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Isolation and Some Properties of Cell Envelope Altered Mutants of *Escherichia coli*

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Mutants of *Escherichia coli* which have a defect in their permeability barrier were selected. The technique used was to employ a strain of *E. coli* having a deletion in the gene for lactose permease and to select for mutants which can grow on lactose at 40 C. Twenty such mutants were isolated and six of these were found to be more sensitive to actinomycin D, sodium deoxycholate, and sodium dodecyl sulfate than was the parental strain. They were also more sensitive to the antibiotics vancomycin and bacitracin, which inhibit peptidoglycan biosynthesis. These mutants were no more sensitive to several different colicins or phages than was the wild-type strain. One of the mutants selected by this technique has an abnormal morphology when grown on certain carbon sources in minimal medium, and this mutant is more extensively studied in the accompanying paper.

During the past several years, various lines of evidence have emerged which implicate the bacterial membrane in the cell division process. On the one hand, biochemical and morphologic criteria exist for a physical association between the bacterial chromosome and the cytoplasmic membrane (reviewed in 2, 9). On the other hand, several temperature-sensitive cell division mutants of *Escherichia coli* have an alteration in their membrane structure. For example, a *dnaA* mutant, affected in initiation of replication, was found to be exquisitely sensitive to the detergent sodium deoxycholate (3) and to produce a membrane protein with altered properties at the nonpermissive temperature (10). Another cell division mutant has been studied which at elevated temperatures produces an excess of membrane-like material in the cytoplasm and ceases its normal growth; this mutant was also found to be sensitive to detergents (4). Since several mutants of this type, which had been selected for defects in deoxyribonucleic acid (DNA) synthesis or some other aspects of cell division, had altered membrane properties, we became interested in whether selection for a membranal alteration would give rise to some mutants affected in the cell division process. Therefore these studies were undertaken in order to establish more conclusively the relationship between cell surface structure and cell division.

The first technique we tried is a rather simple one, and was quite effective in selecting mutants of *E. coli* with an increased permeability. The technique is based on utilization of a strain of *E. coli* with a *lacY* deletion mutation; such a strain will not grow effectively on low concentrations of lactose and does not revert. After mutagenesis, by selecting for bacterial colonies able to grow on lactose, we obtained several mutants with increased permeability to lactose and other compounds. A similar technique has been used successfully by Ricard et al. (8).

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 strain CR34 was the parental strain for these studies. It is F⁻ *leu⁻ thr⁻ thy⁻ thi⁻ lacY⁻*. The colicinogenic strains of *E. coli* employed were given to us by S. Falkow and included CA 58(H); K 235 (K); CA 7 (V+M); CA 23 (D); CA 46 (G); CA 18 (B); CA 31 (A); CA 62 (J+1); and CA 53 (I), as classified by Fredericq (1).

Media and growth of bacteria. Nutrient media employed in this study were either nutrient broth (Difco) or ML (tryptone, 1%; yeast extract, 0.5%; NaCl, 0.5%; pH 7.2). A minimal salts medium was used as previously described (10); this $63B_1$ salts medium was supplemented with carbon sources at 4 mg/ml, and with leucine, threonine and thymine at 50 μg/ml. Actinomycin D (Calbiochem) was used in nutrient agar plates at 7 μg/ml for testing sensitivity to this drug. All experiments with actinomycin D were done in the dark. Detergent plates contained either sodium deoxycholate (3 mg/ml) or sodium

dodecyl sulfate (6 mg/ml). EMB-lactose (Difco) was used in screening the mutants.

Other procedures. Ethyl methanesulfonate was used as the mutagen. The cells were grown in logarithmic phase to a concentration of 5×10^8 cells/ml in medium ML, harvested, washed twice with $63B_1$ medium and resuspended in $63B_1$ without any carbon source at 10^9 cells/ml. Ethyl methanesulfonate was added to a concentration of 1% and incubated with the cells at 30 C for 90 min. The mutagenized cells were then washed with $63B_1$, resuspended in ML, and grown overnight.

