Isolation and characterization of hemin-permeable, envelope-defective mutants of *Salmonella typhimurium*

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From *Salmonella typhimurium* LT2 *hemA* (δ-aminolevulinic acid requiring) 15 mutants were isolated which grew on the hydrophobic compound hemin. All had increased sensitivity to antibiotics such as vancomycin, bacitracin, novobiocin, erythromycin, rifampin, and oleandomycin, and were considered to be envelope mutants (Env$^-$). Half the mutants were rough, based on altered bacteriophage sensitivity and deoxycholate sensitivity, whereas the remainder were smooth; three of the smooth mutants were studied in detail. They gave increased uptake of gentian violet but no increase in leakage of a periplasmic protein, RNase I. The total membranes and fractions from sucrose gradient centrifugations representing inner and outer membranes of the wild type and three mutants were examined by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) and isoelectric focussing – PAGE (IEF–PAGE). The major outer membrane proteins (molecular weights (MW)33 000, 34 000, 35 000, and 36 000) showed no or very little alterations in the Env$^-$ mutants. In SA1926 (*env-52*) one protein spot at MW 48 000, proven to be an outer membrane protein, was missing, whereas a new spot appeared nearby, and other proteins in this area of the gel were reduced. An Env$^+$ transductant selected from this strain had the wild-type protein pattern restored. The two other Env$^-$ mutants had similar but not identical changes in protein composition.

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A partir de *Salmonella typhimurium* LT2 *hemA* (souche dépendante en acide δ-aminolevulinique), on a isolé 15 mutants capables de pousser sur l'hémine, un composé hydrophobe. Tous ces mutants étaient plus sensibles vis-à-vis d'antibiotiques tels la vancomycine, la bacitracine, la novobiocine, l'érythromycine, la rifampicine et l'oléandomycine et ont été considérés comme des mutants d'enveloppe (Env$^-$). La moitié des mutants étaient en phase rugueuse selon les critères d'un changement de sensibilité aux bactériophages et de la sensibilité au déoxycholate alors que l'autre moitié était en phase lisse. Trois de ces mutants en phase lisse ont été étudiés en détail. Chez ces mutants, il y a une augmentation de la captation du violet de gentiane mais pas d'augmentation dans la perte d'une protéine périplasmique, la RNase I. Les membranes complètes et les fractions obtenues par centrifugation en gradient de sucrose représentant les membranes interne et externe du type sauvage et des trois mutants ont été étudiées par électrophorèse en gel de polyacrylamide – dodécyl sulfate de sodium (SDS–PAGE) et électrofocalisation (IEF)–PAGE. Pour les trois mutants Env$^-$, les principales protéines de la membrane externe (poids moléculaire 33 000, 34 000, 35 000 et 36 000) sont très peu ou pas altérées. Chez SA1926 (*env-52*), il manquait une bande de protéine à poids moléculaire 48 000 protéine constituante de la membrane externe, alors qu'il apparaissait une nouvelle bande à proximité et que les autres protéines de cette région du gel étaient réduites. Un transductant Env$^+$ sélectionné à partir de cette souche avait retrouvé le profil protéique de la souche sauvage. Les deux autres mutants Env$^-$ avaient des changements similaires mais non identiques de leur composition protéique.
[Traduit par le journal]

Introduction

The Gram-negative cell envelope has two membranes, the inner or cytoplasmic membrane with properties that resemble the membrane of Gram-positive bacteria and of other cells, and the outer membrane, a unique assemblage containing phospholipids (PL), proteins, and lipopolysaccharide (LPS) (Costerton *et al.* 1974; Stocker and Mäkelä 1978; Nikaido and Nakae 1979). The inner membrane contains the components of numerous active transport systems which accumulate compounds in the cell against concentration gradients. The outer membrane acts as a permeability barrier, resulting in resistance to antibiotics such as actinomycin D, erythromycin, novobiocin, and bacitracin to dyes such as crystal violet, and to detergents such as bile salts and sodium dodecyl sulfate (Nikaido and Nakae 1979). A role for LPS in the outer membrane in this barrier function was indicated, since mutation to produce rough strains with defects in the LPS core (Roantree *et al.* 1977; Schlecht and Westphal 1970; Sanderson, MacAlister *et al.* 1974) or removal of LPS by EDTA treatment (Leive 1974) greatly increased sensitivity to these

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TABLE 1. *Salmonella typhimurium* LT2 strains used in this study

Stock No.	Genotype	Source
SA772	*hemA70*	Săsărman *et al.* (1970)
SA1922	Wild type	SA772 transduction and Hem$^+$ selection[a]
SA1911	*hemA70 env-51*	SA772 mutagenization and hemin-responding selection[b]
SA1925	*env-51*	SA1911 transduction and Hem$^+$ selection
SA1913	*hemA70 env-52*	SA772 mutagenization and hemin-responding selection
SA1926	*env-52*	SA1913 transduction and Hem$^+$ selection
SA1929	Wild type	SA1926 transduction and Env$^+$ selection[c]
SA1914	*hemA70 env-53*	SA772 mutagenization and hemin-responding selection
SA1931	*env-53*	SA1914 transduction and Hem$^+$ selection
SA1355	Wild type (P22)$^+$	Sanderson, Van Wyngaarden *et al.* (1974)
SA1377	*rfaC630* (P22)$^+$	Sanderson, Van Wyngaarden *et al.* (1974)

[a]Transduction was with P22 *int-4*, which had been grown on *S. typhimurium* LT2.
[b]Mutagen was NG and selection for hemin response was on ABA plus 20 μg hemin chloride/mL.
[c]Transduction was with P22 *int-4*, which had been grown on *S. typhimurium* LT2, with selection for Env$^+$ (resistance to vancomycin on ABA).

