THE UNIVERSITY OF CALGARY

Intervening Sequences in 23S Ribosomal RNA in Salmonella

by

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A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOLOGICAL SCIENCES

CALGARY, ALBERTA

JULY, 1996

THE UNIVERSITY OF CALGARY

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled:

Intervening Sequences in 23S Ribosomal RNA in Salmonella

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Date: August 1, 1996

ABSTRACT

Salmonella typhimurium LT2 and *Salmonella typhi* Ty2 were used to investigate 23S ribosomal RNA fragmentation caused by excision of intervening sequences (IVSs) by RNase III. Two IVS regions in the seven 23S rRNA (*rrl*) genes of strains LT2 and Ty2 were sequenced. Multiple IVSs, which were in some cases identical, were found for each strain. IVS possession in strain LT2 was compared to several *S. typhimurium* isolates using the polymerase chain reaction and *Dra*I restriction, and IVS content was found to vary. IVS possession in strain Ty2 was compared to thirty isolates of *S. typhi* using the same method, and it was shown that IVS content was stable. I postulate that IVSs have entered strains LT2 and Ty2 by horizontal transfer, and that they can be gained, lost, or stabilized by gene conversion. An *S. typhimurium* RNase III deficient mutant was viable, showing that ribosomes retaining IVSs are functional.

ACKNOWLEDGMENTS

I wish to thank my supervisor, Dr. K. E. Sanderson for his guidance and support throughout this study.

I thank the members of my supervisory committee, Drs. K. Iatrou, A. B. Schryvers, and S.-L. Wong for their help.

I am grateful to Dr. S.-L. Liu for obtaining and evaluating genomic DNA fragments by pulsed-field gel electrophoresis, and to Ms. Dayle Daines for her assistance in the investigation of IVSs in Salmonella typhi.

I also wish to thank Fiona, my spouse, for her refreshing perspectives and her patience.

DEDICATION

To my Parents,

Don and Rose,

for their love and encouragement

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ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
bp	Base pair(s)
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetate
EtBr	Ethidium Bromide
g	Gram
g	Gravity
IVS	Intervening sequence
kb	Kilobase
kDa	KiloDalton
kV	Kilovolt
l	Liter
mg	Milligram
ml	Milliliter
mM	Millimolar
mm	Millimeter
ng	Nanogram
nm	Nanometer
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pmol	Picomole
PMSF	Phenylmethylsulfonyl fluoride
S	Svedberg unit
SARA	Salmonella Reference Collection A
SDS	Sodium dodecyl sulfate

SGSC	Salmonella Genetic Stock Centre
ul	Microliter
V/cm	Volts per centimeter

INTRODUCTION

The prokaryotic ribosome consists of two subunits, the 30S, or small subunit, and the 50S, or large subunit. The 30S subunit contains the 16S ribosomal RNA (rRNA), and the 50S subunit contains the 23S and 5S rRNAs. Both subunits together contain between 50 to 60 ribosomal proteins (Wittmann-Liebold et al., 1990), and in association make up the 70S ribosome that is responsible for translating messenger RNA into protein.

The 50S ribosomal subunit usually contains contiguous 23S and 5S rRNAs, however, in certain genera of bacteria, such as Campylobacter (Konkel et al., 1994; Linton et al., 1994; Trust et al., 1994), Coxiella (Afseth et al., 1995), Leptospira (Ralph and McClelland, 1993; Ralph and McClelland, 1994; Hsu et al., 1990), Rhodobacter (Lessie, 1965; Kordes et al., 1994), Salmonella (Winkler, 1979; Smith et al., 1988; Burgin et al., 1990; Skurnik and Toivanen, 1991; Hsu et al., 1992; Hsu et al., 1994) and Yersinia (Skurnik and Toivanen, 1991), the 23S rRNA can be fragmented into two or more pieces. In Salmonella typhimurium LT2, 23S rRNA fragmentation is caused by RNase III cleavage, without repair, of novel intervening sequences (IVSs) of about 90-110 bases in length (Burgin et al., 1990). RNase III normally functions in the initial processing steps of the polycistronic transcript that determines the 16S, 23S, and 5S rRNAs. Most IVSs contain neither ORFs that might encode an enzyme to facilitate excision nor terminal consensus sequences that might contribute to autocatalysis as observed for Group 1 and Group 2 introns. For this reason, IVSs are not true introns, but are related elements that disrupt the normal continuity of a gene without affecting its function. The 23S rRNA fragments maintain functionality, presumably through

secondary structure and ribosomal protein interactions in the 50S subunit.

S. typhimurium possesses two distinct types of IVSs based on 23S rRNA fragment stoichiometry (Burgin et al., 1990), one at about bp 550 and another at about bp 1170 in rrl (Escherichia coli rrl numbering [Noller, 1984]). These positions correspond to helix-25, in domain I, and helix-45, in domain II, in the postulated secondary structure of the 23S rRNA of E. coli (Figure 1); both helices represent small tetra-loops (helices with 4 unpaired bases at the tip). IVSs partly replace these small helices with an extended compound helix. Sequencing of two helix-45 IVSs isolated from two independent strains of S. typhimurium, ATCC 23566 (Burgin et al., 1990) and ATCC 13311 (Skurnik and Toivanen, 1991), revealed that both were identical. Helix-25 IVSs were postulated to exist among the seven rrl genes of strain ATCC 23566, but were not isolated. Both IVS types have been sequenced in Salmonella arizonae (Burgin et al., 1990); the helix-45 IVSs of S. arizonae and S. typhimurium have similar nucleotide sequences, suggesting a common ancestry. Further, a comparison of helix-45 IVSs in Yersinia enterocolitica and S. typhimurium showed strong identity in a background of low chromosomal homology (Skurnik and Toivanen, 1991). These data suggest that IVSs are recent evolutionary additions to rrl that have been introduced through horizontal transfer, conceivably by plasmid-mediated conjugation or by phage-mediated transduction, and that IVSs may be functional.

IVSs are clearly unusual genetic elements that merit further study. It has yet to be determined how many and which of the specific rrl genes of S. typhimurium and Salmonella typhi have IVSs in helix-25 or helix-45, and if this IVS possession is stable. Further, it has not been reported whether IVS excision by RNase III is required for the survival of S. typhimurium. I

report experiments that answer these questions and shed light on the mechanism of IVS maintenance and propagation. Speculation on the nature of IVSs, in terms of origin and function, will be made based on the data presented.

LITERATURE REVIEW

A. Ribosomal RNA processing

1. *rrn* operon organization and transcript processing

Prokaryotic 16S, 23S, and 5S rRNAs are typically co-transcribed from one of multiple *rrn* operons into a single 30S precursor rRNA. In *E. coli* and *S. typhimurium* there are seven such *rrn* operons distributed around the chromosome. The 30S precursor rRNA in *E. coli* contains 22% extra sequences that include two intergenic regions. The first intergenic region occurs between the 16S and 23S rRNAs, and can contain one or two tRNAs (spacer tRNAs). The second intergenic region separates the 23S and 5S rRNAs. Beyond the 5S towards the 3′ end of the 30S rRNA, distal tRNAs can be present (Morgan et al., 1980). The 30S transcript is acted upon by various RNases to separate and maturate each of the three rRNAs and the tRNAs. Processing begins before *rrn* operon transcription is complete (Apirion and Gegenheimer, 1984), and can be classified into primary and secondary components (Gegenheimer and Apirion, 1981). This distinction is based upon the ability of primary (endonucleic) RNases to cleave naked RNA; secondary RNases are restricted to a ribonucleoprotein substrate and can be inhibited by chloramphenicol (King et al., 1986). Primary processing RNases are illustrated in Figure 2, secondary processing RNases remain largely unproven.

2. Processing in RNase III mutants

RNase III deficient strains of E. coli have been shown to accumulate 30S (Gegenheimer et al., 1977; King et al., 1984; Apirion et al., 1976) and 25S (23S and 5S) (Ghora and Apirion, 1979; Gegenheimer et al., 1977) precursor rRNAs, and have slower generation times (40% longer) when compared to wild-type isogenic controls (Apirion et al., 1976). The production of the 25S rRNA can be inhibited by chloramphenicol, and therefore the 30S transcript must be cleaved by a non primary less efficient pathway (Gegenheimer et al., 1977). The 16S rRNA was found to be fully matured in RNase III deficient mutants; however, the 23S species was present as a heterogeneous population of differentially processed rRNA (King et al., 1984). Therefore, RNase III is indispensable for attaining fully matured 23S rRNA, but is not an absolute requirement for the production of functional ribosomes.

The reduction of growth rate in rnc mutants may be for several reasons. The most apparent would be a reduction in ribosome translation efficiency. This may stem from a reduction in proficiency of rRNA processing, that in turn leads to a reduced number of ribosomes available for translation; this is supported by the accumulation of 30S and 25S rRNA. In addition, because the 23S rRNA does not fully mature without RNase III, the mechanism of translation may be impaired. RNase III may also control the half-life of certain mRNAs (Belasco and Higgins, 1988). Polynucleotide phosphorylase (PNPase) has a 5′ RNase III site in its mRNA, and without RNase III it accumulates and can be visualized by SDS PAGE as a major band running at approximately 84 kDa (Portier et al., 1987). Since PNPase is a processive 3′ to 5′ exonuclease, certain mRNAs such as trxA experience accelerated decays, and thus, their gene products

would not attain normal physiological levels (Babitzke et al., 1993). In this way, the accumulation of unregulated mRNA may saturate already impaired ribosomes, leaving other more essential mRNAs to be translated at lower efficiency, or may have detrimental activities when over expressed.

B. Intervening sequences

1. Salmonella intervening sequences

Winkler (1979) was the first to identify the fragmented nature of S. typhimurium LT2 23S rRNA. Studies by Burgin et al. (1990) revealed that the 23S rRNA in S. typhimurium LT2 (ATCC 23566) and S. arizonae was fragmented into as many as five discrete segments. The relative stoichiometries were consistent with the total size of the 23S rRNA (2.9-kb). That is, both Salmonella species allowed for two possible cleavage points in the 23S rRNA, one at helix-25 (about bp 550 in rrl (E. coli rrl numbering (Noller, 1984))and one at helix-45 (about bp 1170 in rrl). Clones of an rrn operon from both S. typhimurium LT2 and S. arizonae were isolated and sequenced in the rrl helix-25 and helix-45 regions. This demonstrated that IVSs of 90-110 bp interrupt the normal continuity of the rrl gene at helix-45 in the case of S. typhimurium LT2, and helix-25 and helix-45 in the case of S. arizonae. It was postulated that IVSs were responsible for mediating the observed 23S rRNA fragmentation. Depending on the number and type of IVSs possessed by an rrl gene, three different fragmentation scenarios are possible and are outlined in Figure 3. The observed RNA fragmentation indicated that both S. typhimurium LT2 and S. arizonae are heterogeneous with respect to the number of IVSs harbored by each of the seven rrl genes at each of the two helices (Burgin et al., 1990).

The IVSs sequenced for S. typhimurium and S. arizonae were too small to accommodate an ORF that might encode an enzyme to facilitate IVS excision, and it was presumed that the mechanism was host dependent. Chloramphenicol was used to inhibit protein synthesis in S. typhimurium LT2. The fragmentation was found to persist under such conditions indicating that the cleavage was not mediated through a secondary processing RNase. A better candidate for IVS excision was RNase III. To test this hypothesis, the S. typhimurium LT2 rrn operon clone that contained a helix-45 IVS was propagated in E. coli RNase III deficient strain N2077 (rnc-105) (Apirion and Watson, 1975); no rRNA fragments were recovered from strain N2077. When the the S. typhimurium LT2 rrn operon clone was propagated in an E. coli wild-type, the S. typhimurium 23S rRNA was cleaved to 1.7- and 1.2-kb rRNA fragments. This demonstrated that RNase III was responsible for excision of IVSs (Burgin et al., 1990).

To investigate the possibility that another enzymatic activity was required for IVS excision in conjunction with RNase III, the S. typhimurium LT2 helix-45 IVS was sub-cloned adjacent to a phage T7 promoter; potential substrate RNAs were produced by run-off transcription. The RNA was isolated and treated with purified RNase III; the data showed that RNase III alone was capable of mediating IVS excision (Burgin et al., 1990).

2. Horizontal transfer of intervening sequences

IVSs sequenced in Y. enterocolitica were found to have 87% identity to the helix-45 IVS of S. typhimurium LT2 (Skurnik and Toivanen, 1991). This strong identity, in a background of low chromosomal homology (Brenner et al., 1980), suggests that IVSs are evolutionary recent acquisitions or are strongly conserved over long periods of time. However, the observation that helix-45 IVSs in S. typhimurium LT2 and S. arizonae were only 85%

identical suggests that the former hypothesis is probably correct: IVSs have been introduced into these bacteria late in evolution and possibly by horizontal transmission, conceivably by plasmid-mediated conjugation or by phage-mediated transduction (Skurnik and Toivanen, 1991).

Certain species of Leptospira have 23S IVSs of 485-759 bases that encode a single ORF for a putative peptide of 120 amino acids (Ralph and McClelland, 1993; Ralph and McClelland, 1994). The deduced amino acid homology between four Leptospira IVS ORFs ranges from 49% to 78%, and this degree of amino acid similarity was consistently similar to the amount of DNA conservation in the same region. In the absence of selection, amino acid sequences would be expected to diverge at least twice as fast as nucleotide sequences, and therefore it has been suggested that these peptides function under selective pressure. Parsimony analysis indicated a recent horizontal transfer of an IVS from L. noguchi to L. weillii; both species are considered relatively distant based on 16S gene (rrs) mapped restriction site polymorphisms data. Further, the IVS sequenced from Coxiella burnetii also contains a single ORF that is 70% similar to the ORFs from Leptospira (Afseth et al., 1995). Horizontal transfer has been implicated here as well to explain the ORF conservation between Leptospira spp. and Coxiella.

3. IVSs and other genetic elements

a) Introns and transposons

Group I and group II mobile introns have the ability to catalyze their own splicing in vitro, in addition to being self mobilizing (Sharp, 1987; Lambowitz and Belfort, 1993; Belfort et al., 1995). Group I introns splice via a series of guanosine-

initiated transesterification reactions, while group II introns splice when the 2′ OH of a bulged nucleotide attacks the 5′splice site that generates a characteristic lariat structure. Both intron types rely on short consensus sequences to facilitate splice site recognition. Mobile introns participate by their coding capacity in an active process by which a DNA sequence (the intron) in a gene becomes inserted into another intron-deficient copy of the same gene, and the new copy becomes a functional intron (Dujon et al., 1989; Belfort et al., 1995). In this way, group I introns contain ORFs that encode endonucleases that target intronless alleles of intron-containing genes; I-CeuI, a double-strand endonuclease from the group I intron in the chloroplast large rRNA gene of Chlamydomonas eugametos, is an example. In fact, the 26-bp site that I-CeuI recognizes and cleaves is conserved in the rrl genes of enteric bacteria (Liu et al., 1993c). Group II ORFs encode reverse transcriptase-like proteins, although the exact mechanism of mobility is not yet clear.

IVSs cannot be considered true introns, as defined by group I and II. A mechanism for IVS introduction into the rrl gene is still unknown, and IVS introduction cannot be IVS determined because most IVSs do not contain ORFs. The process of excision from the 23S rRNA is novel, in using RNase III. Further, re-ligation of rRNA fragments does not occur in IVSs as itS does in true introns due to splicing. The fragmentation in the 23S rRNA is tolerated, presumably because of the stabilizing effects of secondary structure and because of ribosomal protein interactions in the 50S subunit. Moreover, intron junctions are defined by short consensus sequences, and this does not compare to the IVS boundaries, which are characterized only by secondary structure with no consensus sequences required. Nevertheless,

IVSs, like introns, interrupt the normal continuity of a gene without affecting its function.

IVSs resemble tRNA introns of yeast to a greater degree than they resemble other introns. The 19 base intron in tRNA is removed by an endogenous nuclease, which generates a linear intron, and a ligase activity joins the fragmented tRNA ends together (Reyes and Abelson, 1988). No consensus sequence has been recognized for the action of this endogenous nuclease activity, although secondary structure adopted by the intron in the tRNA is thought to be critical. This endonuclease-dependent excision of tRNA introns, which requires specific secondary structure, compares to the excision of IVSs by RNaseIII.

Concerning the stem-loop structure of most IVSs, it has been suggested that they are remnants of an inserted and then excised transposon (Burgin et al., 1990). Some transposons generate inverted repeats at the site of excision (Kleckner, 1981). These repeats could give rise to an RNase III-recognized stem structure upon transcription.

b) Eukaryotic expansion segments

The main difference between eukaryotic and eubacterial ribosomes is that eukaryotic rRNAs are considerably larger in size when compared to their bacterial counterparts (Clark et al., 1984; Clark, 1987). The increase in size of eukaryotic rRNA is the result of the presence of descrete RNA stretches not found in eubacterial rRNAs (Clark et al., 1984). These stretches of RNA have been termed expansion segments because they expand on the common functional core of rRNA that is possessed by both eukaryotes and eubacteria. Expansion segments are highly variable in sequence and occurrence in many eukaryotic lineages (Clark, 1987).

The IVSs found in Salmonella have been shown to occur in the same positions as expansion segments in eukaryotic ribosomes, and it been suggested that elements analogous to IVSs may have been inserted into the eukaryotic rRNA genes early in their evolution and have acquired function (Burgin et al., 1990). Certain expansion segments that did not acquire function are removed during rRNA processing like IVSs; these segments are referred to as transcribed spacers, as they usually separate functionally distinct rRNAs. An example of this is the internally transcribed spacer (ITS) that separates the 5.8S and 28S rRNAs in many eukaryotes (Gerbi, 1985).

4. Current theories on IVS function

The maintenance of IVSs in S. typhimurium, S. arizonae and Y. enterocolitica suggests that there must be a positive selective pressure acting on IVSs. Skurnik and Toivanen (1991) have hypothesized a bacteriocin evasion scenario. Although highly speculative, this idea is interesting nonetheless and is based on the fact that the normal habitat for these bacteria is the gut. Bacteriocins are compounds which are produced by bacteria to kill other bacteria. The flora of the gut is thought to be in constant complex competition and bacteriocins are thought to play a part (Mason and Richardson, 1981). It is known that the bacteriocins colicin E3 and DF1 attack and cleave 16S rRNA, inhibiting translation (Luria and Suit, 1987). Skurnik and Toivanen proposed that the discontinuity created by the IVS in the 23S rRNA may be a mechanism for immunizing against such an attack by an unknown bacteriocin.

It has also been reported that in the Salmonella species that contain IVSs, 23S rRNA breaks down rapidly when cells reach the stationary phase of growth, and that the more IVSs present in the rrl genes the faster the degradation (Hsu et al., 1994).

It was proposed that this could be a mechanism for post transcription regulation of rRNA, and may enable Salmonella to rapidly respond to growth conditions in a changing environment.

C. Research objectives

This investigation of the IVSs of S. typhimurium and S. typhi had the following research objectives, and has to a large part been reported in two references (Mattatall and Sanderson, 1996; Mattatall et al., 1996):

1) To isolate, by PFGE, the seven rrl genes of S. typhimurium LT2 and S. typhi Ty2 from genomic cleavage fragments, and to amplify regions that contain IVSs by PCR.

2) To characterize the IVS(s) from each of the seven rrl genes of S. typhimurium LT2 and S. typhi Ty2 by sequence, potential secondary structure analysis, and rRNA stoichiometry comparison.

3) To develop a rapid diagnostic method for determining IVS possession using whole genomic PCR and restriction analysis.

4) To survey selected strains of S. typhimurium and S. typhi for IVS possession using whole genomic PCR and restriction analysis, in conjunction with RNA isolation.

5) To construct an S. typhimurium LT2 RNase III deficient strain and to determine if ribosomes containing IVSs are still functional.

MATERIALS AND METHODS

A. Strains used

All strains used or constructed in this study are available from the Salmonella Genetic Stock Centre (SGSC) and listed in Table 1. Strains were maintained frozen in 15% glycerol at -70°C and were single colony isolated with appropriate antibiotics prior to use.

B. Media

1. Bochner

Curing of Tn10 transposons from mutated genes via selection for imprecise excision can be accomplished using the method of Bochner et al. (1980). The TET protein stops the entry of tetracycline into resistant bacterial cells. It is thought that tetracycline crosses the membrane of bacteria through the binding of metal ions to its polar groups, and TET acts as a competitive chelator of these metal ions and inhibits tetracycline uptake. If these membrane bound metal ions are essential for cell function, then tetracycline resistant cells are hypersensitive to chelating agents such as fusaric and quinaldic acid. Thus, inducing the TET protein in the presence of fusaric acid by growing the bacterial on autoclaved (non killing) chlorotetracycline selects for loss of tetracycline resistance determined by the Tn10 transposon. Purifying selections on 2.5 ug/ml tetracycline LB-agar allowed for the differentiation between resistant cells and non resistant cells as the latter have a smaller colony size. Bochner media contains 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, 2 g/l

glucose, 0.05 g/l chlortetracycline hydrochloride, 10 g/l NaH_2PO_4 H_2O, 6ml/l of 2mg/ml fusaric acid, 5ml/l of 20mM Zinc chloride; sterile fusaric acid and zinc chloride were added after autoclaving.

2. Luria-Bertani (LB)

LB (10 g/l tryptone [BDH], 5 g/l yeast extract [Difco], 10 g/l NaCl, adjusted to pH 7.0 with 10 M NaOH) and LB-agar (LB-broth with 1.5% w/v Bacto-agar [Difco]) with appropriate antibiotic selection were routinely used to grow strains. The selections used as follows: ampicillin, 100 ug/ml; streptomycin, 100 ug/ml; tetracycline, 25 ug/ml; and kanamycin, 50 ug/ml.

3. SOC

Bacterial cells were incubated in SOC broth (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) following electroporation (see below).

C. Enzymes and Chemicals

Endonucleases were obtained from New England Biolabs (I-CeuI and BlnI), Boeringer-Mannheim (SpeI), and Pharmacia (DraI). Taq polymerase was obtained from GIBCO BRL and dNTPs were from Pharmacia. DNase I was from Pharmacia, and rRNasin (placental RNase inhibitor) was from Promega. Most other chemicals, including agarose, were from Sigma Chemical Co.

