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Mutation Preventing Capsular Polysaccharide Synthesis in *Escherichia coli* K-12 and Its Effect on Bacteriophage Resistance

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A mutant strain of *Escherichia coli* K-12 was found in which spontaneous mutation to phage T7 resistance occurred at a very low frequency. T7 resistance in the parental strain from which this mutant was derived resulted from a mutation to excess capsular polysaccharide synthesis. The mutation preventing T7 resistance, *non-9*, inhibited capsule formation when transduced into capsulated strains. The *non-9* mutation was cotransducible with *his*, the gene order in this region being *non-9 his Su-1*.

The T phages of *Escherichia coli* have in common the ability to adsorb to and replicate in *E. coli* strains B and K-12. Compared with many *E. coli* strains found in nature, strains B and K-12 are "rough," i.e., they do not have significant amounts of extracellular polysaccharide capsular material. The T phages adsorb to specific sites on the cell wall (30). Mutants of *E. coli* K-12 and other strains of *E. coli* are found that produce very large amounts of capsular material and are mucoid or "smooth" in appearance (16, 19, 24). Some, but not all of these mutants, are resistant to the T phages and other coliphages because the cell wall phage receptors are covered by the capsule polysaccharide (16, 24).

We have found a mutant of *E. coli* K-12 that mutates at a very low frequency to resistance to phage T7. This work will show that this unusual phenotype is due to a block in capsule formation.

MATERIALS AND METHODS

Bacterial strains, phages, media, and growth conditions. The bacterial strains used are listed in Table 1. Wild-type T phages were obtained from laboratory stocks and grown and assayed on *E. coli* B by using procedures described by Adams (1). The methods for P1kc growth and transduction were previously described (27).

The minimal medium was that of Davis and Mingioli (6) plus 1 μg of thiamine hydrochloride and 0.5 μg of ferrous sulfate per ml. When necessary, amino acids were used at a final concentration of 50 μg/ml. Nutrient broth contained 0.8% (w/v) nutrient broth powder (Difco), 0.1% yeast extract (Difco), and 0.5% sodium chloride. Solid media (nutrient and minimal agar) were made by adding 20 g of agar (Difco) per liter to the liquid medium. Antibiotic medium no. 3 (Difco) was occasionally used in place of nutrient broth. Bromothymol blue medium, used to score fermentation markers, contained 0.8% Difco nutrient broth powder, 1.0% sugar, 0.006% bromothymol blue, and 2% agar.

Overnight, aerated cultures grown at 37 C were used as stationary-phase cultures. Liquid cultures and plates were incubated at 30 C when necessary to increase capsule formation; all other incubations were at 37 C.

Genetic procedures. All matings were uninterrupted. Donor and recipient cultures were mixed and incubated for 1 to 2 hr before plating as described (27). In transductions, a multiplicity of infection of one was used. All recombinants were purified by single-colony isolation on the selective medium.

Unselected auxotrophic and fermentation markers were scored by replica plating. The *Su-1*$^+$ (suppressor containing) and *Su-1*$^-$ alleles were scored by cross-streaking a loopful of a stationary-phase antibiotic medium no. 3 culture against phage T4D amber mutant *amN82* (11) on nutrient agar. After overnight incubation, *Su-1*$^+$ recombinants gave a clearly visible indication of phage growth; *Su-1*$^-$ recombinants yielded a continuous streak. The *non-9* (nonmucoid) mutation was originally scored by its inability to mutate to T7 resistance. Purified recombinants were spotted onto nutrient agar, incubated 2 days, and replica-plated onto a nutrient agar plate spread with 5×10^8 plaque-forming units (PFU) of T7. This plate was incubated for 2 days, and spots yielding T7-resistant mutants were scored as Non$^+$. When *non-9* was scored by its ability to block capsule formation, recombinants were spotted onto minimal medium agar and incubated for 3 days at 30 C. Non$^+$ recombinants became large, convex, and mucoid as compared with Non$^-$ recombinants.

UV sensitivity. The ultraviolet (UV) sensitivity of recombinants was determined by the rapid UV test of Woody-Karrer and Greenberg (33). UV survival curves were made as described previously (27).

