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Use of Polymyxin B, Levallorphan, and Tetracaine to Isolate Novel Envelope Mutants of *Escherichia coli*

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Mutants of *Escherichia coli* were isolated by their resistance to the bacteriocidal effects of the membrane-active drugs polymyxin B, levallorphan, and tetracaine. The mutants were examined for additional changes in cellular physiology evoked by the lesions; many polymyxin-resistant strains had a concomitant increased sensitivity to anionic detergents, and several strains of each type had concomitant alterations in generation time and morphology. Mutants of each class (polymyxin resistant, tetracaine resistant, and levallorphan resistant) were transduced into recipient strains. The levallorphan resistance site (*lev*) was located at approximately 9 min on the *E. coli* chromosome. Polymyxin (*pmx*) and tetracaine (*tec*) resistance loci were also transduced. The *lev* and *tec* strains had a slight prolongation of generation time, in contrast with their isogenic wild-type strains. The *tec* transductant produced long filaments in the absence of tetracaine and had an altered colonial morphology; it reverted at high frequency, with the morphological abnormalities reverting along with the tetracaine resistance. The *pmx* transductant had an increased sensitivity to levallorphan and to anionic detergents. In contrast, both *lev* and *tec* mutants were more resistant to acriflavine than was the wild type or the *pmx* transductant. The *pmx*, *lev*, and *tec* loci differed in sensitivity to mitomycin C; the *lev* strain was more resistant, the *tec* strain was more sensitive, and the *pmx* strain was much more sensitive than the wild type. There was no difference in sensitivity to several other dyes and detergents, colicins, or T bacteriophage between the transductant and isogenic wild-type strains. Thus, *lev*, *tec*, and *pmx* loci confer more subtle alterations in the permeability barrier than do lipopolysaccharide-deficient mutants previously studied.

The envelope of *Escherichia coli* has many roles in cellular physiology, including electron transport (8), oxidative phosphorylation (8), metabolite transport (3), and the biosynthesis of cell surface components (6). It also provides osmotic stability (16), maintains the cell shape (18), and acts as a diffusion barrier (34). Additionally, the cell surface may play a role in deoxyribonucleic acid replication and cell division (23, 40).

A number of cell envelope mutants have been studied, many of which exhibit pleiotropic effects (6). These mutants were found by quite different selection techniques, including sensitivity to lysis at elevated temperature (2, 29), tolerance to colicins (33), increased sensitivity to antibiotics and dyes (10, 11, 35, 43), increased substrate permeability (22), and leakage of periplasmic proteins (26). Several temperature-sensitive *E. coli* cell division mutants had associated membrane defects, including an increased permeability to basic dyes and antibiotics (2). Mutants affected in DNA replication have alterations in membrane protein composition (40). Many colicin-tolerant mutants are sensitive to detergents (9, 33) and have altered metabolite transport properties (33). Mutants with defective lipopolysaccharide (LPS) structure are sensitive to many antibiotics, dyes, and detergents (11, 32, 46), supporting the role of LPS as a surface barrier (24).

The MAD mutant, selected for an increase in permeability to lactose, was found to have an increased sensitivity to detergents and antibiotics, as well as grossly distorted morphology and abnormal cell division (22). This mutant was suppressible; all of its phenotypic properties were reversed upon growth in D-alanine, a constituent of the peptidoglycan layer. Mutants that are resistant to the bacteriocidal effects of ethanol (15) have an abnormal morphology and an increased permeability to lactose. With the exception of the LPS-deficient mutants, the biochemical basis for the pleiotropic phenotypes of mutants with surface alterations has not been found (11, 46).

To explore the relationship between membrane structure and function, we have used some membrane-perturbing agents to isolate resistant mutants with envelope abnormalities. Polymyxin B is a cationic cyclic polypeptide antibiotic that is bacteriocidal for gram-negative cells. Extensive studies on the effects of this compound have shown that it destroys the permeability barrier of *E. coli* cells (13, 37), leads to the release of periplasmic proteins (7), alters membrane morphology (21), and prevents adsorption of phages T3, T4, and T7 (which use LPS as receptors; 20). *E. coli* LPS is broken down into short pieces when exposed to polymyxin (27).

Levallorphan (1,3-hydroxy-*N*-allyl-morphinan), a morphine antagonist, is bacteriocidal for *E. coli* and lyses *E. coli* spheroplasts (4). The agonist levorphanol, which is closely related to levallorphan, leads to a change in the phospholipid composition of *E. coli*, with an increased turnover of phosphatidyl glycerol and accumulation of cardiolipin (48). Tetracaine is a local anesthetic and as such can block nerve transmission (44), alter transport properties of mitochondrial membranes (19), and change the osmotic fragility of erythrocytes (39). Since these agents are bacteriocidal, it should be possible to select resistant mutants and to determine the biochemical changes that lead to the resistance. This manuscript presents the isolation and partial characterization of such mutants, as well as the production of isogenic strains containing the mutational loci.

MATERIALS AND METHODS

Bacterial and bacteriophage strains. Table 1 contains a list of the *E. coli* K-12 strains used in this study. Strain CR34 was the strain used for mutant isolation. Strains DB98 and DB170 were the gift of J. Gallant. The colicinogenic strains used were the gift of P. Fredericq and included CA31(A), R21/Vλ(B), K53(E_1), CA42(E_2), 185M4(E_3)b/23(E_3), CA46(G), CA58(H), CA53(I_a), MR2(I_b), K49(K), 32T19/VT4(M), P15(S4), 1.7a(V), 1855II/S7a(X), TAM588/T4(B+M), and CA23(B+X) as classified by Fredericq (14). Phage P1*vir* was the gift of J. Gallant. T phages were a gift of A. Doermann.

