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New *Salmonella typhimurium* Mutants with Altered Outer Membrane Permeability

SOILA SUKUPOLVI,* MARTTI VAARA, ILKKA M. HELANDER, PETRI VILJANEN AND P. HELENA MÄKELÄ

National Public Health Institute, SF-00280 Helsinki 28, Finland

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We describe three new classes of *Salmonella typhimurium* mutants with increased sensitivity to hydrophobic agents. In contrast to many previously described mutants, the phage sensitivity pattern of these mutants did not give any indication of defective lipopolysaccharide. Furthermore, they had no detectable changes in their phospholipid or outer membrane protein composition, and their growth rate and cell morphology were normal. Class B mutants were nearly as sensitive to novobiocin, fusidic acid, erythromycin, rifampin, and clindamycin as are deep rough (heptoseless) mutants; in addition they were sensitive to methicillin, penicillin (to which heptoseless mutants are resistant), gentian violet, and anionic and cationic detergents. Class A and C mutants had less sensitive, but characteristic phenotypes. None of the three classes were sensitive to serum bactericidal action. The class B mutation mapped between map positions 7 and 11 on the *S. typhimurium* chromosome, and the class C mutation mapped between positions 5 and 7. The map position for the class A mutation remained undefined, but it was separate from the class B and C mutations and, like those, did not correspond to any gene loci known to participate in the synthesis of major outer membrane constituents.

Wild-type *Salmonella* spp. and other gram-negative bacteria are highly resistant to many hydrophobic antibiotics which, even at low concentrations, are toxic to gram-positive bacteria. This is thought to be due to the outer membrane (OM), which forms a selective barrier that allows the permeation of small hydrophilic molecules only (M. Vaara and H. Nikaido, *in* E. Rietschel, ed., *Chemistry of Endotoxins*, in press).

Mutants of *Escherichia coli* and *Salmonella typhimurium* that are sensitive to hydrophobic antibiotics or detergents have been described. The most common among these are mutants defective in the deep core part of lipopolysaccharide (LPS) such as the so-called heptoseless (chemotype Re) mutants of *S. typhimurium* and *E. coli* (19, 26, 32). Also, some mutants defective in the major OM proteins have been described as sensitive to certain agents (34). Other mutations leading to a hydrophobic-sensitive phenotype include the *arcA* mutation at map position 10 (4, 11, 17), the *envA* mutation at map position 2 (8, 22), and the *tolC* mutation at map position 66 (5, 16) in *E. coli* and the *lkyD* mutation at map position 20 (42, 43) in *S. typhimurium*. In addition to hydrophobic sensitivity, these mutations often had other pleiotropic effects, such as reduced growth rate (19), incomplete cell division and chain formation (22, 43), reduced amount of LPS or OM proteins or both (18, 19), or release of periplasmic enzymes (19, 42).

The molecular basis of the increased OM permeability in these mutants is in most cases poorly understood. The best characterized are the deep rough LPS mutants, which also have an increased content of phospholipids in their OM (35). The phospholipids are found also in the outer leaflet of the membrane (13) and the regions of phospholipid bilayer created in this manner could be areas allowing penetration of hydrophobic agents. However, the way in which the defective LPS structure would lead to the altered phospholipid localization is not known.

To better understand the permeability barrier function of the OM and its structural basis we set out to isolate and characterize, both genetically and biochemically, a set of mutants with altered permeability properties. We chose to study *S. typhimurium*, in which both the LPS and the major OM proteins are well characterized and for which mutants with alterations in these properties are available. As a selective agent we used a hydrophobic antibiotic, novobiocin. Novobiocin-supersensitive mutants that did not have defective LPS structure were found to be sensitive to many hydrophobic agents and detergents. On the basis of these sensitivities they fell into three phenotypic classes corresponding to mutations at three separate loci. The mutants differ from many mutants described before in that they have a normal growth rate and a normal cell division process.

MATERIALS AND METHODS

Bacterial strains and bacteriophage used in this study are listed in Table 1. OmpA protein-specific bacteriocin 4-59, produced by *Salmonella canastel* 4-59 (38), was also used for phenotypic characterization of the mutants.

Media, growth conditions, and preservation of strains. The standard medium used for cultivation of the bacteria was Luria broth or Luria plates containing (per liter): tryptone (Difco Laboratories, Detroit, Mich.) 10 g; yeast extract (Difco), 5 g; and NaCl, 5 g (pH 7.0). A 15-g amount of agar was used for the plates. Kanamycin was added at 30 μg/ml and tetracycline was added at 10 μg/ml to score for the presence of transposons Tn*5* and Tn*10*, respectively.

Nutrient agar plates ([per liter]: meat extract, 1.5 g; yeast extract, 1.5 g; peptone, 5 g; glucose, 1 g; NaCl, 3.5 g; K_2HPO_4, 3.68 g; KH_2PO_4, 1.32 g [pH 7.1]; agar, 14 g) with gentian violet (GV), the cationic detergent benzalkoniumchloride (BCL), or the anionic detergent sodium dodecyl sulfate (SDS) were used for testing the sensitivity of the mutants. The concentration of GV in these plates was 10 μg/ml, the BCL concentrations were 0.005, 0.01, 0.02, 0.04, or 0.06%, and the SDS concentrations were 0.1, 0.3, or 1.0%. GV-kanamycin or BCL-kanamycin plates (containing 30 μg of kanamycin per ml and 10 μg of GV or 0.02 μg of BCL per ml) were used in transposon insertion experiments.