D. Primers

PCR was performed using primers from University Core DNA Services (Health Science Centre, University of Calgary): P1 (5'GCGTCGGTAAGGTGATATG3'), P2 (5'GCTATCTCCCGGTTTGATTg3'), P3

(5'CCGATGCAAACTGCGAATAC3'), and P4 (5'TTCTCTACCTGACCACCTG3'); these primers were located at E. coli rrlB base (Noller, 1984) 74-92, 786-805, 901-920, and 1616-1634, respectively. Primer P5 (5'GGCTGTCGTCAGCTCGTGT3') was located at E. coli rrsB base 1056-1074 (Noller, 1984).

E. Plasmids and cosmids

1. pACS21

This pBR322 derived plasmid is a 1.4-kb subclone of the rnc operon that contains the complete rnc gene (Figure 4A) (Takiff et al., 1989). This plasmid has been previously used to complement chromosomal rnc deficiencies in E. coli.

2. pPR1347

This cosmid contains both the rfb gene cluster (rough; required for synthesis of oligosaccharide units) and the rfc gene (rough; O antigen polymerase) of S. typhimurium (Figure 4B) (Neal et al., 1993). It conveys to recipient strains the ability to synthesize the group B long-chain O12 antigen, which is required for P22 adsorption.

F. DNA purification from agarose gel

DNA can be purified away from contaminating protein and nucleotides by adsorbing it to the surface of glass powder fines in the presence of NaI, a chaotropic salt (Vogelstein and Gillespie, 1979). A consequence of NaI incubation is that agarose electrophesed in TAE buffer breaks down and releases DNA held in its matrix; TBE electrophoresed DNA does not breakdown as readily in NaI, but can be purified from agarose with the use of an enhancing buffer. GIBCO BRL provides the GlassMAX

cartridge system, which includes enhancing buffer, for purification of DNA in solution or excised in agarose.

Genomic fragments containing specific rrl genes of S. typhimurium LT2 and S. typhi Ty2 were chosen based on genomic cleavage maps (Figure 5 and 6) (Liu et al., 1993b; Liu and Sanderson, 1995), and on the cleavage map for SpeI in S. typhimurium LT2 (S.-L. Liu, Personal Communication). In Tables 2 and 3, the endonucleases used and fragment sizes for the isolation of S. typhimurium LT2 and S. typhi Ty2 rrl genes are indicated, respectively. Each fragment was excised in agarose under longwave ultraviolet light and purified from the agarose using GlassMAX according to the manufacturer's instructions. This product was also used for the agarose gel purification of amplicons from PCR.

G. Genomic DNA isolation

Genomic DNA isolation by this method was suitable for PCR amplification. The cells were grown overnight with shaking in LB-broth, 5 ml of culture was centrifuged at 5,000 x g for 15 minutes, the pellet of cells was resuspended in 400 ul of proteinase digestion buffer (200 mM Tris-Cl, 25 mM EDTA, 0.3 M NaCl [pH 8.0]), and 100 ul of 10% SDS was added. Then proteinase K was added to a final concentration of 100 ug/ml and the solution was incubated at 42°C until it cleared (usually 1 hour). 500 ul of phenol/chloroform/isoamyl alcohol (25:24:1) was added and the solution was vortexed and centrifuged; this step was repeated twice more with the aqueous phase. DNase-free RNase was added to a final concentration of 20 ug/ml, and the tube was incubated for 30 minutes at 37°C. The genomic DNA was then extracted with an equal volume of chloroform and precipitated with 2.5 volumes of ethanol, washed with 70% ethanol, and resuspended into 500 ul of H_2O.

H. Preparation of genomic DNA embedded in agarose and enzymatic digestion (Liu and Sanderson, 1992)

Cells were grown overnight at 37°C in LB-broth, and harvested by centrifugation. Cells were resuspended in 0.5 ml of Cell Suspension solution (10 mM Tris-HCl, 20 mM NaCl, 100 mM EDTA [pH 7.2]) and mixed with 1.2% agarose. This mixture was then drawn into a 1 ml tuberculin syringe from which the needle adapter had been removed. After solidifying, the agarose disks were cut to a thickness of about 1 mm and placed in 3 ml of Lysing buffer (10 mM Tris-HCl [pH 7.2], 50 mM NaCl, 100 mM EDTA, 0.2% SDS, 0.5% N-lauroyl sarcosine) and heated in a water bath at 65°C for 45-60 minutes. Following lysis of the cells, disks were washed once with Wash solution (20 mM Tris-HCl, 50 mM EDTA [pH 8.0]), then treated with 3 ml of Proteinase solution (100 mM EDTA, 0.2% SDS, 1% N-lauroyl sarcosine, with 0.66 mg of Proteinase K [BRL] added per ml). The disks were then incubated 18-24 hours at 42°C with gentle agitation. Following protein digestions, disks were rinsed twice with Wash solution and treated with 5 ml of a PMSF solution (1 mM PMSF in Wash solution) for 2 hours at room temperature with gentle shaking to remove any remaining proteinase activity. After rinsing disks twice with Wash solution and with Storage solution (1/10 diluted Wash solution), the disks were used for endonuclease digestion or were stored at 4°C.

Genomic DNA embedded in agarose disks was digested by first immersing in a 2X concentration of the buffer supplied by the manufacturer and then incubating at room temperature for 15 minutes. The 2X buffer was then replaced with 30 ul of 1X buffer containing a pre-determined quantity of enzyme per ul sufficient to completely digest the DNA in about 3 hours at 37°C.

I. RNA isolation

Cells were grown with shaking in LB-broth, and were isolated at mid-log phase by centrifugation at 5000 x g for 15 minutes at 4°C. 2 x 10^9 cells were resuspended into 3 ml of extraction buffer (10 mM sodium acetate and 0.15 M sucrose [pH 4.8]) at 4°C. SDS was added to a final concentration of 1% and the solution was vortexed. 3 ml of phenol at 65°C (sodium acetate buffered [pH 4.8]) was added and the solution was vortexed, incubated at 65°C for 5 minutes, incubated at 0°C for 5 minutes, and centrifuged at 5000 x g for 30 minutes at 4°C. The phenol extraction was repeated with the aqueous phase, followed by a final extraction with chloroform. The RNA was precipitated overnight in 2.5 volumes of ethanol at -20°C and centrifuged. The RNA pellet was washed with 75% ethanol and resuspended in DNase I buffer (50 mM Tris-Cl, 10 mM $MgCl_2$ [pH 7.5]) with dithiothreitol to 5 mM. To this solution, 14 units of DNase I and 350 units of rRNasin were added, and the solution was incubated for 30 minutes at 37°C, extracted with an equal volume of chloroform, precipitated with 2.5 volumes of ethanol, and stored at -70°C.

J. Polymerase chain reaction

Refer to Figure 7 for amplicon identification. PCR reactions were carried out according to the instructions accompanying the Taq polymerase on a Techne Gene E Thermal Cycler. PFGE fragment templates were titrated from GlassMAX preparations to optimize amplification while genomic PCR was from 100 ng of purified genomic DNA (see below). Thirty cycles of 1 minute denaturation (94°C), 1 minute annealing (56°C), and either 50 seconds (amplicons A and B), 1 minute 50 seconds (amplicon C) or 3 minutes (amplicon D) extension (72°C) were

carried out. A seven minute extension (72°C) was added as a final step.

K. Cycle sequencing

The sequencing of the PCR products was by the University Core DNA Services (Health Science Centre, University of Calgary). The process utilizes automated Applied Biosystems (ABI) sequencing and a Taq DyeDeoxy Terminator Cycle Sequencing kit (ABI). Conditions were maintained as recommended, which included doubling all reagents and the use of 5% DMSO in some but not all reactions. Nested sequencing primers were used. The forward and reverse primers for amplicon A were 5'TACTCCTGACTGACCGATAG3' and 5'GGCTAGATCACCGGGTTTCG3', and for amplicon B were 5'CCTGCGCGGAAGATGTAACG3' and 5'GCATTCGCACTTCTGATACC3'. All sequencing templates and sequencing primers were gel purified.

L. F plasmid conduction of cosmid pPR1347

100 ul of E. coli SAB4793 (Hessel et al., 1995) donor cells harboring the F plasmid and cosmid pPR1347 from an overnight culture was added to 4.5 ml LB-broth without selection. 100 ul of overnight E. coli HT115 recipient cells were added. The pPR1347 cosmid was conducted by the F plasmid to recipient cells following overnight incubation at 37°C. Kanamycin (the resistance determined by pPR1347) and tetracycline (the resistance determined by the recipient cells) was used for selection. Strain HT115 that contained pPR1347 was used to grow phage P22 HT105/int-102 for transduction of rnc-14::ΔTn10.

M. P22 phage propagation and isolation

P22HT105/int-102, a variant of P22 that selectively packages chromosomal DNA, was grown on S. typhimurium LT2 strains and E. coli strain SAB5300 that contained characterized Tn10 insertions. 10 ul of a stock P22 phage suspension and 100 ul of mid-log cells were added to 5 ml of LB-broth and allowed to incubate at 37°C with vigorous shaking overnight. Lysates were cleared by centrifugation, sterilized by addition of 300 ul $CHCl_3$, and stored at 4°C.

N. Production of electrocompetent cells and electroporation

S. typhimurium LT2 was made electrocompetent as follows. 500 ml of LB-broth was inoculated with 5 ml of overnight culture and grown at 37°C with vigorous shaking for three hours. Cells were chilled on ice and were washed with 1X volume ddH_2O, 0.5X volume ddH_2O, and 0.1X volume 10% glycerol. The cells were then resuspended in a total of 5 ml 10% glycerol, aliquoted, and stored at -70°C until use.

Electroporations were performed as described previously (Binotto et al., 1991) using a Bio-Rad Gene Pulser. 40 ul aliquot of electrocompetent cells was thawed on ice, DNA to be transformed was added, and the mixture transferred to a cuvette containing a 0.2 cm voltage path. Settings selected for the Gene-Pulser were 2.5 kV, 25 uF capacitance, and 200 ohms resistance. Following the pulse, about 1 ml of SOC broth was added, and the suspension was incubated for 1 hr at 37°C, and plated onto appropriate selective media.

O. Agarose gel electrophoresis

DNA bands in agarose were visualized using a shortwave UV transilluminator and photographed using 3000 ASA black-and-white Type 57 Polaroid film with a red filter. If bands were to be cut from the gel, a longwave UV transilluminator was used for visualization.

1. PFGE

Genomic DNA disks from restriction digestions were inserted into wells in 0.7% agarose gels, electrophoresed in 0.5X TBE buffer (1X TBE buffer contains 90 mM Tris-HCl, 90 mM boric acid, 2 mM EDTA [pH 8.0]) with 1 ug of EtBr added per ml. Electrophoresis was performed with either a Bio-Rad CHEF Mapper or DRII PFGE apparatus, and conditions were generally 6 V/cm, 120° angle, with pulse times in accordance with 1 second of pulse time for each 10 kb of DNA size. Run times were approximately 18 hours, depending on regions of interest.

2. Conventional DNA electrophoresis

All PCR products were electrophoresed at 8 V/cm in 0.7% to 1.0% agarose gels containing 0.5x TBE buffer (see above) and 0.5 ug/ml of EtBr.

3. Denaturing RNA electrophoresis

10 ug of RNA was electrophoresed into 1.2% agarose gel using the glyoxal-DMSO denaturation method (Sambrook et al., 1989). RNA can be denatured by heating a sample with a mixture of 1 M glyoxal and 50% DMSO for 1 hour at 50°C. Glyoxylation introduces an additional ring to guanosine residues, which in turn causes steric hindrance of guanosine-cytosine base-

pairings. The denatured RNA is then electrophoresed using 10 mM sodium phosphate buffer (pH 6.8) at 3 V/cm with constant recirculation from anode to cathode. All electrophoresis solutions were treated with 0.1% Diethylpyrocarbonate (RNase inhibitor) and autoclaved prior to use.

P. Northern blotting

Northern blotting was to either Hybond-N or Hybond-N+ membranes (Amersham); the former was used exclusively for blots destined for hybridization as it gave the best signal to noise ratio (data not shown). All Northern blots were stained for RNA using 0.04% methylene blue in 0.5 M sodium acetate (pH 5.2) (Sambrook et al., 1989), washed several times in water, and photographed with 3000 ASA black-and-white Type 57 Polaroid film.

1. Neutral capillary transfer

Neutral blotting was completed as an overnight protocol and is briefly summarized as follows. A single sheet of Whatman 3MM blotting paper was placed on a centered support within a pyrex baking dish; the edges of the sheet were allowed to hang over to reach the bottom of the dish and form wicks. The blotting paper was wetted with 10X SSC (1.5 M NaCl and 300 mM sodium citrate [pH 7.0]), and about 250 ml of SSC solution was added to the baking dish. The gel was placed onto the blotting paper. A section of Hybond-N+ membrane was trimmed to the exact size of the gel and laid directly onto the gel, without trapping air bubbles. Two trimmed, SSC wetted, pieces of blotting paper were then placed on top of the membrane, followed by one non-wetted piece, then sufficient paper towels to absorb 150-200 ml of transfer buffer. Plastic film and Parafilm was placed around

the edges of the agarose gel to prevent a short circuit around the gel. A 500 g of weight was placed on the paper towels. Transfer of RNA to the membrane was complete in 18-20 hours. After transfer, the membrane was stained with methylene blue, photographed, and fixed by incubation at 80°C for 2 hours.

2. Downward alkaline capillary transfer

A rapid alkaline blotting protocol (Chomczynski, 1992) was used for membranes destined for hybridization, and occasionally for other membranes, as it removed glyoxylation (glyoxal disassociates from RNA above pH 8.0),. Also, it has been reported that Northern transfers using this alkaline protocol yield enhanced hybridization signal (Chomczynski, 1992). 2 to 3 cm in height of paper towels were cut 2 cm larger than the gel to be blotted. 5 sheets of Whatman 3MM blotting paper was cut to the same size as the gel; 4 of the sheets were laid down upon the paper towel dry and the last sheet was wetted in the transfer buffer containing 3 M NaCl, 2 mM N-lauroyl sarcosine, 8 mM NaOH (pH 11.4). The membrane, Hybond-N or Hybond-N+, was placed upon the wetted blotting paper and the gel was placed, after soaking in hybridization buffer for 10 minutes, onto the membrane. 3 more pieces of blotting paper, cut to size and wetted with hybridization buffer, were placed onto the gel, followed by 2 long wet strips of blotting paper to for the wick; the wick was completely covered in plastic film. The transfer buffer reservoir was place off to one side, about 3 cm higher than the top of gel. Downward transfer was complete for a 8mm thick 1.2% agarose gel after 3 hours. After transfer, membranes were neutralized with 200 mM sodium phosphate buffer, stained with methylene blue, and photographed. RNA was fixed to Hybond-N membranes by incubation at 80°C for 15 minutes; RNA was fixed

to Hybond-N+ membranes under alkaline conditions and did not require heat fixation.

Q. Oligonucleotide hybridization and chemiluminescent detection of IVSs

RNA from SA5303 was electrophoresed, blotted, methylene blue stained, and photographed as described above. The methylene blue stain was removed by washing the membrane with a solution of 1% SDS and 2X SSPE (0.3 M NaCl, 2 mM EDTA, 20 mM NaH_2PO_4 [pH 7.4]) for 15 minutes. The membrane was then prehybridized for 1 hour with 20 ml of DIG Easy Hyb buffer (Boehringer Mannheim) at 48°C in a hybridization oven (Tek-Star, Bio-Can Scientific). The prehybridization solution was replaced by a solution of DIG (Digoxigenin) Easy Hyb and 10 pmol/ml DIG-end labeled oligonucleotide specific for the helix-45 IVS from S. typhimurium LT2 (5'TAAAGTTGTCTTGGGTGATAC3'). After 2 hours, the membrane was washed twice with 2X SSC, containing 0.1% SDS, for 5 minutes, followed by two 15 minute washes with 0.5X SSC, containing 0.1% SDS.

Chemiluminescent detection of the DIG-labeled oligonucleotide was as recommended by the manufacturer (Boehringer Mannheim). Briefly, the membrane was washed twice with wash buffer (100 mM maleic acid, 150 mM NaCl, 0.3% Tween 20 [pH 7.5]), incubated with blocking solution (Boehringer Mannheim) for 1 hour, and then incubated with anti-digoxigenin antibody alkaline phosphatase conjugate for 30 minutes. The membrane was again washed, and equilibrated with detection buffer (100 mM Tris, 100 mM NaCl [pH 9.5]). The chemiluminescent substrate CSPD (Boehringer Mannheim) was added and the membrane was incubated between two sheets of acetate at 37°C for 15 minutes. The membrane was then exposed to Kodak XAR

film for about 10 minutes and developed according to the film manufacturer's protocol.

R. Computer software and hardware

Each sequence was folded to minimize free energy using the Genetics Computer Group (GCG) version 8 implementation of FoldRNA (Zuker, 1989) using energies defined by Freier et al. (1986). The results were visualized by LOOPVIEWER (Gilbert, 1990) on a Mac II/SI. The GCG GAP program, which minimizes gaps when aligning sequences, was used to generate identity comparisons.

RESULTS

A. IVS analysis of S. typhimurium LT2

1. 23S rRNA fragmentation in S. typhimurium LT2

S. typhimurium LT2 does not contain intact 23S rRNA (2.9-kb) as detected for E. coli K-12 (Figure 8A). Several 23S rRNA fragments were detected, and each correlated to those reported previously for S. typhimurium strains ATCC 23566 (Burgin et al., 1990) and ATCC 13311 (Skurnik and Toivanen, 1991). At least one of the seven rrl genes contains an IVS in helix-25 only, as 2.4- and 0.5-kb fragments were present. Multiple rrl genes contain an IVS in helix-45 only as 1.7- and 1.2-kb fragments were present as major rRNA species, and at least one rrl gene has IVSs in both helices as 1.7-, 0.7-, and 0.5-kb fragments were present (Figure 8B).

2. IVS possession in the seven rrl genes of S. typhimurium LT2.

To further elucidate IVS possession, DNA fragments containing specific rrl genes were isolated following PFGE of genomic DNA from BlnI, I-CeuI, and SpeI digests (Table 2). BlnI, I-CeuI, and SpeI genomic fragments were chosen based on the genomic cleavage map for S. typhimurium LT2 (Figure 5) (Liu et al., 1993a), and genomic cleavage map for SpeI (S.-L. Liu, Personal Communication). All fragments were amplified for amplicons A (which contains helix-25) and B (which contains helix-45), and the products were agarose gel electrophoresed and purified by GlassMAX (Figure 7). Amplicon A that was obtained using DNA containing rrlA, rrlB, rrlC, rrlD, or rrlE as the

template, corresponded to the size of the amplicon resulting when genomic DNA of E. coli was used; this size was predicted to be 731 bp according to the known sequence of rrlB of E. coli (Noller, 1984). This result indicates that these rrl genes do not have a helix-25 IVS. Amplicons obtained using template DNA containing rrlG or rrlH are about 110 bp larger in size (Figure 9A); this suggests that these two rrl genes contain helix-25 IVSs. Amplicon B (containing helix-45) obtained using template DNA containing rrlA, rrlB, rrlC, rrlD, rrlE, or rrlH are larger than the amplicon resulting from amplification of E. coli DNA (calculated from sequence to be 733 bp [Noller, 1984]) by about 90 bp, indicating that each of these rrl genes contains a helix-45 IVS. Amplicon B made with template DNA from rrlG resembles that from E. coli. These data indicate that rrlG has one IVS in helix-25, rrlH has IVSs in both helices, and the other five rrl genes have IVSs only in helix-45. The positions of the seven rrl genes on the genetic map of S. typhimurium LT2 (Liu et al., 1993a), and the IVSs they carry based on data in Figure 9A, is illustrated in Figure 9B.

These results conform with the sizes of 23S rRNA fragments observed for strain LT2 (Figure 8). Stoichiometry of rRNA fragments indicated that a least one rrl gene contains an IVS in helix-25 only, generating a 2.4- and 0.5-kb rRNA fragment; I show this to be rrlG. The presence of a 0.7-kb rRNA fragment indicates that at least one rrl gene carries IVSs in both helices; I show this to be rrlH. The high concentration of rRNA of 1.2- and 1.7-kb fits the conclusion that most rrl genes contain IVSs in helix-45 only. The complete absence of 2.9-kb rRNA, the size of unfragmented 23S rRNA, is consistent with the conclusion that all rrl genes contain at least one IVS.

3. Nucleotide Sequences and potential secondary structures of the helix-25 region.

To confirm the presence or absence of IVSs in the amplicon A products, cycle sequencing of the seven helix-25 regions was completed from both strands. Each resulting sequence was folded into a potential RNA secondary structure to minimize free energy (Figure 10). All helix-25 regions form tight secondary structures where the first and last 8 bases of each structure is conserved, including the E. coli rrlB tetra-loop. The S. typhimurium LT2 helix-25 tetra-loops from rrlA, B, C, D, and E , none of which contain IVSs as predicted by Figure 9A, were identical, but a comparison to E. coli revealed that S. typhimurium contains a 2 nucleotide addition and several base changes in and around the cap of the tetra-loop. rrlG and rrlH are confirmed to contain IVSs, of about 110 bp, inserted in helix-25. These IVSs show a low sequence identity of only 56% (Table 4), and their predicted secondary structure is very different (Figure 10). However, both IVSs maintain identity in the first and last 14 nucleotide, presumably due to conservation of the RNase III excision site, which was shown to occur in the first 16 nucleotide of the helix-45 IVS (Burgin et al., 1990). The ability of RNase III to recognize secondary structure (duplex stems) rather than sequence consensus (Robertson, 1982) would account for the stem of helix-25 and helix-45 IVSs showing limited sequence identity. S. arizonae helix-25 (Burgin et al., 1990) is 56% and 94% identical to rrlG and rrlH, respectively (Table 4). A BLAST search (Altschul et al., 1990) of nucleotide sequences in GenBank (release 84) against each IVS did not identify significant matches with any nucleotide sequences other than the IVSs in Table 4.

