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Two Mutations Which Affect the Barrier Function of the *Escherichia coli* K-12 Outer Membrane

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Two genetically distinct classes of novobiocin-supersensitive mutants were isolated from *Escherichia coli* K-12. One class, given the phenotypic name NbsA, lies at 10 min on the *E. coli* chromosome. The order of the genes in this region, based on transductional analyses, is *proC* NbsA *plsA purE*. The second, NbsB, lies at 80 min. The order of the genes in this region, based on transduction analyses, is *xyl cysE* NbsB *pyrE*. Both classes of mutants show increased sensitivity to hydrophobic drugs but are different: NbsA cells tend to be more sensitive to cationic agents, whereas NbsB cells show the opposite tendency. The sole detectable biochemical alteration in NbsA strain is greater than 90% reduction in the phosphate content of the lipid A region of the lipopolysaccharide. The NbsB mutation results in lipopolysaccharide that contains primarily the stereoisomer D-glycero-D-mannoheptose, rather than L-glycero-D-mannoheptose, and which contains very little of the distal sugars. Since NbsA strains have apparently normal outer membrane proteins and total cellular phospholipids, changes solely in lipopolysaccharide can increase permeability to certain hydrophobic antibiotics. Complementation studies indicate that the NbsA marker is probably allelic with *acrA*. In addition, the NbsB marker is genetically and phenotypically similar to the *rfaD* locus of *Salmonella typhimurium*. For this reason, the phenotypic designations NbsA and NbsB have been changed to the genotypic designations *acrA* and *rfaD*, respectively.

The outer membrane of gram-negative bacteria is a molecular sieve that permits the entry of molecules smaller than approximately 700 in molecular weight (39), while excluding larger molecules such as oligopeptides (42) and even those hydrophobic molecules that can pass most biological membranes (30). The entry of small molecules has been shown by reconstruction experiments (37, 38) to involve lipid, lipopolysaccharide (LPS), Mg^{2+}, and certain outer membrane proteins. The barrier to hydrophobic antibiotics and dyes can be breached when the cells lose half of their LPS on exposure to EDTA (29), suggesting a role for LPS in this barrier. The barrier is also lost when the length of the polysaccharide chain in LPS is drastically reduced by mutation (41, 44); however, this shortening is accompanied by loss of outer membrane proteins (6, 26), so the cause of the increased permeability in such mutants is undetermined.

To determine the minimum outer membrane change required to increase permeability, we need mutants selected for increased permeability whose genetics and chemistry are well defined. Since gram-negative bacteria are resistant to hydrophobic antibiotics (41) at high concentration, we screened for mutants that were sensitive to low concentrations of such antibiotics (e.g., actinomycin D, rifamycin, and novobiocin). By using the technique of Tamaki et al. (54), we found two classes of novobiocin-supersensitive mutants and determined the localization of single-site mutations resulting in novobiocin supersensitivity. We demonstrate that in one mutant class certain permeability changes follow minor changes in LPS without any detectable changes in outer membrane protein or lipid content.

MATERIALS AND METHODS

Bacterial strains and phage. The *Escherichia coli* K-12 strains used in this study are listed in Table 1.

Media and growth conditions. LB broth (34) contained 5 g of yeast extract, 5 g of NaCl, and 10 g of tryptone per liter of distilled water, and the pH was adjusted to 7.4 with 1 M NaOH. LB broth was supplemented where indicated with the following: 5 mM $CaCl_2$; 4.5 μM methyl methane sulfonate; 0.1 to 0.5% sodium dodecyl sulfate; 0.1 to 0.4% sodium deoxycholate; 100 μg of streptomycin sulfate; 14 μg of kanamycin

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

Strain	Sex	Genotype and/or phenotype	Source
CV2	HfrC	*plsA2 glpD3 phoA8 relA1 tonA22 glpR2* T2^r	J. Cronan via CGSC[a]
JM15	F$^-$	*cysE50 tfr-8* Thi	M. Jones-Mortimer via CGSC
N43	F$^-$	*ara-14 lac-85 galK2 malA1 xyl-5 mtl-1 acrA1 rpsL197* λ^r *supE44* ?	N. Nakamura via CGSC
PCO135	F$^-$	*thi-1 purE55 lacY1 malA1 mtl-2 gal rpsL117 supE44* ? *xyl-7* λ^r	P. de Haan via CGSC
PL2	HfrH	*galE28 thi-1 relA1* λ$^-$	M. Gottesman
RK1041	F$^-$	*ilv argH his metB pyrE60 cysE bgl*$^+$ *mtl xyl*	R. Kadner
Lin239	F$^-$	*mtlD leu*	E. Lin
CL2	HfrH	*galE28 thi-1 relA1* NbsA λ$^-$	This paper
CL5	HfrC	*glpD3 phoA8 relA1 tonA22 glpR2*	This paper
CL6	HfrC	*glpD3 phoA8 relA1 tonA22* NbsA *glpR2* T2^r	This paper
CL7	HfrH	*galE28 thi-1 relA1* NbsB λ$^-$	This paper
CL15	F$^-$	*thi lacY1 malA1 xyl-7 mtl-1 gal-3 rpsL117 supE44* ? λ^R	This paper
CL27	HfrH	*galE28 thi-1 relA1* NbsB *rpsL* (P1*clr*100KM) λ$^-$	This paper
CL29	F$^-$	*tfr-8* NbsB *thi-1* ?	This paper
CL30	F$^-$	*tfr-8 thi-1* ?	This paper
CL46	F$^-$	*cysE50 tfr-8 thi* ? *xyl*	This paper
CL51	F$^-$	*thi met-70 lysA trp-38 purE42 plsA2 leu-6 mtl-1 xyl-5 ara-4 lacZ36 azi-6 rpsL109 ton-23 tsx-67* λ$^-$ *supE44*	This paper
CL63	F$^-$	*thi lacY1 malA1 xyl-7 mtl-1 gal-3 rpsL117 supE44* ? NbsA λ^r	This paper
CL66	F′	F508 (*lac*$^+$ NbsA *purE*$^+$)/*thi-1 purE55 lacY1 malA1 mtl-2 gal-2 rpsL117 xyl-7 recA1 supE44* ? λ^r	This paper
CL69	F$^-$	*ara-14 lac-85 galK2 malA1 xyl-5 mtl acrA1 rpsL197* λ^r *supE44 recA1 rpoB*	This paper
CL70	F$^-$	*thi-1 purE55 lacY1 malA1 mtl-2 gal-3 rpsL117 supE44* ? *recA1 rpoB* λ^r	This paper
CL71	F$^-$	*thi-1 lacY1 malA1 xyl-7 mtl-2 gal-3 rpsL117 supE44* ? NbsA *recA1 rpoB* λ^r	This paper
CL72	F$^-$	*tfr-8* NbsB *thi* ? (P1*clr*100KM)	This paper
Phage			
P1*vir*			E. Kline
P1kc			E. Kline
P1*clr*100KM		*clr100* (temperature-sensitive clear plaque mutation); KM (kanamycin resistance)	S. Streicher
Br2		Rough-specific phage	A. Singh
Br10		Rough-specific phage	A. Singh
C21		Rough-specific phage	A. Singh
F0		Smooth- and rough-specific phage	A. Singh