Davis minimal medium (30) with glucose and appropriate amino acids was used as a selective medium in matings and for testing growth requirements.

* Corresponding author.

TABLE 1. Bacterial strains and bacteriophages

Strain or bacteriophage	Genotype or phenotype	Reference or source
SH5014	*ilv-1178 thr-914 his-6116 metA22 trpB2 H1-b H2-e,n,x flaA66 rpsL120 xyl-404 metE551 rfaJ4041* LT2, cured of Fels2.	(38)
SH5346	*purE met-1151 aro-851 rpsL501* HfrH1 (*S. abony*)	(30)
SH6749	*ilv-452 his-6116 metA22 trpB2 H1-b H2-e,n,x flaA66 rpsL120 xyl-404 metE551 hsdL6 hsdSA29 galE496* LT2, cured of Fels2.	(25)
SH7613	*galU939* (from SH5014)	This paper
SH7616	SS mutant[a] class B (from SH5014)	This paper
SH7620	SS mutant class A (from SH5014)	This paper
SH7622	SS mutant class C (from SH5014)	This paper
SH7639	*rfaG4262* (from SH6749)	This paper
SH7640	SS mutant class A (from SH6749)	This paper
SH7641	SS mutant class B (from SH6749)	This paper
SH7796	*hisC3067 galE506* (pR471a)	(10, 20)
SH7913	Smooth, *rfa*$^+$ *z..-901*::Tn*10* derivative of SH7616	This paper
SH7915	Smooth, *rfa*$^+$ *z..-901*::Tn*10* derivative of SH7620	This paper
SH7917	Smooth, *rfa*$^+$ *z..-901*::Tn*10* derivative of SH7622	This paper
SH7931	SS-A$^+$ × Tn*5* derivative of SH7640	This paper
SH7946	SS-B$^+$ × Tn*5* derivative of SH7641	This paper
SH7949	*leu-1151* derivative of SH5346	This paper
SH7965	*proAB*::Tn*10* derivative of SH7913	This paper
SH7967	*proAB*::Tn*10* derivative of SH7917	This paper
SH7969	*proAB*::Tn*10* derivative of SH7915	This paper
SH7981	*pan-540*::Tn*10 pro-688*::Tn*5* derivative of SH7917	This paper
SH8000	*pan-540*::Tn*10* derivative of SH7949	This paper
SL696	*metA22 trpB2 H1-b H2-e,n,x flaA66 rpsL120* LT2	(44)
SL1032	LPS chemotype Rd_1 (*rfaG471*)	(14)
SL1102	LPS chemotype Re (*rfaE543*)	(44)
SL7005	*hisG46 trp-1 uvrB322 z..-901*::Tn*10 cysE1710* Q1	B.A.D. Stocker, personal communication
TT184	*proAB-662*::Tn*10*	J. Roth, University of Utah, Salt Lake City
TT206	*leu-1151*::Tn*10*	J. Roth
TT421	*pan-540*:Tn*10*	J. Roth
TT2382	*pro-688*::Tn*5*	J. Roth
TS736	SH6749 Δ*malE* (F112)	(24)
P22C2	Smooth LPS specific	(44)
9NA	Smooth LPS specific	(44)
Fo	Smooth or chemotype Ra LPS specific	(44)
6SR	Rough LPS specific	(44)
Br60	Rough LPS specific	(44)
Ffm	Rough LPS specific	(44)
Br2	Rough LPS specific	(44)
C21	Rough (chemotypes Rc or Rd_1 LPS specific)	(44)
P221	OmpC protein specific	(23)
PH105	OmpC protein specific	(23)
PH31	OmpD protein specific	(12, 23)
PH51	OmpD protein specific	(12, 23)
Ox2	OmpA protein specific	(9)
ES18	FhuA protein specific	(7)
P22HT105*int*	High-transducing lysate	B.A.D. Stocker, Stanford University, Stanford, Calif. (33)
λ(Km)	*c*I857 *b*221 *rex*::Tn*5*	(24)

[a] SS mutant, Supersensitive mutant.

The bacteria were grown in Luria broth on a rotary shaker (220 rpm) at 37°C into early logarithmic growth phase (50 Klett units; Klett-Summerson colorimeter, red filter) if not stated otherwise. The bacterial strains were preserved at −70°C in 20% skim milk (Difco) (39).

Isolation of supersensitive mutants. The bacteria grown overnight were incubated with 0.5% diethylsulfate for 30 min at 37°C. Supersensitive mutants were then enriched with penicillin in the presence of novobiocin as described by Tamaki et al. (40). The novobiocin sensitivity of the survivors was screened on Luria plates containing 40 μg of novobiocin per ml. Only one mutant was accepted per treated culture to assure their independence.

Sensitivity testing of mutants. One colony from the strain to be tested was picked into 1 ml of sterile water and streaked from this onto GV, BCL, or SDS plates to obtain isolated colonies. The growth after overnight incubation was compared with that on plain nutrient agar.