4. Nucleotide Sequences and potential secondary structures of the helix-45 region.

All rrl genes of S. typhimurium LT2 except for rrlG contained nucleotide sequences with helix-45 IVSs (Figure 11). The helix-45 tetra-loop of E. coli rrlB and S. typhimurium LT2 rrlG are identical except for one nucleotide in the cap. As predicted from Figure 9A, nucleotide sequence shows that rrlA, rrlB, rrlC, rrlD, rrlE, and rrlH have IVSs of about 90 bp inserted in helix-45. These helix-45 IVSs are 100% identical in all these six genes, and they are also identical to those previously reported for two other S. typhimurium strains (Burgin et al., 1990; Skurnik and Toivanen, 1991). S. arizonae helix-45 IVS (Burgin et al., 1990) is 85% identical to S. typhimurium, and Y. enterocolitica helix-45 IVSs from strains of Group 1 and Group 2 (Skurnik and Toivanen, 1991) are 61% and 87% identical, respectively, to helix-45 IVSs of S. typhimurium (Table 5).

5. IVSs in the rrl genes of 21 wild-type strains of S. typhimurium and six separate lines of strain LT2.

a) IVS possession determined by genomic DNA amplification and DraI restriciton

Genomic DNA, which contains all seven rrl genes, was isolated from 21 independent wild-type strains of S. typhimurium from Salmonella Reference Collection A (SARA)(Table 1). Amplicon C (Figure 7), produced with this genomic DNA, was DraI restricted and separated by agarose gel electrophoresis (Figure 12A). Fragment length and intensity variance reveals the number and type of IVSs present throughout the seven rrl genes. The C1-fragments from DraI digestion contain helix-25 (Figure 7); the

shorter fragment estimated from E. coli rrlB nucleotide sequence (Noller, 1984) to be 1008 bp indicated amplification from an rrl gene lacking an IVS, and the longer fragment indicated amplification from an rrl gene possessing an IVS. The high intensity of the shorter fragment and low intensity of the longer fragment obtained with strain LT2 (=SARA2), which has five rrl genes lacking the helix-25 IVS and two possessing the IVS (Figure 12A), are as expected. The C1-fragment patterns of strains of Groups 1 and 3 were similar to strain LT2, suggesting that both have two helix-25 IVSs; however, Group 2 was less intense when compared to Group 1 or 3, and indicated that it possesses only one helix-25 IVS. Strain SARA21 in Group 4 does not carry helix-25 IVSs as it produces only one C1-fragment which is equivalent in size to that produced by amplification of E. coli genomic DNA.

The C2-fragments from DraI digestion contain helix-45 (Figure 7). Strain LT2 DNA shows a low intensity fragment of a size equivalent to the 551 bp fragment expected for amplification of E. coli DNA (Noller, 1984), and a high intensity fragment, about 90 bp larger, indicating amplification from an rrl gene possessing an IVS. As expected, the intensity of the larger fragment is about six times as great, since six of seven rrl genes have the helix-45 IVS (Figure 12A). All the strains in Groups 1 and 4 showed similar C2-fragment patterns indicating that all have six rrl genes with IVSs. Strains in Group 2 contained fewer helix-45 IVSs than Groups 1 and 4, and strains in Group 3 have helix-45 IVSs in all their rrl genes.

b) IVS possession determined by RNA denaturing gel electrophoresis

RNA isolated from the S. typhimurium wild-type SARA Group representatives supports the predicted IVS possession drawn from PCR analysis. Group 1 contains the characteristic LT2 23S rRNA fragmentation pattern, producing 2.4-, 1.7-, 1.2-, 0.7-, and 0.5-kb rRNA fragments; note that no intact 23S (2.9-kb) rRNA is observed. Group 2 is missing one helix-25 IVS when compared to Group 1 (Figure 13A), this is confirmed by a missing 0.5- and 0.7-kb rRNA fragment; the missing 0.5-kb fragment is inferred by a less intense 0.5-kb rRNA band and the stoichiometric relationship with the missing 0.7-kb rRNA fragment. This suggests that Group 2 does not contain any rrl genes which harbor both helix-25 and helix-45 IVSs at the same time, as does rrlH of strain LT2. Further, Group 2 contains fewer helix-45 IVSs than does Group 1, and therefore intact 23S rRNA is expected and observed. Group 3 is missing the 2.4-kb rRNA fragment and contains more 0.7- and 0.5-kb rRNA fragments when compared to strain LT2 (Figure 13A) . This verifies that Group 3 contains helix-45 IVSs in all rrl genes and thus does not produce a 2.4-kb rRNA fragment which would require that only a helix-25 IVS be present in an rrl gene. Group 4 does not contain any helix-25 IVSs but about the same number of helix-45 IVSs when compared to Group 1 (Figure 13A). Intact 23S rRNA without 0.7- or 0.5-kb rRNA fragments was observed for Group 4 with 1.7- and 1.2-kb rRNA fragments; the latter fragments are indicative of helix-45 IVSs.

Amplification of genomic DNA from seven different S. typhimurium LT2 lines followed by DraI digestion yielded indistinguishable fragment sizes and intensities (Figure 12B) for all lines except LB5010. LB5010 contained less of the IVS

containing C1-fragment, when compared to strain LT2, indicating that it lacks one helix-25 IVS. RNA isolated from LB5010 confirms this finding, which is missing a 0.7- and 0.5-kb rRNA fragment similar to Group 2 (Figure 13B); the remaining six S. typhimurium LT2 lines are identical with respect to rRNA fragmentation. Though these seven lines all originated from the same LT2 wild-type strain, they have been maintained in different laboratories since the 1950s, and separated by numerous single colony isolations over 30 years. This shows that IVSs in specific strains are fairly stable.

B. IVS analysis of S. typhi Ty2

1. 23S rRNA fragmentation in S. typhi Ty2

S. typhi Ty2 line H251.1 does not contain intact 23S rRNA (2.9-kb) as detected for E. coli K-12, instead 2.4- and 0.5-kb rRNA fragments (in addition to 16S RNA) were observed (Figure 14A). This fragmentation pattern is consistent with the presence of helix-25 IVSs in all seven rrl genes, as RNase III cutting at the helix-25 site would generate the fragments observed (Figure 14B). S. typhi Ty2 RNA does not contain 1.7-, 1.2-, or 0.7-kb fragments, which supports the conclusion that S. typhi Ty2 does not possess helix-45 IVSs; fragments of these sizes are seen in S. typhimurium RNA, as expected.

2. Isolation and sequencing of the IVS rrl gene regions of S. typhi Ty2

S. typhi Ty2 rrl genes were separated by pulsed-field gel electrophoresis (PFGE) based on the genomic cleavage map for S. typhi Ty2 (Figure 6) (Liu and Sanderson, 1995). Genomic fragments digested with I-CeuI allowed for isolation of rrlA,

rrlB, rrlE, rrlG, and rrlH; rrlC and rrlD were co-purified on a single I-CeuI fragment that was excised in agarose, restricted with SpeI, and electrophoresed (PFGE) to yield two fragments, each containing one rrl gene (Table 3). All fragments were purified by GlassMAX and used as template for PCR to generate amplicons A and B for each rrl gene (Figure 7). Agarose gel electrophoresis of amplicon A from all seven S. typhi Ty2 rrl genes showed that all amplicons were the same size, about 110 bp larger than the amplicon from E. coli whole genomic template (data not shown). This indicates that all S. typhi Ty2 rrl genes contain helix-25 IVSs, as predicted by the RNA fragmentation data (Figure 14A). Amplicon B from all rrl genes was the same size as amplicon B from E. coli (data not shown), indicating, as predicted by RNA data (Figure 14A), that none of the rrl genes of S. typhi Ty2 possess helix-45 IVSs.

Both amplicons A and B were gel purified and cycle sequenced , all seven amplicon A (helix-25 IVS) and amplicon B (helix-45) products, respectively, were found to be 100% identical (Figure 15A and 15B). The S. typhi Ty2 helix-25 IVS was folded into RNA secondary structure. The resultant fold (Figure 15A) was similar in proposed secondary structure to the helix-25 IVS from rrlH of S. typhimurium LT2 (Figure 10). When the nucleotide sequence from the S. typhi Ty2 helix-25 IVS was compared to other species, the helix-25 IVS from rrlH of S. typhimurium LT2 was 97% identical, and the helix-25 IVS from S. arizonae was 94% identical (Table 4). In contrast, the S. typhimurium LT2 rrlG helix-25 IVS (Figure 10) was very different in secondary structure (Figure 15A), and was only 55% identical to the S. typhi helix-25 IVS (Table 4). The helix-45 penta-loop was identical in all seven rrl genes from S. typhi Ty2 (Figure 15B) but differed from the S. typhimurium LT2 rrlG tetra-loop

(Figure 11) by several base changes and one nucleotide deletion in the cap.

3. Distribution of IVSs among wild-type S. typhi strains

To determine IVS possession in S. typhi, whole genomic DNA from 35 strains representing 30 independent wild-type strains (all S. typhi strains in Table 1) was amplified by PCR to produce amplicon C (Figure 7). Amplicon C was restricted by DraI to produce the C1 fragment containing the helix-25 region, and C2 fragment containing the helix-45 region; the amplicons were separated by agarose gel electrophoresis. All DraI restriction patterns were the same for all 35 strains; eight representative strains are in Figure 16 (lanes A to H). These data indicate that all strains possess helix-25 IVSs in all seven rrl genes, and helix-45 in none, as shown earlier for strain Ty2 (lane E). The rrnB operon from randomly-selected S. typhi strains 9032-85 and IP E.88.374 (Table 1) was isolated by pulsed-field gel electrophoresis, amplified by PCR, and the nucleotide sequence of the IVS was found to be 100% identical to the Ty2 helix-25 IVS, confirming the homogeneity of IVSs in S. typhi.

C. Construction and characterization of an RNase III deficient S. typhimurium LT2

Previously, it has been reported that RNase III mutants of E. coli K-12 are viable (Gegenheimer et al., 1977; King et al., 1984). However, since E. coli does not have IVSs in its 23S rRNA, this does not indicate whether ribosomes that have not had IVSs excised are functional. To observe the effects of constructing an RNase III (rnc$^-$) mutant in S. typhimurium, and

thereby stopping IVS excision from the 23S rRNA, the mutation rnc-14::ΔTn10 was transduced from E. coli HT115 to S. typhimurium LT2 as described below.

1. Construction of S. typhimurium LT2 rnc-14::ΔTn10

E. coli HT115 contains the insertion rnc-14::ΔTn10 that has been previously characterized as being RNase III deficient (Table 1). The generalized transducing phage P22 HT105/int-102 that is high transducing and integration deficient, and that propagates on S. typhimurium, was chosen to mediate the tranduction; however, strain HT115 had to be made P22 phage sensitive. That is, to allow for infection of E. coli with P22 phage, the group B long-chain O12 antigen encoded by rfb gene cluster and rfc of S. typhimurium must be present. Cosmid pPR1347 contains rfb and rfc but it is, by itself, non mobilizable. To transfer pPR1347 to strain HT115, conjugative transfer was completed through F factor conduction. When both pPR1347 and the F factor are contained within the same bacterium, the Tn1000 harbored by the F factor can initiate replicative transposition to pPR1347, and as a result a cointegrate structure is formed. If DNA transfer has been initiated during the cointegrate formation, both pPR1347 and the F factor will be transferred to the recipient cell and rapidly resolved. The E. coli SAB4793 (Table 1) was used to conduct cosmid pPR1347 to strain HT115, and strain SAB5300 was isolated; selection was for tetracycline resistance, encoded by the Tn10 insertion, and kanamycin resistance, encoded by pPR1347 (Figure 17A). The phage P22 HT105/int-102 was propagated on strain SAB5300, and a lysate of 10^{10} Pfu/ml was obtained (Figure 17B).

Transduction between E. coli and S. typhimurium was further made problematic by the endogenous methyl-directed

mismatch (mut) repair system possessed by S. typhimurium (Rayssiguier et al., 1989), which reduces inter-species homologous recombination to a low rate when sequence divergence is above 10% (Rayssiguier et al., 1989). Therefore, to facilitate transduction of rnc-14::ΔTn10, S. typhimurium SA3856 (Table 1) that contains mutL-111::Tn10 (mismatch repair deficient) was used as the S. typhimurium transduction recipient. Because strain SA3856 contained a Tn10 element that already encoded tetracycline resistance, selection for rnc-14::ΔTn10 transductants on LB-agar with selection for tetracycline was not feasible. To counter this problem, strain SA3856 was cured of its Tn10 through selection for loss of tetracycline resistance , by spontaneous deletion, through the use of Bochner media (see Materials and Methods)(Figure 17C); strain SA5301 (Table 1) was isolated and confirmed to be tetracycline sensitive. To ensure that SA5301 was still mismatch repair deficient, it was grown on rifampin, an antibiotic known to inhibit RNA polymerase priming activity. Observing growth on rifampin (100 ug/ml) indicated that SA5301 had a high compensating mutation rate in RNA polymerase (Shanabruch et al., 1981), and thus, a mismatch repair deficient phenotype, which produces a high rate of spontaneous mutation.

SA5301 was transduced with the lysate obtained by P22 HT105/int-102 propagation on E. coli HT115 with selection for tetracycline, and S. typhimurium strain SA5302 (Table 1) was isolated and confirmed to be RNase III deficient through observing 30S, 25S, and 23S rRNAs by methylene blue staining of a Northern blot of a glyoxal-DMSO denaturing RNA gel (data not shown) (Figure 17D). To separate the rnc mutation from the mismatch repair deficient background, phage P22 HT105/int-102 was propagated on strain SA5302, and the 10^{10} Pfu/ml lysate obtained was used to transduce S. typhimurium LT2 (wild-type) to

tetracycline resistance resulting in strain SA5303 (Table 1) that was used for RNase III deficiency characterization as described below. Strain SA5303 is S. typhimurium LT2 rnc-14::ΔTn10.

2. Characterization

RNA was isolated from S. typhimurium strains SA5303 (rnc-14::ΔTn10) and LT2 (lanes 1 and 2, Figure 18A), in addition to E. coli strains HT115 (rnc-14::ΔTn10) and K-12 (lanes 3 and 4, Figure 18A). 23S rRNA with 25S and 30S rRNAs were observed for E. coli HT115 (lane 3) as expected, because RNase III deficient strains fail to carry out normal rRNA processing. In S. typhimurium (lane 2) the 23S rRNA is fragmented into 2.4-, 1.7-, 1.2-, 0.7-, and 0.5-kb fragments as observed in earlier results. S. typhimurium SA5303 contained 23S rRNA but none of the 23S rRNA fragments seen in lane 2; this indicated that RNase III is the enzyme that excises IVSs in the wild-type strain (lane 1). The presence of rRNA species that correspond to 25S and 30S rRNA from E. coli HT115 are also visible in strain SA5303 (lane 1).

A careful examination of the 23S, 25S, and 30S rRNAs from S. typhimurium SA5303 (lane 1, Figure 18), showed that they were slightly larger than those of E. coli HT115 (lane 3); this extra size can be attributed to the presence of IVSs.

To determine whether IVSs were present within the 23S, 25S, and 30S rRNAs of strain SA5303, a DIG-labeled oligonucleotide probe specific for the S. typhimurium LT2 helix-45 IVS (5'TAAAGTTGTCTTGGGTGATAC3') was hybridized to the membrane presented in Figure 17A. The oligonucleotide probe was detected by chemilumenescence and showed that IVSs were present in the rRNAs of strain SA5303 (Figure 18B, lane 1), and not in

S. typhimurium LT2 (lane 2), E. coli HT115 (lane 3), or E. coli K-12 (lane 4).

Plasmid pACS21 (rnc$^+$) was electroporated into S. typhimurium SA5303 that contained rnc-14::ΔTn10, and strain SA5304 (rnc-14::ΔTn10/pACS21[rnc$^+$]) was isolated by selecting for tetracycline and ampicillin resistance. RNA isolated from SA5304 did not contain intact 23S rRNA (Figure 19, lane 1), as was present in strain SA5303 (lane 2), and resembled S. typhimurium LT2 (lane 3). This demonstrated that RNase III was responsible for 23S rRNA fragmentation, as was concluded by Burgin et al. (1990); the RNase III activity encoded by the rnc gene carried by pACS21 was able to complement the chromosomal deficiency.

The generation time for E. coli strain HT115 (rnc-14::ΔTn10) was found to be 47 minutes, and the generation time for E. coli W3110 (parent of strain HT115) was 33 minutes (Figure 20A); thus, the generation time of strain HT115 was 42% longer than strain W3110. This reduction in growth rate was in keeping with the 40% increase in generation time previously reported for E. coli rnc deficient strains (Apirion et al., 1976; Gegenheimer et al., 1977; King et al., 1984).

The generation time for S. typhimurium SA5303 (rnc-14::ΔTn10) was 48 minutes, while for S. typhimurium LT2 was 33 minutes (Figure 20B). Thus, the generation time of strain SA5303 was 45% longer than the isogenic wild-type control strain LT2. This reduction in growth rate of the rnc mutant of S. typhimurium was comparable to the rate reported for the E. coli rnc mutant (strain HT115) as described above. In addition, the generation time of S. typhimurium SA5304 (rnc-14::ΔTn10/pACS21[rnc$^+$]) was 42 minutes. Thus, the growth rate was partially restored when compared to SA5303; the generation

time for strain SA5304 was still 27% longer than strain LT2 (Figure 20B).

DISCUSSION

A. Horizontal transfer of IVSs

How are IVSs distributed to different cells, and how are they propagated to more than one of the seven rrl genes of Salmonella? It might be argued that each IVS in a cell evolved independently, due to strong selective pressures, but this seems unlikely as there is no evidence that IVSs have a functional role. IVSs of related nucleotide sequence occur sporadically among many bacterial genera and species, indicating that they have been distributed by horizontal transfer between different cells. This transfer may be by genetic exchange methods, such as conjugation, transduction, or transformation. The only two helix-25 IVSs in S. typhimurium LT2, in rrlG and rrlH, are only 56% identical, but rrlH is 94% identical to the helix-25 IVS from S. arizonae and 96% identical to the helix-25 IVS from S. typhi Ty2 (Table 4). The IVS in helix-45 of S. typhimurium, S. arizonae, and Y. enterocolitica Group 2 all show >80% nucleotide identity, while Y. enterocolitica Group 1 is less related to all the others and to Y. enterocolitica Group 2 (Table 5). These data argue for horizontal transfer of IVSs between different bacterial genera, rather than vertical transfer by replication from parental cells. Thus, two helix-25 IVSs and one helix-45 IVS have entered S. typhimurium LT2 as three independent horizontal transfer events; the single helix-25 IVS of S. typhi Ty2 can be accounted for by a single horizontal transfer. Alternatively, IVSs might have originated in Salmonella and then been distributed to other genera.

B. Gene conversion of IVSs

Propagation of IVSs to different rrl genes within the same cell might be by one of two methods. One is double reciprocal recombination between two genes when they are present on separate sister chromatids immediately after replication. This recombination is normally recA dependent. The rrl genes are good targets for such exchange, since they include at least 500 bp of homologous DNA on each side of the IVSs. This method can exchange IVSs between different rrl genes, but the reciprocal exchange cannot increase the number of IVSs unless it is between an IVS-containing rrl gene and a non IVS-containing rrl gene on separate chromatids.

The second method is gene conversion, which is the non reciprocal transfer of information from one DNA duplex to another, and which may occur by a double-strand-break repair mechanism after replication (Szostak et al., 1983). Gene conversion occurs commonly in eukaryotes such as yeast to maintain homogeneity within a family of repeated sequences and on rare occasions allows a variant sequence to replace existing sequences; it therefore has the potential for directed change and facilitates correction without changing gene dosage (Nagylaki and Petes, 1982). Conversion is less common for genes on the bacterial chromosome, perhaps because bacterial DNA is mostly unique. However, conversion occurs between the rrn operons (Harvey et al., 1988; Harvey and Hill, 1990). Gene conversion of the Salmonella phase 1 flagellin gene fliC to the phase 2 gene fljB on the E. coli K-12 chromosome has been detected (Okazaki et al., 1993). In addition, the catIJF and pcaIJF genes of Acinetobacter calcoaceticus, which share nearly identical nucleotide sequence, convert each other in a recA dependent process at rates of about 10^{-6} (Gregg-Jolly and

Ornston, 1994; Kowalchuk et al., 1995). The following points support the hypothesis that dispersion and sequence maintenance of IVSs through the genome in Salmonella is another example of gene conversion: 1) helix-45 IVSs in six of the seven rrl genes of S. typhimurium LT2 have identical sequence (Figure 11), as do the helix-25 IVSs in all seven rrl genes of S. typhi Ty2 (Figure 15A) (and in the one rrl gene sequenced in each of two other S. typhi wild-type strains). These IVSs do not result from vertical transfer by replication from parent cells, since IVSs occur sporadically. They must have entered a single rrl gene by horizontal transfer, and been propagated by gene conversion. 2) Since there is no proven functional role for IVSs, their sequences would be expected to diverge by random mutation; yet the sequence of all helix-45 IVSs in S. typhimurium LT2, and of all helix-25 IVSs in S. typhi Ty2, are identical, respectively, and diverge when compared to similar IVSs in S. arizonae and Y. enterocolitica (Table 5). This indicates that IVSs are maintained by a continuous process of gene conversion, as is found in the gene families of the eukaryotes (Nagylaki and Petes, 1982). This would also explain why all helix-25 tetra-loops in S. typhimurium LT2, and all helix-45 penta-loops in S. typhi Ty2, are identical as well. 3) IVS sequence variation between different species can therefore be explained by mutation. When an IVS has been initially transferred to a species, and is present in only a single copy, random mutations that do not disrupt the RNase III site could occur resulting in a new mutant IVS; this new sequence could then be propagated to all seven rrl genes by subsequent gene conversion.

It is unlikely that the basis for propagation of IVSs is a transposition mechanism, or that the IVSs closely resemble mobile introns. Mobile introns participate by their coding capacity in an active process by which a DNA sequence (the

intron) in a gene becomes inserted into another intron-deficient copy of the same gene, and the new copy becomes a functional intron (Dujon et al., 1989; Belfort et al., 1995). Since most IVSs are small (90-110 bp in Salmonella) with no large ORFs they cannot code for transposition enzymes. A few IVSs contain ORFs (Ralph and McClelland, 1993; Ralph and McClelland, 1994, Afseth et al., 1995), but they show no evidence of transposition activity. Transposition might be complemented in trans if the cell has genes resembling the double-strand specific endonuclease found in group I mobile introns which generate double stranded breaks at the recipient site, but there is no evidence for such genes on the bacterial chromosome.

