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Functional *Escherichia coli* 23S rRNAs containing processed and unprocessed intervening sequences from *Salmonella typhimurium*

Steven T. Gregory*, Michael O'Connor and Albert E. Dahlberg

Department of Molecular and Cell Biology and Biochemistry, Box G, Brown University, Providence, RI 02912, USA

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ABSTRACT

We have introduced the intervening sequence (IVS) from 23S rRNA of the *rrnD* operon of *Salmonella typhimurium* into the equivalent position of *Escherichia coli* 23S rRNA. *Salmonella typhimurium* 23S rRNA is fragmented due to the RNase III-dependent removal of the ~100 nt stem–loop structure that comprises the IVS. In this study, we have found that insertion of the *S.typhimurium* IVS into *E.coli* 23S rRNA causes fragmentation of the RNA but does not affect ribosome function. Cells expressing the fragmented 23S rRNA exhibited wild-type growth rates. Fragmented RNA was found in the actively translating polysome pool and did not alter the sedimentation profile of ribosomal subunits, 70S ribosomes or polysomes. Finally, hybrid 23S rRNA carrying the A2058G mutation conferred high level erythromycin resistance indistinguishable from that of intact 23S rRNA carrying this mutation. These observations indicate that the presence of this IVS and its removal are phenotypically silent. As observed in an RNase III-deficient strain, processing of the IVS was not required for the production of functional ribosomes.

INTRODUCTION

The 23S rRNA molecules from a number of eubacterial species have been found to be fragmented due to cleavage of intervening sequences or IVSs (1–6). These elements are co-transcribed as part of the rRNA precursor and are removed during maturation, without ligation of the resultant fragments (7). Presumably, the structural integrity of the mature RNA molecule in the 50S ribosomal subunit is maintained by secondary and tertiary structure and by interactions with ribosomal proteins. These IVSs are, therefore, analogous to the eukaryotic internal transcribed spacers found between 5.8S rRNA and 28S rRNA precursors (8). When examined at the molecular level, many eubacterial IVSs have been found to be of the order of 100–200 nt in length and possess the potential to form stable secondary structures (3,4,7,9). In several species, including *Salmonella typhimurium* and *S.arizonae* (7), *Yersinia enterocolitica* (10), *Leptospira* spp. (11) and *Campylobacter* spp. (3,4), a common site of IVSs has been found at the 1164–1185 helix in domain II of the current secondary structure model of 23S-like rRNAs (12). In *S.typhimurium* and perhaps in other species as well, this IVS is excised from the rRNA precursor by RNase III (7).

Based on their sporadic appearance in rRNA operons, it has been argued that the presence of IVSs is a derived rather than a primitive character (7,10,13). 23S rRNA genes of closely related species and even genes within a given species have been found to contain different complements of IVS elements. *Salmonella arizonae* has an IVS in the 1164–1185 helix of some but not all of its *rrn* operons and an additional IVS and fragmentation site is found in some operons at the 533–560 helix. Recently, the *rrnG* and *rrnH* operons of *S.typhimurium* have also been found to encode two distinct IVS elements in the 533–560 helix (in addition to the IVS in the 1164–1185 helix) which are lacking in the *rrnA*, *B*, *C*, *D* and *E* operons (14). *Escherichia coli* lacks all such elements, despite its close evolutionary relationship to *Salmonella* (15). These observations suggest both an unusual mechanism of appearance of such elements and a lack of significant selective pressure for or against their maintenance. No function has been assigned to IVSs.

In order to examine the effects of the introduction of an IVS element into a heterologous system lacking such elements, we inserted the IVS from the 1164–1185 helix of the *S.typhimurium rrnD* operon into the equivalent position of a plasmid-encoded *E.coli rrnB* operon. The 23S rRNA gene on this plasmid carries an A→G transition mutation at position 2058 which allowed us to monitor expression of the IVS-containing rRNA by primer extension analysis (16). High level resistance to macrolide/lincosamide/streptogramin B (MLS) antibiotics conferred by the A2058G mutation (17) also serves as an indicator of plasmid-encoded 23S rRNA function *in vivo*.

High level expression of the RNA bearing the *Salmonella* IVS in *E.coli* produced no growth defect, consistent with a lack of a selective advantage or disadvantage of the IVS. Sucrose gradient profiles of 50S subunits, 70S ribosomes and polysomes from cells containing the fragmented rRNA did not differ from those of cells containing only intact rRNA and primer extension analysis indicated that the IVS did not affect the incorporation of 23S rRNA into each of these fractions. These ribosomes were also found to be functional based on their ability to confer high level erythromycin resistance.

