JOURNAL OF BACTERIOLOGY, Mar. 1971, p. 968-975
Vol. 105, No. 3

Role of Lipopolysaccharides in Antibiotic Resistance and Bacteriophage Adsorption of *Escherichia coli* K-12

SHIGEO TAMAKI, TOMOYASU SATO,[1] AND MICHIO MATSUHASHI
Institute of Applied Microbiology, University of Tokyo, Bunkyo-Ku, Tokyo, Japan

Received for publication 20 October 1970

Novobiocin-supersensitive (NS) mutants which could not grow on plates containing 40 μg or less of novobiocin per ml were isolated from *Escherichia coli* strain JE1011 (derived from *E. coli* K-12). Most of these NS mutants were found to have incomplete lipopolysaccharides (LPS), and they lack phosphate diester bridges in their backbone structure, with or without total loss of heptose, to which the phosphate diester is linked, and consequently lack external outer-core oligosaccharides. The phosphate diester bridges in the LPS backbone are apparently very important in forming a cell surface structure resistant to the penetration of antibiotics such as novobiocin, spiramycin, and actinomycin D. NS mutants, with incomplete LPS, lacking phosphates in their backbone structure were found to be resistant to phage T4, and those which also lacked heptose were resistant to phages T4 and T7. In contrast to the generally accepted idea that resistances to phages T3, T4, and T7 are linked genetically, no NS mutant was found to be resistant to T3. The possible structures of the receptors for T4 and T7 are discussed. The positions of novobiocin-supersensitive genes on the chromosome of several of the NS mutants defective in LPS were mapped. The genes were designated *lpcA* (between *ara* and *lac*) and *lpcB* (between 55 min and 60 min). The latter seemed to be a group of several related genes.

Many antibiotics which are highly toxic for gram-positive bacteria are lethal to gram-negative bacteria only at high concentration. This is thought to be due to the structures of the cell membranes of gram-negative bacteria which resist the penetration of certain antibiotics. A much lower concentration of actinomycin D is required for inhibition of the deoxyribonucleic acid-dependent ribonucleic acid polymerase in *Escherichia coli* than that required for inhibition of growth (7). Similar observations have been obtained with ristocetin (2), vancomycin (2), bacitracin (22), enduracidin (13), and penicillins (8). All these antibiotics inhibit bacterial cell wall synthesis and do so at a much lower concentration than that inhibiting cell growth.

To characterize the structures which resist the penetration of antibiotics in the cell envelopes, we tried to isolate mutants of *E. coli* which were supersensitive to various antibiotics and to identify the changes in their cell composition caused by the mutations.

[1] Present address: Tokyo Central Research Institute, Kyowa Hakko Kogyo Co., Machida-Shi, Tokyo, Japan.

Penicillin G, novobiocin, bacitracin, and enduracidin were used for selection of supersensitive mutants. This paper mainly reports results on novobiocin-supersensitive (NS) mutants. Most of these mutants were found to have defective lipopolysaccharides (LPS). The role of LPS in the adsorption of phages on *E. coli* was also studied.

MATERIALS AND METHODS

Bacteria and isolation of antibiotic-supersensitive mutants. Mutants were isolated from *E. coli* K-12 strain JE1011 (F⁻ *thr*⁻ *leu*⁻ *trp*⁻ *his*⁻ *thy*⁻ *thi*⁻ *ara*⁻ *lac*⁻ *gal*⁻ *xyl*⁻ *mtl*⁻ *str*ʳ). (Genetic symbols are defined in Fig. 1.) Bacteria were usually cultured in nutrient broth at 37 C with shaking. Nutrient broth contained (per liter of deionized water): polypeptone (Daigo Eiyo Kagaku Co., Osaka, Japan), 10 g; Ehrlich meat extract (Kyokuto Seiyaku Co., Tokyo, Japan), 10 g; and NaCl, 2 g. It was adjusted to *p*H 7.4 by addition of 1 ml of 10 N NaOH per liter. For nutrient agar, nutrient broth was solidified by addition of 1.5% agar (Wako Pure Chemical Co., Osaka, Japan).

Cells were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) essentially by the method of Adelberg et al. (1). Cells in the logarithmic phase of growth (5 ml of culture) were collected by centrifugation,

washed twice with 0.85% saline, and suspended in 2 ml of buffer solution (*p*H 6.0) containing 0.05 M tris(hydroxymethyl)aminomethane base, 0.05 M maleic acid, 7.6 mM $(NH_4)_2SO_4$, 0.4 mM $MgSO_4 \cdot 7H_2O$, 30 μM $Ca(NO_3)_2 \cdot 4H_2O$, and 1 μM $FeSO_4 \cdot 7H_2O$. NTG was added at a final concentration of 100 μg/ml. The mixture was allowed to stand for 30 min at 37 C, and then the cells were centrifuged and suspended in 10 ml of nutrient broth. After 17 hr of incubation at 37 C with shaking, 0.1-ml samples were spread on plates of nutrient agar at a concentration giving several hundred colonies per plate (master plates). Colonies were grown at 37 C and replicated on plates of nutrient agar containing penicillin G (potassium salt, Banyu Chemical Co., Tokyo; 5 μg/ml), novobiocin (Meiji Seika Co., Tokyo; 40 μg/ml), enduracidin (Takeda Chemical Co., Osaka; 50 μg/ml), or bacitracin (Sigma Chemical Co., St. Louis, Mo., 200 μg/ml). Colonies which did not grow on at least one of the replica plates at 37 C were collected from the master plates. To isolate antibiotic-supersensitive mutants more efficiently, the penicillin-screening method (10) was tested. It was very useful in enriching NS mutants. Thus, after treatment with NTG and subsequent segregation for 17 hr, cells were transferred to nutrient broth (about 10^8 viable cells per ml) containing 300 μg of penicillin G per ml and 400 μg of novobiocin per ml and were then incubated at 37 C for 16 hr with shaking. The cell suspension was diluted 10^{-3} with nutrient broth, and 0.1-ml samples were spread on nutrient agar plates. Most of the colonies which appeared were novobiocin-supersensitive.

