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Lipopolysaccharide-Deficient, Bacteriophage-Resistant Mutants of *Escherichia coli* K-12

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Bacteriophage-resistant mutants isolated and classified in a previous study were examined for alterations in their lipopolysaccharide (LPS) composition, and properties likely to be affected by alterations in LPS composition were studied. It was found that many of the mutants of the Ktw (K2 resistance), Ttk (T2, T4, or K19 resistance), Bar (bacteriophage), Wrm (wide-range mutants), and miscellaneous resistance groups were altered in their response to a series of antibiotics and to two LPS-specific bacteriophages, C21 and U3. Furthermore, many of the bacteriophages to which these mutants were resistant adsorbed to LPS preparations. By direct sugar analysis of the mutant LPS preparations, it was shown that the mutants fitted into six distinct classes, which are readily derived from an LPS core with a structure resembling that of *Salmonella* or *Escherichia coli* O100. A number of the mutants were shown to map between *pyrE* and *mtl*, which has been previously shown to be the site of a cluster of *rfa* genes in both *Salmonella* and *E. coli*. Outer membrane protein composition was studied in the above mutants using polyacrylamide gel electrophoresis. Some strains were shown to have alterations in the amount of major proteins. The nature of the bacteriophage receptors involved and the alterations leading to resistance are discussed.

The mutation of gram-negative bacteria to bacteriophage resistance often involves a change in some component of the outer membrane, which either is the receptor itself or interacts with it. The components that have been observed to change are either proteinaceous or lipopolysaccharide (LPS) in nature (16). Although it has been shown previously that mutations in *Escherichia coli* K-12 conferring resistance to a bacteriophage with a protein receptor result in alteration or loss of that protein from the outer membrane (5, 25, 30, 37), the nature of mutations conferring resistance to bacteriophages with LPS receptors is less well understood. It is well known for *Salmonella* that there is a correlation between the composition of the LPS and the pattern of sensitivity in certain bacteriophages (44). In *E. coli* a similar correlation between bacteriophage resistance and alterations in LPS composition has been described for bacteriophages U3, C21, P1, T3, T4, T7, ϕ5, ϕW, and ϕ3 (4, 25, 27, 28, 40, 41).

In an earlier paper (12), we described the general pattern of resistance types found when a large collection of bacteriophage-resistant mutant strains was tested against 56 bacteriophages. Study of the resistance patterns of these mutants showed that most fell into 11 distinct groups, several having been previously described, and only five miscellaneous mutants did not fit well into any group. Some of the groups of mutants showed absolute resistance to a specific group of bacteriophages; thus, *tonA*, *tonB*, and *bfe* mutants resist T1- and T5-like, T1-like, and BF23-like bacteriophages only. At the other extreme were groups of mutants which resist to varying degrees several sets of bacteriophages; Wrm (wide-range mutants), Bar (bacteriophage A resistance), Ktw (K2 resistance), and Ttk (T2, T4, and K19 resistance) mutants were of this type. Intermediate groups had both a specific set of resistances to phage K10 (*ktn* mutants), T6 and related phages (*tsx* mutants), K3 and related phages (*con* mutants), or E4 (*efr* mutants) and, in addition, had varying degrees of resistance to phages of other sets.

In this paper we show that some of the bacteriophage-resistant strains described previously have an altered LPS composition. Some of these strains also have an altered outer membrane protein composition. The relationship between the alterations to LPS composition and protein content is discussed, as is the nature of the bacteriophage receptors affected.

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MATERIALS AND METHODS

Media, bacteria, and nomenclature. The media used have been described elsewhere (12). In addition to those strains used previously (12), KL209 (CGSC 4315) (*malB16*, λ^r, λ^-, *sup-53* [unmapped amber suppressor]), HfrJ4 (P10), and KL141 (CGSC 4224) (*thi-1*, *pyrE14*, *argG6*, *thyA25*, *rbs-1*, *malA1*, *strA8* or *strA104*, λ^r, λ^-, *gltS7*) were kindly supplied by B. J. Bachmann. The designation Wrm2 or Bar8, etc., for a strain implies that the strain belongs to subgroup 2 of the Wrm resistance group, or subgroup 8 of the Bar resistance group, etc. (12).

Bacteriophages. Bacteriophages used were as previously described (12), with the addition of C21 and U3, kindly supplied by R. Russell. Bacteriophage C21 was propagated on *E. coli* B, whereas U3 was propagated on *E. coli* K-12 strain AB1133. Bacteriophage P1*kc* was from laboratory stocks.

Extraction of LPS. For neutralization studies, LPS from strain P400 was extracted by the hot phenol-water technique (42). LPS for gas-liquid chromatographic analysis was extracted by the phenol-chloroform-petroleum ether technique (7).

Neutralization studies. Five-tenths milliliter of a given bacteriophage (5×10^5 plaque-forming units per ml) preparation in nutrient broth was added to 0.5 ml of distilled water containing 1 mg, 100 μg, or 10 μg of LPS or to a Triton X-100-insoluble cell wall preparation prepared as described previously (35). The mixtures were then incubated for 3 h at 37 C, diluted 100-fold to stop further reaction, and 0.1 ml was plated with 0.1 ml of an overnight standing culture of strain P400 in a 0.7% agar overlay on nutrient agar, and the plates were incubated overnight at 37 C. For a control 0.5 ml of distilled water was substituted for the LPS or cell wall preparation. A 50% or greater reduction in plaque count relative to the control indicated neutralization.