Media and growth of bacteria. The rich medium routinely used was Difco nutrient agar (1.5%). Special nutrient agars and broths were used for the production of phage lysates and phage sensitivity studies. P1 lysates were prepared in YT broth (8 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter), YT plate agar (YT + 1.5% agar [Difco]), and YT top agar (YT + 0.5% agar [Difco]). Phage sensitivity studies were done by using Hershey broth (8 g of nutrient broth, 5 g of NaCl, 5 g of peptone, and 1 g of glucose per liter), T5 plate agar (12 g of agar [Difco], 13 g of tryptone [Difco], 8 g of NaCl, and 1.3 g of glucose per liter), and T5 top agar (6 g of agar [Difco], 10 g of tryptone [Difco], 8 g of NaCl, and 3 g of glucose per liter). $CaCl_2$ was added to the broths and agar at 1 mM for T phage infection and 5 mM for P1 infection. Colicinogenic strains were grown on nutrient agar containing 0.5% NaCl.

The supplemented minimal salts (SMS63) medium used was $63B_1$ (31) supplemented with 4 mg of glucose per ml, required amino acids at 100 μg/ml, and thymine and adenine at 40 μg/ml, adjusted to pH 7.1. Casamino Acids (0.2%) were also used to supplement $63B_1$ where noted. SMS63 agar was prepared from SMS63 media plus 1.5% Noble agar (Difco). Tris(hydroxymethyl)aminomethane (Tris) minimal agar (pH 7.4) was used with necessary nutrient supplements to test for the presence of the *phoR*$^-$ marker. Colonies possessing this marker growing on Tris minimal agar give a yellow color when sprayed with *o*-nitrophenylphosphate in 0.1 M Tris, pH 8.0. Tris minimal medium (SMST) was also used in transduction (see "P1 transduction," below). Agar plates containing the selective agents were prepared by using SMS63 agar cooled to approximately 50 to 60°C before mixing with the selective

TABLE 1. *List of E. coli K-12 strains used*[a]

Strain	Genotype	Other important phenotypes[b]	Source
CR34	*thr leu thyA thi lacY tonA dra* λ$^-$		Pasteur Institute
3M30P3	*thr leu thyA thi lacY tonA dra* λ$^-$	PxR[a]	This paper
SM4	*thr leu thyA thi lacY tonA dra* λ$^-$	PxR	This paper
1B30P7	*thr leu thyA thi lacY tonA dra* λ$^-$	PxR	This paper
43L303	*thr leu thyA thi lacY tonA dra* λ$^-$	LvR[b]	This paper
13T301	*thr leu thyA thi lacY tonA dra* λ$^-$	TcR[b]	This paper
34T401	*thr leu thyA thi lacY tonA dra* λ$^-$	TcR	This paper
DB170	*proC purE thy lac phoR*		J. Gallant
P335	*proC purE thy lac phoR pmx*	PxR	This paper
L435	*purE thy lac lev*	LvR	This paper
T135	*proC purE thy lac phoR tec*	TcR	This paper
DB98	*proC purE*$^{+rev}$ *thy lac phoR*		J. Gallant

[a] All strains were F$^-$.

[b] PxR, Polymyxin resistant; LvR, levallorphan resistant; TcR, tetracaine resistant.

agent. Thick plates (40 to 50 ml of agar) containing selective agents were poured 1 day in advance, since the agar plates dry out with age, thus changing the concentrations of the critical agents. Liquid cultures were grown with vigorous agitation, with growth monitored as the increase in turbidity at 600 nm, using a Bausch & Lomb Spectronic 88 (1-cm path length).

Chemicals. Polymyxin B sulfate (8,453 U/mg), mitomycin C, acridine orange, methylene blue, cetylpyridinium chloride, cholic acid, and sodium deoxycholate (DOC) were obtained from Sigma Chemical Co., St. Louis, Mo. Tetracaine hydrochloride was the gift of F. Nachod of Sterling-Winthrop Research Institute, Rensselaer, N.Y. Levallorphan tartrate was obtained as a gift from W. E. Scott of Hoffman-LaRoche, Nutley, N.J. Ethyl methane sulfonate was purchased from Eastman Kodak, Inc., Rochester, N.Y. Sodium dodecyl sulfate (SDS) and gentian violet were obtained from Matheson, Coleman and Bell, Los Angeles, Calif. Methyl green, toluidine blue, and eosin Y were obtained from Allied Chemical Co., Morristown, N.J.; Triton X-100 was from Packard Instrument Co., Downers Grove, Ill.; and acriflavine was from Nutritional Biochemicals, Cleveland, Ohio.

Stock solutions of polymyxin were prepared daily in distilled water. Stock solutions of levallorphan and tetracaine were prepared weekly in distilled water and stored at 5°C. Other stock solutions were also prepared in distilled water. All stock solutions, except detergents, were sterilized by membrane (Millipore Corp.) filtration; detergents were sterilized by autoclaving.

