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The *Bacillus subtilis* Ochre Suppressor *sup-3* Is Located in an Operon of Seven tRNA Genes

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Most *Bacillus subtilis* tRNA genes have been isolated from lambda libraries by use of probes that hybridize to tRNA or rRNA sequences. None of those genes map to the region of the *sup-3* mutation. By cloning of the *sup-3* allele, a cluster of seven tRNA genes (the *trnS* operon) that had not been isolated by other methods was identified. In principle, this approach could be used to isolate at least one more predicted tRNA-containing operon in this bacterium. The *trnS* operon was shown to contain tRNA genes for Asn (GUU), Ser (GCU), Glu (UUC), Gln (UUG), Lys (UUU), Leu (UAG), and Leu (GAG). The *sup-3* mutation was found to be a T-to-A transversion that changes the anticodon of the lysine tRNA from 5′-UUU-3′ to 5′-UUA-3′. This result agrees with previous work that determined that the *sup-3* mutation causes lysine to be inserted at ochre nonsense mutations.

Many of the tRNA genes of *Escherichia coli* were identified by mapping and cloning of nonsense suppression mutations (15). Strains with these mutations were shown to have altered tRNA molecules that cause stop codons to be translated. Analysis of the mutations has been important in unraveling various aspects of tRNA function. Nonsense suppression mutations have not been exploited in studies of tRNA function and gene organization in the gram-positive bacterium *Bacillus subtilis*. In this paper, we describe the cloning and sequencing of the first nonsense suppression mutation of *B. subtilis* and report the sequence of a previously unidentified operon of *B. subtilis* that has seven tRNA genes. The operon has been designated *trnS*. The suppressor that was cloned and sequenced is the *sup-3* mutation.

Prior to this study, eight *B. subtilis* operons with tRNA genes were cloned and sequenced (6–9, 18, 22, 24, 27, 28, 30). Hybridization experiments have indicated that most of the tRNA genes of *B. subtilis* are located in these operons (9, 24, 27). This result is surprising, because the total number of different anticodons represented by the sequenced tRNA genes is only 28, compared with 41 that have been identified in *E. coli* (9). It will be interesting to learn whether the *B. subtilis* operons that remain to be identified contain genes for tRNAs with the unrepresented anticodons. It is possible that the translation apparatus of *B. subtilis* is different from that of *E. coli* and that it does not require as many different tRNA species. Researchers have suggested that *B. subtilis* may have less restrictive codon-anticodon pairing rules than does *E. coli* (9). Operons that contain tRNA genes have been cloned from *B. subtilis* by a number of techniques. None of these methods has involved the analysis of suppression mutations. We chose to clone the *sup-3* mutation because it has been mapped to a region in which none of the previously sequenced tRNA genes are located (1). We believed that cloning of the suppressor mutation would probably identify new tRNA genes.

The *B. subtilis* ochre suppression mutation *sup-3* was isolated by C. P. Georgopoulos in the 1960s (5). In 1979, Henner and Steinberg (13) used transduction with phage PBS-1 to locate *sup-3* at about 36° on the *B. subtilis* map (1). In 1981, Lipsky et al. (17) reported isolating a specialized transducing SPβ phage that carries the *sup-3* mutation. They constructed a *B. subtilis* strain that has an integration-defective mutant of phage SPβ inserted into its chromosome at about 38° on the map. Because of the close proximity of the integrated phage and the *sup-3* mutation, induction of the phage produced a lysate that included a phage that carried the nonsense suppressor. They constructed another strain that has the same SPβ insertion with *sup-44*, a different suppression mutation. Phage induction produced a lysate that carried *sup-44*. This result strongly suggests that *sup-3* and *sup-44* are in the same region of the chromosome. These are the only nonsense suppression mutations that have been mapped on the *B. subtilis* chromosome (1).

We used the method of Poth and Youngman (23) and Youngman et al. (33) to isolate a phage clone with the *sup-3* mutation. They constructed a plasmid, pCV-2, that can be used to clone fragments of DNA into phage SPβ. The technique produces recombinant SPβ phage that have sequences derived from pBR322 to facilitate subcloning of DNA fragments from the phage onto *E. coli* plasmids. An SPβ library was constructed with DNA from a *sup-3* strain of *B. subtilis*. Phage carrying *sup-3* were isolated from the library by selecting for suppression of an ochre nonsense mutation in the unlinked *metB* gene.