The sensitivity to different antibiotics was tested by the agar diffusion method, using antibiotic discs (Neo-Sensitabs) from A/S Rosco, Copenhagen, Denmark. The cultures in early logarithmic phase in Luria broth were diluted 1,000-

fold in water, and 0.3 ml of the dilution was spread on Luria plates (diameter, 15 cm). The plates were air dried, and the antibiotic discs were placed on the agar surface. The plates were incubated at 37°C for 18 h. The diameters of the zones of growth inhibition (discs included) were recorded in millimeters.

Phage and bacteriocin sensitivity testing. The sensitivity of the strains to bacteriophages specific for LPS or OM proteins was determined by the drop-on-lawn method on Luria plates as described previously (44). The same method was used for testing sensitivity to bacteriocin 4-59.

Isolation of cell envelopes. Cell envelopes were isolated either by mild sonication modified from the method used by Ames (1) or after lysozyme-EDTA treatment as described in detail by Nurminen et al. (23) and Sabet and Schnaitman (28). In the first method the cells in 0.01 M Tris-hydrochloride buffer (pH 7.2) were subjected to brief sonic oscillation (the microtip of an MSE sonic oscillator, 10 s, 12-μm amplitude), followed by centrifugation at 1,500 × *g* (to remove unbroken cells) and at 8,000 × *g* for 10 min. The pellet was suspended in 0.01 M Tris-hydrochloride buffer (pH 7.2).

SDS-polyacrylamide gel electrophoresis of proteins. SDS-polyacrylamide gel electrophoresis of proteins was carried out in slab gels by the method of Laemmli (15). The acrylamide concentration in the separation gels was 10%. Protein bands in the gel were visualized with Coomassie brilliant blue stain (6).

Electron microscopy. The cells were grown in Luria broth to the logarithmic growth phase, collected, suspended in 0.9% NaCl, and negatively stained with 2% (wt/vol) phosphotungstic acid (pH 6.5). The micrographs were taken with a JEM-100B electron microscope operated at 80 kV.

Phospholipid analysis. For lipid extraction the bacteria were cultivated in 25 ml of Luria broth to the logarithmic phase of growth, collected by centrifugation, and washed once with phosphate-buffered 0.9% saline (pH 7.0). The phospholipids were extracted by the method of Bligh and Dyer (3), with the modification that acidic conditions were employed as described by Nishijima and Raetz (21).

The qualitative analysis of lipid extracts was performed by one-dimensional thin-layer chromatography, using glass-backed plates coated with a 0.25-mm layer of Silica Gel 60 (E. Merck AG, Darmstadt, Federal Republic of Germany). Chloroform-methanol-acetic acid (65:25:10 [vol/vol/vol]) was used as the eluent. Spots were visualized by either iodine vapor or ammonium molybdate-perchloric acid spray (no. 166 as described by Stahl [36]) and identified by comparison with standards.

Release of periplasmic β-lactamase. A conjugative plasmid, R471a, coding for periplasmic β-lactamase was introduced into the mutants by conjugation from strain SH7796 (Table 1). The release of β-lactamase to the growth medium was assayed by the method of Sargent (31). Samples (450 μl) of the supernatant of the bacteria grown into early logarithmic phase were incubated with 3,000 U of penicillin G for 10 min at 37°C in 0.1 M potassium-phosphate buffer (pH 6.5). After this incubation, iodine reagent (0.32 N I_2 + 1.2 M KI) in acetate buffer (pH 4.0) was added, and the mixture was incubated for 30 min at room temperature. Absorbance was measured at 490 nm. A sample of the culture was sonicated as described above and assayed as a control.

Determination of sensitivity to normal serum. Appropriate dilutions of the bacterial suspension were incubated with different concentrations of pooled fresh guinea pig serum in the wells of microtiter plates and plated after 2 h to determine the number of viable bacteria as described in detail by Vaara and Vaara (41).

Transduction of *rfa*$^+$ gene cluster. Transduction was performed with phage ES18 grown on strain SL7005 (Table 1), which has the transposon Tn*10* coding for tetracycline resistance integrated close to the *rfa* gene cluster. A 0.5-ml amount of phage (10^9 PFU) and 0.1 ml (2×10^8 bacteria) of the recipient rough (*rfaJ*) bacteria in early logarithmic growth phase were incubated for 10 min at 37°C. After addition of 5 ml of Luria broth, the incubation was continued for 3 h to allow phenotypic expression before 0.1 ml of the mixture was plated on Luria plates containing tetracycline (10 μg/ml). Phage Ffm (10^8 PFU/ml) was then dropped onto these plates which were then incubated overnight. The tetracycline-resistant, Ffm-resistant transductants growing in the drop area were purified by two successive single-colony isolations on Luria plates.