Sensitivity to colicins was assayed by stabbing colicinogenic strains into nutrient agar plates, allowing them to grow overnight, and then killing the cells with chloroform vapor. The strain to be tested was then poured over the plate containing the colicins, and the size of the halo of inhibition of growth around each stab was determined after overnight growth of such a bacterial lawn. Sensitivity to drugs other than actinomycin D was measured by placing antibiotic discs (Difco) containing three different concentrations of each drug on a lawn of bacteria. The size of the halo around the discs was measured after overnight growth.

RESULTS

Isolation of mutants. The mutagenized culture, after overnight growth on ML, was washed and plated on $63B_1$ containing lactose at a concentration of 2 mg/ml. Plates were incubated at both 30 and 40 C, and the colonies which appeared after 24 hr were picked and purified. None of the colonies which grew at 30 C were later shown to have other surface alterations, probably since lactose can enter at this temperature by the melibiose permease (7). However, six out of twenty mutants which grew on lactose at 40 C were shown to have other surface alterations (*see below*). On EMB lactose plates the colonies were darker than the light pink parental strain, but were not the intense dark color of *lac*-positive strains.

Other criteria were used to determine whether these mutants which could grow in lactose had alterations in their surface properties. The mutants were grown into the exponential phase on ML medium, and then cultured on plates containing either actinomycin D, sodium deoxycholate, or sodium dodecyl sulfate. The results of growth on these agents and on lactose are shown in Table 1. Six of the twenty mutants obtained by growth on lactose at 40 C were shown to be sensitive to actinomycin and the detergents. However, they were not sensitive at 30 C, and two of the strains did not grow well on lactose at 30 C.

The increased permeability, as defined by inhibition of growth on actinomycin- and detergent-containing plates, was not seen in liquid ML but could be detected in liquid $63B_1$. The results of growth on this salts medium containing glucose, in the presence of actinomycin or sodium deoxycholate, are shown in Fig. 1. It can be seen that the mutants vary in their sensitivity to these two agents; in each case they are somewhat sensitive to actinomycin D, whereas the parental strain, CR34, is not. In all the mutants except 7401, there is an increased sensitivity to the detergent sodium deoxycholate.

The cell envelope has been shown to be altered in certain cases of colicin resistance or tolerance (5). To see whether any of our mutant strains have altered sensitivity to colicins, we examined the effect of the colicins listed above. In all cases, the sensitivity was the same in the mutants as in the wild type.

In addition, we tested the sensitivity of the parent and mutant strains to certain antibiotics which seem to act on the bacterial cell surface. As can be seen in Table 2, polymyxin B and colistin, two polypeptide antibiotics which appear to act as detergents on the cell membrane (6), were equally effective in inhibiting the growth of the parent and mutant strains. On the other hand, vancomycin and bacitracin, which inhibit peptidoglycan biosynthesis (11), were more effective on the mutants than on the parent. Nalidixic acid, an inhibitor of DNA synthesis (2), was equally effective on mutant and wild-type strains (not shown), and

TABLE 1. *Association between ability to utilize lactose as a sole carbon source for growth and the inhibitory action of certain agents on mutant derivatives of CR34*[a]

Strain	Growth on:							
	$63B_1$ Agar with lactose (2 mg/ml)		Actinomycin D (7 μg/ml)		Nutrient agar containing:			
					Sodium deoxycholate (3 mg/ml)		Sodium dodecyl sulfate (6 mg/ml)	
	30 C	40 C	30 C	40 C	30 C	40 C	30 C	40 C
CR34	−	−	+	+	+	+	+	+
7401	+	+	+	−	+	−	+	−
2403	−	+	+	−	+	−	+	−
2404	−	+	+	−	+	−	+	−
1040	+	+	+	−	+	−	+	−
5401	+	+	+	−	+	−	+	−
4402	+	+	+	−	+	−	+	−

[a] A loopful of an exponentially growing culture of each strain was inoculated on the above solid media and grown overnight at the indicated temperatures; + indicates growth; −, no growth.