[a] CGSC, *E. coli* Genetic Stock Center, Department of Human Genetics, Yale University School of Medicine, New Haven, Conn. 06510.

sulfate, and 30, 40, or 50 μg of novobiocin per ml. MC buffer (35) contained 5 mM $CaCl_2$ and 100 mM $MgSO_4$ in distilled water. The minimal media used were that of Davis and Mingioli (12), except that citrate was omitted, and the low-phosphate medium was described by Thomas and Abelson (55). Carbon sources were added to a final concentration of 0.5%. Solid media were prepared by adding agar (1.5% final concentration) to the media described above, except for calcium-containing medium which contained 1% agar. Cells were grown at 30 or 37°C on media described above and supplemented when necessary with 100 μg of required nutrient per ml. Kanamycin-resistant lysogens were maintained by growth on kanamycin-supplemented medium at 30°C or room temperature.

Mutant isolation. A *galE* mutant of *E. coli* K-12 (PL2) was mutagenized with ethylmethanesulfonate by the method of Lin et al. (32), counter-selected with penicillin in the presence of novobiocin (54), and subsequently replicated on LB plates plus or minus 40 μg of novobiocin per ml. Colonies that failed to grow in the presence of novobiocin were selected for study (about 2 per 100 survivors). Streptomycin-resistant mutants were isolated as described by Low (33), and *thyA* and *rpoB* mutants were isolated as described by Miller (35).

Genetic analysis. Thermoinducible P1*clr*100KM lysogens of PL2 (wild type) and CL7 and CL29 (NbsB

mutants) were isolated as previously described (9). CL7 and CL29 lysogens (i.e., CL27 and CL72, respectively) were tested for novobiocin and sodium dodecyl sulfate sensitivity. P1*clr*100KM and P1 phage lysates were prepared as previously described (9). The approximate location of the novobiocin-sensitive mutations on the *E. coli* chromosome were first determined by Low's rapid mapping technique (33). Linkage and gene order were based on P1 transductional analysis (31, 44). Reversion analyses, conjugation, episome transfer, and F′ factor isolation (F508) were based on procedures described by Miller (35).

Sensitivity test for antibiotics and other chemical agents. Bacterial cultures were overlaid in 0.6% agar (2.5 ml) on LB plates. The antibacterial agents contained on 6-mm disks were applied after the soft agar solidified; then the plates were incubated for 12 h at 37°C. Sensitivity to various agents was determined by the size of the inhibition zone around a disk. Minimal inhibition concentrations were determined by adding various concentrations of drugs and inhibitory agents to LB broth cultures. The lowest concentrations that completely inhibited growth were recorded.

Phage sensitivity. Bacterial strains and phages to be tested were added (0.1 ml each) to soft agar (2.5 ml) and overlaid on LB plates containing calcium.

Colicin sensitivity. Bacterial strains were overlaid in 0.6% agar (2.5 ml) on LB plates. Dilutions of colicin preparations were applied to plates containing parent or mutant strains and incubated overnight at 37°C, and the highest dilution that completely inhibited growth was recorded.

Preparation of LPS. Cells grown to mid-log phase in LB supplemented with 0.25% glucose and galactose were washed with distilled water and lyophilized. LPS was prepared from the lyophilized cells, following delipidation, by the method of Galanos et al. (17) as modified by Boman and Monner (8).

Preparation of enriched outer membrane fraction. The following procedure is essentially that of Schnaitman (52). The cells were grown at 37°C in 250-ml cultures of LB broth to 4×10^8 per ml. The cells (230 ml) were harvested by centrifugation, washed twice with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.4), and resuspended in the same buffer containing 20 μg each of DNase and RNase per ml. The cell suspension was passed through a French pressure cell at 12,000 lb/in^2 (62,016 cm of Hg). The lysate was adjusted to 0.1 mM Mg^{2+} and then centrifuged at 7,000 × *g* for 5 min. The supernatant was centrifuged in a 60 Ti rotor for 1 h at 200,000 × *g*. The unfractionated envelope fraction (pellet) was washed three times with 10 mM HEPES buffer containing 0.1 mM Mg^{2+}. The crude membrane fraction was then suspended in 10 mM HEPES containing 2% Triton X-100 and incubated for 10 min at 23 to 24°C. This suspension was then chilled and centrifuged at 200,000 × *g* for 1 h in a 60 Ti rotor. The supernatant and pellet were saved. The pellet (outer membrane and murein material) was dissolved in 0.063 M Tris-hydrochloride (pH 6.8). This fraction was used as the source of outer membrane proteins.

Qualitative procedures. The in vivo labeling, isolation, and paper chromatography of intact ^{32}P-labeled LPS was as described by Boman and Monner (8) with the following modifications: (i) 0.4 mCi of [^{33}P]P_i was used instead of $^{32}PO_4^{3-}$ and (ii) the carrier phosphate concentration was increased to 4.8 mM. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Studier (53), as detailed for bacterial membranes by Ames (6).