Gas-liquid chromatography. To 2 mg of LPS in deionized distilled water, 100 μg of arabinose was added as an internal standard. The LPS was then hydrolyzed in a total volume of 2 ml in an ampoule sealed under vacuum. When the LPS of strain P400 was hydrolyzed with 1 N H_2SO_4 for 12 h in a boiling-water bath, neutralized, reduced, acetylated, and run on a gas-liquid chromatograph, the amount of glucose was found to be greater than the amount of heptose (Glu:Gal:Hept = 0.38:0.15:0.30 μM). This was a different result from that found by other workers (6, 19, 28, 38) for *E. coli* K-12. Schmidt et al. (32) showed that when 0.1 N HCl hydrolysis was used, the amount of heptose released from LPS increased with time up to 48 h, at which time it was much greater than the amount released by H_2SO_4 hydrolysis and double the amount released by 0.1 N HCl over 12 h. Jackson and Redmond (15) used an even milder hydrolysis with Dowex 50 (H^+) in 0.01 N HCl for 7 days at 105 C in their studies on the LPS of *Vibrio cholerae*. We decided to use a time of 64 h at 100 C, using 0.1 N HCl as the hydrolyzing agent. This caused the amount of heptose estimated to double compared with that estimated after sulfuric acid hydrolysis. The resultant hydrolysate was then centrifuged ($3000 \times g$, 15 min) to remove lipid; excess acid was neutralized on a column of ion exchange resin, AG1-X2 [$(HCO_3)^-$ form, 100 to 200 mesh, Bio-Rad Laboratories, Richmond, Calif.], and the solution was brought to pH 8.5 with $NaHCO_3$. This mixture of aldoses was reduced by adding 14 mg of sodium borohydride and incubating for 2 h at room temperature. Excess borohydride was destroyed with glacial acetic acid, followed by passage through a column of ion exchange resin, AG50W-X4 (H^+ form, 50 to 100 mesh, Bio-Rad Laboratories). The aldoses thus obtained were converted to their alditol acetates by the technique of Holme et al. (14). These were then dried down and dissolved in a small amount of chloroform and injected into a column of 3% ECNSSM (Applied Science Laboratories Inc., State College, Pa.) on GasChrom Q (100 to 120 mesh, Applied Science Laboratories) in a Varian aerograph series 1700 gas chromatograph. The column temperature was 200 C as suggested by Eriksson-Grennberg et al. (6). As a control, arabinose was added to an equal amount of glucose and subjected to the above procedures, including hydrolysis. LPS from strain P400 or strain AB1133 was included as an additional control in each series of analyses.

When the heptose from *E. coli*, which has been shown to be L-glycero-D-mannoheptose, is reduced and acetylated, it forms a peak X, which runs just after glucose on a gas-liquid chromatograph (32). Under our preparation procedures perseitol and L-glycero-D-mannoheptose form the same heptitol acetate. Equal amounts of perseitol and glucose were run together in a control experiment, and it was found that the area under the glucose peak was equal to the sum of the areas under the heptose and X peaks. Therefore, for each mutant studied we used this sum to represent the total amount of heptose (Table 3).

Glucosamine analysis. "Degraded polysaccharide" was extracted from LPS by treatment with 1% acetic acid (100 C, 2 h), lipid A was removed by centrifugation (20), and the supernatant was further extracted two times with an equal volume of chloroform. All samples were then lyophilized, after which they were hydrolyzed according to the technique of Monner et al. (19). The glucosamine content of the hydrolysate was analyzed according to the technique of Strominger et al. (39).

Antibiotic resistance. Oxoid multodisks 11-14D and 30-9C were used to examine the pattern of resistance or sensitivity to a range of antibiotics, by placing multodisks onto overlays containing 10^7 cells of the strain to be tested. The disks contained chloramphenicol (50 μg), erythromycin (50 μg), tetracycline (50 μg), colistin methane sulfonate (50 μg), kanamycin (5 μg), neomycin (10 μg), and ampicillin (2 μg), to which strain P400 is sensitive; novobiocin (30 μg), to which it is slightly sensitive; and sulphafurazole (500 μg), oleandomycin (10 μg), fusidic acid (10 μg), methicillin (10 μg), cloxacillin (5 μg), and novobiocin (5 μg), to which it is resistant. In the case of erythromycin a decrease or increase in zone size of 50% over the control strain P400 was taken to indicate resistance or sensitivity, respectively. Any lesser change was described as slight (Table 1). All other antibiotics, except for ampicillin, showed absolute changes (i.e., resistance going to sensitivity). Ampicillin resistance was further

TABLE 1. *Alterations in the response to antibiotics, and resistance or sensitivity to bacteriophages C21 and U3*

