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Structure and Organization of Genes for Transfer Ribonucleic Acid in *Bacillus subtilis*

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INTRODUCTION

Information about transfer ribonucleic acid (tRNA) genes in *Bacillus subtilis* has been accumulating rapidly in the last few years. The results demonstrate that some aspects of tRNA gene organization in *B. subtilis* differ from those found in *Escherichia coli*. In light of this new knowledge from *B. subtilis*, we either have to broaden our ideas of what is typical for procaryotes or consider that *B. subtilis*, and perhaps gram-positive organisms in general, may have a unique type of tRNA gene organization. The sporulation process that it undergoes may be responsible for some of the unique properties of *B. subtilis*. This differentiation, leading to the development of an endospore, involves regulation at the levels of transcription and translation and is characterized by a series of biochemical and physiological changes (41). The formation of endospores is dependent on the function of at least 30 genes (32).

This review considers the organization of tRNA genes in *B. subtilis*, sequence analysis of tRNA structural genes, and putative transcriptional control elments for *B. subtilis* tRNA gene sets. Little information apart from sequences of some mature tRNAs or tRNA genes is available for other *Bacillus* species. Some contrasts are offered to tRNA gene organization in *E. coli*, a topic previously reviewed by Ozeki (39) which will be reviewed by Ozeki and Fournier in a future issue of *Microbiological Reviews*. In the future it should be possible to compare these data with those for other procaryotes and particularly for other gram-positive organisms.

ORGANIZATION OF tRNA GENE REGIONS

Mapping of tRNA and rRNA Gene Clusters

One unusual aspect of tRNA gene organization in *B. subtilis* was already recognized in the 1960s: the tRNA genes are highly clustered. Transcriptional mapping studies to elucidate the functional organization of ribosomal ribonucleic acid (rRNA) and tRNA genes in *B. subtilis* indicated that tRNA genes would be clustered in groups in large transcriptional units (2). Density transfer experiments also suggested that the majority of both tRNA and rRNA genes were located in two regions of the chromosome, one near the origin of replication and a second near the terminus (38, 47).

Fine-structure mapping has been much more recent. In *E. coli*, suppressor mutations in specific tRNAs permitted the mapping of many genes. In *B. subtilis*, this source of mapping information has remained underdeveloped. Only

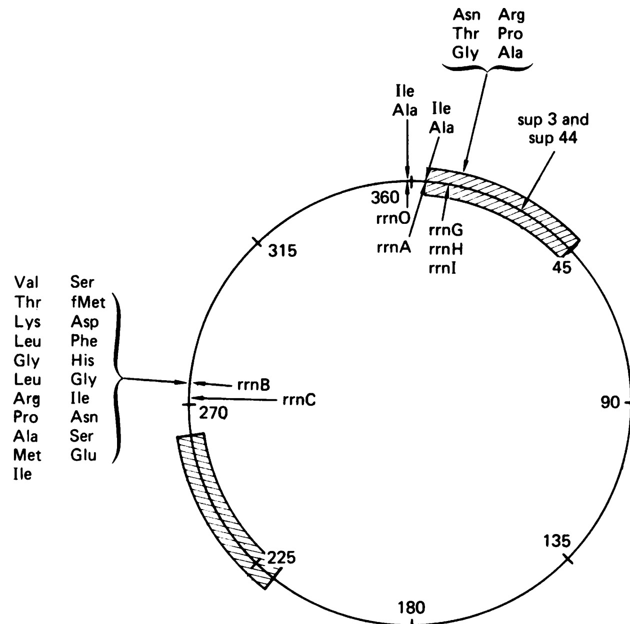

FIG. 1. Map position of tRNA and rRNA genes in *B. subtilis*. Map positions are for a map marked from 0 to 360 when given in this text. For maps of known loci in *B. subtilis*, refer to Henner and Hoch (19) or the *Bacillus* Genetic Stock Center Catalog (9). Loci for tRNA genes are given on the outside of the circle and for rRNA genes on the inside. The shaded area indicates the approximate position of tRNA and rRNA genes demonstrated by early hybridization experiments (38, 47). References are as follows: Ile, Ala, *rrnO*, and *rrnA* (37); *rrnB*, *rrnC*, *rrnG-I* (3); six-tRNA-gene cluster (sequenced published by Wawrousek and Hansen, 57; mapped by K. Bott, personal communication); 21-tRNA-gene cluster (sequence published by Green and Vold [16] and Wawrousek et al. [59]; mapped by Stewart et al. [52]); *sup-3* (20, 31); *sup-44* (31).

two suppressor mutations have been mapped (20, 31), both at position 30, (Fig. 1), and these have not yet been associated with a particular tRNA. Instead, most of the *B. subtilis* tRNA genes have been located somewhat fortuitously because of their association with rRNA gene sets. Figure 1 shows the inferred locations of both tRNA and rRNA genes in the conventional 360° circular map of the *B. subtilis* genome (19). The shaded area on the map indicates the areas rich in tRNA and rRNA genes indicated by early hybridization experiments (38, 47).