Isolation of Tn*5* insertions near the supersensitivity genes. The general method of transposon insertion mutagenesis (manual of the Advanced Bacterial Genetics course at Cold Spring Harbor Laboratory, 1977) was used. Bacteriophage λ was used to introduce Tn*5* into the bacteria. Because *Salmonella* spp. do not naturally have a receptor for λ, the hybrid strain SH9183 was used which had received the *lamB* gene coding for the λ receptor from *E. coli* K-12 and could therefore bind λ (24). SH9183 was grown at 37°C to a density of 4×10^8 cells per ml in Luria broth containing maltose (0.4%) and $MgCl_2$ (10 mM). It was infected with λ(Km) at a multiplicity of infection of 10, incubated for 20 min at 30°C, and then agitated for 1 h at 30°C. Then 0.1 ml was spread on Luria-kanamycin plates and incubated overnight at 42°C. The Tn*5*-mutagenized pool of colonies growing on the kanamycin plates was used to grow the transducing phage P22HT105 *int*. To construct derivatives of supersensitive mutants with Tn*5* near enough to the wild-type allele to allow cotransduction, the mutants were used as recipients in transduction with this phage. Transductants were selected on kanamycin plates, and the pooled kanamycin-resistant transductants were then plated on GV- and BCL-kanamycin plates. Individual colonies growing on these plates were picked, restreaked on Luria plates, and tested for phage, antibiotic, and detergent sensitivities. Transducing phage was then grown on transductants that were antibiotic and detergent resistant and also kanamycin resistant. Transduction to the original supersensitive mutant ascertained that Tn*5* was integrated near the wild-type allele of the supersensitivity gene in question.

Mating procedures. Matings were performed by using Hfr donor strains (30) with a recipient-donor proportion of 10:1 in tubes of Luria broth incubated at 37°C for 1 h. After the incubation, 0.1 ml of dilutions of the mixture were spread on appropriate selective plates.

Chemicals. Diethylsulfate and SDS were from Fluka Ag, Buchs, Switzerland. Novobiocin and kanamycin sulfate were from Sigma Chemical Co., St. Louis, Mo. Penicillin was from Hoechst-Pharma AG, Zürich, Switzerland. Tetracycline chloride was from Leiras, Turku, Finland. The 10% BCL solution (Sterilan) was made by Medica, Helsinki, Finland.

RESULTS

Isolation and sensitivity of the mutants. Supersensitive mutants were isolated from two strains: SH5014 (*rfaJ*, determining chemotype Rb_2 of rough LPS) and SH6749 (*galE* mutant that has LPS of the Rc chemotype when grown without galactose, but smooth LPS in the presence of

TABLE 2. Sensitivity of the mutants and some reference strains to GV, detergents, and antibiotics

Strain	Description	Sensitivity to[a]:			Inhibition zones caused by antibiotics[b]								No. of mutants with resulting phenotype
					Group I				Group II			Group III	
		GV (10 μg/ml)	BCL (50 μg/ml)	SDS (1 mg/ml)	Novobiocin (100 μg)[c]	Fusidic acid (400 μg)	Erythromycin (78 μg)	Rifampin (30 μg)	Clindamycin (25 μg)	Penicillin (5 μg)	Methicillin (29 μg)	vancomycin (70 μg)	
SH5014	Parent *rfaJ*	R	R	R			14	16					
SH7620	Class A	S	R	R	20	23	23	27				15	8
SH7616	Class B	S	S	S	19	27	24	18	29	23	27		3
SH7622	Class C	S	S	R[d]	21	27	27	35	17				1
SH7613	*galU*[e]	S	S	S	23	23	25	23	24				1
SH6749	Parent *galE*	R	R	R				20					
SH7640	Class A	S	R	R	18	17	15	27				13	2
SH7641	Class B	S	S	S	25	30	27	25	29	30	24		3
SH7639	*rfaG*[e]	S	S	S	18	24	22	25	25				1
SL1032	Rd_1 (*rfaG*)	S	S	S	20	18	25	27	19	14		16	
SL1102	Re (*rfaE*)	S	S	S	34	32	34	33	29	16		16	
SL696	Smooth	R	R	R				17					

[a] R, resistant; S, sensitive. All strains were sensitive to chloramphenicol and tetracycline and resistant to streptomycin (because of the *rpsL* mutation).
[b] Measured in millimeters; the diameter of the antibiotic disc (9 mm) was not subtracted.
[c] The diffusible amount of the antimicrobial agent in the discs according to the manufacturer.
[d] Sensitive to 10 mg/ml, whereas all the other strains resistant to SDS grew in the presence of 10 mg of SDS per ml.
[e] Differing from the parent in sensitivity to LPS-specific phages.

galactose). Mutants with novobiocin-sensitive phenotypes were enriched by penicillin killing in the presence of novobiocin. A total of 15 independent mutants that in preliminary tests were sensitive to novobiocin (40 μg/ml) on Luria plates were selected from each parental strain, tested for their sensitivity to the hydrophobic dye GV, to the detergents BCL and SDS, as well as to a number of antibiotics, and compared to smooth and rough reference strains. All strains, including the parents, were sensitive to standard broad-spectrum antibiotics (see footnote *d*, Table 2) except streptomycin (because of the *rpsL* mutation present in all strains) without much strain-to-strain variation (data not shown). Differing sensitivities were recorded for GV, detergents, and hydrophobic antibiotics (Table 2).