Quantitative procedures. Carbohydrate analyses were performed by gas-liquid chromatography as described by Holme et al. (23) and Monner et al. (36). Mild acid hydrolysis of the LPS was as described by Schmidt et al. (50). To determine the phosphate concentration of the polysaccharide and lipid A fractions of LPS, 100 mg (or less) of LPS was hydrolyzed in 1.2 N acetic acid at 100°C for 1.5 h. The acetic acid was removed by lyophilization. The lyophilized material was resuspended in deionized water and extracted three times with equal volumes of chloroform at room temperature. The chloroform phases were combined, taken to dryness in a rotary evaporator at 35°C, and washed twice with deionized water to remove traces of degraded polysaccharide. The aqueous phase (polysaccharide fraction) and the chloroform phase (lipid A fraction) were lyophilized. 2-Keto-3-deoxyoctulosonic acid concentrations were determined using LPS samples hydrolyzed in 0.02 N H_2SO_4 for 20 min, by the method of Weissbach and Hurwitz (56). Phospholipid content was determined as described by Ames (4). Total phosphate was assayed by the method of Ames (2). Determination of total sugar was by the phenol-H_2SO_4 method of Dubois et al. (13). Partition coefficients were determined as described by Nikaido (41).

Source of chemicals. Antibiotic-impregnated disks were obtained from Difco. Dyes, sodium deoxycholate, and sodium dodecyl sulfate were obtained from Sigma. All chemicals were reagent grade. Alditol acetate sugar standards were obtained from Supelco. The column packing material, 5% ECNSS-M (polyester-silicone), was obtained from Applied Science Laboratories, Inc. Perseitol, L-glycero-D-mannoheptitol, was obtained from Pfanstiehl. Perseitol was completely acetylated to hepta-*O*-acetyl-L-glycero-D-mannoheptitol. In this form it was used as the L-glycero-D-mannoheptose standard. Hepta-*O*-acetyl-D-glycero-D-mannoheptitol, used as the D-glycero-D-mannoheptose standard, was obtained from M. B. Perry, National Research Council of Canada, Ottawa, Canada. The colicin preparations were supplied by John Foulds of the National Institutes of Health.

RESULTS

Novobiocin-sensitive mutants. Thirteen novobiocin-sensitive mutants were isolated as described in Materials and Methods. Preliminary genetic analysis of these mutants by the rapid mapping method of Low (33) indicated that two distinct chromosomal markers were involved. These were given the phenotypic names NbsA and NbsB. Two isolates, CL2 (of the 7 NbsA mutants isolated) and CL7 (of the 6 NbsB mutants isolated), were arbitrarily chosen for further study.

Location of novobiocin-supersensitive

markers. The mating experiments indicated that the NbsA marker lies between *proC* and *lip* (9- to 14-min interval) on the *E. coli* chromosome (7), and the second chromosomal marker (NbsB) lies in the interval *xyl* to *ilv* (79- to 83-min interval). NbsA was localized more precisely at 10 min by determining cotransduction frequencies of this marker with *proC, plsA, purE,* and *lip*. Transductional analyses of *proC,* NbsA, *plsA*, and *purE* indicated the gene order *proC* NbsA *plsA purE* (Table 2 and Fig. 1).

The NbsB mutants are resistant to P1*vir*. P1*vir* resistance was overcome by selecting thermoinducible, kanamycin-resistant P1 lysogens (9) of NbsB mutants (CL7 and CL29), using phage P1*clr*100KM. These lysogens resulted from host range mutants of P1*clr*100KM since the novobiocin-hypersensitive characteristic was not lost. P1 lysates (3.6×10^9 plaque-forming units) were prepared from lysogenic cultures of NbsB mutants after thermoinduction at 42°C.

Preliminary transductions of markers in the *xyl* to *ilv* region of the chromosome demonstrated that NbsB was cotransducible with *xyl, mtl, cysE,* and *pyrE* (Fig. 2). The location of NbsB was based on its cotransduction frequency with several chromosomal markers (Table 3 and Fig. 2). Further support for this order comes from P1 transductions in which xyl^+ $cysE^+$ and $cysE^+$ $pyrE^+$ transductants were selected. A total of 23% of the xyl^+ $cysE^+$ transductants inherited the NbsB phenotype, whereas 87% of the

TABLE 2. *Three-factor crosses of NbsA with plsA and purE*[a]

Selected marker	Nonselected chromosomal markers	No. found
$purE^+$	NbsA$^+$ $plsA^+$	22
	NbsA$^+$ *plsA*	289
	NbsA $plsA^+$	1
	NbsA *plsA*	0
$plsA^+$	NbsA$^+$ $purE^+$	37
	NbsA$^+$ $purE^+$	129
	NbsA $purE^+$	10
	NbsA *purE*	73

[a] Cross between donor CL2 NbsA $plsA^+$ $purE^+$ and recipient strain CL51 NbsA$^+$ *plsA purE*.

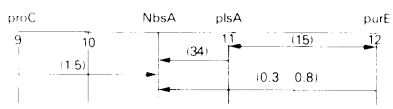

FIG. 1. *Gene order of the NbsA region. Numbers in parentheses indicate percent cotransduction frequencies with points of each arrow designating the unselected marker. The double-pointed arrow indicates the average value from reciprocal crosses.*

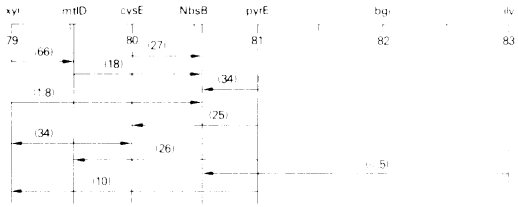

FIG. 2. *Order of loci in xyl-ilv region. The symbols are the same as for Fig. 1.*

$cysE^+$ $pyrE^+$ transductants inherited the NbsB phenotype (Table 3). Similar results were obtained when a P1*clm*100KM lysogenic derivative of another NbsB transductant (CL72) was used as the donor strain. As an additional control, we scored donor markers known to lie between the selected markers or to the left or right of the selected markers (7, 25). We found that 93% of the xyl^+ $cysE^+$ transductants (Table 3, cross 1) received the donor marker mtl^+, whereas 2% of these transductants received the donor marker $pyrE^+$. In the case of the $cysE^+$ $pyrE^+$ transduction (Table 3, cross 2), 10% of the transductants received the donor marker xyl^+. These results are consistent with the gene order indicated in Fig. 2.