P1 transduction. Transducing phage were harvested from the donor strain infected with P1*vir* by treatment with several drops of chloroform at room temperature. This lysate was centrifuged at 12,000 $\times$ *g* for 5 min at 5°C. The supernatant of the first centrifugation was recentrifuged, and the final supernatant was used as the phage preparation. It was stored over chloroform at 5°C in a tightly capped tube. For transduction, P1 phage were added to a culture of the recipient suspended in SMS63 and, after 20 min at 37°C, the cells were diluted 25-fold in SMS containing 10 mM citrate. The cell suspension was centrifuged, and the pellet was resuspended in a small volume of SMS63. One-third of the cell suspension was plated directly onto selective medium, and the remainder was diluted 25-fold with SMS63 for overnight growth, after which aliquots were plated on selective medium. Colonies appearing on the selection plates were purified on nutrient agar and then on selective agar. As controls, the phage preparation alone and the uninfected recipient culture were always plated. Cells infected with transducing particles grown on one donor strain were plated on a selective agent to which the donor strain was not resistant, as an additional confirmation that the specific marker was uniquely responsible for the increased number of colonies found on plates after transduction.

Replica plating to determine cross-resistance and detergent sensitivity. Small patches of the strains to be tested were grown on SMS63 plates for replica plating. After overnight incubation, this master plate was used for replication onto agar plates containing either selective agents or detergents. These plates were examined daily for 7 days to record growth. The last plate in each series of replications was a nonselective plate to serve as a control.

Test of sensitivity to phages of the T series. Overnight cultures of the various strains were grown in Hershey broth at either 30 or 40°C, concentrated by resuspension in one-fourth volume fresh Hershey broth, and kept on ice until used. Freshly poured plates containing T5 plate agar, 1 mM in Ca^{2+}, were layered with 2.5 ml of T5 top agar, 1 mM in Ca^{2+}, containing 0.5 ml of the concentrated bacterial suspension. Ten-microliter droplets of phage suspension were placed on each plate. Droplets of each of the T phages T_1, T_2, T_3, T_4, T_5, T_6, and T_7, containing 10^1 and 10^4 plaque-forming units, were plated on each strain. After overnight incubation at 30 and 40°C, the size and clarity of each spot was judged.

Colicin sensitivity. The colicinogenic strains were grown for 48 h at 37°C on nutrient agar plates containing 0.5% NaCl to allow colicin production. These plates were treated with chloroform vapor for 15 min. Then the strain to be tested, grown to late log phase at 37°C, was mixed with nutrient soft agar (0.75%) containing 0.5% NaCl and was poured over the colicin-containing plate. Zones of clearing were measured after 24 h at 37°C.

Other procedures. The mutants and transductants were challenged with several agents other than the selective ones, including acridine orange, mitomycin C, methylene blue, gentian violet, SDS, DOC, cholic acid, cetylpyridinium chloride, Triton X-100, acriflavine, methyl green, toluidine blue, and eosin Y. Filter disk and gradient plate methods were used to measure the degree of sensitivity or resistance of the mutants to the agents. The filter disk method involved placing a filter paper disk impregnated with a known amount of one of the agents on a lawn of bacteria freshly spread on a SMS63 agar plate. The diameter of the zone of inhibition was measured after 18 h of growth. The gradient plate method used was a slight modification of the method of Nakamura (35). The base agar used in this study was SMS63, and the rectangular plates used were plastic petri dishes (9 by 9 by 1.3 cm; Kimble no. 52900). The content of agent in the upper layer is specified in each experiment. Each plate was poured to a total of 80 ml of agar (40.0 ml in upper and 40.0 ml in lower layer). The angle of the sloping bottom layer was controlled by pouring each plate at the same position on an inclined plane. Reliability of data from each plate was further controlled by streaking all strains on the same plate. Two small loops of midlog-phase cells (optical density at 600 nm $\cong$ 0.5) in SMS63 at 37°C were streaked along the gradient. The plates were incubated for 24 h at 37°C, after which the length of the bacterial stripe was measured.

RESULTS

Mutant selection. Mutants were isolated from cultures of CR34 after mutagenesis with

ethyl methane sulfonate according to the method of Lin et al. (25) or Osborn et al. (38). The second dilution for growth in the method of Lin et al. was omitted to increase the probability of isolating slowly growing mutants.

Mutants were selected as being resistant to the lethal effects of one of the surface-active agents by their ability to form colonies on SMS63 agar containing the agent. The selective concentration of each agent was that which reduced the viable count of CR34 by a factor greater than 10^5. Selective concentrations were found for each agent at both 30 and 40°C in order to detect the appearance of possible temperature-sensitive mutants. The selective concentration of polymyxin (2 μg/ml) was the same at both 30 and 40°C. Levallorphan was used at 2.75 mM (30°C) and 2.5 mM (40°C); tetracaine was used at 2.35 mM (30°C) and 2.1 mM (40°C). The concentrations of the latter agents were really threshold ones; a slight increase in concentration led to killing of all the cells in the culture. This threshold phenomenon was seen previously with levallorphan effects on *E. coli* cells (4).

Preliminary characterization of the mutants. By examining 30 independently mutagenized cultures, 56 resistant mutants were isolated and found to be stable to repeated challenge with the selective agent. Of these, 30 were polymyxin resistant (PxR), 19 were levallorphan resistant (LvR), and 7 were tetracaine resistant (TcR). In a preliminary analysis, aimed at detecting gross surface alterations or abnormalities in cellular division, the strains were screened for the effects of growth temperature on resistance, cross-resistance to the other two selective agents, sensitivity to T phages, sensitivity to the anionic detergents SDS and DOC, generation time, and morphology. The strains which were most unlike the wild type were selected for further examination. The preliminary characterization of these strains is summarized in Table 2.

