

# Charged tmRNA but not tmRNA-mediated proteolysis is essential for *Neisseria gonorrhoeae* viability

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**tmRNA, through its tRNA and mRNA properties, adds short peptide tags to abnormal proteins, targeting these proteins for proteolytic degradation. Although the conservation of tmRNA throughout the bacterial kingdom suggests that it must provide a strong selective advantage, it has not been shown to be essential for any bacterium. We report that tmRNA is essential in *Neisseria gonorrhoeae*. Although tagging *per se* appears to be required for gonococcal viability, tagging for proteolysis does not. This suggests that the essential roles of tmRNA in *N.gonorrhoeae* may include resolving stalled translation complexes and/or preventing depletion of free ribosomes. Although derivatives of *N.gonorrhoeae* expressing *Escherichia coli* tmRNA as their sole tmRNA were isolated, they appear to form colonies only after acquiring an extragenic suppressor(s).**

**Keywords:** *Escherichia coli*/10Sa RNA/SsrA RNA/trans-translation

## Introduction

The *ssrA* gene of *Escherichia coli* encodes a small, abundant and unusual RNA (Ray and Apirion, 1979; Chauhan and Apirion, 1989; Oh *et al.*, 1990), named tmRNA (also known as 10Sa RNA and SsrA RNA) based on its having characteristics of both tRNA and mRNA (Williams and Bartel, 1996). A relationship of tmRNA to tRNA was first suggested by structural models showing that tmRNA could fold into a tRNA-like configuration with a T $\Psi$ C stem-loop and an acceptor stem with a 3' end CCA aminoacylation sequence (Komine *et al.*, 1994). The acceptor stem contains a G:U wobble, which, like the G3:U70 wobble in the acceptor stem of alanyl tRNA (Hou and Schimmel, 1988; McClain *et al.*, 1988), is configured to serve as the signal recognized by alanyl-tRNA synthetase (Komine *et al.*, 1994). *In vitro* studies showed that *E.coli* tmRNA could be charged with alanine (Komine *et al.*, 1994). A relationship of tmRNA to mRNA was first suggested by the finding of Tu *et al.* (1995) that

interleukin-6 expressed in *E.coli* acquired an 11 amino acid C-terminal tag (AANDENYALAA). A search of *E.coli* genomic sequence revealed the *ssrA* gene as the probable gene encoding the last 10 amino acids of the tag (Tu *et al.*, 1995). Based on these and their own studies, Keiler *et al.* (1996) proposed that tmRNA removes prematurely terminated polypeptide from truncated mRNA while adding an 11 amino acid tag that targets the polypeptide for proteolysis. Referred to as *trans*-translation or co-translation, this model postulates that tmRNA, charged with alanine and functioning as tRNA, enters the acceptor position of the ribosome, which stalls on mRNA lacking an in-frame stop codon. The alanine is added to the C-terminus of an uncompleted polypeptide; then, serving as an mRNA, tmRNA provides the template for the addition of the tag. At least three proteases, Tsp, HflB (FtsH) and Clp, recognize this C-terminal tag (Keiler *et al.*, 1996; Gottesman *et al.*, 1998; Herman *et al.*, 1998). Consistent with the idea that tmRNA is involved in some aspect of translation is the finding that tmRNA is associated with 70S ribosomal particles, but not with the 50S or 30S subunits (Komine *et al.*, 1996; Tadaki *et al.*, 1996).

The fact that *ssrA* is highly conserved in the eubacteria, homologs being identified in all but one of the nearly 70 bacterial species examined (Williams, 1999), suggests that tmRNA provides an important activity to bacteria. It is surprising, then, that in the three bacterial species previously examined, *E.coli* (Oh and Apirion, 1991), *Vibrio cholerae* (C.Huang, V.DiRita and D.I.Friedman, unpublished data) and *Bacillus subtilis* (Muto *et al.*, 1998), tmRNA was shown not to be essential. *Escherichia coli* mutants that do not express tmRNA, however, exhibit some phenotypic characteristics, such as slower growth rates and altered motility at high temperature, and slower recovery from carbon starvation (Oh and Apirion, 1991; Komine *et al.*, 1994). Also, *E.coli* mutants lacking a functional tmRNA fail to support growth of certain hybrid phages (Strauch *et al.*, 1986; Retallack *et al.*, 1994; Withey and Friedman, 1999). These phages, as a group called  $\lambda$ immP22, have varying amounts of genetic material from coliphage  $\lambda$  and *Salmonella* phage P22 (Gemski *et al.*, 1972; Botstein and Herskowitz, 1974). As recently reported, the parental P22 phage fails to grow in *Salmonella typhimurium* derivatives lacking tmRNA activity (Karzai *et al.*, 1999). Although growth of some  $\lambda$ immP22 hybrids is supported effectively by mutant tmRNAs which add tags that do not contain the signal for protein degradation, growth of these hybrids is not supported by a mutant tmRNA that cannot be charged with alanine (Withey and Friedman, 1999). These observations led to the proposal that tagging for proteolysis might not be the primary role for tmRNA; rather, in some cases, tmRNA may only be required to remove the stalled ribosome from intact mRNA, with degradation of polypeptides serving only an

ancillary role (Withey and Friedman, 1999). Recent studies have shown that a protein encoded by the *smpB* gene, located in *E.coli* immediately upstream of the *ssrA* gene (*ssrA<sup>Ec</sup>*), is also required for tmRNA activity (Karzai *et al.*, 1999; J.Withey, C.Huang and D.I.Friedman, in preparation).

Although studies of tmRNA have focused on *E.coli* *ssrA* variants where tmRNA is not essential, it is plausible to consider that studies in other bacteria might uncover more information on the physiological role of this unique and ubiquitous RNA. Because *Neisseria gonorrhoeae* (gonococcus) differs from *E.coli* in having an extremely limited ecological niche and a relatively small genome size, we chose to determine the role of tmRNA in the growth of *N.gonorrhoeae*. We identified and cloned the *ssrA* homolog from *N.gonorrhoeae* (*ssrA<sup>Ng</sup>*) and found that tmRNA is essential for growth of *N.gonorrhoeae*. Consistent with the results of studies with  $\lambda$ immP22 phages, our studies show that while charging of tmRNA, and presumably tagging *per se*, are required, degradation of the tagged polypeptides is not required for the growth of *N.gonorrhoeae*. Further, these studies suggest that *E.coli* tmRNA supports the growth of *N.gonorrhoeae* only when the bacterium has acquired an as yet unidentified extragenic suppressor mutation(s).

## Results

### Identification and cloning of the *ssrA* gene of *N.gonorrhoeae*

We identified a candidate for the *ssrA* gene of *N.gonorrhoeae* strain N400 (Freitag *et al.*, 1995) based on the results of a homology search of the genome sequence of *N.gonorrhoeae* strain FA1090 (Roe *et al.*) using the sequence of the *ssrA<sup>Ec</sup>* gene as the search template. The putative *ssrA<sup>Ng</sup>* gene shares 59% nucleotide identity with the *ssrA<sup>Ec</sup>* gene. Based on the defined 5' and 3' ends of the *E.coli* tmRNA (Chauhan and Apirion, 1989; Komine *et al.*, 1994), we have determined that the *ssrA<sup>Ng</sup>* gene encodes an RNA of 363 nucleotides, equal in length to that of the mature *E.coli* tmRNA (Figure 1).

The putative *ssrA<sup>Ng</sup>* gene, like other identified *ssrA* homologs, possesses conserved sequences in regions thought to be essential for tmRNA function, including those at the 5' terminus, in the sequence encoding the peptide tag and at the 3' terminus (Figure 1). The putative tag sequence encoded by *ssrA<sup>Ng</sup>* differs by only one codon from that encoded by *ssrA<sup>Ec</sup>*, having a threonine at position 5 instead of an asparagine (Figure 1). We cloned a 1.8 kb PCR product (GCtm) containing the *ssrA<sup>Ng</sup>* gene from strain N400 into pUP6, a plasmid with two gonococcal uptake sequences (GCU) in the polylinker (Wolfgang *et al.*, 1999), yielding plasmid pGCtm.

