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Escherichia coli Mutant Containing a Large Deletion from *relA* to *argA*†

ALAN G. ATHERLY

Department of Genetics, Iowa State University, Ames, Iowa 50011

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A mutant of *Escherichia coli* has been isolated that contains a large deletion (about 3×10^7 daltons of deoxyribonucleic acid) encompassing *argA*, *fuc*, and *relA*. This mutant strain (AA-787) is also cold sensitive for growth at 18°C. Strain AA-787 was obtained fortuitously as a cold-sensitive pseudorevertant of a strain having a heat-sensitive peptidyl-transfer ribonucleic acid hydrolase. Genetic analysis, using transduction and interrupted mating, showed the cold sensitivity mutation to be located adjacent to *relA*. Further analysis demonstrated loss of *relA*, *fuc*, and *argA* gene functions but retention of *eno* and *recB*, closely linked genes adjacent to *relA* and *argA*, respectively. Unusually high cotransduction of flanking markers (*cysC* and *thyA*) indicated loss of approximately 1 min of the *E. coli* genetic map in strain AA-787. Guanosine 3'-diphosphate 5'-diphosphate (ppGpp) was synthesized in mutant strain AA-787 at basal levels, and ppGpp synthesis was stimulated by carbon-source downshift. No ppGpp synthesis could be obtained using ribosomes isolated from strain AA-787. These findings, taken together, show that deletion of *relA* in *E. coli* does not completely abolish ppGpp synthesis and suggests that another enzyme system must also be responsible for ppGpp synthesis.

Guanosine 3'-diphosphate 5'-diphosphate (ppGpp) has been implicated in the regulation of a number of metabolic events in bacteria: the synthesis of rRNA, tRNA, r-protein, α-subunit of RNA polymerase, elongation factor Tu, and phospholipids to mention a few (for review, see reference 8). This compound is synthesized by a ribosome-bound enzyme (stringent factor) from GTP and ATP in the presence of mRNA and a codon-specific uncharged tRNA (16, 21).

The stringent factor is coded for by the *relA* gene, which maps at min 59 of the recalibrated *Escherichia coli* linkage map (3). Using many independently isolated mutants of *relA*, an excellent correlation was found between the synthesis of rRNA and in vitro stringent factor activity (3). Other genes involved in ppGpp synthesis and metabolism are *relC*, *relX*, and *spoT*. *relC* codes for the 50S ribosomal protein L11 (12), and *spoT* codes for an enzyme that hydrolyzes ppGpp to GMP and pyrophosphate (24). *relX* is involved somehow in determining basal levels of ppGpp (19), although the role of *relX* is not yet clear. Another gene, *relB*, has been shown not to be involved in ppGpp metabolism (10). All of these genes, except *relX*, are located in separate areas of the *E. coli* linkage map distinct from *relA*.

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A puzzling observation in all *relA* mutants so far isolated is that ppGpp is still present at basal levels and its accumulation can be stimulated, but only by carbon source downshift (15). The residual synthesis of ppGpp in *relA* mutant may be because the *relA* gene product is absolutely required for normal growth, but only at very low concentrations. Consequently, all *relA* mutants would be leaky and contribute just enough gene product for survival. This interpretation is probably not correct since, herein, I describe a mutant of *E. coli* that has a deletion in the *relA* gene which can still synthesize ppGpp. The existence of this mutant suggests that another enzyme also must be responsible for ppGpp synthesis. This conclusion is reinforced by a report, describing isolation of insertion and nonsense mutants of *relA*, published during preparation of this manuscript (13).

MATERIALS AND METHODS

Growth conditions and bacterial strains. All strains are derivatives of *E. coli* K-12 and are listed in Table 1. Chemically defined media were made by supplementing a minimal salt buffer (1) with amino acids at 10 to 50 μg/ml, depending upon their ratio in proteins (or by adding 0.2% Casamino Acids), thiamine at 1 μg/ml, and glucose at 0.2%. Broth media were described previously (1). Liquid cultures were grown aerobically on a rotary-action shaker at various tem-

