JOURNAL OF BACTERIOLOGY, Aug. 1982, p. 529–533
0021-9193/82/080529-05$02.00/0

Vol. 151, No. 2

New Pleiotropic Alkaline Phosphatase-Negative Mutants of *Escherichia coli* K-12

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Received 8 January 1982/Accepted 14 April 1982

Escherichia coli K-12 mutants showing reduced alkaline phosphatase activity were isolated as 5-fluorouracil-plus-adenosine-resistant derivatives of a *upp pho* (either *phoS* or *phoT*) strain. One class of these mutants displayed a temperature-sensitive alkaline phosphatase-negative phenotype, a pleiotropic defect for growth on some substrates, an increased sensitivity to toxic compounds (e.g., EDTA, mitomycin, and chloramphenicol), and alterations in the expression of some membrane proteins. It phenotypically differed from previously described mutants. The mutation was located at min 8.5 close to the *phoA* gene and defines a new genetic locus we called *napA* (for negative alkaline phosphatase pleiotropic phenotype). As these mutants have lost the ability to grow on lactose and galactose, Lac^+ and Gal^+ revertants were isolated that simultaneously recovered the parental phenotype.

Cell envelope proteins of *Escherichia coli* are synthesized in the cytoplasmic compartment. They have to be exported into or across the cytoplasmic membrane to reach the extracytoplasmic compartments (4, 15, 19). A genetic analysis of protein export should help in understanding the molecular mechanisms involved in the export process.

E. coli alkaline phosphatase is a well-documented exported protein (8, 18). Its synthesis is derepressed after inorganic phosphate starvation. Four genes are related to its synthesis: *phoA* is its structural gene (13), *phoB* and *phoM* genes code for positive regulatory proteins, and the *phoR* gene encodes for a product which acts as a positive and negative regulatory protein (6, 18, 21).

Here, we describe the isolation of a new class of *E. coli* pleiotropic alkaline phosphatase-negative mutants. They were selected as fluorouracil-plus-adenosine-resistant derivatives of a *upp* strain. Fluorouracil-resistant *upp* strains can be sensitized again to fluorouracil if a source of ribose 1-phosphate, such as adenosine, is available and metabolized by exported enzymes involved in nucleoside uptake and metabolism. Using this selection procedure, we looked for resistant strains displaying an alkaline phosphatase-negative phenotype. Several classes of pleiotropic mutants were isolated by this method. In this paper, we present the properties of mutations closely linked to the *phoA* locus.

MATERIALS AND METHODS

Bacterial strains. All bacterial strains used in this study and listed in Table 1 were *E. coli* K-12 derivatives.

Media. Cells were grown either in rich L medium (12), in minimal Tris medium (T) (20) or in 63 medium (12). Minimal media were supplemented with 0.4% glucose (G), 1 μg of thiamine hydrochloride per ml, and appropriate amino acids. To derepress cells for alkaline phosphatase synthesis, we used TG medium supplemented with 0.25% proteose peptone (TGLP medium).

Sensitivity to drugs and phages. Sensitivity to drugs was tested by spotting 10 μl of a drug solution on 2×10^8 bacteria spread on L agar plates. After 16 h of incubation at 42°C, the growth was scored. Phage sensitivity of mutants was tested by cross-streaking.

Growth on carbon sources. Growth analyses were performed at 42°C on 63 medium plates containing either sugars (5 mM), nucleosides (5 mM), or nucleotides (3 mM).

Crude cellular extracts. Cells were disrupted by sonication with a Sorensen oscillator (80 kC) for 10 min, while the suspension was maintained below 10°C. Sonic extracts were purified by centrifugation (12,000 × *g* for 20 min at 4°C), and supernatants were used to assay soluble enzyme activities.