MATERIALS AND METHODS

Bacterial strains and plasmid. The bacterial strains used in this study are listed in Table 1. Plasmid pCV-2 was obtained from P. Youngman.

Media. Bacterial strains were grown on plates of tryptose blood agar base (Difco) or Spizizen minimal medium with 0.5% glucose, 10 mg of tryptophan per liter, 1 μg of biotin per liter, 10 μM $MnCl_2$, and 17 g of purified agar per liter (11). Additives included 50 μg of ampicillin, 5 μg of chloramphenicol, or 40 μg of methionine per ml. Liquid cultures were grown in Luria-Bertani broth (11). Cultures were aerated at 37°C.

DNA restriction, ligation, and transformation. DNA restriction and ligation were performed as described by the manufacturers. Restriction enzymes were purchased from New England Biolabs, United States Biochemicals (USB), and Amersham. T4 DNA ligase was obtained from USB. *E. coli*

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TABLE 1. Bacterial strains used in this study

Strain	Genotype	Source
B. subtilis		
CU1050	*leu met thr sup-3 att*SPβ	34
CU1064	*metB5 attSP*β	34
CU1065	*trpC2 att*SPβ	34
CU1962[a]	*sup-3* [*metB5*]	Laboratory collection
CU1142	*dal-1 metB5*	17
CU3068	*metB5 thr-5 att*SPβ	Laboratory collection
CU4058	*metB5 recE4 att*SPβ	Laboratory collection
CU4831	*metB5* (SPβ *c2 del-2*::Tn*917*)	Laboratory collection
CU4931[a,b]	*sup-3 zai*::*cat* [*metB5*]	This work
E. coli		
Jm109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-pro*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*)	31
Jm2R−	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-pro*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*) *mcrAB serB*::Tn*5*	29

[a] The *metB5* mutation is present in CU1962 and CU4931 but is suppressed by the *sup-3* mutation.
[b] *zai*::*cat* indicates the insertion of the chloramphenicol resistance gene of pC194 into the *Sal*I site located 3′ to the tRNA operon described in this paper.

transformations were performed as described by Hanahan (10). The method described by Yasbin et al. (32) was used for *B. subtilis* transformations.

PCR. The polymerase chain reaction (PCR) was used to amplify sequences from *B. subtilis* chromosomal DNA. The primers used were A11 (5′-GAAGATCTCGCAATGTAGT GGAC-3′) and A7 (5′-GAAGATCTGCTTATTCCAC CCAC-3′). The 3′ end of primer A11 corresponds to the nucleotides from positions 42 to 57. The 3′ end of primer A7 is complementary to the nucleotides from positions 2316 to 2301. One hundred-microliter reaction mixtures included 200 ng of chromosomal DNA, 1 μM each primer, 10 μM each deoxynucleotide triphosphate, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, 16 mM $MgCl_2$, and 2.5 U of *Thermus aquaticus* DNA polymerase (Promega). Each reaction was cycled 30 times in a Perkin-Elmer Cetus DNA thermocycler at 92°C for 1 min, 35°C for 2 min, and 72°C for 4 min.

Cloning of a DNA fragment with the *sup-3* allele into the *B. subtilis* bacteriophage SPβ. DNA was isolated from a *sup-3* strain of *B. subtilis* and cloned into phage SPβ by the method of Poth and Youngman (23). Fragments of DNA were isolated from a partial *Mbo*I digest of CU1962 *sup-3* [*metB5*] and ligated to fragments derived from plasmid pCV-2 digested with *Bam*HI and *Sst*I (33). Plasmid pCV-2 includes DNA from SPβ and from transposon Tn*917*. It contains a cloning site, a *cat* gene that gives rise to chloramphenicol resistance (Cm^r), and DNA from pBR322 that permits the isolation of cloned DNA directly in *E. coli*. The *metB5* allele is suppressible by *sup-3* (17). CU4831 *metB5* (SPβ *c2 del-2*::Tn*917*) was transformed with the resulting concatamer. Met$^+$ colonies of bacteria that received the *sup-3* allele were selected on minimal medium plates with chloramphenicol.

