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Isolation and Characterization of a New Globomycin-Resistant *dnaE* Mutant of *Escherichia coli*

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We isolated a globomycin-resistant, temperature-sensitive mutant of *Escherichia coli* K-12 strain AB1157. The mutation mapped in *dnaE*, the structural gene for the α-subunit of DNA polymerase III. The in vivo processing of lipid-modified prolipoprotein was more resistant to globomycin in the mutant strain 307 than in its parent. The prolipoprotein signal peptidase activity was also increased twofold in the mutant, and there was a threefold increase in the activity of isoleucyl-tRNA synthetase. The results suggest that a mutation in *dnaE* may affect the expression of the *ileS-lsp* operon in *E. coli*. In addition, strain 307 showed a reduced level of streptomycin resistance compared with its parental strain AB1157 (*rpsL31*). Strain 307 was killed by streptomycin at a concentration of 200 μg/ml, which did not affect the rate of bulk protein synthesis in this mutant. A second mutation which was involved in the reduced streptomycin resistance in strain 307 was identified and found to be closely linked to or within the *rpsD* (*ramA*, ribosomal ambiguity) gene. Both *dnaE* and *rpsD* were required for the reduced streptomycin resistance in strain 307.

Globomycin is a cyclic peptide antibiotic which acts as a specific inhibitor of prolipoprotein signal peptidase (SPase II). The accumulation of modified prolipoprotein in globomycin-treated cells causes a morphological change from rod-shaped to spherical cells, with eventual cell lysis (8, 11). Due to its specific mode of action, globomycin has been an invaluable tool in studies of lipoprotein biogenesis in *Escherichia coli*. Globomycin-resistant mutants can be classified into three groups: (i) mutants with greatly reduced amounts of or no lipoprotein (15, 37); (ii) mutants with structurally altered lipoproteins (6, 15, 37); and (iii) mutants with increased levels of SPase II activity (32).

In the present study, we sought conditionally lethal globomycin resistance mutations in essential genes. One such mutant had a defect in *dnaE*, the structural gene for the α-subunit of DNA polymerase III (35). Genetic and biochemical studies indicate that the *dnaE* mutation results in increased expression of the *ileS-lsp* operon in *E. coli*. In addition, the *dnaE* allele together with a preexisting *rpsD* (or *ramA*, for ribosomal ambiguity, the structural gene for ribosomal protein S4) causes a reduction in streptomycin resistance in strain 307 compared with its parental strain AB1157 (*rpsL31*).

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* strains and plasmids are described in Table 1 and the legend to Fig. 2, respectively. Media included proteose peptone beef extract broth, L broth, and M9 minimal medium (38), which was supplemented with a carbon source and amino acids as required. The mutant carrying the conditionally lethal mutation was grown at 30°C as the permissive temperature and at 42°C as the nonpermissive temperature.

Genetic techniques. Conjugation and P1 transduction were carried out as described by Miller (21, 22). P1 *cml clr* 100 bacteriophage was used in transductions. Transformation with plasmid DNA was performed by the method of Maniatis et al. (17). *E. coli* strains containing transposon Tn*10* near the *rpsE* gene were isolated by using λNK561 as described by Silhavy et al. (29).

In vivo studies of inhibition of prolipoprotein processing by globomycin. Overnight cultures were inoculated into M9-glucose medium and incubated at 30°C. At the mid-logarithmic phase of growth (A_{600} = 0.4), globomycin was added to a final concentration of 100 μg/ml. Following additional incubation for 30 min at 30°C, the culture was pulse labeled for 2 min with 40 μCi of [^{35}S]methionine and the labeling was terminated by the addition of trichloroacetic acid (TCA; final concentration 10%). The TCA precipitate was collected, washed with acetone, solubilized, and immunoprecipitated with antiserum against purified lipoprotein as described previously (38). The lipoprotein immunoprecipitates were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10), and the extent of prolipoprotein processing was estimated by quantitative determination of radioactivity in the region of the gel corresponding to prolipoprotein and lipoprotein by liquid scintillation counting.

Incorporation of [^{3}H]thymine. Thymine-requiring derivatives were isolated from strains 307 and AB1157 as described by Miller (23) and used for the measurement of [^{3}H]thymine incorporation. Cells were grown in 5 ml of M9-glucose medium supplemented with required amino acids, thiamine, and thymine (50 μg/ml) at 30°C. At the early logarithmic phase of growth (A_{600} = 0.25), cultures were divided into two portions, one kept at 30°C and the other shifted to 42°C. [^{3}H]thymine (0.2 μCi) was added to each portion, and samples of cultures were withdrawn at regular time intervals into tubes containing TCA (final concentration, 5%). The TCA-insoluble materials were collected by filtration through Millipore filters, and the radioactivity was determined by liquid scintillation counting.

In vitro assay of SPase II activity. The procedures for the purification of [^{35}S]cysteine-labeled lipid-modified prolipoprotein and the preparation of cell envelope fraction for SPase II assay have been described previously (33). The