Quantitative evaluation of antibiotic sensitivity. Samples (0.1 ml) of suspensions of the parent and mutant strains from overnight cultures in nutrient broth were inoculated into 5 ml of the same medium in 25-ml L-shaped tubes containing different concentrations of the test antibiotics. Test cultures were shaken at 37 C for several hours, and cell growth was estimated from the absorption at 660 nm. The concentrations of antibiotics required to inhibit growth to half that in the control tube containing no antibiotic were calculated.

Coumermycin was kindly supplied by Bristol-Banyu Chemical Co., Tokyo, and spiramycin by Kyowa Hakko Co., Tokyo.

Mating experiments. The following Hfr strains were used in mating experiments: W2252 [Hfr C (*O-lac-leu-ara-thr*) *met- str*^{s}], JE1031 (Hfr H (*O-thr-ara-leu-lac*) *met*$^-$ *thi*$^-$ *str*^{s}], AB313 [Hfr (*O-mtl-xyl-str-thy*) *thr*$^-$ *leu*$^-$ *thi*$^-$ *lac*$^-$ *str*^{r}], and AB312 [Hfr (*O-str-xyl-mtl*) *thr*$^-$ *leu*$^-$ *thi*$^-$ *lac*$^-$ *str*^{r}]. The methods used were essentially those of Taylor and Thoman (27). However, because of the low efficiency of mating of most mutant strains, as described below, extensive dilution of mixtures after pair formation was not possible. In typical experiments, male and female cells in the logarithmic phase of growth (titer 2×10^8 cells per ml) in nutrient broth were mixed to a ratio of 1:4. Samples (0.25 ml) of the mixture were placed in separate test tubes, and the tubes were incubated at 37 C with very gentle shaking. Mating was interrupted at 2- to 5-min intervals by diluting the mixture with 9 volumes of 0.85% saline and blending it in a Vortex mixer for 1 min. Exconjugant donor cells were then killed on selective plating media containing 200 μg of dihydrostreptomycin (Kyowa Hakko Co., Tokyo) per ml if the donor strains were streptomycin-sensitive. Treatment with male-specific phage MS2 was also applied (27) if the donor strains were streptomycin-resistant.

Portions (0.1 ml) of each sample (undiluted or diluted 1:10) were spread on several selection plates. Nutrient broth or a defined medium with suitable additions was used for selecting antibiotic-resistant (wild type) recombinants. The defined medium contained (per liter of deionized water): K_2HPO_4, 7 g; KH_2PO_4, 2 g; $MgSO_4$, 0.1 g; $(NH_4)_2SO_4$, 1 g; sodium-citrate $\cdot 2H_2O$, 0.5 g; and glucose, 2 g. To solidify the medium, 1.5% agar (Wako Pure Chemical Co., Osaka, Japan) was added. Amino acids were added at a concentration of 50 μg/ml each, thymine at 10 μg/ml, and thiamine at 2 μg/ml. Defined media were also used for selecting prototrophic recombinants of requirement markers. Eosine-methylene blue medium was used for selection of recombinants fermenting sugar. It contained (per liter of deionized water): K_2HPO_4, 4 g; $MgSO_4 \cdot 7H_2O$, 1 g; $(NH_4)_2SO_4$, 5 g; NaCl, 1 g; eosine Y, 0.3 g; methylene blue, 0.065 g; agar, 15 g; sugar, 10 g (arabinose, 5 g), and requirement marker substances as described for defined medium.

Preparation and analysis of lipopolysaccharides. Lipopolysaccharides were isolated from the parent and mutant cells by the phenol extraction method of Westphal (17). The isolated lipopolysaccharides were hydrolyzed by treatment with 0.1 M acetic acid at 100 C for 1 hr, and the resulting polysaccharides in the aqueous supernatant were purified by the method of Risse et al. (19) by paper electrophoresis in acetic acid-pyridine-water (10:4:286, v/v/v) at 500 v per 30 cm for 3 hr. A strong negatively charged band reducing silver nitrate consisting of 2-keto-3-deoxyoctonate and phosphate (band 1) and a less negatively charged band consisting of core-saccharides containing phosphate and a small amount of 2-keto-3-deoxyoctonate (band 2) were separated. Analyses were performed directly on the supernatant after hydrolysis with acetic acid and on the bands separated. 2-Keto-3-deoxyoctonate was measured by the thiobarbituric acid reaction (30), heptose by the cystein-sulfuric acid reaction (18), and ester phosphate by the method of Lowry et al. (11). For analysis of glucose and galactose, polysaccharides were hydrolyzed with 1 N H_2SO_4 at 100 C for 4 hr. Then glucose was measured enzymatically with a glucose oxidase (EC 1.1.3.4)-peroxidase (EC 1.11.1.7) system (Glucostat, Worthington Biochemical Corp., Boston, Mass.) or with hexokinase (EC 2.7.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49, Boehringer, Mannheim, Germany), and galactose was measured with galactose dehydrogenase (EC 1.1.1.48, Boehringer). The ethyl ester of 2-keto-3-deoxyoctonate used as a standard was a gift from E. Heath, Johns Hopkins University, Baltimore, Md., and heptose was a gift from O. Lüderitz, Max Planck Institut für Immunbiologie, Freiburg, Germany.