Phage lysates and DNA isolation. Ten-milliliter and 100-ml SPβ lysates of the *sup-3* lysogens were made by heat and mitomycin induction as described by Weiner (29). CU4058 *metB5 recE4 att*SPβ was infected with one of the lysates. Colonies were isolated on minimal medium with chloramphenicol. These Met$^+$ bacteria had received a recombinant SPβ phage carrying the *sup-3* mutation. The phage was named SPβS. A 100-ml lysate made from the RecE4 (SPβS) strain was used to infect a logarithmically growing culture of the SPβ-sensitive strain CU1050. The culture was incubated for 3 h at 37°C and kept at 4°C overnight. The remaining cells were removed by centrifugation. Phage SPβS was precipitated with polyethylene glycol, and DNA was extracted as described by Fink et al. (4). Attempts to isolate phage SPβS DNA from a Rec$^+$ strain were not successful; phage DNA isolated from a wild-type strain was found to have a deletion that had removed the *sup-3* allele.

Isolation of plasmid pDG200. A 10-kb DNA fragment was isolated from a *Bgl*II digest of phage SPβS DNA with Geneclean (Bio 101). The DNA was circularized by ligation and used to transform *E. coli* Jm2R−. Plasmids that can replicate in *E. coli* were isolated because the vector used to construct SPβS (pCV-2) contains sequences derived from *E. coli* plasmid pBR322 (33). These sequences were contained in the 10-kb fragment isolated from the phage. Colonies were isolated on tryptose blood agar base with ampicillin. The resulting 10-kb plasmid was designated pDG200 (Fig. 1).

Cloning of the chloramphenicol acetyl transferase gene of

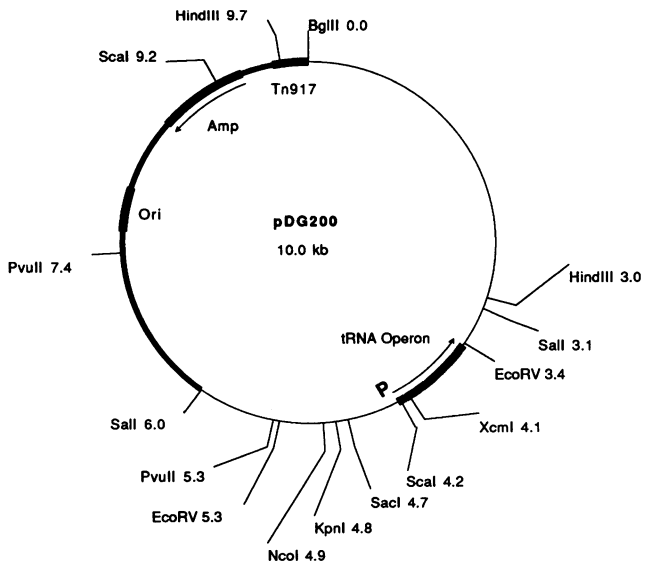

FIG. 1. Restriction map of pDG200. Sequences from the *Bgl*II site at 0 kb to the *Sal*I site at 6 kb were derived from the DNA fragment that was cloned in phage SPβS. The rest of the plasmid was derived from cloning vector pCV-2.

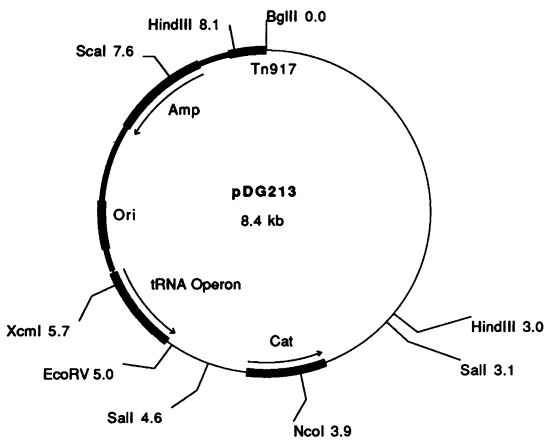

FIG. 2. Restriction map of pDG213. Sequences of plasmid pDG200 from the *Sca*I site at 4.2 kb to the *Pvu*II site at 7.4 kb were deleted spontaneously. This deletion removed the first tRNA gene of the operon and all upstream sequences, including the putative promoter. To construct pDG213, the plasmid with the deletion was linearized at the *Sal*I site and ligated to a 1.5-kb fragment that was derived from pC194 and that includes the chloramphenicol resistance gene.

pC194 into a derivative of pDG200. Plasmid pDG200 was cut with *Pvu*II and religated. A spontaneous deletion occurred, yielding a plasmid with a deletion from the *Sca*I site at 4.2 kb to the *Pvu*II site at 7.4 kb. This deletion removed the first tRNA gene in the operon, part of the second tRNA gene, and over 1.8 kb of upstream sequences. We believe that the deletion was isolated because it removed the promoter of the operon. This plasmid was cut with *Sal*I and ligated to a 1.5-kb *Sal*I fragment containing the chloramphenicol acetyltransferase gene of pC194. The resulting plasmid was designated pDG211. This plasmid was shown to have two tandem copies of the pC194 fragment. It was cut with *Nco*I and religated to remove the extra sequence. The resulting plasmid was designated pDG213 (Fig. 2).