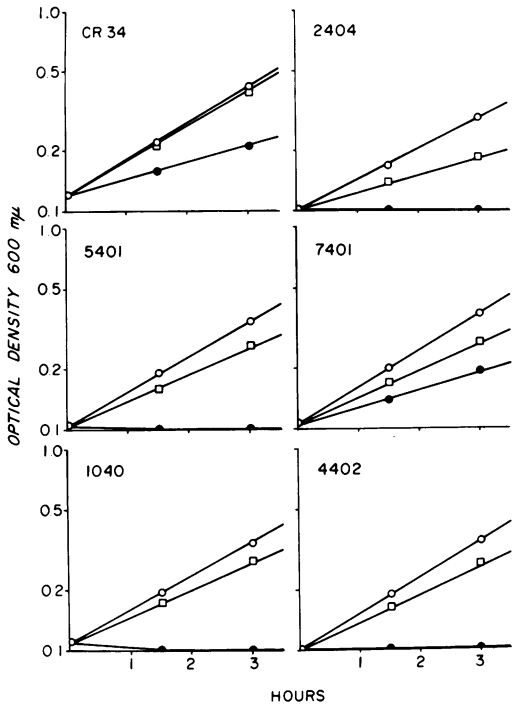

FIG. 1. *Growth of mutant and wild-type strains. Cultures of the indicated strains growing logarithmically at 40 C on 63B$_1$ salts medium containing 0.4% glucose were divided into three parts. To one was added actinomycin D (10 μg/ml), □; to another, sodium deoxycholate (2 mg/ml), ●; the third had no additions,* O.

TABLE 2. *Sensitivity of the mutant strains to antibiotics*[a]

Strain	Diameter of zone of growth inhibition (mm)[b] in the presence of:			
	Colistin (10 μg)	Polymyxin B (100 μg)	Vancomycin (30 μg)	Bacitracin (5 μg)
CR34	8	7	11	None
7401	8.5	7	13	7
2404	8	8	15	8
5401	8	7.5	15	6.5
4402	9	7	15	8
104012	8.5	7	15	9

[a] Similar data were obtained for two different concentrations of each antibiotic; the relative differences between the effects on CR34 and the mutant derivatives remained.

[b] Antibiotic discs (6 mm; Difco) were placed on a lawn of each strain, and the zones of inhibition were measured after overnight growth. The measurement was of the diameter of the inhibition zone.

several sulfonamide derivatives were without effect on any of the strains.

In addition, all of the strains were tested for their sensitivity on ML to phages T2, T4, T7, φX, R17, fd, and lambda, (the virus strains were a gift of T. Young). The wild type was sensitive only to T2, T4, T7, and lambda, and there was no difference between the sensitivities of the mutants and wild type to these virus strains.

DISCUSSION

The mutants isolated by this technique, although having an increased permeability, are really not drastically altered in this regard from the wild-type strain. They grow well in minimal salts media and in nutrient broth and, with the exception of 2404 growing on certain carbon sources (Fig. 1; and accompanying paper), have the same generation time as the wild type on all media. Thus, there appears to be no marked physiological impairment. Nonetheless, the strains are more sensitive to detergent; they do not grow at all at a concentration of sodium deoxycholate which inhibits the growth rate of the wild type by 55%. They are also slightly sensitive to actinomycin D, unlike the parent strain, and these strains are more sensitive to two antibiotics which inhibit peptidoglycan biosynthesis. Thus, by selecting for increased permeability to a carbon source, lactose, whose permease is lacking in the parental strain, we were able to obtain a series of mutants altered in several other surface properties. One of these mutants, 2404, has a disturbed morphology when grown on certain specific carbon and energy sources. A characterization of the physiology of this strain is the basis of the accompanying paper.

Ricard et al. (8) have used a technique similar to the one employed here for the isolation of surface altered strains. They selected for mutants using a parental strain in which there was a double permease deficiency; the mutants they found also had an increased sensitivity to sodium deoxycholate. This technique seems to be a rather simple and general method for selecting for surface altered mutants of *E. coli*.

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