TABLE 1. *Strains of E. coli used in this study*

Strain	Genotype	Reference or source
NF-58	*argA metB thi relA*$^+$	11
NF-59	*argA metB thi relA*	11
CP-78	*argH thr leu his thi relX1 relA*$^+$	12
AA-7852	Derivative of CP-78, *pth-1*	2, 15
AA-787	Derivative of AA-7852, cold-sensitive *relA14 argA fuc relX1*	This paper
AA-38	Derivative of NF-58 *metB thi relA14 fuc relX1 argA*	Transduction with AA-787
KL-16	Hfr, *rps*$^+$ (counterclockwise near *lysA*)	3
P4X	Hfr, *rps*$^+$ (clockwise near proB)	3
NF-305	*pyrB43 argG6 metB1 lysA29 his-1 leu-6 recA1 rpsL104 specA13 supE44*	11
NF-306	F160 *lysA* to *cysC* in host NF-305	B. J. Bachmann 11
NF-178	*cysC13 spc*^{r} *pyrB leu-2 his-1 argG6 metB relA*$^+$ *thyA*	J. Friesen
KLF16/KL110	F116 *metC* to *fuc* in host KL110, *argG6 metB1 leu-6 his-1 thyA23 recA1 supE44*	B. J. Bachmann

peratures. Growth was measured turbidimetrically on a Klett-Summerson colorimeter.

Isolation of mutant and genetic crosses. No mutagen was used for isolation of mutant strain AA-787. From overnight culture of strain AA-7852 (carrying *pth*) grown from a single colony, several thousand revertants to 42°C growth were selected. Cells that did not grow at 18°C but did grow at 42°C were selected and restreaked. These occurred at a rate of about 1 in 200.

A cross was performed between strain KL-16 (Hfr, *rps*$^+$ *thyA*$^+$) and the cold-sensitive strain AA-787 (F$^-$ *rpsL thyA relA14*). The procedure has been fully described (1). To detect *thyA*$^+$ *rpsL* recombinants, samples were spread on minimal agar lacking thymine and containing 200 μg of streptomycin per ml and incubated at 30°C for 36 h. To detect recombinants that were not cold sensitive, samples of the mating mixture were spread on broth agar containing 100 μg of streptomycin per ml and incubated for 48 h at 18°C.

To transfer the cold sensitivity mutation to another strain and to perform a four-factor cross, a lysate of phage P1 was prepared on strain AA-787 (carrying *thyA*$^+$, and cold sensitive) by the procedure of Miller (20). The recipient strain NF-58 (carrying *argA metB* and *relA*$^+$) was converted to *argA*$^+$ by mating with Hfr KL-16 and selecting recombinants that did not require arginine for growth and were *relA*$^+$. A *thyA* mutant strain was then selected from this strain (20). After transduction, *thyA*$^+$ recombinants in strain NF58 were obtained by spreading on minimal agar containing methionine and arginine, followed by growth at 30°C for 48 h. These colonies were tested for arginine requirements and cold sensitivity by restreaking on similar plates lacking arginine at 30°C and broth agar plates grown at 18°C. Each colony was identified by a number and recorded. *relA* gene activity was tested in each recombinant by a procedure described by Cashel (personal communication). This procedure involves growing cells overnight on broth agar plates and picking enough cells with a toothpick to give turbidity in 200 μl of 40 μM phosphate minimal-glucose (with 50 μg of thymidine per ml, 1 mg of serine hydroxamate per ml, 300 μg of L-valine per ml, and 5 μCi of $^{32}PO_4^{+3}$ per ml). This mixture is incubated for 1 h at 37°C, 50 μl of 13 N formic acid is added, and the mixture is frozen for 15 min; 10 μl is spotted 1 cm apart on polyethyleneimine thin-layer sheets and eluted 10 cm with 1.5 M KH_2PO_4 (pH 3.4). The spots are visualized by exposure to X-ray film overnight, and the presence or absence of ppGpp is established by the presence of a darkened area at an R_f of 0.20.