DNA isolation. Small amounts of plasmids were isolated by the boiling method of Holmes and Quigley (14). Larger amounts were purified by CsCl gradient centrifugation as previously described (19).

DNA sequencing. Sequencing was performed by the method of Sanger et al. (26). [α-^{35}S]dATP (specific activity, 3,000 Ci nmol^{-1}) and [γ-^{32}P]dATP (specific activity, 3,000 Ci nmol^{-1}) were purchased from Amersham. Plasmid sequencing was performed with Sequenase kits as specified by the manufacturer (USB). Sequencing of PCR products was done according to the procedures supplied with Promega fmol sequencing kits.

Nucleotide sequence accession number. The sequence reported here has been submitted to the GenBank data base under accession number L13170.

RESULTS

Isolation of a recombinant SPβ phage expressing the gene with the *sup-3* mutation. Fragments of the *B. subtilis* chromosome carrying the *sup-3* allele were cloned into the temperate phage SPβ by use of plasmid vector pCV-2 (33). The vector carries SPβ sequences and pBR322 sequences. Selection for Sup-3 was done with *metB5* as the suppressible marker. The *sup-3* mutation suppresses both the *metB5* and the *thr-5* mutations (17). To verify that the recombinant phage, which we named SPβS, expresses the *sup-3* allele, the phage was used to infect CU3068 *metB5 thr-5*. The resulting colonies were able to grow on minimal medium plates without methionine or threonine.

Cloning of the *sup-3* mutation into a plasmid. Plasmid pDG200 was isolated from phage SPβS (Fig. 1.) The plasmid was shown to contain the *sup-3* mutation by transforming CU1064 *metB5* and isolating transformants that did not require methionine for growth.

Sequence analysis. A 2.3-kb region of pDG200 was sequenced (Fig. 3). Seven tRNA genes were identified. Sequences that match the consensus sequence for *B. subtilis* vegetative promoters (20) were found just upstream of the first tRNA gene. A potential transcriptional terminator was found just downstream of the last tRNA gene (Fig. 3). One of the genes has a sequence that is identical to those of two previously sequenced *B. subtilis* lysine tRNA genes, except that it has the anticodon sequence 5′-UUA-3′ instead of 5′-UUU-3′ (6, 8, 28). This anticodon sequence allows the tRNA to translate the ochre stop codon 5′-UAA-3′. To determine whether this tRNA gene is the site of the *sup-3* mutation, the region was sequenced from a PCR product derived from a *sup*$^+$ strain of *B. subtilis*. The sequence of the anticodon of the wild-type strain was found to be 5′-UUU-3′.

Location and orientation of the operon on the *B. subtilis* chromosome. The *sup-3* mutation has been shown to be located close to the region of the alanine racemase gene (*dal*) on the chromosome of *B. subtilis* (13). To verify that the DNA cloned in pDG200 was derived from this region, we inserted a selectable marker into a derivative of pDG200, moved it into the *B. subtilis* chromosome, and used it to map the cloned region with respect to a mutation in the *dal* gene. Plasmid pDG213 was linearized at the *Bgl*II site and used to transform strain CU1064 *metB5*. Colonies were selected on minimal medium with chloramphenicol. DNA was extracted from one of the transformants, designated CU4931 *cat sup-3* [*metB5*]. This DNA was used to transform CU1142 *dal-1 metB5*. Colonies were selected for Sup-3, Dal$^+$, or Cm^r and tested for the acquisition of unselected markers (Table 2). All three markers were cotransformable, indicating that they are in the same region of the chromosome. The relative frequencies for the different classes of transformants suggest that the gene order is *cat–sup-3–dal*.

Search for *sup-44*. PCR was used to amplify sequences from strain CU1964 *sup-44 metB5*. *sup-44* is known to insert leucine into ochre codons (18a). Sequence analysis of the product did not identify any mutations in the Leu (UAG) or Leu (GAG) tRNA genes of *trnS*.

