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DNA Sequence and Complementation Analysis of a Mutation in the *rplX* Gene from *Escherichia coli* Leading to Loss of Ribosomal Protein L24

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A mutation in *Escherichia coli* leads to the loss of ribosomal protein L24, severely impaired growth, and a temperature-sensitive phenotype. The mutation was shown to be in *rplX*, the gene for protein L24, and was due to the alteration of an AAA codon to a TAA stop codon at position 61 in *rplX* that resulted in a 20-amino acid peptide instead of the 104 amino acids of wild-type L24 protein. *rplX* genes from three temperature-resistant and fast growing pseudorevertants of the mutant were cloned and sequenced. They were found to have different base substitutions in the TAA codon, resulting in the reappearence of a full-sized protein L24 moiety. Complementation of the slow growth in *trans* could be achieved with several plasmids containing at least the *spc* promoter and intact L14 and L24 genes. Plasmids containing genes distal to *rplX* could further stimulate growth, and the wild type arose when the entire *spc* operon and the alpha operon were present. In all cases, protein L24 was expressed by the plasmids. Therefore, slow growth could be explained by polarity extending to the alpha operon. However, temperature sensitivity could not be complemented by any of the plasmids in *trans*, although we found that this phenotype was caused by the mutation in the *rplX* gene.

Mutational analysis provides a powerful tool in investigating the function of cellular components. However, although mutants of *Escherichia coli* have been isolated that harbor alterations in most ribosomal proteins (6, 14), very few of these alterations give rise to a conditionally lethal phenotype such as cold or temperature sensitivity (20, 26). Most of these mutants were found to have alterations in ribosomal proteins S4 and S5 (12).

In contrast, mutants lacking a ribosomal protein frequently possess a conditionally lethal phenotype as a result of lesions (7, 8). Mutants lacking any one of 15 of the 53 *E. coli* ribosomal proteins have been described, including one lacking protein L24 (9). Since protein L24 is one of two proteins implied as being essential in the initiation of a large ribosomal subunit assembly (2, 19), it was of interest to further characterize this mutant. In agreement with the important role ascribed to this protein, the lesion resulted in a severe reduction in growth rate and deficiency in the amount of the large ribosomal subunit as well as a temperature-sensitive phenotype (9). No L24 cross-reacting material was detected either in the ribosome or in the postribosomal supernatant (10). Since hitherto the nature of the mutation responsible for the absence of a ribosomal protein has not been determined, we considered it worthwhile to analyze the L24-lacking mutant.

We also investigated several temperature-resistant secondary mutants in which protein L24 was present in the ribosome, albeit in a form different in electrophoretic mobility to that in the wild type. Further, we investigated the phenotypes of temperature sensitivity and slow growth arising from lesions to determine whether they could be explained solely by the absence of protein L24.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in these studies are listed in Table 1. The plasmids (Fig. 1) are either derivatives of pRE432 (11) or pACYC184 (4). pRE432 is a 13.5-kilobase large mini-F plasmid with a copy number of ca. 1 to 2 copies per cell.

Media and genetic analysis. Bacteria were grown in L-broth. Antibiotics and their respective concentrations were added as follows: chloramphenicol, 10 μg/ml; ampicillin, 50 μg/ml; and tetracycline, 10 μg/ml. Transductions with phage P1 were carried out as described previously (13).

Enzymes and nucleotides. Restriction endonucleases, T4 DNA ligase, *E. coli* DNA polymerase, and its "large" Klenow fragment were purchased from Boehringer and Bethesda Research Laboratories; deoxy- and dideoxynucleotides were purchased from Pharmacia P-L Biochemicals, Inc., [α-^{32}P]dATP (400 Ci/mmol) was purchased from Amersham.