### Functional analysis of *N.gonorrhoeae* tmRNA in *E.coli*

We tested whether gonococcal tmRNA could complement an *E.coli* strain lacking tmRNA for growth of  $\lambda$ immP22hy25 (Hilliker and Botstein, 1976), a phage requiring tmRNA for growth (Retallack *et al.*, 1994; Withey and Friedman, 1999). Plasmid pGCtm was introduced into an *E.coli* derivative, K8619 (Withey and Friedman, 1999), that carries an *ssrA<sup>Ec</sup>* allele inactivated with a chloram-

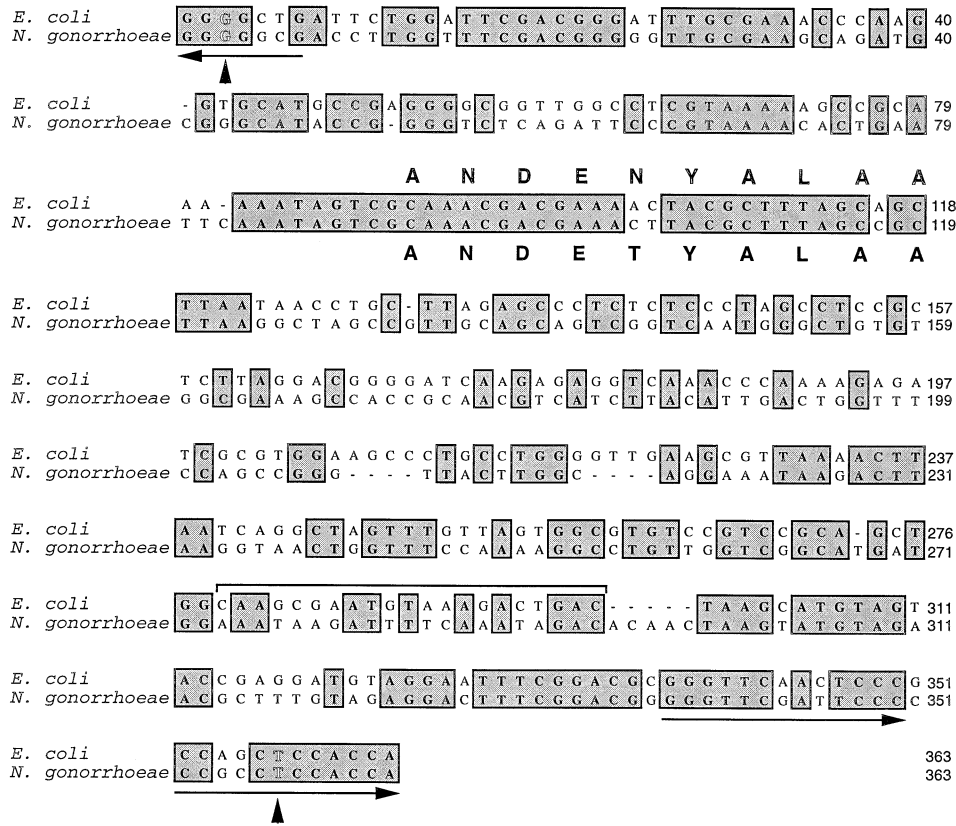
phenicol acetyltransferase (*cat*) gene insertion (*ssrA<sup>Ec</sup>::cat*) (Kirby *et al.*, 1994), creating strain K8745. Northern blot analysis using the coding sequence of *ssrA<sup>Ec</sup>* as the probe showed that pGCtm in K8745 expressed an RNA having the size of and homology with *E.coli* tmRNA (Figure 2).

Phage growth was measured as efficiency of plating (EOP). When the EOP of  $\lambda$ immP22hy25 on the bacterial lawns formed from K37 (wild type for *ssrA<sup>Ec</sup>*) was taken as 1, the EOP of  $\lambda$ immP22hy25 on the bacterial lawns formed from the *ssrA<sup>Ec</sup>::cat* strain K8619 was  $3.8 \times 10^{-5}$ , while the EOP on lawns formed from the strain, K8745, which only expresses tmRNA from the plasmid-encoded *ssrA<sup>Ng</sup>* gene, was 0.5 (Table I). Thus, based on its capacity to support growth of  $\lambda$ immP22hy25, the gonococcal tmRNA expressed from pGCtm is functional.

### Evidence that tmRNA is essential for the growth of *N.gonorrhoeae*

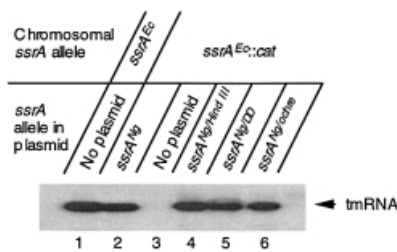
To determine if tmRNA is essential in *N.gonorrhoeae*, we attempted to construct a derivative with an insertionally inactivated *ssrA* gene. pGCtm/*ssrA<sup>Ng</sup>::cat*, a derivative of pGCtm with a *cat* gene inserted into the *ssrA<sup>Ng</sup>* gene, was used in these experiments. It would be expected that when *N.gonorrhoeae* is transformed with the linear GCtm/*ssrA<sup>Ng</sup>::cat* fragment, chloramphenicol-resistant (Cm<sup>r</sup>) transformants (i.e. recombinants) will form by a double crossover event that results in an allelic replacement of the wild-type *ssrA<sup>Ng</sup>* gene with the *ssrA<sup>Ng</sup>::cat* allele. We were unable to obtain stable Cm<sup>r</sup> recombinants following transformation with the GCtm/*ssrA<sup>Ng</sup>::cat* fragment when the recipient *N.gonorrhoeae* was haploid for *ssrA<sup>Ng</sup>* (strain N400). However, we were able to obtain stable Cm<sup>r</sup> recombinants when the recipient *N.gonorrhoeae* was diploid for *ssrA<sup>Ng</sup>* (strain C101). The latter observation ruled out a number of possible technical reasons for our failure to obtain allelic replacement with *ssrA<sup>Ng</sup>::cat* in the haploid strain, including problems in the DNA preparation, defects in transformation or failure of recombination in the region of the genome containing the *ssrA<sup>Ng</sup>* gene. Thus, the generation of stable recombinants in the diploid strain suggested that the failure to obtain allelic replacements in the haploid strain with *ssrA<sup>Ng</sup>::cat* is due to the essential nature of the product of the *ssrA* gene in *N.gonorrhoeae*.

We next determined whether insertion mutations adjacent to but not in *ssrA<sup>Ng</sup>* could be crossed into the haploid strain. A library of mini-transposon insertions in the GCtm insert in pGCtm was constructed by shuttle mutagenesis (Seifert *et al.*, 1986) using the m-Tn<sub>cm</sub> transposon, a derivative of Tn3 with a *cat* gene (Drake *et al.*, 1997). Insertion mutations were located upstream of the *ssrA<sup>Ng</sup>* gene (group A), in the *ssrA<sup>Ng</sup>* gene (group B) or downstream of the *ssrA<sup>Ng</sup>* gene (group C) (Figure 3). Thirteen representative m-Tn<sub>cm</sub> insertion mutations were tested for their ability to complement the *ssrA<sup>Ec</sup>::cat* mutation for  $\lambda$ immP22hy25 phage growth in *E.coli* as well as the formation of Cm<sup>r</sup> colonies when transformed into *N.gonorrhoeae* either haploid or diploid for the *ssrA<sup>Ng</sup>* gene. As shown in Figure 3, for all insertion mutations having m-Tn<sub>cm</sub> in the *ssrA<sup>Ng</sup>* gene (group B),  $\lambda$ immP22hy25 growth in *E.coli* was not supported and Cm<sup>r</sup> recombinants could not be isolated from attempted transformations of an *N.gonorrhoeae* strain haploid for the *ssrA<sup>Ng</sup>*. However, Cm<sup>r</sup> colonies could be isolated from



- ← 5' end sequence encoding part of the acceptor stem
- 3' end sequence encoding part of the acceptor stem and the TψC stem loop
- ▲ Nucleotides encoding the GU pair in the acceptor stem
- ▭ Sequence corresponding to the *E. coli* tmRNA region hybridized by the probe specific for *E. coli* tmRNA

**Fig. 1.** Sequence alignment of *E. coli* and *N. gonorrhoeae* (strain N400) *ssrA* genes. Boxes indicate positions of sequence identity. Letters denoting amino acid tag sequences are placed either above (for *E. coli*) or below (for *N. gonorrhoeae*) the codons of the tagging sequences. The tag sequences of the two *ssrA* genes differ at only one codon; codon 5 encodes asparagine in *ssrA<sup>Ec</sup>* and threonine in *ssrA<sup>Ng</sup>*.