As shown in the table, resistance to polymyxin was to higher concentrations of the agent than was resistance to levallorphan or tetracaine. The PxR mutants had a high incidence (80%) of sensitivity to anionic detergents, suggesting that there may be a substantial alteration in their outer membrane. There was no marked cross-resistance between the three selective agents, although several of the LvR mutants showed a slightly increased resistance to tetracaine, and vice versa. A few of the strains had abnormalities in their morphology or in their generation time, and we wanted to determine whether these growth alterations were related to resistance to the surface-active agents. The multiple phenotypes seen with some of the resistant strains might have been caused by several different mutations. For example, in a recently studied envelope mutant that had surface alterations as well as an increased generation time (47), the strain was found to have two lesions, at the *envA* and the *sloB* loci, which were responsible for the surface and growth abnormalities, respectively. To have nearly isogenic mutant and wild-type strains for subsequent analysis, we elected to transduce some interesting mutants into recipient strains.

TABLE 2. *Some characteristics of isolated resistant strains*

Selective agent	Maximum resistance[a]	Cross-resistance[b]	Detergent sensitivity[c]	Growth characteristics
Polymyxin B (2 μg/ml)	10×	None	Abnormal: 80% of strains sensitive to either DOC, SDS, or both at 40°C	Essentially normal: only 1/3 had lengthened generation time
Levallorphan (2.5 mM)	1.6×	Few (2/19) resistant to tetracaine.	Essentially normal: 1/19 sensitive to DOC and 3/19 to SDS at 40°C	Essentially normal: one ts at 40°C
Tetracaine (2.1 mM)	1.3×	Many (6/7) resistant to levallorphan.	Only one slightly sensitive to SDS	Two strains had elongated generation time and bizarre cellular morphology

[a] Maximum resistance was determined by replica plating on plates containing various concentrations of the selective agent. The value shown is the concentration that kills the mutant divided by the concentration that kills the wild type.

[b] Cross-resistance was determined by replica plating on selective concentrations of all agents.

[c] Detergent sensitivity was measured by replica plating on plates containing 12 mg of SDS or DOC per ml, concentrations of detergent that do not kill the wild type.

Before transduction was attempted, several strains chosen for further analysis were screened for their efficiency of plating on selective agar at 37°C (Table 3). Concentrations of selective agents were chosen to given an efficiency of plating for the mutants of about 1 but to kill the wild-type strains DB170 and CR34. While examining wild-type strains to find suitable recipients for transduction, we found that some wild-type strains differed in their response to levallorphan and tetracaine, with several being more resistant than CR34. This difference among wild-type strains may be due to the abrupt increase in killing seen over a small range of concentration of the agents; slight differences in bacterial physiology may mediate this resistance. Strains DB98 and DB170 were found to be the best recipient strains, since both of these strains were sensitive to killing by the selective agents at the same concentration as CR34. Additionally, the genetic markers of these two strains are in a region of the *E. coli* map rich in sites that are thought to affect envelope structure (see Fig. 1); Godson (17) has reported other membrane biosynthetic mutants near 9 min that are not shown on the map. We are grateful to J. Gallant for pointing this fact out to us.

Transduction of the resistance loci. The LvR marker of strain 43L303 was found by P1 transduction to map in the *proC-purE* region of the *E. coli* map (Table 4) and was closely linked to *phoR* and *proC*. Although the number of transductants examined was small, an analysis of the phenotypes of the transductants indicated that the LvR mutation lies around 9.0 min (Fig. 1) on the *E. coli* chromosome. We term this mutation *lev*.

Using DB98 and DB170 as recipients, the polymyxin resistance of three PxR strains has been successfully transduced. In each of these crosses, the transductants appeared only after overnight growth, suggesting that a growth-dependent modification of the cell was neces-

TABLE 3. *Efficiency of Plating (EOP) and other characteristics of resistant strains selected for further study*

Strain	EOP on selective media[a]			Properties
	Polymyxin B (2 μg/ml)	Tetracaine (2.1 mM)	Levallorphan (2.7 mM)	
Wild type				
DB170	2.1×10^{-8}	6.2×10^{-7}	5.3×10^{-8}	Wild-type recipient
CR34	$<3.2 \times 10^{-8}$	7.6×10^{-7}	1.6×10^{-8}	Wild-type parent
PxR				
SM4	1			Resistant to polymyxin B >20 μg/ml (30°C); sensitive to SDS and DOC at 40°C, resistant at 30°C.
3M30P3	0.8			Resistant to polymyxin B <10 μg/ml (30°C); sensitive to SDS and DOC at 30 and 40°C.
1B30P7	0.9			Resistant to polymyxin B >20 μg/ml (30°C); sensitive to SDS at 40°C, resistant at 30°C; sensitive to DOC at 30 and 40°C; grows P1 phage very poorly.
LvR				
43L303			1	Resistant to 3.6 mM levallorphan (30°C); ts for growth at 40°C; generation time prolonged at 30°C; slight cross-resistance to tetracaine.
TcR				
13T301		0.6		Resistant to 2.8 mM tetracaine (30°C); more resistant to levallorphan than wild type; prolonged generation time.
34T401		0.6		Resistant to 2.8 mM tetracaine at 30°C and 2.3 mM tetracaine at 40°C; generation time 2× wild type; abnormal, bumpy cells at 30 and 40°C.

[a] Colonies found on selective media divided by colonies found on nutrient media. Cells used in this experiment were grown at 37°C in SMS63 into midlog phase and then diluted and plated immediately onto selective and nonselective plates. Selection plates were incubated at 30°C. Nonselective plates were incubated at 30, 37, and 40°C. The average number of colonies appearing was used in the EOP determination.