Preparative and analytical techniques. DNA manipulations were generally carried out as described by Maniatis et al. (16). Chromosomal DNA was isolated with Sarkosyl as described by Chow et al. (5). DNA fragments were separated by horizontal agarose gel electrophoresis (17). For preparative gels, low melting agarose (Biorad) was used. Ligation of DNA fragments was done as described by Weiss et al. (25). DNA was sequenced with M13mp10 and mp11 (18) by the dideoxy chain termination method of Sanger et al. (21).

RESULTS

Fine mapping of the mutation. Since the mutant TA109, which lacks protein L24, is rather unstable at every temperature, we isolated a stable but still temperature-sensitive derivative, TA109-130, which showed an additional S11 alteration (*rpsK*). To establish that the conditionally lethal mutation which eliminates L24 synthesis was located close to or in the protein L24 gene, we first constructed plasmid pKN11. This contained the whole *rplX* gene on the mini-F plasmid pRE432, together with the entire protein L14 gene, about two-thirds of the protein L5 gene, and the *spc* operon promoter (Fig. 1). The insert was a 1.9-kilobase *Sal*I-*Bam*HI fragment derived from lambda *fus*3. The location of the

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TABLE 1. Bacterial strains used in this work

Strain	Characteristics	Source or reference
KL14	Hfr *thi-1 rel-1*	K. Isono
A19	Hfr *metB relA1 rna-19*	L. Gorini
AM109	As A19 *argE argC argB* or *argH rplD14 rplX3* EryD[a]	9
TA109	As A19 *rplX3 rpsL*	9
TA109-130	As TA109 *rpsK13*	This work
TA109-131	As TA109-130 *srl*::Tn*10 recA* transduced from UH748	This work
TA109-310	As TA109 *rplX310*	This work
TA109-313	As TA109 *rplX313*	This work
TA109-314	As TA109 *rplX314*	This work
UH748	F506/UH69 *aroD man pps rpsL his supF*? *trg*::Tn*5 recA srl*::Tn*10*	S. Harayame

[a] The map position of the EryD mutation has not been determined; the locus is not, however, cotransducible with markers in the main cluster of ribosomal protein genes at 72 min on the *E. coli* chromosome.

restriction endonuclease cutting sites was inferred from the DNA sequence of the *spc* operon previously published (3). Mutant TA109-130 was transformed with pKN11 and with the same plasmid lacking the insert. Selection was done on agar without antibiotics at the nonpermissive temperature 42°C and on selective media at 30°C. Numerous temperature-resistant colonies were obtained when this *recA*$^+$ strain was transformed with pKN11 but not when it was transformed with pRE432. Transformants obtained at 30°C were checked for growth at 42°C. Clones transformed with pKN11 grew faster at 30°C and were temperature resistant, whereas this was not the case with those transformed with pRE432. A similar result was obtained when plasmid pKN1, a derivative of pACYC184 having the same insert, was compared with pACYC184. From these results we drew the conclusion that the mutation was indeed close to or within *rplX*, the gene coding for protein L24. Not only temperature sensitivity but also slow growth disappeared after introducing the wild-type allele.

To determine whether the mutation was an insertion or deletion, chromosomal DNA of the parental wild-type strain A19 and mutant AM109 was digested with the enzymes *Sal*I and *Bam*HI, separated on an agarose gel, and blotted on a nitrocellulose filter. A Southern blot with lambda *fus*3 DNA as a probe was performed. There was no difference in the size of the fragments (data not shown). From this we concluded that either a very small insertion or deletion or, alternatively, a base alteration had occurred.

Cloning and DNA sequence of the mutant gene. Having established that the mutation was covered by the 1.9-kilobase *Sal*I-*Bam*HI fragment, we cloned the corresponding DNA from mutant AM109. Chromosomal DNA was isolated, digested with *Sal*I and *Bam*HI, and separated on a 0.8% agarose gel. DNA corresponding in size to the wild-type fragment was eluted and ligated into pACYC184. A total of 120 transformants were pooled in groups of five. DNA of the plasmids was extracted, and one clone was identified by a dot blot hybridization test with the wild-type fragment as a radioactive probe. The plasmid DNA of this clone was purified, and an *rplX3* strain was transformed with it. No temperature-resistant or faster growing transformants were obtained.

