

Isolation and Characterization of a Phosphonomycin-Resistant Mutant of *Escherichia coli* K-12

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A mutant was isolated from *Escherichia coli* K-12 which showed increased resistance towards phosphonomycin, a new bactericidal antibiotic recently isolated from strains of *Streptomyces*. Evidence is presented which suggests that this mutant is resistant to lysis by phosphonomycin because of a lower affinity of phosphoenolpyruvate:uridine diphospho-*N*-acetylglucosamine enolpyruvyl transferase for this antibiotic. This mutant was also found to be temperature-sensitive in growth. At 42 C mutant cells grew poorly, and the rate of incorporation of ³H-diaminopimelic acid into trichloroacetic acid-insoluble material was also greatly reduced. Genetic studies indicate that the increased resistance toward phosphonomycin and temperature sensitivity in growth of this mutant are probably the consequences of a single mutation.

It has been shown that the first unique reaction in the biosynthesis of uridine diphosphate (UDP)-*N*-acetylmuramyl peptides in microorganisms is the transfer of enolpyruvate from 2-phosphoenolpyruvate (PEP) to the C-3 position of glucosamine in UDP-*N*-acetylglucosamine (UDPGlcNAc) (3, 12). Anwar and his co-workers have purified this enzyme, PEP:UDPGlcNAc enolpyruvyl transferase, from *Enterobacter cloacae* (4). The second enzyme in the pathway of biosynthesis of cell wall precursors, UDP-*N*-acetyl enolpyruvylglucosamine reductase, has also been characterized (13).

Phosphonomycin (1,2-epoxypropylphosphonic acid), a bactericidal antibiotic recently isolated from strains of *Streptomyces*, has been shown to be a potent and irreversible inhibitor of PEP:UDPGlcNAc enolpyruvyl transferase (1, 5). Phosphonomycin presumably enters the bacterial cell via either of at least two inducible transport systems for phosphate esters, namely the L- α -glycerophosphate transport system (*glpT*) and the hexose phosphate transport system (*uhp*) in *Escherichia coli* (7). Thus, most mutants resistant to phosphonomycin were found to be defective in the uptake of L- α -glycerophosphate or glucose-6-phosphate, thus unable to grow on one of these two compounds as the sole carbon source (F. Kahan, *personal communication*). However, in addition to this decreased penetration of a

drug as a mechanism of drug resistance in bacteria, we can envisage several other biochemical mechanisms, including an increased degradation of the drug (or decreased conversion of an inactive to an active principle), an increased concentration of a metabolite antagonizing the drug, derepressed synthesis of the target enzyme, activation of an alternative metabolic pathway bypassing the drug-inhibited reaction, a decreased requirement for the final product, and an altered enzyme with a reduced affinity for the drug (2).

Among the above-mentioned types of drug-resistant mutants, we are mainly interested in those in which the drug resistance is either due to derepression in the synthesis of the target enzyme or due to decreased affinity of the mutant enzyme towards the drug. Thus one might expect among *E. coli* mutants resistant to phosphonomycin ones with over-production of PEP:UDPGlcNAc enolpyruvyl transferase and those having modified transferase with decreased affinity for phosphonomycin or PEP or both. In our preliminary experiments, we have confirmed that the most frequently isolated mutants of *E. coli* resistant to phosphonomycin were those which had lost their ability to grow on minimal media with L- α -glycerophosphate or glucose-6-phosphate as sole carbon source, while remaining capable of growing on minimal media containing glucose or glycerol. The drug resistance in these mu-

tants is presumably due to defects in the entry of the drug into the mutant cells. In this paper, we shall describe the isolation of a phosphonomycin-resistant mutant from *E. coli* K-12 which grows normally in minimal media containing L- α -glycerophosphate or D-glucose-6-phosphate as sole carbon source. Preliminary characterization of this mutant suggests that the resistance toward phosphonomycin is due to a decreased affinity of PEP:UDPGlcNAc enolpyruvyl transferase toward this analogue of PEP. Likewise, there appears to be a corresponding increase in the K_m of this enzyme for PEP, which may explain the decreased rate of peptidoglycan synthesis and of growth and division of the mutant cells at 42 C.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* K-12, strain AB1157, (F⁻*arg*⁻*thr*⁻*his*⁻*pro*⁻*leu*⁻*thi*⁻*lac*⁻*gal*⁻*ara*⁻*xyl*⁻*mtl*⁻*strA*⁺*tsx*⁺) was used as the parental wild-type strain in this study. The genetic markers of this strain and those of HfrC and HfrH were described previously (14). The growth conditions and the composition of the rich media (L broth and PPBE broth) were the same as previously described (14). The minimal medium used in the present study was M₉ medium which contains 0.6% Na₂HPO₄, 0.3% KH₂PO₄, 0.05% NaCl, 0.1% NH₄Cl, 0.024% MgSO₄, and 0.011% CaCl₂.

Mutagenesis. Mutagenesis of *E. coli* cells with ethyl methane sulfonate (EMS) was carried out by the method of Lin et al. (8).