TABLE 1. *Bacterial strains*[a]

Strain	Sex	Genotype	Source
JC355	F⁻	*argG his leu metB gal lac malA mtl xyl tonA* λ⁻	J. G. Flaks
DS-3	?	*Su-1⁺ his phoA-S26 thi*	R. C. Wilhelm
M99	F⁻	*Su-1⁺ strA proA lac*	M. Malamy
AT2427	Hfr (H type)	*thi-12 cysC39*	A. L. Taylor
MC100	F⁻	*leu proC ade trp strA*	A. Markovitz
MC102	F⁻	*capR9 leu ade trp strA*	A. Markovitz, ProC⁺ transductant of MC100
ES129	F⁻	As JC355 but *non-9*	NTG mutagenesis
ES365	F⁻	As MC102 but *his*	NTG mutagenesis
ES366	F⁻	As ES129 but His⁺	AT2427 × ES129 conjugation
ES367	?	As DS-3 but *cap* (mucoid)	NTG mutagenesis
ES368	F⁻	*capR9 non-9 leu ade trp strA*	ES366 × ES365 transduction
ES369	F⁻	As M99 but *his*	EMS mutagenesis
ES370	F⁻	As JC355 but *cap*	NTG mutagenesis

[a] Symbols: *ade*, adenine; *arg*, arginine; *cap*, capsule; *cys*, cysteine; *gal*, galactose; *his*, histidine; λ , non-lysogenic for phage λ; *lac*, lactose; *leu*, leucine; *mal*, maltose; *met*, methionine; *mtl*, mannitol; *non*, nonmucoid; *pho*, alkaline phosphatase; *pro*, proline; *str*, streptomycin; *Su-1⁺*, amber suppressor-containing; *tonA*, resistance to phages T1, T5; *trp*, tryptophan; *xyl*, xylose.

Mutagenesis. Treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was by the procedure of Adelberg et al. (2). Ethyl methanesulfonate (EMS) mutagenesis was carried out according to Schwartz (25). Mucoid mutants were found by incubating the survivors of mutagenesis on minimal agar for 3 days at 30 C.

Determination of nondialyzable methylpentose. The cultures were grown to stationary phase in minimal medium at 30 C with aeration. As described by Kang and Markovitz (15), the unwashed cultures were boiled 15 min, centrifuged at 3,000 × *g* for 10 min, the supernatant dialyzed at 4 C against several changes of distilled water, and recentrifuged at 27,000 × *g* for 10 min. The superantant fluid was analyzed for methylpentose with the Dische-Shettles reaction (10-min boiling; 8).

Extraction of nucleotides. Cultures were grown as described for the methylpentose determination. Hot water-extractable material was obtained by the procedure of Lieberman et al. (18), and the optical density (OD) was determined at 260 and 280 nm.

RESULTS

Characteristics of the non-9 mutant. Strain ES129 was found after NTG treatment of JC355. The survivors of the mutagenesis were replica-plated to detect clones that formed few or no T7-resistant mutants. ES129 gave no T7-resistant growth on replica plating. When 0.1 ml of a stationary-phase nutrient broth culture of ES129 (ca. 2×10^8 cells) was spread on nutrient agar previously spread with 10^8 PFU of T7, no T7-resistant colonies were found. In repeated determinations, the frequency of T7 resistance in ES129 was $< 2 \times 10^{-9}$ mutants/cell. In the parent, JC355, the frequency was approximately 10^{-6} mutants/cell. The mutation frequencies of resistance to phages T2, T6, and the metabolic inhibitor sodium azide were similar for JC355 and ES129, indicating that the latter strain was not an antimutator.

