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Loss of the Spacer Loop Sequence from the *rrnB* Operon in the *Escherichia coli* K-12 Subline That Bears the *relA1* Mutation

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A polymorphism affecting the spacer region of the *rrnB* rRNA operon is described. Strains from a major *Escherichia coli* K-12 subbranch are missing a 106-nucleotide portion of the *rrnB* 16S-to-23S spacer, and a 20-nucleotide sequence is found in its place. We have called this mutant operon *rrnB2*. The *rrnB2* spacer was most probably derived from either *rrnC* or *rrnE*. This alteration of *rrnB* may have occurred by a recombinational exchange or by gene conversion. In the genealogy of *E. coli* K-12 strains, the appearance of *rrnB2* is associated with the spontaneous occurrence of the first relaxed mutation, but attempts to show a selective relationship between the two mutational events have had negative results. The sequences of the *rrnG* and *rrnC* 16S-to-23S spacers have also been determined and their comparisons to the other *rrn* operons encoding $tRNA^{Glu2}$ are presented.

There are seven rRNA operons in *Escherichia coli*, each of which consists of genes for 16S, 23S, and 5S rRNA. In addition, a spacer between the 16S- and 23S-coding regions contains genes for either $tRNA^{Glu2}$ (*rrnB*, *rrnC*, *rrnE*, and *rrnG*) or for both $tRNA^{Ile1}$ and $tRNA^{Ala1B}$ (*rrnA*, *rrnD*, and *rrnH*). Brosius et al. (5) compared the spacer regions of two of the $tRNA^{Glu2}$-coding operons, *rrnB* and *rrnE*. The spacers were similar except that the *rrnB* spacer contained 86 additional nucleotides. They further noted the presence within the additional sequence of an interrupted dyad symmetry that is indicative of a possible secondary structure. No function has been discovered for this sequence. When a fragment from within this sequence was used to probe a restriction enzyme digest of *E. coli* genomic DNA, Brosius et al. found that the sequence hybridized to a second locus, presumed to be *rrnG*. We will refer to this sequence as the *rsl* (ribosomal spacer loop) sequence.

In contrast to these findings, one of us (C. L. Squires) found that *rrnG*, but not *rrnB*, contained the *rsl* sequence (results cited in reference 5). An essential difference in the two sets of experiments was the source of the *rrnB* operon. Brosius et al. used the transducing bacteriophage λ*rif*^{d}18 (11), whereas Squires used pLC34-34 from the Clarke-Carbon bank (6). We will refer to the *rrnB* operon on plasmid pLC34-34, which lacks the *rsl* sequence in its spacer, as *rrnB2*. Explanations for these different results include the possibility that one of these cloned genes underwent a rearrangement between the time of its isolation and its analysis. In that case, the cloned gene would not provide a true representation of *rrnB*. Alternatively, it might be that the *rrnB* and *rrnB2* operons already differed in the *E. coli* K-12 strains used for the original isolation of these cloned sequences. Our results show that this second explanation is correct.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* K-12 prototypes, i.e., CGSC 4401 and CGSC 5073, and strains 58, 58-161, and W6 were obtained from the *E. coli* Genetic Stock Center. The Cavalli Hfr *trpA36* strain (PB153) (8) and the HfrH *trpA36* strain (CH326) were from the collection of C. Yanofsky. Plasmids pLC23-30, pLC34-34, and pLC22-36 were obtained from M. Nomura. Phage P1 transduction techniques and other microbial genetic techniques were as described previously (7).

DNA extraction and Southern analysis. Techniques for DNA isolation, *Bam*HI and *Pst*I restriction endonuclease digestion, gel electrophoresis and transfer to diazotized paper, and hybridization with ^{32}P-oligonucleotide probes were as described previously (12). The oligonucleotide probes were prepared by using an Applied Biosystems DNA Synthesizer. Radiolabeling of these oligonucleotides was accomplished by using 10 U of T4 polynucleotide kinase (Bethesda Research Laboratories, Gaithersburg, Md.) and 135 ng of oligonucleotide in a 20-μl reaction volume. The conditions were 50 mM glycine (pH 9.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.2 mM spermidine hydrochloride, 1 mM [γ-^{32}P]ATP (3,000 Ci/mmol; supplied by Amersham Corp.), and 25% glycerol.