Isolation of phosphonomycin-resistant mutants. An overnight culture of the wild-type strain AB1157 in PPBE broth was diluted 50-fold with fresh media and incubated at 37 C for 2 hr. EMS was then added at a concentration of 0.03 ml of EMS per 2 ml of culture. After reincubation at 37 C for 2 hr, the mutagenized cells were centrifuged, washed, and allowed to grow out in PPBE broth at 30 C overnight for phenotypic expression. Phosphonomycin was then added to this culture to a final concentration of 0.3 mM. Killing of the sensitive cells by this drug was allowed to proceed at 30 C for 5 to 6 hr. The remaining viable cells were then centrifuged, washed, and allowed to grow in PPBE broth at 30 C overnight. The survivors were found to be mostly transport mutants of any of the following three types: *glpT*⁻, *uhp*⁻, or pleiotrophic *ctr*⁻ (7). To eliminate these transport mutants, we inoculated a sample of the PPBE broth culture into minimal medium (M₉ containing, per milliliter of culture, 20 μ g each of histidine, proline, arginine, threonine, and leucine, and 5 μ g of thiamine, with 0.04 M DL- α -glycerophosphate as the sole carbon source, followed by a similar cycle of counter-selection with 0.01 M D-glucose-6-phosphate as the sole carbon source. This selection procedure, with phosphonomycin in PPBE broth, followed by growth in minimal media with L- α -glycerophosphate (or D-glucose-6-phosphate) as sole carbon sources, was repeated. Finally the survi-

vors of this procedure were plated on M₉ plates containing amino acid supplements and L- α -glycerophosphate as the carbon source. The colonies were picked and replica-plated for temperature sensitivity in growth on PPBE plates. Those with poorer growth at 42 than at 30 C were selected for further studies.

Assay of PEP:UDPGlcNAc enolpyruvyl transferase activity. Cells were grown in L broth at 30 C and harvested at the early stationary phase of growth [optical density at 600 nm (OD₆₀₀) = 1–1.2]. Cells were washed once with distilled water (approximately 25 times the volume of the wet cells), followed by washing with ice-cold acetone (approximately 25 times the volume of the wet cells). The acetone-washed cells were dried in vacuo at room temperature for 2 to 3 hr. Acetone-treated powder was stored at –80 C and retained the activity of this enzyme for at least 6 months. The crude enzyme preparation was made by extracting the acetone powder (200 mg) with 1.2 ml of tris(hydroxymethyl) aminomethane (Tris)-Hydrochloride (pH 7.4) containing 4 mM dithiothreitol.

¹⁴C-labeled PEP was prepared enzymatically from 3-phospho-D-glycerate-*U*-¹⁴C essentially according to the procedure of Gunetileke and Anwar (4). The product was separated from the reaction mixture by paper chromatography on Whatman no. 3 MM paper, using a solvent system of *n*-butanol-formic acid (88%)-diisopropyl ether (3:2:1).

PEP:UDPGlcNAc enolpyruvyl transferase activity was assayed according to a procedure kindly made available to us by F. Kahan. Each assay mixture in a final volume of 100 μ liters contains 10 μ moles of sodium maleate (pH 6.8), 1 μ mole of KF, 8 nmoles of dithiothreitol, 30 nmoles of PEP-*U*-¹⁴C (300 counts per min per nmole) and 500 nmoles of UDP-GlcNAc. The reaction mixture was incubated at 37 C for varying periods of time (usually 5 to 20 min), using amounts of crude extracts containing 50 to 200 μ g of protein. The reaction was stopped by the addition of 0.5 ml of 5% trichloroacetic acid containing sodium pyrophosphate. The precipitated proteins were removed by centrifugation at 1,000 \times *g* at room temperature for 10 min, and the supernatant fluid was transferred to a tube containing 0.1 ml of a suspension of charcoal (100 mg/ml). The pellet was washed with 0.5 ml of 0.05 M ammonium acetate, and the washing was added to the charcoal suspension. The charcoal suspension was kept in ice for 30 min with occasional mixing. The suspension then was passed through a small column packed with glass wool and washed with 10 ml of 0.05 M ammonium acetate, pH 3.8. The charcoal column was then eluted with 2 ml of a mixture of 95% ethanol-0.05 M NH₄OH (1:1). The eluate was counted in a Beckman scintillation counter LS-230, using 10 ml of Patterson-Greene's scintillation solution (10).

The rate of the transferase reaction assayed by the method described here was constant for at least 20 min; the rate was also constant with increasing amounts of crude extracts up to 500 μ g of protein.