In subsequent experiments biochemical and physiological characteristics of both markers were compared with those of isogenic control strains and constructed by transduction as follows: CL5 and CL6 were transductants of CV2 and differ only in that CL6 carries NbsA from CL2. Similarly, CL15 and CL63 were transductants of PCO135 and differ only in that CL63 carries the NbsA marker from CL2. CL29 and CL30 were transductants of JM15 and are genetically the same except that CL29 carries the NbsB marker from CL27. The complete genotypes of these strains are shown in Table 1. Later tests indicated that all relevant characteristics were the same for the original parent PL2 and the isogenic control stains that were constructed as described above.

Reversion studies. Novobiocin-resistant revertants of the mutants were obtained by plating samples of overnight cultures onto LB plates containing novobiocin which were then incubated for 2 days at 37°C. Spontaneous reversion occurred at a frequency of 1 colony per 10^8 cells plated. The novobiocin-resistant revertants exhibited wild-type sensitivities to other antibiotics, detergents, dyes, phages, and colicins tested. In addition, the relevant biochemical characteristics were restored by reversion; thus, the phosphate content of the lipid A of NbsB revertants was normal, and the normal heptose was restored in NbsB mutants (see below). The number of revertants was increased by diethyl sulfate

TABLE 3. *Gene order in the NbsB region based on P1 transduction*

Cross[a]	Strain				Selected marker(s)	Recombinant marker(s)		No. found	Frequency (%)
1	CL72 (donor)	+	+	NbsB	*xyl*$^+$ *cysE*$^+$		NbsB$^+$	109	77
	CL46 (recipient)	*xyl*	*cysE*	+			NbsB	32	23
2	CL27 (donor)	+	NbcB	+	*cysE*$^+$ *pyrE*$^+$		NbsB$^+$	13	13
	RK1041 (recipient)	*cysE*	+	*pyrE*			NbsB	86	87
3	CL27 (donor)	+	+	NbsB	*xyl*$^{+\,b}$	cysE$^+$	NbsB$^+$	20	18
	RK1041 (recipient)	*xyl*	*cysE*	+		cysE$^+$	NbsB	2	2
						cysE	NbsB$^+$	90	80
						cysE	NbsB	0	0
4	CL27 (donor)	+	NbsB	+	*pyrE*$^+$	cysE$^+$	NbsB$^+$	17	8
	RK1041 (recipient)	*cysE*	+	*pyrE*		cysE$^+$	NbsB	31	15
						cysE	NbsB$^+$	118	57
						cysE	NbsB	43	21
5	CL27 (donor)	+	NbsB	+	*pyrE*$^+$	mtl$^+$	NbsB$^+$	15	8
	RK1041 (recipient)	*mtl*	+	*pyrE*		mtl$^+$	NbsB	32	17
						mtl	NbsB	110	60
						mtl	NbsB	27	15

[a] Gene order indicated in this table is consistent with the distribution of genotypic classes of transductants.
[b] The *pyrE* marker was scored in this cross and showed a 1% cotransduction frequency.

and nitrosoguanidine (35), suggesting that single-point mutations were responsible for the phenotypes described for the NbsA and NbsB mutants.

Relationships of NbsA and NbsB to known *E. coli* genotypes. The NbsA mutation lies at 10 min on the *E. coli* chromosome. Another marker in this region, *acrA*, has been reported (24, 40) to cause increased sensitivity to acridine and lipophilic substances. The results of genetic analyses of *acrA* (40) were quite similar to those reported here for NbsA, and we found that *acrA* strains are supersensitive to novobiocin. To understand the relationship of these alleles, complementation tests were performed. We isolated an F′ strain (CL66) and partially characterized its episome by mating CL66 with strains CL70 (*lac purE*) and CL71 (*lac nbsA*). By mating F′ strain CL66 with the *acrA* strain CL69, merodiploids were constructed with *acrA* as the chromosomal marker and NbsA as the episomal marker. Complementation was not observed between these markers. Therefore, *acrA* and NbsA are concluded to represent point mutations in a single functional unit, and the gene will be termed *acrA*. Since the majority of the experiments were done with our strains and not with the original *acrA* strain, the phenotypic designation NbsA will be retained for such experiments to avoid confusion. In addition, certain key experiments were repeated with an authentic *acrA* strain, as will be described below.

The NbsB mutation falls in the region of the chromosome tentatively indicated to contain the *rfa* gene cluster in *E. coli* (7) and shows the same biochemical phenotype as previously isolated (28) *Salmonella rfaD* mutants (see below). We shall therefore give it the genetic designation *rfaD*.

Antibiotic, dye, and detergent sensitivities in *nbs* mutants. The minimal inhibitory concentration was determined for novobiocin and several other drugs and inhibitory agents (Table 4). These data indicated that novobiocin sensitivity was increased 80-fold for the NbsA strain and 40-fold for the NbsB strain. It was also observed that the NbsA and NbsB strains were sensitive to concentrations of other agents at 8- to 300-fold-lower concentration than that which inhibited wild-type strains (Tables 4 and 5). The hydrophobicity of these substances was measured by their partition into 1-octanol per 0.5 sodium phosphate (pH 7.0) (41). If substances showing a partition >0.02 are taken to be hydrophobic (41), then the sensitivity of both NbsA and NbsB strains is increased predominantly to hydrophobic substances. The spectra of sensitivities, while overlapping, show differences (Tables 4 and 5). NbsA strains are more sensitive to cationic agents (i.e., mitomycin C, chlortetracycline, neomycin, oleandomycin, tetracycline, and clindamycin). The higher sensitivity of NbsA to methylene blue (a cationic cye) also fits with this idea (Tables 4 and 5). The NbsB mutant was sensitive to a larger number of hydrophobic agents, both anionic and cationic.