C. IVS gain and loss

The observed variation in the number of IVSs in different wild-type strains of S. typhimurium, and in the LB5010 line of strain LT2, supports the idea that IVSs have no essential function and, as such, are dispensable. The time over which these changes occurred probably varies, and is a result of relatively sporadic conversion events; depending on the type of template used for gene conversion (IVS-containing or non IVS-containing) an rrl gene can either gain or lose an IVS. Nonetheless, most SARA set members and six out of seven separate lines of S. typhimurium LT2 all possess the same number of IVSs in both helix-25 and helix-45; the S. typhimurium LT2 lines have been separated from each other by many single colony isolations over more than 30 years, and show divergence in some characters.

In contrast, thirty different wild-type strains of S. typhi from various sources contain helix-25 IVSs in all seven rrl genes. IVS homogeneity in S. typhi may occur, first, because restriction to growth in humans limits occupation of

different ecological niches and consequently reduces genetic variability by selecting for a clonal population; this is unlike S. typhimurium, which grows in many different hosts. Secondly, to generate IVS heterogeneity in S. typhi, a helix-25-containing rrl gene from another (Salmonella) species would need to be horizontally transferred and recombined with a helix-25 IVS-containing rrl gene; this event should be rare due to niche restriction of S. typhi. In addition, given that all seven S. typhi rrl genes are presently identical in IVS content, even if horizontal transfer or mutation were to change one of these IVSs, gene conversion using the remaining six unaltered rrl genes as templates should operate to restore the original IVS.

D. RNase III deficient S. typhimurium

An RNase III deficient mutant of S. typhimurium (strain SA5303) produces rRNA that is not fragmented and that contains IVSs in the 23S rRNA in the ribosome (Figure 18). The presence of IVSs within the 23S rRNA is tolerated, and does not seriously interfere with translation, as strain SA5303 does not show any appreciable growth depression above that of E. coli strain HT115, when both were compared to their isogenic controls (Figure 19).

It has been reported that helix-25 and helix-45 cap nucleotides in E. coli can be chemically cut by dimethyl sulfate while in the 50S ribosome (Egebjerg et al., 1990). Thus, helix-25 and helix-45 are accessible to chemical cleavage by being on the outer surface of the ribosome. In this way, S. typhimurium IVSs probably do not seriously interfere with translation because they extend out and away from the surface of the ribosome.

E. Speculation on the origin and function of IVSs

Many ideas have been reported in the literature to explain the origin and possible function of IVSs. Some considerations that must be taken into account to formulate such speculation are: 1) IVSs are excised from the 23S rRNA by RNase III, 2) IVSs occur sporadically in several genera of bacteria, and can be gained or lost at the level of the cell line, 3) Sequence consensus can be very high among related IVSs, 4) IVSs seem to be horizontally transferred because they occur in such diverse bacterial genera that independent evolution of IVSs could not account for their sequence conservation.

1. Speculation from the literature

Burgin et al. (1990) suggested that IVSs could be a footprint of an inserted and then excised transposon, which generated an inverted repeat at the site of excision that is recognized and cleaved by RNase III. This idea addresses both the origin of RNase III cleavage of IVSs and possibly their horizontal transfer; in light of the promiscuous nature of transposons, horizontal transfer of IVSs would be credible. However, this theory does not account for IVS sequence conservation beyond what is required for RNase III cleavage. Skurnik and Toivanen (1991) argued that the conservation of IVSs was to preserve the fragmentation of rRNA that serves as a novel mechanism for evasion of bacteriocins. Hsu et al. (1994) suggested that IVS conservation may occur to enable bacteria to rapidly turn over rRNA when they are fluctuating between growth cycles. These last two ideas address RNase III cleavage of IVSs through the implication of function, but again, neither serve to involve all qualities possessed by IVSs. Furthermore, in implying that IVSs confer a selective advantage on their host,

these two ideas cannot explain IVSs are not propagated to every rrl gene in those cells that contain IVSs.

2. Speculation from this study

The data presented in this study may shed light on the nature of IVSs. Several new ideas are presented to account for IVS characteristics.

a) IVS origin through palindromic-slippage of replicating DNA

To look at the possible origin of IVSs, we should first consider the helix-25 and helix-45 sequences, which do not contain IVSs. That is, both helix-25 and helix-45 cap nucleotide sequences are variable among strains of S. typhimurium, S. typhi, and E. coli studied (Figures 10, 11, and 15). This is consistent with the finding that 42 eukaryotic, archeabacterial, eubacterial, and chloroplast sequences also show variability in homologous helices (Egebjerg et al., 1990). Keeping in mind that the rrn operons of S. typhimurium and E. coli are about 97% identical, the sequence variation observed for these helices is high. This implies that helix-25 and helix-45 may be "hot spots" for mutation that may result from DNA replication errors.

It has been suggested that DNA replication errors occur through a DNA template slippage mechanism. Frameshift mutations often result from the addition or deletion of a repeated unit of DNA sequence; a unit can be a single nucleotide or multiple nucleotides of specific sequence. During replication, template misalignment along stretches of repeated DNA sequence is believed to be responsible; this effect is referred to as

template-slippage and is expected to occur in both bacteria and eukaryotes (Steisinger et al., 1966; Hancock, 1995).

In addition to this form of slippage, a second form can occur that does not require repeated units of DNA sequence. Here, palindromic sequences that misalign during DNA replication by forming alternative secondary structures (stem-loops) are thought to provide templates for base substitution and frameshift mutations (Boer and Ripley, 1984). Each unpaired base in the stem of a stem-loop structure provides a potential site for mutation that will either cause removal of the unpaired base or a frameshift to introduce a complementary base. Mutations of this type result in the restoration of the complementarity of both strands that comprise the stem of a stem-loop. Palindromic-slippage has been observed for the phage T4 rII gene (Boer and Ripley, 1984). Therefore, IVSs, being quasipalindromic sequences, may have their origin in DNA replication errors that are caused by palindromic-slippage initiated by helix-25 and helix-45 sequences. This would account for the extensive duplex structure of IVSs. The assumption that IVSs are on the surface of the ribosome probably accounts for the tolerance of helix extension through slippage mutation. The expansion segments of eukaryotes are also characterized by extensive secondary structure, and therefore, the postulate of Burgin et al. (1990) that elements analogous to IVSs may have participated in expansion segment evolution may be correct.

b) Ribosome adaptation and RNase III excision of IVSs

IVS removal from the 23S rRNA of S. typhimurium LT2 is not absolutely required for functional ribosomes to be produced, as IVSs were shown to remain within the 23S rRNA isolated from S.

typhimurium RNase III deficient strain SA5303 (Figure 18); all rrl genes of S. typhimurium LT2 have IVSs, thus ribosomes containing IVSs must be functional. Although RNase III mutants in E. coli have a characteristically slow growth rate in comparison to the wild-type, the growth rate of strain SA5303 was not reduced in comparison to E. coli RNase III deficient mutants (Figure 19). That is, the secondary structure of the IVSs in the 23S rRNA of S. typhimurium SA5303 does not apparently prevent the ribosome from carrying out translation; this does not preclude there being a slight negative selection that I could not detect. I postulate that IVS secondary structure may have been adapted to the shape of the ribosome, and this would have occurred before IVSs acquired the capacity for RNase III excision. For IVSs to have been adapted to accommodate to the shape of the ribosome, they would need to be subject to a selective pressure, and this pressure was probably the effect of IVS secondary structure on ribosome translation efficiency. IVSs would only affect ribosome translation if they were initially not excised from the 23S rRNA by RNase III. Thus, the consistent selective pressure on non excised IVSs to accommodate to ribosome shape would explain why tight stem-loop structures are formed by IVSs (Figures 10, 11, and 15), even when the sequence identity between similar IVSs is very low. For instance, the two helix-25 IVSs from rrlG and rrlH of S. typhimurium contain extensive duplex structure, but only 56% sequence identity. Therefore, short extensions of helix-25 or helix-45, probably produced by DNA slippage during replication, would be selected against if they adversely affected ribosome function, but would be selectively neutral if they accommodated to the shape of the ribosome. More specifically, palindromic-slippage would be expected to drive the extension of a perfect stem in a stem-loop structure. The fact that helix-25 and

helix-45 IVSs in S. typhimurium have distinct secondary structures would imply that a selective pressure to maintain specific IVS structure was present at one time.

Two possible scenarios arise to account for IVSs acquiring RNase III cleavage sites, as the nature of RNase III cutting still remains unproven. Robertson and Dune (1975) suggested that there are RNA features besides double-strandedness that are required for RNase III cutting to occur; however, it was also conceded that it may be equally true that any RNA stem-loop structure can serve as a substrate for RNase III cleavage, but only a few are large and stable enough to be recognized.

Therefore, the first scenario would assume that IVSs had to evolve to acquire RNase III cleavage sites. Once an IVS started to become large, and therefore sterically interfered with ribosome function, even if only slightly, pressure to either 1) delete the IVS from the DNA, 2) adapt the IVS for deletion from the rRNA, or 3) adapt the IVS for function would be great. Deletion of the IVS from the DNA would need to be precise to effectively remove only the IVS and not the surrounding 23S rRNA; and if the IVS had been propagated to multiple rrl genes, DNA deletion of one IVS would be prone to a high rate of repair by gene conversion using IVS-containing rrl templates. The second solution, selecting for adaptation of the IVS for excision from the rRNA, seems more probable. If one IVS was to acquire the secondary structure necessary for RNase III excision, gene conversion could distribute the change to other rrl genes containing the IVS, and thereby remove any adverse effects that IVSs had on ribosome function, while not removing the IVSs themselves from the rrl genes. The last solution, selecting for functional adaptation of the IVS, would probably require coevolution of both ribosomal RNA and protein, and thus, would be complex in nature and require much evolutionary time.

The second senario would assume that RNase III potentially recognizes all RNA stem-loop structures, and that any stable double-stranded RNA extending from the surface of the ribosome would be cut. In this case, IVSs, being on the surface of the ribosome, would be excised from the 23S rRNA without the need for structural adaptation to acquire an RNase III site.

The scenario that IVSs evolved to acquire an RNase III cleavage site due to negative selective pressure exerted by the IVS on the ribosome, or that IVSs are natural substrates for RNase III cleavage, would both be consistent with RNase III excision of IVSs as not conferring a selective advantage over other cells that do not contain IVSs. RNase III excision of IVSs would only be advantageous insomuch as it would remove the potential disadvantage of leaving IVSs in the ribosome. This would allow IVSs to be sporadically gained or lost without affecting cell viability.

c) Gene conversion of IVSs

Gene conversion of IVSs would account for their ability to be gained or lost, as well as for their sequence conservation among different genera and species. When a single cell of a species contains rrl genes with and without IVSs, as does S. typhimurium LT2, gene conversion of one rrl gene by another could proceed by either an IVS-containing or non IVS-containing template; this could result in the gain or loss of an IVS, respectively. Sequence conservation of IVSs can also be attributed to gene conversion. When the number of IVS containing rrl genes in a cell is greater than those that do not carry IVSs, I predict that more conversion events will involve IVS-containing templates. Therefore, there will be a tendency for IVS-containing rrl genes to convert each other, and thereby homogenize and maintain IVS sequence identity. This is observed

in the extreme for S. typhi Ty2, which contains identical helix-25 IVSs in all seven rrl genes.

TABLE 1. Selected strains used in this study.

Strain designation	Species	Description	Locality	Source
HT115	*E. coli* K-12	*rnc-14*::ΔTn*10*, derived from strain W3110	Lab strain	(Takiff et al., 1989)
SAB4793	*E. coli* K-12	(del)*lacX74* *trp*(-) *sup*(+) lambda(+) / F42 *finP301* *lac*(+) (EDFL51)/pPR1347 (*rfb*$^+$ *rfc*$^+$)	Lab strain	(Neal et al., 1993)
SAB5300	*E. coli* K-12	*rnc-14*::ΔTn*10*/pPR1347 (*rfb*$^+$ *rfc*$^+$)	Lab strain	This study
SA1332[a]	*E. coli* K-12	Wild-type	Lab strain	SGSC
W3110	*E. coli* K-12	LAM(-) Inversion (*rrnD*-*rrnE*) *rph-1*	Lab strain	J. Lederberg
1196-74	*S. typhi*	Wild-type	Mexico	(Reeves et al., 1989)
1707-81	*S. typhi*	Wild-type	Liberia	(Reeves et al., 1989)
25T-35	*S. typhi*	Wild-type	Canada	R. Khakhria, LCDC[b]

Strain designation	Species	Description	Locality	Source
25T-36	*S. typhi*	Wild-type	Canada	R. Khakhria, LCDC[b]
25T-37	*S. typhi*	Wild-type	Canada	R. Khakhria, LCDC[b]
25T-38	*S. typhi*	Wild-type	Canada	R. Khakhria, LCDC[b]
25T-39	*S. typhi*	Wild-type	Canada	R. Khakhria, LCDC[b]
25T-40	*S. typhi*	Wild-type	Canada	R. Khakhria, LCDC[b]
25T-41	*S. typhi*	Wild-type	Canada	R. Khakhria, LCDC[b]
3137-78	*S. typhi*	Wild-type	India	(Reeves et al., 1989)
3815-73	*S. typhi*	Wild-type	unknowm	(Reeves et al., 1989)
383-82	*S. typhi*	Wild-type	Marshal Islands	(Reeves et al., 1989)

Strain designation	Species	Description	Locality	Source
415Ty	S. typhi	Wild-type	Netherlands	(Edwards and Stocker, 1988)
417Ty	S. typhi	Wild-type	New-Caledonia	(Edwards and Stocker, 1988)
9032-85	S. typhi	Wild-type	Taiwan	(Reeves et al., 1989)
9228-77	S. typhi	Wild-type	El Salvador	(Reeves et al., 1989)
H238.1	S. typhi	aroC1019, derived from strain ISP-1820	Chile	(Hone et al., 1991)
H251.1[c]	S. typhi	aroC1019, derived from strain Ty2	USSR	(Hone et al., 1991)
In14	S. typhi	Wild-type	Indonesia	T. Pang
In15	S. typhi	Wild-type	Indonesia	T. Pang
IP E.88.353	S. typhi	Wild-type	Dakar	(Boyd et al., 1993)
IP E.88.374	S. typhi	Wild-type	Dakar	(Boyd et al., 1993)

Strain designation	Species	Description	Locality	Source
ISP-1820	*S. typhi*	Wild-type	Chile	(Hone et al., 1991)
SA4828	*S. typhi*	Wild-type	Canada	C. Anand, Alberta Provincial Lab
ST1002	*S. typhi*	Wild-type	Malaysia	T. Pang
ST143	*S. typhi*	Wild-type	Malaysia	T. Pang
ST145	*S. typhi*	Wild-type	Malaysia	T. Pang
ST168	*S. typhi*	Wild-type	Malaysia	T. Pang
ST24	*S. typhi*	Wild-type	Malaysia	T. Pang
ST495	*S. typhi*	Wild-type	Malaysia	T. Pang
ST60	*S. typhi*	Wild-type	Malaysia	T. Pang
Ty2 LCDC	*S. typhi*	Wild-type, independent isolate of strain Ty2	USSR[e]	R. Khakhria, LCDC[b]
Ty21a	*S. typhi*	Wild-type, independent isolate of strain Ty2	USSR[e]	B. Stocker
Ty2	*S. typhi*	Wild-type, independent isolate of strain Ty2	USSR[e]	(Hone et al., 1991)

Strain designation	Species	Description	Locality	Source
Ty514[d]	S. typhi	Wild-type, independent isolate of strain Ty2	USSR[e]	(Edwards and Stocker, 1988)
EB360	S. typhimurium	wild-type isolate of strain LT2	Lab strain	E. Barrett
LB5010	S. typhimurium	metA22 metE551 ilv-452 leu-3121 trpC2 xyl-404 galE856 hsdL6 hsdSA29 hsdSB121 rpsL120 H1-b H2-e,n,x fla-66 nml(-) Fel-2(-)	Lab strain	(Bullas and Ryu, 1983)
LT2A	S. typhimurium	wild-type isolate of strain LT2	Lab strain	B. Ames
SA4254	S. typhimurium	wild-type isolate of strain LT2	Lab strain	C. Higgins
SA3856	S. typhimurium	metA22 trpC2 metE551 xyl-404 H1-b H2-e,n,x fla-66 (cured of Fels2) rpsL120 hsdL6 hsdSA29 ilv-452 galE496 mutL-111::Tn10	Lab strain	(Rayssiguier et al., 1989)

Strain designation	Species	Description	Locality	Source
SA5301	S. typhimurium	metA22 trpC2 metE551 xyl-404 H1-b H2-e,n,x fla-66 (cured of Fels2) rpsL120 hsdL6 hsdSA29 ilv-452 galE496 ΔmutL	Lab strain	This study
SA5302	S. typhimurium	metA22 trpC2 metE551 xyl-404 H1-b H2-e,n,x fla-66 (cured of Fels2) rpsL120 hsdL6 hsdSA29 ilv-452 galE496 ΔmutL rnc-14::ΔTn10	Lab strain	This study
SA5303	S. typhimurium	rnc-14::ΔTn10	Lab strain	This study
SA5304	S. typhimurium	rnc-14::ΔTn10/pACS21 (rnc^+)	Lab strain	This study
SARA1	S. typhimurium	Wild-type, electrophoretic type 1	Mexico	(Beltran et al., 1991)
SARA2[d]	S. typhimurium	Wild-type, electrophoretic type 1, strain LT2	Lab strain	(Liu et al., 1991)
SARA3	S. typhimurium	Wild-type, electrophoretic type 1	Rhode Island	(Beltran et al., 1991)

Strain designation	Species	Description	Locality	Source
SARA4	S. typhimurium	Wild-type, electrophoretic type 1	Indiana	(Beltran et al., 1991)
SARA5	S. typhimurium	Wild-type, electrophoretic type 1	Mongolia	(Beltran et al., 1991)
SARA6	S. typhimurium	Wild-type, electrophoretic type 2	Ohio	(Beltran et al., 1991)
SARA7	S. typhimurium	Wild-type, electrophoretic type 3	Norway	(Beltran et al., 1991)
SARA8	S. typhimurium	Wild-type, electrophoretic type 5	Finland	(Beltran et al., 1991)
SARA9	S. typhimurium	Wild-type, electrophoretic type 7	California	(Beltran et al., 1991)
SARA10	S. typhimurium	Wild-type, electrophoretic type 9	California	(Beltran et al., 1991)
SARA11	S. typhimurium	Wild-type, electrophoretic type 10	Thialand	(Beltran et al., 1991)
SARA12	S. typhimurium	Wild-type, electrophoretic type 11	Louisiana	(Beltran et al., 1991)

Strain designation	Species	Description	Locality	Source
SARA13	S. typhimurium	Wild-type, electrophoretic type 12	France	(Beltran et al., 1991)
SARA14	S. typhimurium	Wild-type, electrophoretic type 13	Panama	(Beltran et al., 1991)
SARA15	S. typhimurium	Wild-type, electrophoretic type 14	Texas	(Beltran et al., 1991)
SARA16	S. typhimurium	Wild-type, electrophoretic type 15	North Carolina	(Beltran et al., 1991)
SARA17	S. typhimurium	Wild-type, electrophoretic type 16	Yugoslavia	(Beltran et al., 1991)
SARA18	S. typhimurium	Wild-type, electrophoretic type 17	Iowa	(Beltran et al., 1991)
SARA19	S. typhimurium	Wild-type, electrophoretic type 21	Mexico	(Beltran et al., 1991)
SARA20	S. typhimurium	Wild-type, electrophoretic type 22	France	(Beltran et al., 1991)
SARA21	S. typhimurium	Wild-type, electrophoretic type 23	Oregon	(Beltran et al., 1991)

Strain designation	Species	Description	Locality	Source
SL937	S. typhimurium	wild-type isolate of strain LT2	Lab strain	B. Stocker
TN3618	S. typhimurium	wild-type isolate of strain LT2	Lab strain	L. Miller

[a]This E. coli K-12 strain was used as the wild-type reference strain, unless otherwise noted.

[b]LCDC, Laboratory Centre for Disease Control, Ottawa.

[c]S. typhi H251.1 was used as the S. typhi reference strain.

[d]S. typhimurium wild-type strain LT2 is from a set of 22 S. typhimurium strains isolated by Lilleengen (Liu et al., 1991); this isolate has been substituted for SARA2 and used as the S. typhimurium LT2 wild-type reference strain.

[e]USSR, countries of the former Soviet Union.

Table 2. Genomic cleavage fragments containing *S. typhimurium* LT2 *rrl* genes.[a]

rrl gene locus	Cleavage enzyme	Fragment Size (kb)
A[b]	I-*Ceu*I	92
B[b]	I-*Ceu*I	145
C[b]	*Bln*I	90
D[c]	*Spe*I	407
E[b]	I-*Ceu*I	44
G[c]	*Spe*I	226
H[b,d]	*Bln*I	1580

[a]Separation of fragments by PFGE was as described (Liu et al., 1993b).

[b]From *S. typhimurium* LT2 genomic cleavage map for *Bln*I and I-*Ceu*I (Liu et al., 1993b).

[c]From *S. typhimurium* LT2 genomic cleavage map for *Spe*I (S.-L. Liu, Personal Communication).

[d]Note that the *Bln*I 1580-kb fragment contains both *rrlE* and *rrlH*, however, a partial *rrl* gene of *rrnH* was retrieved through the use of amplicon D (see Figure 7).

TABLE 3. Genomic cleavage fragments containing *S. typhi* Ty2 *rrl* genes[a,b]

rrl gene locus[c]	Cleavage enzyme	Fragment Size (kb)
A	I-*Ceu*I	146
B	I-*Ceu*I	44
C[d]	I-*Ceu*I/*Spe*I	159
D[d]	I-*Ceu*I/*Spe*I	343
E	I-*Ceu*I	136
G	I-*Ceu*I	828
H	I-*Ceu*I	724

[a]Separation of fragments by PFGE was as described (Liu and Sanderson, 1992).