**Fig. 2.** Northern blot analysis identifying tmRNAs of *E. coli* and *N. gonorrhoeae*. The probe was synthesized using *ssrA<sup>Ec</sup>* as template. Strains examined were: K37, wild-type for *ssrA<sup>Ec</sup>* (lane 1), K8619, isogenic with K37 except that it has the *ssrA<sup>Ec</sup>::cat* allele (lane 3), and variants of K8619 carrying pGCTm (lane 2) or derivatives of pGCTm with *ssrA<sup>Ng</sup>* mutant alleles (lanes 4–6).

transformations of a strain diploid for *ssrA<sup>Ng</sup>*. For all variants having m-Tncm insertions outside the *ssrA<sup>Ng</sup>* gene (groups A and C), *λimmP22hy25* growth in *E. coli* was supported and Cm<sup>r</sup> recombinants were obtained with *N. gonorrhoeae* haploid or diploid for the *ssrA<sup>Ng</sup>* gene. These results argue that m-Tncm insertions in *ssrA<sup>Ng</sup>* affect gonococcal viability directly, not by interfering in a polar manner with expression of downstream genes.

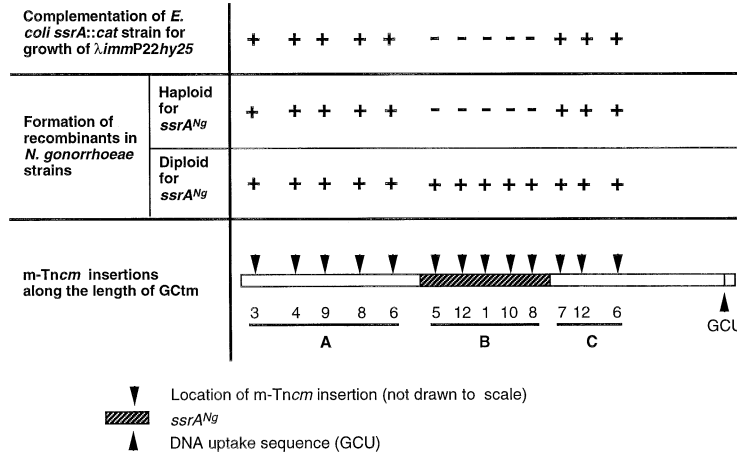
**Table I.** Ability of *ssrA* alleles to support the growth of *λimmP22hy25*

Strain	<i>ssrA</i> alleles	EOP <sup>a</sup>
K37	<i>ssrA<sup>Ec</sup></i>	1
K8619	<i>ssrA<sup>Ec</sup>::cat</i>	3.8 × 10 <sup>-5</sup>
K8745	<i>ssrA<sup>Ec</sup>::cat/ssrA<sup>Ng</sup></i>	0.5
K9335	<i>ssrA<sup>Ec</sup>::cat/ssrA<sup>Ng/ochre</sup></i>	5.4 × 10 <sup>-5</sup>
K9336	<i>ssrA<sup>Ec</sup>::cat/ssrA<sup>Ng/DD</sup></i>	4.0 × 10 <sup>-5</sup>
K9337	<i>ssrA<sup>Ec</sup>::cat/ssrA<sup>Ng/HindIII</sup></i>	0.4
K9101	<i>ssrA<sup>Ec</sup>::cat/ssrA<sup>Ng/-StyI</sup></i>	0.5
K9102	<i>ssrA<sup>Ec</sup>::cat/ssrA<sup>Ng/UG</sup></i>	5.3 × 10 <sup>-5</sup>

<sup>a</sup>EOP represents the titer of the phage on a bacterial lawn formed from the indicated strain divided by the titer on a lawn formed on K37, our standard *E. coli* strain with a wild-type *ssrA<sup>Ec</sup>* allele.

**Use of mutant *ssrA<sup>Ng</sup>* alleles to determine activities of tmRNA required by *N. gonorrhoeae***

To explore a possible role(s) of tmRNA in growth of *N. gonorrhoeae*, we constructed mutant *ssrA<sup>Ng</sup>* alleles based on the set of *ssrA<sup>Ec</sup>* mutants used in the studies examining the requirement for *λimmP22* growth in *E. coli* (Withey and Friedman, 1999). The DNA sequences encoding the acceptor stem and the tag in *N. gonorrhoeae* tmRNA are

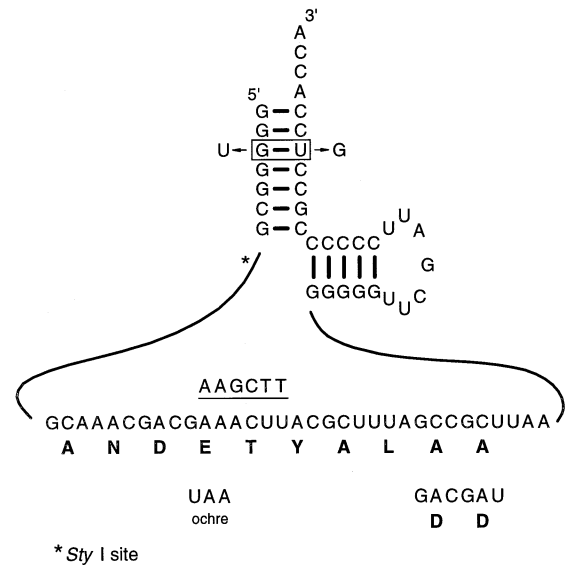


**Fig. 3.** Functional analysis of *ssrA<sup>Ng</sup>* alleles with transposon insertions in and surrounding *ssrA<sup>Ng</sup>*. A library of 13 m-Tncm insertions was created in plasmid pGCTm. The positions (shown by downward pointing arrows) in the GCTm fragment of these insertions were located by DNA sequencing. Insertions were located in three regions: A, upstream of *ssrA*; B, within *ssrA*; and C, downstream of *ssrA*. The effect on tmRNA activity of each insertion was determined by assessing growth of  $\lambda$ *immP22hy25* in derivatives of the *ssrA<sup>Ec</sup>::cat* *E. coli* strain K8619 carrying variants of pGCTm with the respective 13 insertions (row 1); (+) support of phage growth and (-) no support of phage growth. Formation of stable recombinants with mini-transposon inserted *ssrA<sup>Ng</sup>* alleles in *N. gonorrhoeae* either haploid (row 2) or diploid (row 3) for *ssrA<sup>Ng</sup>* was determined by selecting Cm<sup>r</sup> colonies; (+) Cm<sup>r</sup> colonies were isolated and (-) Cm<sup>r</sup> colonies were not isolated.

nearly identical to those encoding *E. coli* tmRNA. We assumed that changes affecting *E. coli* tmRNA action should, when duplicated in *N. gonorrhoeae* tmRNA, similarly affect the action of that tmRNA. The construction of *ssrA<sup>Ng</sup>* mutant alleles, and the subsequent selection of *N. gonorrhoeae* recombinants carrying those mutant alleles, was facilitated by the use of the m-Tncm transposon insertion C7, located 16 nucleotides downstream of the 3' end of the *ssrA<sup>Ng</sup>* gene (see Figure 3). This insertion was tightly linked to *ssrA<sup>Ng</sup>*, but did not interfere with expression of functional tmRNA (see previous section). Thus, we could use chloramphenicol resistance as a linked selectable marker for allelic replacement of the chromosomal *ssrA<sup>Ng</sup>* gene with the desired *ssrA<sup>Ng</sup>* mutant alleles.

We looked first at the effect of *ssrA<sup>Ng</sup>* mutations designed to allow tagging, but not subsequent degradation of tagged proteins. The *ssrA<sup>Ng/ochre</sup>* allele has a glutamic acid codon, GAA, at position 4 of the tagging sequence changed to the ochre translation termination codon, TAA (Figure 4). The ochre mutation should result in premature termination of translation, producing a tag lacking the terminal ALAA required for proteolytic degradation (Keiler *et al.*, 1996). The *ssrA<sup>Ng/DD</sup>* allele has the two alanine codons at positions 9 and 10 changed to two aspartic acid codons (Figure 4). This change, referred to as DD, results in a tag in which the critical terminal alanines are replaced with negatively charged amino acids shown to interfere with degradation (Parsell *et al.*, 1990; Keiler *et al.*, 1996). Thus, the presence of either of these mutant tags should not signal proteolysis.