A cross-resistance relationship exists involving resistance to phages T3, T4, and T7 (5, 7). Therefore, an attempt was made to select mutants of ES129 resistant to these phages and to determine the cross-resistance patterns. A stationary-phase nutrient broth culture of ES129 was spread on nutrient agar with either 10^8 PFU of T3, T4, or T7 and on minimal agar with T7. T3 resistant mutants of ES129 formed at about $1/_{50}$th the frequency found with JC355. These mutants grew very slowly. After three consecutive single-colony purifications to free the mutants of phage, they were grown in broth and cross-streaked against T3, T4, and T7 on nutrient agar. The mutants selected for T3 resistance were resistant only to T3. Of sixteen mutants selected for T4 resistance, only one after purification was resistant to any phage, and that was resistant only to T4. The instability of T4 resistance in *E. coli* K-12 was previously found by Curtiss (5). No T7-resistant mutants of ES129 were found on nutrient agar. On minimal agar, they formed at a frequency of 5×10^{-8} mutants/cell. After purification, these mutants were sensitive to T7 as determined by cross-streaking on minimal and nutrient agar, sensitive to T4, but resistant to T3. Within the T3, T4, and T7 group, only resistance to T3 is stable in spontaneous mutants of ES129.

A culture of ES129 was treated with the mutagen EMS. The survivors were grown to stationary phase and plated on nutrient agar with

phage T7. As a control, a portion of the same culture was used without EMS mutagenesis. The nonmutagenized culture gave no T7-resistant mutants. However, T7-resistant mutants were found at a frequency of 10^{-7} mutants/cell in the mutagenized culture. These mutants were purified three consecutive times. All were nonmucoid, unlike T7-resistant mutants of JC355. Phage resistance was determined by cross-streaking. All the mutants were T2 and T6 sensitive. Of 16 such mutants, two had become T7 sensitive after purification. One of these was T3 resistant. The remaining 14 mutants gave three distinct phage-resistance phenotypes; resistance to T7 and T3, resistance to T7 and T4, and resistance only to T7. Five of the 14 resistant mutants were tested for T7 adsorbance. All five did not adsorb the phage, indicating that a change in the phage receptor rather than phage tolerance had occurred.

All T7-resistant mutants of JC355 selected on nutrient agar were mucoid. After purification, they remained T7 resistant and were cross-resistant to T3 and T4. Only a slight indication of phage growth was seen when these mutants were cross-streaked against T2 and T6. The receptors for phages T3, T4, and T7 are in the lipopolysaccharide layer of the cell wall, and the T2 and T6 receptors are in the lipoprotein (30, 31). In *E. coli* B, which cannot become mucoid (9), no cross-resistance is found among the T2, T6, and the T3, T4, T7 groups. It has been shown in *E. coli* B that resistance to phages T3, T4, and T7 (B/3, 4, 7) is due to a change in the lipopolysaccharide (32). Resistance to T7 in JC355 probably results from extracellular capsular material covering the phage receptors. This explains the partial cross-resistance to T2 and T6. ES129 cannot form a capsule and become T7-resistant in this manner.

Genetic analysis. To map *non-9*, the mutation affecting T7 resistance in ES129, an uninterrupted mating was made with AT2427 as donor and ES129 as recipient. The *non-9* mutation was scored by the inability of recombinants to form T7-resistant colonies. The results (Table 2) placed *non-9* near *his* on the bacterial chromosome.

The inability of *non-9* strains to form T7-resistant mutants was thought to be due to a block in capsule formation. If this were so, the transfer of *non-9* to a mucoid, capsulated recipient would result in a recombinant with a nonmucoid phenotype. To test this hypothesis and the possibility of the linkage of *non-9* to *his* by transduction, phage P1kc was grown on ES366, a His^+ *non-9* strain. It was originally planned to use a T7-resistant, mucoid mutant of JC355 as a recipient. However, all T7-resistant mutants were also resistant to P1kc. Therefore, JC355 was treated with NTG, and a mucoid mutant (ES370) was selected on minimal medium at 30 C as described above. This mutant was P1kc sensitive and also T7 sensitive. The mutation to mucoid (excess capsule formation) can occur at two distinct loci: *capR* (synonomous with *lon*; see references 3, 14, 19) and *capS* (21). The *capR* mutants are also UV sensitive (3, 9, 14). ES370 was not UV sensitive and assumed to be a *capS* mutation. In the ES366 × ES370 transduction, 46 of 142 His^+ transductants (31%) were nonmucoid. This cross demonstrated the linkage of *non-9* to *his* and the mutation's ability to prevent capsule formation.