DNA sequencing. The *rrn* 16S-to-23S spacer regions from pLC23-30 (*rrnG*), pLC34-34 (*rrnB2*), and pLC22-36 (*rrnC*) (6, 10) were isolated on *Sal*I restriction fragments and ligated into phage M13mp73 DNA. Clones of both orientations were identified and were sequenced by the dideoxy method with two synthetic primers: a sequence from the 3′ end of the 16S gene (GGTAACCGTAGGGGAACCT) and an antisequence from the 5′ end of the 23S gene (GCCAGGGCATCCA CCGTGTA).

RESULTS

To determine whether the *rrnB* polymorphism arose before or after the initial gene isolations by Clarke and Carbon (6) and by Brosius et al. (5), we tested several *E. coli* K-12 strains for the presence of the *rsl* sequence in *rrnB*. Our approach took advantage of the fact that neither *Bam*HI nor *Pst*I cuts within any of the seven *rrn* operons, with the consequence that each operon occurs on a fragment of unique size (3, 9). Genomic DNA was digested with a combination of *Bam*HI and *Pst*I restriction endonucleases, fractionated by gel electrophoresis, and transferred to dia-

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	X-ray		X-ray		spont.		spont.	
K-12(λ)	→	58 F+	→	58-161F+	→	W6 F+	→	Cavalli Hfr
		bio-1		*bio-1*		*bio+*		*metB1*
		spoT1		*metB1*		*metB1*		*spoT1*
				spoT1		*spoT1*		*relA1*
						relA1		

FIG. 1. Derivation of Cavalli Hfr from *E. coli* K-12 as described by Bachmann (1). spont., Spontaneous.

zotized paper. The papers were hybridized successively with ^{32}P-oligonucleotide probes specific for 23S rRNA and for the *rsl* sequence. Among the strains examined was Cavalli Hfr, the progenitor of the strain used to prepare the Clarke-Carbon plasmid bank. Significantly, we found that the restriction fragment containing *rrnB* from Cavalli Hfr showed no homology with the *rsl*-specific probe, whereas K-12 prototypes, CGSC 4401 and CGSC 5073, did (data not shown). Both strains showed homology between *rrnG* and the *rsl*-specific probe. Thus the discrepancy concerning the structure of the *rrnB* spacer can be explained by a genetic polymorphism differentiating Cavalli Hfr (*rrnB2*) from other *E. coli* K-12 (*rrnB*) strains.

Bachmann (1) has described the lineage of many of the important early K-12 derivatives, and the derivation of Cavalli Hfr is shown in Fig. 1. Cavalli Hfr was derived from K-12 in four steps. One of these steps was the unintentional, spontaneous conversion of strain 58-161 to strain W6. W6 differs from 58-161 by the *relA1* mutation as well as a reversion of the *bio* mutation (2). We tested these progenitors of Cavalli Hfr to see where the *rrnB* polymorphism arose. The results (Fig. 2) showed that strains 58 (lane 2 and 6) and 58-161 (lane 3 and 7) are similar to K-12 (lane 1 and 5) in that both retain *rsl* in *rrnB*. However, W6 (lane 4 and 8), the immediate progenitor of Cavalli Hfr, has lost *rsl*. Hfr-H, another W-6 derivative (1), also lacks *rsl* at *rrnB* (data not shown). Therefore, the loss of *rsl* from *rrnB* is correlated with the *relA1* mutation, and the association of these alleles has been maintained in several strains.

We have used DNA sequencing to determine more about how the *rsl* sequence was lost from the *rrnB* operon. *Sal*I restriction fragments that span the spacer regions of *rrnB2*, *rrnC*, and *rrnG* were isolated from plasmids pLC34-34, pLC22-36, and pLC23-30 (6, 10) and then cloned and sequenced. These newly sequenced tRNA^{Glu2} spacers were compared with the already published sequences of *rrnB* (5) and *rrnE* (14) (Fig. 3). The *rrnB2*, *rrnC*, and *rrnE* sequences were very similar but not identical. This similarity between the *rrnB2*, *rrnC*, and *rrnE* spacer sequences contrasted to the substantial divergence of the two *rsl*-encoding operons, *rrnB* and *rrnG*. The *rrnB* and *rrnG* sequences were not the same size (440 and 431 nucleotides, respectively) and differed from each other at 25 positions. This divergence is contrary to the statement, attributed to one of us (C. L. Squires) that the spacers of *rrnG* and *rrnB* are identical (5). Of particular importance to the issue at hand, the *rrnB* and *rrnB2* sequences differed by more than the simple presence or absence of the *rsl* sequence. This has implications for the probable origin of the *rrnB* polymorphism (see Discussion).