The parent strains SH5014 and SH6749 were resistant to GV and SDS, as was the smooth reference strain SL696. They were also resistant to BCL at a concentration of 50 μg/ml. At BCL concentrations above 200 μg/ml, the strains were at least partly inhibited, and the *galE* mutant SH6749 was slightly more sensitive than SH5014 (data not shown). SH5014, SH6749, and SL696 were also resistant to almost all the hydrophobic antibiotics (Table 2).

The two deep rough reference strains of chemotypes Rd_1 and Re were sensitive to GV, BCL, SDS, and most of the antibiotics, very slightly sensitive to penicillin and vancomycin, and not at all sensitive (by this method) to methicillin.

Most of the supersensitive mutants fell, on the basis of their sensitivities, into two groups which we termed classes A and B. In addition, three mutants differed from these in some respects. One of them, SH7622, was assigned to sensitivity mutant class C, whereas two appeared to be altered in their LPS in a manner characteristic of deep rough mutants (see below). In total, we obtained 10 class A mutants (8 from SH5014 and 2 from SH6749) and 6 class B mutants (3 from each parent strain). The only class C mutant (SH7622) originated from SH5014.

Class A mutants were the least sensitive. They were equally as resistant to SDS, BCL, and penicillin as were the parents but sensitive to GV and to several of the hydrophobic antibiotics. Class B mutants were the most sensitive, being susceptible both to GV, detergents, and all antibiotics except vancomycin. The class C mutant was an intermediate between these, resistant to SDS and penicillin but sensitive to GV and BCL. However, the SDS resistance of this class was less complete (growth at ≤1 mg/ml) than that of class A mutants or the parent strains, which grew in SDS concentrations over 10 mg/ml. The two deep-rough-like mutants closely resembled the reference Rd_1 strain.

A closer look at the antibiotic sensitivities of the mutants suggested grouping of the antibiotics as follows: (i) novobiocin, fusidic acid, erythromycin, and rifampin, the parents were resistant to these antibiotics but all the mutants were sensitive. (ii) Penicillin, methicillin, clindamycin, and a close analog, lincomycin, class B but not class A mutants were sensitive to these antibiotics. The class C and the deep rough mutants were sensitive to clindamycin but not to penicillin or methicillin. (iii) Vancomycin, most of the mutants were resistant but the least-sensitive class A mutants were sensitive to this antibiotic. However, the growth inhibition zones obtained with this mutant were rather small, compared with the very large inhibition zones seen with the other antibiotics. (iv) Chloramphenicol and tetracycline, wild-type *Salmonella* spp. are sensitive to these antibiotics. The most-sensitive class B mutants gave even larger inhibition zones with these antibiotics.

Morphology and growth characteristics of the mutants. The growth rate of the mutants in Luria broth was similar to that of the parents. The numbers of viable bacteria at the end of log phase were also similar for all the mutants and their parents. The mutants were indistinguishable from their parents in colony morphology on Luria plates or on Davis minimal agar.

Phase-contrast microscopy could not reveal any alterations in the cell morphology of the mutants. No defects in cell division, such as growth in chains, could be observed.

Electron microscopy with negatively stained samples, also, did not show any alterations in the cell surface of the mutants.

Sensitivity of the mutants to bacteriophages specific for LPS or OM proteins. The sensitivity of the mutants to bacteriophages, which use different OM components (LPS or major OM protein) as their receptors, was determined with the results shown in Table 3. The class A, B, or C mutants originating from SH5014 were, like their *rfaJ* parent, sensitive to the rough LPS-specific phages Br60, Ffm, and Br2. The class A and B mutants originating from SH6749, on the other hand, were sensitive to Ffm, Br2, and C21 and resistant to the other LPS-specific phages when tested on Luria plates. The addition of galactose and glucose to the plates caused these mutants to be sensitive to the phages P22C2, 9NA, and FO that use either the O side chain or the complete LPS core as their receptors. In all these characteristics they were indistinguishable from their *galE* parent SH6749.

All the mutants and their parents were sensitive to phages PH105 and P221 (23) specific for the OM porin protein OmpC, to PH51 and PH31 (12, 23) specific for OmpD protein, and to phage Ox2 (9) and bacteriocin 4-59 (38) both specific for OmpA protein. Thus, all these proteins were probably present in the OM of the mutants. They were also sensitive to ES18 (7), which uses the *fhuA* gene product as its receptor.

The two mutants SH7613 (from SH5014) and SH7639 (from SH6749) had phage sensitivity patterns (Table 3) and phenotypic properties (Table 2) closely similar to those of deep rough strains of chemotype Rd_1. This phenotype is expected to arise from a mutation either in the *rfaG* gene, in the *rfa* cluster, or in the *galU* gene at map position 34 (37). Transduction from an *rfa*$^+$ donor produced a number of smooth antibiotic- and detergent-resistant transductants from SH7639, which were therefore inferred to be *rfaG* mutants. The mutation in SH7613 did not map in the *rfa* locus, and was inferred to be *galU*. These two mutants were not studied further.