Measurements of DAP incorporation. To cells at early logarithmic phase of growth (OD₆₀₀ = 0.1) in PPBE broth or L broth were added L-lysine and ³H-labeled diaminopimelic acid (DAP) (65 mCi/mmole)

to final concentrations of 0.5 mg/ml and 1 μ Ci/ml of culture, respectively. Samples (1 ml) of cell suspension were taken at regular intervals of time into chilled tubes containing 0.5 ml of 50% trichloroacetic acid and 1 ml of bovine serum albumin (1 mg/ml). The acid precipitates were collected by filtration through 24-mm glass fiber paper (Whatman GF/c), and washed with 10 ml of 10% trichloroacetic acid followed by 10 ml of 95% ethyl alcohol. The filters were dried with an infrared lamp, and the radioactivity was measured in a Beckman scintillation counter with toluene-based scintillation solution. At the end a sample of the acid precipitate was also hydrolyzed in 6 N HCl at 100 C for 20 hr. The hydrolysate, after removal of HCl, was spotted on Whatman no. 1 paper and subjected to high-voltage electrophoresis at pH 3.5 in pyridine-acetic acid-water (1:10:440) for 30 min at 70 v/cm to separate DAP and lysine. Over 90% of the incorporated radioactivity was recovered as DAP.

Assays. Protein concentrations were determined by the method of Lowry et al. (9) or by the biuret method (6).

Chemicals. All chemicals used in the present study were reagent grade, purchased from commercial sources. 3-Phospho-D-glycerate- U - 14 C (65 mCi/mmole) was purchased from Calatomic Corp.; 2,6-diaminopimelic acid- G - 3 H (200 μ Ci/ μ mole) was purchased from Amersham Searle Corp. Phosphonomycin was a generous gift of F. Kahan, Merck Sharp and Dohme Res. Lab., Rahway, N.J.

RESULTS

Isolation of phosphonomycin-resistant mutants of *E. coli*. We have selected a number of phosphonomycin-resistant mutants of *E. coli* which were also temperature-sensitive in growth. That the drug resistance is not simply due to a mutation in *glpT* or *uhp* is evidenced by their abilities to grow on L- α -glycerophosphate and glucose-6-phosphate in the absence of derepressed alkaline phosphatase. Most of the mutants that we selected on this basis turned out to be slow growers at both 30 and 42 C, and they might have been partially selected by the phosphonomycin treatment since the drug probably kills growing cells only. Most of them showed distinct morphological aberrations at 42 C (filamentation, bulging, spheroplast, bizarre shape, etc.). None of these mutants had an elevated level of PEP:UDPGlcNAc enolpyruvyl transferase activity in the crude extract. Only one mutant, strain E187, showed increased resistance of this enzyme towards phosphonomycin in vitro.

Growth properties of mutant strain E187. The minimal concentration of phosphonomycin to lyse cells of mutant E187 in PPBE broth was very high in comparison with that of the wild-type strain AB1157 (Table 1). As ex-

pected, this minimal concentration for the lysis of the mutant cells could be lowered by prior growth in media containing L- α -glycerophosphate or D-glucose-6-phosphate, presumably due to induction of these two transport systems (*glpT* and *uhp*, respectively). Strain E187 retained all the genetic markers of AB1157, and showed a phage susceptibility pattern identical to that of AB1157. Like the wild-type strain, E187 cells grew on minimal media containing glucose, glycerol, DL- α -glycerophosphate, or D-glucose-6-phosphate as sole carbon source and on DL-glycerophosphate as sole phosphate source. However, E187 cells grew poorly in rich media at 42 C and became elongated and less rigid before gradually undergoing lysis to become ghosts (Fig. 1). At 30 C, especially in the presence of glucose, E187 formed mucoid colonies on enriched or minimal media. As can be seen from Fig. 2 and 3, both the rate of growth (increase in turbidity) and that of cell wall synthesis (3 H-DAP incorporation into trichloroacetic acid-insoluble material) were unaffected by 0.3 mM phosphonomycin in PPBE broth culture at both 30 and 42 C, whereas the wild-type cells were lysed rapidly by this concentration of phosphonomycin. The lysis of the wild-type cells was accompanied by cessation of 3 H-DAP incorporation into trichloroacetic acid-precipitable materials, as well as by degradation of preexisting cell walls. As can be seen in Fig. 3, the reduced rate of growth of the mutant cells at 42 C in the absence of phosphonomycin was accompanied by a concomitant decrease in 3 H-DAP incorporation into trichloroacetic acid-insoluble materials.

Inhibition of PEP:UDPGlcNAc enolpyruvyl transferase by phosphonomycin. When a crude extract of the wild-type strain was preincubated with 0.05 mM phosphonomycin at 37 C, the activity of PEP:UDPGlcNAc enolpyruvyl transferase was irre-

TABLE 1. Inhibition of growth by phosphonomycin

Pregrown on	Minimal phosphonomycin concn for lysis in PPBE broth (mM)	
	Wild type	Mutant E187
M ₉ + a.a. ^a + glucose	0.006	0.3
M ₉ + a.a. + DL- α -glycerophosphate	0.006	0.03
M ₉ + a.a. + D-glucose-6-phosphate	0.003	0.03

^a Amino acids.