antibiotics. Nikaido and Nakae (1979) postulated two main pathways through the outer membrane. The hydrophilic pathway, which accommodates compounds that partition into phosphate buffer rather than 1-octanol, is not much influenced by the LPS composition of the cell, and is effective only with molecules smaller than molecular weight (MW) 600–660 (Decad and Nikaido 1976). Reconstitution studies with mixed PL–LPS bilayers to which outer membrane proteins were added indicate that the "peptidoglycan-associated" major proteins or porins, called Ia and Ib in *Escherichia coli* (Schmitges and Henning 1976), and 34K, 35K, and 36K[4] in *Salmonella typhimurium* (Nikaido and Nakae 1979), have a crucial role in this mechanism, probably forming water-filled pores with specific exclusion limits (Nakae 1975, 1976*a*, 1976*b*). The hydrophobic pathway for the penetration of compounds that partition into 1-octanol rather than phosphate buffer is almost inactive in wild-type strains of *S. typhimurium* and *E. coli*; Nikaido and Nakae (1979) proposed that there is no PL, but there is LPS in the outer leaflet of the outer membrane (Smit and Nikaido 1978; Mühlradt and Golecki 1975) and hydrophobic compounds will not penetrate. They further postulated that the loss of LPS in rough mutants, which results in loss of protein as well (Ames *et al.* 1974; Koplow and Goldfine 1974), results in PL in the outer leaflet to produce a PL bilayer through which hydrophobic compounds can penetrate. The appearance of PL on the outer surface of the cell was detected by covalent labelling with cyanogen bromide activated dextran (Kamio and Nikaido 1976).

This report describes a positive selection method for isolation of mutants that no longer exclude certain hydrophobic compounds; we chose to investigate those mutants that are smooth (i.e., normal in their LPS). The method uses a hemin nonsynthesizing strain of *S. typhimurium*; cells with the normal barrier function are permeable to and will grow on a small hydrophilic molecule, δ-aminolevulinic acid (δ-ala) (MW 131) but are not permeable to the larger, hydrophobic hemin (MW 651) (Săsărman *et al.* 1970). Mutants that grew on hemin were selected and characterized for growth pattern, permeability to a variety of compounds, phage sensitivity, and leakage of periplasmic enzymes, and the proteins were examined with sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) and isoelectric focussing – PAGE (IEF–PAGE).

Materials and methods

Bacterial strains and phages

The strains used are listed in Table 1. The phages used to distinguish smooth and rough mutants and the methods used were those described by Wilkinson and his colleagues (1972). P22 *int-4* phage, a nonlysogenizing mutant, was used in transduction.

Media

Tests were done using the following media: Difco nutrient broth (NB) and Difco nutrient agar (NA) (NB plus 1.5% Difco Bacto-agar), both of which contained no sodium chloride; Difco Penassay broth (PB) (also called antibiotic medium No. 3), and Penassay broth solidified with 1.5% Difco Bacto-agar (ABA); Oxoid blood agar base (CM55); and Davis modified

[4]Proteins 33K (elsewhere in text), 34K, 35K, and 36K are designated as such in the literature cited and are proteins with approximate molecular weights of 33 000, 34 000, 35 000, and 36 000, respectively.

minimal media (MM) (Davis and Mingioli 1960), containing 0.2% glucose. Media were supplemented where necessary with δ-ala (20 μg/mL), hemin chloride, (10 μg/mL), or amino acids (20 μg/mL). All growth was at 37°C unless otherwise stated.

Chemicals

The following chemicals were used: hemin chloride and δ-aminolevulinic acid (δ-ala) (Calbiochem); [U-^{14}C]polyadenylic acid (Miles Laboratories, Inc.); erythromycin, vancomycin, tetracycline, novobiocin, gentamicin, nalidixic acid, clindamycin, nafcillin, rifampin, oleandomycin, streptomycin, chloramphenicol, ampicillin, carbenicillin, and cephalothin sensitivity discs (Baltimore Biological Laboratories); bacitracin sensitivity discs (Difco); gentian violet (GV) (Fisher); sucrose ultrapure (Schwartz-Mann); urea (Baker); ampholines (LKB); nicotinamide adenine dinucleotide phosphate (NADP), bovine serum albumin (BSA), DNase, RNase I, ovalbumin, chymotrypsinogen A, *N*-methyl(*N'*-nitro)-*N*-nitrosoguanidine (NG), sodium deoxycholate (DOC), acrylamide, and bisacrylamide (Sigma Chemical Co.).

Mutagenesis

Mutagenesis was performed on NA supplemented with hemin. Cells were grown in NB by fermentation, or NB supplemented with δ-ala in which case they were washed before use. Cells were spread on a plate and a solution containing 100 μg of the mutagen, NG, was spotted in the center of the plate.

Transduction

Where Hem$^+$ transductants were desired, Hem$^-$ cells were inoculated by flooding broth cultures onto NA plates, then P22 *int-4* phage grown on Hem$^+$ LT2 was spotted on top. The plates were incubated overnight and the Hem$^+$ transductants were streaked for single-colony isolation.

Initial characterization of Env$^-$ mutants

Phage sensitivity was tested by placing a drop of the phage suspension (at a titer of ca. 10^8 plaque-forming units/mL) onto a lawn of cells on either NA or ABA plates. The plates were incubated overnight. Antibiotic discs were placed on NA or CM55 plates that had been inoculated by flooding with broth cultures and dried. After overnight incubation, sensitivity was measured as the radius of the circle of growth inhibition around the antibiotic disc. Sensitivity or resistance to DOC was determined as growth inhibition on NA plates containing a final concentration of either 0.1 or 0.4% DOC. GV uptake was determined using a modification (Gustafsson *et al.* 1973) of the Normark and Westling (1971) technique.

Enzyme assays

Samples (8–10 mL) of log phase cultures grown to 80 Klett units in nutrient broth with no NaCl were removed and centrifuged at 10 000 × *g* for 10 min at 4°C. The supernatants were assayed without further treatment. The pellet of cells was resuspended in the same volume of 0.01 *M* Tris–HCl, pH 7.0, and sonicated extracts (nine 10-s bursts, with 1-min cooling periods, using a Branson sonicator set at the No. 3 reading) were assayed for enzyme activity. The assays for RNase I (Lopes *et al.* 1972) and succinic dehydrogenase (King 1967) have been described.