It has been shown in a previous study from our laboratory (Withey and Friedman, 1999) that *E. coli* having *ssrA<sup>Ec</sup>* alleles with either the ochre or DD changes (*ssrA<sup>o</sup>* or *ssrA<sup>DD</sup>*, respectively) as their only functional *ssrA<sup>Ec</sup>* gene support the growth of  $\lambda$ *immP22hy25* nearly as well as an isogenic strain with a wild-type *ssrA<sup>Ec</sup>*. Therefore, we assumed that the *ssrA<sup>Ng</sup>* ochre and DD alleles (*ssrA<sup>Ng/ochre</sup>* and *ssrA<sup>Ng/DD</sup>*, respectively) would also function in *E. coli* to support the growth of  $\lambda$ *immP22hy25*, but



**Fig. 4.** Variants used in determining the properties of gonococcal tmRNA required for *N. gonorrhoeae* growth. The DD mutation was generated by replacing the two terminal alanine codons with two aspartic acid codons, while the ochre mutation was generated by changing the fourth codon from GAA to the sequence encoding the ochre translation terminator UAA. Both mutants also carry a single base substitution (from AAACTT to AAGCTT) that provides an identifying *Hind*III site, as indicated by the underlined sequence above the tag sequence. Two changes were made in the sequences encoding the acceptor stem: these result in the change of the G:U base pair required for recognition of alanyl-tRNA synthetase to a U:G base pair. This mutant also carries a single base substitution (from CCTTGG to CGTTGG) that provides an identifying marker by eliminating a *Sty*I site that is only five nucleotides away from the G3 position, indicated by the asterisk.

this was not the case. As shown in Table I, neither variant *ssrA<sup>Ng</sup>* allele complemented the *E. coli* strain K8619 for the growth of  $\lambda$ *immP22hy25*. Nevertheless, *E. coli* carrying either of these *ssrA<sup>Ng</sup>* mutant alleles expressed stable tmRNA comparable in amount with that expressed by the wild-type *ssrA<sup>Ng</sup>* allele as detected by Northern blotting (Figure 2). Therefore, it is unlikely that instability of these

**Table II.** *ssrA* allele and *N.gonorrhoeae* viability

<i>ssrA</i> allele linked to Cm <sup>r</sup> marker <sup>b</sup>	Allelic replacement <sup>a</sup> (No. of Cm <sup>r</sup> colonies)	
	N400 ( <i>ssrA</i> <sup>Ng</sup> haploid)	C101 ( <i>ssrA</i> <sup>Ng</sup> diploid)
<i>ssrA</i> <sup>Ng</sup>	118	120
<i>ssrA</i> <sup>Ng/HindIII</sup>	124	122
<i>ssrA</i> <sup>Ng/DD</sup>	130	140
<i>ssrA</i> <sup>Ng/ochre</sup>	41	28
<i>ssrA</i> <sup>Ng/-SryI</sup>	58	84
<i>ssrA</i> <sup>Ng/UG</sup>	0	38

<sup>a</sup>One of three experiments that had essentially the same results. Approximately  $3 \times 10^6$  bacteria transformed with  $\sim 3 \mu\text{g}$  of DNA were plated for each transformation.

<sup>b</sup>The Cm<sup>r</sup> marker is located in the linked transposon 16 nucleotides downstream of the *ssrA*<sup>Ng</sup> gene.

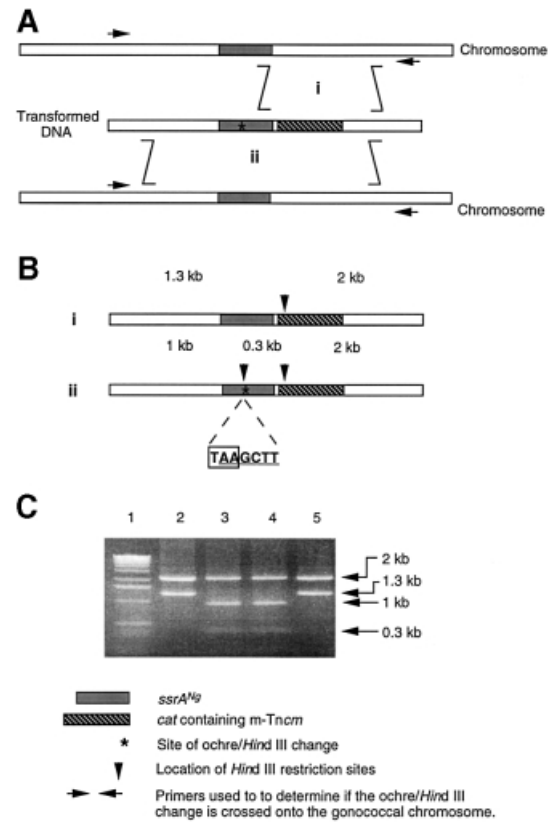
tmRNA molecules can explain the failure to support phage growth.

Derivatives of *N.gonorrhoeae* with either the *ssrA*<sup>Ng/ochre</sup> or the *ssrA*<sup>Ng/DD</sup> allele were obtained by replacement of the wild-type *ssrA*<sup>Ng</sup> with the appropriate mutant allele (Table II and Figure 5, see also Materials and methods). These results suggest that directing proteins toward degradation is unlikely to be an essential activity for tmRNA in *N.gonorrhoeae*.

We next examined a mutation altering the G:U base pair in the acceptor stem that blocks the alanyl aminoacylation of tmRNA and, in turn, blocks *trans*-translation at the earliest step. Like the G:U in the acceptor stem of alanyl tRNA, this G:U base pair is essential for charging the *E.coli* tmRNA with alanine (Komine *et al.*, 1994). A mutant allele of the *ssrA*<sup>Ng</sup> gene, *ssrA*<sup>Ng/UG</sup>, was constructed so that the G:U base pair in the acceptor stem of the tmRNA (G3 and U357) is changed to U:G (U3, G357, respectively) (Figure 4). We made changes as conservative as possible to minimize any alteration in the tmRNA structure (Komine *et al.*, 1994; Williams and Bartel, 1996; Felden *et al.*, 1997; Zwieb *et al.*, 1999) that might result from the mutations. An *ssrA*<sup>Ec</sup> gene similarly mutated to alter the U:G charging signal encodes a tmRNA that fails to support growth of  $\lambda$ immP22 phages (Withey and Friedman, 1999). Not surprisingly, we find that when placed in *E.coli*, the *ssrA*<sup>Ng/UG</sup> mutant allele of *N.gonorrhoeae* also fails to support growth of  $\lambda$ immP22hy25 (Table I). We next determined whether *N.gonorrhoeae* with *ssrA*<sup>Ng/UG</sup> as the sole *ssrA* gene is viable by attempting to construct a strain with that allele as the only *ssrA* gene. To this end, a DNA fragment carrying *ssrA*<sup>Ng/UG</sup> with the linked Cm<sup>r</sup> marker was transformed into *N.gonorrhoeae* either haploid or diploid for *ssrA*<sup>Ng</sup>, and Cm<sup>r</sup> colonies were selected. We failed to obtain Cm<sup>r</sup> recombinants following transformation of *ssrA*<sup>Ng</sup> haploid strain N400. However, we did obtain viable Cm<sup>r</sup> colonies following transformation of the *ssrA*<sup>Ng</sup> diploid strain C101 (Table II). These results indicate that alanyl aminoacylation of tmRNA is required for the essential role(s) that tmRNA plays in supporting growth of *N.gonorrhoeae*.