DISCUSSION

Significant differences in tRNA gene organization have been noted for *E. coli* and *B. subtilis* (9). Fewer operons containing tRNA genes have been found in *B. subtilis*. Several of these have larger numbers of tRNA genes than have been found in *E. coli* operons. Forty operons that contain tRNA genes have been identified in *E. coli* (16). While the maximum number of tRNA genes found in a single operon in *E. coli* is 7 (16), clusters of 21 and 16 tRNA genes have been reported for *B. subtilis* (6, 28). The tRNA genes of *E. coli* have been found in operons that contain rRNA genes and in operons with genes that encode proteins (16). Other tRNA genes are located in operons without other genes. Most of the *B. subtilis* tRNA genes that have been sequenced have been found in operons with rRNA genes (9). So far, *B. subtilis* tRNA genes have not been found in operons that have genes for proteins. This work

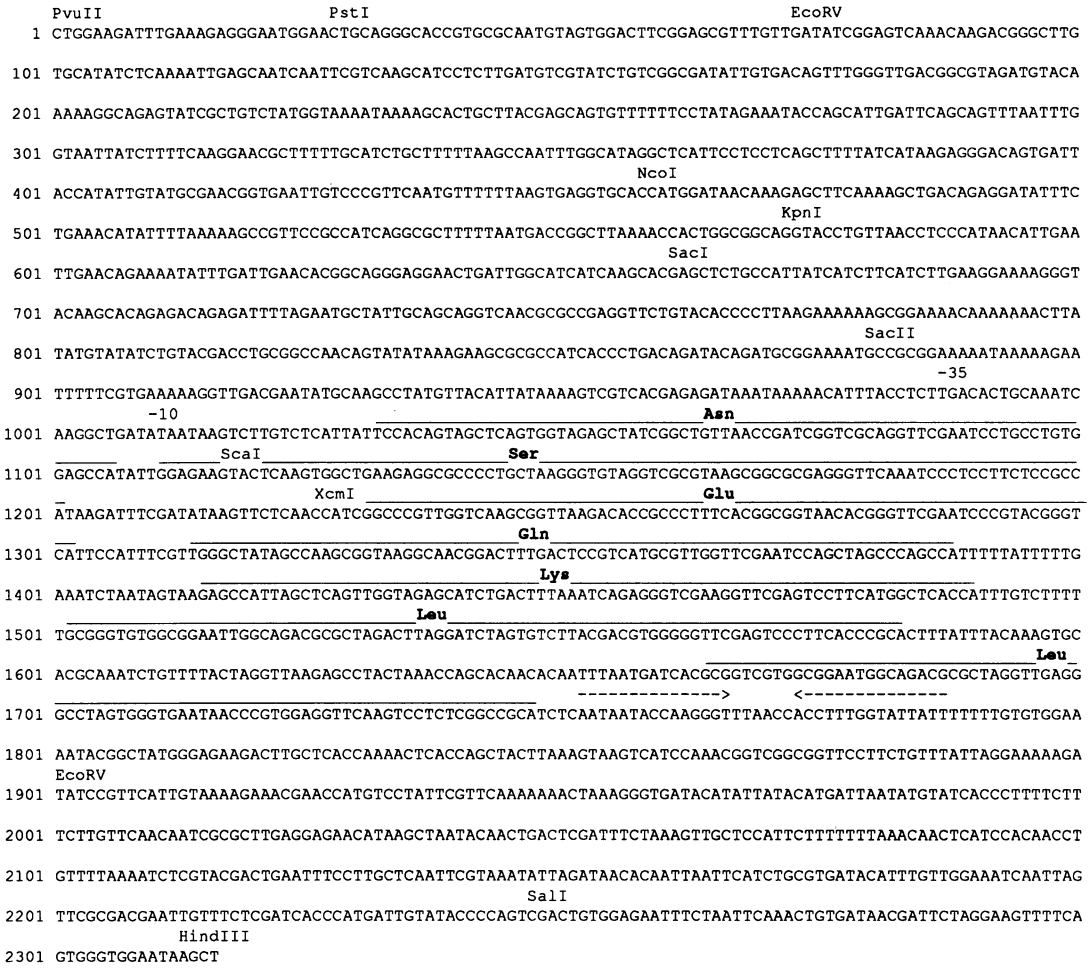

FIG. 3. Nucleotide sequence of a fragment of pDG200 that includes the tRNA operon. The tRNA genes are designated by lines above the sequence. The genes are identified by three letters representing amino acids above the anticodons. Sequences that are perfect matches to the −10 and −35 consensus sequences for *B. subtilis* promoters are indicated above the positions at which they occur in the fragment. Restriction endonuclease sites are identified over their sequences. A potential transcription termination sequence is suggested by convergent arrows.

reports the sequence of the ninth operon cloned from *B. subtilis* that contains tRNA genes. The operon does not contain genes for rRNA or a protein.