Loss of the *rsl* from *rrnB*, giving rise to the *rrnB2* operon, is correlated with the spontaneous occurrence of the first *relA* mutation (Fig. 1 and 2). Since the *relA* locus affects the control of ribosomal RNA synthesis, the possibly coincidental loss of *rsl* and acquisition of *relA1* suggested a possible selective relationship between the two events. For example, although no function of the *rsl* sequence has been established, it might be that two copies of the sequence are severely disadvantageous to a *relA* mutant. If this were true, then occurrence of the *relA1* mutation would have produced a situation in which the *rsl* loss was selected. Such a selective situation would have important implications for *rsl* function.

In an attempt to detect such an effect, we constructed a set of congenic derivatives of Cavalli Hfr that were *relA1* and had *rrnB* with and without its associated *rsl* sequence. The construction started with PB153, a Cavalli Hfr made *trpA36*. PB153 was modified through a series of phage P1 transductions by using *zij-116*::Tn*10*, which is inserted near *rrnB* (12). The constructions were designed to produce pairs of *relA1* strains which were either *rrnB*$^+$ Tet^r and *rrnB2* Tet^s or *rrnB*$^+$ Tet^s and *rrnB2* Tet^r. Scoring of the *rrnB* alleles for these constructions was done by Southern analysis. No growth advantages for any of the strains were observed by simple inspection of colony size or of growth in liquid culture. A much more sensitive test was performed by mixing Tet^r and Tet^s cultures of opposite *rrnB* genotypes in Luria broth. The mixed cultures were grown for 144 generations by 12 successive serial transfers, each made after the cultures had reached stationary phase. The ratios of Tet^r and Tet^s cells were periodically measured. In no case did the pairs differ by more than 4% in their apparent growth rates (calculated as described in reference 9), and the direction of even this small bias did not consistently favor either of the *rrnB* genotypes. Liquid cultures were also stored for a month at room temperature, and survival of the *relA1 rrnB*$^+$ and *relA1 rrnB2* cultures was compared. No significant differences were found. It appears that under the conditions tested, presence of the *rsl* sequence in *rrnB* has little or no effect on the growth or survival of an *relA1* mutant.

DISCUSSION

Several mechanisms for the loss of the *rsl* sequence from *rrnB* can be imagined. Brosius et al. (5) have noted the two

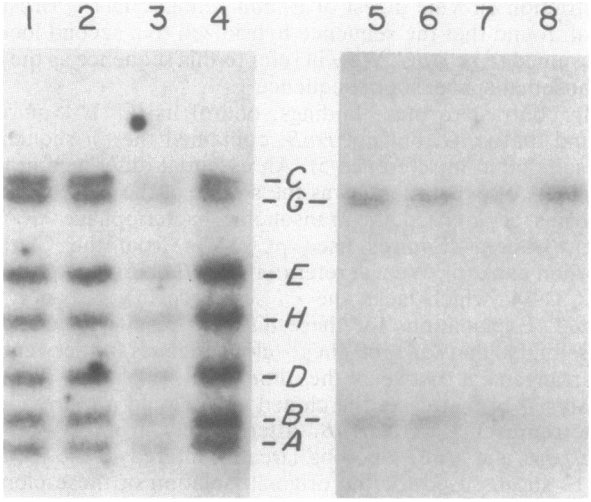

FIG. 2. *Bam*HI-*Pst*I fragments coding for *rsl*. DNA isolation, enzymatic digestion, and analysis were as indicated in Materials and Methods. The uppercase letters designate restriction fragments containing the corresponding *rrn* operons. Lanes: 1 and 5, strain K-12 (CGSC 5073); 2 and 6, strain 58; 3 and 7, strain 58-161; 4 and 8, strain W6. Lanes 1 to 4 were probed with an oligonucleotide specific for 23S rRNA; lanes 5 to 8 were probed with an oligonucleotide specific for the *rsl* sequence. The 23S probe was equivalent to nucleotides 6147 to 6164 and the *rsl* probe was equivalent to nucleotides 3129 to 3147 of the *rrnB* sequence published by Brosius et al. (5).