Phages. T4 was obtained from Y. Masamune, Institute of Applied Microbiology of the University of Tokyo, and T3 was from T. Watanabe, Keio University Medical School. Two preparations of T7 were obtained from T. Watanabe and F. W. Studier, Brookhaven National Laboratory, Upton, N.Y., and used in duplicate experiments. P1 was obtained from Y. Sugino, Osaka

University, and Mul from A. L. Taylor, University of Colorado, Denver.

RESULTS AND DISCUSSION

Nature of NS mutants. Six mutants (NS1 to NS6) were isolated by the direct replica method and others (NS7 to NS22) were obtained after penicillin screening. The sensitivities of NS1 to NS6 to novobiocin and seven other arbitrarily selected antibiotics was studied extensively (Table 1). The LD_{50} values with novobiocin, coumermycin, spiramycin, and actinomycin D of these mutants were $1/10$ to $1/100$ of those of the parent strain, JE1011. However, the LD_{50} value of NS4 for novobiocin was exceptional in being only a little less than that of the parent. Coumermycin, a compound related to novobiocin, had a completely different effect from novobiocin on NS6.

The mutants were only slightly sensitive to a second group of antibiotics (i.e., ristocetin, enduracidin, and bacitracin). However, the sensitivities of different strains to the two groups of antibiotics varied.

The mutants showed no increase in sensitivity to penicillin G.

It is interesting to compare these results with those obtained previously for penicillin-supersensitive (PS) mutants (24; M. Matsuhashi, T. Sato, H. Matsuzawa, and B. Maruo, Abstr. 6th Int. Congr. Genet. Tokyo, p. 69, 1968). The PS mutants (PS1 and PS3) were 10 times more sensitive to penicillin G, ampicillin, and cephalothin than the parent strain, JE1011, but their sensitivities to antibiotics other than penicillins and cephalosporins were not increased significantly (Table 1). Results on a mutant of another type, EBS1, obtained as an enduracidin- and bacitracin-supersensitive mutant, are also given in Table 1.

These results strongly suggest that the supersensitivities of these mutants are due to defects of certain structures in the cell surface which specifically resist the penetration of certain antibiotics. NS mutants lack some barrier structures against novobiocin, coumermycin, spiramycin, actinomycin D, etc., and PS mutants lack some barrier structures against penicillins and cephalosporins. To test this, chemical analyses were made of the mutant cells. It was found that most NS mutant cells (18 of 19 NS mutants) had incomplete LPS. The points of mutation in the other NS mutant and in the PS- and EBS mutants remain to be identified.

Analysis of cell wall LPS. Samples of LPS were prepared from the cells of the parent and mutant strains. Results of analyses of these samples are shown in Table 2.

In the parent strain (JE1011), the proportions of heptose, organic phosphates, glucose, and galactose in the aqueous phase after hydrolysis of LPS with acetic acid were 2:2:1:0.5. No rhamnose was found, and glucosamine was not measured. LPS of the mutants contained significantly different levels of heptose, phosphate, glucose, and galactose. In all the NS mutants except NS6, the supernatants of acetic acid hydrolysates contained essentially no phosphate, glucose, or galactose (Table 2). Strains NS1 to NS3 also contained little or no heptose. The heptose content of NS4 was about one-quarter and that of NS5 was about one-half of that of the parent. The small amounts of phosphate in the acetic acid supernatants from mutants NS1 to NS5 are probably due to the phosphates bound to 2-keto-3-deoxyoctonate or lipid A. Upon paper electrophoresis of these supernatants, essentially no phosphate was found in band 2, the position of core saccharides, although band 2 from NS4 and NS5 contained almost the same amount of heptose as their supernatants (Table 2). Similar results were later obtained with other NS mutant strains: NS9 to NS11, NS14, and NS16 to NS20

TABLE 1. *LD_{50} of E. coli strain JE1011 and its antibiotic-supersensitive mutants*[a] *(μg per ml)*

Strain	Antibiotic							
	Novobiocin	Coumermycin	Spiramycin	Actinomycin D	Ristocetin	Enduracidin	Bacitracin	Penicillin G
Parent JE1011	410	22	9	70	140	200	>1,000	20
NS1	18	0.5	0.2	3.5	9	5.5	190	13
NS2	3	4	<0.1	5	140	400	500	>20
NS3	5	4.5	0.2	7.4	140	200	860	>20
NS4	180	5	0.5	10	7.2	300	500	19
NS5	6	3	0.3	14	12	>400	140	20
NS6	4	>22	0.4	7	>140	>400	>1,000	>20
EBS1	100		2	1.2	2	15	30	16
PS1	180	7				100	>1,000	2.5
PS3	140	14	5.6		120	125	>1,000	1.7

[a] Results expressed as micrograms per milliliter.