TABLE 4. *Minimal inhibitory concentrations of various agents*

Strain	Phenotype	Antibacterial agent (μg/ml)						
		Novobiocin	Acriflavine	Sodium deoxycholate	Sodium dodecyl sulfate	Crystal violet	Methylene blue	Nalidixic acid
Wild type[a]		>400	>40	>80,000	>80,000	50	>40	>10
CL63	NbsA	5	5	8,900	250	5	5	2
CL29	NbsB	10	15	1,000	250	5	10	5

[a] These values are the same for wild-type strains PL2, CL15, and CL30.

TABLE 5. *Antibiotics, dyes, and detergent sensitivity of NbsA and NbsB strains*[a]

Drug sensitivity	Concn (μg/disk)	Inhibition zone diameter (mm)			Partition coefficient
		Wild type[b]	NbsA CL6	NbsB CL29	
Increased sensitivity to:					
Novobiocin	30	<6[c]	16	13	>20[d]
Chloramphenicol	10	10	21	16	12.4[d]
Crystal violet	2	<6	15	16	14.4[d]
Nalidixic acid	1,700	14	21	21	3.16[e]
Deoxycholate	1,700	<6	<6	14	1.09[e]
Erythromycin	5	<6	12	9	0.79[e]
Acriflavine	5	<6	12	<6[c]	0.12[e]
Sodium dodecyl sulfate	1,700	<6	17	16	0.02[e]
Methylene blue	2	<6	20	<6	0.02[e]
Actinomycin D	35	<6	<6	14	>20.0[d]
Rifamycin SV	5	<6	<6	12	8.8[d]
Clindamycin	2	<6	14	<6	0.70[e]
Chlortetracycline	5	10	16	12	0.31[d]
Mitomycin C	10	21	34	25	0.21[e]
Oleandomycin	15	<6	18	<6	0.07[e]
Tetracycline	5	10	14	10	0.07[e]
Neomycin	5	11	15	11	0.01[d]
Ampicillin	10	15	22	15	0.01[d]
No change in sensitivity to:					
Kanamycin	5	14	15	15	0.16[d]
Bacitracin	10	<6	<6	<6	0.12[d]
Oxytetracycline	5	<6	<6	<6	0.09[e]
Cloxacillin	1	<6	<6	<6	0.02[e]
Penicillin G	5	<6	<6	<6	0.02[d]
Vancomycin	30	<6	<6	<6	0.01[d]

[a] Average value of three separate tests. The first group of substances in this table are those to which both NbsA and B showed increased sensitivity. The second group are those to which only NbsB showed increased sensitivity, and the third group are those to which only NbsA showed increased sensitivity.

[b] The wild-type values are the average of values obtained for isogenic wild-type strains CL5 and CL30.

[c] The diameter of the disk was ca. 6 mm and has not been subtracted, thus <6 means no inhibition. Although acriflavine showed no inhibition due to the low disk concentration, NbsB cells had increased sensitivity as measured by minimum inhibitory concentration (Table 4).

[d] Partition coefficients were taken from reference 41.

[e] Partition into 1-octanol/0.05 M sodium phosphate (pH 7) was tested, and a substance was designated hydrophilic if its partition coefficient was less than 0.02 (41).

Colicin sensitivity. Some colicin-resistant and colicin-tolerant mutants have altered sensitivity to antibiotics, detergents, and dyes (10, 11). Since these changes in sensitivity have been related to surface changes (10, 11, 15, 22), we tested the sensitivity of these strains to selected group A and group B colicins (10, 11). These results (Table 6) indicate that both NbsA and NbsB show a reduced sensitivity to group A colicins and enhanced sensitivity to group B colicins.

LPS of Nbs mutants. We tested the sensitiv-

TABLE 6. *Quantitation of colicin sensitivity of Nbs mutants*

Strain	Phenotype	Endpoint dilution of colicin prepn[a]							
		Group A					Group B		
		A	E1	E2	L-246	K	B	M	D
PL2[b]	Wild type[b]	400	104,800	200	1,600	6,400	800	6,400	100
CL6	NbsA	100	104,800	<10[c]	800	1,600	12,800	25,600	400
CL29	NbsB	10	104,800	<10	<10	10	100	3,200	100

[a] The colicin preparations were diluted and tested as described in the text.

[b] PL2 and wild-type strains CL5 and CL30 isogenic to Nbs strains were used to determine the wild-type response, and they gave equivalent results.

[c] <10, Resistant to 10-fold dilution.

ity of the parent and NbsA and NbsB strains to LPS-specific phages (Br2, C21, Br10, F0, and P1*vir*) since the sensitivity of *E. coli* to these phages has been correlated with the presence or absence of certain LPS components (16, 44, 48, 51). The parent strains (including PL2 when grown with galactose, as it is *galE*) exhibited a pattern of phage sensitivity consistent with a complete, wild-type LPS structure of *E. coli* K-12 (Fig. 3). The phage sensitivity of the NbsA mutant suggested that its LPS was similar. On the other hand, NbsB cells were resistant to phages P1 and C21, even in the absence of galactose. Franklin showed (16) that a mutation that prevents the addition of glucose to LPS blocks phage P1 infection. It is also known that cells with LPS containing galactose are resistant to phage C21 (47). When galactose is lost, they become sensitive whether or not glucose is still present, but the loss of more proximal sugars, heptose, or 2-keto-3-deoxyoctonic acid, result in regaining C21 resistance (47). Therefore, these results suggested that the NbsB strain is missing glucose, galactose, and heptose (16, 44, 45). However, NbsB cells were sensitive to Br10 and to F0, both of which require core components (phosphate and heptose, respectively) which should be missing in heptoseless organisms (51). These apparently contradictory findings were resolved by further analysis (see below and discussion).