#### Action of *E.coli* tmRNA in *N.gonorrhoeae*

To determine whether *E.coli* tmRNA supports growth of *N.gonorrhoeae*, we constructed an *ssrA*<sup>Ng/ssrA</sup><sup>Ec</sup> hetero-



**Fig. 5.** Details of the allelic replacement that placed *ssrA*<sup>Ng</sup> mutant alleles on the gonococcal chromosome. Construction of a recombinant chromosomal *ssrA*<sup>Ng</sup> gene with the ochre mutation is used as an example of the procedure. (A) The double crossover recombination events resulting in allelic replacement. Selection is for Cm<sup>r</sup> carried by the linked m-Tncm. The m-Tncm will be crossed onto the chromosome without the ochre mutation if the recombination occurs as shown in (i). The ochre mutation (indicated by the asterisk) will be crossed with the m-Tncm onto the chromosome if the recombination occurs as shown in (ii). Arrows indicate the positions of the primers used in (B) and (C). (B) Schematic representation of the PCR products of the allelic replacements diagrammed in (A). The downward pointing arrows indicate the locations of the *Hind*III sites used in the analysis of the PCR products shown in (C). The location of the ochre codon is shown in (ii). (C) Cleavage of PCR products confirming proper allelic replacement. Cm<sup>r</sup> transformant colonies were used as the source of template DNA for the PCRs. PCR products, generated using the primers indicated in (A), were cleaved with *Hind*III and the products analyzed by electrophoresis in an agarose gel [source of DNA: lane 1, DNA 1 kb ladder (Gibco); lane 2, wild-type allele; lane 3, plasmid with cloned *ssrA*<sup>Ng/ochre</sup> allele; lanes 4 and 5, transformants]. Because an engineered *Hind*III site was positioned adjacent to the ochre mutation, PCR products from transformants that have an extra *Hind*III site, as shown in lane 4, are likely to carry the ochre mutation. Therefore, these transformants were chosen for sequence analysis to determine definitively if the transformant had the desired mutation.

diploid strain (C102) that has the *ssrA*<sup>Ec</sup> gene located in the non-essential *IgA1* protease gene (*iga*) (Figure 6A). Northern blot analysis, employing a probe specific for *E.coli* tmRNA, showed that the level of tmRNA of *E.coli* origin expressed in the heterodiploid gonococcal strain was approximately equal to that expressed in the *E.coli* strain K37 (Figure 6B). We then determined if the endogenous *ssrA*<sup>Ng</sup> gene in the heterodiploid strain could be replaced with an *ssrA*<sup>Ng</sup> allele insertionaly inactivated by an m-Tncm transposon without affecting viability. For this purpose, two GcTm derivatives with m-Tncm inserted at

**Table III.** Use of allelic replacement to study the requirement for an *ssrA* allele in *N.gonorrhoeae*

Selective marker used in allelic replacement	Antibiotic selection marker	Recipient strain and <i>ssrA</i> allele(s)			
		N400 ( <i>ssrA</i> <sup>Ng</sup> )	C101 ( <i>ssrA</i> <sup>Ng</sup> / <i>ssrA</i> <sup>Ng</sup> )	C102 ( <i>ssrA</i> <sup>Ng</sup> / <i>ssrA</i> <sup>Ec</sup> ) <sup>a</sup>	C103 ( <i>ssrA</i> <sup>Ng</sup> :: <i>cat</i> / <i>ssrA</i> <sup>Ec</sup> ) <sup>a</sup>
m-Tn <sub>cm</sub> in <i>ssrA</i> <sup>Ng</sup> (B12)	Cm <sup>r</sup>	–	+	+ <sup>b</sup>	NA
m-Tn <sub>cm</sub> downstream of <i>ssrA</i> <sup>Ng</sup> (C12)	Cm <sup>r</sup>	+	+	+	NA
<i>kn</i> <sup>r</sup> in <i>iga</i>	Kn <sup>r</sup>	NA	NA	+	–

<sup>a</sup>The *ssrA*<sup>Ec</sup> gene is located in the *iga* gene.

<sup>b</sup>These Cm<sup>r</sup> recombinant colonies appeared ~3 days following plating, while other recombinant colonies appeared ~1 day following plating.

(+) indicates that antibiotic-resistant recombinants were isolated.

(–) indicates that antibiotic-resistant recombinants were not isolated.

NA, not applicable.

different locations were employed, B12 within the *ssrA*<sup>Ng</sup> gene and C12 immediately 3' of the *ssrA*<sup>Ng</sup> gene (see Figure 3).

Although Cm<sup>r</sup> recombinants were obtained following transformation of the heterodiploid strain C102 with DNA carrying the B12 insertion, these transformants (*ssrA*<sup>Ng</sup>::*cat*/*ssrA*<sup>Ec</sup>) were observed only after ~3 days of incubation (Table III). This was in contrast to the results of transformations either with DNA carrying the B12 insertion into the homodiploid strain C101 (*ssrA*<sup>Ng</sup>/*ssrA*<sup>Ng</sup>) or with DNA carrying the C12 insertion into the heterodiploid strain C102 (*ssrA*<sup>Ng</sup>/*ssrA*<sup>Ec</sup>), all of which produced Cm<sup>r</sup> colonies in ~1 day (Table III). Surprisingly, once isolated, the heterodiploid transformants with the B12 insertion (e.g. C103) now grow normally, i.e. form colonies in ~1 day. One explanation for the initial delay in colony formation is that transformants expressing only *ssrA*<sup>Ec</sup> are viable but grow very slowly. More rapid growth occurs when they acquire some type of mutation(s) that allows the foreign tmRNA to function in *N.gonorrhoeae* either by making a change in *E.coli* tmRNA *per se* or by altering the expression or activity of an *N.gonorrhoeae* function(s). Alternatively, a mutation(s), by altering the expression or activity of a gene product(s) normally regulated by tmRNA, could eliminate the requirement for tmRNA.

To determine if a mutation(s) located in the *ssrA*<sup>Ec</sup> gene is responsible for this normal growth phenotype, we constructed a new heterodiploid strain by introducing the *ssrA*<sup>Ec</sup> gene from strain C103 into strain N400. Although this recombinant, C104, has the *ssrA*<sup>Ec</sup> gene from strain C103, we still observed a delay in colony formation (~3 days) following a transformation in which the wild-type *ssrA*<sup>Ng</sup> gene was replaced with the B12 insertion.

The next set of experiments tested whether a mutation(s) at a second site on the gonococcal chromosome eliminates the requirement for tmRNA. This was done by determining if we could replace the *ssrA*<sup>Ec</sup> of strain C103 located in *iga* successfully by a kanamycin resistance gene (*kn*<sup>r</sup>) cassette similarly cloned in *iga*, yielding a strain without a functional *ssrA* gene. Two *ssrA* heterodiploid strains were used in the experiment; strain C102 with both *ssrA* alleles (*ssrA*<sup>Ng</sup> and *ssrA*<sup>Ec</sup>) intact and strain C103 with *ssrA*<sup>Ec</sup> as the only functional *ssrA* allele. As shown in Table III, the *ssrA*<sup>Ec</sup> gene could be replaced with *iga*::*kn*<sup>r</sup> in C102, where there still was a functional *ssrA*<sup>Ng</sup> gene, but *ssrA*<sup>Ec</sup> could not be replaced in C103 where the *ssrA*<sup>Ng</sup> gene had already been inactivated insertionally by a *cat* gene. Therefore, if the normal growth phenotype when

*ssrA*<sup>Ec</sup> is the only functional *ssrA* allele is due to a second site mutation(s), that mutation(s) does not eliminate the requirement for an intact *ssrA* gene.

Finally, we addressed the question of whether a mutation somewhere in the *N.gonorrhoeae* genome is required for effective action of the *E.coli* tmRNA in that bacterium. To do this, we reconstructed the heterodiploid strain by replacing the inactivated *ssrA*<sup>Ng</sup>::*cat* allele in C103 with the wild-type *ssrA*<sup>Ng</sup> allele, creating *N.gonorrhoeae* derivatives heterodiploid for *ssrA* (*ssrA*<sup>Ng</sup>/*ssrA*<sup>Ec</sup>) (see Materials and methods). When these heterodiploids, such as C105, were transformed with DNA carrying the B12 insertion, Cm<sup>r</sup> recombinants were obtained in ~1 day, not the ~3 days observed following transformation of the original heterodiploid strain C102, a result expected if the C103 strain had acquired a mutation(s) that allows normal colony growth when *E.coli* tmRNA is the only functional tmRNA.

## Discussion

In this study, we report the identification of the *ssrA* gene of *N.gonorrhoeae* and show that this gene, which shares 59% homology with the *ssrA* gene of *E.coli*, encodes a tmRNA essential for *N.gonorrhoeae* growth.