Preparation of total membranes

A slight modification of the technique of Smit *et al.* (1975) was used. All procedures were done at 0–4°C except where noted. The cells were grown in 1 L of NB, with shaking, to 80 Klett units and harvested in a Sorvall centrifuge at 12 000 × *g* for 15 min, washed once in 10 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, pH 7.4, and the pellets stored frozen. Thawed pellets were suspended in 25 mL HEPES, with 1 mg each of DNase and RNase A. The suspension was passed twice through a cooled French pressure cell (Aminco) at 14 000 – 16 000 lb/in.^{2} (1 lb/in.^{2} = 6.895 kPa) and centrifuged at 12 000 × *g* for 15 min to remove unbroken cells and debris. The supernatants were centrifuged at 226 000 × *g* in a Spinco fixed-angle Ti60 rotor (Beckman) for 90 min. The pellets were resuspended (using a tissue grinder) and washed twice with HEPES buffer. This suspension was referred to as total membranes and contained 6–10 mg protein/mL.

Separation of inner and outer membranes

This was done according to the method of Koplow and Goldfine (1974).

PAGE

Slab gels were prepared according to the method of Laemmli (1970) and consisted of an 11-cm lower separating gel and a 1-cm upper spacing gel. Separating gels were either 10 or 15% acrylamide. The electrode buffer and the sample buffer of Laemmli were used and the samples were dissolved by boiling them for 3 min in the sample buffer. Protein (100–200 μg) was loaded in each sample well. The gels were run at 50 mA (four slabs at a time) in a Pharmacia gel electrophoresis G-4 apparatus until the bromophenol marker dye had entered the separating gel, at which time the current was increased to 100 mA. The gels were fixed in 25% isopropanol and 10% acetic acid overnight, then stained in 0.2% Coomassie brilliant blue, 45% methanol, and 10% acetic acid for 2 h. Destaining was done electrophoretically at 36 V for 20 min in 7% acetic acid.

Two-dimensional separation (IEF–PAGE)

A slight modification of the techniques of Ames and Nikaido (1976) and O'Farrell (1975) was used. Samples were prepared according to Ames and Nikaido (1976) with the exception that the samples were solubilized by boiling for 3 min. Protein (200–300 μg) was loaded on each tube. The IEF gels were made as described by O'Farrell (1975) using a 2% ampholine solution comprising the following pH ranges: 4–6, 6–8, and 3.5–10 in a 2:2:1 ratio. The electrophoresis was carried out at 300 V for 18–19 h and then at 400 V for 1.5 h. At the end of the run, gels were removed from tubes and either frozen or run in the second dimension immediately with no equilibration. The second dimension was performed on either 10 or 15% acrylamide SDS slab gels prepared as previously described with the exception that no wells were formed in the stacking gel. The IEF gel was placed on the top of the stacking gel between the glass plates and held there by adding a warm 1% agar solution dissolved in sample buffer. The whole process from removal of the IEF gel from the tube to the start of the second-dimensional run was done as swiftly as possible to

TABLE 2. Generation times of Env$^+$ and Env$^-$ strains

Stock No.	Genotype	Growth (generation time in minutes)[a] NB	NB plus δ-ala (20 μg/mL)	NB plus hemin chloride (50 μg/mL)
SA772	*hemA70*	∝[b]	144	∞
SA1914	*hemA70 env-53*	∞	360	240
SA1922	Wild type	42	NT[c]	NT
SA1931	*env-53*	50	NT	NT

[a]Growth was measured in a Klett-Summerson colorimeter using a red filter (No. 66). HemA$^-$ strains were grown in 5 mL of broth in Klett tubes and were aerated by bubbling air through glass tubes; HemA$^+$ strains were grown in 10 mL of broth in 250-mL sidearm flasks and were aerated by shaking on a New Brunswick Scientific incubator at 150 rpm. Generation time is the time for a doubling of optical density.
[b]The sign ∝ indicates no increase in optical density during the time of the experiment.
[c]Not tested.

prevent diffusion of the protein bands. The SDS gels were run as previously described. The pH gradient was determined as described in Ames and Nikaido (1976).

Total protein determination

This was performed using the method of Lowry *et al.* (1951) with BSA as standard.

Results

Isolation of hemin-responding mutants

Envelope-defective (Env$^-$) mutants were selected directly using HemA$^-$ strains, which grow in the presence of δ-ala (MW 131) but not on the larger end product hemin (MW 651), as hemin cannot penetrate into the cell (Săsărman *et al.* 1970). HemA$^-$ cells (SA772, *S. typhimurium* LT2 *hemA70*) (Table 1) were mutagenized with NG, placed on NA plus 10 μg/mL hemin, and those few colonies that grew were selected; these were assumed to have a defect in their permeability barrier. This assumption was confirmed by the fact that every colony isolated in this manner had an increased sensitivity to one or more antibiotics.

Fifteen hemin-responding isolates were obtained; of these, eight were classed as smooth, with normal LPS based on sensitivity to phages P22 and FO and resistance to phage Ffm, whereas seven were considered rough (O-somatic sugar deficient LPS) based on resistance to phages P22 and FO and sensitivity to phage Ffm (Wilkinson *et al.* 1972). The classification was confirmed because the smooth parental line, SA772, and all mutants classed as smooth were DOC resistant (able to grow in 0.4% DOC), whereas all the strains classed as rough were unable to grow in 0.4% DOC; rough mutants with greatly enhanced permeability, which is usually due to defects in the heptose component of the LPS, are usually sensitive to DOC (Roantree *et al.* 1977; Sanderson, Van Wyngaarden *et al.* 1974; Wilkinson *et al.* 1972). In preliminary experiments with Hem$^-$ strains, all 15 hemin-responding mutants had increased sensitivity to one or more of the antibiotics vancomycin, bacitracin, novobiocin, or erythromycin, so all were inferred to have cell envelope defects. No further work was done on the rough mutants. Of the eight smooth hemin-responding mutants, three were selected for detailed analysis.