Preparation of membrane fractions. Cells were labeled with 50 μCi of sulfate-35 during three generations of growth, at 42°C in TGLP medium. They were harvested during the mid-logarithmic phase of growth. Inner and outer membranes were separated by isopycnic centrifugation as described previously (14), except that spheroplast formation was induced with 130 μg of lysozyme per ml (17,000 U/mg), and spheroplast dis-

TABLE 1. Bacterial strains

Strain	Genotype[a]	Source/reference
188	Hfr *thi metB1 lacI phoS,T*	Spontaneous arsenate-resistant derivative of strain Gal5 (11)
323	Hfr *thi metB1 lacI phoS,T deoC*	Spontaneous revertant of strain 188 *thyA* able to grow with 2 μg of thymine per ml (7)
49D	Hfr *thi metB1 lacI phoS,T deoC upp*[b]	Spontaneous 5-fluorouracil (2.5 μg/ml)-resistant derivative of strain 323 (1)
459	Hfr *thi metB1 lacI phoS,T deoC upp napA*	This work
459R1	Hfr *thi metB1 lacI phoS,T deoC upp*	Spontaneous Lac$^+$ revertant of strain 459
459R2	Hfr *thi metB1 lacI phoS,T deoC upp*	Spontaneous Gal$^+$ revertant of strain 459
LEA145	F$^-$ *lacZ proC tsx trp rpsL xyl mtl*	E. Yagil
LEA145.1	F$^-$ *lacZ proC tsx trp rpsL xyl mtl upp*	5-Fluorouracil-resistant transductant of strain LEA145 with P1 grown on strain 49D
678	F$^-$ *proC tsx trp rpsL xyl mtl upp ΔphoA8*	Lac$^+$ AP$^-$ transductant of strain LEA145.1 with P1 grown on strain E15 (*ΔphoA8*)

[a] Genetic nomenclature is from Bachmann and Low (2), except for *napA*, which stands for negative alkaline phosphatase pleiotropic phenotype.

[b] The mutation responsible for resistance to 5-fluorouracil was identified as a *upp* mutation by cotransduction with the *purC* marker.

ruption was performed with a Branson Sonifier at 40 W with 4 pulses of 15 s each.

Polyacrylamide gel electrophoresis and fluorography. Sodium dodecyl sulfate (SDS)-polyacrylamide gel (10% acrylamide) electrophoresis was performed as described by Laemmli (10). Electrophoresis was carried out at room temperature during 4 h at 150 V with 12-cm-long slabs or overnight at 90 V with 18-cm-long slabs. Gels were stained and destained as previously described (11). For fluorography experiments, gels were soaked in En^{3}Hance scintillator solution for 1 h. The excess of scintillator was precipitated with water. Dried gels were exposed to RP Royal X-Omat film at −70°C. Films were scanned with a Vernon Phi5 densitometer (Vernon, Paris, France).

Chemicals. Mitomycin C, chloramphenicol, 5-fluorouracil, nucleosides, nucleotides, and alkaline phosphatase substrates were purchased from Sigma Chemical Co., St. Louis, Mo. Nitrosoguanidine and lysozyme were obtained from Fluka A.G., Buchs, Switzerland and Merck E., Darmstadt, West Germany, respectively. Radioactive inorganic sulfate (42.8 Ci/mg) and En^{3}Hance were purchased from the Radiochemical Centre, Amersham, England and New England Nuclear Corp., Boston, Mass., respectively. Other reactants used were of analytical grade.

TABLE 2. Sensitivity of mutant 459 to growth inhibitors[a]

Strain	EDTA[b]			Mitomycin				Chloramphenicol		
	50	100	200	0.1	0.25	0.5	1	2.5	5	10
49D (wild type)	+	+	−	+	+	+	−	+	+	−
459	+	−	−	−	−	−	−	−	−	−

[a] Sensitivity to growth inhibitors was tested at 42°C as described in the text. +, Growth; −, no growth around spots.

[b] Growth inhibitor concentrations are expressed in micrograms per 10 μl.