Paper and gas-liquid chromatography indicated that the LPS of both the NbsA and NbsB strains might be altered. The LPS of the mutant and wild-type strains was labeled with [^{33}P]P$_i$ in vivo. The intact LPS was isolated and chromatographed on paper as indicated in Materials and Methods. The lowest R_f value (0.44) was found for LPS from CL5 and CL30, the wild-type strains. An R_f value of 0.49 was found for LPS extracted from the NbsA mutant, and two spots were observed for the LPS of the NbsB mutant, with R_f values of 0.56 and 0.72.

The defect in the NbsA strains (CL6, CL63) was detected when ^{33}P incorporation and inorganic phosphate determinations indicated that the total phosphate content in its LPS is reduced at least 50%, relative to the wild type. Experiments which involved mild acid hydrolysis, separation of LPS into lipid A and polysaccharide fractions, and phosphate determination, indicated that the phosphate content is reduced more than 90% in the lipid A region of the LPS of the NbsA strain and is normal in the heptose region (Table 7 and Fig. 3).

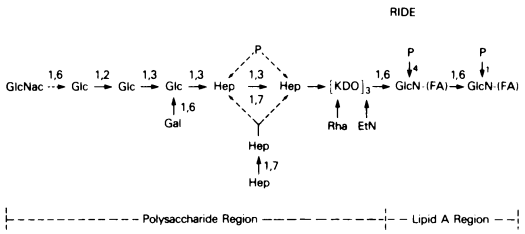

FIG. 3. *Tentative structure of the E. coli K-12 LPS. Details of the structure have been provided by the groups of O. Westphal, H. G. Boman, and other authors referenced in this paper (23, 25, 41, 42, 47). Symbols: GlcNaC, N-acetylglucosamine; Glc, glucose; Gal, galactose; Hep, L-glycero-D-mannoheptose; KDO, 2-keto-3-deoxyoctulosonic acid; Rha, rhamnose; EtN, ethanolamine; GlcN, glucosamine; P, phosphate; and FA, fatty acids.*

Finally, the LPS of the parent and mutant strains was isolated and analyzed by combined gas-liquid chromatography–mass spectromety. The neutral sugar content of the LPS of the NbsA mutant and wild-type strains (PL2, CL5, and CL30) were the same (Fig. 4). The LPS of the NbsB strain, however, contained little glucose, galactose, or L-glycero-D-mannoheptose (Fig. 4). Quantitative analysis of gas chromatograms of wild-type and mutant LPS samples confirmed the apparent reduction of the glucose, galactose, and heptose contents of the NbsB mutant (Table 7). Instead, it contained a compound whose mass spectrum indicated it was heptose, but its retention time was different from L-glycero-D-mannoheptose although identical to

TABLE 7. *Analysis of LPS components of Nbs mutants*[a]

Strain	LPS yield	Gal	Glc	D-Glycero-D-manno-heptose	L-Glyero-D-mannohep-tose	Rha	KDO[b]	Polysaccha-ride phos-phate	Lipid A phos-phate
Wild type[c]	142	0.18	0.59	ND[d]	0.84	0.08	0.90	0.40	0.40
CL6	144	0.24	0.49	ND[d]	0.86	0.09	1.15	0.40	<0.006
CL29	86	0.05	0.19	0.546	Trace[e]	0.04	2.51	0.38	0.46

[a] All values are expressed as micromoles per milligram of LPS, except LPS yield which is expressed as milligrams per gram of lyophilized cells. These values are the average of two determinations.

[b] KDO, 2-keto-3-deoxyoctulosonic acid.

[c] The wild-type values are the average of CL5 and CL30 isogenic to mutant strains except of the Nbs loci and the original wild type PL2.

[d] ND, Not detected.

[e] Trace, peak just observable.