For easier growth and handling, the HemA$^-$ strains, both SA772 (Env$^+$) and hemin responding (Env$^-$), were transduced to HemA$^+$ with P22 *int-4* phage. The Hem$^+$ Env$^-$ transductants retained the antibiotic and phage sensitivity of their Hem$^-$ Env$^-$ parents. The P22 *int-4* phage does not form stable lysogens; nonlysogenic lines were selected by replica plating single-colony isolates of the lysogenic transductants onto a lawn of *S. typhimurium* LT2 (a P22-sensitive strain) and selecting colonies that failed to give lysis of the indicator strain.

To isolate isogenic Env$^+$ lines of the Env$^-$ mutants, P22 phage grown on *S. typhimurium* LT2 Env$^+$ was adsorbed to SA1926 Env$^-$ in PB for 30 min; the cells were then plated on NA plus 220 μg vancomycin/mL and a vancomycin-resistant transductant, SA1929, was isolated. SA1929 was restored to the wild-type phenotype for resistance to erythromycin and novobiocin as well as to vancomycin indicating that a single gene controlled sensitivity to all three antibiotics.

Growth of hemin-responding mutants

The growth of a hemin-responding mutant, SA1914, was compared with that of its parent, SA772 (Table 2). Neither strain grew in NB, but both grew slowly when supplemented with δ-ala; the Env$^-$ mutant, SA1914, grew as well in hemin chloride as in δ-ala, whereas SA772 did not grow in hemin chloride. The HemA$^+$ transductants of both strains, SA1922 and SA1931, grew in NB.

TABLE 3. Properties of *S. typhimurium* strains

Stock No.	Genotype	Rough or smooth phenotype[a]	%GV uptake[b]	Antibiotic sensitivity[c,d] Ery (15)	Bac (10)	Van (30)	Nb (30)	Rif (15)	Ol (15)	Cl (2)	Nf (1)
SA1922	Wild type	S	22	1	0	0	1	1	0	0	0
SA1925	*env-51*	S	56	3	3	3	3	4	3	0	0
SA1926	*env-52*	S	53	4	4	4	2	5	3.5	0	0
SA1931	*env-53*	S	61	6	6	0	7	2.5	3.5	3.5	2
SA1929	Wild type	S	43	0	0	0	1.5	1.5	0.5	0	0
SA1355	Wild type	S	26	1	0	1	2				
SA1377	*rfaC630*	R	70	2	4	2	3				

[a]Rough (R) or smooth (S) phenotype determined according to phage sensitivity. Smooth, P22 and FO sensitive and Ffm resistant; rough, Ffm and P22 sensitive and FO resistant. SA1355 and SA1377 are both exceptions, being P22 resistant (Sanderson, Van Wyngaarden *et al.* 1974).

[b]GV uptake is measured as described in Materials and methods. The values given are the averages of at least three separate determinations.

[c]Antibiotic sensitivity is given as the radius of the zone of growth inhibition around the disc. Ery, erythromycin; Bac, bacitracin; Van, vanomycin; Nb, novobiocin; Rif, rifampin; Ol, oleandomycin; Cl, clindamycin; Nf, nafcillin. Antibiotic disc concentration is given as micrograms per disc (in parentheses). Env$^-$ and Env$^+$ strains showed no significant difference in sensitivity to antibiotics (micrograms per disc) gentamicin (10), streptomycin (10), chloramphenicol (30), ampicillin (10), carbenicillin (50), cephalothin (30), nalidixic acid (30), and tetracycline (30).

All the HemA$^+$ derivatives of the strains grew at 37°C on MM plus glucose; this indicates that no additional auxotrophies were introduced. This same observation was made on the mutants while they were still in the HemA$^-$ state, i.e., they grew at 37°C on MM plus δ-ala without any other supplements. There is no temperature sensitivity of growth of any of the envelope mutants at elevated temperatures (42 or 44°C).

Phage and bacteriocin sensitivity

All three Env$^-$ mutants, as well as the parent, are sensitive to phages ES18, PH54, and PH 105, indicating that these mutants retain the receptors for these phages; these receptors are thought to be outer membrane proteins (Graham and Stocker 1977; Siitonen *et al.* 1977). Both the Env$^+$ and the Env$^-$ strains are resistant to the phage PH51, as the LPS of the smooth strains is thought to block the phage receptor (Siitonen *et al.* 1977). SA1922 (Env$^+$), SA1925 (Env$^-$), and SA1931 (Env$^-$) are resistant to the bacteriocin 4-59 produced by *S. canastel* SL1702; this is to be expected, as the strains are all smooth and it is believed that the LPS shields the bacteriocin receptor (Graham and Stocker 1977). SA1926, which like the other Env$^-$ strains was classed as smooth based on phage sensitivity, shows a partial sensitivity to the bacteriocin, which may indicate some defect in LPS synthesis or assembly.

Sensitivity to antibiotics, DOC, and GV

It is known that certain *env* mutants (Egan and Russell 1973), *tol* mutants (Bernstein *et al.* 1972), and rough mutants (Roantree *et al.* 1977; Sanderson, Van Wyngaarden *et al.* 1974) have altered sensitivity to detergents such as DOC. However, the strains SA1922, 1925, 1926, and 1931 grow normally in NA plates with 0.1 and 0.4% of the detergent.