RESULTS

Isolation of mutants. Strain 49D was mutagenized with nitrosoguanidine (12), and mutants were selected at 42°C for resistance to 2.5 μg of 5-fluorouracil per ml in the presence of 1.5 mM adenosine on 63-glucose medium plates. These mutants were tested for pleiotropy by examining their alkaline phosphatase activity by a qualitative test previously described (5) and their resistance to 7.5 μg of 5-fluorouracil per ml in the presence of 2 mM AMP (3′-AMP or 5′-AMP). Those which simultaneously became alkaline phosphatase deficient and resistant to fluorouracil in the presence of AMP were studied further.

We analyzed previously described mutants isolated as resistant to 5-fluorouracil plus a nucleotide (*ush*, *ompB*, and *ompF* mutants [3]) and showed that they were not altered for alkaline phosphatase activity. On the other hand, a *upp phoB* strain (*phoB23* mutation [21]) remained sensitive to fluorouracil plus a nucleoside or a nucleotide.

TABLE 3. Alkaline phosphatase activity in mutant 459

Strain	Alkaline phosphatase sp act[a] after growth at (°C):	
	37	42
49D	810	323
459	137	2

[a] Cells grown in TGLP medium were harvested in late-exponential growth. Quantitative assay for alkaline phosphatase (with 10^{-3} M *p*-nitrophenyl-phosphate as the substrate) was performed as described (17). One unit of alkaline phosphatase is defined as the amount of enzyme which hydrolyzes 1 nmol of substrate per min. Specific enzyme activities are expressed as units per milligram of bacterial dry weight.

TABLE 4. Genetic characterization of *napA* mutations

Donor strain	Recipient strain	No. of Pro$^+$ recombinants analyzed[a]	Unselected characters[b]					
			AP$^-$	AP$^+$	β-gal (Con)		β-gal$^-$	
					AP$^-$	AP$^+$	AP$^-$	AP$^+$
459	LEA145.1	122	95	27	26	0	69	27
459	678 (Δ*phoA8*)	69	69	0	—	—	—	—

[a] Transduction with bacteriophage P1 *kc* was carried out as described previously (12). Pro$^+$ recombinants were selected at 37°C on 63 medium supplemented with glucose and tryptophan and purified twice by single-colony isolation before further analysis.

[b] Mutant 459 did not grow on lactose, but synthesized β-galactosidase constitutively, as did parental strain 49D. β-gal (Con), β-Galactosidase constitutive synthesis; β-gal$^-$, no β-galactosidase activity; AP$^+$, derepressible alkaline phosphatase activity; AP$^-$, no alkaline phosphatase activity. These phenotypes were tested as described in the text.

Phenotypic properties of pleiotropic mutants. Several classes of pleiotropic alkaline phosphatase-negative mutants were isolated. Properties of 459-like mutants will be described. Mutant 459 was more sensitive to EDTA, mitomycin C, and chloramphenicol (Table 2) and had lost the ability to grow on glycerol, arabinose, lactose, galactose, maltose, xylose, ribose, adenosine, uridine, 3'-AMP and 5'-AMP. Tests for phage sensitivity revealed that it was more resistant to bacteriophage λ *vir*. Mutant 459 and parental strain 49D were equally sensitive to lipopolysaccharide-requiring phages and to phages that require the major outer membrane proteins.

Quantitative assays for alkaline phosphatase activity showed that mutant 459 was deficient and not cryptic for this enzyme (Table 3) (3). Moreover, its alkaline phosphatase content was