OM protein composition of the mutants. Cell envelopes were prepared from mutant and parent strains in exponential growth phase and analyzed for their protein composition by SDS-polyacrylamide gel electrophoresis. No differences could be detected in protein composition between parent strains and their mutants (Fig. 1). The finding was the same irrespective of whether the envelopes were prepared by the lysozyme-EDTA method or by sonication (see above). In addition, envelopes were also prepared from the mutant and parent bacteria grown to stationary growth phase; again, no differences could be observed between the mutants and the parent strains (data not shown). We conclude that the hydrophobic sensitivity of the mutants was not due to loss or electrophoretic alteration of a major OM protein or to any other alteration in the major protein composition of their cell envelopes.

Phospholipid analysis. Phospholipid analysis by thin-layer chromatography showed that both the mutants and the parents contained phosphatidylethanolamine, cardiolipin, and phosphatidylglycerol. However, this does not exclude the possibility that the strains may to some extent differ in total lipid content or in the relative proportions of individual phospholipids. No additional spots were seen in the chromatograms of any of the strains.

Leakage of periplasmic β-lactamase from the mutants. To test whether our supersensitive mutants had OM alterations which would lead to the leakage of periplasmic proteins, the plasmid R471a coding for periplasmic β-lactamase was introduced into the supersensitive mutants by conjugation from SH7796. The amount of penicillinase released from the mutants was determined in supernatants of growing cultures at early logarithmic growth phase. The penicillin-hydrolyzing activity present in the supernatants of exponentially growing cultures of supersensitive mutants was very small (<7%) compared with the activity of sonicated control preparations. The class C mutant SH7622 was not used in this experiment.

Sensitivity of the mutants to normal serum complement. To test whether the mutations leading to supersensitivity also made the mutants susceptible to killing by serum complement, we prepared smooth derivatives of them, because rough strains as a rule are sensitive to serum complement (18, 27). This was done by transducing the *rfa*$^+$ locus from SL7005 into the mutants with bacteriophage ES18. The complement source used was pooled fresh guinea pig serum. The numbers of viable cells were determined in cultures of the smooth derivatives after incubation for 2 h with different concentrations of guinea pig serum. Both the smooth control strain and the derivatives of the three supersensitive mutant classes were able to multiply in the presence of 1, 3, 10, or 30% serum. This was true even of the class B mutant, which was the most sensitive to antibiotics and detergents. Thus

TABLE 3. Bacteriophage and bacteriocin sensitivities of supersensitive mutants and some reference strains

Strain	Description	Sensitivity to phages specific for[a]:														
		Smooth LPS		Smooth or Ra	Rough LPS					OmpC		OmpD		OmpA		FhuA (ES18)
		P22C2	9NA	FO	6SR	Br60	Ffm	Br2	C21	P221	PH105	PH31	PH51	Ox2	Bacteriocin 4-59	
SH5014	Parent	R	R	R	R	S	S	S	R	S	S	S	S	S	S	S
SH7620	Class A	R	R	R	R	S	S	S	R	S	S	S	S	S	S	S
SH7616	Class B	R	R	R	R	S	S	S	R	S	S	S	S	S	S	S
SH7622	Class C	R	R	R	R	S	S	S	R	S	S	S	S	S	S	S
SH7613	*galU*	R	R	R	R	R	R	R	S	S	S	S	S	S	S	S
SH6749	Parent *galE*	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S
SH7640	Class A	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S
SH7641	Class B	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S
SH7639	*rfaG*	R	R	R	R	R	R	R	S	S	S	S	S	S	S	S
SL1032	Rd_1 (*rfaG*)	R	R	R	R	R	R	R	R	S	S	S	S	S	S	S
SL1102	Re (*rfaE*)	R	R	R	R	R	R	R	R	S	R	R	S	R	R	S
SL696	Smooth	S	S	S	R	R	R	R	R	R	R	R	R	R	R	S

[a] Tested with the drop-on-lawn method; R, resistant; S, sensitive.

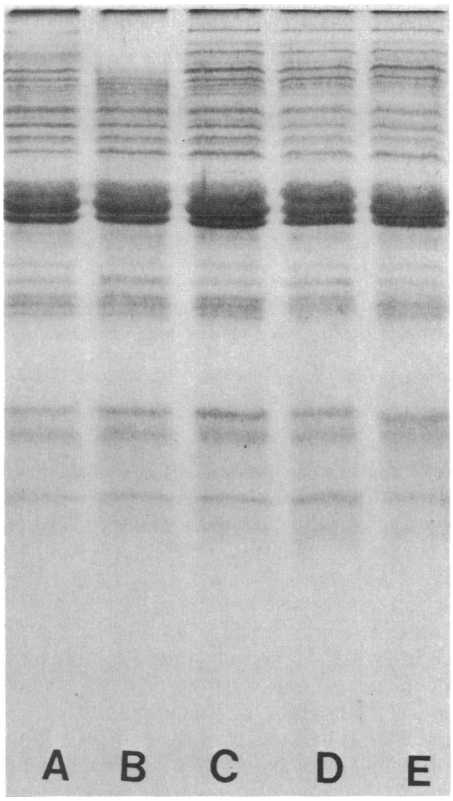

FIG. 1. SDS-polyacrylamide gel electrophoresis of cell envelopes of supersensitive mutants and parent strains. Lanes: A, SH6749, parent *galE*; B, SH5014, parent *rfaJ*; C, SH7616, class B mutant; D, SH7622, class C mutant; E, SH7620, class A mutant. The strongest bands correspond to porins (36,000 and 34,000) and the OmpA protein (33,000).

the mutations leading to supersensitive phenotypes in the three classes of mutants did not sensitize them to serum complement.