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TABLE 1. Bacterial strains

Strain	Genetic markers	Source[a] or reference
AB1157	*thr-1 ara-14 leuB6* Δ(*gpt-proA*)*62 hisG4 lacY1 tsx-33 supE44 galK2 rfbD1 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1*	CGSC
307	*dnaE307* mutant of AB1157	This work
KL14	Hfr *thi-1 relA1 spoT1*	CGSC
KL723	F'104 *thr-1 leuB* Δ*6* (*gpt-proA*)*62 his-4 recA13 argE3 thi-1 ara-14 lacY galK2 xyl-7 mtl-1 rpsL31 tsx-33 supE44*	CGSC
E5014	F'128 Δ(*gpt-lac*)*5 relA1 thi-1 mal-24 rpsE2112*	CGSC
JW353	*thr-1 leuB6 zae-502*::Tn*10 thyA6 met-86 thi-1 deoC1 lacY1 rpsL67 fhuA21 supE44*	CGSC
RK4375	*thi aroB pan-6 malA fhuB477*::Tn*10*	13
GM10	*thr-1 leuB6 tonA2 pro-33 lacY1 dnaY10*(Ts) *supE44 gal-6 hisG1 rfbD1 galP63 rpsL9 malA1 xyl-7 mtlA2 argH1 thi-1*	CGSC
GM36	*thr-1 leuB6 fhuA2 pro-33 lacY1 dnaX36*(Ts) *supE44 gal-6 hisG1 rfbD1 galP63 rpsL9 malA1 xyl-7 mtlA2 argH1 thi-1*	CGSC
AX727	*dnaZ2016 thi lac rpsL*	CGSC
SK922-AB	*rpsL31* transductant of SK922	This work
JC5029-ABX	*rpsL31 x* transductant of JC5029	This work
AB1157(307)	*zae-502*::Tn*10* 307 transductant of strain AB1157 [donor, 307(zae-502::Tn10)]	This work
AB1157X(307)	*zae-502*::Tn*10* 307 transductant of strain AB1157X [donor, 307(zae-502::Tn10)]	This work
AB1157(dnaX)	*zba-3000*::Tn*10 dnaX* transductant of strain AB1157 [donor, GM36(zba-3000::Tn10)]	This work
AB1157X(dnaX)	*zba-300*::Tn*10 dnaX* transductant of strain AB1157X [donor, GM36(zba-3000::Tn10)]	This work
AB1157(dnaY)	*purE*::Tn*10 dnaY* transductant of strain AB1157 [donor, GM10(purE::Tn10)]	This work
AB1157X(dnaY)	*purE*::Tn*10 dnaY* transductant of strain AB1157X [donor, GM10(purE::Tn10)]	This work
AB1157(dnaZ)	*zba-3000*::Tn*10 dnaZ* transductant of strain AB1157 [donor, AX727(zba-3000::Tn10)]	This work
AB1157X(dnaZ)	*zba-3000*::Tn*10 dnaZ* transductant of strain AB1157X [donor, AX727(zba-3000::Tn10)]	This work
AB1157(dnaN)	*tnaA*::Tn*10 dnaN* transductant of strain AB1157 [donor, HC194(tnaA::Tn10)]	This work
AB1157X(dnaN)	*tnaA*::Tn*10dnaN* transductant of strain AB1157X [donor, HC194(tnaA::Tn10)]	This work
307(zae-502::Tn10)	*zae-502*::Tn*10* transductant of strain 307 (donor, JW353)	This work
GM10(purE::Tn10)	*purE*::Tn*10* transductant of strain GM10 (donor, NK6051)	This work
GM36(zba-3000::Tn10)	*zba-3000*::Tn*10* transductant of strain GM36 (donor, KL743)	This work
AX727(zba-3000::Tn10)	*zba-3000*::Tn*10* transductant of strain AX727 (donor, KL743)	This work
HC194(tnaA::Tn10)	*tnaA*::Tn10 transductant of strain HC194 (donor, Me8375)	This work
KL743	*zba-3000*::Tn*10*	CGSC
NK6051	Δ(*gpt-lac*)*5 purE79*::Tn*10 relA1 spoT1 thi-1*	CGSC
Me8375	*tnaA*::Tn*10 couA2* Δ(*lac-pro*) *supE*/F'-*lacZ*::Tn*5-pro*$^+$	NIG

[a] CGSC, Coli Genetic Stock center, Yale University, New Haven, Conn.; NIG, National Institute of Genetics, Mishima, Japan.

reaction mixture (10 μl) contained [^{35}S]cysteine-labeled modified prolipoprotein (2×10^4 cpm), 0.25% Nikkol, 50 mM Tris hydrochloride (pH 7.4), 0.6 mM $MgCl_2$, 0.25% β-mercaptoethanol, and various amounts of cell envelope proteins from strain 307 or AB1157. Incubation was carried out at 37°C for 30 min with gentle shaking. The conversion of modified prolipoprotein to mature lipoprotein was determined by SDS gel electrophoresis of the reaction mixture, followed by measurement of the radioactivity in the regions of the gel corresponding to prolipoprotein and mature lipoprotein.

Assay of isoleucyl-tRNA synthetase activity. The S-100 fractions which were used as the source of this enzyme were prepared from strains 307 and AB1157 as follows. Cells were grown in L broth and collected at the mid-logarithmic phase of growth. The cell pellets were washed with 50 mM Tris hydrochloride buffer (pH 7.5) containing 50 mM KCl, 10 mM $MgCl_2$, and 0.1 mM dithiothreitol (TKMD buffer). Washed cells were ground with alumina and treated with DNase I by the addition of 2 volumes of TKMD buffer containing 10 μg of DNase I per ml. The mixture was centrifuged at 200,000 × *g* for 3 h at 4°C, and the supernatant fraction was dialyzed against TKMD buffer containing 30% glycerol. For the assay of isoleucyl-tRNA synthetase activity, the reaction mixture (150 μl) contained 50 mM Tris hydrochloride (pH 7.5), 4 mM ATP, 40 mM KCl, 10 mM $MgCl_2$, 50 μM [^{14}C]isoleucine (specific radioactivity, 50 μCi/μmol), 300 μg of *E. coli* tRNA, and S-100 fraction (20 or 60 μg of protein). At regular time intervals, 30 μl of the reaction mixture incubated at 37°C was withdrawn and spotted on Whatman 3MM filter paper. The filter papers were soaked in cold 5% TCA, washed with ethanol, and dried. The radioactivity retained on the filter papers was determined by liquid scintillation counting (31).