*To whom correspondence should be addressed. Tel: +1 401 863 3652; Fax: +401 863 1182; Email: Steven_Gregory@Brown.edu

As in *S.typhimurium*, *E.coli* 23S rRNA containing the IVS was fragmented in an RNase III-dependent manner. Moreover, removal of the IVS was not required to confer erythromycin resistance. We interpret these results to indicate that insertion of an IVS element into rRNA genes at the 1164–1185 helix is not deleterious and that removal of the IVS is not a prerequisite for the production of functional 23S rRNA.

MATERIALS AND METHODS

Bacterial strains and plasmids

Construction of plasmids was performed in EF41 [F⁻ Δ(*lac-pro*) *thi recA1*] (18) or DH1 [F⁻ *supE44 recA1 endA1 gyrA96 thi1 hsdR17 relA1 spoT1*? *rfbD1*?]. CAG1636 [F⁻ *his bio* RNase I⁻ RNase III⁻ *relA recA56 srl*::Tn*10*] is an RNase III-deficient strain derived from BL107 kindly provided by Dr Alan Grossman. Plasmid pST1 (7) contains the *rrnD* operon of *S.typhimurium* cloned into pBR322 and was a kind gift of Dr Norman Pace. Plasmid pSH3 is a derivative of pSTL102 (19) carrying the *E.coli rrnB* operon in which the *Hpa*I–*Sph*I fragment in the 23S rRNA gene has been replaced by the homologous fragment from *S.typhimurium rrnD*. Plasmid pGQ66 is a derivative of plasmid pGQ7 (18) in which the *Hpa*I–*Sph*I fragment (extending from position 608 to 1232) in the 23S rRNA gene has been replaced by the homologous fragment from *S.typhimurium rrnD*. The rRNA operon of this plasmid is under the control of the bacteriophage λ P_L promoter. The temperature-sensitive 857 allele of the phage λ cI repressor is expressed from the pSC101-derived kanamycin resistance plasmid pLG857 (20).

Preparation of total RNA

Total RNA was prepared from 50 ml mid log phase cells grown in LB medium plus 100 μg/ml ampicillin at 37°C in the case of cells carrying pSH3 or 100 μg/ml ampicillin and 50 μg/ml kanamycin at 42°C in the case of cells carrying pGQ66 and pLG857. RNA was extracted by the freeze–thaw lysozyme method described by DeStasio and Dahlberg (21).

Electrophoresis and gel purification of plasmid-encoded 23S rRNA fragments

Total RNA was fractionated on 4% polyacrylamide–7 M urea, TBE-buffered gels and either stained with methylene blue or visualized by UV shadowing. For gel purification, RNA fragments were visualized by UV shadowing, excised and eluted in 10 mM Tris–HCl, pH 7.6, 1 mM EDTA, 0.3 M NaOAc.

Polysome preparation and primer extension analysis of mutant rRNA

Polysomes were prepared as described previously (22) and rRNA was extracted and analyzed by primer extension analysis using the A2058G mutation as a marker for plasmid-encoded 23S rRNA (16). Primer extension products were quantitated using a Fuji phosphorimager.

RESULTS

Construction of a *S.typhimurium–E.coli* hybrid rRNA operon

Two systems for the expression of 23S rRNA containing the *S.typhimurium* IVS were constructed. One was derived from the plasmid pSTL102 (19), a pBR322-based construct containing the *rrnB* operon transcribed from the tandem P_1P_2 *rrn* promoters. This plasmid contains a C→U transition mutation at position 1192 of 16S rRNA which confers resistance to spectinomycin (23) and an A→G transition mutation at position 2058 of 23S rRNA which confers resistance to erythromycin (17). These mutations allow the fate of plasmid-encoded rRNA to be followed by primer extension analysis (16) and the functionality of the RNA to be determined by expression of drug resistance phenotypes *in vivo*. A second construct was derived from the pBR322-based plasmid pGQ7 (18), which contains the *rrnB* operon under the control of the phage λ P_L promoter. This promoter is repressible by the λ cI repressor. Conditional expression of plasmid-encoded rRNA is achieved by inactivation of a temperature sensitive cI repressor at 42°C. This system was used to correlate the appearance of fragmented 23S rRNA with the induction of plasmid-encoded IVS-containing rRNA synthesis.