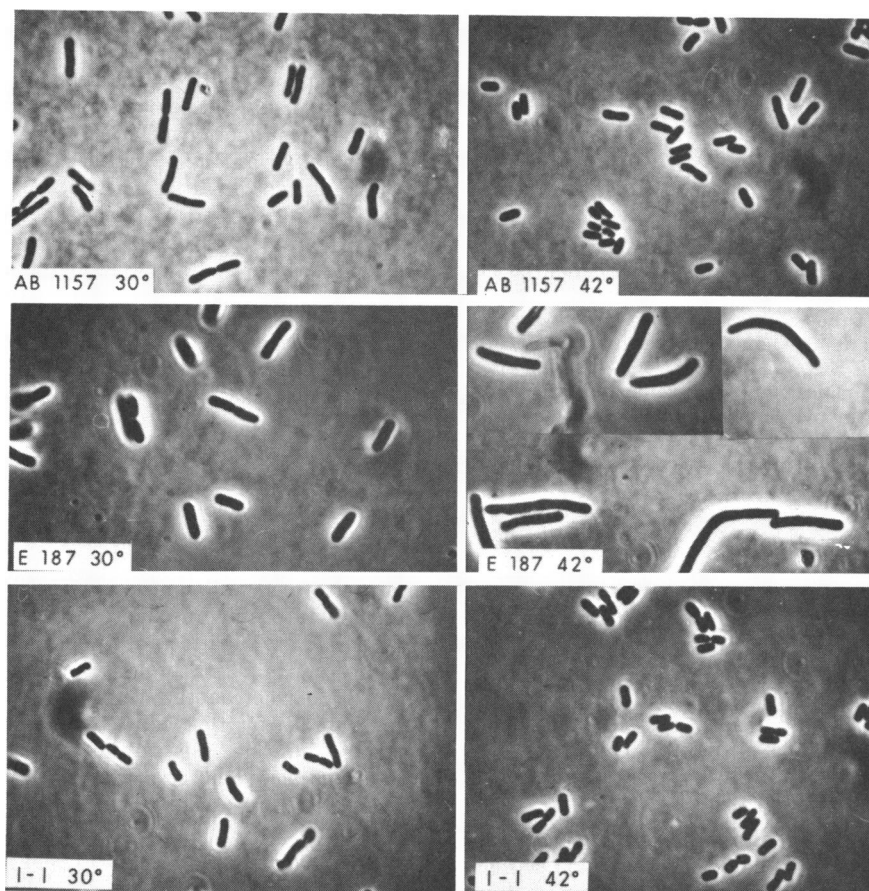


FIG. 1. Phase-contrast micrographs of cells of strain AB1157, E187, and temperature-resistant E187 recombinant (1-1), grown in *L* broth at 30 and 42 C. Cells were grown in *L* broth at 30 and 42 C, respectively, for 3 to 4 hr, and phase-contrast micrographs were taken at a magnification of 1,000 \times .

versibly inactivated, as shown in Fig. 4. We have confirmed the observation by Kahan and his co-workers (F. Kahan, *personal communication*) that the inactivation of this enzyme by phosphonomycin required the presence of UDPGlcNAc (Table 2). On the other hand, the activity of this enzyme in the crude extract of E187 cells was more resistant to this inactivation by phosphonomycin (Fig. 4). Mixing experiments (Table 3) indicated that this increased resistance of E187 crude extract to phosphonomycin inhibition is probably not due to a soluble factor protecting the transferase from inhibition by phosphonomycin (e.g., elevated level of PEP) nor because of inactivation of phosphonomycin by E187 crude extract. A mixture of PEP:UDPGlcNAc enolpyruvyl transferase active fractions of an ammonium sulfate fractionation of the crude extracts from AB1157 and from E187 showed

intermediate sensitivity toward phosphonomycin (Table 3).

Affinity of PEP:UDPGlcNAc enolpyruvyl transferase for PEP and for phosphonomycin in AB1157 and E187. When increasing amounts of phosphonomycin were added to the crude extracts of wild-type and mutant cells in the presence of a saturating concentration of UDPGlcNAc but varying amounts of PEP, the kinetic data shown in Fig. 5 were obtained. Thus the mutant enzyme appeared to have fourfold lower affinity toward phosphonomycin than that of the wild-type enzyme. This might account for the apparent resistance of this mutant toward phosphonomycin both *in vitro* and *in vivo*. Likewise, there was a three- to fourfold increase in the K_m for PEP of the mutant enzyme as compared to that of the wild type (Fig. 6).