TABLE 2. *Analysis of LPS and phage sensitivity of E. coli strain JE1011 and its antibiotic-supersensitive mutants*[a]

Strain	LPS (acetic acid supernatant)					Band 2		Phage sensitivity				
	2-Keto-3-deoxy-octonate	Heptose	Phosphate	Glucose	Galactose	Heptose	Phosphate	T3	T7	T4	P1	Mul
Parent JE1011	120	520	550	280	140	450	330	s	s	s	s	s
NS1	220	0	90	0	10	0	0	s	r	r	r	r
NS2	340	20	60	0	20	0	10	s	r	r	r	r
NS3	340	70	40	0	20	30	10	s	r	r	r	r
NS4	240	170	60	0	20	130	20	s	s	r	r	r
NS5	210	320	80	0	20	260	20	s	s	r	(r)	r
NS6	120	570	650	250	130	270	280	s	s	s	s	s
EBS1	90	640	410	150	120	470	270	s	s	s	s	s
PS3	110	500	530	240	120	200	330	s	s	s	s	s

[a] Results expressed as nanomoles for 1 mg of LPS.

were like NS1 to NS3, whereas NS7, NS12, and NS13 were like NS4 and NS5. (NS22 was exceptional in containing some phosphate and heptose, as described later, although its behavior with phage T4 was like that of other mutants. The nature of the phosphate residues in this mutant is unknown.)

As recently shown in *Salmonella minnesota*, the backbone structure of LPS consists of heptosyl-α-(1 $\rightarrow$ 3)-heptose strands (4) linked through glycosidic bonds to the nonreducing terminal of (2-keto-3-deoxyoctonate)$_x$. The latter is further attached through ketosidic bonds to glucosamine moieties in lipid A (6). The diheptose strands are believed to be cross-linked through phosphodiester bridges (5), forming a network of LPS on the cell surface. The outer core oligosaccharides [D-*N*-acetylglucosaminyl-α-] D-glucosyl-α-D-galactosyl-α-(1 $\rightarrow$ 3)-[D-galactosyl-α-(1 $\rightarrow$ 6)-] D-glucose are attached through glycosidic bonds at the nonreducing ends of the backbone polysaccharides.

In *E. coli*, the diheptose linkage was reported to be α-(1 - 2) not α-(1 - 3) as in *Salmonella* (9), but the general structure of the core LPS in *E. coli* is believed to be very similar to that of *Salmonella*.

If we assume that LPS of *E. coli* K-12 has the same backbone structure as that of *Salmonella* LPS, our NS mutants, with the exception of NS6, all lack phosphate in the LPS backbone which forms a network of diheptose-phosphate polymer on the cell surface.

These phosphate linkages may coordinate with other components of the cell envelope, very likely membrane lipoproteins, to form a structure at the cell surface which resists the penetration of antibiotics. However, the differences in sensitivities of the mutants to different antibiotics (Table 1) suggest that the surface structures of the bacteria are very complicated.

Schlecht and Westphal (20, 21) recently reported using a series of rough (R) mutants of *S. minnesota* ranging from those with a complete core (Ra) to those with complete deletion of outer core oligosaccharides and heptose (Re). They found that the sensitivities of these mutants to erythromycin, rifamycin, actinomycin D, and bacitracin increased progressively with loss of sugar moieties from the outside of the R core of LPS, the highest sensitivity being seen in those completely lacking heptose moieties (Re). However, our results with *E. coli* suggest that the most important factor for sensitivity to novobiocin, coumermycin, spiramycin, and actinomycin D is phosphate diester bridges and that the sugar moiety in the LPS core is unimportant. This difference may indicate a delicate difference between the structures of the cell envelopes of *E. coli* and *Salmonella*.

Schlecht and Westphal (21) also found that tetracycline resistance is highest in Re mutants (complete heptose deletion) and lowest in Rb mutants (GlcNAc deletion), and resistance to erythromycin varies in approximately the reverse way. We did not study the effect of tetracycline on our NS mutants, but the results of Schlecht and Westphal on penicillin sensitivity are somewhat contradictory to ours. In *Salmonella* R strains, the highest penicillin resistance was obtained in Rc (galactose deletion), and sensitivity increased either on further deletion (Rd_1, Rd_2, and Re) or elongation (Rb, Ra) of core saccharide chains (21). These results are compatible with those obtained by Stocker et al. (Bacteriol. Proc., p. 79 1969). In *E. coli*, however, deletion of heptose and phosphate did not affect the sensitivity of cells to penicillins. These discrepancies require further study.

Unlike most NS mutants, the structure of LPS of mutant NS6 seemed to be similar to that of the parent (Table 2). Thus, it is believed to differ

in a component of the cell envelope other than LPS. This mutant was the only strain isolated which was sensitive to methylene blue. Two other methylene-blue sensitive strains, JE2 and JE16, were reported previously by Sugino (23). We found that these two strains were also novobiocin-supersensitive (*unpublished data*).

For comparison, Table 2 also shows results on EBS1 and PS3, an enduracidin- and bacitracin-supersensitive mutant and a PS mutant, respectively. No defects in the LPS of either mutant have yet been found.

Genetic analyses of NS mutants. It was difficult to determine the positions of novobiocin supersensitivity genes on the chromosome. This was because the frequency of recombination of the mutant chromosome with those of Hfr's was generally too low to give a precise time course of chromosome transfer from Hfr's by mating. Another difficulty was that all the NS mutants except NS6 were resistant to infection with the transducing phage P1. Both difficulties may have been due to incomplete LPS of the mutants.