The Env$^-$ strains demonstrated increased sensitivity to a number of antibiotics (Table 3). Erythromycin (MW 773), bacitracin (MW 1460), vancomycin (MW 3300), and novobiocin (MW 610) were used as the initial discriminators of sensitivity because they are more effective against Gram-positive bacteria than Gram-negative bacteria and because they are more effective on rough strains than smooth strains (Roantree *et al.* 1977; Sanderson, Van Wyngaarden *et al.* 1974; Schlecht and Westphal 1970). With only a few exceptions, all three Env$^-$ mutants were more sensitive to the antibiotics erythromycin, bacitracin, vancomycin, novobiocin, rifampin, and oleandomycin than were Env$^+$ strains, whereas only SA1931 (*env-53*) was more sensitive to clindamycin and nafcillin (Table 3). These data report the sensitivity of the HemA$^+$ transductants, but the sensitivity of the HemA$^-$ strains originally isolated is similar (data not shown). There were no significant differences in sensitivity with several other antibiotics (Table 3).

Gustafsson *et al.* (1973) found that Env$^+$ strains of *E. coli* instantaneously take up about 20% of a 10 μg/mL solution of GV at 37°C, which they attribute to binding of the positively charged dye molecule to the cell envelope. In Env$^-$ mutants, but not in Env$^+$ strains, this initial binding of dye was followed by uptake into the cell and binding to the ribosomal fraction (Gustafsson *et al.* 1973). Env$^+$ smooth strains of *S. typhimurium* bound 20–30% of the dye (Table 3), whereas a rough mutant, SA1377, bound 70%. The three Env$^-$ mutants took up 50–60% of GV, i.e., two to three times that of their parents, whereas the Env$^+$ transductant, SA1929, showed reduced uptake (43%).

Leakage of a periplasmic enzyme

Leakage of RNase I into the medium during growth in

NB at 37°C was 1–3% of the total, cell-bound enzyme in both the Env$^+$ parent, SA1922, and in the three Env$^-$ mutants; thus, leakage of this periplasmic enzyme is not increased in the Env$^-$ mutants.

Membrane proteins of the parental line

The total membranes of the parent, SA1922, were examined using both PAGE and IEF–PAGE. IEF–PAGE resolved 50–60 protein spots (shown in a composite drawing, Fig. 1) in 15% gels with a pH gradient from 6.5 to 5.0, whereas PAGE showed 30–40 bands. There are four major outer membrane proteins in SA1922 visible in the densitometry tracings of PAGE (Fig. 2), and in IEF–PAGE (Fig. 3) where three of the proteins, the 34K, 35K, and 36K "porins," run close together in the acid end of the gel and the fourth (the 33K protein) is at the more basic end of the gel (about pH 6.2). The acidic isoelectric point for the higher MW porins, and a more basic one for the low MW (33K) or II* protein, agrees with that found by Sato *et al.* (1977) for *E. coli*.

Comparison of total, inner, and outer membranes of SA1922

Inner and outer membranes were separated by sucrose density gradient centrifugation, and two distinct peaks were obtained (data not shown). The higher density peak was enriched in the major outer membrane proteins in the MW 33 000 – 36 000 region; this is called the outer membrane fraction (Fig. 2). Only traces of these proteins remained present in the lower density peak, which is called the inner membrane fraction. Contamination of the outer membrane fraction with inner membrane proteins was indicated by a reduced but significant level (19% of the activity found in total

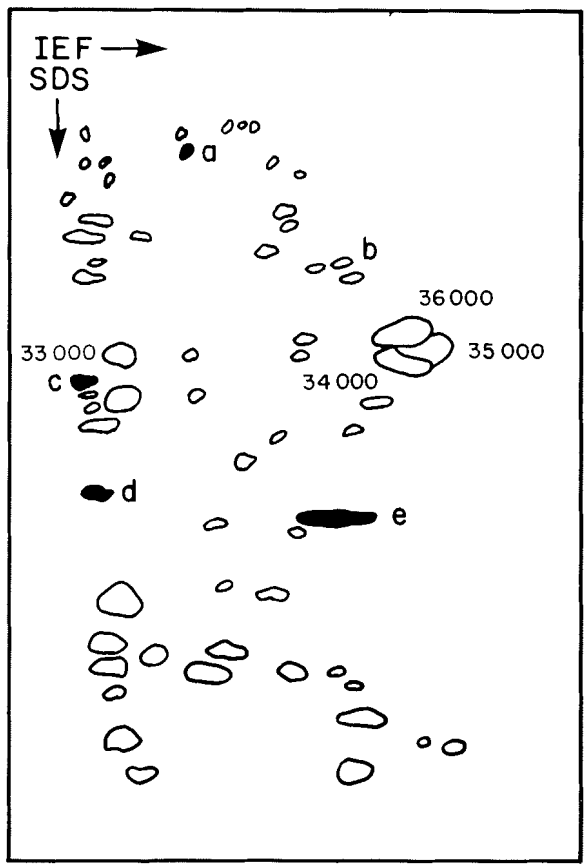

FIG. 1. A composite drawing of an IEF–PAGE of total membranes of *S. typhimurium*. The proteins of a typical LT2 wild type, SA1922, are shown as open circles; additional proteins present in one or more of the mutant strains are shown as closed circles. The IEF gel was run first (horizontally as shown), then the SDS–PAGE was run (vertically as indicated by the arrow).