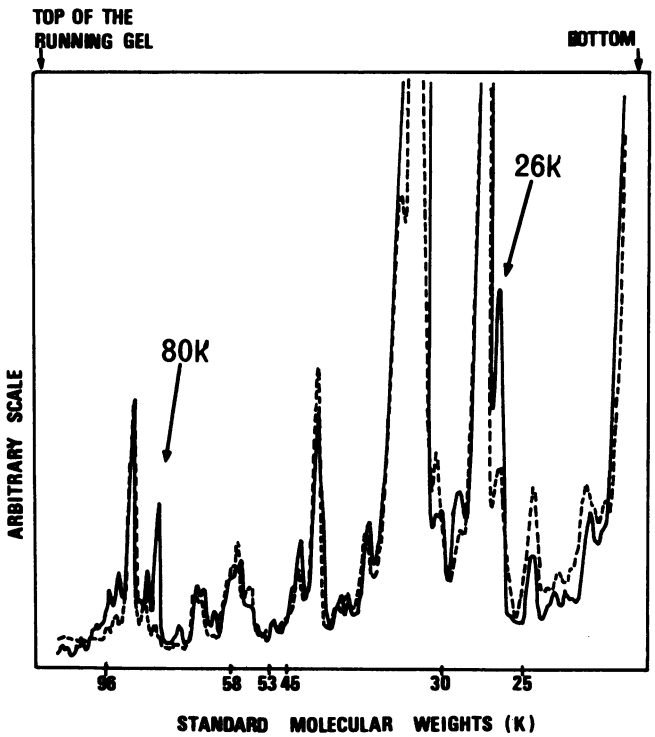

FIG. 1. SDS-polyacrylamide gel electrophoresis patterns of outer membrane proteins from strains 49D (——) and 459 (– – –). A sample of outer membrane proteins equivalent to 2 × 10^7 cells was subjected to electrophoresis. The slab gel was 18 cm long. The autoradiography was performed by exposing the gel for 218 h. Molecular weights, as indicated on the figure, were determined using phosphorylase *a* (96,000), catalase (58,000), glutamic dehydrogenase (53,000), *E. coli* alkaline phosphatase (monomer 45,000), carbonic anhydrase (30,000), chymotrypsinogen (25,000), and lysozyme (14,000) as standards.

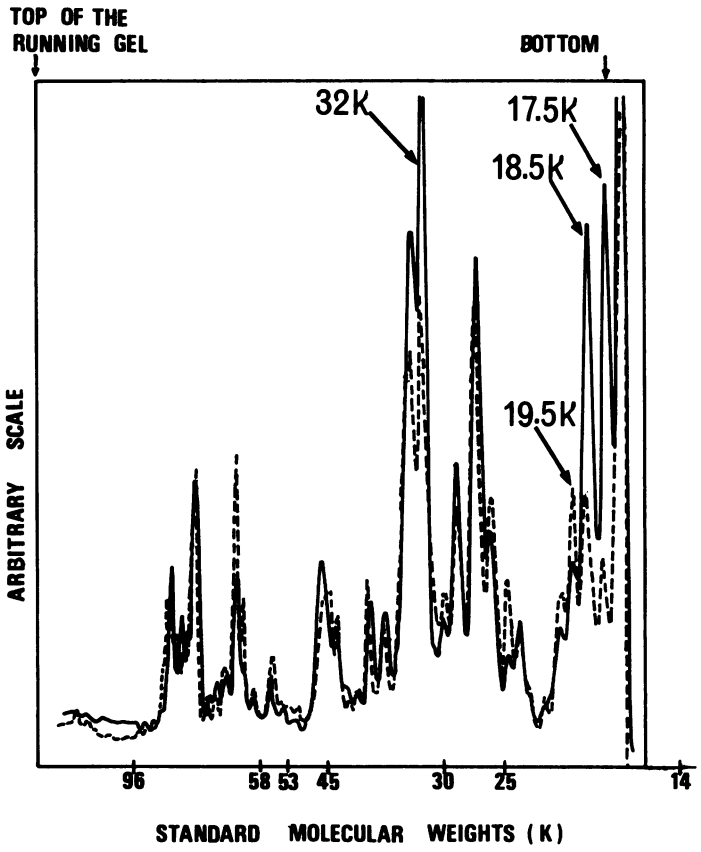

FIG. 2. SDS-polyacrylamide gel electrophoresis patterns of inner membrane proteins from strains 49D (——) and 459 (– – –). A sample of inner membrane proteins equivalent to 5×10^7 cells was subjected to electrophoresis. The slab gel was 12 cm long. For autoradiography, gel exposition was 240 h long. Molecular weights, as indicated on the figure, were determined using standards as described in the legend to Fig. 1.

temperature sensitive. Quantitative assays of other periplasmic activities (5'-nucleotidase, 3'-nucleotidase, RNase I, chromosomal β-lactamase, thymidine phosphorylase, uridine phosphorylase, and purine phosphorylase) showed these enzymes were present in normal amounts in mutant 459.