An additional transduction utilized the same donor (ES366) and ES365, a *his capR9* strain, as the recipient. Of 151 His^+ transductants, 41 or 27% were nonmucoid. As determined by the rapid UV test, all recombinants were UV sensitive, i.e., still contained *capR9*. The survival curve of one *capR9 non-9* transductant (ES368) was identical to ES365. To further establish the *non-9 capR9* genotype of the nonmucoid transductants, a P1kc lysate was prepared as ES368. The lysate was used to transduce ES370 to His^+. Twenty-seven per cent of the transductants were nonmucoid, close to the previous value for the *non-9* - *his* linkage. The presence of *capR9* was verified by using MC100, a *proC* mutant, as a recipient; *capR9* and *proC* are cotransducible (19). As determined by the mucoid phenotype, 15% of the Pro^+ transductants had received *capR9*.

A three-factor transduction was used to determine the location of *non-9* in relation to the *his* and *Su-1* loci, *Su-1* being cotransducible with *his* (28). ES366, the prospective donor, was permissive for the T4 amber mutation *amN82* used to score for $Su\text{-}1^+$. However, it was expected that the suppressor mutation in ES366 was at a site

TABLE 2. *Genetic analysis of recombinants from the mating of AT2427 × ES129*

Selected markers	Total recombinants tested	Unselected markers		
		Marker	No. found	Per cent of total
Leu^+ Cys^+	81	Lac^+	29	36
		Gal^+	13	16
		Non^+ [a]	2	2.5
		His^+	2	2.5
His^+ Cys^+	79	Leu^+	32	41
		Lac^+	27	37
		Gal^+	32	41
		Non^+ [a]	56	71

[a] Non^+ scored by ability to mutate to T7 resistance.

distinct from *Su-1*. To test this, transducing phage grown on ES366 was used to transduce ES369, an *Su-1*$^+$ strain, to His$^+$; 23% of the recombinants did not permit growth of T4D *amN82*, indicating the presence of the *Su-1*$^-$ allele in ES366. Table 3 gives the results of the three-factor transduction in which ES367, a mucoid, *Su-1*$^+$ *his* strain was the recipient. There was no cotransduction of the donor *non-9* and *Su-1*$^-$ markers indicating that they were too distant on the chromosome to be included in a single transducing fragment of deoxyribonucleic acid. The most probable order is *non-9 his Su-1*.

Determination of methylpentose. The polysaccharide composing the capsule of *E. coli* K-12 contains the methylpentose fucose (19, 24) that can be quantitatively determined by the Dische-Shettles reaction (8). The determinations for strains JC355 (Non$^+$ Cap$^+$), ES129 (*non-9*), ES368 (*non-9 capR9*), and ES365 (*capR9*) are shown in Table 4. Only ES365 was mucoid. The results confirm that the mucoid or nonmucoid phenotypes represent the presence or absence of a large amount of polysaccharide capsular material.

Nucleotide accumulation. Lieberman et al. (18) isolated a nonmucoid mutant, *non-2*, that, when present in the same genome with *capR9*, resulted in a large accumulation of nucleotides. ES368, a *capR9 non-9* strain, repeatedly gave an increased value of OD_{260} per 10^9 viable cells. A representative experiment is shown in Table 5. The OD_{260}: OD_{280} ratio of the extracted material was 2, indicative of nucleotides. The nucleotide accumulation in ES368 was not as large as that found by Lieberman et al. for their mutant.