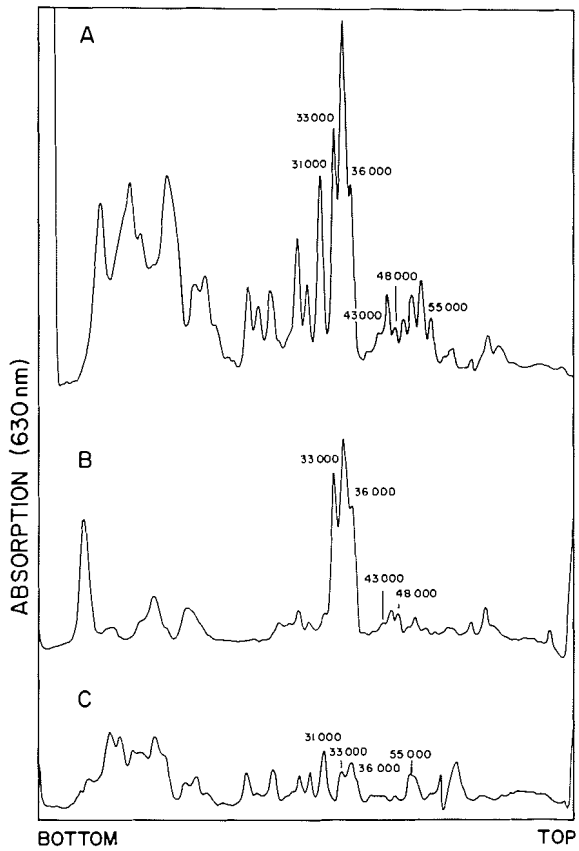

FIG. 2. Densitometry tracings at 630 nm, in a Beckman Acta III spectrophotometer, of Coomassie blue stained proteins separated on a 15% polyacrylamide gel. (A) Total membranes of SA1922; (B) outer membrane of SA1922; (C) inner membrane of SA1922. The values represent approximate molecular weights calculated from the following standards: BSA (68 000), ovalbumin (43 000), chymotrypsinogen (25 000), and RNase I (13 500).

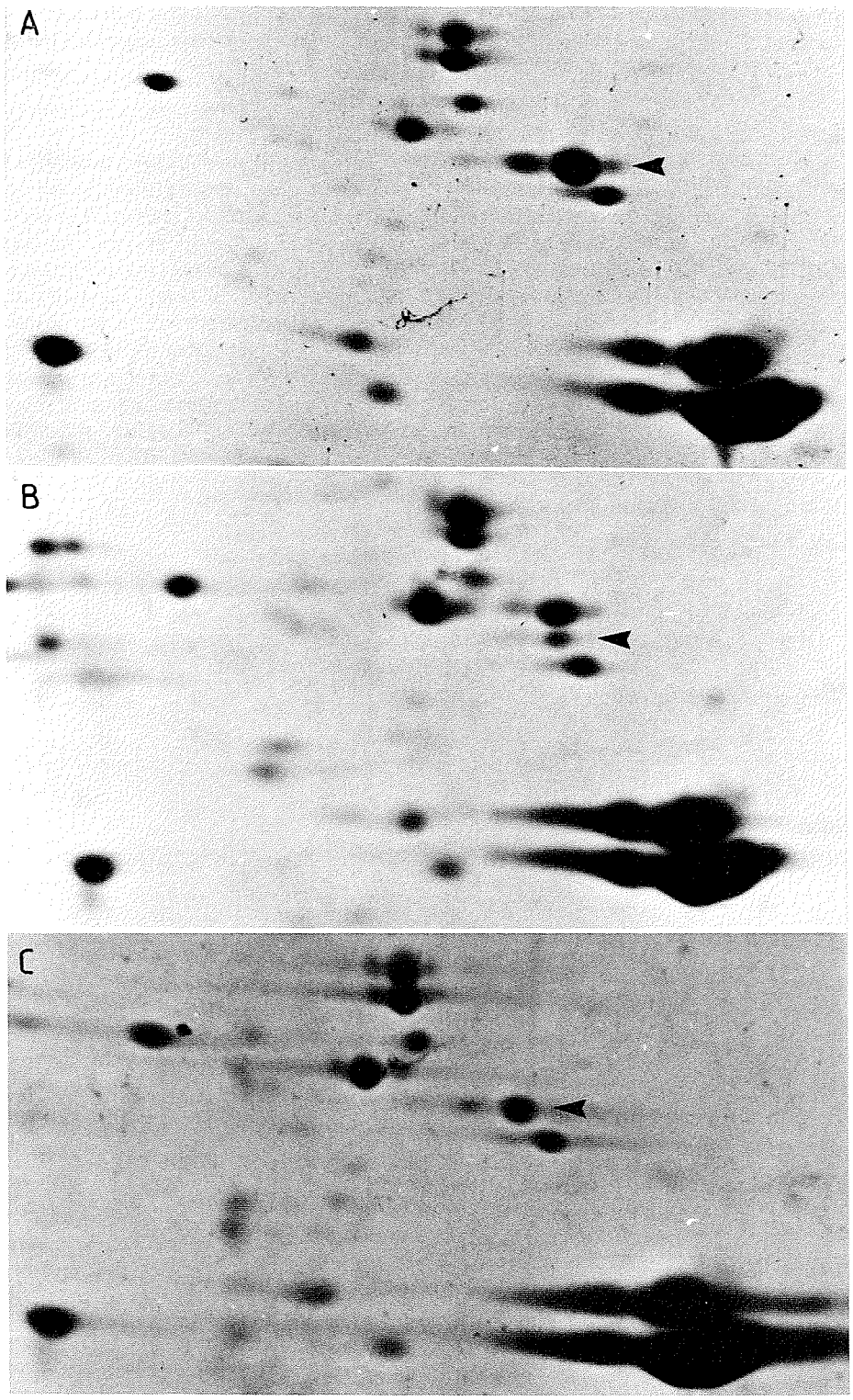

FIG. 3. Part of the gel of an IEF–PAGE separation of total membranes using a 10% polyacrylamide gel. (A) SA1922 *env*$^+$; (B) SA1926 *env-52*; (C) SA1929 *env*$^+$ transductant. Major outer membrane proteins are at the bottom of the gel. The arrow at the top points to a protein that is present in *env*$^+$ but greatly reduced in *env-52*; a new protein spot appears above this spot in *env-52*.