To determine the location of the mutation on the fragment, mutant and wild-type fragments were digested with *Alu*I and *Sal*I, and the resulting *Alu*I-*Sal*I fragment was cloned into phage M13mp11. The DNA was sequenced, and a single base alteration was observed which changed an AAA lysine codon to a TAA stop codon (Fig. 2). The expected length of the peptide resulting from this mutation would be 20 amino acids compared with 104 residues for the whole protein. A consequence of the mutation should be the acquisition of a second *Hpa*I restriction site, which was confirmed. The surrounding sequence extending into the L14 gene, and on the other side to the end of the L24 gene, did not show any base alteration. Thus, we concluded that we had identified the mutation responsible for the loss of protein L24.

Cloning and DNA sequence of the *rplX* gene of three revertants. A total of 20 temperature-resistant secondary mutants were isolated from strain TA109. They were analyzed by two-dimensional gel electrophoresis. In all 20, a protein spot in a position similar to protein L24 was observed, and all mutants had growth properties which were indistinguishable from those of wild-type strain A19. None had a protein L24 identical in electrophoretic mobility to that of the wild type. Two major classes of pseudorevertants, as distinguished by electrophoretic mobility, were obtained. In addition, one pseudorevertant in which L24 was considerably more acidic than in the wild type was observed. We isolated chromosomal DNA from a representative of the

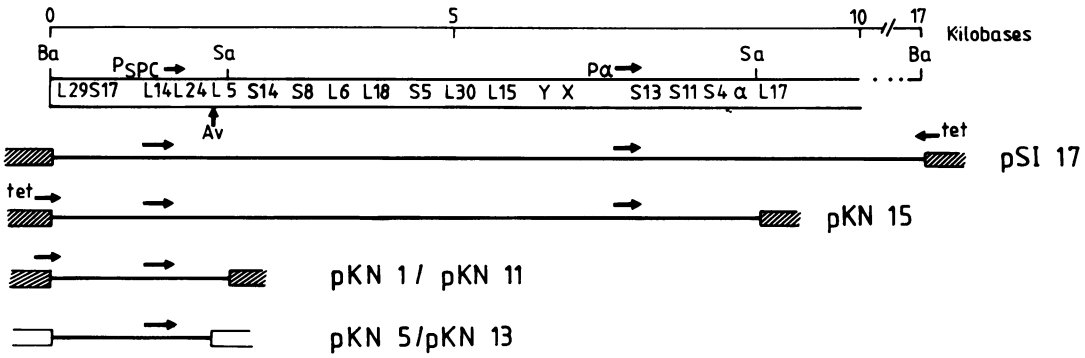

FIG. 1. The chromosomal region around the *spc* operon as deduced from lambda *fus*2 (15) and the plasmids which were constructed. Plasmids pKN11, pKN13, pKN15, and pSI17 are derivatives of the mini-F plasmid pRE432. Plasmids pKN1 and pKN5 were derived from pACYC184. The arrows indicate the direction of transcription. The orientation of the fragments in the plasmids is indicated by the *tet* gene whose orientation was recently described (24). Only plasmids pKN5 and pKN13 were not analyzed in this respect. The restriction sites are abbreviated as follows: Av, *Ava*I; Ba, *Bam*HI; and Sa, *Sal*I.