Assay for antibiotic sensitivity. *E. coli* cells were grown in M9 medium at 30°C on a rotary shaker overnight. Approximately 10^6 cells of an overnight culture were inoculated into 100 μl of M9 medium containing various amounts of antibiotic in a 96-well microtiter plate and incubated at 30°C for 48 h. The lowest concentration of antibiotic inhibiting cell growth was determined by visual inspection of the microtiter plate.

Chemicals. [^{35}S]methionine (specific activity, 300 mCi/mmol) was purchased from New England Nuclear Corp. Amino acids and streptomycin were purchased from Sigma Chemical Co. Globomycin was a generous gift from M. Arai (Sankyo Co., Tokyo). Tryptone, yeast extract, and agar were purchased from Difco Laboratories.

RESULTS

Isolation of globomycin-resistant and temperature-sensitive mutants of *E coli*. To isolate globomycin-resistant mutants altered in an essential gene, we selected spontaneous globo-

mycin-resistant mutants of *E. coli* and tested them for temperature-sensitive growth. Samples (1 ml) of overnight cultures of strain AB1157 were harvested by centrifugation, suspended in 200 μl of L broth, spread on an L agar plate containing globomycin (80 μg/ml), and incubated at 30°C for 4 days. Approximately 100 globomycin-resistant colonies were obtained from 10^9 cells. These colonies were purified and tested for temperature-sensitive growth at 42°C. Approximately 1% of the globomycin-resistant mutants showed temperature-sensitive growth.

The phenotypes of one of the globomycin-resistant and temperature-sensitive mutants, strain 307, are summarized in Table 2. Unlike its parental strain AB1157, strain 307 cells did not form colonies on agar plates at 42°C. In liquid culture, the growth of strain 307 at 42°C as measured by A_{600} increased at the same rate as the parental strain, and phase-contrast microscopic examination of 307 cells indicated that 307 cells elongated as filaments but did not divide at 42°C. Strain 307 was about three times more resistant to globomycin than its parental strain. Unexpectedly, it was more sensitive to streptomycin than strain AB1157, even though they both contain the *rpsL31* allele.

Mapping the mutation in strain 307. Conjugation with Hfr strain KL-14 and strain 307 revealed that the temperature-sensitive mutation in strain 307 was located between 2 min (*leu*) and 8 min (*lac*) of the *E. coli* chromosome (data not shown). A spontaneous rifampin-resistant mutant was isolated from strain 307 and used as the recipient in conjugation experiments with *E. coli* strains harboring F′ plasmids covering this region of the *E. coli* genome (Fig. 1). F′-ductants with F′104 or F′128 donors showed normal growth at 42°C and wild-type levels of globomycin sensitivity (30 μg/ml) and streptomycin resistance (1 mg/ml). Thus, the recessive mutation in strain 307 responsible for globomycin resistance and temperature-sensitive growth was complemented by the overlapping region of F′104 and F′128.

Based on these preliminary mapping data, we proceeded to locate the mutation in strain 307 by P1 transduction with various strains carrying Tn*10* in the 4-min region of the *E. coli* chromosome. The temperature-sensitive mutation in strain 307 was found to be linked to *fhuB*::Tn*10* (3.7 min) (33 to 34% cotransduction) and to *zae*::Tn*10* in strain JW353 (50 to 55% cotransduction). The Tn*10* at 4 min in strain JW353 has been shown to be cotransducible with *dnaE* at 55% (J. A. Wechsler, personal communication via B. Bachmann). These results strongly suggest that the temperature-sensitive mutation in strain 307 is located near or at the *dnaE* gene.

Complementation analysis of strain 307 with plasmids carrying cloned genes. Plasmids carrying cloned genes from the 4-min region of the *E. coli* chromosome were obtained. Plasmid pMWE103 carries both *dnaE* and *pgsB* (Fig. 2) (3, 35). pMWE203 and pMWE303 carry *dnaE* and part of *pgsB*, whereas pDC4 carries *pgsB* and part of *dnaE* (3). Complementation analysis clearly indicated that plasmids containing an intact *dnaE* gene could complement the temperature-sensitive growth defect in strain 307 and also restore its globomycin sensitivity to the wild-type level. To eliminate the possibility that the flanking sequence of the *dnaE* gene in pMWE303 encoded the gene product that was defective in strain 307, we constructed two subclones of the *dnaE* gene in pMWE303 by cloning the *Cla*I-*Hin*dIII and *Hin*dIII-*Bam*HI fragments into pUC8 to make pTW1 and pTW2, respectively (Fig. 2). Whereas pMWE303 complemented the temperature-sensitive growth defect in strain 307, pTW1 and pTW2 did not. From these results, we conclude that the temperature-sensitive mutation in strain 307 resides in the *dnaE* gene. The phenotype of reduced resistance to streptomycin of strain 307 was also complemented by F′104, F′128, and pMWE303 (data not shown). These results suggest that the *dnaE* mutation in strain 307 is responsible for the temperature-sensitive growth defect, reduced streptomycin resistance, and increased globomycin resistance and that the mutation is recessive for all these phenotypes.

TABLE 2. Phenotype comparison of strains 307 and AB1157

Strain	Colony formation		Sensitivity[a] (μg/ml)	
	30°C	42°C	Globomycin	Streptomycin
AB1157	+	+	30	1,000
307	+	−	100	100

[a] Maximal antibiotic concentration at which the bacterial cells grew.