Ten rRNA gene sets occur in *B. subtilis*. Seven of these have been mapped, and their locations are indicated in Fig. 1 (for a discussion of the mapping of rRNA genes in *B. subtilis*, refer to Bott et al. [3]). Three ribosomal operons designated *rrnG*, *rrnH*, and *rrnI* map at position 10, and there are probably more yet to be mapped near this region. The ribosomal protein genes also are found predominantly in this area, at about 10° on the linkage map, although some ribosomal protein genes map outside the principal cluster (8). Two rRNA operons, *rrnB* and *rrnC*, map near position 270. Three rRNA operons remain unmapped. Two of these may map near position 225, in the same general location as the cluster of 16 tRNA genes (K. Bott, personal communication).

In addition, some tRNA gene regions have been isolated in cloned DNA fragments and sequenced but not yet located on the genome (see Table 1 for a list of all sequenced tRNA genes). Two rRNA gene sets, *rrnA* and *rrnO*, are positioned very close to the origin of replication in Fig. 1 (37). These are the only two of three analyzed rRNA gene sets which contain tRNAs in the 16S to 23S spacer region, and in both cases the tRNA genes code for an isoleucine and an alanine tRNA (3, 37). Another group of tRNA genes located downstream from the rRNA gene set designated *rrnB* (at 280° on the map) has been mapped in the area between *argA* and *thr* (51). This group of tRNA genes has been sequenced and contains 21 tRNA species (16). Another group of six tRNA genes (57) is probably located at map position 10, with still another group of 16 tRNA genes (58) around position 225 (K. Bott, personal communication).

The proximity of tRNA and rRNA genes found by the density transfer hybridization experiments and transcriptional mapping experiments mentioned above had led to the expectation that tRNA genes would be found in association with rRNA genes, possibly on the same transcriptional units. This idea was further supported by hybridization of tRNAs and rRNAs to *Eco*RI fragments of total genomic DNA from *B. subtilis* (57); the results of these hybridization experiments are illustrated in Fig. 2. This figure shows hybridization of tRNAs to about six to nine different restriction fragments. An analysis of *E. coli* similar to that shown in Fig. 2 for *B. subtilis* reveals locations of tRNA genes on at least 26 different restriction fragments (5). The assumption from such experiments is that tRNA genes in *B. subtilis* are found close together, whereas in *E. coli* they are scattered.

Several *Eco*RI fragments hybridize simultaneously to rRNA and tRNA probes. The 1.2- and 1.4-kilobase (kb)

TABLE 1. Summary of DNA regions containing tRNA genes in *B. subtilis*

tRNA genes	Association with rRNA genes	Designation by preceding rRNA gene set[a]	Designation from *Eco*RI fragments (57)	Reference(s)
Ile, Ala	Spacer between 16S and 23S rRNA genes	*trnA* and *trnO*		33, 37
Asn, Thr, Gly, Arg, Pro, Ala	Between two rRNA gene sets	*trnH*	*trrnB*	57
Lys, Glu, Asp, Phe	None known	*trnY*		61
Val, Thr, Lys, Leu, Gly, Leu, Arg, Pro, Ala, Met, Ile, Ser, fMet, Asp, Phe, His, Gly, Ile or Met, Asn, Ser, Glu	Promoter-distal to an rRNA gene set	*trnB*	*trrnE*	16, 58
Asn, Ser, Glu, Val, Met, Asp, Phe, Thr, Tyr, Trp, His, Gln, Gly, Cys, Leu, Leu	Promoter-distal to an rRNA gene set	—[b]	*trrnD*	58

[a] Since most *B. subtilis* tRNA genes are found in association with rRNA gene sets, a logical system for designating tRNA gene regions is to name them after the preceding rRNA gene set.

[b] Map position not published. Altman and Bott tentatively map this at about position 225 (personal communication).