The *S.typhimurium* IVS was introduced into both plasmids as an *Hpa*I–*Sph*I fragment from the *S.typhimurium rrnD* operon of plasmid pST1 (7). This fragment is 708 bp in length and contains the entire domain II of 23S rRNA (Fig. 1). A restriction fragment spanning this segment of the 23S rRNA gene was also cloned into M13mp vectors and both strands were sequenced in their entirety to confirm the presence of the IVS and to identify sequence differences between *E.coli* and *S.typhimurium* in this region. A total of 10 sequence differences were identified outside the IVS in the *Hpa*I–*Sph*I fragment (shown in Fig. 1). Five of the variations were located in helical elements; these retained Watson–Crick or G-U pairings. Five others were located in unpaired regions at non-conserved positions.

Table 1. Doubling times of plasmid-containing DH1 and distribution of plasmid-encoded RNA in 50S subunits, 70S ribosomes and polysomes

rRNA plasmid	Doubling time (min)	Plasmid-encoded RNA (%) 50S	70S	Polysomes
pSTL102 (WT)	38 ± 1	64.8 ± 5.0	55.1 ± 5.7	55.0 ± 2.3
pSH3 (IVS)	39 ± 2	68.1 ± 5.1	57.8 ± 4.7	54.7 ± 2.1

The A2058G mutation in 23S rRNA, which confers resistance to erythromycin, was used as a marker for plasmid-encoded rRNA expression. Relative proportions of plasmid-encoded and chromosomally encoded rRNAs were determined by three to five independent primer extension assays as described by Sigmund *et al*. (16), using a primer complementary to nucleotides 2080–2061 of 23S rRNA.

