P$-labeled LPS relative to labeled lipid and labeled nucleic acid, respectively. In the *envA1 sefA1* double mutant D2201 the percentage of $^{32}P$ label in LPS and phospholipids relative to nucleic acids was 35 and 10% lower, respectively, when compared with those of D21. The $^{32}P$ label in LPS and in phospholipids was also decreased in the *sefA1* mutant D2101.

## DISCUSSION

The *envA1* mutant, in contrast to heptose-deficient mutants, is hypersensitive to both hydrophobic and hydrophilic antibiotics (19). Here we demonstrate that the permeability coefficient for a hydrophilic compound, cephalosporin C, was about four to five times higher for *envA1* than for the wild type. Thus, unlike heptose-deficient mutants, the *envA1* mutation leads to an increased rate of influx not only for hydrophobic but also for hydrophilic compounds.

No significant qualitative differences in any OM components were found in the *envA1* mutant when compared with its wild-type parent. The LPS of the *envA1* mutant showed the same mobility in chromatography as that of the wild type LPS, in contrast to that of the LPS in all described mutants. The agreement between the phosphate labeling of the LPS and the 2-keto-3-deoxyoctulosonic acid contents in the *envA1* mutant and the wild type shows that the *envA1* mutant is phenotypically distinct from NbsA (6). Two-phase partitioning studies revealed that the cell surface of the *envA1* mutant was more hydrophobic than that of the wild type. However, the degree of hydrophobicity displayed was not

as marked as in a heptose-deficient *E. coli* mutant. In the latter type of mutant an increased amount of phospholipid is found in the OM (27). Exposure of lipids on the outer leaflet of the OM has been claimed to enable passage of hydrophobic compounds (27). However, the phospholipid content of the *envA1* OM is not increased. This, together with the observed resistance of the OM of an *envA1*-carrying strain towards phospholipase C (J. Starka, personal communication) argues against large phospholipid-containing areas on the outer leaflet of *envA1* OM. Instead, it is tempting to suggest that the reduced amount of LPS found in an *envA1* strain is directly related to the permeability defects and the increased hydrophobicity of the OM.

The observed LPS reduction in *envA1* is only partially compensated for by an increase in OM protein. However, the recovery of protein, LPS, and phospholipids was 7% lower in *envA1* compared with the other strains. The possibility of an increase in *envA1* OM of some other component, e.g., glycolipid, can therefore not be excluded. The decrease in LPS in the OM of *envA1* strains would lead to an increased distance between hydrophilic LPS sugar chains in the outer leaflet. It is possible that hydrophobic molecules could be exposed which might facilitate the solubilization of a hydrophobic compound into the lipid interior of the membrane. The biochemical reason for the increase in penetration rate for intermediate-sized hydrophilic compounds is not understood at present. However, it most likely reflects a changed pore function.

The *sefA1* mutation suppresses the EnvA1 phenotype apparently by introducing more protein into the OM. The OM protein/LPS ratio was 85% higher in the *envA1 sefA1* double mutant in comparison with the *envA1* mutant and 30% higher in comparison with the corresponding wild-type strain. Thus, our data clearly indicate that the argued exposure of hydrophobic moieties caused by the low amount of OM LPS found in the *envA1* strain is suppressed by insertion of extra amounts of OM proteins in the *envA1 sefA1* strain. No qualitative differences were found in the OM protein profiles of *sefA1*-carrying strains. This suggests that *sefA1* leads to a uniform increase in different OM proteins rather than an increased production in any particular OM protein. The *sefA1 envA1* double mutant did not show an increased amount of LPS per cell mass when compared with the *envA1* mutant. Nevertheless, *sefA1* suppressed the high diffusion rate for cephalosporin C caused by the *envA1* mutation. This suggests that the LPS molecule per se does not contribute to the pore function. Rather, our data favor the hypothesis that either the functional diameter of the pore or its activity (26) can be altered, depending on the amount of protein present in the OM.

## ACKNOWLEDGMENTS

We express our gratitude to Christina Fors for skillful technical assistance. The stimulating discussions with John K. Davies and Hans Wolf-Watz are gratefully acknowledged.

This work was supported by Swedish Natural Science Research Council grant Dnr B 3373-101.

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