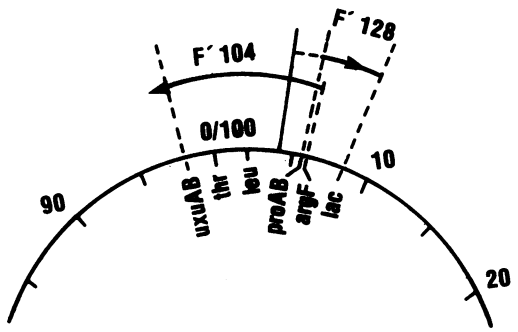

FIG. 1. Partial genetic map of *E. coli* (1) showing the region covered by F′104 and F′128. Results presented in this study show that F′128 contains the 4-min region of the *E. coli* chromosome.

DNA synthesis in strain 307. To verify our mapping data, which indicated that strain 307 carried a defect in *dnaE*, we measured DNA synthesis in strain 307 and its parent. To do this, thymine-requiring derivatives of strains AB1157 and 307 were used. The rate of [^{3}H]thymine incorporation into TCA-insoluble materials in strain 307 was about half of that of strain AB1157 at the permissive temperature (30°C) (Fig. 3). At 42°C, strain 307 was severely impaired in the incorporation of [^{3}H]thymine into TCA-insoluble materials compared with the wild-type strain. These results strongly support the conclusion that strain 307 contains a conditionally lethal mutation in *dnaE*.

Effect of globomycin on in vivo processing of prolipoprotein in strain 307. Globomycin resistance was studied by examining the kinetics of prolipoprotein processing in vivo. The processing of prolipoprotein was completely inhibited by globomycin at 80 μg/ml in strain AB1157, but only partially (40%) inhibited in strain 307 (Fig. 4). These results indicate that the increased resistance to globomycin in strain 307 was correlated with a lesser inhibition of prolipoprotein processing by globomycin.

SPase II activity in strain 307 and the effect of globomycin in vitro. To ascertain whether the increased resistance to globomycin in strain 307 was due to an increased level of SPase II activity, we compared SPase II activity in the crude cell envelopes of strain 307 and strain AB1157. The SPase II activity in strain 307 was twofold higher than in the AB1157 cell envelope (Fig. 5). We also compared the effect of globomycin on SPase II activities in vitro with the cell envelope fractions from strain 307 and strain AB1157. The crude membrane of strain 307 contained higher SPase II activity than strain AB1157 (Fig. 6). Consequently, more SPase II activity remained in the 307 mutant cell extract than

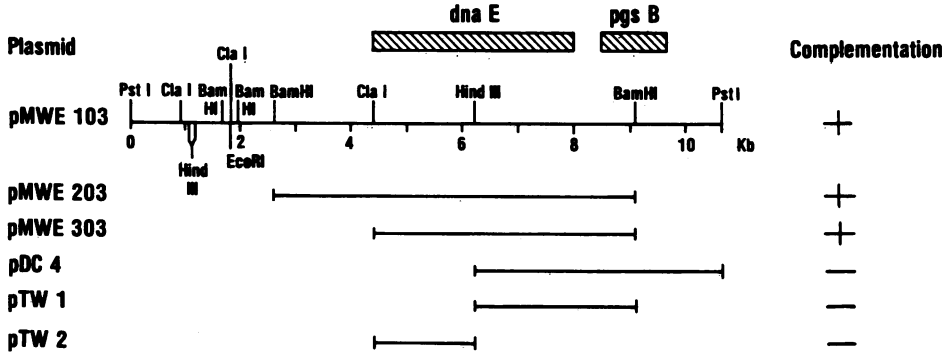

FIG. 2. Complementation of the temperature-sensitive mutation in strain 307 with plasmids carrying the genes at the 4-min region of the *E. coli* genome. Symbols: +, wild-type phenotype (normal growth at 42°C and normal level of globomycin sensitivity); −, mutant phenotype (temperature-sensitive growth and increased globomycin resistance). The physical map of plasmid pMWE103 was described by Welch et al. (35). Hatched bars indicate the approximate locations of *dnaE* and *pgsB* based on data previously reported by Shepard et al. (28) and Crowell et al. (3).

in the AB1157 cell extract in the presence of globomycin, with the percentage of SPase II activity inhibited by a given concentration of globomycin being approximately the same in strains 307 and AB1157. These results indicate that the increased resistance of strain 307 to globomycin is due to an increase in the level of SPase II activity and not to any change in the intrinsic properties of the enzyme.

Increased isoleucyl-tRNA synthetase activity in strain 307. We have shown previously that the *lsp* gene is cotranscribed with the *ileS* gene in *E. coli* (23a, 24, 31). To determine whether the apparent increase in the SPase II activity in strain 307 was accompanied by a concomitant increase in the activity of isoleucyl-tRNA synthetase, we compared the activity of this enzyme in the S-100 fractions prepared from strains 307 and AB1157. The isoleucyl-tRNA synthetase activity in strain 307 was threefold higher than in strain AB1157 (Fig. 7). These results suggest that there is increased expression in the *ileS-lsp* operon as a result of the *dnaE* mutation in strain 307.

Reduced streptomycin resistance of strain 307. Strain 307 was less resistant to streptomycin than its parental strain AB1157, which contains the *rpsL31* allele. When cells of strain 307 were incubated with 200 μg of streptomycin per ml, viable cell counts were reduced to one-tenth of the original value in 3 h, although the OD_{600} of the culture continued to increase during the 3-h incubation (Fig. 8A and D). The growth and viability of the parental strain AB1157 were not affected by streptomycin at the same concentration (Fig. 8B and E). Strain 609 was used as a streptomycin-sensitive control. Both the viable cell counts and OD_{600} were severely affected by streptomycin at 20 μg/ml (Fig. 8C and F). Apparently, strain 307 is less resistant to streptomycin than AB1157 but more resistant than a strain with a wild-type $rpsL^+$ allele such as strain 609. While the increase in the OD_{600} continued for several generations after the addition of streptomycin, cells of strain 307 failed to form colonies on solid medium containing 200 μg of streptomycin per ml. On the other hand, incorporation of [^{35}S]methionine into proteins in strains 307 and AB1157 was not affected after a 2-h incubation with streptomycin (200 μg/ml) (data not shown).