Correlation of temperature sensitivity in

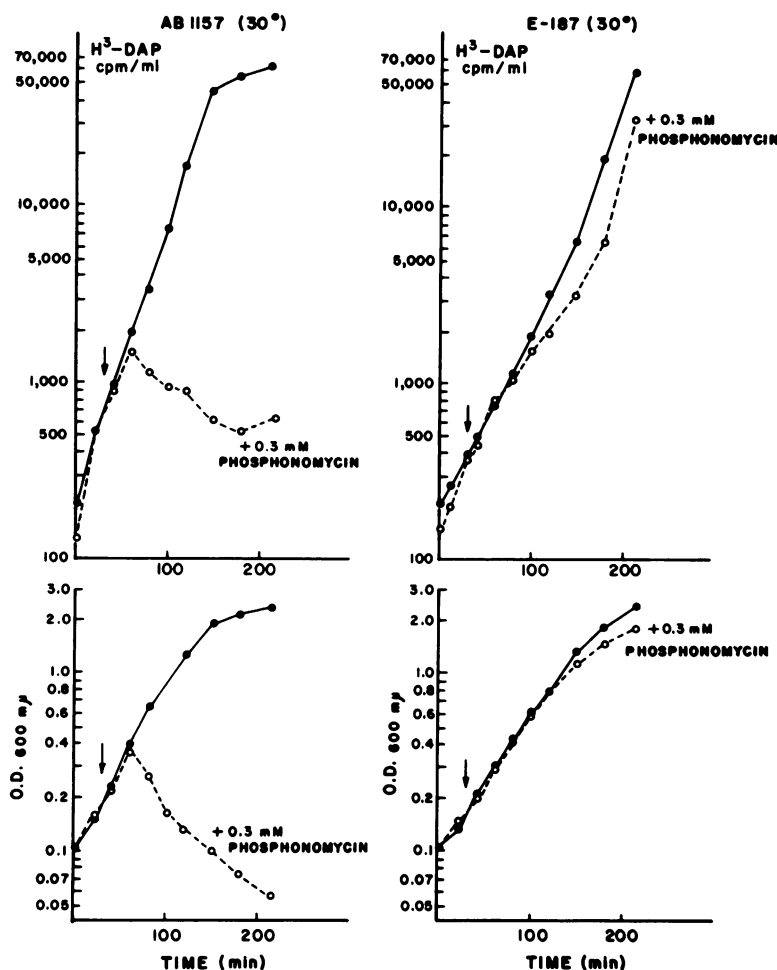


FIG. 2. Effect of phosphonomycin on growth and ^3H -DAP incorporation into trichloroacetic acid-insoluble material of AB1157 and E187 cells at 30 C. Cells were grown in 25 ml of PPBE broth at 30 C. Growth was followed by measurements of the optical density of the culture at 600 nm in a Zeiss spectrophotometer. Diaminopimelic acid- G - ^3H (DAP) (200 $\mu\text{Ci}/\mu\text{mole}$) and L -lysine were added to final concentrations of 1 $\mu\text{Ci}/\text{ml}$ and 500 $\mu\text{g}/\text{ml}$, respectively. Phosphonomycin was added to one of a pair of identical cultures to a final concentration of 0.3 mM, at the time indicated by arrow. At regular intervals of time, incorporation of ^3H -DAP into acid-insoluble material was measured as described in Materials and Methods.

growth and phosphonomycin resistance in strain E187. Since strain E187 was obtained following EMS mutagenesis, it is conceivable that these two phenotypes, i.e., resistance to phosphonomycin and temperature sensitivity in growth, might be totally unrelated phenomena resulting from separate mutational events. To examine this possibility, spontaneous temperature-resistant revertants of strain E187 were isolated. Temperature-resistant revertants were found to be as sensitive to phosphonomycin as the wild-type strain, both in vivo and in vitro (Table 4 and Fig. 4). It thus appeared likely that the temperature sen-

sitivity in growth and phosphonomycin resistance were the consequences of a single mutational event.

Preliminary data on the mapping of the E187 mutation suggested the chromosomal location to be near the *argECBH* cluster at minute 77. Temperature-resistant recombinants could be obtained readily by conjugation between HfrC and E187 but not between HfrH and E187. The distribution of unselected markers among these *t'⁺strA^r* recombinants is given in Table 5. Four temperature-resistant *strA^r* recombinants were studied for phosphonomycin sensitivity. All four recombinants (Table