Genetic differentiation of supersensitive mutants. To investigate whether the three phenotypically different mutant classes were due to mutations at the same locus or in separate loci, we constructed strains which had the transposon Tn*5* near the wild-type allele of the supersensitivity genes. This made the easy transfer and selection of the wild-type allele possible by transduction with P22. Several derivatives of class A and B mutants were isolated in which cotransduction between Tn*5* and the wild-type allele of the supersensitivity gene was 90% or more. However, despite repeated attempts we did not obtain such a derivative of the class C mutant.

When the wild-type allele of the supersensitivity gene in the class A mutant was transduced into class A, B, and C mutants, both class A mutants regained wild-type resistance to GV, detergents, and antibiotics, whereas the class B and C mutants remained sensitive (Table 4). Correspondingly, the transfer of the class B wild-type allele made the two class B mutants, but not class A or C mutants, resistant to antibiotics and detergents. Neither class A nor class B wild-type alleles could cause the class C mutant, SH7622, to be resistant to antibiotics, detergents, or GV. Thus, the supersensitive mutants have mutations at three different genetic loci in a manner corresponding to the phenotypic classification of the mutants.

Mapping of the supersensitivity genes. Preliminary conjugation experiments with different Hfr donors indicated that the mutations leading to supersensitivity might be located in the early part of the *S. typhimurium* chromosome. A donor suitable for further conjugational mapping was prepared from *Salmonella abony* SH5346, which is *purE* (map position 11), by transducing into it *leu*::Tn*10* (map position 3) from TT206. SH5346 is a derivative of HfrH1 which injects the chromosome clockwise starting near *pyrB* (map position 98) (30). Recipients were prepared by transducing *pro-662*:Tn*10* (map position 7) into the supersensitive mutants.

Selection was made for Pro^+, and about 100 recombinants from each mating were tested for unselected markers (Table 5). Selection plates did not contain aromatic amino acids to counterselect the donor (which is *aro-851*). The *leu* marker was not transferred into the recipients in any matings, probably because it is transferred very early by the HfrH1 donor and would be excluded by any crossing over necessary to integrate a piece of the donor chromosome.

Of 88 Pro^+ recombinants in a mating in which the class B mutant SH7965 was the recipient, 24 were resistant to GV, BCL, and novobiocin, like the wild-type strain. Of these 24 recombinants, 17 (71%) had received the *purE* marker at the same time as the wild-type supersensitivity gene ($SS\text{-}B^+$). (In this paper the preliminary symbols SS-A, SS-B, and SS-C are used to describe the genes coding for supersensitivity; for example SS-A is the gene defined by the mutation of a class A mutant. We hope to be able to replace the preliminary designations with more descriptive ones once the function of the genes is known.) This would suggest that the class B mutation lies near the *purE* gene (map position 11). It was not, however, cotransduced with *purE* in P22HT-mediated transduction (50 transductants tested). Our results (Table 5) suggest that the most probable gene order is *pan–pro*–SS-B–*purE*.

In the mating with the class C supersensitive mutant SH7967 as a recipient, 47 of 98 Pro^+ recombinants (48%) tested had received the wild-type supersensitivity gene ($SS\text{-}C^+$). Only 10 of the $SS\text{-}C^+$ recombinants (21%) had also received the *purE* marker. The class C mutation therefore seems to lie closer to *proAB* (map position 7) than *purE*.

To investigate further on which side of *proAB* the class C mutation is located, we chose the marker *pan* at map position 5. The strain SH7981, constructed by transducing *pan*::Tn*10* and *pro*::Tn*5* into the class C mutant, was used as a recipient in a mating with SH7949 (Table 6). Of 92 recombinants selected as Pan^+, 43 had also received the

TABLE 4. Numbers of kanamycin-resistant transductants with wild-type phenotypes of different supersensitive mutant classes after transduction by P22 grown on mutants with Tn*5* linked to the wild-type allele of supersensitivity genes

Donor[a]	No. of kanamycin-resistant transductants with wild-type phenotype[b]				
	Class A		Class B		Class C (SH7917)
	SH7640	SH7915	SH7641	SH7913	
Class A SH7931 (derivative of SH7640)	18	17	0	0	0
Class B SH7946 (derivative of SH7641)	0	0	16	18	0

[a] With Tn*5* linked to the wild-type allele of the supersensitivity gene.
[b] Of 20 tested transductants.