Phenotype-resistant group	Sub-group	Representative mutants	Response to antibiotics[a]							Bacteriophage resistance or sensitivity[b]	
			NV5	NV30	PN	E	OL	FD	CB[c]	C21	U3
Ton A		P417				SL					
Ton B		P442									
Bfe		P445									
Tsx	1	P407									
	2	P433									
Con		P460									I
Efr		P448									
Ktn		P466									
Misc	1	P491			SL						R
	2	P443									
	3	P498				R				I	P
	4	P237								S	R
	5	P493								S	R
Ktw	1	P456, P457				R					
	2	P476				R					
	3	P240			SL					S	I
Ttk	1	P429								S	R
	2	P423								P	I
	3	P425									R
	4	P474			SL						R
Bar	1	P455		S						P	R
	2	P492	S	S	S	SL		S		S	R
	3	P409, P413		S	S					S	R
		P404	S	S						S	R
		P495		S		S				S	R
		P497	S	S		S				S	R
		P494	S	S		S	S	S		S	R
		P496, P415	S	S		S		SL		S	R
	4	P405	S	S		S	S			S	R
	5	P402		S				SL		S	R
	6	P451	S	S		SL			SL	S	R
	7	P487	S	S	S	S		S		S	R
	8	P489	S	S				SL			R
Wrm	1	P435	S	S		S	S	S			R
		P479	S	S	R	S		S	S		R
	2	P416	SL	S	SL	SL	SL	SL		S	R
		P424		S		SL		SL		S	R
		P239	SL	S		SL		SL		S	R

[a] Only alterations in the mutant response to antibiotics are included. The wild-type (P400) response is full resistance to 5 μg of novobiocin, 10 μg of fusidic acid, 10 μg of methicillin, and 10 μg of oleandomycin, and sensitivity to 30 μg of novobiocin (zone size, <0.5 mm), 2 μg of ampicillin (zone size, 3 mm), and 50 μg of erythromycin (zone size, 1.5 mm). For all other antibiotics tested (see Materials and Methods), the response was identical for all strains. Abbreviations: R, Resistant, i.e., producing a smaller zone of inhibition than strain P400; S, sensitive or supersensitive, i.e., larger zone of inhibition than strain P400; SL, slight increase in sensitivity, with the exception of strain P417, which shows a slight decrease in sensitivity to erythromycin.

[b] All alterations to the wild-type response (being sensitivity to bacteriophage U3 and resistance to C21) are recorded as: R, full resistance; S, full sensitivity; I, inhibition with turbid plaques; and P, partial resistance with efficiency of plating reduced. All terms are defined in reference 12.

[c] Symbols from multodisks: NV5, 5 μg of novobiocin; NV30, 30 μg of novobiocin; PN, ampicillin; E, erythromycin; OL, oleandomycin; FD, fusidic acid; and CB, methicillin.

tested by plating approximately 1,000 cells on nutrient agar plates containing 0.5, 1, 2, 3, 4, 5, 15, 30, or 75 μg of ampicillin per ml (Beecham Research Laboratories, Australia). Strain P400 was found to be resistant to 1.0 μg/ml but sensitive to 2.0 μg/ml.

Genetic analysis. The techniques of conjugation and gradient transfer analysis of mutants were those of de Haan et al. (8). Bacteriophage P1*kc* stocks for transduction were harvested from confluent lysis plates, and transduction was carried out by incubating (37 C, 30 min) bacteriophages (2×10^7/ml) and recipient bacteria (2×10^8/ml) in nutrient broth containing 2 mM Ca^{2+}. Single recombinant colonies were picked off, spread on a nutrient agar plate, and grown overnight at 37 C, and a single colony was then spread with a platinum loop on one-ninth of a dried nutrient agar plate. A drop (0.01 ml) of bacteriophage lysate containing 10^6 or more plaque-forming units was applied to the spread area and, after incubation at 37 C overnight, an area of lysis indicated sensitivity. The resistant mutant used in the study and its parent strain P400 acted as controls. Growth factor requirements of recombinants were scored by replica plating onto suitably supplemented minimal media.

Preparation of membrane samples and polyacrylamide gel electrophoresis. The preparation and sodium dodecyl sulfate (SDS) solubilization of membrane samples and the polyacrylamide gel running techniques are as described previously (34, 37), with the exception that samples prepared by method (iii) were not dialyzed against SDS-urea. Sample preparation method (i) was that of Neville (21) and is essentially solubilization in SDS with mercaptoethanol at 37 C; method (i) samples were run on SDS gels based on the Neville (pH 8.64) discontinuous buffer system. Sample preparation methods (ii) and (iii) were essentially SDS solubilization at 100 and 37 C, respectively; method (ii) samples were run on SDS-urea gels based on the Bragg and Hou (pH 11.4) system (34, 35); and method (iii) samples were run on SDS-urea gels based on the Maizel (pH 7.2) system (34, 35).

RESULTS

Antibiotic resistance and sensitivity. Tamaki et al. (40) have shown that LPS-deficient strains selected as novobiocin supersensitive may be resistant to bacteriophages T4 and T7. Eriksson-Grennberg and co-workers (6) also showed that many of their class II ampicillin-resistant mutants were LPS deficient. We thought some of our bacteriophage-resistant mutants would have altered LPS, and, because of the above correlations between antibiotic sensitivity and LPS composition, we tested a range of our mutants for altered antibiotic sensitivity. Mutants of the Ktw, Ttk, Bar, Wrm, and miscellaneous groups frequently had an altered response to novobiocin, fusidic acid, oleandomycin, ampicillin, and erythromycin (Table 1). However, none of our mutants were resistant to high levels of ampicillin as described previously for other LPS-altered strains (6), and in fact only one strain, P479, was partially resistant to 2 μg of ampicillin per ml.