TABLE 4. *Transduction of lev locus*[a]

P1 donor	Recipient	Analysis of transductant genotypes
43L303 (*proC*$^+$ *phoR*$^+$ *purE*$^+$ *lev*^{r})	DB170 (*proC*$^-$ *phoR*$^-$ *purE*$^-$ *lev*^{s})	*proC*$^+$ *phoR*$^+$ *purE*$^-$ *lev*^{r}, 57% *proC*$^-$ *phoR*$^+$ *purE*$^-$ *lev*^{r}, 29% *proC*$^-$ *phoR*$^-$ *purE*$^-$ *lev*^{r}, 14% *proC*$^+$ *phoR*$^-$ *purE*$^-$ *lev*^{r}, 0%
43L303 (*proC*$^+$ *phoR*$^+$ *lev*^{r})	DB98 (*proC*$^-$ *phoR*$^-$ *lev*^{s})	*proC*$^+$ *phoR*$^+$ *lev*^{r}, 66% *proC*$^-$ *phoR*$^+$ *lev*^{r}, 33%

[a] Transductants were selected for resistance to 2.5 mM levallorphan as described in the text. With DB170 as recipient, 14 stable transductants were analyzed for cotransduction of other markers; with DB98, three stable transductants were analyzed.

sary to allow the PxR phenotype to appear. The transduction of TcR markers from 13T301 and 34T401 into DB170 has been successful, but, as with the PxR mutants, there was no linkage of any of the markers present in DB98 or DB170.

Properties of transductant strains. (i) Growth properties. We examined the growth properties of the transduced strains to see whether any of the abnormalities in the original isolates persisted in the transductants. For example, the LvR isolate 43L303 had a prolonged generation time at 30°C and did not grow at 40°C. As shown in Table 5, the *lev* and *tec* lesions led to an increased generation time when the mutated loci were present in either the original isolate or the transduced derivatives, but the effect was less dramatic in the derivatives. The morphology of the transduced derivatives containing *pmx* or *lev* (P335 or L435) was identical with that of the wild type. The *tec* transductant T135 made elongated cells in liquid media, with frequent filaments more than 30 cell equivalents in length (Fig. 2). Strain T135 reverted at high frequency to tetracaine sensitivity, with a concomitant reversion of the morphological abnormality. Colonies of T135 had an irregular outline, in contrast to the smooth colonial form of the isogenic wild type (or revertant). The *pmx* and *lev* mutants did not differ from the wild type in cell or colonial morphology.

(ii) Extent of resistance to the selective agents. The transductants were examined for their resistance to different concentrations of the selective agents. This examination was most conveniently carried out with the gradient plate technique: a small amount of logarithmically growing bacterial culture is placed on a square plate containing a gradient of the agent of interest. Figure 3 shows how the wild-type recipient, DB170, and the transduced derivatives behaved when growing on gradient plates containing the different selective agents. The only strain that could grow at all on 5 μg of polymyxin B per ml was the derivative, P335, containing the polymyxin resistance locus *pmx* from 3M30P3. The LvR mutant, L435, containing the levallorphan resistance locus *lev* from 43L303 could grow in the presence of substantially higher concentrations of levallorphan than could either the wild type or the tetracaine-resistant mutant. The *pmx* locus conferred increased sensitivity to levallorphan; whereas the wild type, DB170, grew at low concentrations of levallorphan, strain P335 was killed. The TcR transductant T135 grew on substantially higher concentrations of tetracaine than did either the wild type or the PxR transductant. As with the original isolates, resistance to polymyxin was a much more dramatic difference from the wild-type condition than was resistance to either levallorphan or tetracaine. These data are summarized quantitatively in Table 6, where the length of heavy growth on each of the plates is given for each of the agents used.

(iii) Sensitivity to other agents. When the anionic detergents DOC and cholate were incorporated into gradient plates, the only strain sensitive to these was the polymyxin-resistant transductant P335 (Table 6). Neither DB170 nor the *lev* and *tec* transductants were sensitive to these detergents. This is the same situation that was seen with the original PxR isolates (Table 1), which were the only detergent-sensitive strains. None of the strains was sensitive to the nonionic detergent Triton X-100 at concentrations up to 20 mg/ml, and all were killed by the cationic detergent cetylpyridinium chloride at concentrations below 0.05%.

Sensitivity and resistance to acriflavine and other basic dyes is mediated by the locus *acrA*, which is in the region of the *E. coli* chromosome in which *lev* maps. It has been postulated that *acrA* is a site which determines plasma membrane structure in *E. coli* (36). To test whether the strains we isolated were affected at this locus, we examined sensitivity to acriflavine and a number of other basic and acidic dyes, as well as to mitomycin C, another agent for

which sensitivity is mediated by this locus. Resistance to acriflavine is the wild-type condition, as shown by DB170 and P335, which were similarly resistant (Table 6). However, both the *lev* mutant and the *tec* mutant were even more resistant to acriflavine than the wild type, DB170, and could grow at 30 μg/ml. Thus, none of the strains had become sensitive to acriflavine, although the level of resistance differed, depending on whether or not DB170 possessed either the *lev* or *tec* lesion. When sensitivity to mitomycin C was explored, the strains behaved