Streptomycin sensitivity of strains carrying the *dnaE307* mutation. The *dnaE* allele in strain 307 was transduced into various *rpsL* mutants by P1 transduction by using a Tn*10* insertion near the *dnaE* gene as the selected marker, and the sensitivity of these *rpsL dnaE307* mutants to streptomycin

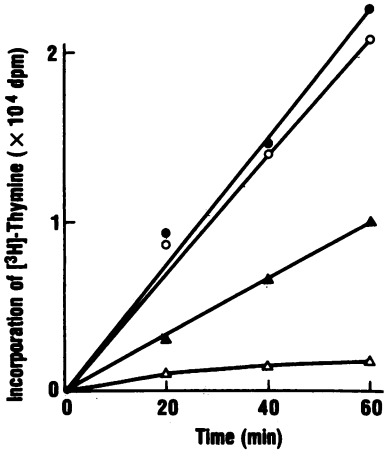

FIG. 3. Incorporation of [^{3}H]thymine into TCA-insoluble materials in wild-type (AB1157) and mutant (307) cells. The rates of DNA synthesis in the wild-type and mutant strains were assessed by measuring the incorporation of [^{3}H]thymine into TCA-insoluble materials at both the permissive (30°C) and nonpermissive (42°C) temperatures. After the addition of [^{3}H]thymine, samples of the cultures were withdrawn at the time intervals indicatd in the figure, and the ^{3}H radioactivity in the TCA-insoluble material was determined. Symbols: ●, strain AB1157 at 30°C; ○, strain AB1157 at 42°C; ▲, strain 307 at 30°C; △, strain 307 at 42°C.

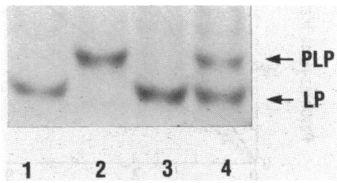

FIG. 4. Effects of globomycin on the processing of prolipoprotein (PLP) in the wild-type (AB1157) and mutant (307) strains in vivo. Wild-type and mutant cells were treated with globomycin (80 μg/ml) for 30 min and then pulse labeled with [^{35}S]methionine for 2 min. Mature lipoprotein (LP) was immunoprecipitated and analyzed by SDS-12.5% polyacrylamide gel electrophoresis as described in Materials and Methods. Lanes: 1, strain AB1157 without globomycin; 2, strain AB1157 treated with globomycin (80 μg/ml); 3, strain 307 without globomycin; lane 4, strain 307 treated with globomycin (80 μg/ml).

and globomycin was determined (Table 3). All transductants showed increased globomycin resistance compared with their respective parental strains. Streptomycin sensitivity in these strains was not changed, except in strain AB1157 *dnaE307*. When the *dnaE307* mutation was reintroduced into strain AB1157 from strains 609 *rpsL31 dnaE307*, D10 *rpsL31 dnaE307*, PCO291 *rpsL125 dnaE307*, and MX419 *rpsL183 dnaE307*, all temperature-sensitive transductants of strain AB1157 showed lower streptomycin resistance (100 μg/ml) than strain AB1157 (1,000 μg/ml). It should be noted that the level of streptomycin resistance in strain AB1157 was lower than those of strain 609 *rpsL31* and D10 *rpsL31* (10,000 μg/ml), even though the latter strains had the same *rpsL31* allele present in strain AB1157 after P1 transduction. These results suggest that strain 307, and most likely strain AB1157 as well, contains another mutation, designated *x*, in addition to the *dnaE307* mutation, and the coexistence of *x* and *dnaE* lowers the streptomycin resistance level in strain 307.

Mapping of *x* mutation in strain 307 by transduction. Since *rpsL* x^+ strains were more resistant to streptomycin than *rpsL x* strains (Table 3), it was possible to distinguish between *rpsL* x^+ and *rpsL x* strains on an agar plate containing 1 mg of streptomycin per ml; *rpsL* x^+ strains grew well on this medium, while *rpsL x* mutants grew poorly. Tn*10* was inserted near the *x* gene as described by Silhavy et al. (29). The *x* gene was found to be cotransducible with *rpsL* and with *rpsE*. To determine the relative position of gene *x* in the *rpsE* gene cluster, a three-factor-cross analysis was performed (Table 4). Gene *x* was located between *rimE* and *rpsE* at 72 min on the *E. coli* genetic map (Fig. 9) and may correspond to one of the ribosomal protein genes.

Correlation between *x* mutation and leaky threonine requirement in strain 307. x^+ and *x* derivatives of strain 307 were constructed by P1 transduction with a donor strain with a Tn*10* insertion near the *x* gene. *rpsL*$^+$ derivatives were also constructed. *x dnaE rpsL* strains (307-22 and 307-23) showed streptomycin sensitivity equal to that in strain 307 (0.1 mg/ml), whereas x^+ *dnaE rpsL* strains (307-2 and 307-21) showed a high level of streptomycin resistance (10 mg/ml) (Table 5). These results clearly indicate that the coexistence of *dnaE* and *x* was responsible for the phenotype of reduced streptomycin resistance in strain 307. When we checked other genetic markers known to be present in strain AB1157,

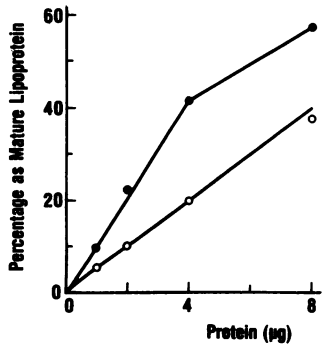

FIG. 5. SPase II activity in wild-type and mutant cell envelopes. The cell envelope fractions from the wild-type strain AB1157 (○) and the mutant strain 307 (●) were assayed for SPase II activity in vitro, with [^{35}S]cysteine-labeled lipid-modified prolipoprotein as the substrate. The percentage of conversion of prolipoprotein into lipoprotein following incubation was determined by SDS-polyacrylamide gel electrophoresis and scintillation counting of the regions of the gel corresponding to prolipoprotein (substrate) and mature lipoprotein (product).