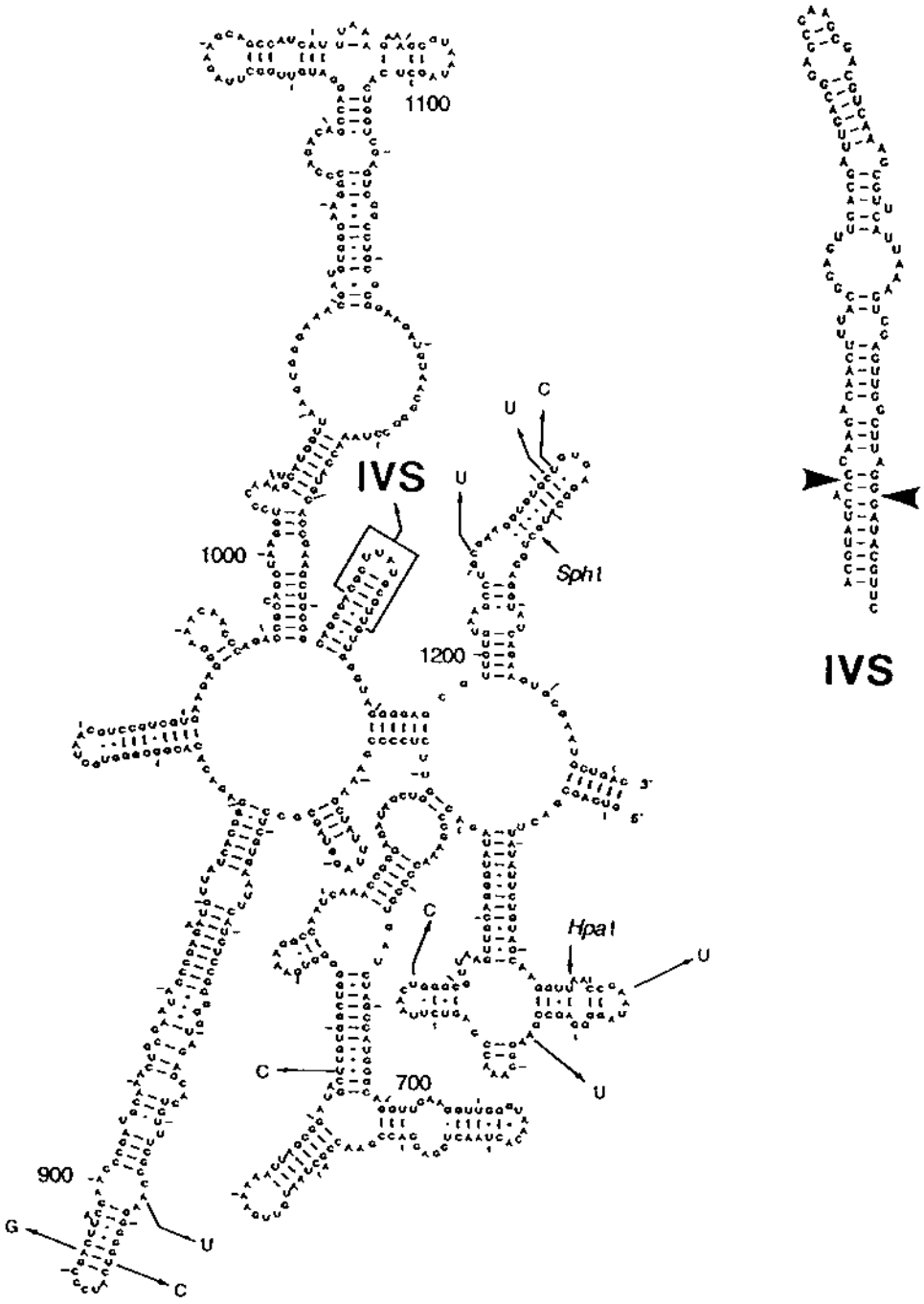

Figure 1. Secondary structure of *E.coli* 23S rRNA domain II indicating sequence differences in *S.typhimurium* 23S rRNA domain II and the location of the IVS. The current secondary structure model of the IVS (7) is also shown.

The *S.typhimurium* IVS is phenotypically silent in the *E.coli rrnB* operon

Introduction of the *S.typhimurium* IVS into *E.coli* 23S rRNA produced no detectable growth phenotype. Doubling times of DH1 containing plasmid pSTL102 or pSH3 were determined to be 38 and 39 min respectively (Table 1). These doubling times are effectively identical within experimental limits.

Sucrose gradient profiles of 50S subunits, 70S ribosomes and polysomes from DH1 containing pSTL102 or pSH3 were indistinguishable (Fig. 2). No fragmentation of 50S subunits or alteration in the ratio of 70S ribosomes to 50S subunits was observed.

To ensure that the absence of a detectable growth phenotype was not due to abnormally low steady-state levels of IVS-containing rRNA, 50S subunits, 70S ribosomes and polysomes were fractionated on sucrose gradients and plasmid-encoded 23S rRNA from each fraction was quantitated by primer extension analysis using the A2058G mutation as a marker (16). IVS-containing rRNA was found in subunit, ribosome and

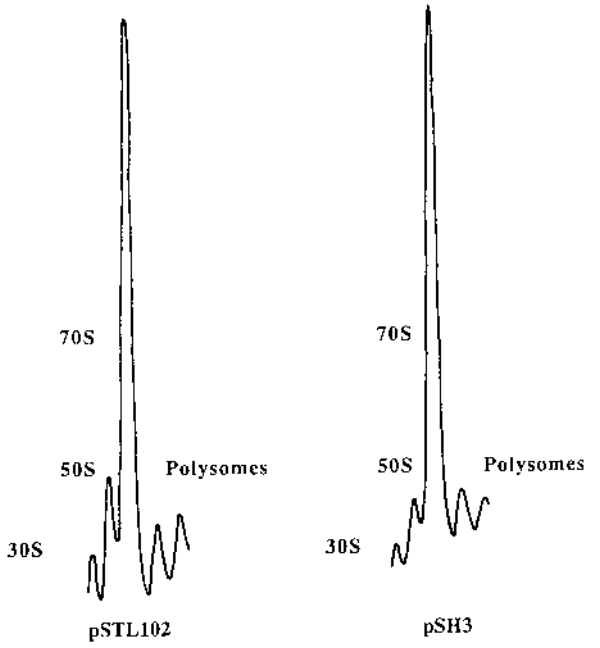

Figure 2. Sucrose gradient profiles of 30S and 50S subunits, 70S ribosomes and polysomes. Lysates from DH1 containing either pSTL102 (left) or pSH3 (right) were centrifuged through a 10–40% sucrose gradient.

polysome fractions at levels which were indistinguishable from those of RNA from cells containing pSTL102 (Table 1).

The IVS did not interfere with the ability of 23S rRNA containing the A2058G mutation to confer high level resistance to erythromycin (Fig. 3). Cultures of DH1 containing pKK3535 (wild-type at position 2058), pSTL102 (A2058G) or pSH3 (A2058G) were streaked for single colonies on LB plates containing either 100 μg/ml ampicillin (left) or 100 μg/ml ampicillin plus 200 μg/ml erythromycin (right) and incubated at 37°C for 2 days (Fig. 3). This level of erythromycin was sufficient to completely inhibit growth of the wild-type control. However, no difference in growth between pSTL102 and pSH3 was observed. Based on these three criteria, we concluded that the IVS had no phenotypic effect.