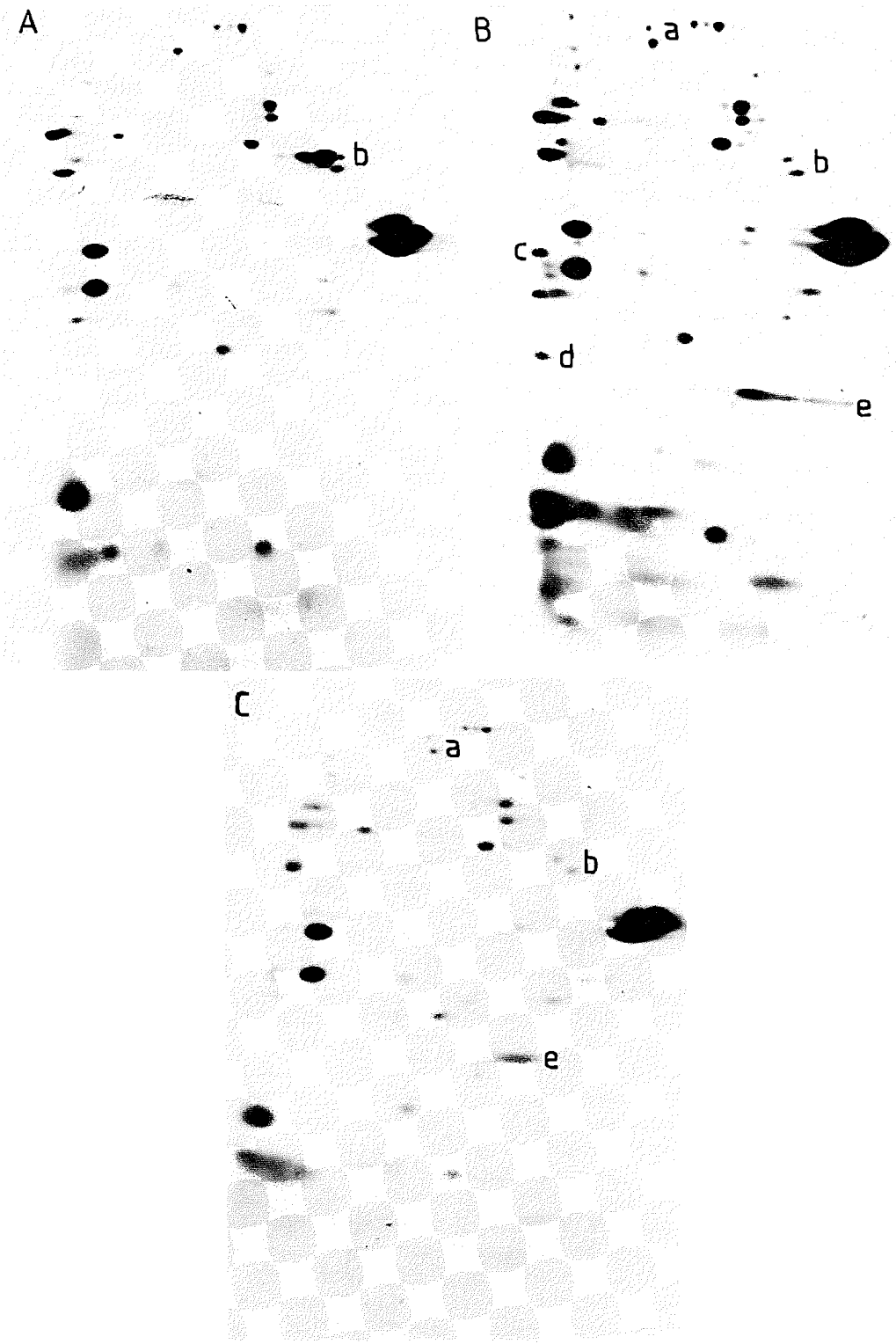

FIG. 4. IEF–PAGE separation of total membranes using a 15% polyacrylamide gel. (A) SA1922 *env*$^+$; (B) SA1925 *env-51*; (C) SA1931 *env-53*. Strain SA1925 has extra proteins not present in SA1922 at *a*, *c*, *d*, and *e*, and a reduction of the proteins in area *b*. SA1931 has extra proteins at *a* and *e* and a reduction of proteins in area *b*.

membranes) of the inner membrane enzyme succinic dehydrogenase in the outer membrane fraction (data not shown). The densitometry tracings of the PAGE of SA1922 (Fig. 2) show that the proteins in the MW 43 000 – 48 000 region (called region "*b*" on the two-dimensional gels) are mostly outer membrane proteins, whereas the proteins in the MW 48 000 – 49 000 region appear to be a mixture of inner and outer membrane proteins.

Comparison of membranes of mutant lines

In SA1926 (*env-52*), one protein spot visible in the Env$^+$ parent, SA1922, disappears, whereas a new spot appears nearby, and several other proteins in this area are reduced, whereas a transductant, SA1929, selected for wild-type vancomycin resistance, was found to have a simultaneous restoration of the wild-type level of antibiotic sensitivity and the wild-type protein pattern (Fig. 3), indicating that the antibiotic sensitivity and the protein changes are due to the same genetic event. As noted earlier, proteins in this area of the gels (*b*) are outer membrane proteins. In SA1925 (*env-51*), as in SA1926, proteins in area *b* (i.e., the MW 43 000 – 48 000 region) are greatly reduced when compared with those of SA1922, with one protein spot undetectable (Fig. 4). In addition, new proteins occurred in SA1925 at positions *a*, *c*, *d*, and *e* (Fig. 4), which are undetectable in SA1922.

In SA1931 (*env-53*), there is reduction in 36K protein and increase in 35K and 34K proteins, reduction in proteins in area *b*, with the protein missing from SA1925 again undetectable, and two new minor protein spots at positions *a* and *e* on the gel.

Discussion

The isolation of hemin-permeable envelope mutants was based on the premise that the barrier layer, which prevents the penetration of molecules through the cell wall, may be disturbed by a variety of mutational events to produce strains with altered permeability. A positive selection for the penetration of the required metabolite, hemin, was used such that growth was possible only if the hemin was able to penetrate these barrier layers.