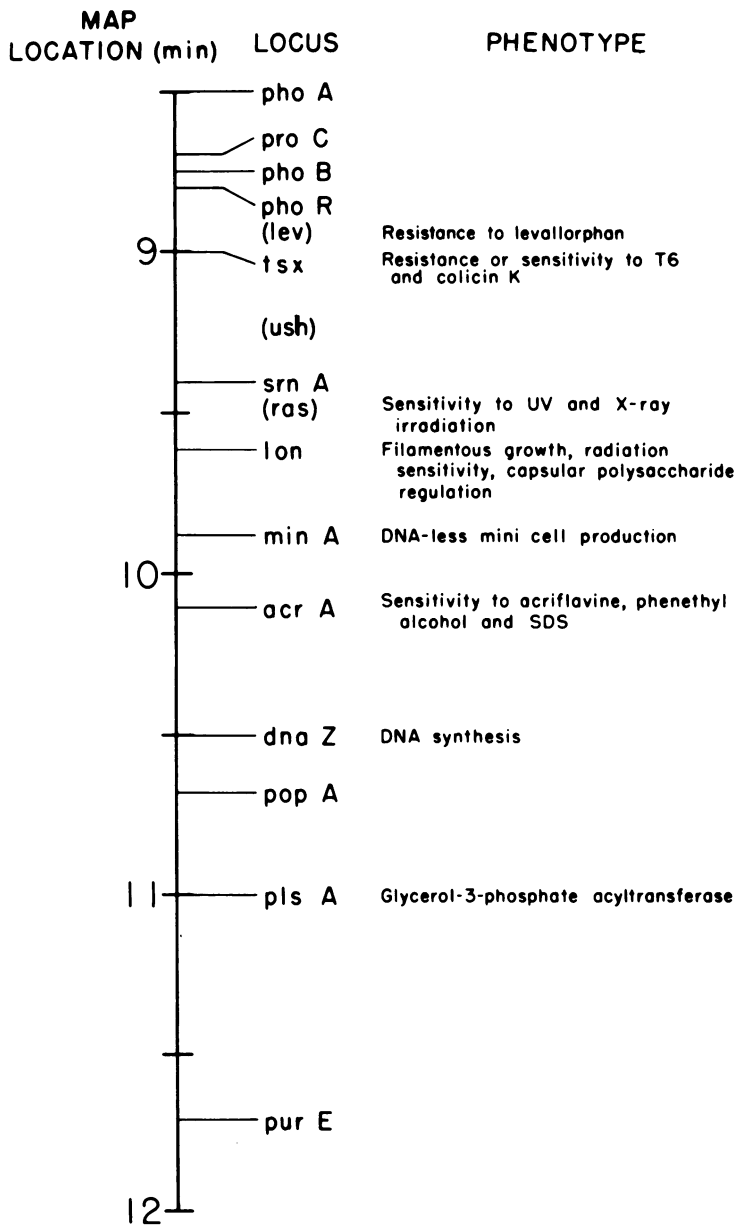

FIG. 1. *Chromosome map of E. coli showing relative positions of genes in the region of 8.5 to 12 min. The map locations of genes in parentheses are only approximate. The genes with known or suspected effects on the E. coli surface are briefly described. A new gene, lev, described in this paper, has been included in this section of the map from Bachmann et al. (1).*

TABLE 5. *Comparison of generation times of strains containing resistance loci*[a]

Resistance locus	Generation time (min) with locus in strain	
	CR34	DB170
Wild type	85	63
pmx	84	63
lev	124	75
tec	111	77

[a] The generation time for the strains was determined at 37°C in SMS63 supplemented with 100 μg threonine, proline, and leucine per ml and 40 μg of adenine and thymine per ml. The loci were in the following strains of isolate and recipient, respectively: *pmx*, 3M30P3 and P335; *lev*, 43L303 and L435; and *tec*, 13T301 and T135.

somewhat differently (Table 6). The *lev* transductant L435 was slightly more resistant to mitomycin than was the wild type, whereas the *tec* transductant T135 was slightly more sensitive. The *pmx* strain P335 was killed by mitomycin C. All of the strains were slightly more sensitive to gentian violet than was the parent. As discussed below, these differences in sensitivity may reflect relatively subtle alterations of either the outer or inner membrane of *E. coli*. The following agents were tested against DB170 and the other transductants examined in Table 6, with no effect on growth of any strain: methyl green, 400 μg/ml; acridine orange, 100 μg/ml; toluidine blue, 300 μg/ml; methylene blue, 500 μg/ml; and eosin Y, 500 μg/ml.

(iv) Sensitivity to colicins and bacteriophage. Many mutants previously studied with membrane alterations have been resistant to strains of T phage, due to loss of the phage receptor, or to colicins, due to either loss of the receptor or a defect in transmission of the killing effect of the colicin. To test whether the *pmx*, *lev*, or *tec* mutants were affected at either phage or colicin receptor sites, or had a colicin-tolerant phenotype, we grew them in the presence of either colicin or T phages.

The transduced strains did not differ from DB170 in sensitivity to colicins A, B, E_1, E_2, E_3, G, H, I_a, I_b, K, J, S, V, or X, as determined with a series of colicinogenic strains generously provided by P. Fredericq. The colicin sensitivity determinations were performed as described in Materials and Methods. No difference in sensitivity to the T phages was found between the wild type and the transduced derivatives. Thus, the *pmx*, *lev*, and *tec* loci that were examined were wild type for colicin and T phage sites on the cell surface. Mathieu and Legault-Hetu (28) reported that colicin K-tolerant cells of *E. coli* were less sensitive to the effects of polymyxin in the presence of colicin K; however, the *pmx* lesion studied above did not affect the sensitivity of the strain to colicin K.