RNase III-dependent fragmentation of *E.coli–S.typhimurium* hybrid 23S rRNA by the *S.typhimurium* IVS

To examine fragmentation due to the IVS, total RNA isolated from mid log phase cultures was electrophoresed on a 4% polyacrylamide–urea gel and stained with methylene blue as shown in Figure 4. RNA from EF41 containing wild-type pGQ7 in lane 1 indicates the positions of intact 23S and 16S rRNAs. Lane 2 shows RNA from EF41 containing both plasmids pGQ66 and pLG857 grown at 30°C, while RNA from the same cell line grown at 42°C is shown in lane 3. Expression of IVS-containing 23S rRNA from pGQ66 is repressed at 30°C and maximal at 42°C. Lane 3 shows the appearance of two new RNA species. One of these RNAs migrating between intact 23S and 16S rRNA corresponds to the larger 3′ fragment of 23S rRNA containing domains III–VI. The identity of this fragment was confirmed by gel purification and sequencing with AMV reverse transcriptase (24) using an oligonucleotide primer complementary to positions 2254–2273 of *E.coli* 23S rRNA (data not shown). A second, faster migrating species was observed, corresponding to the 5′ terminal fragment containing domains I and II. These results demonstrate the fragmentation of *E.coli–S.typhimurium* hybrid 23S rRNA due to the presence of the *Salmonella* IVS.

Because previous studies have noted the susceptibility of domain II to RNase cleavage (25,26) and a weak phosphodiester bond between nucleotides U1915 and A1916 (27), which would produce fragments of similar size to those produced by RNase III cleavage at the IVS, we mapped the 5′-end of the large 23S rRNA fragment by primer extension using an oligonucleotide complementary to positions 1246–1262. Primer extension using either the gel purified large RNA fragment or total RNA from cells containing a mixture of intact and fragmented 23S rRNA produced a strong stop at the expected position, as previously

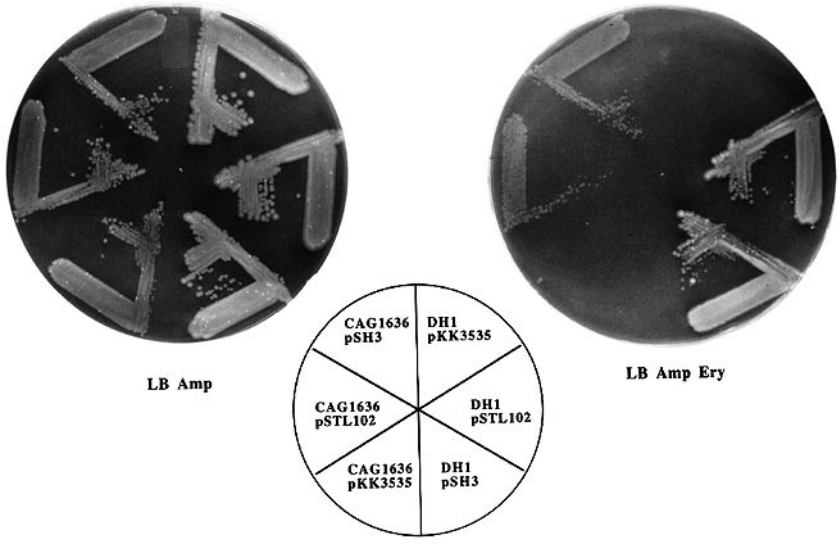

Figure 3. Erythromycin resistance conferred by the A2058G mutation in the presence and absence of the *S.typhimurium* IVS. (Left) Growth on LB ampicillin; (right) growth on LB ampicillin plus 200 μg/ml erythromycin. The identities of cell lines are as indicated in the figure.