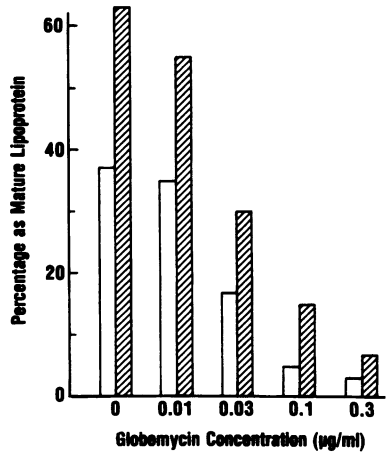

FIG. 6. Globomycin sensitivity of SPase II activity in the cell envelopes of the wild-type (open bars) and mutant (hatched bars) strains in vitro. SPase II activity in the cell envelopes of strain AB1157 (wild type) and strain 307 (mutant) was measured in the presence of increasing concentrations of globomycin in the reaction mixture.

we found a correlation between *x* and a leaky threonine requirement. In the presence of the *rpsL31* allele, a mutation in *x* was associated with a leaky threonine requirement, while x^+ derivatives were less leaky. In addition, the difference in the leakiness of threonine requirement was independent of the mutation in the *dnaE* gene. All *rpsL*$^+$ derivatives, strains 307-31, 307-32, 307-33 and 307-34, exhibited a leaky threonine requirement irrespective of the mutation in gene *x* (Table 5).

Streptomycin sensitivity of *dna* derivatives of AB1157 and AB1157X. To ascertain whether the sensitization of strain AB1157X toward streptomycin by the presence of a *dnaE* allele was unique for *dnaE* mutations, we constructed isogenic pairs of AB1157 and AB1157X carrying various other *dna* mutations and determined their streptomycin sensitivity. The coexistence of *x* and any of these *dna* alleles,

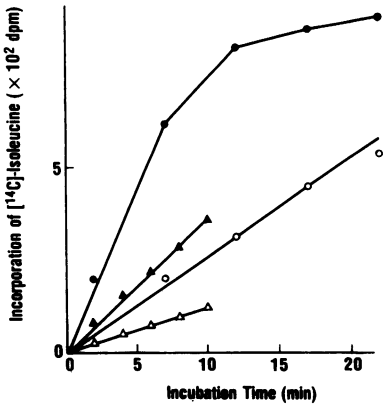

FIG. 7. Isoleucyl-tRNA synthetase activities in strain AB1157 and strain 307. The S-100 fractions prepared from AB1157 and 307 cells were used as the source of isoleucyl-tRNA synthetase. [^{14}C]isoleucine radioactivity bound to isoleucyl-tRNA was measured as described in Materials and Methods. Symbols: ●, S-100 fraction (60 μg of protein) from strain 307; ○, S-100 fraction (60 μg of protein) from strain AB1157; ▲, S-100 fraction (20 μg of protein) from strain 307; △, S-100 fraction (20 μg of protein) from strain AB1157.

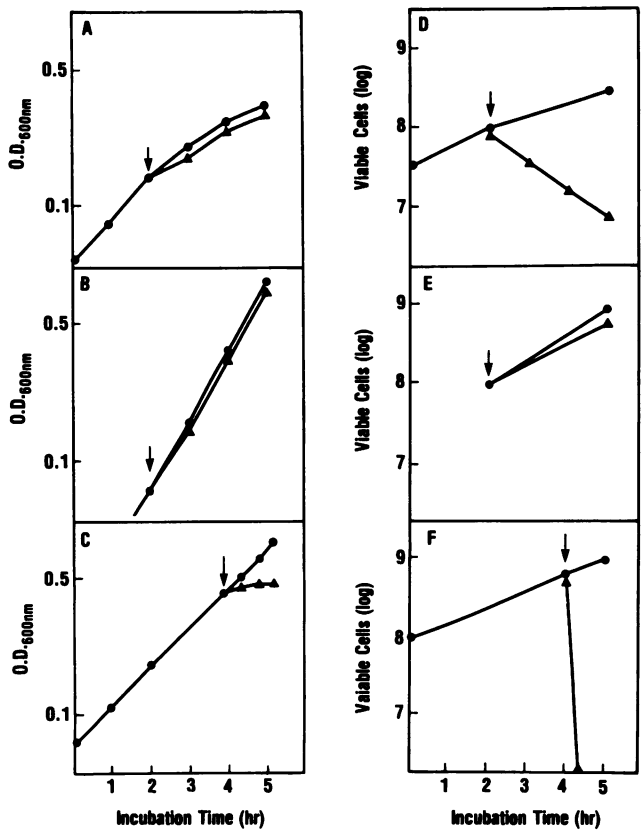

FIG. 8. *E. coli* cells were grown overnight in M9-glucose medium supplemented with required amino acids and thiamine. One-tenth milliliter of the overnight culture was inoculated into 5 ml of fresh medium in a 25-ml test tube and incubated at 30°C on a rotary shaker. At the indicated time (arrows), streptomycin (200 μg/ml for 307 and AB1157, 20 μg/ml for 609) was added, and incubation was continued. At intervals, the OD_{600} and viable cell number were determined. L agar plates were used for the determination of viable cell number. (A and D) Strain 307; (B and E) strain AB1157; (C and F) strain 609. Symbols: ●, without streptomycin; ▲, with streptomycin.

including *dnaX*, *dnaY*, *dnaZ*, and *dnaN*, greatly reduced the sensitivity to streptomycin in strains carrying the *rpsL31* allele (Table 6). Thus, the reduction in streptomycin resistance in strain 307 due to the presence of *x* and *dnaE* may be attributable to a combined effect of defective DNA replication and the mutational alteration in a ribosomal protein gene.