It has been proposed that the tagging of the C-terminus of unfinished polypeptides by tmRNA, through *trans*-translation, serves to direct those polypeptides to a degradative pathway (Keiler *et al.*, 1996). Our studies show that although tagging for proteolysis is unlikely to be the essential function of tmRNA in *N.gonorrhoeae*, *trans*-translation is likely to be an essential function. To target polypeptides effectively for proteolysis, it was shown in *E.coli* that the tag requires specific amino acids (ALAA) at its C-terminus (Keiler *et al.*, 1996). Since the tag encoded by *ssrA*<sup>Ng</sup> varies by only one amino acid from that of *ssrA*<sup>Ec</sup>, it seems plausible to assume that the action of tmRNA as well as the associated processes are the same in the two organisms. By changing the tag sequence to one that either terminates translation upstream of the important terminal amino acids (*ssrA*<sup>Ng</sup>/*ochre*) or to one with changes in the terminal amino acids known to result in a faulty signal (*ssrA*<sup>Ng</sup>/*DD*) in *E.coli*, tmRNA should no longer provide a mechanism for targeting polypeptides for proteolysis (Parsell *et al.*, 1990; Keiler *et al.*, 1996). The fact that we could construct viable derivatives of *N.gonorrhoeae* that have *ssrA*<sup>Ng</sup> genes with either the ochre or DD tagging sequences as their sole intact *ssrA* gene suggests that the essential role of tmRNA in

*N.gonorrhoeae* is in a step prior to degradation of the tagged polypeptide. On the other hand, attempts to construct derivatives of *N.gonorrhoeae* in which the wild-type *ssrA*<sup>Ng</sup> gene is replaced with a mutant allele that encodes a tmRNA defective in amino acid charging (*ssrA*<sup>Ng/UG</sup>) were unsuccessful. Thus, it appears that *N.gonorrhoeae* requires charged tmRNA, even though it does not require that tmRNAs encode the signal appropriate to direct polypeptide degradation.

Based on the results obtained from studying the role of tmRNA in growth of certain  $\lambda$ immP22 phages, Withey and Friedman (1999) proposed that the major activity of tmRNA was removal of stalled ribosomes from mRNA. They postulated that some sequence or structure in the mRNA causes ribosome stalling, resulting in a block in translation. If this block is not removed, presumably by tmRNA, expression from the affected mRNA is reduced and if this, in turn, affects the synthesis of a protein(s) that is normally present in limited amounts, phage viability is impaired. It is plausible to assume that an analogous scenario occurs in *N.gonorrhoeae*. Accordingly, ribosome stalling results in lower than normal levels of an essential protein(s) normally synthesized in limited amounts, to levels below those needed for viability. Thus, release of the ribosome from the mRNA would be the important function of tmRNA, with degradation having, at most, an ancillary role. Consistent with this idea is the recent finding that the presence of rare codons in mRNA coupled with a scarcity of the cognate tRNA initiates the *trans*-translation mechanism (Roche and Sauer, 1999).

Relief of stalling could be important for another reason; by reducing sequestration of ribosomes, it could ensure that ribosomes are recycled adequately. The number of ribosomes in the bacterial cell appears ultimately to be regulated by the level of rRNA transcription (Sarmientos *et al.*, 1983; Gourse *et al.*, 1986). Hence, the number of rRNA operons (*rrn*) in a bacterium is likely to influence the nature of the response to a reduction in free ribosomes. *Neisseria gonorrhoeae* has four *rrn* operons (Dempsey and Cannon, 1994) compared with seven in *E.coli* (Kiss *et al.*, 1977) and 10 in *B.subtilis* (Bott *et al.*, 1984; LaFauci *et al.*, 1986), *ssrA* being essential in *N.gonorrhoeae* but not in the latter strains. Thus, *N.gonorrhoeae*, with a relatively low number of *rrn* operons, may be more heavily dependent on ribosome recycling to maintain an adequate level of free ribosomes than those bacteria with a higher copy number of rRNA genes.

A major consideration in assessing the function of tmRNA is the fact that the tagging sequences of all known *ssrA* genes are very similar and have terminal hydrophobic amino acids (Williams and Bartel, 1996; Williams, 1999). The *E.coli* and *N.gonorrhoeae* tagging sequences differ at only one codon, while there is less homology in other regions of these two tmRNAs. These observations are consistent with the argument that the tagging sequences are likely to be central to the activity of tmRNAs. Thus, it was surprising to find that results from two of our experiments suggested that there could be some level of specificity of the bacterium for its own tmRNA.

The first indication of such specificity derived from the results of the complementation assays testing phage plating in *E.coli* when the only tmRNAs are the tmRNA variants encoded either by *ssrA*<sup>Ng/ochre</sup> or *ssrA*<sup>Ng/DD</sup>. Previous studies

from our laboratory had shown that the *ssrA*<sup>Ec</sup> variants that encode tmRNAs with the ochre and DD tag sequences complemented the *E.coli* strain with the insertionally inactivated *ssrA*<sup>Ec</sup> gene for supporting the growth of  $\lambda$ immP22hy25. Although the tmRNA encoded by a wild-type *ssrA*<sup>Ng</sup> gene supports growth of  $\lambda$ immP22hy25, the products encoded by the *ssrA*<sup>Ng</sup> ochre and DD mutant alleles fail to support the growth of this phage. Moreover, as also reported here, the ochre and DD variants of *ssrA*<sup>Ng</sup> support growth of *N.gonorrhoeae*. The second indication of such specificity derived from our observation that colonies of *N.gonorrhoeae* derivatives that had *ssrA*<sup>Ec</sup> as the sole functioning *ssrA* allele on the chromosome appeared to have acquired a mutation(s) during their initial growth that increased growth rate and led to colony formation. Northern blot analysis showed that the levels of *E.coli* tmRNA expressed in *N.gonorrhoeae* were comparable to levels expressed in K37 (an *E.coli* strain wild-type for *ssrA*<sup>Ec</sup>) or to levels of gonococcal tmRNA expressed in gonococcal strain N400. Therefore, it seems unlikely that differences in levels of tmRNA can explain the apparent need for a mutation(s) for *E.coli* tmRNA to function fully in *N.gonorrhoeae*. It is more likely that there is some qualitative difference between these two tmRNAs that explains the inability of *E.coli* tmRNA to support gonococcal growth effectively without additional changes. Our studies suggest that if this increase in growth rate is due to a mutation(s), the mutation(s) is not located in the *ssrA*<sup>Ec</sup> gene, but somewhere in the *N.gonorrhoeae* genome.

In considering possible targets for such a mutation, one obvious candidate is the *smpB* gene. SmpB protein is required for tmRNA activity both in *E.coli* (Karzai *et al.*, 1999) and *N.gonorrhoeae* (J.Withey, C.Huang and D.I.Friedman, in preparation). These SmpB proteins, like their cognate tmRNAs, share significant sequence identities (45% amino acid identity for the SmpB proteins), but obviously have differences. Nevertheless, a mutant SmpB cannot explain our results since DNA sequence analysis of the *smpB* gene of gonococcal strain C103 revealed no differences from the sequence of the wild-type gonococcal *smpB* gene (data not shown). This result does not rule out the possibility that a mutation affecting another protein influences SmpB–tmRNA interaction.

Since the *E.coli* and gonococcal tmRNAs have nearly identical tag sequences, sequences outside of the region encoding the tag may influence tmRNA function. Obviously, the tmRNA structure is important (Komine *et al.*, 1994; Williams and Bartel, 1996; Felden *et al.*, 1997; Zwieb *et al.*, 1999), and our studies with the *ssrA*<sup>Ng/UG</sup> mutant show that for growth of both  $\lambda$ immP22hy25 and *N.gonorrhoeae*, amino acid charging is necessary for tmRNA function. Since both *E.coli* and *N.gonorrhoeae* *ssrA* encode similar signals for amino acid charging, we must look elsewhere to explain the functional differences between these two tmRNAs.