DISCUSSION

In general, the *lev*, *tec*, and *pmx* mutants examined here were less pleiotropic than previously studied envelope mutants (6, 9–11). For example, sensitivity to detergents or drugs was much less marked with the *pmx*, *lev*, and *tec* strains than with LPS-defective mutants, which have a generalized defect in the envelope barrier properties. Sensitivity to colicins or bacteriophages remains unaltered, although there are discrete examples of surface modifications, both in the primary resistance to the selective agent and in other properties.

The PxR strains were most pleiotropic, with several types of barrier defects being present. In addition to being sensitive to anionic detergents, the *pmx* transductant is more sensitive than the wild type to levallorphan, mitomycin C, and gentian violet. Detergent sensitivity is characteristic of other classes of envelope mutants. LPS-deficient mutants (11) and most group A colicin-tolerant mutants (9) are sensitive to one or more anionic detergents. The increased sensitivity of the *pmx* transductant P335 to such unrelated compounds as levallorphan, mitomycin C, and gentian violet, and its wild-type resistance to other dyes and tetracaine, suggest that only a limited defect in the barrier exists in this strain. Note that the strain is resistant to polymyxin, in contrast to other LPS-deficient mutants (10). Thus, the *pmx* lesion confers a selective alteration in the barrier function of the *E. coli* cell, in addition to resistance to the detergent-like polypeptide polymyxin B.

In contrast to the *pmx* locus, both *lev* and *tec* lead to subtle changes in cell physiology, aside from the drug resistance itself. Both *lev* and *tec* gave L435 and T135, respectively, a small increase in resistance to acriflavine, and slightly affected the strains' resistance to mitomycin C and gentian violet. The *lev* lesion increased mitomycin C resistance, whereas *tec* decreased this resistance. Both *lev* and *tec* conferred greater sensitivity to gentian violet, although not as much as *pmx*. The nature of these alterations and the properties of the agents used in the selection support the hypothesis that the strains are indeed envelope mutants. Both *lev* and *tec* transductants had a slight increase in generation time in comparison with the iso-

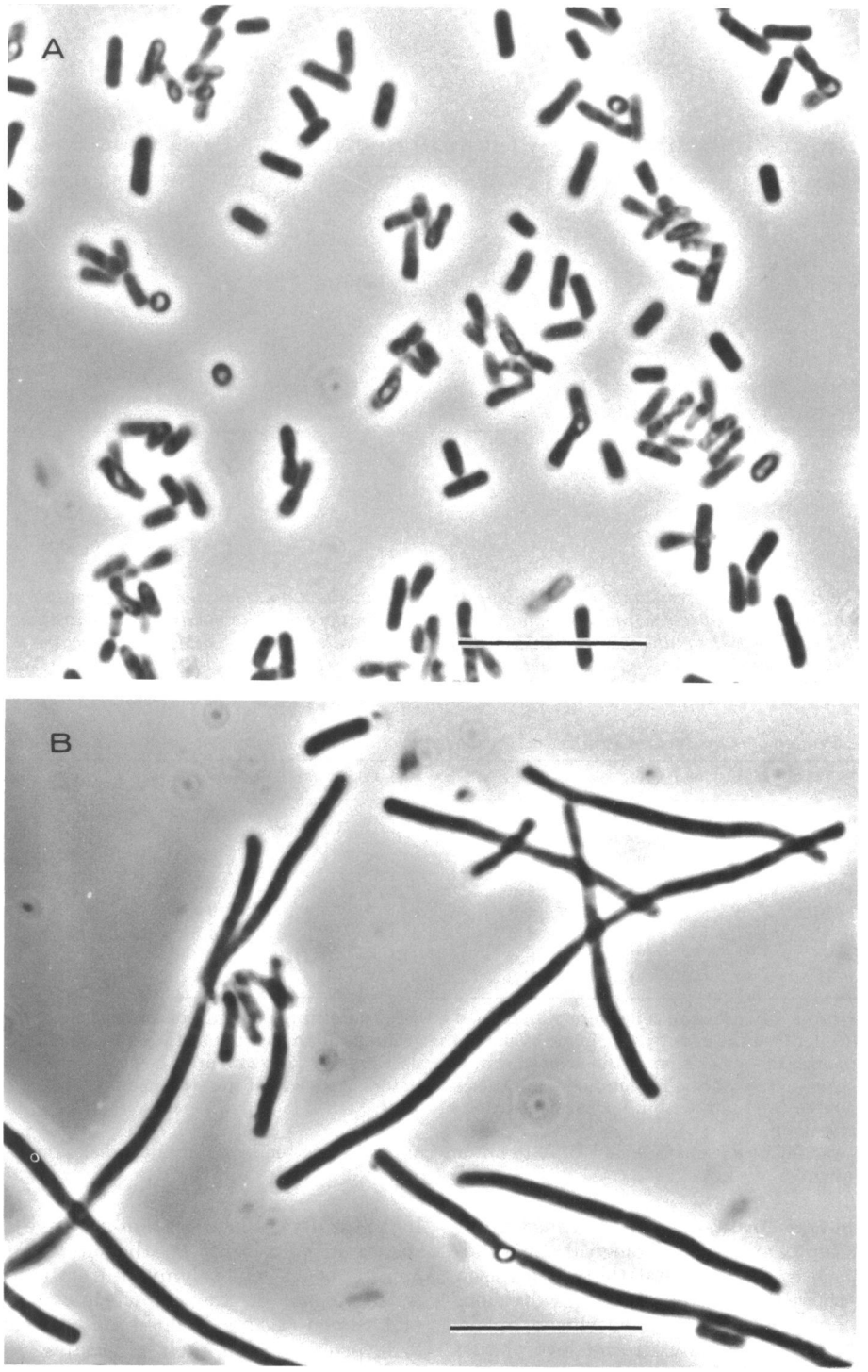

FIG. 2. *Morphology of the tec transductant T135 (B) in comparison with the wild type, DB170 (A). These cells were in midlog growth phase in Hershey broth + 40 μg of thymine per ml at 37°C before fixation in 3% glutaraldehyde for microscopy. The bar indicates 10 μm.*