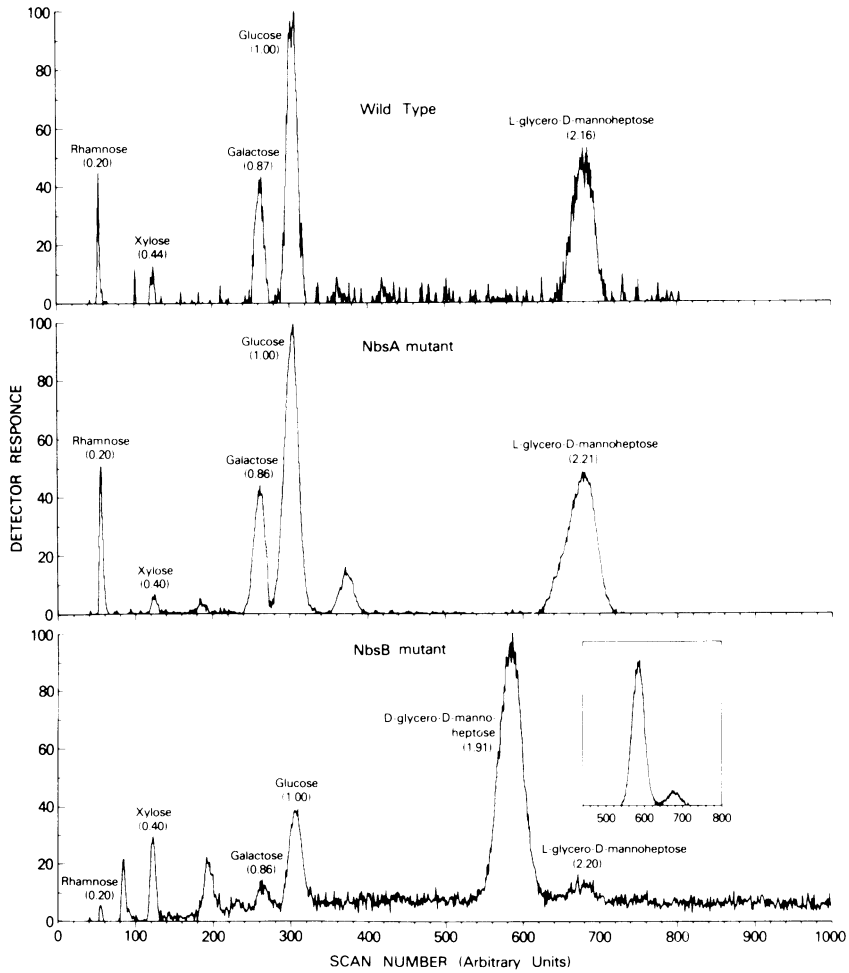

FIG. 4. *Gas chromatograph-mass spectrometry of LPS from E. coli K-12 parental (PL2) and mutant strains (CL6, CL29). Column: 5% ECNSS-M on Chromosorb Q.; injector temperature, 270°C; column temperature, 210°C; detector temperature, 265°C; nitrogen flow, 36 ml/min. An equal amount of xylose was added as an internal standard to each sample. Detector response means total ion current over an electronic mass range of 100 to 750. The numbers in parentheses are retention times relative to that of glucose. The inset is a trace of ion current at an electron mass of 524. Note that the Finnigan MS data system 6000 allows the graphic display of mass spectrometry data.*

authentic D-glycero-D-mannoheptose. D-Glycero-D-mannoheptose is the putative penultimate compound proposed for the biosynthesis of L-glycero-D-mannoheptose (14, 19). Rhamnose was detected in the LPS of both mutant and wild-type strains, but appeared to be less in the NbsB strain. There are several minor peaks in Fig. 4 that have not been identified. The ratio of components in the LPS of NbsB mutants suggests that D-glycero-D-mannoheptose is present on about half of the LPS molecules in the cell and that most of the remainder may terminate in 2-keto-3-deoxyoctulosonic acid (note the higher amount, by weight, of this compound in the analyses of NbsB LPS in Table 6). The phosphate content was not significantly changed in the NbsB strain. A few percent (perhaps 5%) of the molecules may have normal L-glycero-D-mannoheptose, and these could account for some of the glucose and galactose observed.

Phospholipids and outer membrane proteins. Biochemical analysis of the total phospholipid content and disk gels of proteins of these mutants indicate no significant change in major phospholipids (i.e., phosphatidylethanolamine, phosphatidyl glycerol, and cardiolipin) of either mutant class (Table 8). Disk gel electrophoresis revealed that outer membrane protein components were the same in the NbsA strain but altered in the NbsB strain (Fig. 5). Major outer membrane proteins in the molecular weight range of 30,000 to 36,000 of the NbsB mutant are reduced, and the protein banding patterns were changed in the 60,000 and 20,000 molecular weight ranges. The total protein of the NbsB strain's outer membrane fraction was reduced approximately 50% relative to the wild-type strain (Fig. 5 legend).

Biochemical relationship of NbsA and original acrA mutant. As mentioned above, *acrA* and the NbsA strains are allelic by complementation tests. However, *acrA* has been reported to have inner membrane protein changes and no LPS changes. When the *acrA* strain used in the above complementation test was analyzed by gel chromatography for inner or outer membrane protein changes, none was detected. However, when the LPS of this strain was analyzed, it showed a 50% reduction in phosphate content (data not shown), in accord with the genetic evidence that these strains are allelic.

TABLE 8. *Phospholipid content of wild-type and mutant strains*

Strain	Phenotype	Phosphatidylethanolamine (% of total)	Phosphatidylglycerol (% of total)	Cardiolipin (% of total)
PL2[a]	Wild type	58	37	5
CL63	NbsA	53	39	8
CL29	NbsB	64	31	6

[a] The phospholipid content of the isogenic strains (CL15 and CL30) of the Nbs mutants were the same as that of PL2.

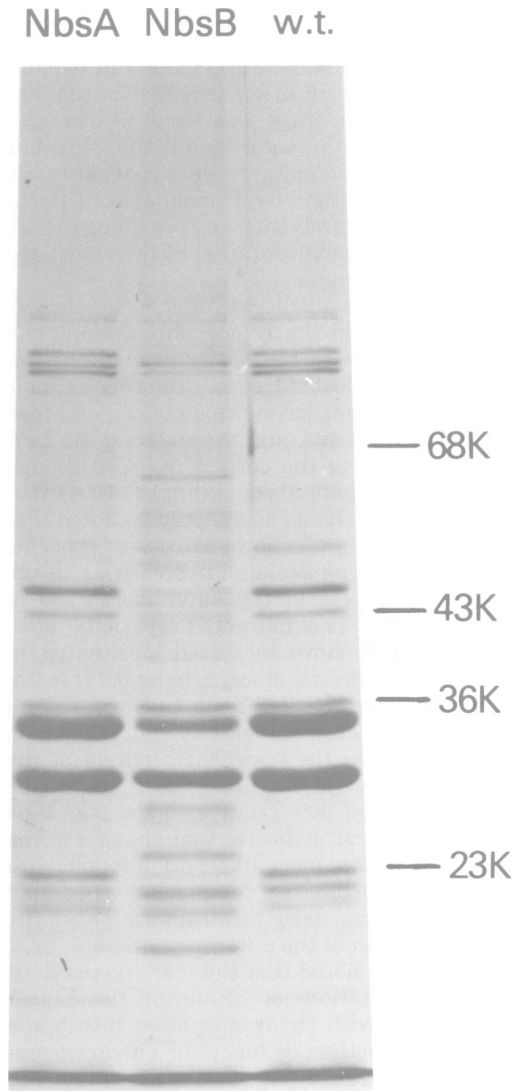

FIG. 5. *Outer membrane proteins of the Nbs mutants and parental strain. Each lane contains 40 μg of enriched outer membrane protein. Cultures containing 1.3 × 10^{11} cells yielded 1.35 mg of outer membrane protein from the wild type, 1.30 mg from NbsA, and 0.65 mg from NbsB (average of three experiments). Strains: w.t., PL2; NbsA, CL6; and NbsB, CL29. The protein banding pattern of CL5 and CL30 was identical to that of PL2.*

DISCUSSION

This paper demonstrates changes in two distinct loci that result in supersensitivity to novobiocin and other drugs, as a result of increased permeability of the outer membrane. Novobiocin-supersensitive mutants were previously isolated by this method by Tamaki et al. (54); the current work extends theirs by providing precise mapping and characterization of the associated biochemical changes.