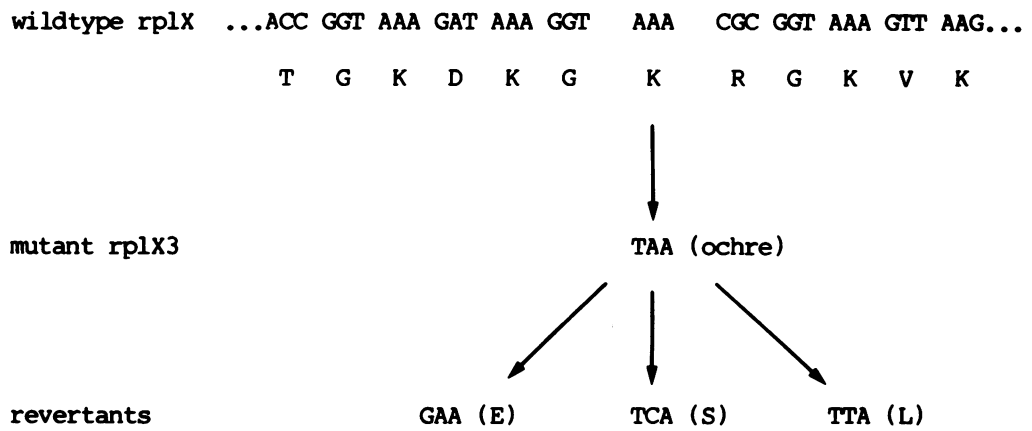

FIG. 2. The DNA sequence and the corresponding amino acid sequences of wild-type gene *rplX*, mutant gene *rplX3*, and three revertant genes which regained temperature resistance: *rplX310* (GAA), *rplX313* (TCA), and *rplX314* (TTA). The GAA, TCA, and TTA codons corresponded to glutamic acid (E), serine (S), and leucine (L), respectively.

major classes of pseudorevertants (TA109-313 and TA109-314) and from the one pseudorevertant with a more acidic L24 (TA109-310). DNA was digested with *Sal*I and *Bam*HI and cloned into pRE432.

From complementation studies (see below), we knew that a fragment containing the wild-type allele (pKN11) could stimulate the growth rate of strain TA109-131. We expected this to be also true for the revertants. Therefore, after ligation of the *Sal*I-*Bam*HI fragments from the DNA of the pseudorevertants into pRE432 and transforming mutant TA109-131, we used positive selection to obtain the fragment bearing *rplX*. DNA sequence analysis (Fig. 2) showed three different amino acid triplets where previously there had been a stop codon. The three amino acids were different from the original one, i.e., lysine. In secondary mutant TA109-310, there was a GAA codon corresponding to glutamic acid. In mutant TA109-313, there was a TCA codon for serine, and in TA109-314 there was a TTA codon for leucine.

Complementation analysis of the mutation in *rplX*. We investigated whether the observed phenotypes of very slow growth (about one-sixth of that of the wild type at 30°C) and temperature sensitivity were due only to the absence of a normal sized protein L24 moiety or whether there were other factors involved. For this purpose, a *recA* derivative of the mutant TA109-130, TA109-131, was constructed. This strain was transformed with a series of plasmids which all possessed an intact protein L24 gene together with the *spc* promoter and the gene which lies between these two and codes for ribosomoal protein L14. The plasmids varied principally in the number of ribosomal protein genes located downstream of *rplX*. Transformants were selected at 30°C and analyzed for growth rate and temperature sensitivity.

All transformants grew significantly faster than did the mutant transformed with plasmids without insert. This was 330 min for the mutant transformed with pRE432 alone, 200 min for pKN13, 95 min for pKN11, 60 min for pKN15, and 40 min for pSI17, which is equal to the growth of wild-type A19. The growth rate increased with the increased length of the insert of ribosomal protein genes. It was interesting that transformants with plasmid pKN11, with about two-thirds of the gene for L5, grew much better than the one transformed with plasmid pKN13. However, we could not detect any protein corresponding to an altered L5 by using two-dimensional gel electrophoresis.

None of the plasmids conferred temperature resistance on the *recA* strain TA109-131. Since one explanation could be that this was because of an unidentified temperature-sensitive mutation in lambda *fus*3, we cloned the corresponding fragments from chromosomal DNA of Hfr strain KL14. However, we obtained the same result. Faster growth was observed, but a temperature-sensitive phenotype was maintained. Only in a mating of KL14 with TA109-131, selecting for temperature resistance and *rpsL*, to obtain episomes covering that part of the chromosome including *rplX* could we obtain a high number of F′-containing strains which could grow at the nonpermissive temperature. This indicated that complementation was only achieved in *trans* when a larger DNA fragment covering the whole 72-min region was introduced into the mutant.