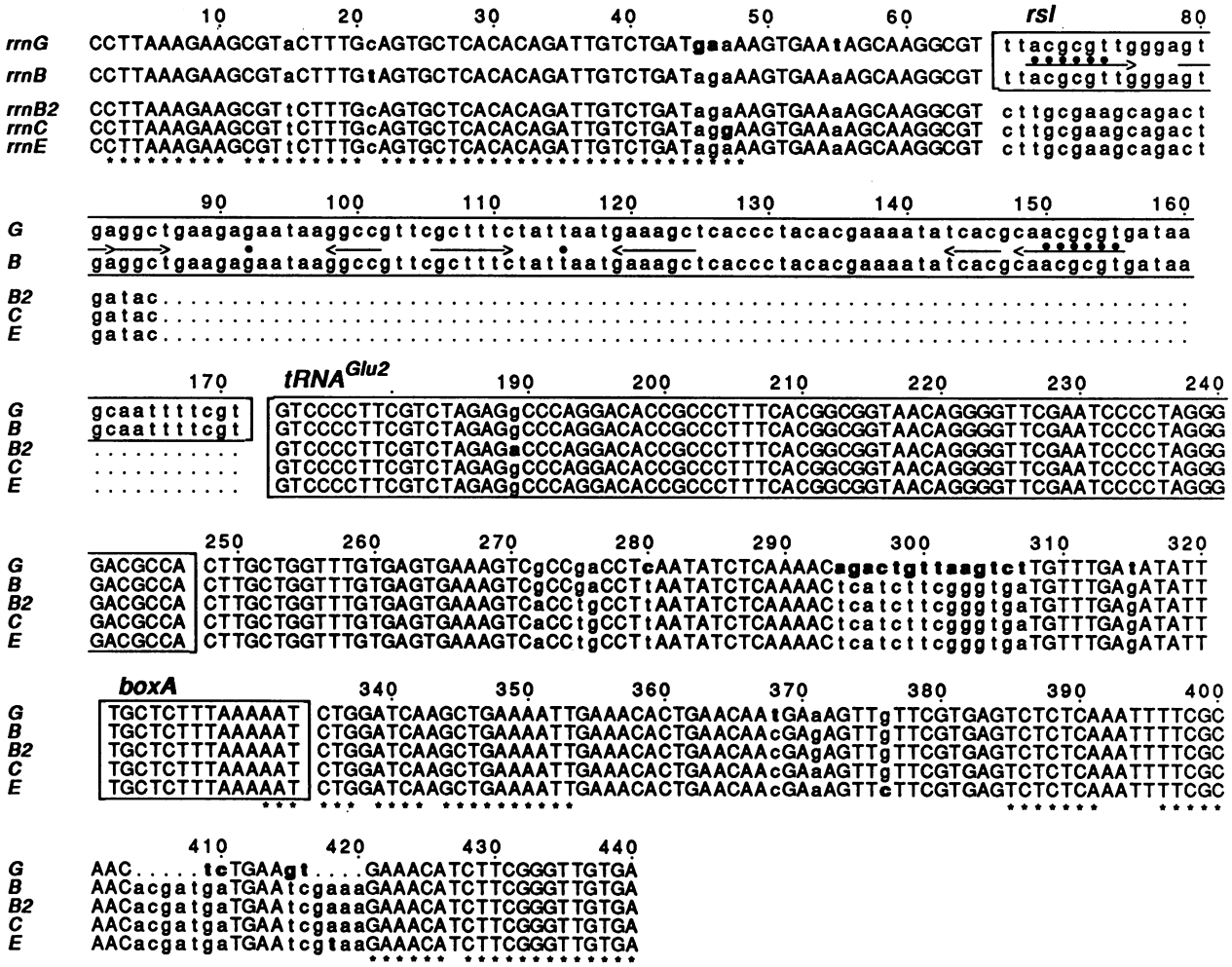

FIG. 3. Comparison of $tRNA^{Glu2}$ spacer region sequences. The *rrn* 16S-to-23S spacer regions from *rrnG*, *rrnC*, and *rrnB2* were determined as indicated in Materials and Methods. The spacers for *rrnB* (5) and *rrnE* (14) are from published sequences. Nucleotides that are conserved in all of the *rrn* operons presented here are designated by uppercase letters. Nucleotides that are divergent in one or more operons are designated by lowercase letters. Heterogeneous nucleotides that are unique to a single operon are indicated by boldface lowercase letters. · · · ·, Absence of a nucleotide at a particular position in the alignment. The *rsl*, $tRNA^{Glu2}$, and boxA sequences are enclosed in solid boxes. The two 6-base-pair palindromic repeats (•) in the *rsl* region enclose an interrupted dyad symmetry, indicated by the arrows below the *rrnG* sequence. Nucleotides involved in the predicted base pairing for the 16S and 23S precursor rRNA processing stalks are marked (*) under the sequences.