Alterations in the sensitivity to bacteriophage U3 and resistance to bacteriophage C21. It has been shown that the receptors for bacteriophages C21 (26, 33) and U3 (41) are in the LPS rough core and that mutants in which the composition of the LPS is altered may be changed in their response to these bacteriophages. Our mutants were tested by spotting these bacteriophages onto an agar layer containing the mutants (Table 1). Mutants of the Ktw, Ttk, Bar, Wrm, and miscellaneous groups again frequently had an altered response.

Adsorption of bacteriophages to LPS. Beumer et al. (3) showed that bacteriophages T3, T4, T7, H^+, and V adsorbed to LPS extracted from *E. coli* B by the hot phenol-water technique. We tested 21 bacteriophages for their ability to adsorb to a similar extract from strain P400 and to a Triton X-100-insoluble cell wall preparation (Table 2). The bacteriophages were mostly representatives of those sets of bacteriophages affected by Ktw, Ttk, Bar, Wrm, and miscellaneous mutations, but some others were tested. Bacteriophages H3 (incapable of lysing *tsx* mutants and very similar to bacteriophage T6) and K26 (not distinguishable from bacteriophage T1) were not neutralized by the cell wall preparation, a similar result to that obtained with some other bacteriophages suspected of having LPS receptors. However, they were also not neutralized by the LPS preparation. Of the other bacteriophages failing to absorb to LPS, namely E4, Ox5, K15, and T2, only T2 was appreciably neutralized by a cell wall preparation. Thus, the nature of the receptor for bacteriophages E4, Ox5, and K15 remains obscure, although Ox5 might have as its receptor the protein deleted in *con* mutants (37).

A number of bacteriophages were more efficiently neutralized by LPS than by cell wall preparations. This is partly accountable for by the fact that the cell wall contains only 20% LPS. However, this does not explain the 100-fold difference in neutralization efficiency observed for bacteriophages T3, φII-T, W31, and E11. This effect might be due to other cell wall components limiting, in some way, the interaction of bacteriophage and receptor in the whole cell or cell wall.

Analysis of the rough core sugars of the LPS. Analyses of the neutral sugars of AB1133, P400, and 15 mutant strains were carried out as described in Materials and Methods. The results (Table 3) show heptose, glucose, and ga-

TABLE 2. *Adsorption of bacteriophages to LPS and cell wall preparations*

Bacteriophage	Resistance groups[a]	Adsorption by cell wall (%)[b]			Adsorption by LPS (%)[b]		
		1.0 mg/ml	0.1 mg/ml	0.01 mg/ml	1.0 mg/ml	0.1 mg/ml	0.01 mg/ml
K26	Ton A, Ton B	0	0	0	0	0	0
K10	Ktn, Wrm	0	0	0	55	8	0
H3	Tsx	32	0	0	0	0	0
E4	Efr, Bar, Wrm, Misc	0	0	0	0	0	0
K2	Ktw, Bar, Wrm	0	0	0	65	12	0
K20	Ktw, Bar, Wrm	54	0	0	53	0	0
Ox5	Con, Bar, Wrm, Ttk, Misc	18	0	0	17	0	0
K16	Ttk, Bar, Wrm, Misc	91	60	12	100	100	100
F27	Ttk, Bar, Wrm, Misc	100	92	52	100	100	100
E7	Ttk, Bar, Wrm, Misc	0	0	0	55	12	0
A	Bar, Wrm	0	0	0	51	0	0
T3	Bar, Wrm	10	0	0	100	100	83
T4	Ttk, Bar, Wrm, Misc	100	81	64	95	53	10
K19	Ttk, Bar, Wrm	0	0	0	75	28	0
T2	Ttk, Wrm	100	95	75	10	0	0
T7	Wrm	96	87	80	100	100	100
φI	Wrm	76	21	0	100	95	46
W31	Wrm	65	25	0	99	99	93
E11	Wrm	56	0	0	99	99	86
K15	Wrm	57	18	0	0	0	0
φII-T		70	0	0	100	90	65

[a] See previous paper (reference 12).

[b] Represents the percentage of plaque-forming units (initially 5×10^5/ml) adsorbed by the given concentrations of LPS or cell wall.

TABLE 3. *Analysis of neutral sugars of LPS of strain P400, some of its phage-resistant mutants, and strain AB1133*

Strain	Resistance group[a]	LPS[b]		
		Glucose	Galactose	Heptose
AB1133		0.39	0.24	0.56
P400		0.39	0.26	0.58
P460	Con	0.40	0.19	0.55
P456	Ktw1	0.32	0.15	0.32
P457	Ktw1	0.41	0.14	0.41
P429	Ttk1	0.21	0.18	0.38
P426	Ttk3	0.23	0.19	0.37
P474	Ttk4	0.19	0.18	0.35
P404	Bar3	0.23	0	0.35
P415	Bar3	0.21	0	0.40
P495	Bar3	0.14	0.03	0.31
P405	Bar4	0.04	0.03	0.39
P436	Bar4	0.02	0	0.33
P416	Wrm2	0.02	0.01	0.26
P424	Wrm2	0.09	0	0.27
P489	Bar8	0.02	0.04	0.12
P435	Wrm1	0	0	0

[a] Subgroup of each resistance group is included where appropriate.