In a separate experiment testing the linkage distance between *cysC* and *thyA*, a P1 lysate was prepared on AA-787 or CP-78 (carrying *cysC*$^+$ and *thyA*$^+$). The recipient strain NF-178 (carrying *cysC* and *thyA*$^+$) was converted to *thyA* (20). After transduction, recombinants not requiring thymine were selected on minimal agar plates containing all amino acids and uracil but lacking thymine. Colonies appeared after 48 h at 30°C. These colonies were replicated onto similar minimal agar plates but lacking cysteine. After several days of growth the number of cysteine-requiring transductants was recorded. For measurement of reversion frequency of *argA*, strain AA-787 had to be converted to *argH*$^+$. This was done by conjugation with Hfr strain P4X that enters counterclockwise near *proB* (Table 1). Recombinants were selected that grew on ornithine and the other required amino acids. The reversion frequency for several genes was measured in strain AA-787. For arginine requirements (*argA*), 10^{10} cells were spread on several minimal agar plates containing no arginine. The other required amino acids were present. Growth of colonies was seen as an indication of a reversion to no arginine requirement. *fuc* was tested by spreading 10^{10} cells on minimal agar plus required amino acids with fucose as the carbon source (0.5%). Reversion from cold sensitivity was tested by spreading 10^{10} cells on broth agar and incubating at 18°C.

Enzyme and gene activity assays. The presence of *lysX* gene activity was determined as described by Jenkins (7). Strains that are *lysX* excrete large amounts of lysine and thus stimulate the growth of lysine-requiring strains. Strain NF305 was mixed, at 100 cells per ml, with minimal agar containing a carbon source and all required amino acids except lysine. This mixture was poured into petri dishes and allowed to solidify. The test strains were streaked across the agar surface and incubated for several days until growth on

the surface was clearly established. The presence of lysine excretion was determined by the appearance of small colonies within the agar and near the growth of the test strain. A control was done by streaking a solution of 5-mg/ml lysine across the agar surface which resulted in growth of strain NF305 colonies.

Enolase activity was measured as previously described (22), and protein was determined by the method of Bradford (6). The presence of a *rec* mutation in strain AA-787 was tested as described by Miller (20). Peptidyl tRNA hydrolase was assayed by J. Menninger as previously described (2, 18).

For the measure of the cell-free synthesis of ppGpp, the reaction mixture in 5 μl contained: GTP, 2 mM; ATP, 4 mM; [^{3}H]GTP, 10 μCi (10 Ci/mmol), 400 μg of S-30 extract (5 μl); 50 mM Tris-acetate (pH 7.8); 5 mM dithiothreitol; 10 mM Mg acetate$_2$; 70 mM NH_4 acetate. Incubation was done for 3 h at 18°C. The reaction was stopped by addition of 1 μl of 24 N formic acid. The products were separated on polyethyleneimine-cellulose chromatography sheets, eluting with 1.5 M phosphate buffer (pH 3.4). The radioactive spots were cut out and counted in a scintillation spectrophotometer.

Synthesis of ppGpp in whole cells was measured as described by Cashel (7). Cells were grown in low-phosphate media (40 μM), required amino acids, and 0.03% glucose and 0.3% succinate with 0.32 mCi of ^{32}P per ml.

RESULTS

Mutation selection and growth rates. The mutant strain (AA-787) was fortuitously isolated (see Materials and Methods) as a pseudorevertant of a heat-sensitive peptidyl tRNA hydrolase strain (2, 18). The grandparent strain is strain CP-78. The parent strain, AA-7852, grows poorly at 40°C but not at all at 42°C. The mutant strain, AA-787, which is also cold sensitive, grows well at 40 and 42°C, but not at 18°C on either broth or minimal media (Table 2).

Genetic studies. The mutation in strain AA-787 was located by interrupted mating with Hfr strain KL-16 by using *thyA* as a reference marker. The cold sensitivity mutation is slightly distal (counterclockwise) from *thyA* with reference to the entry point of KL-16 (*lysA* at min 61, reference 3). This suggested a point near *argA*, *fuc*, or *relA*. Subsequent tests showed all three gene activities to be absent in strain AA-787. Separately determined reversion frequencies for *fuc*, *argA*, and cold sensitivity were less than 10^{-10} (see Materials and Methods). The nearest enzyme activities coded by genes mapping clockwise (*recB*) and counterclockwise (*eno*) to *argA* and *relA*, respectively, were assayed (see Materials and Methods) and found normal (data not shown). The absence of these three adjacent gene activities (*argA*, *fuc*, and *relA*) and their extremely low reversion frequency suggested that a deletion may be present from near *argA* to *relA*.