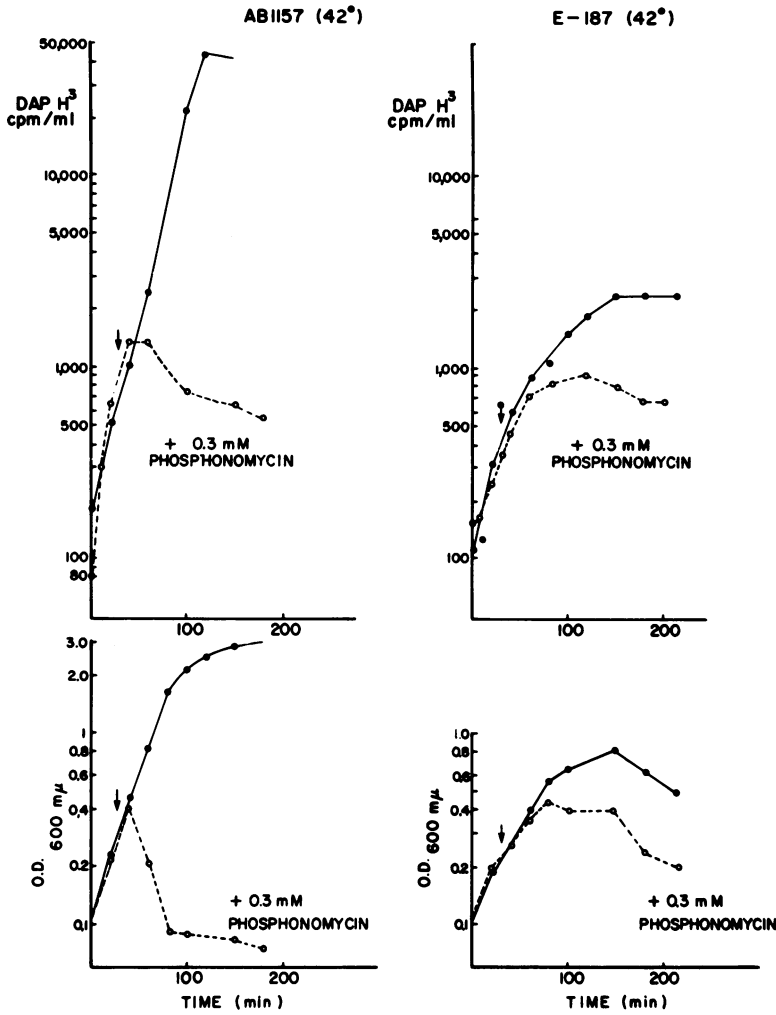


FIG. 3. Effect of phosphonomycin on the growth and ^3H -DAP incorporation into trichloroacetic acid-insoluble material of AB1157 and E187 cells at 42 C.

7) were sensitive to phosphonomycin, both in vivo and in vitro (Table 6). However, mapping of the E187 mutation by P1 transduction showed no cotransduction between *argH* and the E187 mutation.

DISCUSSION

The elucidation of the complex pathway leading to the biosynthesis of peptidoglycan is intimately related to the discovery of many potent antibiotics and the studies of their mechanisms of action. Phosphonomycin, like most bactericidal antibiotics, inhibits the biosynthesis of the bacterial cell wall, which is singularly important in the maintenance of the mechanical integrity and osmotic stability of a

bacterial cell. Moreover, peptidoglycan is unique to bacterial cells as compared to animal and human host cells and hence is an ideal target for selective chemotherapy.

The present work concerns the isolation and characterization of a phosphonomycin-resistant mutant of *E. coli*. As previous work on D-cycloserine-resistant mutants has demonstrated, drug-resistant mutants are useful in at least two respects: in offering yet more evidence that the inhibition of a given enzyme activity is the biochemical basis of drug action; and secondly, in providing a tool for study of the regulation of the synthesis of the given enzyme (11).

The data presented in this paper are consistent with the notion that mutant E187 is

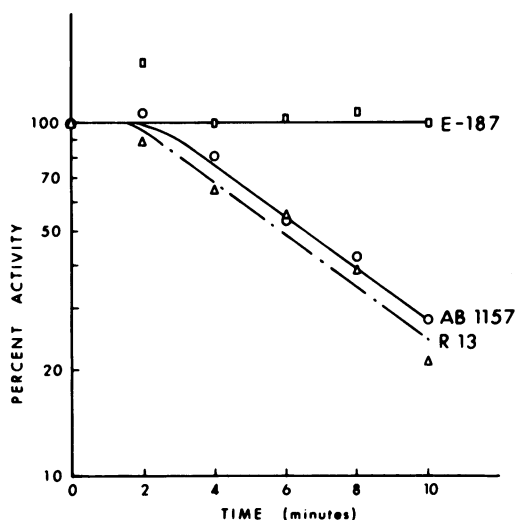


FIG. 4. Inhibition of PEP:UDPGlcNAc enolpyruvyl transferase by preincubation with 0.05 mM phosphonomycin. The crude extracts from AB1157, E187, and a temperature-resistant revertant of E187 (R-13) were preincubated at 37 C in the presence of 100 mM sodium maleate (pH 6.8), 10 mM KF, 0.8 mM dithiothreitol, 4 mM UDPGlcNAc, and 0.05 mM phosphonomycin for varying periods of time. At intervals of 2 min, samples of the preincubation mixture were taken into tube containing ^{14}C -PEP to start the reaction, the final concentration of PEP being 0.24 mM. Activity of this enzyme was plotted against the time of preincubation with phosphonomycin.