Watanabe et al. showed, from mating experiments using Hfr and F^- strains of *S. typhimurium*, that cells with an incomplete R core of LPS (either male or female) have reduced frequencies of chromosome transfer by mating (28). This could also be the case with *E. coli*, and therefore only rough maps of gene positions were made.

Figure 1 shows the kinetics of gene transfer in mutant strain NS14 by HfrC (A) and by HfrH (B). The transfer of the *nov*^{r} gene (for symbols see legend to Fig. 1) appeared between *ara* (1 min) and *lac* (10 min) in both cases. Determination of the frequency of switches between *ara* and *nov* and *nov* and *lac* in mating with both Hfr's in tryptophan and xylose selections was carried out as shown in Table 3, and the distance from *ara* to *nov* was roughly calculated as 0.53 of the distance between *ara* and *lac*. We designated this gene *lpcA* (LPS-core synthesis).

However, the *nov* genes of most NS mutants were on the opposite side of the chromosome. Their positions were located roughly between *xyl* and *thy* by mating with Hfr AB313, an Hfr strain transferring chromosome in the order *mtl-xyl-str-thy*. An example of the kinetics for a cross to recipient strain NS20 is illustrated in Fig. 2. Similar results were obtained on the transfer of *nov*^{r} by Hfr AB313 with the other NS mutant strains, NS7, NS9, NS10, NS17, and NS19. Genes of sugar and phosphate (14) transfer in the R-core of LPS are located (*rfa*) in the corresponding area on the chromosome of *S. typhimurium* (12) and, therefore, the *nov* genes of our mutants could be identical to several genes of the *rfa* group of *Salmonella*. However, the transfer of *nov*^{r} by Hfr AB312, a strain transferring its chromosomes clockwise starting from 60 min (26), could not be demonstrated in any of the above mutants, suggesting that the positions of *nov* genes are between 55 min (*thyA*) and 60 min. We designate this group of genes *lpcB* (LPS-core synthesis). The *envB* gene recently reported by Normark (15), which is supposed to be related to the formation of cell envelopes, was found to be situated close to *strA* (64 min) and could also be related to, or involved in, the *lpcB* group. The mutant carrying the *envB* gene is supersensitive to several antibiotics and is also resistant to phage P1 (15). However, this mutant has a different cell morphology (spherical cells) from the wild strain, like NS strains carrying the *lpcB* gene, and also differs from the wild type or NS strains with the *lpcB* gene in its sensitivity to penicillins.

There is some information on the positions of genes of other supersensitive mutants which do not seem to be LPS defective. The enduracidin- and bacitracin-sensitivity gene of EBS1 was transferred either by HfrC or AB313 with high frequency of linkage with *xyl*. The penicillin sensitive gene PS3 was located close to *thr-leu*, as previously reported (24). A very similar gene (*envA*), at 2 to 4 min, was reported by Normark et al. (16). However, the cell morphology of the *envA* mutant not forming complete septa is different from that of PS3.

The approximate positions of the antibiotic-supersensitive genes are summarized in Fig. 3.

Role of LPS of E. coli in phage adsorption. With the exception of NS6, all the NS mutants so far obtained were defective in the LPS backbone structure. These LPS mutants were also resistant to T4 and several other coliphages unlike the wild strain. The sensitivities of the mutants to phages T3, T4, T7, and P1*kc* and Mul of Taylor (25) are summarized in Table 2.

Table 2 shows clearly that all the supersensitive mutants lacking phosphates in the LPS backbone were resistant to phage T4 and, when they also lacked heptose completely, they were also resistant to T7. This relationship between the LPS backbone structure and T-phage sensitivity was confirmed in results on a number of other NS mutants isolated later and not listed in Table 2: NS9 to NS11, NS14, and NS16 to NS20 had LPS like NS1 to NS3 and were also resistant to both T4 and T7; NS12 and NS13 had LPS like NS4 or NS5 and were resistant to T4 but sensitive to T7. Exceptions were NS22, which contained heptose and some phosphate in the LPS backbone but was T4-resistant and T7-sensitive, and NS7, with an LPS backbone like that of NS4, which was only partially resistant to T4 and

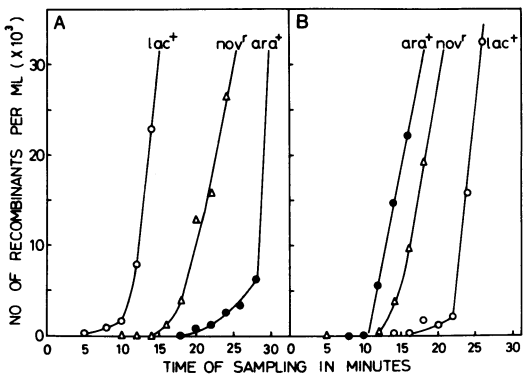

FIG. 1. *Kinetics of chromosome transfer by donor strains (A) W2252 (Hfr C ara⁺ lac⁺ strˢ novʳ) and (B) JE1031 (Hfr H ara⁺ lac⁺ strˢ novʳ) to recipient strain NS14 (F⁻ ara⁻ lac⁻ strʳ novˢ). Novobiocin (15 μg/ml) was present in the seclective plating media for novobiocin resistance. The following symbols are used in this paper: thr, threonine; leu, leucine; trp, tryptophan; his, histidine; met; methionine; thy, thymine; thi, thiamine; ara, arabinose; lac, lactose; gal, galactose; xyl, xylose; mtl, mannitol; mal, maltose; str, streptomycin; novˢ, novobiocin-supersensitive; novʳ, novobiocin-resistant (wild type); tfr, resistance to phage T3, T4, T7, and λ. The time scale of reference markers is according to Taylor (26).*