[b] Values given as micromolar concentration of sugar per milligram of LPS. All figures given are the average of two or three results, with the exception of P400 (six results) and AB1133 (five results).

lactose as the only neutral sugars in the LPS of strains AB1133 and P400. The absence of rhamnose, found in some other strains of *E. coli* K-12 (6, 19, 22, 28), is explained by the fact that AB1133 is directly derived from strain Y10 (2), which is deficient in the biosynthesis of thymidine diphosphate-L-rhamnose (22). We were also unable to find glucosamine attached to the polysaccharide portion of the LPS of strains P400, P460 (*con*), and P425 (Ttk3), despite the previous finding of Schmidt (31) that his strain of *E. coli* K-12 has a complete core. Glucosamine appears to be an essential part of the completed rough cores of smooth strains of *Salmonella typhimurium* (9, 17, 23, 38) and *E. coli* O100 (10), but not of *E. coli* O71 (20). Rooney and Goldfine (29) have shown that their *E. coli* K-12 strain CR34 has no glucosamine in the polysaccharide portion of its LPS, and it is interesting that both this strain and strain AB1133 are derived from strain Y53 (2). However, whether or not other *E. coli* K-12 strains have glucosamine in their LPS other than that attached to the lipid A portion is uncertain.

It was interesting to note that the *non* mutation (block in capsular polysaccharide synthesis) in strain P400 made no difference to the sugar composition. The *con* strain P460, which

has a major cell wall protein defect (37), also had an unchanged sugar composition.

Strains P456 and P457 from the Ktw resistance group have reduced levels of heptose and galactose. Strains P429, P425, and P474 from the Ttk resistance group are deficient in glucose and heptose, whereas the three Bar3 mutants are additionally deficient in galactose. The other strains tested (P436 and P405 [Bar4], P489 [Bar8], P416 and P424 [Wrm2], and P435 [Wrm1]) had little or no glucose or galactose in their LPS. The Wrm group mutants and P489 (Bar8) also showed lower levels of heptose than other mutants tested, with the Wrm1 mutant, P435, being totally deficient in heptose and in all other neutral sugars. When the other Wrm1 mutant, P479, was tested, it reverted on two separate occasions and showed not only a wild-type bacteriophage sensitivity pattern but a sugar composition similar to strain P400.

Membrane protein composition. The membrane proteins of strain P400 and mutant strains were examined by SDS-polyacrylamide gel electrophoresis. The strains chosen were representative of mutant groups showing LPS defects and also included representatives of those other groups whose membrane proteins have not been reported on previously.

Method (i) preparations of whole and outer membrane of each strain listed in Table 4 were run on Neville system gels. Method (ii) and method (iii) preparations of each strain were run on gels of pH 11.4 and pH 7.2, respectively. These studies indicated that there were no proteins missing from the outer or cytoplasmic membranes of the mutants examined. However, we are unable to make a firm conclusion regarding this, as some of the cell envelope proteins may be either masked by other proteins of similar electrophoretic mobility or present in undetectable amounts in both parent and mutant strains.

Several of the strains appeared to have a reduced amount of protein 1 (in the Schnaitman nomenclature [35, 36]), but only in the case of strains P456, P495, and P489 was the reduction sufficient to be clearly outside the experimental variation of duplicate membrane preparations. In each case the level of protein 1 was reduced to 20 to 70% of the control level (Fig. 1 and 2; Table 4). In some experiments the level of protein 3 was reduced in strain P489.

Strain P424 as originally isolated had greatly reduced amounts of proteins 1 and 3. However, in later experiments this result could not be repeated, and it was also noted that the strain had become sensitive to bacteriophages K20 and K21, although the sugar composition of the LPS was still defective.

TABLE 4. *Alterations in the levels of the major proteins of E. coli K-12 in the outer membranes of P400 and some of its phage-resistant derivatives*

Strain	Resistance phenotype	Levels of[a]		Protein in outer membrane (%)[b]
		Protein 1	Protein 3	
P400		100	100	72
P448	Efr	100	100	
P466	Ktn	100	100	
P493	Misc5	100	100	
P456	Ktw1	50-70	100	66
P476	Ktw2	100	100	
P240	Ktw3	100	100	
P429	Ttk1	100	100	63
P423	Ttk2	100	100	74
P425	Ttk3	100	100	70
P474	Ttk4	100	100	62
P495	Bar3	50-70	100	67
P405	Bar4	100	100	84
P436	Bar4	100	100	
P489	Bar8	20	50-100	77
P435	Wrm1	100	100	68
P424	Wrm2	50-100	100	63

[a] The major proteins are labeled 1 and 3, in accordance with the nomenclature of Schnaitman (36) for Bragg-Hou (pH 11.4) gels. Results are expressed as the percentage of the peak remaining in the given membrane preparation when compared with the control (P400), which is taken to have 100% of the level of each protein.