TABLE 2. *Growth characteristics*

Strain	Medium	Doubling time (min)[a] at:		
		18°C	30°C	40°C
CP-78	L broth	+	37 ± 4	25 ± 4
AA-7852	L broth	+	69 ± 4	180 ± 10
AA-787	L broth	−	70 ± 6	30 ± 3
CP-78	Minimal	+	54 ± 4	44 ± 3
AA-7852	Minimal	+	59 ± 6	310 ± 10
AA-787	Minimal	−	118 ± 5	56 ± 5

[a] Symbols: +, growth; −, no growth.

The cold sensitivity mutation was more carefully located by a four-factor cross using P1 phage cotransduction of *relA*, *argA*, and cold sensitivity, selecting for *thyA*$^+$. Only two of eight possible classes appeared in 86 *thyA*$^+$ recombinants selected; 36 were *relA argA* and cold sensitive, and 50 were *relA*$^+$ *argA*$^+$ and not cold sensitive. A cotransduction freqeuncy of 42% between *thyA*, cold sensitivity, and *argA* indicates a map position near *argA* (3). *relA*, *argA*, and cold sensitivity all cotransduce at the same frequency and thus suggest a deletion encompassing all three genes.

If the area encompassing *argA* to *relA* is deleted in mutant strain AA-787, cotransduction of *cysC* and *thyA* (flanking markers) should occur more frequently than the approximately 2 to 5% normally found (3). *cysC* and *thyA* are 1.7 map units apart on the recalibrated *E. coli* genetic map (3), which is approaching the length of the P1 DNA transduction fragment (3). To test this conclusion a P1 phage lysate was prepared on strain AA-787 (*cysC*$^+$ *thyA*$^+$) and used in the transduction of strain NF-178 (*cysC thyA*). Cells not requiring thymine (*thyA*$^+$) were selected, and these recombinants were tested for cotransduction of *cysC*$^+$. Of 101 *thyA*$^+$ transductants, 35 were *thyA*$^+$ *cysC*$^+$, giving a cotransduction frequency of 35%. This value is considerably greater than the 2 to 5% obtained in a normal cross (using CP-78, data not shown). Using the equation of Wu (25), with these data a distance of 0.56 map units can be calculated between *cysC* and *thyA* in AA-787. Thus, 1 to 1.2 map units have been deleted between *thyA* and *cysC* in mutant strain AA-787.

The cold sensitivity phenotype was recessive to wild type since, upon introduction of episome F160 from strain NF-306, cold sensitivity was recessive along with mutant functions in *fuc*, *relA*, and *argA*. Introduction of F116, however, which partly covers the deleted area (counterclockwise from *fuc* to *metC*), did not abolish cold sensitivity. It must be concluded that the gene

responsible for cold sensitivity is located between *fuc* and *relA*.

ppGpp synthesis. Stringent-factor activity was determined by measuring the cell-free conversion of GTP to ppGpp by using ribosomes from the cold-sensitive mutant (AA-787) and its grandparent strain (CP-78). A 38.6% conversion of GTP to ppGpp was found using strain CP-78 ribosomes, but only 0.3% conversion was observed using strain AA-787 ribosomes. These data, hence, indicate that no structural gene product of the *relA* gene is active in strain AA-787.

Basal levels of ppGpp and the ability to synthesize ppGpp upon downshift were determined using whole cells. It is known that *relA* strains can synthesize appreciable quantities of ppGpp upon downshift, but *relA* point mutant strains used in all previous studies are likely missense mutants (4, 12). If *relA* alone is responsible for the synthesis of ppGpp, then this strain should not have any ppGpp present due to the deletion present in AA-787. As seen from the data in Fig. 1, the basal levels of ppGpp, in a strain possessing this deletion, are not different from that found in point mutant *relA* strains. Furthermore, upon shift from a glucose to succinate carbon source, considerable ppGpp was synthesized in the deletion strain. Results from *relA* and *relA*$^+$ strains (NF-58 and NF-59) are given for comparison.