DISCUSSION

A spontaneous mutation in the gene for ribosomal protein L24 has previously been described as causing a temperature-sensitive phenotype and leading to a growth rate of only about one-sixth of that of the wild type. In addition, protein L24 was undetectable both on the ribosome and in whole-cell extracts investigated either by two-dimensional polyacrylamide gels or by a battery of immunological techniques (10). All other ribosomal proteins were present in the mutant in amounts indistinguishable from that of the wild type.

Sequencing the segment of DNA from the mutant carrying the protein L24 gene showed that there had been a single base change of A to T in nucleotide 61 of the reading frame. In this way, an AAA triplet coding for lysine had been converted into a chain termination TAA triplet. On the basis of the DNA sequence data, we might expect a peptide to be generated from the *rplX3* gene which is 20 residues long compared with one 104 residues long in the wild-type case. Immunological tests with whole-cell extracts or purified ribosomes failed to show any anti-L24 cross-reacting material, although some of the methods used have been shown to be able to detect moieties of only 4 residues long (23). It may be that a polypeptide of 20 residues is produced, but the antigenic determinants which are recognized by antibodies against wild-type protein L24 are lost. Another possibility may be a rapid degradation of the peptide, since nonsense peptides are usually very unstable. This is the most likely

explanation, especially when the peptide is not incorporated into ribosomes. A third possibility may be the stability of RNA caused by the mutation so that the peptide is produced in negligible amounts. In any case, our data show that the molecular event leading to the lack of protein L24 occurred in the structural gene *rplX*.

There is also the question of whether our findings can be extrapolated to other mutants lacking ribosomal proteins. In at least some cases, Southern blot analysis with DNA from these mutants revealed that the segment carrying the particular gene for the protein which was lacking was larger than in the wild type (E. R. Dabbs and N. P. Fiil, unpublished data). This suggested that the loss of the gene product was either due to an insertion element entering the gene or to some other insertional event. Both events have already been observed for mutations in the genes for protein S5, *rpsE* (22), and protein S4, *rpsD* (22a). However, in these cases the different sized proteins are not missing. Perhaps significantly, in no instance has the loss of ribosomal protein been due to deletion of the gene.

When a *recA*$^+$ *rplX3* strain was transformed with even the shortest of the plasmids described here, recombination occurred between chromosomal and plasmid DNA. The resulting recombinants were wild type with respect to temperature response, growth rate, and form of protein L24. Also, the analysis of pseudorevertants led to the same result, except that in all three cases analyzed there was a different form of protein L24 obtained. This clearly shows that all phenotypes are associated with the nonsense mutation in *rplX*.

Temperature sensitivity, on the other hand, could not be complemented by any of the plasmids in *trans*, although protein L24 was expressed in all cases. Our results discount the other mutation in *rpsK* (S11) as being responsible. Therefore, one possibility could be that the mutation in *rplX* is causing an imbalance in the synthesis of ribosomal proteins. This imbalance may cause further secondary effects because of the lack of the protein and the nonsense mutation. On this basis, our plasmids may partly complement some of the effects but cause different lesions like a slide increase in other ribosomal proteins.

The question of whether the protein or the genetic location of the mutation or both are responsible for the phenotypes can be partly answered by complementation analysis. The short plasmids containing the genes for proteins L14 and L24 stimulate growth to a considerable extent (e.g., 330 min for the mutant compared with 95 min for the mutant transformed with pKN11). In this case, we can conclude that the expression of the protein L24 is responsible. The fact that plasmids having larger inserts with several genes downstream of *rplX* can stimulate growth even more (e.g., 40 min for the mutant transformed with pSI17) indicates polarity caused by the nonsense mutation in *rplX*. We are presently investigating this question further.

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