One mutation, allelic with *acrA* (see below), lies at 10 min on the *E. coli* chromosome and results in increased sensitivity to many hydrophobic antibiotics and dyes, primarily cationic ones. This mutant has normal outer membrane protein components, and the sugars of its LPS are all present in the correct ratios. Although the total phospholipid content of the NbsA mutant is also not changed, it should be noted that our analysis does not allow us to observe specific changes in the phospholipid content or in the fatty acid content of the outer membrane of these mutants. This mutant is especially interesting, since it shows increased sensitivities to drugs without drastic changes in its outer membrane structure. However, its LPS did have a different R_f on chromatography, and the reason was found when the phosphate content of the lipid A portion of its LPS was found to be reduced at least 90% relative to the wild-type strain. This result indicates that changes in the LPS in a region most likely to interact with other outer membrane components (i.e., lipid and protein) can lead to an alteration of the barrier function of the outer membrane.

It should be noted that there are no previous reports of mutations at 10 min on the *E. coli* chromosome with changes in outer membrane synthesis, structure, or function. The *acrA* mutation was previously reported to affect only the inner membrane of *E. coli* (24, 40); however, as mentioned above, we have not been able to duplicate those results and instead find reduced phosphate in LPS, as in NbsA strains. However, we have shown that *acrA* and NbsA mutations are noncomplementary, so the proper name of the locus is *acrA*.

The second mutation causes an increase in sensitivity to some of the hydrophobic substances tested that were anionic, including deoxycholate, and to fewer of the cationic substances. Such increased sensitivity is known to be typical of *Salmonella* and *E. coli* mutants that are extremely defective in core synthesis (41, 48, 51). Tamaki et al. (54) presented evidence that their novobiocin supersensitivity mutants produced defective LPS, which included changes in the neutral sugar content and phage sensitivity patterns.

It was therefore not surprising that this mutant contained a defect in its LPS core. D-Glycero-D-mannoheptose is substituted for L-glycero-D-mannoheptose in the lipopolysaccharide. Adams et al. first reported that the predominant aldoheptose found in most LPS of gram-negative bacteria is L-glycero-D-mannoheptose and that D-glycero-D-mannoheptose is a minor component (1). The biosynthesis of L-glycero-D-mannoheptose in coliforms has been postulated to occur by racemization of the latter molecule to the former (14, 19, 28), and the NbsB mutant is therefore presumed to be blocked in this reaction. Similar mutants have been isolated in *Salmonella* (28), but their LPS contained a very large amount of the normal isomer (about 50%); the block in the mutant described herein is therefore presumably more complete. In preliminary work we have shown that the mutated gene codes for the relevant gene product (presumably an epimerase) by isolating temperature-sensitive mutants with the NbsB phenotype (data not shown). The composition of the mutant LPS suggests that the D-glycero-D-mannoheptose is added to some but probably not all of the nascent chains, some of which may terminate at 2-keto-3-deoxyoctulosonic acid. The D-glycero-D-mannoheptose apparently does not usually serve as an efficient acceptor of distal sugars since the glucose and galactose are reversibly reduced. The phosphorylation of this heptose seems normal—an observation also made in the *rfaD* mutants of *S. typhimurium* (28).

The abnormal heptose also changes the pattern of phage sensitivity. Resistance to phages C21 and P1 is usually characteristic of a heptoseless core, but sensitivity to phage F0 and Br10 usually indicates the presence of heptose and phosphate, respectively (51). We may therefore postulate that C21 and P1 cannot, but F0 and Br10 can, use D-glycero-D-mannoheptose as a receptor. Another result of the mutation is a change in outer membrane protein composition very similar to that observed in mutants lacking heptose altogether (6, 26), suggesting that whatever the mechanism that requires heptose for normal insertion of protein does not recognize D-glycero-D-mannoheptose as sufficient.

This class of mutants lies at 80 min on the *E. coli* chromosome. This region has previously been implicated as the site of *rfa* (LPS core biosynthesis) genes in *E. coli* K-12. By using hemagglutination tests and phage sensitivity, Schmidt demonstrated that *rfa* genes were inherited by 60% of the recipients in mating ex-

periments that received the *mtl*$^+$ allele (49). Hancock and Reeves isolated mutants in the *mtl pyrE* region that showed changes in the LPS core and in associated bacteriophage sensitivity (20). Havekes et al. (21) isolated conjugation-deficient *E. coli* K-12 F$^-$ mutants with heptose-less LPS. Preliminary genetic analysis of these mutants indicated that one mutation was closely linked to *xyl*. These data indicated that the *rfa* gene cluster of *E. coli* K-12 lies in this region homologous with the position of the *rfa* gene cluster in *Salmonella* (27). When combined with the existence of the above-mentioned *Salmonella rfaD* mutants (28), these data strongly suggest that the NbsB mutant deserves the genotype *rfaD*. Although the *Salmonella* mutants were mapped, the previously isolated relevant *rfa E. coli* K-12 mutants (see above) have not been mapped, and so complementation with NbsB strains was not attempted.

The barrier mutants described herein, *acrA* and *rfaD*, provide useful tools for studying LPS core biosynthesis, aldoheptose biosynthesis, a subject about which little is known, and certain in vivo metabolic processes. For example, one of the barrier mutants isolated by us (an *acrA* mutant) has already been used by Gellert et al. to demonstrate an in vivo effect of novobiocin and coumermycin on DNA replication (18). We would like especially to point out that an *acrA* mutant might be a useful addition to strains available for testing carcinogenic substances, in the test devised by Ames et al. (3). The *rfaD* mutant gives antibiotic sensitivities similar to those of the *Salmonella* strains already in use for such tests, but the *acrA* gives a different spectrum of sensitivities. Thus, this mutant class might detect mutagenic substances that would yield false negatives with the strains now in use.

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