It is important to note for this consideration that the gonococcal *ssrA*<sup>Ng/ochre</sup> and *ssrA*<sup>Ng/DD</sup> mutant genes are functional in *N.gonorrhoeae*. This suggests that these variants of *ssrA*<sup>Ng</sup> may function more effectively in *N.gonorrhoeae* than in *E.coli*. We can offer three explanations for such an effect of the bacterial environment. Firstly, a difference in the sequence outside the tag region

**Table IV.** Bacterial strains

Strain	Genus	Parental strain	Plasmid	Relevant genotype	Source/reference
K37	<i>E.coli</i>			<i>ssrA<sup>Ec</sup></i>	this laboratory
K8619	<i>E.coli</i>	K37		<i>ssrA<sup>Ec</sup>::cat</i>	Withey and Friedman (1999)
K8745	<i>E.coli</i>	K8619	pGCTm	<i>ssrA<sup>Ng</sup></i>	this work
K9101	<i>E.coli</i>	K8619	pGSmu1	<i>ssrA<sup>Ng</sup>/-SryI</i>	this work
K9102	<i>E.coli</i>	K8619	pGSmu2	<i>ssrA<sup>Ng</sup>/UG</i>	this work
K9335	<i>E.coli</i>	K8619	pGSmu3	<i>ssrA<sup>Ng</sup>/ochre</i>	this work
K9336	<i>E.coli</i>	K8619	pGSmu4	<i>ssrA<sup>Ng</sup>/DD</i>	this work
K9337	<i>E.coli</i>	K8619	PGSmu5	<i>ssrA<sup>Ng</sup>/HindIII</i>	this work
VD300	<i>N.gonorrhoeae</i>			<i>ssrA<sup>Ng</sup></i>	Koomey <i>et al.</i> (1987)
N400	<i>N.gonorrhoeae</i>	VD300		<i>ssrA<sup>Ng</sup> recA6(tetM)<sup>a</sup></i>	Freitag <i>et al.</i> (1995)
C101	<i>N.gonorrhoeae</i>	N400		<i>ssrA<sup>Ng</sup>/ssrA<sup>Ng</sup></i>	this work
C102	<i>N.gonorrhoeae</i>	N400		<i>ssrA<sup>Ng</sup>/ssrA<sup>Ec</sup></i>	this work
C103	<i>N.gonorrhoeae</i>	N400		<i>ssrA<sup>Ng</sup>::cat/ssrA<sup>Ec</sup></i>	this work
C104	<i>N.gonorrhoeae</i>	N400		<i>ssrA<sup>Ng</sup>/ssrA<sup>Ec</sup>b</i>	this work
C105	<i>N.gonorrhoeae</i>	C103		<i>ssrA<sup>Ng</sup>/ssrA<sup>Ec</sup></i>	this work

<sup>a</sup>The expression of the *recA* gene is IPTG inducible.

<sup>b</sup>This *ssrA<sup>Ec</sup>* gene is derived from that of C103.

could influence some interaction of tmRNA. Secondly, a sequence difference could result in differences in structure that could influence such an interaction. Thirdly, the variant tmRNAs could function similarly in *E.coli* and *N.gonorrhoeae*, but the required levels of tmRNA activity necessary for *N.gonorrhoeae* viability may be less than those required for phage growth.

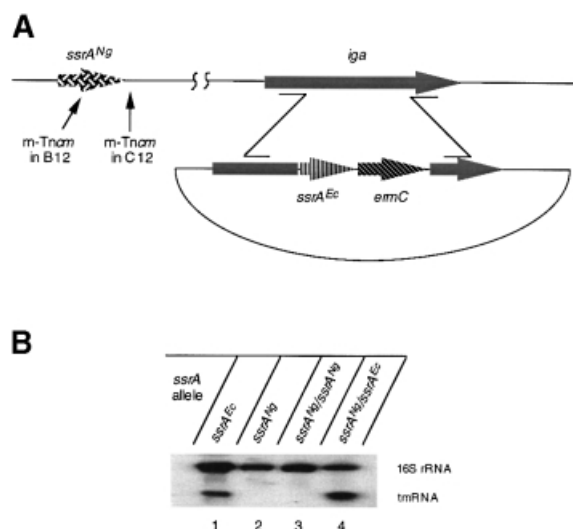
Why is tmRNA essential for *N.gonorrhoeae* but not for *E.coli*? There are two obvious differences between these bacteria. First, the *N.gonorrhoeae* genome is about half the size of the *E.coli* genome (Roe *et al.*). Secondly, *N.gonorrhoeae* only has one niche, the human host, while *E.coli* has two, existing within as well as without an animal host (Britigan *et al.*, 1985). These two characteristics are likely to be related. Because it need only encode functions for survival in one niche, *N.gonorrhoeae* is likely to require a smaller array of gene products and, thus, have fewer redundant functions. Based on these considerations, we suggest three possible reasons why *ssrA* is essential in *N.gonorrhoeae*. (i) The activity supplied by tmRNA is essential and, unlike *E.coli*, a function capable of substituting for *ssrA* is not present to maintain proper levels of some essential protein(s) normally present in limited amounts. (ii) *Neisseria gonorrhoeae* is missing a function(s) that can substitute for the protein(s) that are present at sub-physiological levels in the absence of tmRNA. (iii) Ribosome stalling is more severe in *N.gonorrhoeae*.

Even though previous studies have shown that those bacteria in which the *ssrA* gene is inactivated are viable, the nearly universal presence of tmRNA (Williams, 1999) indicates that tmRNA provides an important survival advantage to bacteria. Thus, a bacterium such as *N.gonorrhoeae*, in which *ssrA* is an essential gene, should prove to be extremely useful in determining the critical role or roles of tmRNA.

## Materials and methods

### Bacterial strains and phage

Bacterial strains are listed in Table IV.  $\lambda$ immP22hy25 was constructed by an *in vivo* cross between  $\lambda$  and P22 (Hilliker and Botstein, 1976).



**Fig. 6.** Substitution of the *N.gonorrhoeae* tmRNA with *E.coli* tmRNA in *N.gonorrhoeae*. (A) Insertion of *ssrA<sup>Ec</sup>* in the *N.gonorrhoeae* genome at the *iga* locus. PCR fragments containing *ssrA<sup>Ec</sup>* and the *ermC* gene (encoding resistance to erythromycin) were cloned into the gonococcal non-essential *iga* gene and the entire fragment was cloned in plasmid pUP6. The resulting plasmid, pIGA::*ssrA<sup>Ec</sup>*, was transformed into N400 and erythromycin-resistant (*Em<sup>r</sup>*) transformants were isolated. These *Em<sup>r</sup>* colonies, shown to be heterodiploid for *ssrA* (*ssrA<sup>Ng</sup>/ssrA<sup>Ec</sup>*), were formed by a double crossover within the *iga* sequences flanking the *ssrA<sup>Ec</sup>-ermC* insert. Strain C102 is one of these heterodiploid derivatives of N400 that was chosen for further study. Two derivatives of GCTm carrying m-TnOm insertions, B12 (in *ssrA<sup>Ng</sup>*) and C12 (downstream of *ssrA<sup>Ng</sup>*), were used to transform C102 to determine if *E.coli* tmRNA is sufficient to support gonococcal viability. (B) Northern blot analysis to identify *E.coli* tmRNA in *N.gonorrhoeae*. RNA was isolated from bacterial strains whose genotypes are listed at the top of each lane. Two probes were used, one specific for *E.coli* tmRNA and the other specific for 16S rRNA; the latter served as a control to indicate the relative amounts of RNA loaded in each lane.

### Plasmids

pUP6 (Wolfgang *et al.*, 1999) contains two gonococcal uptake sequences (GCU) required for *N.gonorrhoeae* transformation and two *NotI* sites that flank both the polylinker and the uptake sequences. Cleavage at the *NotI* sites provided fragments for transformation. Plasmid pGCTm was constructed by cloning the *ssrA<sup>Ng</sup>*-containing GCTm PCR product into the pUP6 vector. Plasmids pGSmu1–GSmu5 are derivatives of pGCTm



that, respectively, carry the mutant alleles *ssrA<sup>Ng/Sil-</sup>*, *ssrA<sup>Ng/UG</sup>*, *ssrA<sup>Ng/ochre</sup>*, *ssrA<sup>Ng/DD</sup>* and *ssrA<sup>Ng/HindIII</sup>* (see 'Construction of *ssrA<sup>Ng</sup>* mutant alleles' for details). Plasmid pIGA, a derivative of pUP6 with a 4 kb fragment containing the gonococcal IgA1 protease gene (*iga*) cloned into the *HindIII* site of the vector, served as the precursor for construction of two plasmids. Each of these plasmids carries an insert cloned into the *SacI* site linked to an *ermC* gene in the *iga* gene; pIGA::*ssrA<sup>Ec</sup>* with the *ssrA<sup>Ec</sup>* gene and pIGA::*kn<sup>r</sup>* with a gene encoding resistance to kanamycin (*kn<sup>r</sup>*).