[b] Calculated as a percentage after dividing the amount of protein in the outer membrane preparation (estimated as described previously [37]) by the total amount of material in the preparation (estimated by dry-weight measurements).

The protein composition of strain P435 was normal (Fig. 2), yet LPS from the same preparation was totally deficient in heptose, glucose, and galactose.

Genetic analysis. When strain P404 was mated with various Hfr strains, it was found that only KL209 (Hfr J4, point of origin 81 min, anticlockwise transfer) gave appreciable transfer of sensitivity to bacteriophage A. A de Haan gradient transfer analysis (8) was performed by mating strains CGSC 4315 (*malB argE*$^+$/Hfr J4) and P404 (*malB*$^+$ *argE*/F$^-$) using *argE* as the selective marker and *malB* as the counter-selection against the donor. Recombinants (200) were tested for sensitivity to streptomycin, fermentation of mannitol and xylose, and bacteriophage sensitivity. By plotting $\log_{10}$ of the number of recombinants against the *E. coli* K-12 genetic map position, the bacteriophage resistance gene was shown to be at approximately 72 min (Fig. 3). By P1*kc* transduction into strain CGSC 4224, it was shown that bacteriophage A resistance was 55% cotransducible with *pyrE*$^+$, which was itself 20% cotransduci-

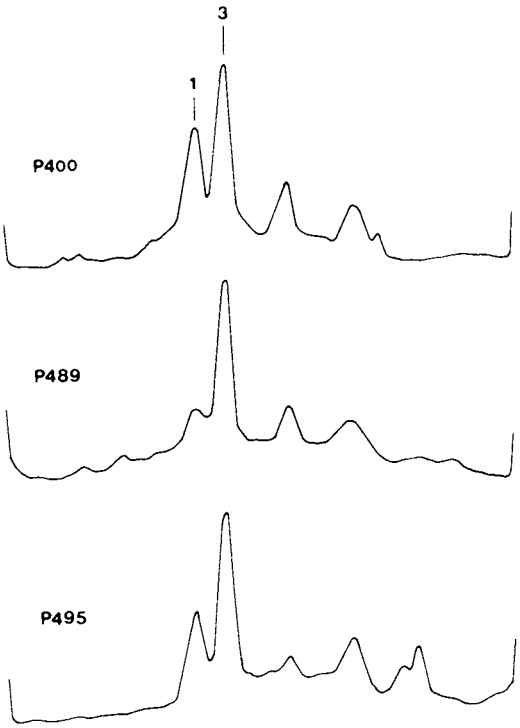

FIG. 1. *Comparison by densitometry of stained bands of the outer membrane proteins of strain P400 and two of its bacteriophage-resistant mutants, P489(Bar8) and P495(Bar3). The technique used was the Schnaitman modification of the Bragg-Hou system (35), and the peak nomenclature is as described in reference 35.*

ble with *mtl*, and that the bacteriophage resistance gene lay between these two markers (Table 5). Thus, using Wu's (45) relationship between map distance and cotransduction frequency, we can place this bacteriophage resistance gene at approximately 71.8 min. Similar but smaller-scale conjugation or transduction experiments indicated that the bacteriophage-resistant strains P455, P492, P413, P490, P405, P402, P451, P487, P489, P416, and P239 all had mutations in this region of the chromosome. These mutants represented all of the subgroups of the Bar group and subgroup 2 of the Wrm group, and it is likely that they are part of an *rfa* gene cluster. We tested strain P479, a Wrm1 mutant, and also representative mutants from the Ttk (P423, P474), Ktw (P240, P456, P458, P476, P477), miscellaneous (P237), and Efr (P448) groups and were able to show that they did not map in this region of the chromosome.

DISCUSSION

Previous studies on the LPS of *E. coli* K-12 have differed in the estimated amounts of some of the sugars, in particular that of heptose (4, 6, 19, 28, 29, 40). We have adopted the improved techniques developed by Schmidt, Fromme, and Mayer (32) for good release of heptose and for inclusion of heptose-degradation products and obtained a value for strain P400 of 58 μmol of LPS per ml, which is higher than some of the earlier reported values.

We find the glucose level to be almost identical to those previously reported, but the galactose level is slightly higher than most previously reported values (6, 19, 28, 40). The molar ratio we obtain for our parent strain P400 approximates for heptose-glucose-galactose a ratio of 3:2:1.3 (wt/wt/wt). Six to seven neutral sugars is a common value for an LPS core, making this the likely molar composition.