6-base-pair repeats that lie near the ends of the *rsl* sequence (Fig. 3), and they have suggested that this region might have been involved in the deletion of the *rsl* sequence. The DNA sequences show that this was not the case, however, because the 120-base-pair *rsl* region of *rrnB2* was totally replaced by an entirely different 20-base-pair sequence, and there was no remnant of the 6-base-pair repeats from the *rsl* or their flanking sequences. Since the 20-base-pair sequence (bases 66 to 85 in Fig. 3) is identical to sequences in *rrnC* and *rrnE*, it is more likely that an interaction between one of those operons and *rrnB* was responsible for loss of *rsl* from the *rrnB* operon. This interaction could have been of two distinct sorts. A double recombinational event between *rrnB* and either *rrnC* or *rrnE* could result in the reciprocal exchange of spacer types. If this unequal exchange occurred between operons on sister chromatids, the result would be the apparent loss of the *rsl* sequence from the genome, since segregation of the chromatids would occur immediately. Alternatively, there could have been a gene conversion between *rrnB* and either *rrnC* or *rrnE* from the same chromosome. This gene conversion could nonreciprocally replace the spacer of *rrnB* with that of *rrnC* or *rrnE*. The net effects of these reciprocal and nonreciprocal exchanges would be the same. A natural precedent for the reciprocal exchange of spacers between *rrn* operons has been described. It has been shown that an exchange of spacers between the *rrnB* and *rrnD* operons has occurred since the divergence of *Salmonella typhimurium* and *E. coli* (12).

The *rrnG* spacer differs from *rrnB* at 25 positions in the alignment. It is also 9 nucleotides shorter than the *rrnB* spacer because a 17-nucleotide sequence (positions 404 to 420 in Fig. 3) in *rrnB* is replaced by an 8-nucleotide sequence in *rrnG*. This portion of *rrnG* is identical to the *rrnD** and *rrnX* spacer sequences (described in reference 4), suggesting *rrnG* as a possible origin of part of these anomalous operons. The other major difference between *rrnG* and *rrnB*, 14 nucleotides at positions 294 to 307, is part of an interrupted dyad symmetry (not shown) that is conserved in both

spacers. This possible hairpin structure pairs 30 (*rrnG*) and 32 (*rrnB*) bases in a 40-base region (281 to 320). Considering the many differences between *rrnB* and *rrnG*, it is interesting that the two *rsl* sequences themselves are completely conserved. This conservation might indicate a selection for function, but what this function might be remains obscure. At least four other conserved areas within the 16S-to-23S spacers shown in Fig. 3 are functional. They are the 3′ end of the 16S processing stalk (5, 18), the 5′ end of the 23S processing stalk (4, 5), the $tRNA^{Glu2}$ gene, and a boxA sequence (K. Berg, C. Squires, and C. L. Squires, unpublished data) that has in vivo antiterminator activity in an *rrn* operon antitermination test system (13). The processing-stalk structures, first noted in the *rrnD** and *rrnX* operons (4, 18), also hold for the *rrnB* operon (5) and for the 16S processing stalk of the *rrnG* operon (15; this work). The few heterogeneities that do occur within processing-stalk regions have little or no effect on the stability of the predicted stalk.

There is one difference in the *rrnB2* spacer sequence that is not found in any other $tRNA^{Glu2}$ spacer; the A at position 189 replaces a G that is highly conserved in the D-loop of tRNAs (16). This change almost certainly would have a negative effect on the function of the tRNA (17). At present, we do not know whether this mutation occurs in all *rrnB2* operons or is unique to pLC34-34.

Our initial observation of the loss of *rsl* from *rrnB* in *relA1* mutants suggested a selective relationship between the two mutations. If such a relationship existed, it was hoped that it could be used to establish the function of the *rsl* sequence. However, reconstruction of *relA1* cultures with *rsl* present in *rrnB* failed to reveal any selective advantages that were dependent on the *rrnB* allele. Therefore, the question of whether the occurrence of the *relA1* mutation and the loss of the *rsl* sequence were simple coincidences, or whether some other more subtle selection was operating, remains open.

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