DISCUSSION

We have isolated a novel globomycin-resistant mutant of *E. coli* which is temperature sensitive in growth. Our initial goal was to isolate mutants carrying conditionally lethal globomycin resistance mutations in essential genes, the products of which participate in the biogenesis of lipoproteins in *E. coli*. Unexpectedly, we isolated a *dnaE* mutant defective in DNA replication, especially at the nonpermissive temperature. This mutant, strain 307, was globomycin resistant due to increased expression of the *ileS-lsp* operon. Consequently, strain 307 cells contained elevated levels of both isoleucyl-tRNA synthetase and SPase II activity compared with the parental strain AB1157. The twofold increase in SPase II activity in strain 307 would account for the increased globomycin resistance in this mutant.

TABLE 3. Globomycin and streptomycin sensitivity of *E. coli* strains carrying *dnaE307*

Strain (relevant genotype)	Sensitivity[a] (μg/ml)	
	Globomycin	Streptomycin
AB1157X (*rpsL31 x*)	30	1
307 (*rpsL31 x dnaE307*)	100	0.1
AB1157X-307 (*rpsL31 x dnaE307*)	100	0.1
609-AB (*rpsL31*)	30	10
609-307 (*rpsL31 dnaE307*)	80	10
D10-AB (*rpsL31*)	30	10
D10-307(*rpsL31 dnaE307*)	60	10
PCO291 (*rpsL125*)	10	8
PCO291-307 (*rpsL125 dnaE307*)	60	8
MX419 (*rpsL183*)	20	10
MX419-307 (*rpsL183 dnaE307*)	40	10

[a] Maximal antibiotic concentration in which these strains grew.

We have previously shown that the *lsp* gene is cotranscribed with the *ileS* gene, even though the *lsp* gene can also be transcribed from an internal promoter located in the *ileS* gene 230 base pairs upstream of the *lsp* translation initiation codon (9, 23a, 24, 31). The observation of a concomitant increase in the level of isoleucyl-tRNA synthetase and SPase II activity in strain 307 provides independent evidence for cotranscription of the *ileS* and *lsp* genes in *E. coli*. Regulation of the *ileS* gene is complex and poorly defined (25). It has been reported that the level of isoleucyl-tRNA synthetase activity can be derepressed by isoleucine starvation (19, 20). Mutations in *ilvU* and *ilvA*, as well as deletions in *ilvDAC* genes, would affect the level of isoleucyl-tRNA synthetase activity and the derepressibility by isoleucine limitation (2, 5, 16, 36). Mutants resistant to ketomycin or trifluoroleucine and valine are altered in the regulation of expression of the *ileS* gene (12). The regulation of expression of *ileS* by threonine deaminase (encoded by *ilvA*) and by 2-ketobutyrate appears to be complex (30). Finally, there appears to be growth rate-dependent regulation of the expression of *ileS* superimposed on the regulation by isoleucine or enzymes involved in the biosynthesis of isoleucine (26). All these studies were completed prior to the discovery that the *ileS* gene is part of an operon containing five cistrons (*x-ileS-lsp-orf-149-orf-316*), including three genes of unknown function (14, 23a, 24, 31). In all likelihood, this will turn out to be an operon of mixed function. The relationship between defective DNA replication and regulation of the *x-ileS-lsp-orf-149-orf-316* operon remains to be elucidated (23a, 24).

We have shown that the coexistence of *dnaE* and *x* mutations in strain 307 results in greatly reduced resistance to streptomycin despite the presence of *rpsL31*. Derivatives of strain AB1157 with the genotype *rpsL x* exhibited a leaky threonine requirement compared with *rpsL* x^+ derivatives. This phenotype is similar to the phenotype caused by the *ramA* mutation (7, 27). Streptomycin-resistant mutants are

TABLE 4. Mapping of gene *x* by P1 transduction[a]

Unseleted marker	No. of transductants
rimE$^+$ *x*	104
rimE$^+$ x^+	0
rimE x	16
rimE x^+	6

[a] The donor strain was JC5029-ABX (*rimE*$^+$ *x rpsE*), and the recipient strain was SK922-AB (*rimE* x^+ *rpsE*$^+$). The selected marker was *rpsE*.

known to exhibit restriction of translation ambiguities (7, 27). Starting with a leaky arginine-negative amber mutant which was made less leaky by the introduction of an *rpsL* allele, Rosset and Gorini selected for secondary mutations that restored the original leaky arginine auxotrophy. Such mutations, which counteracted the increased translational fidelity caused by the *rpsL* allele, were called *ramA* for ribosomal ambiguity (7, 27). *ramA* is now known to be allele to *rpsD*, which lies between *rimE* and *rpsE* (Fig. 9). Our results suggest that the *thr-1* allele in strains AB1157 and 307 is an amber mutation and can be suppressed by the *supE* allele in both strains. It is known that *rpsL* mutations not only restrict the phenotypic suppression and misreading induced by streptomycin, but also affect the genetic suppression of amber mutations by the nonsense suppressors (7). The observation that *rpsL* x^+ strains were less leaky in threonine requirement than *rpsL*$^+$ (x^+ or *x*) strains can be attributed to the restriction of *supE* by the *rpsL* mutation. Based on these considerations and the mapping data for the *x* mutation in strain 307, we therefore postulate that the *x* mutation in strain 307 may be identical to *rpsD* (or *ramA*).