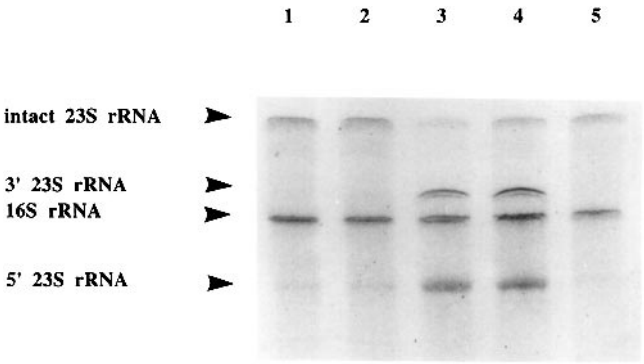

Figure 4. Gel electrophoresis of rRNA. 4 µg RNA were loaded into each of six lanes, electrophoresed at 200 V for 19 h and stained with methylene blue. Lane 1, wild-type rRNA; lane 2, RNA from pGQ66 grown at 30°C; lane 3, RNA from pGQ66 grown at 42°C; lane 4, RNA from pSH3 grown in DH1; lane 5, RNA from pSH3 grown in the RNase III⁻ strain CAG1636. Arrows mark the positions of RNA species. 3′ 23S rRNA, the fragment of 23S rRNA consisting of domains III–VI; 5′ 23S rRNA, the fragment of 23S rRNA consisting of domains I and II.

determined by Burgin *et al.* (7) for *S.typhimurium* 23S rRNA, whereas primer extension using total RNA from wild-type cells produced no such stop (data not shown). Thus, the downstream cleavage site in the IVS is the same in both the hybrid rRNA and *S.typhimurium* 23S rRNA (7).

Fragmentation of hybrid 23S rRNA was found to be RNase III-dependent, as shown for *S.typhimurium* 23S rRNA (7). RNA from DH1 containing pSH3 exhibited the fragmentation pattern established for the hybrid 23S rRNA (lane 4), whereas RNA from the RNase III-deficient strain CAG1636 containing pSH3 consisted exclusively of unfragmented RNA (lane 5).

Removal of the IVS is not required for ribosome function

The absence of fragmented rRNA in the RNase III-deficient strain is consistent both with degradation of the IVS-containing RNA in the RNase III-deficient strain and with its incorporation intact into functioning ribosomes. As shown in Figure 3, when cultures of DH1 or CAG1636 containing pKK3535 (WT), pSTL102 (A2058G) or pSH3 (A2058G) were streaked onto plates containing either 100 µg/ml ampicillin (left) or 100 µg/ml ampicillin plus 200 µg/ml erythromycin (right), the erythromycin resistance phenotypes conferred by pSTL102 and pSH3 were indistinguishable. This indicated that unfragmented rRNA containing the intact IVS is functional *in vivo*. Thus, removal of the IVS is not a prerequisite for the production of functional ribosomes.

DISCUSSION

The *S.typhimurium* 1164–1185 helix IVS in the context of *E.coli–Salmonella* hybrid 23S rRNA is phenotypically silent; it does not alter growth, expression of a drug resistance phenotype or incorporation of 23S rRNA into the actively translating pool of ribosomes. Most interestingly, ribosomes remain functional without excision of the IVS.

The site of insertion of IVSs in many eubacteria seems to be non-random. Many are found at the 1164–1185 helix (4,7,14), while others are found at the 533–560 helix (7,14). This may reflect either preferences for insertion at certain DNA sequences or selection against the presence of IVSs at other sites in rRNA. Substantial selection probably exists against the presence of IVS elements at rRNA functional sites, ribosomal protein binding sites or where a discontinuity in the RNA molecule would lead to instability of the 50S subunit.

Our most striking finding is that removal of this IVS is not needed for the production of functional ribosomes. Large extensions of the 1164–1185 helix presumably do not perturb functional sites within the ribosome. The equivalent helix in 28S rRNAs of eukaryotic cytoplasmic ribosomes is a site of expansion segments and an evolutionary relationship between IVSs and expansion segments has been suggested (7). Our finding is consistent with the idea that some expansion segments may have initially evolved from IVS elements via the loss of RNase processing sites. Such elements could subsequently attain functional importance (28).

The presence of this IVS has no detectable physiological consequence in *Salmonella*, other than fragmentation of 23S rRNA. Conceivably, the initial insertion of this IVS into *Salmonella* 23S rRNA genes could have been deleterious, with subsequent acquisition of additional mutations (either in rRNA or ribosomal proteins) resulting in the current lack of phenotype. However, the absence of phenotype upon the *de novo* introduction of this element into *E.coli* suggests that the IVS structure in its current form and location is inherently silent. Given the extremely close phylogenetic relationship of *Escherichia* and *Salmonella* (15), it is also unlikely that genetic differences between the two organisms are responsible for the lack of phenotype. It therefore seems most likely that the innocuous nature of IVSs is intrinsic to such elements.

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