[b]From *S. typhi* Ty2 genomic cleavage map (Liu and Sanderson, 1995).

[c]Although each *rrl* gene is denoted by a single letter, the *S. typhi* Ty2 genomic cleavage map (Liu and Sanderson, 1995) indicates some of the genes as hybrids to represent postulated genomic recombination events. I have substituted the hybrid *rrl* gene letters with single gene letters corresponding to their order on the *S. typhimurium* LT2 genome (Liu et al., 1993b).

[d]*rrlC* and *rrlD* were co-purified on the I-*Ceu*I 502-kb fragment. This fragments was restricted at a single *Spe*I site and separated by PFGE into the fragment sizes shown.

TABLE 4. Comparison of helix-25 IVSs.[a]

Helix-25 IVS source	% Nucleotide sequence identity with helix-25 IVS of:			
	S. arizonae	*S. typhimurium* LT2 *rrlG*	*S. typhimurium* LT2 *rrlH*	*S. typhi* Ty2
S. arizonae	-			
S. typhimurium LT2 *rrlG*	56	-		
S. typhimurium LT2 *rrlH*	94	56	-	
S. typhi Ty2	94	55	97	-

[a]GCG GAP was used to generate identity comparisons.

Table 5. Comparisons of helix-45 IVSs.[a]

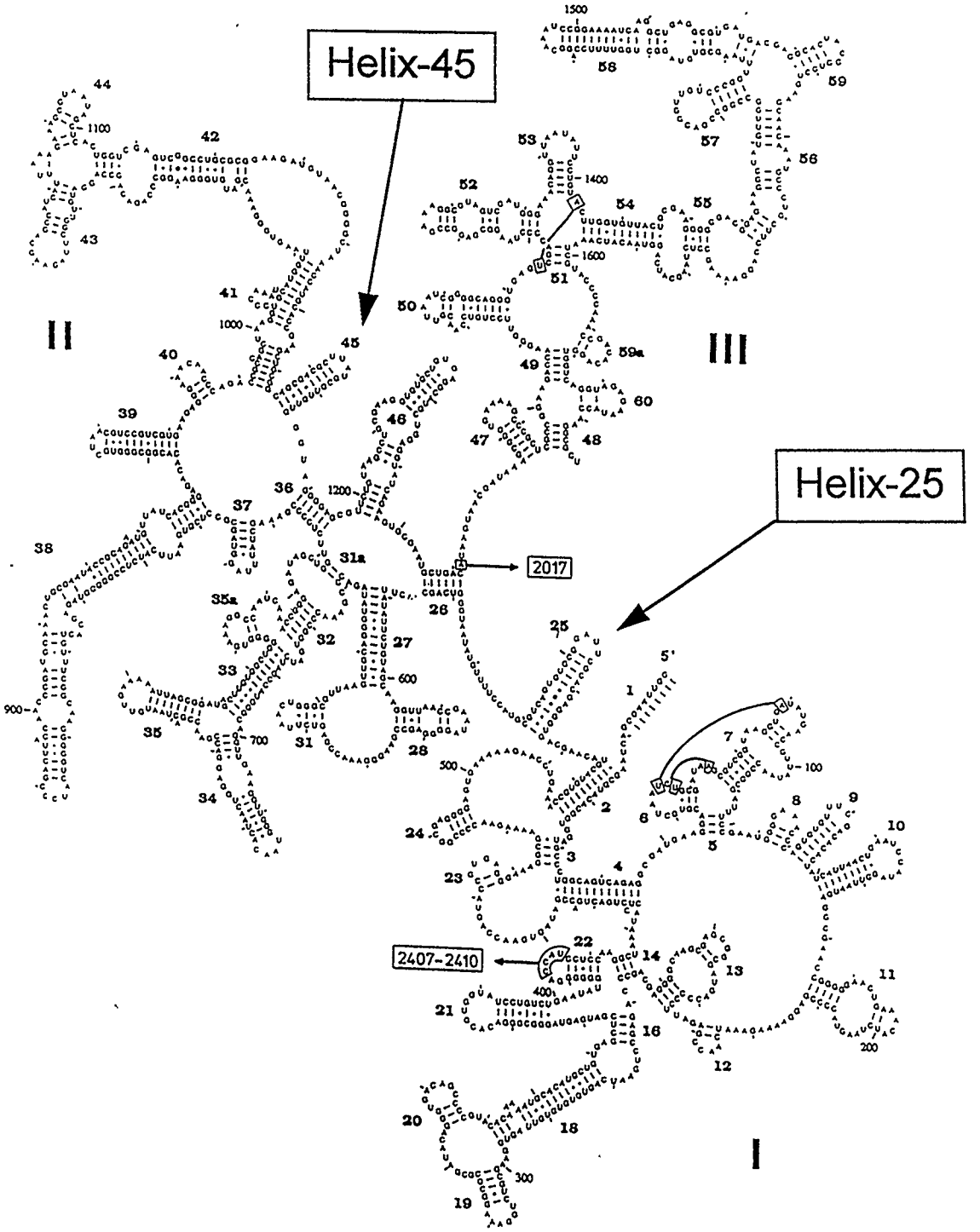
Helix-45 IVS source	% Nucleotide sequence identity with helix-45 IVS of:			
	S. arizonae	*S. typhimurium* LT2	*Y. enterocolitica* Group 1	*Y. enterocolitica* Group 2
S. arizonae	-			
S. typhimurium LT2	85	-		
Y. enterocolitica Group 1	66	61	-	
Y. enterocolitica Group 2	83	87	59	-

[a]GCG GAP was used to align sequences for identity comparisons.

Figure 1. 23S rRNA structural model showing the location of the six primary domains (I to VI), helix-25, and helix-45.

This 23S rRNA model, which is from Egebjerg et al. (1990), has evolved through several refinements, the most recent of which was derived from Hopfl et al. (1989). The six domains of the 23S rRNA, which are defined by long-range double helices, are indicated (I to IV). Base pairs that are supported by phylogenetic comparison, or base pairs within putative helices for which there is no evidence against their existence are joined by a line or a dot; the latter represent guanosine-uridine base pairings. Helices have been numbered according to the Desulfurococcus mobilis 23S rRNA model (Leffers et al., 1987). Every 10th nucleotide from the 5′ end is indicated with a short line; every 50th nucleotide is indicated with a longer line. **A.** 23S rRNA folded from base 1 to base 1647 is shown. Helix-25, in domain I, and helix-45, in domain II, that can contain IVSs are labeled with an arrow. **B.** 23S rRNA folded from base 1648 to base 2904 is shown. The rRNA fold was created with EDSTRUC (N. Larson, unpublished data).

A



B

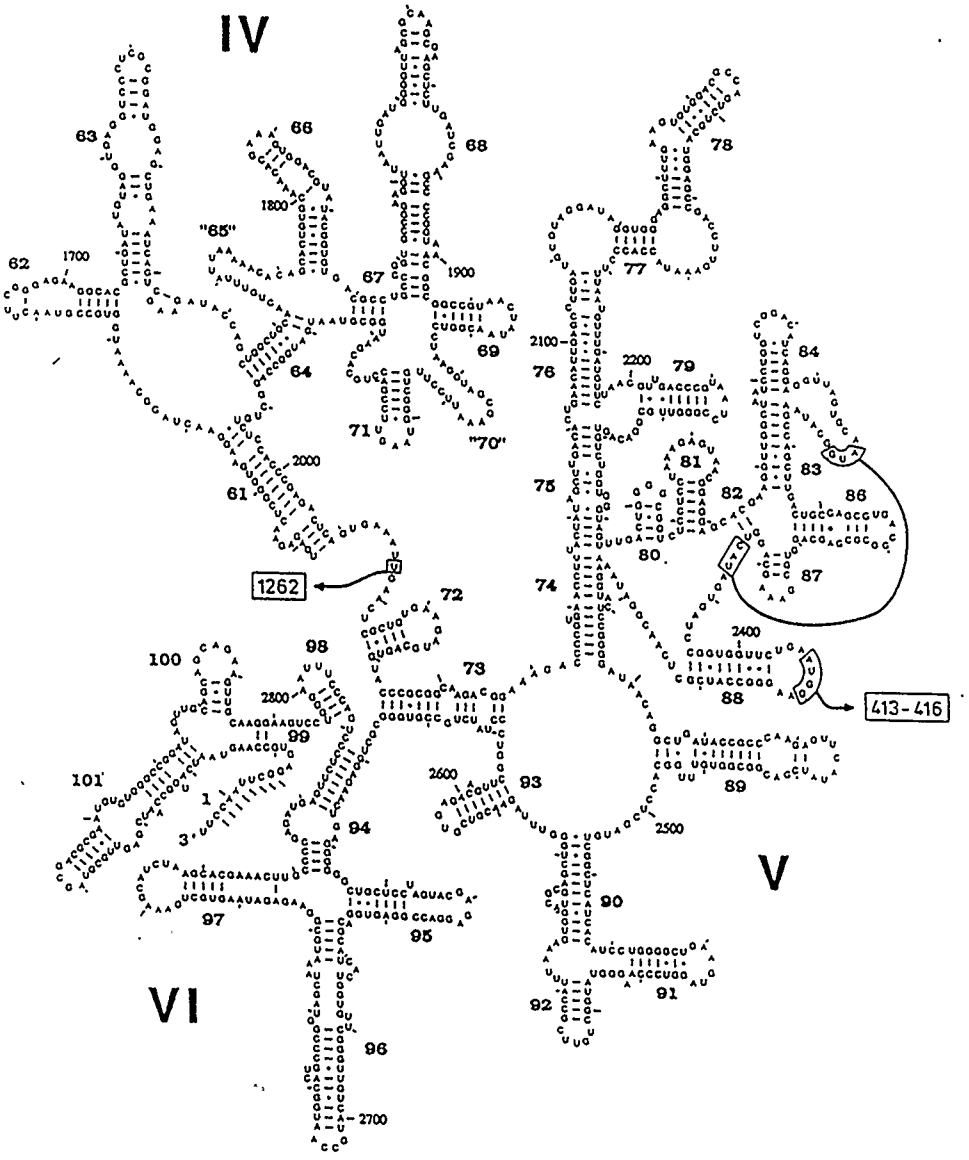


Figure 2. Primary processing sites within the 30S rRNA.

A typical 30S rRNA transcribed from an rrn operon is shown with 16S, 23S, and 5S rRNAs, in addition to a spacer tRNA, of which there may be one or two, and a distal tRNA (shown in brackets), which is not present in all rrn operons. The enzyme responsible for primary processing of the nascent 30S rRNA in E. coli (Dune, 1976; Robertson, 1982) and S. typhimurium (Suryanarayana and Burma, 1975), is RNase III. A consensus for RNase III cleavage has not been reported, but it tends to produce single stranded cuts next to RNA bulges or mispairs in double stranded regions of 20-30 bp (Robertson, 1982). Two double-stranded stems loop out the 16S and 23S rRNAs as shown, and each strand of each stem is cut by RNase III (1a, 1b, 4a, and 4b) to release precursor rRNAs. The 5S rRNA is processed by RNase E to release the precursor 5S rRNA (5a and 5b) (Roy et al., 1983). Primary processing of spacer and distal tRNAs uses RNase P at the 5′ end (2 and 6), and an uncharacterized 3′ endonuclease (3 and 7) (King et al., 1986).

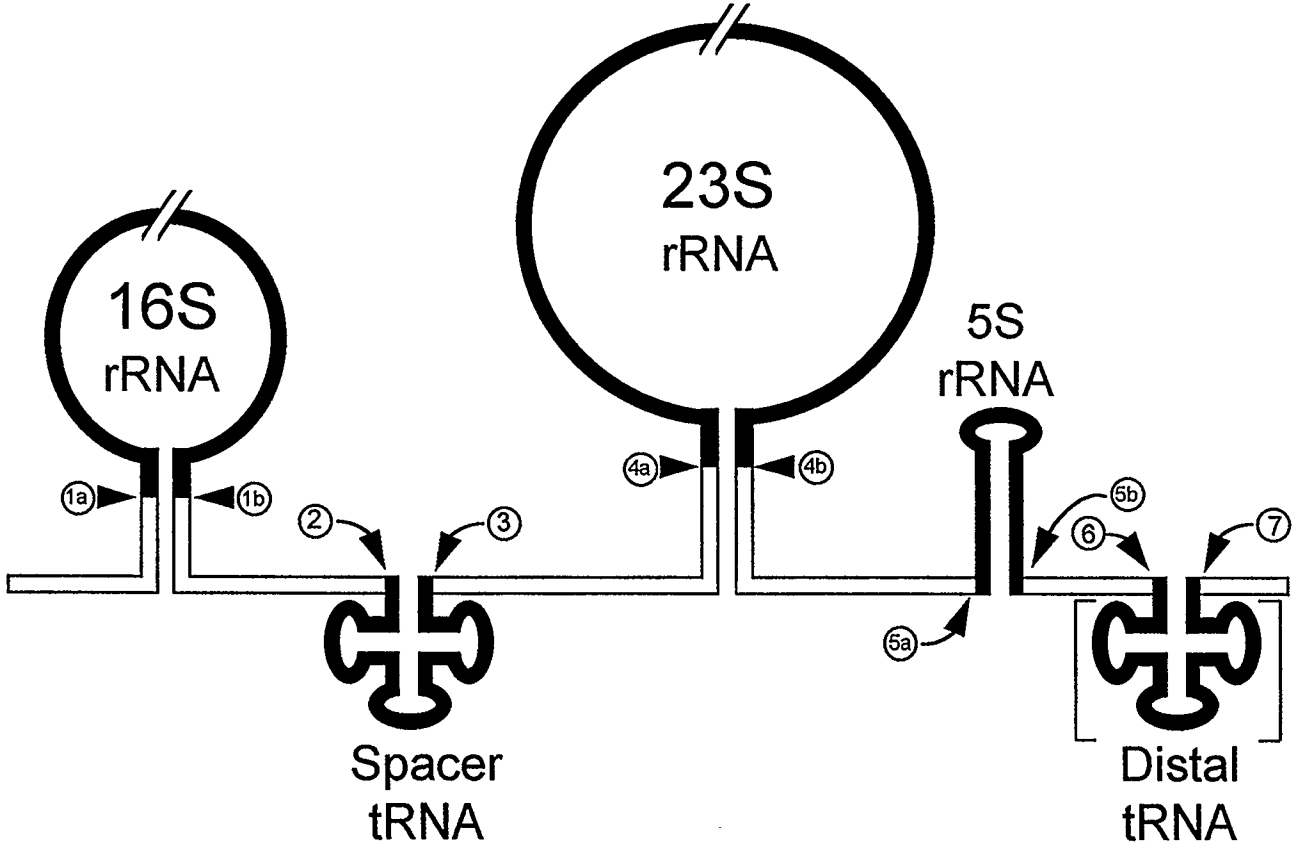

16S
rRNA
23S
rRNA
5S
rRNA
1a
1b
2
3
4a
4b
5a
5b
6
7
Spacer
tRNA
Distal
tRNA

Figure 3. RNase III processing of rRNA and IVSs.

RNase III releases precursor 16S and 23S rRNA from the 30S transcript. It is also responsible for IVS excision from helix-25 and helix-45 of the 23S rRNA. All possible points of RNase III cleavage are shown. Depending on the presence of a helix-25 IVS and/or a helix-45 IVS, three different modes of 23S rRNA cleavage are possible. **A.** If only a helix-25 IVS is present (about bp 550 in rrl) then 0.5- and 2.4-kb 23S rRNA fragments are formed. **B.** If only a helix-45 IVS is present (about bp 1170 in rrl) then 1.2- and 1.7-kb 23S rRNA fragments are formed. **C.** If both helix-25 and helix-45 are present in an rrl gene then 0.5-, 0.7-, and 1.7-kb 23S rRNA fragments are formed.

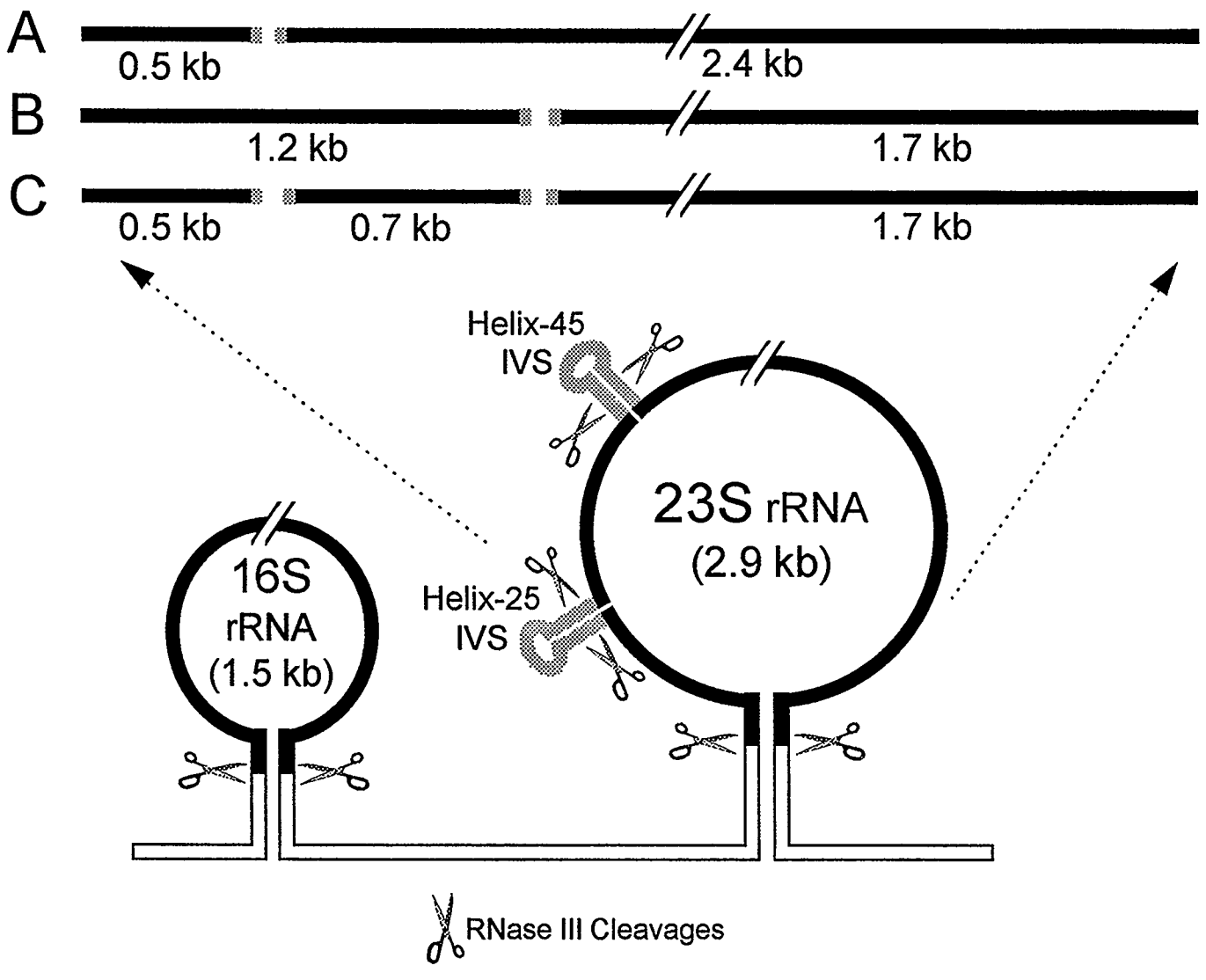

A
0.5 kb
2.4 kb
B
1.2 kb
1.7 kb
C
0.5 kb
0.7 kb
1.7 kb
Helix-45
IVS
23S rRNA
(2.9 kb)
16S
rRNA
(1.5 kb)
Helix-25
IVS
RNase III Cleavages

Figure 4. Schematic of plasmid pACS21 and cosmid pPR1347.

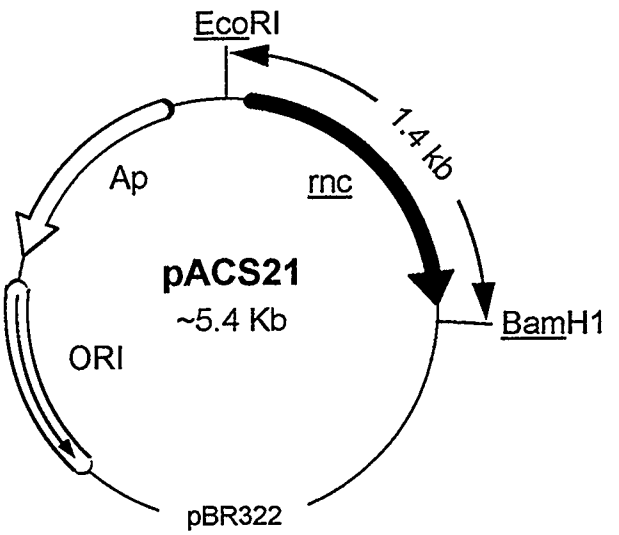
A. pACS21

Plasmid pACS21 is a subclone of the plasmid pACS1, a plasmid pBR322 derivative, which contains the entire rnc operon for RNase III, Era, and RecO synthesis (Takiff et al., 1989). pACS21 contains a 1.4-kb EcoRI - BamHI fragment that contains the rnc gene. Plasmid selection is for ampicillin resistance (Ap).

B. pPR1347

Cosmid pPR1347 contains the rfb gene cluster and rfc, from S. typhimurium. This cosmid allows for the mobilization of ability to synthesize the O12 somatic antigen used as the receptor for phage P22. The construction of pPR1347 has been reported (Neal et al., 1993). Cosmid selection is for kanamycin (Kan).