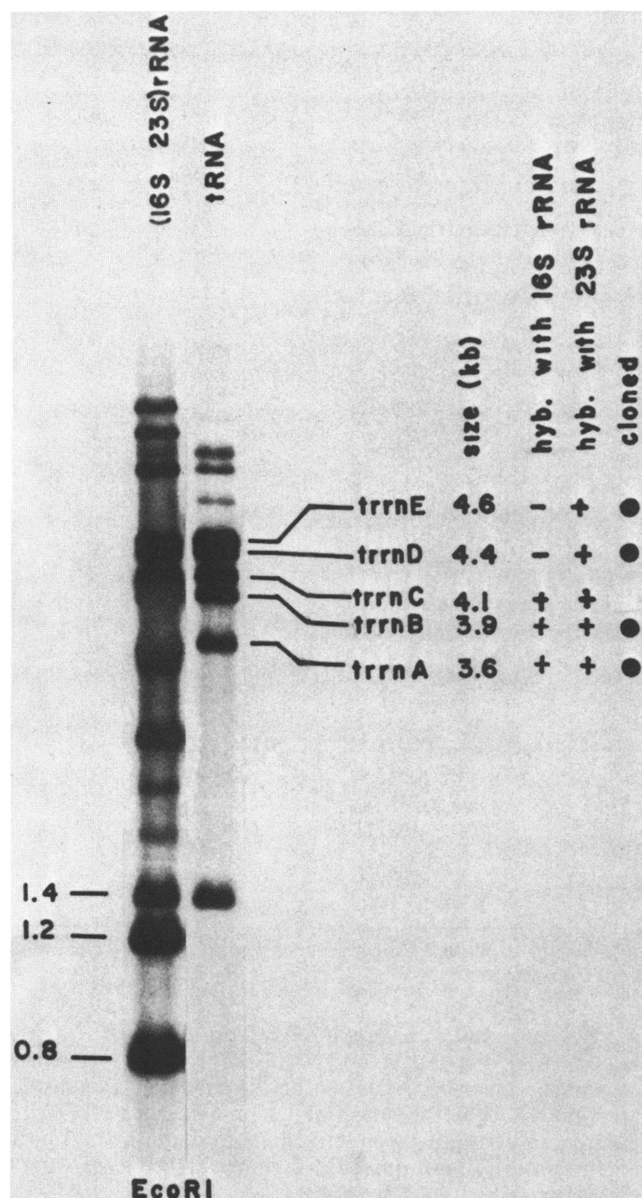

FIG. 2. Hybridization of tRNA and rRNA to restriction fragments from total genomic DNA from *B. subtilis*. Southern blot analysis of total genomic *B. subtilis* DNA restricted with *Eco*RI, electrophoresed on agarose, transferred to nitrocellulose, and hybridized to rRNA and tRNA probes (figure from Wawrousek and Hansen [57]).

fragments span the space between 16S and 23S rRNA; the 1.4-kb fragment contains spacer tRNAs in this region, whereas the 1.2-kb piece does not. Wawrousek and Hansen (57) designated the five other bands which hybridized prominently to both rRNA and tRNA as *trrnA* through *trrnE*. The *trrnA*, *B*, and *C* fragments hybridized with both 16S and 23S rRNA probes and therefore might occur between tandem rRNA gene sets. Bands D and E hybridized only to the 23S rRNA probe, suggesting that they might follow an rRNA gene set.

The complete sequence of *trrnB* was given by Wawrousek and Hansen (57); a preliminary sequence of that region had also been done (P. Zuber, Ph.D. thesis, University of Virginia, Charlottesville, 1982). It contains six tRNA genes occurring between tandem rRNA gene sets, probably *rrnH* and *rrnI* (K. Bott, personal communication). The sequence of *trrnD* (58) and *trrnE* (16, 58) has also been completed. They contain 16 and 21 tRNA genes, respectively, each occurring distal to an rRNA gene set. The sequence of *trrnA* and *C* has not yet been reported; it has been estimated that these regions will contain two to three and 8 to 12 tRNA genes, respectively (57). Table 1 lists the sequenced regions containing tRNA genes in *B. subtilis* and specifies associations with rRNA gene sets. Two methods have been used to designate tRNA gene regions for *B. subtilis* in Table 1. The *trrnA-E* designation was based on the lengths of *Eco*RI restriction fragments containing those regions (57). A system which may prove more logical in the future is to name the tRNA regions after the rRNA gene set which immediately precedes them, or in the case of the spacer tRNA genes, after the rRNA gene set in which they occur.