TABLE 2. UDPGlcNAc-dependent inhibition of PEP:UDPGlcNAc enolpyruvyl transferase by phosphonomycin

Enzyme source	Per cent inhibition ^a	
	+ UDPGlcNAc	- UDPGlcNAc
Strain AB1157	67.2	9.8

^a After preincubation for 10 min with 0.05 mM phosphonomycin. Preincubation of the crude extract was carried out at 37 C in the presence of 100 mM sodium maleate (pH 6.8), 10 mM KF, 0.08 mM dithiothreitol, and 0.05 mM phosphonomycin, with or without 4 mM UDPGlcNAc. After 10 min of preincubation, the reaction was started by the addition of PEP- ^{14}C to a final concentration of 0.24 mM, or by the addition of both PEP- ^{14}C (0.24 mM) and UDPGlcNAc (4 mM).

resistant to phosphonomycin, not because of a permeability barrier to this drug, but rather due to an intrinsic change in the kinetic properties of the target enzyme. There are a number of biochemical mechanisms by which bacterial mutants may become resistant to a

given antibiotic. The normal level of PEP:UDPGlcNAc enolpyruvyl transferase activity in the crude extract of this mutant and its increased resistance toward phosphonomycin in vitro eliminate mechanisms of drug resistance such as derepression of the synthesis of the wild-type enzyme, deficient uptake of this drug, and a new metabolic pathway for the synthesis of UDP-*N*-acetylmuramic acid. It is conceivable, however, that E187 enzyme is protected from inhibition of phosphonomycin either because of an accumulation of PEP in E187 cells, or due to a lack of UDPGlcNAc, the prior binding of which to the enzyme is a prerequisite for the inhibition by phosphonomycin. The former possibility is unlikely in view of the fact that the enzyme activity in E187 after ammonium sulfate fractionation remained resistant to phosphonomycin. Preliminary experiments also indicated that the levels of PEP in both strains are similar. The latter possibility that a lack of UDPGlcNAc results in the apparent resistance of this enzyme toward phosphonomycin is ruled out by the in vitro experiments in which saturating amounts of UDPGlcNAc were used. Although we could not yet conclusively eliminate the possibility that phosphonomycin is inactivated by an unknown enzymatic activity present in E187, the data from a mixed incubation experiment shown in Table 2 argue against this possibility. Genetic studies of this mutant strongly suggest that the phosphonomycin resistance and temperature-sensitive defect in

TABLE 3. Inactivation of mixed enzymes^a of wild type and mutant by phosphonomycin

Time after preincubation with 0.05 mM phosphonomycin (min)	Relative activity		
	AB1157	E187	AB1157 + E187
0	100	100	100
3	56	95	89
6	45	73	66
9	28	65	53
12	14	57	30

^a Enzymes used in this experiment were 40 to 60% ammonium sulfate fraction of crude extracts from wild-type strain (AB1157) and from mutant (E187). The crude enzyme preparation was precipitated by saturation with ammonium sulfate and centrifuged. The pellet was redissolved successively with 80, 60, 40, 20, 0% ammonium sulfate in 0.05 M Tris, pH 7.5. The 40% ammonium sulfate-soluble fraction containing maximal PEP:UDPGlcNAc enolpyruvyl transferase activity was used to determine the inactivation by preincubation with phosphonomycin under conditions described in the legend to Fig. 4.

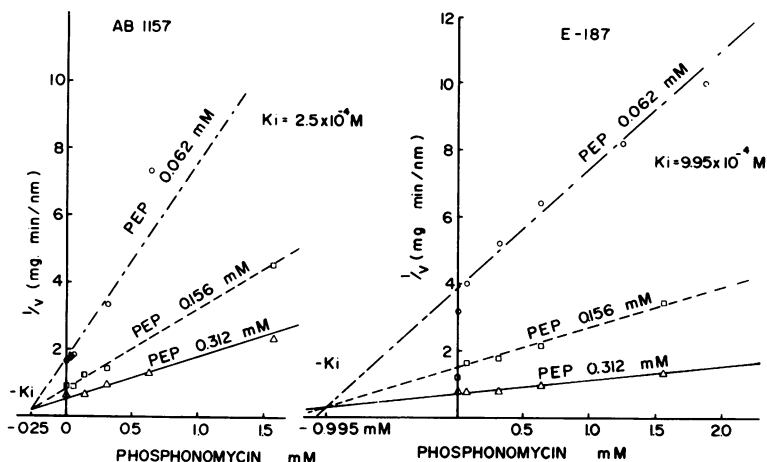


FIG. 5. K_i for phosphonomycin of PEP:UDPGlcNAc enolpyruvyl transferase in AB1157 and E187. The reciprocals of initial velocities ($1/V$) were plotted against concentrations of phosphonomycin in the presence of varying amounts of PEP. An apparent K_i for phosphonomycin could be obtained as the value on the x axis below the point of intersection of the three curves.