A


EcoRI
1.4 kb
Ap
rnc
pACS21
~5.4 Kb
ORI
BamH1
pBR322

B

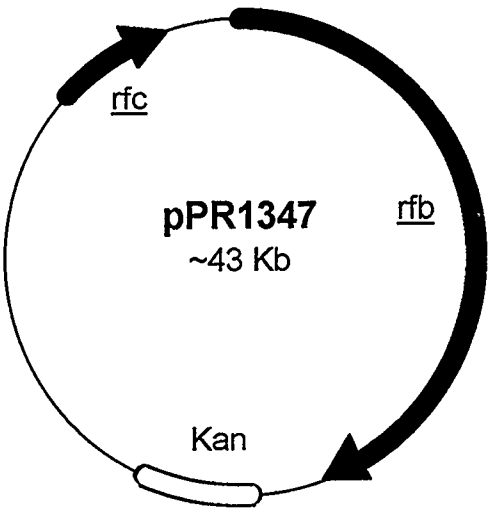

rfc
pPR1347
~43 Kb
rfb
Kan

Figure 5. Genomic cleavage map of S. typhimurium LT2 for XbaI, BlnI, and I-CeuI (Liu et al., 1993b).

Cleavage sites for each enzyme are indicated around the S. typhimurium LT2 chromosome. Each fragment, resulting from each enzyme cleavage, is lettered in a clockwise manner. The location of the seven rrn operons is shown and expanded to greater detail around the outer surface of the chromosome. Each rrn operon detailed map shows the direction of transcription and the location of cleavage sites within each gene in the operon (refer to Figure 2 for a description of the rrn operon, and the genes it contains). The known location of selected genes, derived by Tn10 mapping, are indicated. The chromosome itself is measured on the outer surface in centisomes (1 cs equals about 48.1 kb), and on the inner surface in kb.

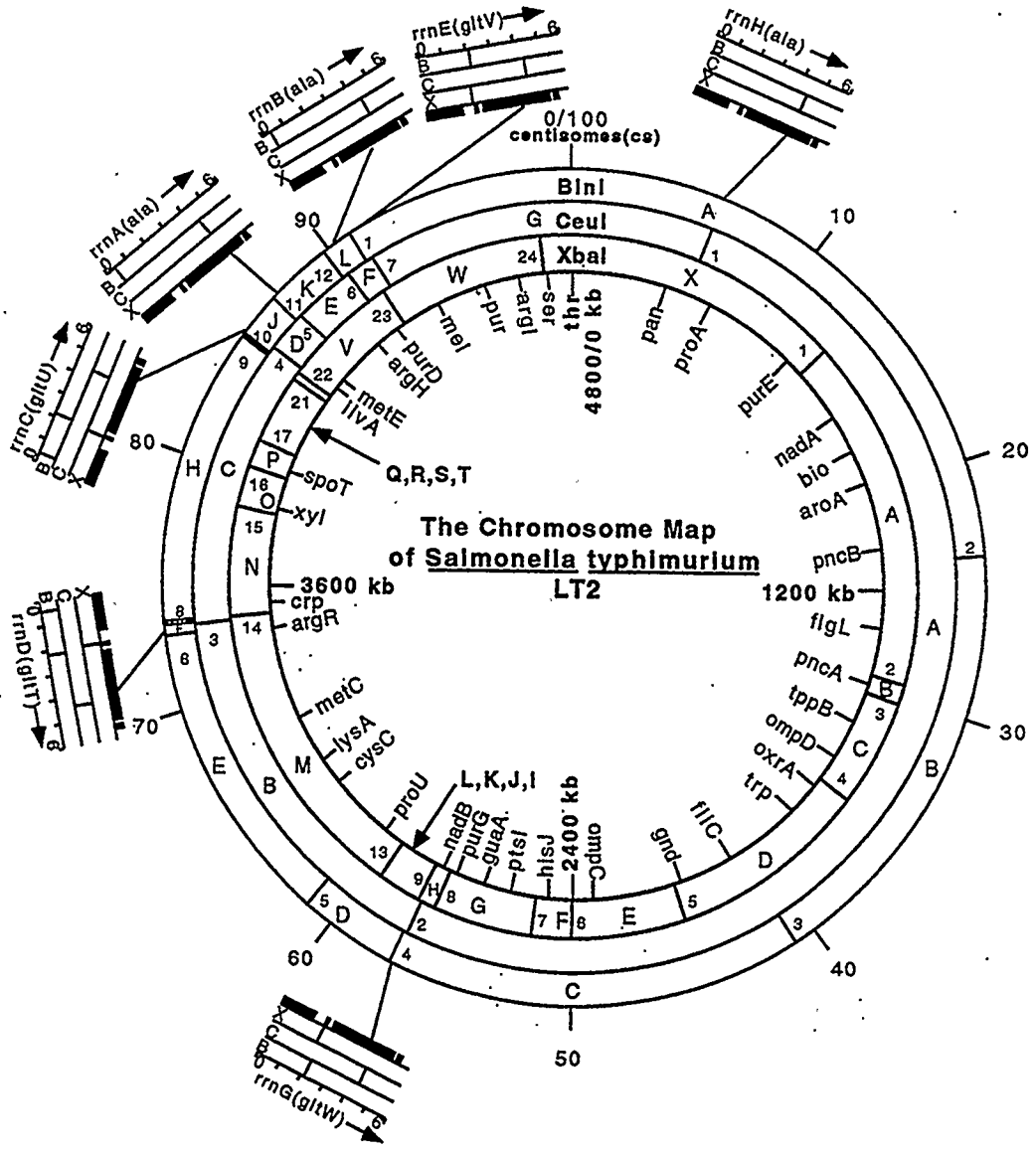

The Chromosome Map
of Salmonella typhimurium
LT2
0/100
centisomes(cs)
BlnI
CeuI
XbaI
4800/0 kb
2400 kb
1200 kb
3600 kb
rrnE(gltV)
rrnB(ala)
rrnH(ala)
rrnA(ala)
rrnC(gltU)
rrnD(gltT)
rrnG(gltW)
Q,R,S,T
L,K,J,I
thr
ser
argI
pur
mel
purD
argH
metE
ilvA
spoT
xyl
crp
argR
metC
lysA
cysC
proU
nadB
purG
guaA
ptsI
hisJ
ompC
gnd
fliC
trp
oxrA
ompD
tppB
pncA
flgL
pncB
aroA
bio
nadA
purE
proA
pan

Figure 6. Genomic cleavage map of S. typhi Ty2 for XbaI, BlnI, I-CeuI, and SpeI (Liu and Sanderson, 1995).

Cleavage sites for each enzyme are indicated around the S. typhi Ty2 chromosome. Each fragment, resulting from each enzyme cleavage, is lettered in a clockwise manner. The location of the seven rrn operons is shown and expanded to greater detail around the outer surface of the chromosome. Each rrn operon detailed map shows the direction of transcription and the location of cleavage sites within each gene in the operon (refer to Figure 2 for a description of the rrn operon, and the genes it contains). The known location of selected genes, derived by Tn10 mapping, are indicated. The chromosome itself is measured on the outer surface in centisomes (1 cs equals about 48.1 kb), and on the inner surface in kb.

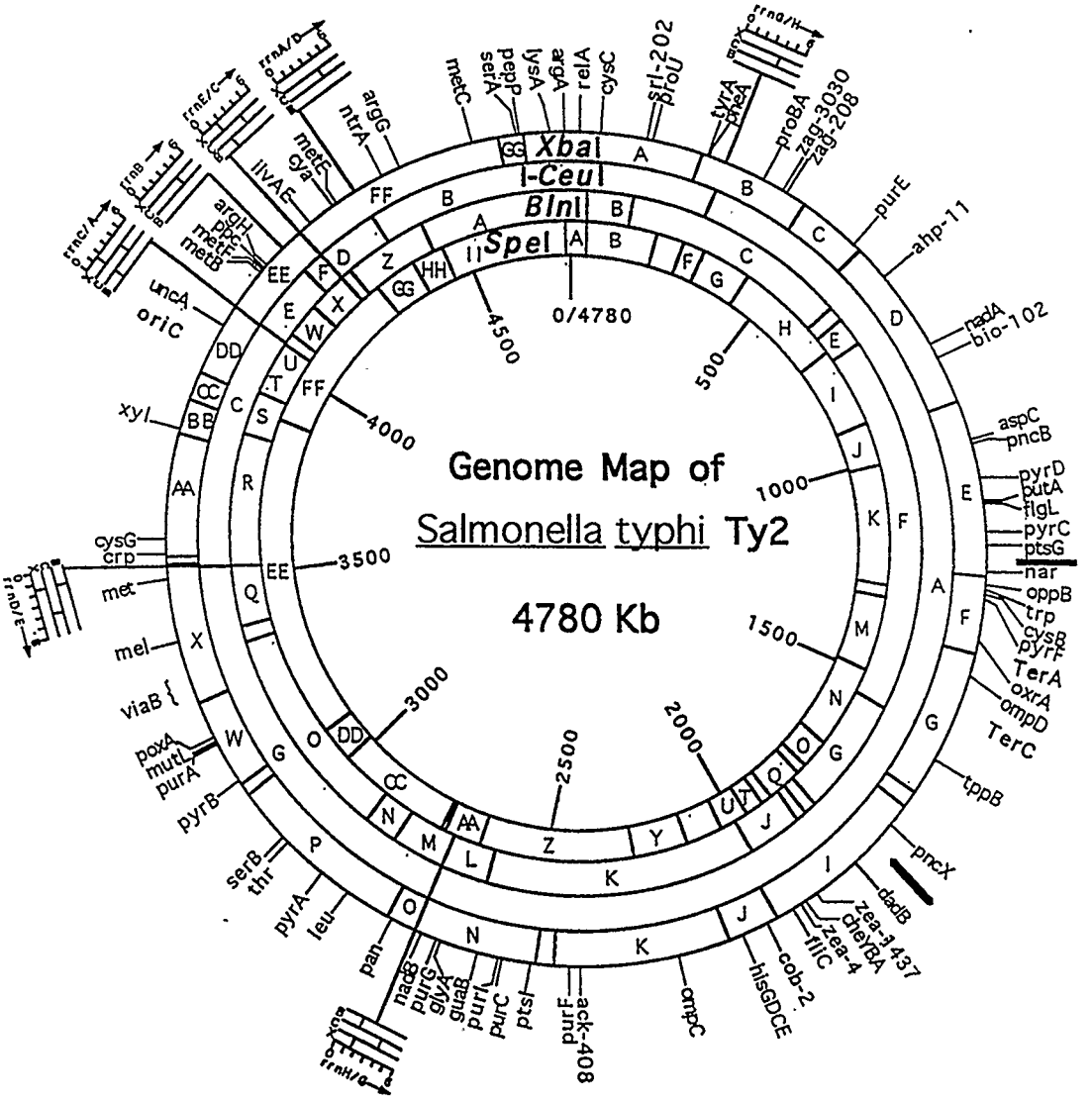

Genome Map of
Salmonella typhi Ty2
4780 Kb
XbaI
I-CeuI
BlnI
SpeI
0/4780
500
1000
1500
2000
2500
3000
3500
4000
4500
oriC
terA
terC
dadB
pncX
tppB
ompD
oxrA
TerA
pyrF
cysB
trp
oppB
nar
ptsG
flgL
butA
pyrD
aspC
pncB
bio-102
nadA
ahp-11
purE
cob-2
hisGDCE
ampC
ack-408
purF
ptsI
purC
purI
guaB
glyA
purG
nadB
pan
leu
pyrA
thr
serB
pyrB
purA
mutL
poxA
viaB
mel
met
crp
cysG
xyl
uncA
cyaA
metE
ntrA
argG
metC
serA
pepP
lysA
argA
relA
cysC
srl-202
proU
tyrA
pheA
proBA
zag-3030
zag-208
rrnA/D
rrnE/C
rrnB
rrnC/A
rrnG/H
rrnH/G
rrnE/B

Figure 7. Schematic of *rrl* PCR strategy.

One *rrn* operon determines one 16S rRNA (*rrs*), one or two tRNAs, one 23S rRNA (*rrl*), and one 5S rRNA (not shown). Considering several *E. coli rrn* operon sequence identities, four primer sets were constructed to PCR amplify amplicons A, B, C, and D. Amplicon A used primers P1 and P2; amplicon B used primers P3 and P4; amplicon C used primers P1 and P4; amplicon D used primers P5 and P4. Amplicon A (731 bp without IVS, according to *rrnB* gene sequence [Noller, 1984]) and B (733 bp without IVS) were sequencing templates for nested primers in the helix-25 and helix-45 regions, respectively. Amplicon C was used to determine IVSs in the whole genome. Restricting C with *Dra*I yielded two fragments from each *rrl* gene: the C1-fragment (1008 bp without IVS) reported the presence of the helix 25-IVS (about 110 bp) and the C2-fragment (551 bp without IVS) reported on the presence of the helix 45-IVS (about 90 bp). Therefore, the genomic distribution of IVSs among the seven *rrl* genes was determined from length and intensity variance of four fragments. Amplicon D (2560 bp without IVSs) was used to retrieve *rrlH* amplicons A and B from a mixed template; the rrl gene of *rrnE* was co-purified with *rrlH* on the *Bln*I-1580-kb fragment. Amplification of D required the 3′ end of the *rrs* (16S rRNA) gene, that effectively eliminated *rrlE* (incomplete *rrn* operon) but included *rrlH* (complete *rrn* operon). *rrlH* amplicon D was gel purified and isolated in the same manner as the PFGE fragments and used as a template for amplicons A and B.

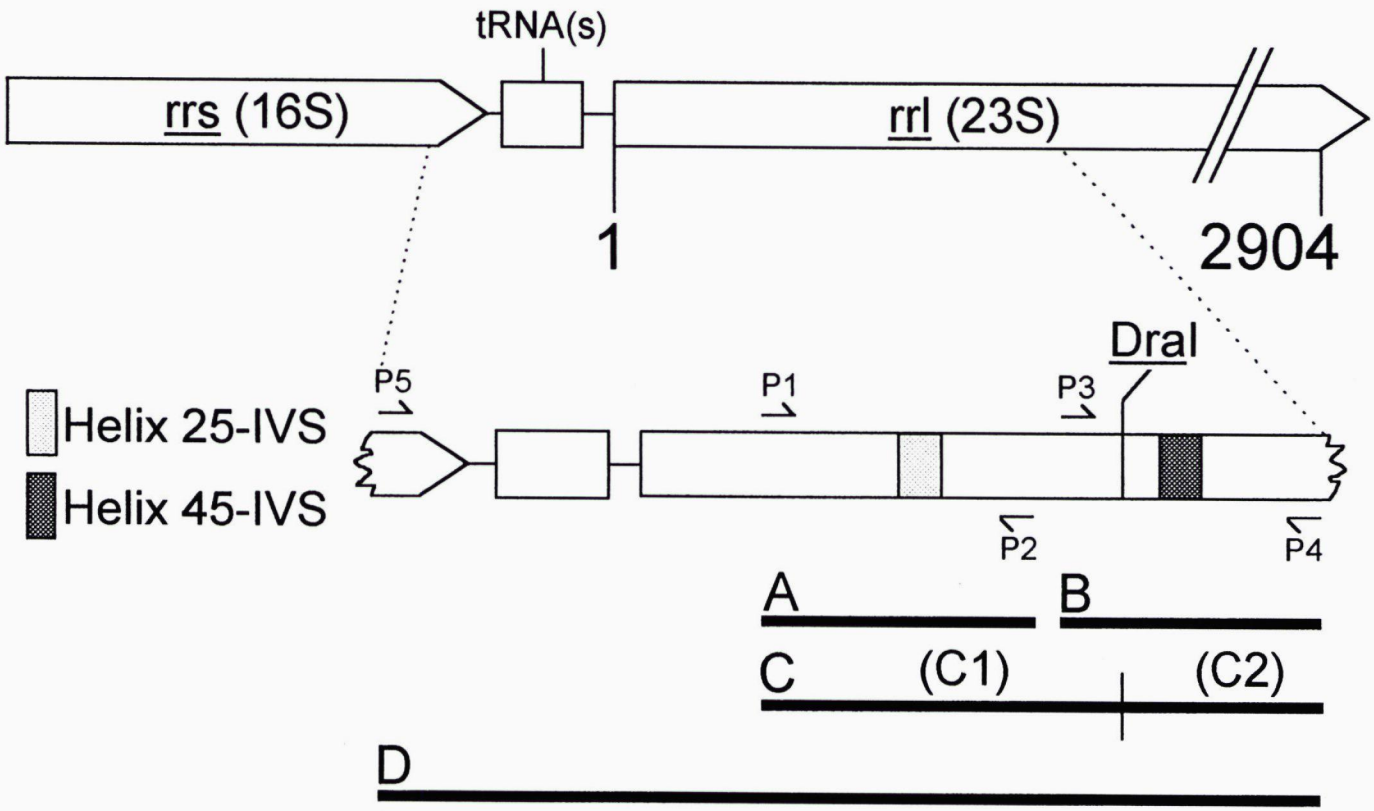

tRNA(s)
rrs (16S)
rrl (23S)
1
2904
DraI
P5
P1
P3
Helix 25-IVS
Helix 45-IVS
P2
P4
A
B
C
(C1)
(C2)
D

Figure 8. 23S rRNA fragmentation in S. typhimurium LT2.

A. 23S rRNA fragmentation in S. typhimurium LT2 (lane Tm) and E. coli (lane Ec) was observed by isolation of RNA, followed by glyoxal-dimethylsulfoxide (DMSO) 1.2% agarose gel electrophoresis of 10 ug of the RNA, blotting to Hybond-N+, and staining with methylene blue. At least one of the seven rrl genes of S. typhimurium contains an IVS in helix-25 only, as 2.4- and 0.5-kb fragments were present in comparison to the location of the E. coli 23S and 16S rRNAs. Multiple rrl genes contain an IVS in helix-45 only as 1.7- and 1.2-kb fragments were present, and at least one rrl gene has IVSs in both helices as 1.7-, 0.7-, and 0.5-kb fragments were present.

B. 23S rRNA fragmentation schematic. 23S rRNA fragments of 2.4- and 0.5-kb indicate that one rrl gene carries an IVS in helix-25 (about bp 550 in rrl). 23S rRNA fragments of 1.7- and 1.2-kb indicate that one rrl gene carries an IVS in helix-45 (about bp 1170 in rrl). 23S rRNA fragments of 1.7-, 0.7-, and 0.5-kb indicate that one rrl gene carries IVSs in both helices.

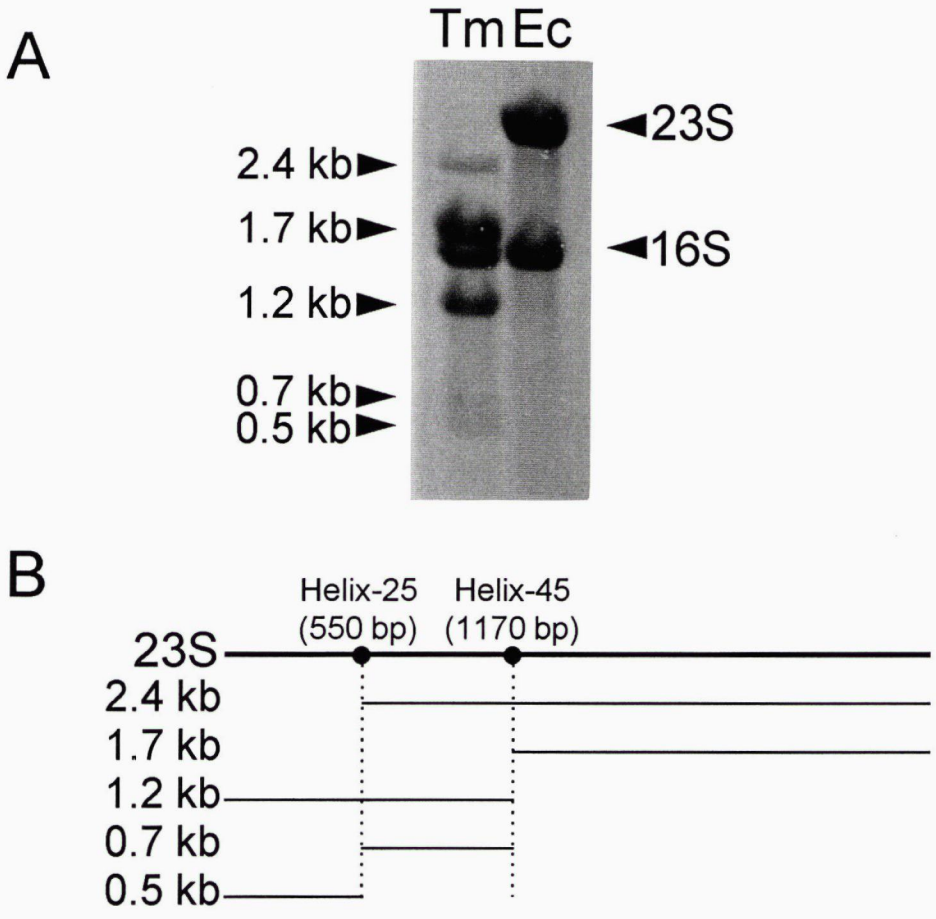
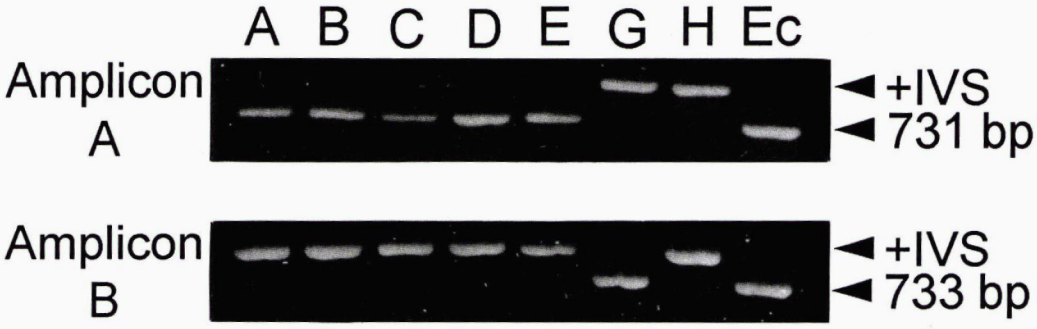

A
Tm Ec
23S
16S
2.4 kb
1.7 kb
1.2 kb
0.7 kb
0.5 kb
B
Helix-25
(550 bp)
Helix-45
(1170 bp)
23S
2.4 kb
1.7 kb
1.2 kb
0.7 kb
0.5 kb

Figure 9 Amplicon A and B isolated from each *rrl* gene from *S. typhimurium* LT2.