The fractional value for galactose may be due

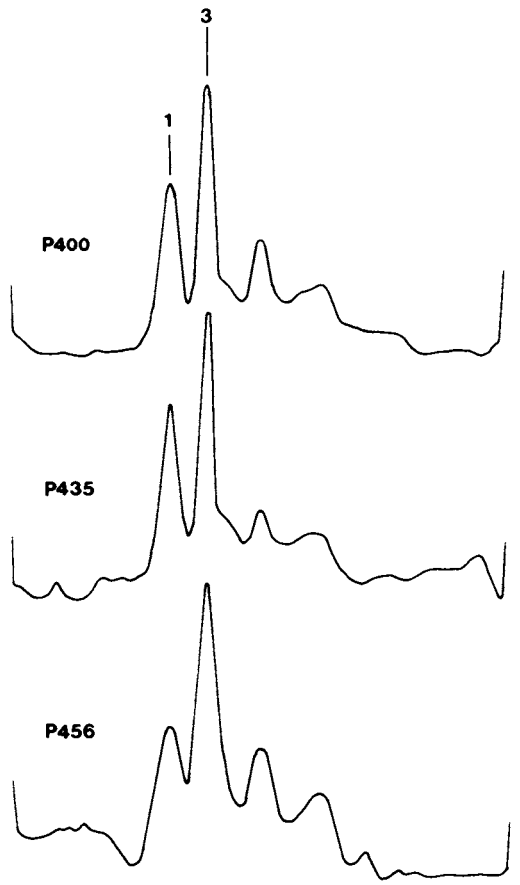

FIG. 2. *Comparison by densitometry of stained bands of the outer membrane proteins of strain P400 and two of its bacteriophage-resistant derivatives, P435(Wrm1) and P456(Ktw1). The technique used was the Schnaitman modification of the Bragg-Hou system (35), and the peak nomenclature is as described in reference 35.*

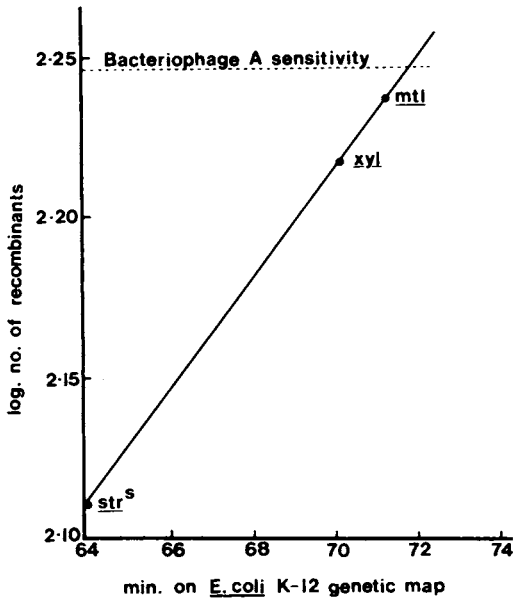

FIG. 3. *de Haan gradient transfer analysis of conjugation between strain P404 (bacteriophage A^r, argE mtl xyl malB$^+$ str^r/F$^-$) and strain KL209 (bacteriophage A^s, argE$^+$, mtl$^+$ xyl$^+$ malB str^s/Hfr J4), with analysis of malB$^+$ argE$^+$ recombinants. Results indicate that the bacteriophage resistance gene maps at approximately 72 min.*

to incomplete substitution of the main chain by a side chain galactose, as proposed previously for *Salmonella* (9, 13). In this paper we studied representative strains of the various groups of bacteriophage-resistant mutants identified in an earlier study (12). Antibiotic sensitivity and the sensitivity patterns to bacteriophages C21 and U3 suggested that many of the mutants had altered LPS, and this was confirmed by direct study of the sugars of the LPS core. There have been extensive studies of similar mutants of *Salmonella*, which show that individual mutations affect the specific transfer ases involved in the sequential addition of sugars to the growing core (17, 43). *E. coli* is very closely related to *Salmonella*, and one would expect a similar situation to occur.

The compositions of the LPS of our mutants are compatible with the hypothesis that *E. coli* K-12 has an LPS core structure similar to that of *Salmonella* or *E. coli* O100, although lacking glucosamine at least in strain P400, and that as for *Salmonella* the various mutations block the biosynthesis of this structure at specific points. Figure 4 shows how specific blocks could account for the sugar compositions found (Table 3). Although other structures for the core would be compatible with the data, it seems probable that the *E. coli* K-12 LPS core closely resembles that of *Salmonella* and *E. coli* O100. However, our studies allow no conclusions regarding the sugar linkages involved. Evidence that the defect for strain P435 does not extend into the 2-keto-3-deoxyoctonic acid region of the LPS is provided by the fact that both the 2-keto-3-deoxyoctonic acid/protein ratios (unpublished data) and the patterns and amounts of proteins (Table 4; Fig. 2) in the outer membranes of strains P435 and P400 are quite similar.

The low heptose levels in Ktw, Bar3, Bar4, and Ttk mutants resemble the situation recently reported for *Salmonella* (9), where only the strains with more complete structure have the full side chain heptose substitution, and other studies have reported reduced heptose levels in mutants retaining other neutral sugars (6, 33).

Some of the LPS-altered mutant strains also had reduced amounts of protein 1 in their outer membrane. However, one of our mutant strains, P435 (Wrm1), which has no detectable heptose, glucose, or galactose in its LPS core, has a normal outer membrane protein composition. We conclude that LPS core is not required for the presence of major outer membrane proteins in normal amounts, as had previously been suggested (1). It should be noted that the P435 mutation is the only one of the Bar and Wrm mutations studied that did not map in the *rfa* region.

Since *E. coli* and *Salmonella* genes for LPS synthesis commonly map in the same region of the chromosome (6, 31, 33, 38, 44), it is likely that the Bar 1 to 8 and Wrm2 mutations, mapping near *pyrE*, resemble the *rfa* mutations of *Salmonella* and affect specific transferases (38, 43, 44). It appears then that absence of some of the transferases can, directly or indirectly, affect the outer membrane protein composition.