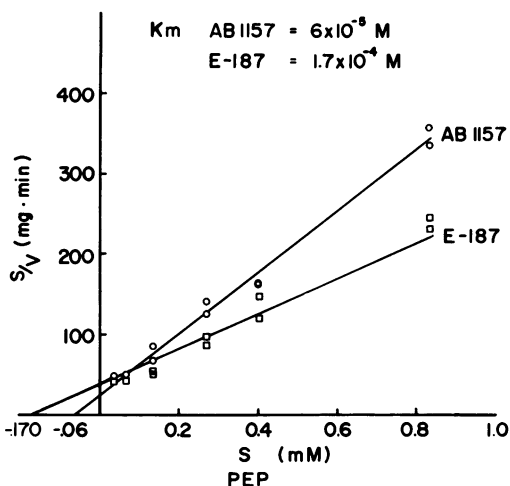


FIG. 6. K_m for PEP of PEP:UDPGlcNAc enolpyruvyl transferase activities in AB1157 and E187. S/V was plotted against S (PEP concentration) to obtain the apparent affinity of this enzyme for PEP.

the growth and division of this mutant are the consequences of a single mutational event.

We are currently analyzing the cell wall precursors in E187 as well as in AB1157 grown at 30 and 42 C. Preliminary data indicated that there was a significant reduction in the level of UDP-*N*-acetylmuramyl peptides in E187 as compared to the wild-type strain AB1157 at 42 C. Work is in progress concerning the fine genetic mapping of this mutation and the purification and characterization of this enzyme

TABLE 4. Phosphonomycin sensitivity in temperature-resistant revertants of strain E187

Strain	Lysis by phosphonomycin in PPBE broth		PEP:UDPGlcNAc enolpyruvyl transferase (nmol/mg of protein/hr)		
	0.12 mM	0.3 mM	-Phosphonomycin	+Phosphonomycin (1 mM)	Percent inhibition
AB1157	+	+	71.35	22.56	68
E187	-	±	71.46	65.34	9
<i>tr</i> Revertants					
R6	+	+	81.48	20.16	75
R13	+	+	103.32	33.84	67
R15	+	+	94.38	25.86	73
R16	+	+	62.82	25.86	59

from this mutant and from the wild-type strain.

Anwar and his co-workers have reported the inhibition of PEP:UDPGlcNAc enolpyruvyl transferase from *Enterobacter cloacae* by UDP-*N*-acetylmuramyl-L-alanyl-D-glutamate-*meso*-DAP (Anwar, Sodek, and Zemell, Fed. Proc. 30: 1284, 1971). In collaboration with B. Lugtenberg, we have confirmed this observation with the *E. coli* enzyme and further found that UDP-*N*-acetylmuramyl pentapeptide (UDP-*N*-acetylmuramyl-L-alanyl-D-glutamate-*meso*-DAP-D-alanine) is also a very potent inhibitor of this en-

TABLE 5. Linkage of *ts* mutation in E187 in a cross between HfrC and E187; selected marker: temperature-resistant *strA*^r

Unselected marker from donor	Frequency (%)
<i>tsx</i> ^a	14
<i>pro</i> ⁺	60
<i>lac</i> ⁺	65
<i>leu</i> ⁺	71
<i>ara</i> ⁺	61
<i>thr</i> ⁺	76
<i>arg</i> ⁺	94
<i>mtl</i> ⁺	11
<i>xyl</i> ⁺	3
<i>his</i> ⁺	0
<i>gal</i> ⁺	0

TABLE 6. Phosphonomycin sensitivity in temperature-resistant recombinants of strain E187

Recombinant ^a	Lysis by phosphonomycin in PPBE broth		PEP: UDPGlcNAc enolpyruvyl transferase (nmoles/mg of protein/hr)		
	0.12 mM	0.3 mM	- Phosphonomycin	+ Phosphonomycin (1 mM)	Percent inhibition
1-1	+	+	66.3	13.92	79
1-7	+	+	45.84	14.22	69
1-13	+	+	79.08	13.44	83
1-19	+	+	92.94	16.74	82
AB1157	+	+	64.68	7.74	88
E187	-	±	80.04	41.64	48

^a Genotypes of recombinants and parental strains are given in Table 7.

TABLE 7. Genotypes of recombinants and parental strains listed in Table 6

Strain	Selected markers		Unselected markers										
	Growth at 42 C	<i>strA</i>	<i>tsx</i>	<i>lac</i>	<i>pro</i>	<i>leu</i>	<i>ara</i>	<i>thr</i>	<i>arg</i>	<i>mtl</i>	<i>xyl</i>	<i>his</i>	<i>gal</i>
HfrC	+	s	s	+	+	+	+	+	+	+	+	+	+
E187	-	r	r	-	-	-	-	-	-	-	-	-	-
1-1	+	r	s	+	+	+	+	+	+	-	-	-	-
1-7	+	r	r	-	-	-	-	-	+	-	-	-	-
1-13	+	r	r	-	-	+	+	-	-	-	-	-	-
1-19	+	r	s	+	+	-	-	-	-	-	-	-	-

zyme (*unpublished data*). It would be interesting to see whether this very first enzyme involved in the biosynthesis of peptidoglycan is truly a critical point of metabolic control of this pathway. We hope to test whether the ehzyme isolated from mutant strain E187 would show desensitization toward the feedback control by UDP-*N*-acetylmuramyl peptides. Isolation of more structural gene mutants of this key enzyme may provide valuable information concerning the metabolic control of peptidoglycan synthesis.

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