About half of the hemin-permeable strains isolated are rough mutants, with defective LPS, as defined by their phage sensitivity; these strains were all DOC sensitive, and probably would have shown the many differences from smooth mutants, which are characteristic of deep rough mutants with defects in the inner core LPS region (Roantree *et al.* 1977; Sanderson, Van Wyngaarden *et al.* 1974; Schlecht and Westphal 1970). These strains were not tested further. These findings are analogous to the work of Tamaki *et al.* (1971), who isolated novobiocin-supersensitive mutants in *E. coli* and found that approximately half were rough, the remainder smooth.

The hemin-permeable smooth mutants resembled the wild type for properties frequently altered in mutants with modified permeability. None of the Env$^-$ mutants showed the temperature sensitivity for growth seen in deep rough mutants (Chatterjee, Sanderson *et al.* 1976). None allowed leakage of the periplasmic enzyme, RNase I, as seen in rough (Chatterjee, Ross, and Sanderson 1976) and smooth strains (Weigand and Rothfield 1976). None showed the sensitivity to DOC frequently found in rough and other envelope mutants (Antón 1972; Egan and Russell 1973).

All the mutants showed increased sensitivity to most of the antibiotics vancomycin, novobiocin, erythromycin, bacitracin, rifampin, and oleandomycin. These antibiotics are normally ineffective on Gram-negative cells and, as their target sites are inside the barrier layer of the cell, sensitivity indicates disruption of this barrier. Because some envelope mutants are sensitive to these antibiotics, e.g., mutants with rough LPS (Roantree *et al.* 1977; Sanderson, Van Wyngaarden *et al.* 1974) and some are insensitive, e.g., lipoprotein (*lpo*) mutants (Nikaido, Bavoil, and Hirota 1977), the range of sensitivities seen in our mutants was not surprising. Wild-type cells bind about 20% of the dye in a GV solution; our Env$^-$ mutants, and a rough mutant (SA1377), as well as previously described rough (Gustafsson *et al.* 1973) and *envA* mutants (Normark and Westling 1971), give increased uptake.

The presence of certain outer membrane proteins can be tested by using phages that use these proteins as receptors. Two major outer membrane proteins of *S. typhimurium*, the 36K and 34K proteins are believed to be the primary receptors of phages P221 and PH54 and of phages PH51 and PH52, respectively (Nurminen *et al.* 1976; Siitonen *et al.* 1977). In *S. typhimurium*, the gene *sid*, corresponding to *tonA* of *E. coli*, determines an outer membrane protein able to absorb ES18 (Graham and Stocker 1977). The phages PH51, PH52, and P221 were not useful in examining SA1922 and its mutants, as the smooth LPS normally hinders phage attachment (Siitonen *et al.* 1977); however, the sensitivity of SA1922 and its mutants to PH54 indicates they have the phage receptor corresponding to the 36K protein. The sensitivity to phage ES18 indicated that these strains have the protein receptor for this phage, perhaps a protein of about MW 85 000, equivalent to the analogous *E. coli tonA* gene product (Graham and Stocker 1977; Wayne and Neilands 1975). The Env$^+$ strain, SA1922, like other LT2 lines, has 33K, 34K, 35K, and 36K outer membrane proteins (Ames 1974; Nakae and Ishii 1978; Nikaido, Song *et al.* 1977). The Env$^-$ mutants derived from it have

similar amounts of these major proteins, although in SA1931 the 36K band is reduced, whereas the 34K and 35K bands are increased, so there is no measureable difference in the total amounts. The 34K, 35K, and 36K proteins, or "porins," form diffusion pores for the entry of low molecular weight hydrophilic substances (Nikaido, Song *et al.* 1977), but the molecules to which these Env$^-$ mutants show increased permeability, i.e., novobiocin, GV, bacitracin, and vancomycin, are classed by Nikaido (1976) as belonging to the hydrophobic group of substances that enter the cell by a mechanism different from the hydrophilic pore mechanism. Therefore, the unchanged porins in the Env$^-$ mutants, which show greater permeability to novobiocin, bacitracin, GV, and vancomycin, are not surprising.

Examination of the mutant lines by IEF–PAGE revealed alterations in several of the minor proteins of the cell envelope. All three Env$^-$ mutants were altered, although to different extents, in outer membrane proteins in the MW 43 000 – 48 000 region. SA1926 (*env-52*) was transduced to vancomycin resistance; the Env$^+$ transductant regained wild-type antibiotic resistance to all the antibiotics tested as well as the wild-type protein pattern in the MW 43 000 – 48 000 region. This indicates that the increased penetration of antibiotics and the altered protein pattern are both due to the same genetic change, and suggests that the altered proteins may cause the alteration in penetration. However, changes in one component of the envelope may result in secondary changes in other components; e.g., *ompA* (*tolG*) mutants have a major alteration in protein II* but also have changes in several of the minor proteins (Ames and Nikaido 1976) and LPS mutants, where the primary change is to the LPS, also have changes in the membrane proteins (Ames *et al.* 1974; Van Alphen *et al.* 1976).

Nikaido (1976) and Nikaido and Nakae (1979) suggested that hydrophobic molecules enter the cell by dissolving into the lipid bilayer of the cell envelope. Thus, wild-type cells would not be permeable to these hydrophobic substances either because the sugar side chains of the LPS give a hydrophilic region through which the molecules cannot pass or there are no exposed phospholipids on the cell surface in which the hydrophobic molecules can dissolve. Further evidence is that changes in the phospholipid content of the outer membrane can be related to changes in antibiotic resistance (Suling and O'Leary 1977). In this respect, one possible way the alterations in the minor outer membrane proteins seen in the hemin-permeable mutants could affect the permeability of the cell is to permit reorganization of the membrane to leave some phospholipids exposed on the surface of the cell. There is evidence that about 20 minor outer membrane proteins are associated with the LPS (Schweizer *et al.* 1978); alteration in this type of protein could promote some membrane reorganization.

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