Strains with defective LPS are also altered in their sensitivity to certain antibiotics (compare Tables 1 and 3), and increased sensitivity to novobiocin may be a particularly good indicator

TABLE 5. *Analysis of three-factor transduction involving the pyrE and mtl markers and the gene coding for the Bar3 phenotype in strain P404*

Marker[a]			No. of transductants
pyrE	Bacteriophage A resistance	*mtl*	
+	+		51
+	+	+	29
+			65
+		+	0

[a] Selection for *pyrE*$^+$ using donor P404 and recipient KL141. Symbol: + represents the donor characteristic, which was *pyrE*$^+$, A^r, and *mtl*$^-$.

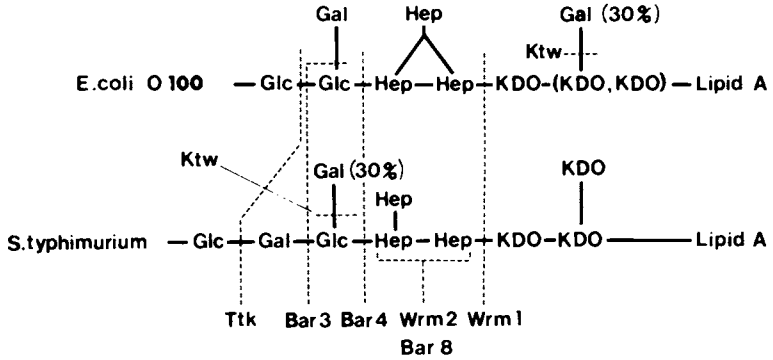

FIG. 4. *Visualization of the stages at which the LPS-deficient, bacteriophage-resistant mutants of E. coli K-12 are altered. The LPS models used are the partial rough core structures of E. coli O100 (10) and S. typhimurium (9). Phosphate and ethanolamine bound to the rough cores have been omitted. Abbreviations: KDO, 2-keto-3-deoxyoctonate; Hep, L-glycero-D-mannoheptose; Glc, glucose; and Gal, galactose.*

of alterations in LPS composition (40). Presumably the altered sensitivity reflects altered permeability of the outer membrane, but our data do not allow a simple orering in terms of increasing permeability. The mutant strains which have altered LPS are all in the Ttk, Bar, Wrm, Ktw, and miscellaneous groups of bacteriophage-resistant mutants. The bacteriophages which, in general, have reduced the plaque-forming ability only among these mutant types (bacteriophages with activity spectra of types 4 or 5 [12]) can often be shown to adsorb to LPS (Table 2). However, bacteriophage K15, with a similar type of activity spectrum, was not neutralized by LPS but was neutralized by cell walls, leaving the nature of its receptor in doubt (see below). The neutralization of bacteriophage K10 by LPS but not by cell wall was unexpected, as it has an activity spectrum of type 3, and other bacteriophages with this type of activity spectrum have protein receptors (12).

One might expect each bacteriophage to require a specific sugar or linkage as a receptor, and that mutations lacking this part of the LPS core would be resistant. However, comparison of resistance patterns with the LPS composition of the strains does not suggest any simple relationship. Often a mutation giving a relatively small change in the LPS composition of a strain will lead to resistance to a given bacteriophage, whereas larger changes do not affect sensitivity and very large changes lead again to resistance. Examples include bacteriophage C21 as described previously (33) for *E. coli* O8 and bacteriophages T4 (40; Table 3), K2, K20, K21, and K29 for *E. coli* K-12 (see Results). Other examples can be seen by comparing data described earlier (12) and Fig. 3. Thus it appears that for many bacteriophages the receptor requirements are complex. It seems likely to us that the conformation of the LPS (which would be altered by loss of one or more sugars) has an important determinative role in its ability to act as a receptor and, thus, in whether a strain will be resistant to a given bacteriophage.

Furthermore, mutants with similar but not identical resistance patterns (e.g., strains P456 and P457, P425 and P474, P415 and P495, P405 and P436, and P416 and P424 [12; Table 3]) often have similar amounts of core sugars. The variation in the resistance patterns of individual mutants is too great to be accounted for simply by the small number of possible defects in LPS biosynthesis indicated in Fig. 3 and 4, and there must be some method of introducing microheterogeneity. Hämmerling et al. (9) have suggested that their own work and that of Hellerqvist and Lindberg (13) provide evidence for considerable heterogeneity of the basal core structure of lipopolysaccharides within a strain, and this may result in the heterogeneity noticed in bacteriophage resistance patterns (12).

We are approaching a situation where we can at least partially define the receptors for the 56 bacteriophages used in this study. Thus bacteriophages with similar resistance patterns to T6, T5, BF23, K10, and K3 (12) bind to a protein receptor that is inactive or missing in *tsx* (manuscript in preparation), *tonA* (5), *bfe* (30), *lamB* (18) and *con* (37) mutants, respectively. T1-like bacteriophages bind reversibly to *tonA*-protein and subsequently require energy from the energized membrane state for irreversible adsorption (11). The remaining bacteriophages, with the possible exceptions of T2, E4, and K15, seem to use specific parts and possibly specific conformations of the LPS as their receptors.

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