### Media

*Escherichia coli* strains were grown on LB plates or in LB broth (Sambrook *et al.*, 1989). The *N.gonorrhoeae* strains were grown on clear solid medium (Gc agar) at 37°C in 5% CO<sub>2</sub> or liquid Gc medium pre-incubated in 5% CO<sub>2</sub> (Gc broth) (Kookey *et al.*, 1987). Antibiotics were used at the following final concentrations: 50 µg/ml of kanamycin for both *E.coli* and *N.gonorrhoeae*; 12.5 and 10 µg/ml of chloramphenicol for *E.coli* and *N.gonorrhoeae*, respectively; 300 and 8 µg/ml of erythromycin for *E.coli* and *N.gonorrhoeae*, respectively.

### Cloning procedures

The GcTm fragment containing *ssrA<sup>Ng</sup>* was isolated for cloning by PCR, using Gc1 (5'-CGTTCGAGCATATCGGTTTC-3') and Gc4 (5'-CCGA-GATACTGAAAGGTGCG-3') as primers, and genomic DNA of strain N400 as the template.

The *ssrA<sup>Ec</sup>* gene was isolated by PCR using Ec/Gc1 (5'-GATCGAGCTCGGCTATCACATCCGACAC-3') and Ec/Gc2 (5'-GATCGAGCTCGCTTACTGCCACTGGACTT-3') as primers, and *E.coli* genomic DNA as the template. The synthesized fragment was digested with *SacI* and, as described above, cloned into the *SacI* site of plasmid pIGA.

To introduce the *ssrA* gene of *E.coli* into the chromosome of *N.gonorrhoeae*, the non-essential gonococcal IgA1 protease (*iga*) gene cloned into pUP6 plasmid was used as the crossover sequence. The *ermC* gene, encoding erythromycin resistance and located adjacent to the cloned *ssrA<sup>Ec</sup>* gene, served as the selective marker (Figure 6).

### In vivo assay for tmRNA function

Functional activities of the cloned *N.gonorrhoeae* *ssrA* wild-type and mutant genes were assessed by testing for support of growth of *λimmP22hy25*. Plasmids with the cloned *ssrA* genes to be tested were transformed into K8619, a derivative of our standard *E.coli* strain K37 that carries the *ssrA::cat* allele (Withey and Friedman, 1999). Overnight cultures of the K8619 derivatives carrying the plasmid constructs to be tested were used to form lawns on LB plates containing, when indicated, the appropriate antibiotic. Phage growth was assessed by measuring efficiency of plating (EOP) as described by Bear *et al.* (1984).

### Northern blot analysis and DNA sequencing

Northern blot analysis was performed essentially as described by Sambrook *et al.* (1989). Total bacterial RNA samples were prepared using the Qiagen RNA kit (Qiagen). One probe that hybridizes tmRNAs from *E.coli* and *N.gonorrhoeae* was synthesized employing the *ssrA<sup>Ec</sup>* gene as a template and labeled with <sup>32</sup>P using the DNA random labeling kit from Boehringer Mannheim. The other probe that specifically detects *E.coli* tmRNA was an oligonucleotide, end-labeled with <sup>32</sup>P using T4 polynucleotide kinase (New England Biolabs), whose sequence (5'-GTCAGTCTTTACATTCGCTTG-3') corresponds to a region of *ssrA<sup>Ec</sup>* that differs from *ssrA<sup>Ng</sup>* (see Figure 1).

DNA sequencing was performed as previously described (Sanger *et al.*, 1977). Gonococcal colonies were used as the source of template DNA to confirm appropriate allelic replacement. In other cases, purified plasmid or chromosome DNA was used as template DNA.

### Shuttle mutagenesis

The procedures as described by Seifert *et al.* (1986) were employed to generate a library of transposon insertion mutations along the length of the GcTm fragment. The positions of the m-Tncm transposon insertions were identified by PCR and confirmed by DNA sequencing.

### Transformation of *N.gonorrhoeae*

See Table IV for a description of the strains. Transformation of *N.gonorrhoeae* was performed essentially as described by Freitag *et al.* (1995). Since *recA* is under the control of the plac promoter in N400 and its derivatives, isopropyl-β-D-thiogalactopyranoside (IPTG) was used to induce the transient expression of RecA during transformation. Strain C101, which is diploid for *ssrA<sup>Ng</sup>*, was generated by transforming N400

with pGcTm and selecting for kanamycin-resistant transformants. Since the bacterium does not support replication of this plasmid (Drake *et al.*, 1997), stable transformants will only be formed if the plasmid integrates into the gonococcal chromosome. Strain C102 was constructed by allelic replacement of the *iga* gene with the *iga::ssrA<sup>Ec</sup>* allele by transforming the *iga::ssrA<sup>Ec</sup>* fragment released from pIGA::*ssrA<sup>Ec</sup>* into the N400 strain and selecting for erythromycin-resistant transformants (Figure 6A). C104, *ssrA<sup>Ng/ssrA<sup>Ec</sup></sup>*, was made by transforming the genomic DNA of C103 into N400 and isolating erythromycin-resistant colonies. C105 was constructed from C103 by replacing the *ssrA<sup>Ng::cat</sup>* allele with the wild-type *ssrA<sup>Ng</sup>* allele. C103 cells were placed on Gc plain plates (no antibiotics added) pre-impregnated with IPTG to induce RecA expression, then a drop of pGcTm DNA solution was placed on the bacteria. Bacteria from the pGcTm DNA-treated area were transferred to fresh Gc plain plates to isolate single colonies. Colonies were tested for growth on Gc plain plates and Gc plates containing chloramphenicol. Colonies that had become sensitive to chloramphenicol were tested by PCR to determine if the wild-type *ssrA<sup>Ng</sup>* allele had replaced the *ssrA<sup>Ng::cat</sup>* allele.

### Construction of *ssrA<sup>Ng</sup>* mutant alleles

Mutant *ssrA<sup>Ng</sup>* alleles were constructed using splicing by overlap extension (SOE) (Horton *et al.*, 1989). The following are brief descriptions of the GcTm variants (nucleotide numbering is based on the sequence of the deduced mature tmRNA). (i) *ssrA<sup>Ng/UG</sup>* has nucleotide substitutions that change the G:U base pair at the acceptor stem of tmRNA and a substitution that eliminates a *SlyI* restriction site near the 5' terminus of the *ssrA<sup>Ng</sup>* gene. The UG change was engineered by two substitutions, G3T and T357G. The *SlyI* change, five nucleotides away from the G3T substitution, was engineered by a nucleotide substitution at position 10 (a change in the recognition site from CCTTGG to C~~C~~T~~T~~GG) (Figure 4). (ii) *ssrA<sup>Ng/ochre</sup>* has a nucleotide substitution that changes the fourth codon of the putative tagging sequence (GAA) to a nonsense codon (TAA) (Figure 4). (iii) *ssrA<sup>Ng/DD</sup>* has nucleotide substitutions that change codons 9 and 10 of the putative tagging sequences from alanine codons (GCA and GCT) to aspartic acid codons (GAC and GAT) (Figure 4). The GcTm variants with changes in the tagging sequence also have a substitution at nucleotide 13 of the tagging sequence that introduces a *HindIII* site (Figure 4). The changes creating the *HindIII* or eliminating the *SlyI* sites do not affect tmRNA function; tmRNA expressed from genes having one or the other of those changes supported *λimmP22hy25* growth in *E.coli* and *N.gonorrhoeae* growth (Tables I and II).

The C7 DNA used as template in the PCRs has the m-Tncm insertion located immediately downstream of *ssrA<sup>Ng</sup>* (Figure 3). Chloramphenicol resistance conferred by the transposon facilitated selection of allelic replacements. Cm<sup>r</sup> transformants were screened for the appropriate allelic replacement by restriction enzyme analysis of PCR products. DNA sequencing was employed to establish conclusively that the appropriate allelic replacement had occurred.

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