A. The DNA fragments resulting from amplification using the rrl genes (lanes A to H) of *S. typhimurium* LT2 as templates. Amplicon A contains helix-25 and B contains helix-45 (see Figure 7). Amplicons A and B each may be a shorter fragment (indicating no IVS in the template *rrl* gene) or a longer fragment (indicating an IVS). Lane Ec indicates that genomic DNA of *E. coli* K-12 was used as template. 731 bp is the size predicted for the *E. coli* amplicon A, and 733 bp for the amplicon B, based on sequence from *E. coli rrlB* (Noller, 1984).

B. The orientation and position of each *S. typhimurium* LT2 *rrl* gene on the chromosome (derived from ref [Noller, 1984]) and the type(s) of IVS(s) detected in each *rrl* gene. The approximate locations of the origin of replication (Ori) and terminus (Ter) are indicated.

A


A B C D E G H Ec
Amplicon A
+IVS
731 bp
Amplicon B
+IVS
733 bp

B

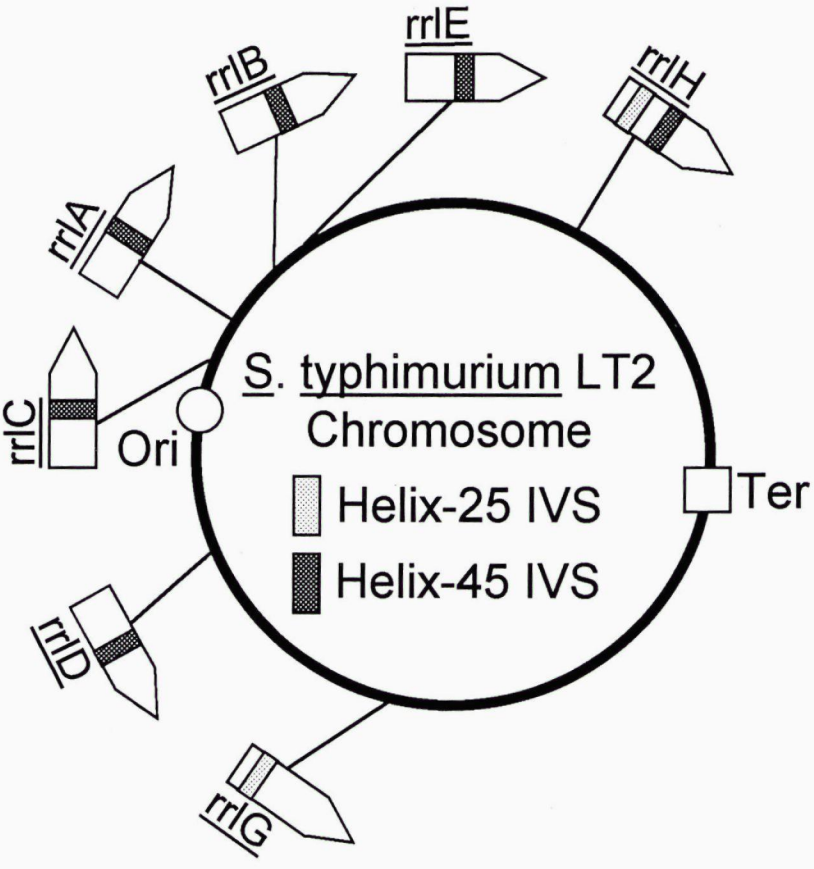
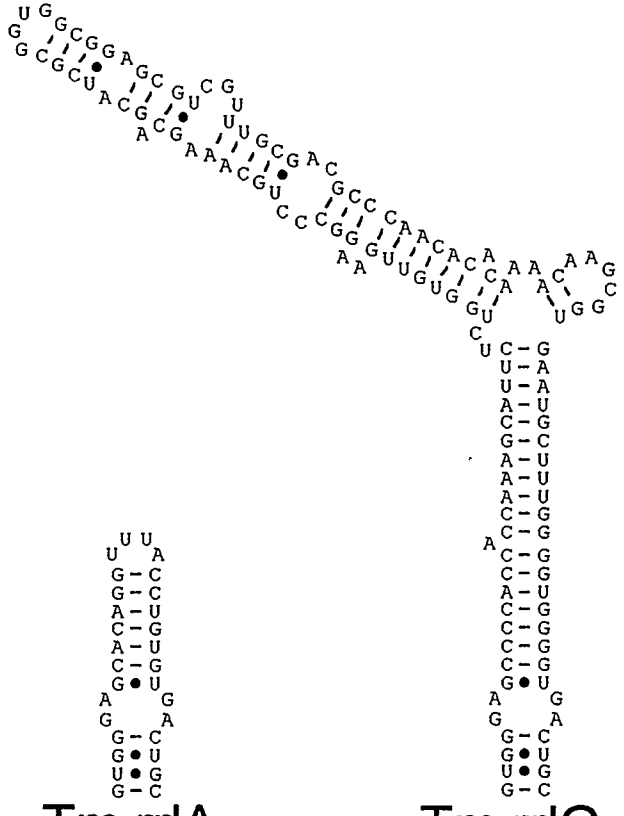

rrlE
rrlB
rrlH
rrlA
rrlC
Ori
S. typhimurium LT2
Chromosome
Helix-25 IVS
Helix-45 IVS
Ter
rrlD
rrlG

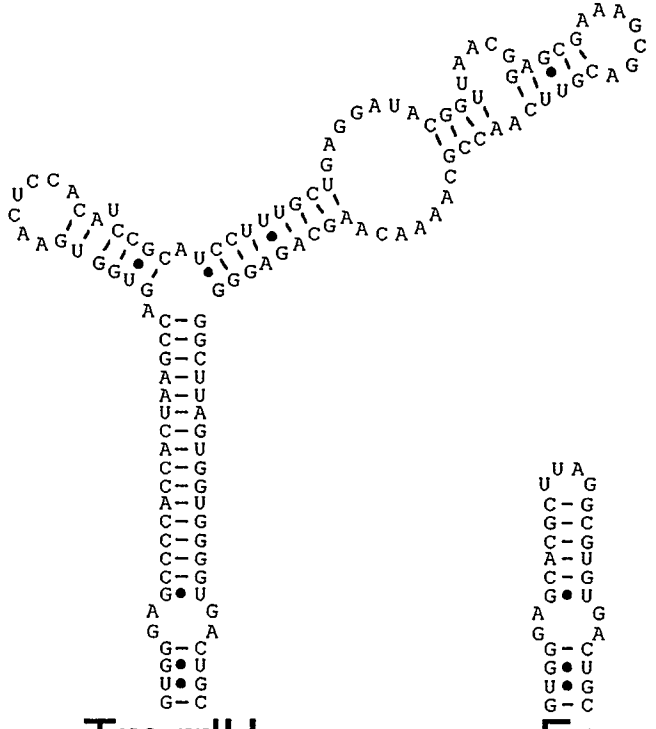
Figure 10. Potential RNA secondary structures of all helix-25 nucleotide sequences from S. typhimurium LT2.

S. typhimurium LT2 (lane Tm) rrl genes that are grouped together are 100% identical. E. coli (lane Ec) sequence is indicated for helix-25 of rrlB (Noller, 1984). The free energy calculated at 37°C for the rrlG helix-25 IVS was -54.5 kcal/mol, and for the rrlH helix-25 IVS was -52.2 kcal/mol. Filled circles represent guanosine-uridine base pairings. GenBank accession numbers: U43669, helix-25 rrlG IVS; U43670, helix-25 rrlH IVS.



Tm rrlA, B,C,D,E

Tm rrlG

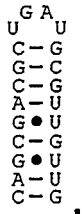


Tm rrlH

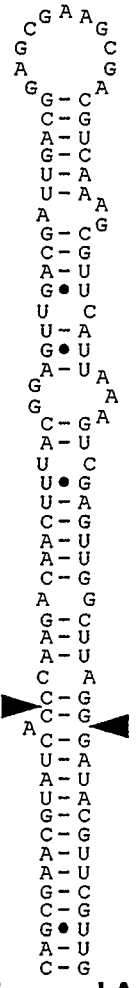
Ec

Figure 11. Potential RNA secondary structures of all helix-45 nucleotide sequences from S. typhimurium LT2.

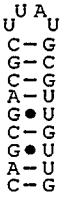
S. typhimurium LT2 (lane Tm) rrl genes that are grouped together are 100% identical. The RNase III helix-45 IVS cleavage sites, reported by Burgin et al. (1990), are shown by arrows. E. coli (lane Ec) sequence is indicated for helix-45 of rrlB (Noller, 1984). The free energy calculated at 37°C for the helix-45 IVS was -37.9 kcal/mol. Filled circles represent guanosine-uridine base pairings. GenBank accession numbers: U49921, helix-45 rrlA; U49922, helix-45 rrlB; U49923, helix-45 rrlC; U49924, helix-45 rrlD; U49925, helix-45 rrlE; U49926, helix-45 rrlH.



Tm rrlG



Tm rrlA, B, C, D, E, H



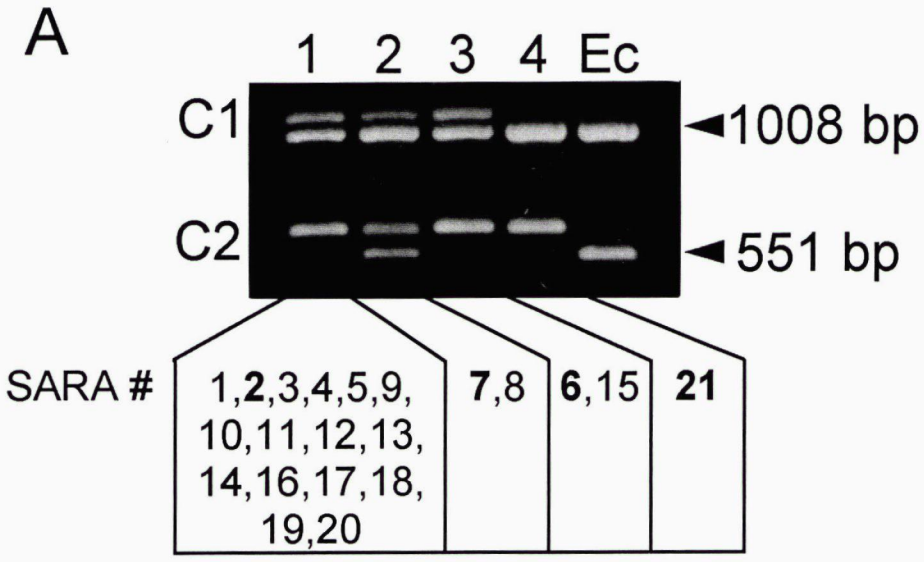
Ec

Figure 12. DraI restriction patterns observed for various S. typhimurium strains and S. typhimurium LT2 lines.

DraI restriction of amplicon C which was obtained using whole genomic DNA as template. PCR products were GlassMAX purified, quantified by spectrophotometer, then 600 ng was digested by DraI.

A. Amplification of genomic DNA of 21 S. typhimurium wild-type strains of SARA (Table 1); these can be separated to four visually distinct groups (1-4). The SARA set numbers in boldface were used as representatives of each group. SARA2 represents strain LT2. The restriction patterns were standardized using E. coli (lane Ec) genomic DNA as template: the C1-fragment (calculated to be 1008 bp according to rrnB gene sequence [Noller, 1984]) and the C2-fragment (551 bp).

B. Amplification of genomic DNA of seven different lines of S. typhimurium LT2 (lanes A to G) and E. coli K-12 (Ec). (A) SGSC 1412, the strain used as the wild-type of LT2 at the SGSC; stocked in 1955 as a lyophil, obtained at the SGSC in 1985 from J. Lederberg, stored since then in lyophil and at -70°C. (B) LB5010 (multiply auxotrophic, host-restriction-deficient) obtained from L. Bullas (Bullas and Ryu, 1983). (C) TN3618 wild-type obtained from L. Miller. (D) SA4254 wild-type obtained from C. Higgins. (E) SL937 wild-type obtained from B. Stocker. (F) LT2A wild-type obtained from B. Ames. (G) EB360 wild-type obtained from E. Barrett.


A
1 2 3 4 Ec
C1
C2
1008 bp
551 bp
SARA #
1,2,3,4,5,9,
10,11,12,13,
14,16,17,18,
19,20
7,8
6,15
21

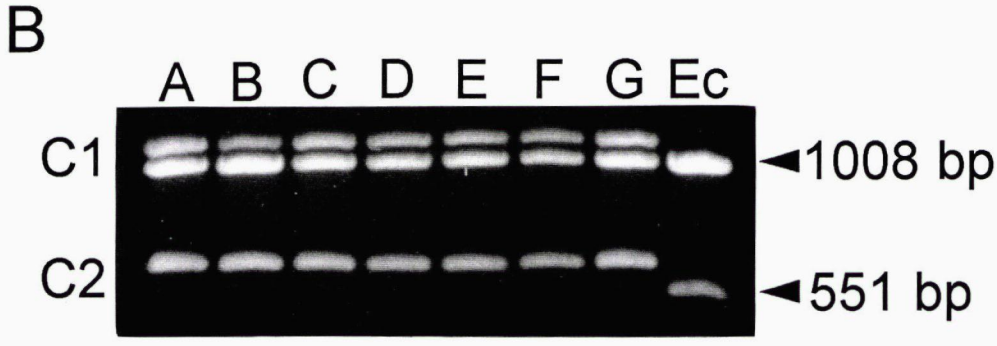

B
A B C D E F G Ec
C1
C2
1008 bp
551 bp

Figure 13. rRNA of strains of S. typhimurium and lines of strain LT2.

10 ug of RNA was separated using 1.2% agarose glyoxal-DMSO denaturing gel electrophoresis, blotted to Hybond-N+, and stained with methylene blue.

A. rRNA from Group 1(SARA2), 2(SARA7), 3(SARA6), and 4(SARA21) of the SARA set, as defined in Figure 4A. Group 1 contains the characteristic LT2 23S rRNA fragmentation pattern, producing 2.4-, 1.7-, 1.2-, 0.7-, and 0.5-kb rRNA fragments. E. coli K-12 (Ec) indicates the positions of the 23S and 16S rRNA.

B. rRNA isolated from seven different lines of S. typhimurium LT2 (lanes A to G) and E. coli K-12 (lane Ec). (A) SGSC 1412. (B) LB5010. (C) TN3618. (D) SA4254. (E) SL937. (F) LT2A. (G) EB360.

C. 23S rRNA fragmentation schematic. 23S rRNA fragments of 2.4- and 0.5-kb indicate that one rrl gene carries an IVS in helix-25 (about bp 550 in rrl). 23S rRNA fragments of 1.7- and 1.2-kb indicate that one rrl gene carries an IVS in helix-45 (about bp 1170 in rrl). 23S rRNA fragments of 1.7-, 0.7-, and 0.5-kb indicate that one rrl gene carries IVSs in both helices.

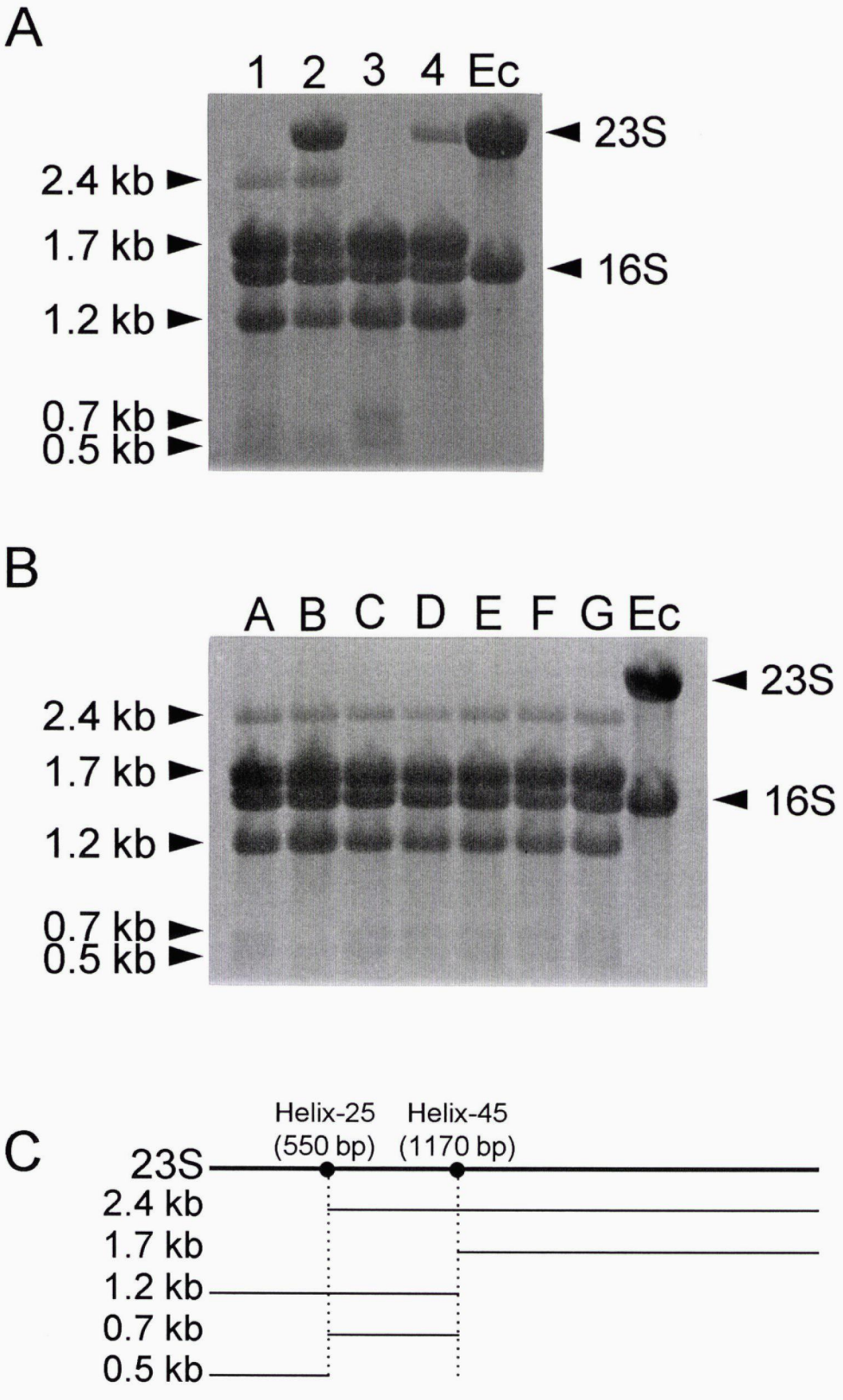
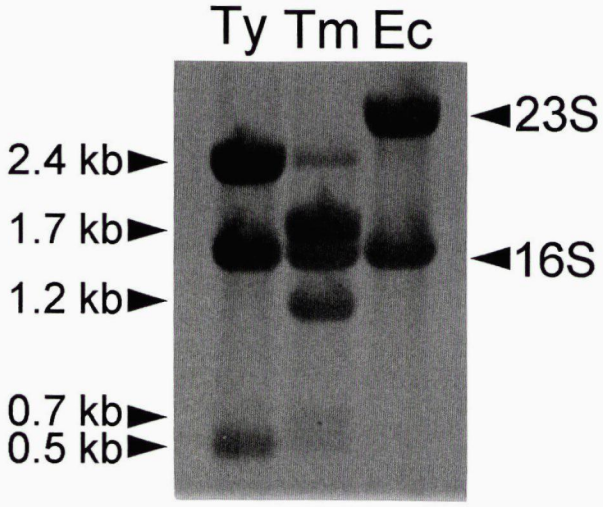

A
1 2 3 4 Ec
23S
16S
2.4 kb
1.7 kb
1.2 kb
0.7 kb
0.5 kb
B
A B C D E F G Ec
23S
16S
2.4 kb
1.7 kb
1.2 kb
0.7 kb
0.5 kb
C
Helix-25 (550 bp)
Helix-45 (1170 bp)
23S
2.4 kb
1.7 kb
1.2 kb
0.7 kb
0.5 kb

Figure 14. 23S rRNA fragmentation in S. typhi Ty2.

A. 23S rRNA fragmentation in S. typhi Ty2 (lane Ty) was observed by isolation of RNA, followed by glyoxal-DMSO 1.2% agarose gel electrophoresis of 10 ug of the RNA, blotting to Hybond-N+, and staining with methylene blue. S. typhi Ty2 produced 2.4- and 0.5-kb rRNA fragments, and no intact 23S rRNA. S. typhimurium LT2 (lane Tm) indicates the positions of all possible 23S rRNA fragments; it contains one of the seven rrl genes with an IVS in helix-25 only (2.4- and 0.5-kb fragments), five with an IVS in helix-45 only (1.7- and 1.2-kb fragments), and one that has IVSs in both helices (1.7-, 0.7-, and 0.5-kb fragments) (Figure 8). E. coli K-12 (lane Ec) reveals only 23S and 16S rRNA.

B. 23S rRNA fragmentation schematic. 23S rRNA fragments of 2.4- and 0.5-kb indicate that one rrl gene carries an IVS in helix-25 (about bp 550 in rrl). 23S rRNA fragments of 1.7- and 1.2-kb indicate that one rrl gene carries an IVS in helix-45 (about bp 1170 in rrl). 23S rRNA fragments of 1.7-, 0.7-, and 0.5-kb indicate that one rrl gene carries IVSs in both helices.