TABLE 5. Frequencies of different recombinant classes in the cross between HfrH1 donor and class B mutant[a]

Genetic constitution of recombinants[b]				No. of recombinants with this genotype	Minimum no. of quadruple crossovers required to produce the recombinants observed, assuming the gene order:			
pan	*pro*	SS-B	*pur*		SS-B–*pan*–*pro*–*pur*	*pan*–SS-B–*pro*–*pur*	*pan*–*pro*–SS-B–*pur*	*pan*–*pro*–*pur*–SS-B
1	1	0	0	9		9		
1	1	1	1	8				
0	1	0	0	31				
0	1	1	1	25	25			
0	1	1	0	16	16			16
1	1	1	0	3				3
0	1	0	1	2			2	

[a] The HfrH1 donor SH8000 *leu pan*::Tn*10 purE* and SH7965 *pro*::Tn*10* class B mutant were crossed, and the selection was made for Pro$^+$.
[b] With most probable gene order, *pan*–*pro*–SS-B–*pur*. Donor allele, 1; recipient allele, 0.

wild-type allele of the class C supersensitivity gene, and of these 43 recombinants, 16 (37%) had also received the donor *proAB*$^+$. Of 66 recombinants selected as Pro$^+$, 33 were SS-C$^+$ and 13 of them (39%) had also received the donor *pan*$^+$, suggesting that the class C supersensitivity gene lies between *pan* and *proAB*. An analysis of the frequencies of different recombinant classes indicated *pan*–SS-C–*pro*–*purE* as the most probable gene order (Table 6). The class C mutation was not cotransduced with either *pan* or *proAB* when tested by P22HT-mediated transduction (50 transductants tested from each transduction).

We have not yet been able to define the map position of the class A mutation, but it does not lie in the same area as the other supersensitivity genes. (In the cross with SH7949 as a donor and SH7640 derivative with *pro*:Tn*10* as a recipient, we did not find any SS-A$^+$ recombinants among 96 Pro$^+$ recombinants tested.)

DISCUSSION

We have isolated *S. typhimurium* mutants which are sensitive to a number of hydrophobic compounds, indicating that their OM permeability barrier is defective. To our surprise, most of these mutants (17 of a total of 19; classes A, B, and C separable by phenotype and locus of mutation) did not correspond to any previously characterized permeability mutants in *Salmonella* spp. Their LPS phage sensitivity patterns did not give any indication of altered LPS. They had no detectable changes in phospholipid or OM protein composition. They did not leak periplasmic proteins. Their cell morphology and cell division were unaltered. In contrast to the deep core LPS mutants (19) their growth rate was unimpaired.

The isolation method used in this work selects for sensitivity to novobiocin. Tamaki et al. (40), who first described the method for *E. coli* K-12, mainly found mutants with defective LPS deep core. It is not known why we found only two such mutants (LPS chemotype Rd_1, see above). A possibility is that the selection method, which includes a long segregation time after mutagenesis, favors rapidly growing mutants such as ours.

The class B mutation resulted in the most-sensitive mutant phenotype. Thus, class B mutants were nearly as sensitive to hydrophobic antibiotics as the mutants with the deep rough Re chemotype of *Salmonella* spp. Importantly, they were also very sensitive to penicillin and methicillin, which are not inhibitory to Re strains. The class B mutation was located between *pro* (map position 7) and *purE* (map position 11) on the *Salmonella* chromosome. The *acrA* mutation of *E. coli*, also resulting in a hypersensitive phenotype (11, 17), is located at map position 10 (2). The mapping data thus suggest that our class B mutation may correspond to the *acrA* mutation, and this should be tested in further work on the mutants.

The phenotype of the class C mutant was intermediate

TABLE 6. Frequencies of different recombinant classes in the crosses between HfrH1 donor and class C mutant[a]

Selection	Genetic constitution of recombinants[b]				No. of recombinants with this genotype	Minimum no. of quadruple crossovers required to produce the recombinants observed, assuming the gene order:		
	pan	SS-C	*pro*	*pur*		*pan*–SS-C–*pro*–*pur*	*pan*–*pro*–SS-C–*pur*	*pan*–*pro*–*pur*–SS-C
A	1	1	1	0	9			9
	1	1	1	1	4			
	0	1	1	1	2			
	0	1	1	0	5	5	5	5
	0	0	1	1	3		3	3
	0	0	1	0	43			
B	1	0	0	0	42			
	1	1	0	0	27		27	27
	1	1	1	0	7			7
	1	0	1	0	3	3		
	1	1	1	1	9			
	1	0	1	1	1	1	1	
	1	0	0	1	3	3	3	3

[a] The HfrH1 donor SH7949 *leu purE* and SH7981 *pan*::Tn*10 pro*::Tn*5* class C mutant were crossed, and the selection was made for Pro$^+$ (A) and Pan$^+$ (B).
[b] With the most probable gene order, *pan*–SS-C–*pro*–*pur*. Donor allele, 1; recipient allele, 0.

between class A and B mutants. The class C mutation maps between the loci *pan* and *pro* at map positions 5 and 7, respectively, and does not correspond to any of the previously defined loci participating in the synthesis of OM components (2, 29).

The class A mutants were the least sensitive, and differed from the others, being resistant to detergents. Although the map position of the class A mutation is so far not accurately defined, it is not located in the same area in the chromosome as that of the class B or C mutations and not close to known LPS or OM protein genes.

What is the significance of these mutants? Both biochemical and genetic evidence suggest that in the mutants described thus far unknown structures or metabolic pathways are affected. The sensitivity properties strongly suggest that these structures or functions affect the OM, which is responsible for the unique hydrophobic and detergent resistance of gram-negative enteric bacteria. The affected structures or functions still remain unknown, but the mutants are expected to provide useful tools for their study and thereby for a study of the structure-function relationships in the OM.

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