Revertants of mutant 459 appeared spontaneously with frequencies of 10^{-6} and 10^{-7}, respectively. Most of them recovered a parental phenotype, which strongly suggests that the mutation responsible for pleiotropic properties of mutant 459 is a single mutation.

Location of *napA* mutations. The mutation responsible for mutant 459 properties was found to be 78% cotransducible with the *proC* gene, which is located close to the *phoA*, *phoB*, and *phoR* genes (Table 4) (9, 13). However, unlike any known pleiotropic alkaline phosphatase-negative mutation, situated on the *tsx* side of the *proC* gene (21), the strain 459 mutation was located by three-factor crosses on the *lac* side of *proC* (Table 4). We denoted this new locus as *napA* (for negative alkaline phosphatase pleiotropic phenotype). *napA* mutations were more precisely localized very close to the *phoA* gene, as no Pro^+ AP^+ recombinants could be isolated with strain 459 as a donor and strain 678 (Δ*phoA proC*) as a recipient (Table 4).

Effect of *napA* mutations on cytoplasmic and cell envelope proteins. Using stained SDS-polyacrylamide slab gels, the only detectable effect of *napA* mutations on soluble cytoplasmic and periplasmic proteins after electrophoresis was the absence of the alkaline phosphatase monomer. However, modifications of the inner and outer membrane protein profiles could be identified after ^{35}S labeling of cell proteins (Fig. 1 and 2). Two outer membrane proteins of apparent molecular weights 80,000 and 26,000 and three inner membrane proteins of apparent molecular weights of 32,000, 18,500 and 17,500 were present in reduced amounts in mutant 459 extracts. One inner membrane protein of apparent molec-

ular weight 19,500 was present in an increased amount.

DISCUSSION

Mutant 459, described in this study, was selected for its ability to grow in the presence of fluorouracil plus adenosine or AMP. This selection has been used previously (3) and yielded mutants with altered membranes, carrying mutations in the *ompF* and *ompB* genes at min 21 and 74.5 respectively. Mutant 459, however, had a single mutation located at min 8.5 in the *napA* locus between *lac* and *proC* genes. Undoubtedly, the identification of this new class of mutants was made possible by the requirement for both fluorouracil-plus-adenosine-resistant and alkaline phosphatase-negative phenotypes.

Clearly, the gene altered in 459-like mutants confers new properties on the host: a temperature-sensitive alkaline phosphatase-negative phenotype, an inability to grow on many carbon sources, an increased sensitivity to growth inhibitors, and decreased amounts of inner and outer membrane proteins.

Mutant 459, which lost the ability to grow on lactose, 3'-AMP, adenosine and uridine, but contained the same β-galactosidase, cyclic phosphodiesterase, purine phosphorylase, and uridine phosphorylase activities as its parental strain, might be altered in the transport of these compounds. Moreover, mutant 459 did not grow on maltose and was more resistant to bacteriophage λ *vir*. These phenotypes cannot be simply explained by a deficiency in the *lamB* product, since mutant 459 did not grow on 5 mM maltose (16).

Perhaps, mutant 459 has a general membrane defect causing a variety of mutant phenotypes. Additional experiments are in progress to determine the nature and the function of the *napA* gene product.

ACKNOWLEDGMENTS

We gratefully acknowledge our many colleagues for their gifts of bacterial strains and bacteriophages. We are indebted to Danièle Atlan for excellent technical assistance and to Simone Ottomani for preparing microbiological media.

This work was supported by research grants from the Centre National de la Recherche Scientifique (LP 05421 and ATP A651-3074), the Institut National de la Santé et de la Recherche Médicale (ATP 72-79-104), and the Fondation pour la Recherche Médicale. M.H. was supported, in part, by a predoctoral fellowship from the Délégation à la Recherche Scientifique et Technique.

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