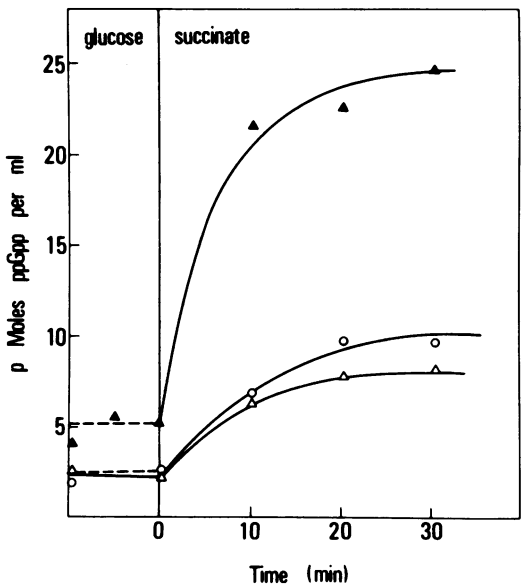

FIG. 1. *Accumulation of ppGpp in cells shifted from glucose to succinate carbon source. Growth media and procedures for measuring ppGpp were done as previously described (7). Symbols: ▲, NF-58 (relA$^+$); ○, NF-59 (relA); △, AA-38 (ΔrelA, cold sensitive).*

DISCUSSION

Several interesting conclusions can be made from investigation of strain AA-787. The presence of a basal level of ppGpp and the stimulation of ppGpp synthesis upon carbon-source downshift in other *relA* strains may be due to a partly active stringent factor. However, strain AA-787, which is deleted in *relA*, still synthesizes ppGpp similar to archetype *relA* strains (5). These findings strongly argue for the existence of another enzymatic pathway for ppGpp synthesis other than with stringent factor; however, it is possible that the delection in AA-787 includes a regulatory gene for *relA* and not the structural gene. This alternative seems unlikely, however, since no stringent factor activity could be found associated with the ribosomes. In support of this conclusion, Zabos et al. (26) and Sy (23) reported ribosome-independent synthesis of ppGpp and, more recently, Friesen et al. (13) found nonsense and insertion mutants in *relA* that still synthesize ppGpp.

A few words should be said about *relX*, which is also deleted in strain AA-787. The concentrations of ppGpp, both basal and induced, during downshift were not significantly different between strain NF-58 (*relX*$^+$ *relA*$^+$), NF-59 (*relX*$^+$ *relA1*), and AA-38 [Δ(*relX2-relA14*)] (see Fig. 1). This is in contrast to what was observed by Pao and Gallant (19), using the same genetic background but a different *relX* (*relX1*) mutant. Other features of Δ*relX2* and *relX1* are different. *relX1* (in an NF background) is temperature sensitive (at 42°C) but Δ*relX2* is not; however, strain AA-787 [Δ(*relA14-relX2*)] has a greatly reduced plating efficiency onto minimal media with 100 μg of leucine per ml, similar to *relA1 relX1* mutants characterized by Pao and Gallant. The *relX1* mutation was naturally occurring in a strain when found by Pao and Gallant (19). They ascribe a key role for this gene in restricting ppGpp accumulation during carbon source downshift. Observations with strain AA-787 and its NF derivative AA-38 do not bear this conclusion out.

Bachmann et al. (3) report the position of the gene *pyrG*, coding for cytidine triphosphate synthetase, as being between *relA* and *argA*. This map position probably is incorrect inasmuch as this enzyme activity was present in strain AA-787. *pyrG* is likely located near min 59, proximal to *relA*. Further, *lysX* is reported in this area of the genome (14). Strain AA-787 was found not to be mutant for *lysX* (19) (see Materials and Methods). Thus, *lysX* also may be incorrectly

located on the *E. coli* genetic map (3). It can be concluded that no genes are present between *relA* and *argA* that are necessary for growth on minimal media with glucose, glycerol, or succinate as a carbon source.

Just exactly why this strain is cold sensitive is not known. The cold sensitivity phenotype can be retained when transferred to other strains, thus it is not dependent upon the parent strain gene makeup. From results of partial diploids covering the deletion or part of the deletion in strain AA-787, it can be concluded that the cold sensitivity function is located between *relA* and *fuc* genes. However, it cannot be distinguished whether cold sensitivity is due to deletion of *relA*.

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