The sequence reported in this work brings the total number of tRNA genes identified in *B. subtilis* to 69. Seventy-eight tRNA genes have been found in *E. coli* (16). The *E. coli* genes code for tRNAs with 41 different anticodons. Genes coding for tRNAs with 29 different anticodons are now known in *B. subtilis*.

The fact that fewer tRNA species have been found in *B. subtilis* than in *E. coli* has led some researchers to hypothesize that *B. subtilis* may have fewer anticodons represented by its tRNAs (9). They have suggested that special codon-anticodon pairing rules could allow *B. subtilis* to translate its codons with fewer tRNA species. More flexible pairing rules have been established for the translation of mitochondrial genes in certain eukaryotes (2, 3, 12). There is evidence that similar pairing rules may be involved in the translation of genes in mycoplasmas, a group of bacteria with small genomes, many of which are intracellular parasites of eukaryotic cells (25). Similarities have been noted in tRNA gene organization in *B. subtilis* and *Mycoplasma capricolum* (9). It has been suggested

TABLE 2. Transformation of CU1142 *dal-1 metB5* with DNA from CU4931 *sup-3 zai::cat* [*metB5*][a]

Marker selected for:	Phenotype	No. of transformants
Sup-3	Cm^r Dal^+	22
	Cm^r Dal^-	25
	Cm^s Dal^+	11
	Cm^s Dal^-	46
Dal^+	Cm^r Sup-3	42
	Cm^r Sup^+	3
	Cm^s Sup-3	21
	Cm^s Sup^+	84
Cm^r	Dal^+ Sup-3	123
	Dal^+ Sup^+	0
	Dal^- Sup-3	14
	Dal^- Sup^+	0

[a] The phenotypes were scored as follows: Sup-3, does not require methionine; Sup^+ requires methionine; Cm^r, resistant to chloramphenicol; Cm^s, sensitive to chloramphenicol; Dal^+, does not require D-alanine; Dal^-, requires D-alanine.

that *Bacillus* spp. and the mycoplasmas may have descended from an ancestral bacterium in which translation required fewer tRNA species than are required in *E. coli*. Alternatively, the special translation rules observed in the mycoplasmas may have evolved as a consequence of the reduction in the size of their genomes when they became parasites of other cells.

The gene for leucine tRNA (GAG) is the only gene in the *trnS* operon that has an anticodon not represented by previously sequenced genes of *B. subtilis*. It should be noted that this gene codes for a tRNA species that is not found in the mycoplasmas. There are now five tRNA species that have been found in *B. subtilis* but not in *M. capricolum*. It is obvious that there are differences in the translation apparatus of *B. subtilis* and *M. capricolum*. It will be interesting to learn what other tRNA species are encoded by the remaining uncloned *B. subtilis* tRNA genes.

Our finding that the *sup-3* mutation is in a gene for a lysine tRNA is in agreement with the work of Mulbry et al. (21). They sequenced proteins derived from a chloramphenicol acetyltransferase (*cat*) gene in which they had inserted an ochre nonsense mutation. When they sequenced the Cat protein from cells carrying the *sup-3* mutation, they found that lysine was inserted at the nonsense codon. When they sequenced the Cat protein from a strain with the *sup-44* mutation, they observed that leucine was inserted at the codon (18a).

Because of the close map positions of *sup-3* and *sup-44* (17), we thought that the two mutations might be in genes within the same operon. Our preliminary evidence shows that they are not. Since *sup-44* appears to affect a leucine tRNA, we prepared and sequenced a PCR product, derived from a *sup-44* strain, that would include the two leucine tRNA genes of the *trnS* operon. We did not find any mutation in either of the genes. Genetic evidence also suggests that the *sup-44* mutation is not in *trnS*. If the *sup-44* mutation is in the region that was cloned into recombinant phage SPβS, it should be possible to transfer the mutation to the phage by homologous recombination. Transformation of DNA from a *sup-44* strain into an SPβS lysogen did not produce cells that had *sup-44* inserted into the prophage. Our conclusion that *sup-44* is not in *trnS* suggests that there might be another cluster of tRNA genes in this region. Cloning of the *sup-44* mutation may identify some of the remaining tRNA genes of *B. subtilis*.

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