TABLE 3. *Frequency of switches in xyl⁺ and trp⁺ selections from Hfr's (strˢ novʳ) × NS14 (F⁻ ara⁻ lac⁻ trp⁻ xyl⁻ strʳ novˢ)*

Crosses	NS14 × HfrC		NS14 × HfrH	
Primary selection	*xyl⁺ strʳ*[a]		*trp⁺ strʳ*[b]	
A *ara⁺ lac⁻*	50	98	79	103
ara⁻ lac⁺	48		24	
B *ara⁺ novˢ*	32	88	43	69
ara⁻ novʳ	56		26	
C *novʳ lac⁻*	48	70	53	67
novˢ lac⁺	22		14	
B/(B + C)	0.56		0.51	

[a] Number tested, 295.
[b] Number tested, 404.

sensitive to T7. None of these mutant cells adsorbed T4.

From these results, it is concluded that the cross-linking phosphate in the LPS backbone is responsible for the adsorption of phage T4 by the cells or, based only on the above results, it may be the receptor of T4 itself. But for the adsorption of phage T7 at least one heptose moiety is necessary, and phosphate cross-linkings are dispensable.

However, the nature of the receptors for T4 and T7 seem to be more complex. The resistances of *E. coli* strain B to phage T3, T4, and T7 have

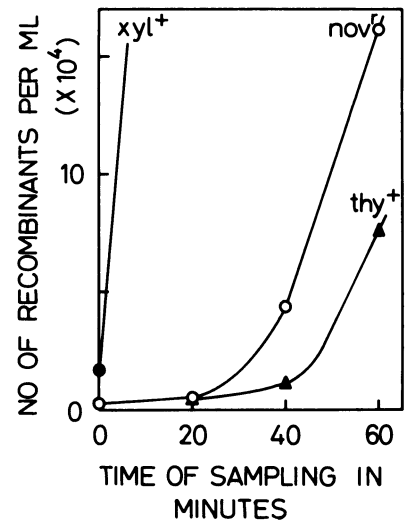

FIG. 2. *Kinetics of chromosome transfer by donor strain AB313 (Hfr thr⁻ leu⁻ xyl⁺ thy⁺ novʳ) to recipient NS20 (F⁻ thr⁺ leu⁺ xyl⁻ thy⁻ novˢ). The recipient carrying originally the same auxotrophic markers as JE1011 was converted thr⁺ leu⁺ with JE1031 (Hfr H) prior to the mating experiment. Novobiocin (30 μg/ml) was present in selective plating media for novʳ.*

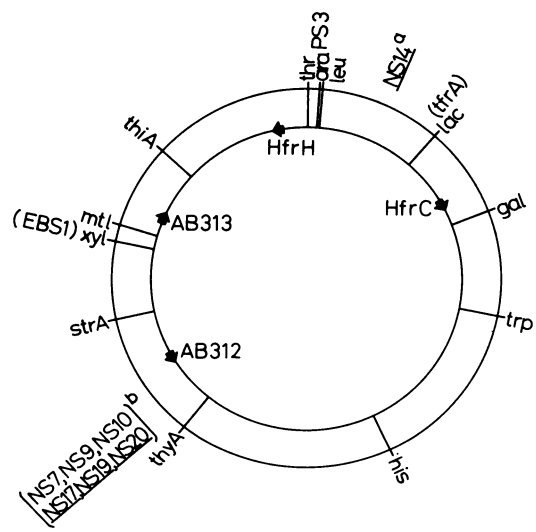

FIG. 3. *Linkage map of Escherichia coli K-12 showing NS mutations. Arrowheads on the inner circle indicate the point of origin and the direction of chromosome transfer for Hfr strains. a, lpcA: Resistance to phage T4 and T7, but not to T3. b, lpcB: Resistance to phage T4 and T7, or to T4 only, not to T3.*

been thought to be genetically indistinguishable, and Weidel supposed them to be a single genetic event (29). He also found that the LPS of the resistant mutant B/3,4,7 was completely deficient in heptose. In contrast, in *E. coli* K-12, the linkage of three kinds of resistances is not so close (3). Our experiments using NS mutants showed differences in these resistances more clearly. Adsorption of T4 is dependent on the presence of phosphate diester bridges in LPS, and that of T7 depends on heptose. Moreover, adsorption of T3 was unaffected by deletion of these two components, as seen in Table 2. The adsorptions of T3 and T7 were not checked directly, but the sensitivity of a mutant to a phage unequivocally implies that the phage is adsorbed.

The resistances to the three T phages were clearly separate as shown by isolating NS mutants differing in their resistances. However, they were usually linked, as shown by isolating phage-resistant mutants (3). This indicates that the receptor sites for these phages are not simply residues of LPS but complex structures coordinated with other components of the cell surface, very likely lipoproteins. To confirm this, phage-resistant mutants were isolated, and their novobiocin-sensitivity, LPS composition, their ability to adsorb T4 phage, and their phage sensitivity were examined. Table 4 shows results on 16 T4-resistant mutants formed spontaneously in a culture in nutrient broth. They were divided in two groups: novobiocin-supersensitive (ns) and normal (nr). All nine strains in the ns group were defective in phosphate in the LPS backbone and did not adsorb T4, but some were sensitive and some resistant to T7 and T3. One strain (T431) was T7-sensitive, and this strain contained heptose, in agreement with the results obtained with NS mutant strains. Eight other strains were T7-resistant; of these, six lacked heptose and two (T452, T472) contained heptose. This result strongly suggests that some factor in LPS other than heptose is also required for adsorption of T7. Heptose residues seem to be indispensable for adsorption, as no T7-sensitive mutants lacking heptose were isolated. Four strains (T411, T422, T471, and T431) were T3-sensitive, as in the case of NS mutants, but five strains (T413, T421, T442, T452, and T472) were T3-resistant. Resistance to T3 was not related to the presence or absence of heptose in LPS. T3-resistant strains (five strains) were also T7-resistant. These results show that the adsorption of T3 requires some factor related to LPS, and this factor is also related to that required for adsorption of T7.