A


Ty Tm Ec
23S
16S
2.4 kb
1.7 kb
1.2 kb
0.7 kb
0.5 kb

B

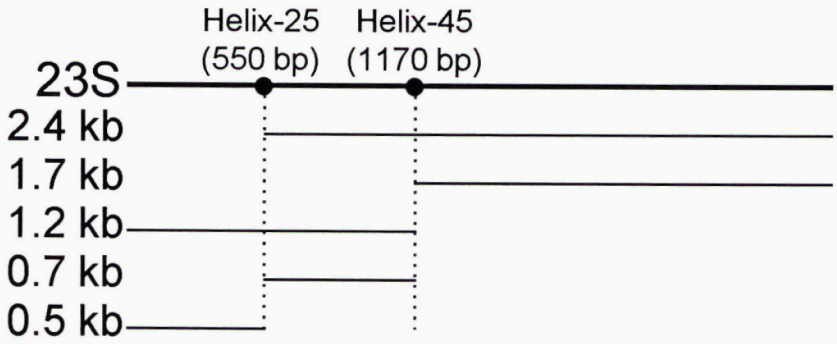
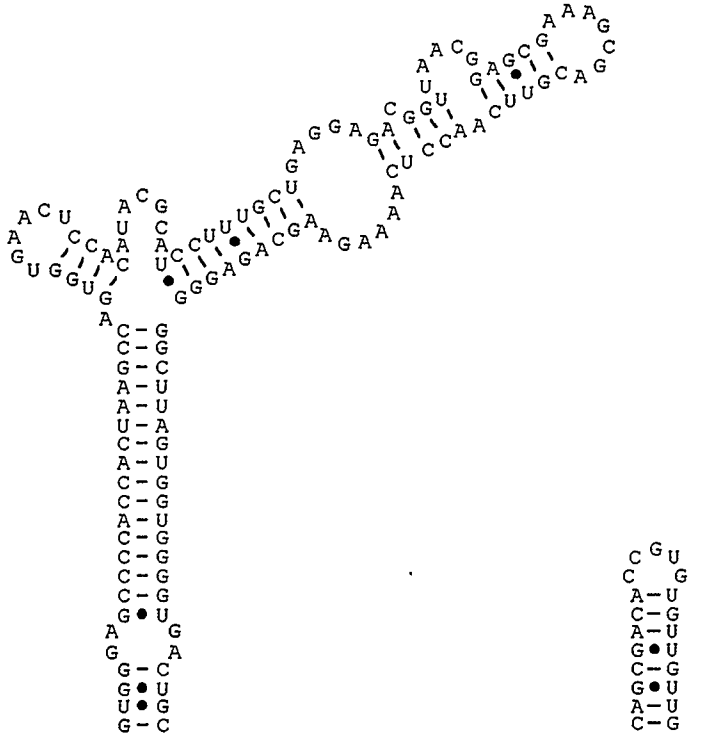

Helix-25
(550 bp)
Helix-45
(1170 bp)
23S
2.4 kb
1.7 kb
1.2 kb
0.7 kb
0.5 kb

Figure 15. Potential RNA secondary structures of all helix-25 and helix-45 nucleotide sequences from S. typhi Ty2.

Potential RNA secondary structure for IVSs. The secondary structure of S. typhi Ty2 (Ty) helix-25 IVS (free energy, -49.8 kcal/mol) resembles that of S. typhimurium (Tm) rrlH (Figure 10). The RNA fold of the helix-45 from S. typhi Ty2, into a penta-loop, is also shown. GenBank accession numbers: U54692, helix 25 IVS rrlA; U54693, helix 25 IVS rrlB; U54694, helix 25 IVS rrlC; U54695, helix 25 IVS rrlD; U54696, helix 25 IVS rrlE; U54697, helix 25 IVS rrlG; U54698, helix 25 IVS rrlH.



A

B

Figure 16. DraI restriction patterns observed for various S. typhi strains.

DNA from 35 S. typhi strains (all S. typhi strains in Table 1), isolated from embedded agarose blocks (Liu and Sanderson, 1992) using GlassMAX, was amplified to amplicon C. Each amplicon was purified, quantitated by spectrophotometer, and 600 ng was restricted with DraI; the resultant DNA was electrophoresed in 1% agarose, and stained with 0.5 ug/ml EtBr. Lanes A (1196-74), B (1707-81), C (382-82), D (9228-77), E (H251.1), F (IP E.88.374), G (IP E.88.353), and H (ST168) are representatives of the 35 strains, all of which gave indistinguishable results. Genomic DNA of S. typhimurium LT2 (Tm) gave a heterogeneous distribution of fragments, as expected based on its known IVS possession (Figure 8). PCR of genomic DNA of E. coli K-12 (Ec) yielded the C1 and C2 fragments, calculated to be 1008 bp and 551 bp, respectively, according to E. coli rrlB gene sequence (Noller, 1984).

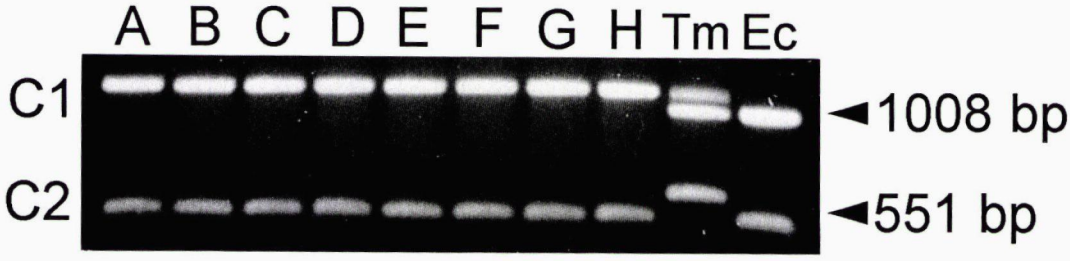

A B C D E F G H Tm Ec
C1
C2
1008 bp
551 bp

Figure 17. Construction of RNase III deficient *S. typhimurium*.

Method used to construct *S. typhimurium* RNaseIII deficient strain SA5303 is as follows. **A.** *E. coli* HT115 contained the *rnc-14*::ΔTn*10* insertion that was to be transduced by phage P22 to *S. typhimurium*. Because phage P22 requires the group B long-chain O12 antigen of *S. typhimurium* for adsorption, the *rfb rfc* genes were conducted by *E. coli* strain SAB4793 to HT115 and strain SAB5300 was isolated. **B.** Phage P22 was propagated on strain SAB5300 and the lysate was collected. **C.** To provide a mismatch repair background for the homologous recombination of *rnc-14*::ΔTn*10* from *E. coli* to *S. typhimurium*, *S. typhimurium* SA3856 that contained *mutL-111*::Tn*10* was used. Because SA3856 Tn*10* was tetracycline resistant, this created a final selection problem in that *rnc-14*::ΔTn*10* also confers tetracycline resistance. SA3856 was therefore cured of its tetracycline resistance by growing on Bochner media, which selected for tetracycline sensitivity. Strain SA5301 that contained Δ*mutL* was isolated. **D.** SA5301 was incubated with the P22 lysate that contained *rnc-14*::ΔTn*10* from strain SAB5300, and *S. typhimurium* SA5302 was isolated. **E** Phage P22 was propagated on strain SAB5302 and the lysate was collected. **F.** *S. typhimurium* LT2 was incubated with the P22 lysate that contained *rnc-14*::ΔTn*10* from strain SA5302, and *S. typhimurium* SA5303 was isolated.

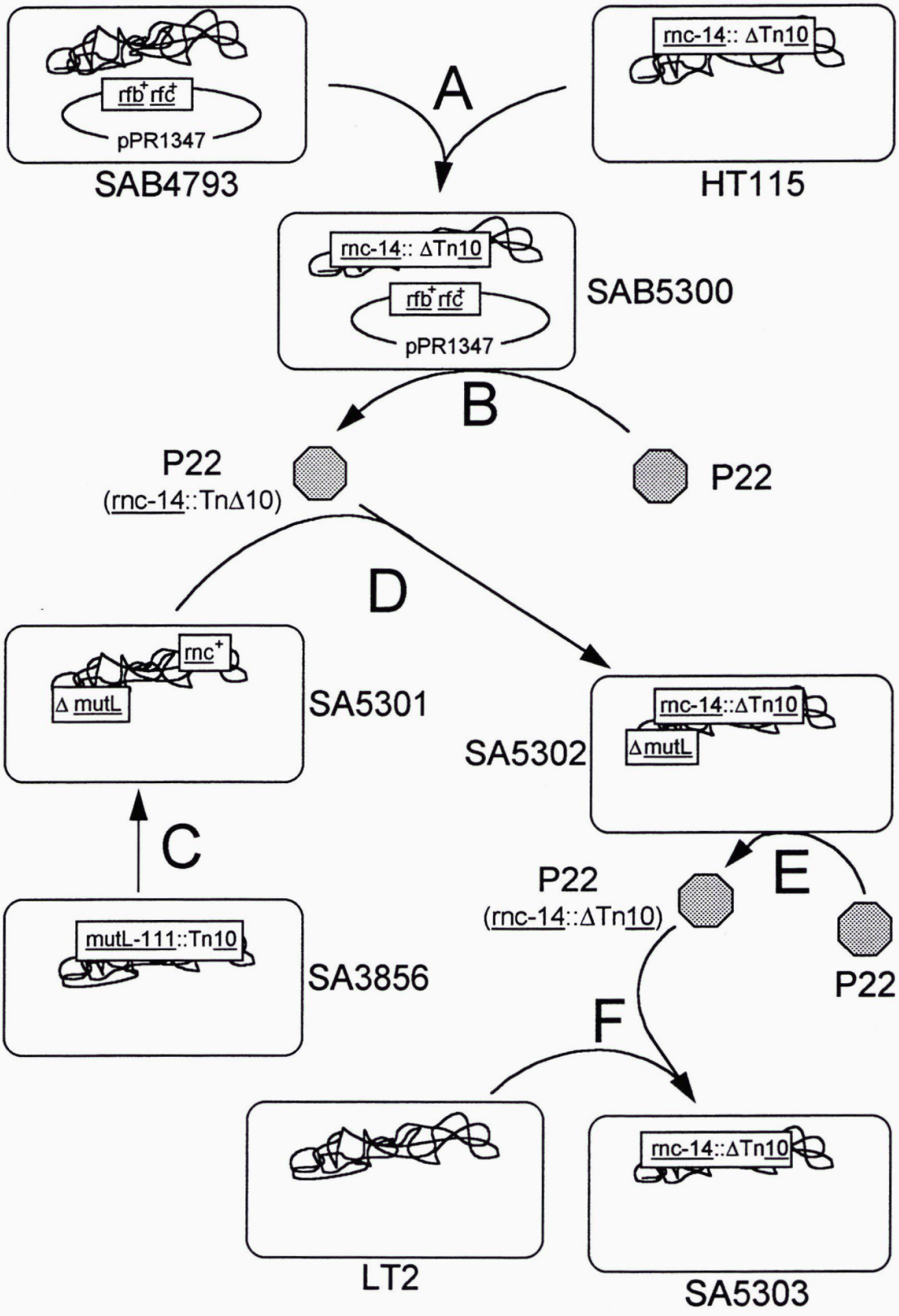
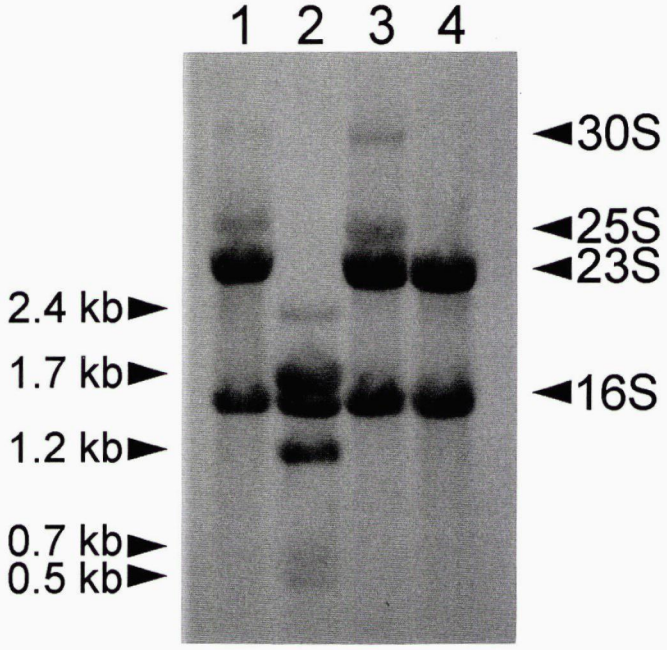

rfb+ rfc+
pPR1347
SAB4793
A
rnc-14:: ΔTn10
HT115
rnc-14:: ΔTn10
rfb+ rfc+
pPR1347
SAB5300
B
P22
(rnc-14::TnΔ10)
P22
D
rnc+
Δ mutL
SA5301
rnc-14::ΔTn10
ΔmutL
SA5302
C
mutL-111::Tn10
SA3856
P22
(rnc-14::ΔTn10)
E
P22
F
LT2
rnc-14::ΔTn10
SA5303

Figure 18. rRNA isolated from strains of S. typhimurium and E. coli.

A. rRNA was observed following glyoxal-DMSO 1.2% agarose gel electrophoresis of 10 ug of the RNA, blotting to Hybond-N, and staining with methylene blue. RNA from S. typhimurium strain SA5303 (rnc-14::ΔTn10) shows intact 23S and 16S rRNA, with 25S and 30S rRNAs (lane 1). S. typhimurium LT2 shows the characteristic 23S rRNA fragmentation pattern (lane 2). E. coli strain HT115 (rnc-14::ΔTn10) shows 16S, 23S, 25S, and 30S rRNAs (lane 3). E. coli K-12 shows the position of the 16S and 23S rRNAs (lane 4).

B. Hybridization of the membrane (above) with an oligonucleotide specific for the helix-45 IVS from S. typhimurium LT2 was detected by chemiluminescence. Only 23S, 25S, and 30S rRNAs from S. typhimurium SA5303 were detected; this shows that IVSs are present within this rRNA.

A


1 2 3 4
30S
25S
23S
16S
2.4 kb
1.7 kb
1.2 kb
0.7 kb
0.5 kb

B

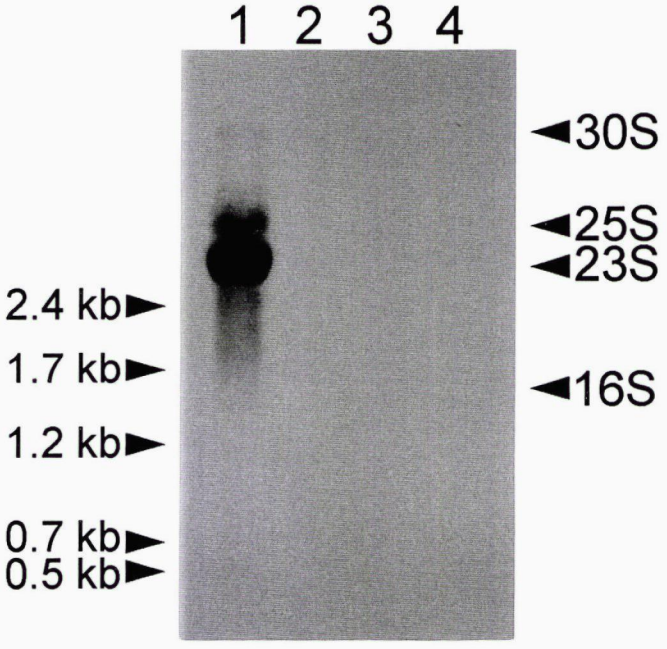

1 2 3 4
30S
25S
23S
16S
2.4 kb
1.7 kb
1.2 kb
0.7 kb
0.5 kb

Figure 19. rRNA isolated from strains of *S. typhimurium* and *E. coli*.

rRNA was observed following glyoxal-DMSO 1.2% agarose gel electrophoresis of 10 ug of the RNA, blotting to Hybond-N, and staining with methylene blue. RNA from *S. typhimurium* strain SA5304 (*rnc-14*::ΔTn*10*/pACS21[*rnc*$^+$]) shows a restoration of 23S rRNA fragmentation as observed for *S. typhimurium* LT2 (lane 1). RNA from *S. typhimurium* SA5303 (*rnc-14*::ΔTn*10*) contains 16S, 23S, 25S, and 30S rRNAs. *S. typhimurium* LT2 shows the characteristic 23S rRNA fragmentation pattern (lane 3). *E. coli* K-12 shows the position of the 16S and 23S rRNAs (lane 4).

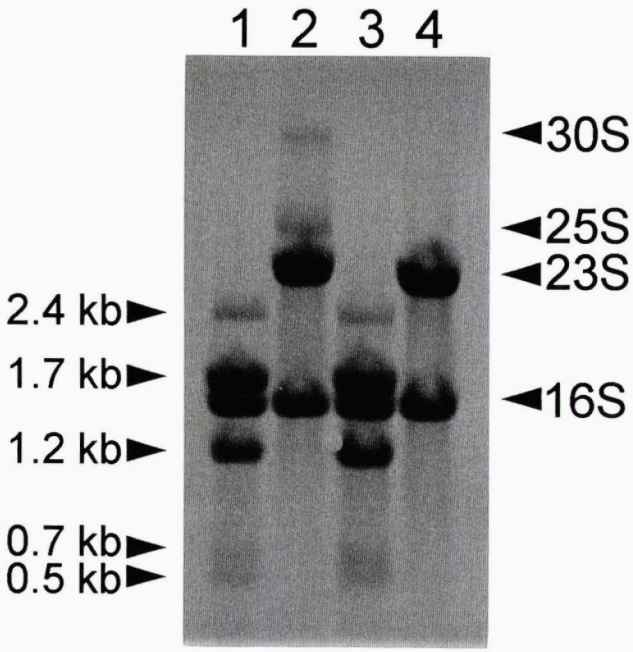
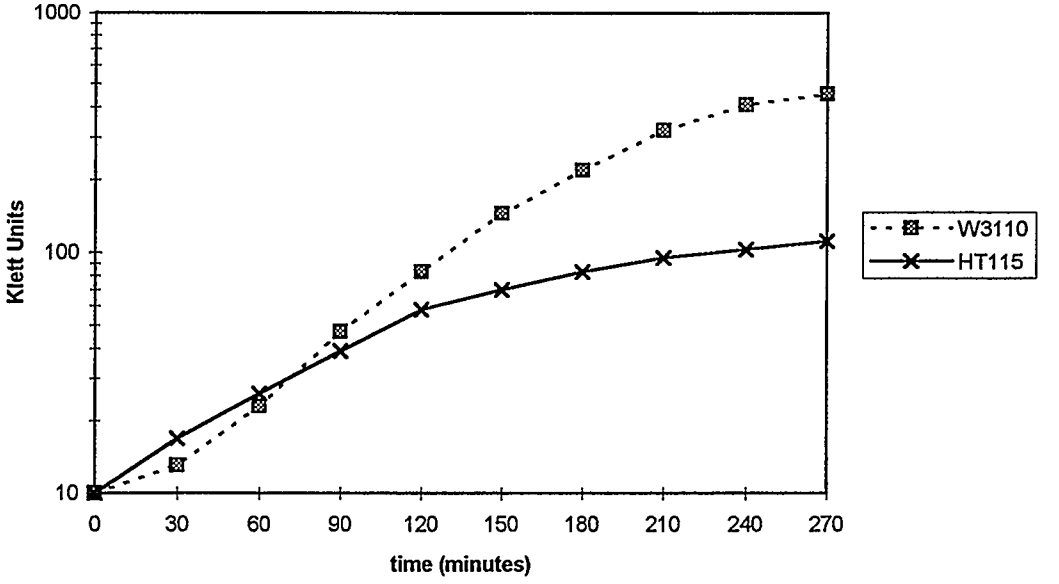

1 2 3 4
30S
25S
23S
16S
2.4 kb
1.7 kb
1.2 kb
0.7 kb
0.5 kb

Figure 20. Growth of strains of E. coli and S. typhimurium.

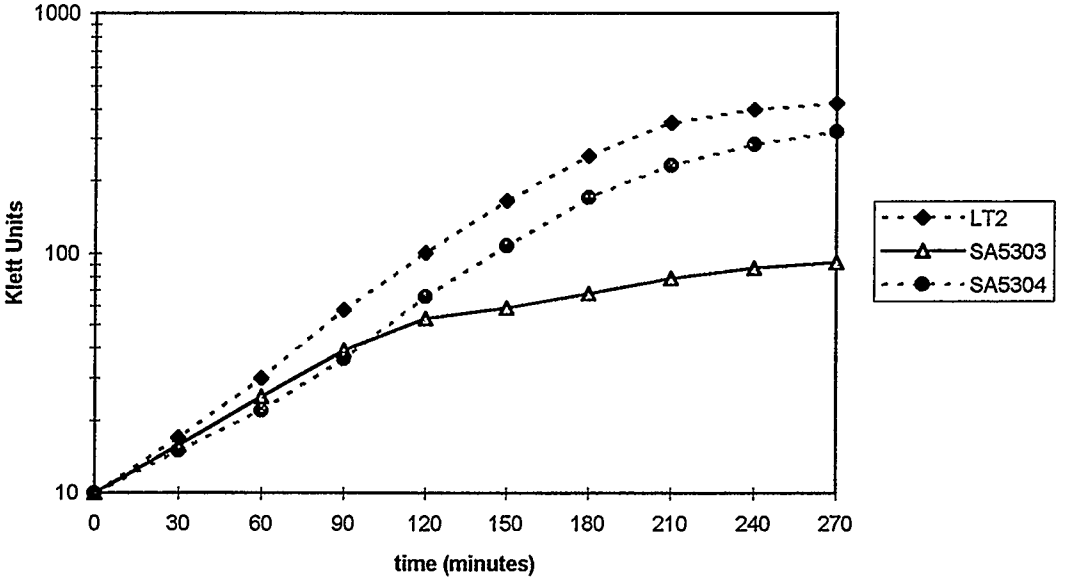
A. Growth was observed over several hours by optical density using a Klett colorimeter for LB-broth cultures incubated at 37°C with aeration. The generation time calculated for E. coli W3110 (parent of strain HT115) was 33 minutes. The generation time for strain E. coli HT115 (rnc-14::ΔTn10) was 47 minutes. These generation times were measured over the initial 120 minutes.

B. Growth was observed as described above. The generation time calculated for S. typhimurium SA5303 (rnc-14::ΔTn10) was 48 minutes. The generation time for S. typhimurium LT2 was 33 minutes. The generation for strain SA5304 (rnc-14::ΔTn10/pACS21) was 40 minutes.

A


1000
100
10
Klett Units
0
30
60
90
120
150
180
210
240
270
time (minutes)
W3110
HT115

B


1000
100
10
Klett Units
0
30
60
90
120
150
180
210
240
270
time (minutes)
LT2
SA5303
SA5304

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