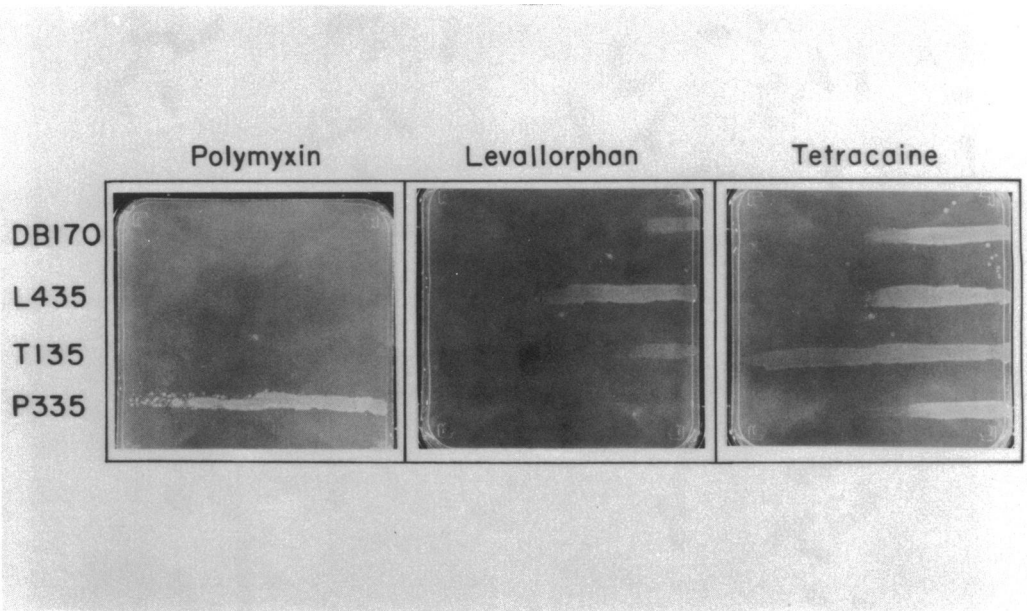

FIG. 3. *Extent of cross-resistance of pmx, lev, and tec transductants. Gradient plates containing polymyxin, levallorphan, and tetracaine were made as described in the text. The maximal concentration of each drug, at the left side of the plate, was: polymyxin B, 5 μg/ml; levallorphan, 4.5 mM; and tetracaine, 2.8 mM. Two loopfuls of logarithmically growing wild type (DB170), lev (L435), tec (T135), and pmx (P335) were streaked on the plate after the gradient was established (see text).*

TABLE 6. *Growth of transductant strains and the isogenic wild type in the presence of several selective agents*[a]

Strain	Selective agents			Other agents				
	Polymyxin B (5 μg/ml)	Levallorphan (4.5 mM)	Tetracaine (2.8 mM)	DOC (1%)	Cholate (1%)	Acriflavine (30 μg/ml)	Mitomycin C (5 μg/ml)	Gentian violet (20 μg/ml)
DB170 (WT)	0	2	4.5	9	9	5	2.2	7.5
L435 (*lev*^{r})	0	5.2	4.9	9	9	9	3.0	6.3
P335 (*pmx*^{r})	7.5	0	4.5	0	1	4.5	0	5.2
T135 (*tec*^{r})	0	2.5	8.6	ND[b]	ND	9	1.8	5.5
CR34 (WT)	0	0.5	ND	9	9	2	ND[c]	2.5

[a] Logarithmically growing cells were plated upon SMS63 containing varying concentrations of the agent shown. Gradients of the agents were made by using the gradient plate technique of Nakamura (35), with the agent present in the upper agar at the level shown. The plates sat for 12 to 24 h after being poured, in order to allow the gradients to form. The numbers are the centimeters of growth of the streaked bacteria, measured from the end of the plate with a low concentration of agent.

[b] ND, Not done.

[c] CR34 was about as sensitive as DB170 to mitomycin C when tested by using filter disks impregnated with the agent.

genic wild-type strain, and the *tec* transductant had an accompanying morphological abnormality, supporting the idea that there is a liason between the cell surface and cellular division.

Other bacterial strains have shown an altered response to polymyxin and levorphanol (the agonist analogue of levallorphan). Meyers et al. (30) have reported the isolation of polymyxin-resistant variants of *E. coli*, but little is known about the mechanism of resistance (12). In naturally resistant gram-negative bacilli, such as *Proteus mirabilis*, the outer membrane appears to prevent polymyxin from reaching a sensitive site on the cytoplasmic membrane (45). Simon (41) has isolated mutants resistant to levorphanol, but their properties were not explored. The effect of levallorphan on bacterial cells seems to be in altering permeability, for spheroplasts lyse in the presence of the drug (4), and this lysis is prevented by Ca^{2+} and

Mg^{2+}. The transport of putrescine in *E. coli* is inhibited by levorphanol (42), again suggesting the cytoplasmic membrane as a target for these drugs. Other evidence (5) implicates levallorphan as an inhibitor of the interconversion of ppGpp (MSI) and pppGpp (MSII), which itself may be catalyzed by a membrane enzyme system.

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