Seven other T4-resistant strains in the nr group were apparently not LPS-defective. Three of these strains did not adsorb T4. These three strains (T481, T4121, and T4131) seem to be defective in some part of the T4 receptor other than the LPS backbone, but the nature of their defect is unknown. These mutants were all sensitive to T3 and, therefore, the structure which is defective is not necessary for the adsorption of T3. The other four strains which could adsorb T4 (T482, T491, T4111, and T4141) do not seem to have defective receptors and will not be discussed here.

Similarly T3-resistant mutants were formed spontaneously and were investigated. Among 42 T3-resistant mutants isolated, none was novobiocin-supersensitive, as could be expected from the previous finding that NS mutants were all T3-sensitive. Among 42 T3-resistant mutants, 21 strains were resistant to both T4 and T7, 18 strains were resistant to T4 but sensitive to T7, and 3 strains were sensitive to both T4 and T7.

The mode of interaction of the cell surface with phages seems to be complex, and it may be worth mentioning that Watanabe (*personal communication*) has shown in the rough strains of *S. typhimurium* that T4 phage was not adsorbed onto the cells of mutants at any stage of deletion of LPS, although T3 and T7 were adsorbed.

As illustrated in Fig. 3, the locus on the chromosome of the linked resistance genes to T3, T4, T7, and λ in *E. coli* K-12 was previously mapped by Curtiss at 9 min (*tfrA;* reference 3). His paper

TABLE 4. *Nature of T4 phage-resistant mutants*

Strain	Novobiocin sensitivity	LPS		T4-phage adsorption	Sensitivity to phage	
		Heptose	Phosphate		T3	T7
Parent JE1011	nr	+	+	+	s	s
T411, T422, T471	ns	−	−	−	s	r
T431	ns	+	−	−	s	s
T413, T421, T442	ns	−	−	−	r	r
T452, T472	ns	+	−	−	r	r
T481	nr	+	+	−	s	r
T4121, T4131	nr	+	+	−	s	s
T482	nr	+	+	+	r	r
T491, T4111, T4141	nr	+	+	+	r	s

did not show whether this gene was related to the receptor or to some mechanism after phage adsorption. As summarized in Fig. 3, the T4 receptor genes were present in at least two loci of NS14 (*lpcA*) and of NS7 and other NS mutants (*lpcB*). Their loci will increase in number on further mapping of the genes of NS strains and T4-resistant strains.

ACKNOWLEDGMENTS

We are very much indebted to Yoshinobu Sugino of Osaka University, Faculty of Science, for providing strains of *E. coli* from his stock and for advice on genetic methods. We also thank O. Westphal and S. Schlecht of Max Planck Institut für Immunbiologie, Freiburg, Germany, for telling us of their unpublished results and T. Yamakawa and N. Ueda of University of Tokyo, Medical School, for advice on sugar analysis. One of us, T. S., is very grateful to Bunji Maruo and Hiroshi Matsuzawa of Institute of Applied Microbiology, University of Tokyo, for their help in starting this work during his undergraduate course.