DISCUSSION

E. coli B, the original host of the T phages and the strain in which most work concerning T phage cross-resistance has been done, is presumably a *capR non* double mutant. Donch and Greenberg (9) showed that strain B is UV sensitive because of a *capR* mutation. Although B itself is not mucoid, the *capR* allele of B produced a mucoid phenotype when transduced to strain K-12. This indicates a *non* mutation in *E. coli* B. B can mutate in one step to resistance to phages T3 and T4 (B/3, 4) or to resistance to T3, T4, and T7 (B/3, 4, 7). These mutants are nonmucoid, form at a frequency of about 10^{-6}, and the B/3, 4, 7 mutants have an altered cell wall lipopolysaccharide, the receptor site of phages T3, T4, and T7 (32). A more complex situation exists in *E. coli* K-12. Curtiss (5), selecting for T3 resistance and scoring for resistance to phages T4, T7, and λ and proline auxotrophy, found 10 different phenotypes in strain K-12. One of the phenotypes found by Curtiss, resistance to phages T3, T7, and λ and Pro$^-$, appeared to be caused by a deletion at minute 8 on the *E. coli* map of Taylor (29). Some of the 10 phenotypes were mucoid. In JC355, the parent of the *non-9* strain, all T7-resistant mutants were mucoid. The resistance is presumed to be caused by the covering of the phage receptor sites with excess capsular polysaccharide. In ES129, the original *non-9* strain, the *non-9* mutation prevented capsule formation and spontaneous T7-resistant mutants were not found. T7-resistant mutants were found in ES129 only after EMS mutagenesis. Because these mutants were sensitive to T2 and T6 and nonmucoid, they were considered to be altered in the lipopolysaccharide layer of the cell wall analogous to the B/3, 4, 7 mutation in *E. coli* B. The low frequency of T7-resistant mutations in ES129 and the difference in phenotypes of ES129 and *E. coli* B T7-resistant cells suggest that the

TABLE 3. *Analysis of the three-factor transduction involving the his, non-9, and Su-1 markers*[a]

Donor ES366, Recipient, ES367. His$^+$ transductants selected

Marker			No. of transductants
non-9	*his*	*Su-1*	
1[b]	1	0	74
0	1	0	51
0	1	1	15
1	1	1	0

[a] Marker *non-9* scored by mucoid or nonmucoid phenotype.

[b] 1, donor; 0, recipient phenotype.

TABLE 4. *Polysaccharide formation*

Strain	Relevant genotype	Polysaccharide[a]
JC355	Wild type	<1
ES129	*non-9*	<1
ES368	*non-9 capR9*	<1
ES365	*capR9*	74

[a] Expressed as micrograms of nondialyzable methylpentose per milliliter per unit of cell turbidity (optical density at 600 nm).

TABLE 5. *Nucleotide accumulation in stationary-phase cultures*

Strain	Relevant genotype	Viable cells/ml	OD_{260}[a]
ES366	*non-9*	1.8×10^9	0.773
MC100	Wild type	1.7×10^9	1.01
ES365	*capR9*	1.1×10^9	1.08
ES368	*capR9 non-9*	1.0×10^9	2.40

[a] Per 10^9 viable cells per milliliter.

cell wall lipopolysaccharide of *E. coli* K-12, the ancestor of ES129, differs from that of *E. coli* B.

The pathway of capsule polysaccharide biosynthesis in *E. coli* K-12 is thought to involve 11 enzymes (17, 21). Mutants of four of these have been identified and mapped: uridine diphosphogalactose 4-epimerase (*galE*) at minute 17 (4), uridine diphosphoglucose pyrophosphorylase (*galU*) at minute 25 (26), phosphomannose isomerase at minute 33 (22), and phosphoglucoisomerase at minute 79 (12). Grant et al. (13) showed that *galE* or *galU* mutants could not form a capsule. The location of *non-9* at minute 39 is distinct from these four. However, Donch and Greenberg (10) recently found a *his*-linked mutation, possibly allelic to *non-9*, that inhibited capsule formation. Lieberman et al. (18) isolated four Non$^-$ mutants (*non-1* to *4*) in a *capR9* strain. None has been mapped yet. The *non-3* was guanosine diphosphate-mannose pyrophosphorylase deficient (17); *non-2*, when present in the same genome as *capR9*, resulted in the accumulation of large amounts of nucleotides (18). In this respect, *non-9* resembles *non-2*, although the nucleotide accumulation was not as great as that found with *non-2*. Lieberman et al. suggested that the *non-2* strain contained an inhibitor of guanosine diphosphate-L-fucose synthetase. Enzymatic studies are needed to determine which step in the synthesis of capsular polysaccharide is affected by the *non-9* mutation.

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