Streptomycin is known to have pleiotropic effects, and the molecular mechanism of its lethal action remains to be elucidated. In the present paper, we have demonstrated the lethal action of streptomycin in the absence of significant inhibition of protein synthesis. Since the bactericidal effect of streptomycin and kanamycin can be prevented by chloramphenicol, tetracycline, and erythromycin, it has been postulated that the lethal action of streptomycin and kanamycin requires a normal ribosomal cycle and that misreading is required for the bactericidal effects of aminoglycoside antibiotics (39). It is worth noting that the *dnaE* mutation in strain 307 rendered this mutant more sensitive to kanamycin, amikacin, and gentamicin in addition to streptomycin, but not to spectinomycin (data not shown). Like streptomycin, kanamycin, amikacin, and gentamicin are known to cause misreading, whereas spectinomycin does not (4, 18). These results suggest that misreading may be important in the bactericidal effect of streptomycin in strain 307. The lethal action of streptomycin in strain 307 is not simply caused by a total blockade of protein synthesis, since bulk protein synthesis was not affected by streptomycin in this strain. Instead, it is related to increased ribosomal misreading caused by streptomycin due to an *rpsD* mutation and the presence of a *dnaE* mutation (or one of the various *dna* alleles, including *dnaE*, *dnaN*, *dnaX*, *dnaY*, and *dnaZ*). The relationship between the *dnaE* mutation (or other *dna* mutations) and the lethality of streptomycin in strain 307 remains to be investigated. It should be noted that the presence of an *rpsD* mutation is a prerequisite for the reduction of streptomycin resistance in an *rpsL* strain by a mutation in the *dna* genes (Tables 3, 5, and 6). This observation strongly suggests that streptomycin may affect DNA replication, which has been rendered partially defective even at the permissive temperature by the *dna* mutation, through *rpsD*-enhanced translational misreading of the mRNA encoding the defective *dna* gene product. In other words, the combined action of streptomycin and an *rpsD* mutation in strain 307, which contains *rpsL31* and *dnaE307*, results in the conversion of a conditionally lethal phenotype due to *dnaE307* at 42°C to streptomycin-induced lethality at 30°C, i.e., reduced streptomycin resistance.

It was not known previously that strain AB1157 harbors a mutation in the *rpsD* gene. As a result of the characterization of strain 307, we checked the level of streptomycin resistance of AB1157 clones which had been stored in our

TABLE 5. Correlation between streptomycin sensitivity and leaky threonine requirement and the mutation in gene *x*[a]

Strain	Relevant genotype			Streptomycin sensitivity[b] (mg/ml)	Threonine requirement
	rpsL	*x*	*dnaE*		
AB1157X	−	−	+	1	Leaky
307	−	−	−	0.1	Leaky
307-2	−	+	−	10	Less leaky
307-21	−	+	−	10	Less leaky
307-22	−	−	−	0.1	Leaky
307-23	−	−	−	0.1	Leaky
307-31	+	+	+	0.003	Leaky
307-32	+	−	+	0.002	Leaky
307-33	+	+	−	0.002	Leaky
307-34	+	−	−	0.002	Leaky

[a] *E. coli* cells were grown in M9-glucose medium supplemented with required amino acids and thiamine at 30°C on rotary shaker overnight. One loopful of the culture was inoculated into 5 ml of fresh M9-glucose medium without threonine and incubated at 30°C. The OD_{600} of the culture was measured. Strains AB1157, 307, 307-22, 307-23, and all *rpsL*$^+$ strains grew well (OD_{600} of 0.8 to 0.9) in 48 h, whereas strains 307-2 and 307-21 did not (OD_{600} of <0.04).

[b] Maximum streptomycin concentration in which these strains grew.

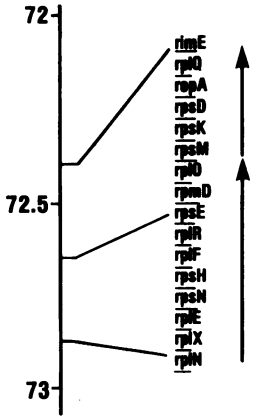

FIG. 9. Map position of the *x* mutation on the *E. coli* chromosome (1). Arrows indicate the direction of transcription of the operons. Numbers represent minutes.

TABLE 6. Streptomycin sensitivity of *dna* derivatives of AB1157 and AB1157X

Strain	Streptomycin sensitivity[a] (mg/ml)
AB1157	≧10
AB1157X	6
307	0.1
AB1157(307)	≧10
AB1157X(307)	0.2
AB1157(dnaX)	≧10
AB1157X(dnaX)	0.4
AB1157(dnaY)	≧10
AB1157X(dnaY)	0.6
AB1157(dnaZ)	≧10
AB1157X(dnaZ)	0.2
AB1157(dnaN)	≧10
AB1157X(dnaN)	0.6

[a] Maximum concentrations of streptomycin in which these strains grew. Concentrations tested: 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6, 8, and 10 mg/ml.

laboratory. Surprisingly, we found two kinds of strain AB1157. One AB1157 strain, represented by the strain used in the present study, shows low-level streptomycin resistance (1,000 μg/ml) and a leaky threonine requirement. The other AB1157 strain is characterized by a high level of streptomycin resistance (10 mg/ml) and less leaky threonine auxotrophy. The latter clone is presumably *rpsD*$^+$ (or *x*$^+$). Recently, Verma and Egan reported phenotypic variation in AB1157 cultivars from different sources; AB1157 East and AB1157 West differ in their susceptibility to host infection by coliphage 186 after UV irradiation of the host cell (34). The relationship between the status of the *rpsD* gene and the phenotypic variation observed by these workers remains to be determined.

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K.S. and T.W. contributed equally to this work.

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