LITERATURE CITED

1. Adelberg, E. A., M. Mandel, and G. C. C. Chen. 1965. Optical conditions for mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in *Escherichia coli* K-12. Biochem. Biophys. Res. Commun. **18**:788–795.
2. Anderson, J. S., M. Matsuhashi, M. A. Haskin, and J. L. Strominger. 1967. Biosynthesis of the peptidoglycan of bacterial cell walls. II. Phospholipid carriers in the reaction sequences. J. Biol. Chem. **242**:3180–3190.
3. Curtiss, R., III. 1965. Chromosomal aberrations associated with mutations to bacteriophage resistance in *Escherichia coli*. J. Bacteriol. **89**:28–40.
4. Dröge, W., O. Lüderitz, and O. Westphal. 1968. Biochemical studies on lipopolysaccharides of *Salmonella* R mutants. 3. The linkage of the heptose units. Eur. J. Biochem. **4**:126–133.
5. Dröge, W., H. Ruschmann, O. Lüderitz, and O. Westphal. 1968. Biochemical studies on lipopolysaccharides of *Salmonella* R mutants. 4. Phosphate groups linked to heptose units and their absence in some R lipopolysaccharides. Eur. J. Biochem. **4**:134–138.
6. Gmeiner, J., O. Lüderitz, and O. Westphal. 1969. Biochemical studies on lipopolysaccharides of *Salmonella* R mutants. 6. Investigations on the structure of the lipid A component. Eur. J. Biochem. **7**:370–379.
7. Hurwitz, J., J. J. Furth, M. Malamy, and M. Alexander. 1962. The role of deoxyribonucleic acid in ribonucleic acid synthesis. III. The inhibition of the enzymatic synthesis of ribonucleic acid and deoxyribonucleic acid by actinomycin D and proflavin. Proc. Nat. Acad. Sci. U.S.A. **48**:1222–1230.
8. Izaki, K., M. Matsuhashi, and J. L. Strominger. 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. XIII. Peptidoglycan transpeptidase and D-alanine carboxypeptidase: penicillin sensitive enzymatic reactions in strains of *Escherichia coli*. J. Biol. Chem. **243**:3180–3192.
9. Kuriki, Y., and K. Kurahashi. 1965. Phosphomannoheptose isolated from cell wall of uridine diphosphate glucose pyrophosphorylase-less mutant of *Escherichia coli* K-12. J. Biochem. (Tokyo) **58**:308–311.
10. Lederberg, J. 1950. Isolation and characterization of biochemical mutants of bacteria. Methods Med. Res. **3**:5–22.
11. Lowry, O. H., N. R. Roberts, K. Y. Leiner, M. Wu, and A. L. Farr. 1954. The quantitative histochemistry of brain. I Chemical methods. J. Biol. Chem. **207**:1–17.
12. Mäkelä, P. H., and B. A. D. Stocker. 1969. Genetics of polysaccharide biosynthesis. Annu. Rev. Genet. **3**:291–322.
13. Matsuhashi, M., I. Ohara, and Y. Yoshiyama. 1970. Inhibition of bacterial cell wall synthesis in vitro by enduracidin, a new polypeptide antibiotic, p. 226–229. Progress in antimicrobial and anticancer chemotherapy (Proc. 6th Int. Congr. Chemother., vol. 1. University of Tokyo Press, Tokyo.
14. Mühlradt, P., H. J. Risse, O. Lüderitz, and O. Westphal. 1968. Biochemical studies on lipopolysaccharides of *Salmonella* R mutants. 5. Evidence for a phosphorylation of *Escherichia coli* with anomalous cell division and abilchem. **4**:139–145.
15. Normark, S. 1969. Mutation in *Escherichia coli* K-12 mediating spherelike envelopes and changed tolerance to ultraviolet irradiation and some antibiotics. J. Bacteriol. **98**: 1274–1277.
16. Normark, S., H. G. Boman, and E. Matsson. 1969. Mutant of *Escherichia coli* with anomalous cell division and ability to decrease episomally and chromosomally mediated resistance to ampicillin and several other antibiotics. J. Bacteriol. **97**:1334–1342.
17. Osborn, M. J. 1966. Preparation of lipopolysaccharide from mutant strains of *Salmonella*, p. 161–164. *In* V. Ginsburg and E. Neufeld (ed.), Methods in enzymology, vol. 8. Academic Press Inc., New York.
18. Osborn, M. J. 1963. Studies of the gram-negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of *Salmonella typhimurium*. Proc. Nat. Acad. Sci. U.S.A. **50**:499–506.
19. Risse, H. J., W. Dröge, E. Ruschmann, O. Lüderitz, and O. Westphal. 1967. Eine neue Gruppe von *Salmonella* R-Mutanten. Serologische und biochemische Analyse des Heptosekerns von Lipopolysacchariden aus *S. minnesota*- und *S. ruiru* Mutanten. Eur. J. Biochem. **1**:216–232.
20. Schlecht, S., und O. Westphal. 1968. Antibiotica-Empfindlichkeit bei S- und R-Formen von *Solmonella minnesota*. Naturwissenschaften **10**:494–495.
21. Schlecht, S., und O. Westphal. 1970. Untersuchungen zur Typisierung von *Salmonella*-R-Formen IV. Typisierung von *S. minnesota*-R-Mutanten mittels Antibiotica. Zentrabl. Bacteriol. Parasitenk. Infektionskr. Hyg. Abt. 1 Orig. **213**:356–381.
22. Siewert, G., and J. L. Strominger. 1967. An inhibitor of the dephosphorylation of lipid pyrophosphate, an intermediate in the biosynthesis of the peptidoglycan of bacterial cell walls. Proc. Nat. Acad. Sci. U.S.A. **57**:767–773.
23. Sugino, Y. 1966. Mutants of *Escherichia coli* sensitive to methylene blue and acridines. Genet. Res. **7**:1–11.
24. Tamaki, S., T. Sato, Y. Sugino, H. Matsuzawa, and M. Matsuhashi. 1970. Studies on the mutants of *Escherichia coli* sensitive to antibiotics. p. 405–409. Progress in Antimicrobial and Anticancer Chemotherapy (Proc. 6th Int. Congr. Chemother.) vol. 1. University of Tokyo Press, Tokyo.
25. Taylor, A. L. 1963. Bacteriophage-induced mutation in *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **50**:1043–1051.
26. Taylor, A. L. 1970. Current linkage map of *Escherichia coli*. Bacteriol. Rev. **34**:155–175.
27. Taylor, A. L., and M. S. Thoman, 1964. The genetic map of *Escherichia coli* K-12. Genetics. **50**:659–677.
28. Watanabe, T., T. Arai, and T. Hattori. 1970. Effects of cell wall polysaccharide on the mating ability of *Salmonella typhimurium*. Nature (London) **225**:70–71.
29. Weidel, W. 1958. Bacterial viruses (with particular reference to adsorption/penetration). Annu. Rev. Microbiol. **12**:27–48.
30. Weissbach, A., and J. Hurwitz. 1959. The formation of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli* B. Identification. J. Biol. Chem. **234**:705–709.