The one tRNA gene region which shows no apparent association with rRNA genes (61) is designated *trnY* in Table 1. No rRNA gene sequences were found on a 1-kb *Eco*RI-*Hin*cII fragment containing these four tRNA genes. This block of genes includes putative promoter and terminator sequences and probably represents an independent transcriptional unit. The DNA region sequenced originally came from a 3-kb *Eco*RI fragment, identified in the experiment illustrated in Fig. 2. The 3-kb fragment may have been too close to the 3.2-kb fragment in the gel to permit its detection.

Large Clusters of tRNA Genes—Evolutionary Considerations

Two immediate questions are raised by these recent observations of two large clusters of tRNA gene regions in *B. subtilis*. Why are these genes arranged in clusters? Do other organisms have the same type of arrangement?

Other examples of clustering of tRNA genes can be found in yeast mitochondria and in bacteriophage T4. About 21 tRNA genes are found in yeast mitochrondrial DNA. They are all coded by the same DNA strand, and they are found in close proximity, separated by anywhere from a few to as many as several hundred nucleotides (62). A 9-kilobase pair region between the 21S rRNA and *oxiI* genes contains 16 tRNA genes, and the existence of polygenic transcripts carrying at least five to seven tRNA gene sequences has been demonstrated (40). None contain intervening sequences, nor do they encode the CCA end of the tRNA. In the *E. coli* phage T4, eight tRNA genes are found together (c.f. the review by Schmidt [44]). There are no repeated gene sequences in this cluster, and these genes are transcribed as a unit (14).

Large tRNA gene clusters are clearly not a necessity since that type of tRNA gene organization is not found in *E. coli* (39). Little is known about tRNA gene organization in procaryotes besides *E. coli* and *B. subtilis*, so that the comparison cannot yet be extended very far. However, one very interesting study has shown that tRNA gene organization in *Spiroplasma* sp. strain BC3 is related to that found in *Bacillus* (41a). They have found a segment of DNA from *Spiroplasma* sp. strain BC3 containing the tRNA genes for Cys(GCA), Arg(ACG), Pro(UGG), Ala(UGC), Met(CAU), Met/Ile(NAU), and Ser(UGA). This segment is preceded by a putative promoter sequence. The part of the segment starting with the $tRNA^{Arg}$ gene through the $tRNA^{Ser}$ gene is very similar to a segment in the *B. subtilis* tRNA region containing 21 tRNA genes (Fig. 3). Sequence homologies in the tRNA structural genes range from about 87 to 91%.

```
Region                          tRNA GENE SEQUENCE                                        SPACER LENGTH
                                                                                             (bases)
-------------------------------------------------------------------------------------------------------
5'-flanking                 B. subtilis    6 tRNA genes       →                               - 9 -
  region                    Spiroplasma    tRNA (Cys,GCA)     →                               - 41 -

Arg (ACG)     GCGCCCGTAGCTCAATTGGATAGAGCGTTTGACTACGGATCAAAAGGTTAGGGGTTCGACTCCTCTCGGGCGCGCCA    - 16 -
                    *   *             *                        **        *    ***
              GCGCCCATAGATCAATTGGATAGATCGTTTGACTACGGATCAAAAGGTTGAGGGTTCGATTCCTTCTGGGCGCGCCA    - 23 -

Pro (UGG)     CGGGAAGTAGCTCAGCTTGGTAGAGCACATGGTTTGGGACCATGGGGTCGCAGGTTCGAATCCTGTCTTCCCGACCA    - 5 -
                 *        *               **           **                          *
              CGGAAAGTAGCTTAGCTTGGTAGAGCACTCGGTTTGGGACCGAGGGGTCGCAGGTTCGAATCCTGTCTTTCCGACCA    - 16 -

Ala (UGC)     GGGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCGGTTCGATCCCGCTAGGCTCCA        - 19 -
                 *  *                  *     *           *    **             * *  *
              GGGCCCGTAGCTCAGCTGGGAGAGCACCTGCCTTGCACGCAGGGGGTCGACGGTTCGATCCCGTTCGGGTCCACCA     - 32 -

Met (CAU)     GGCGGTGTAGCTCAGCTGGCTAGAGCGTACGGTTCATACCCGTGAGGTCGGGGGTTCGATCCCCTCCGCCGCTACCA    - 2 -
                   **            *       **   *         *      ** *    * ** *  ****
              GGCGGGATAGCTCAGCTGGTTAGAGCGCTCGGCTCATACCCGGGAGGTCAAGAGTTCAAGTCTCTTTCTCGCTACCA    - 11 -

Met (CAU)     GGACCTTTAGCTCAGTTGGTTAGAGCAGACGGCTCATAACCGTCCGGTCGTAGGTTCGAGTCCTACAAGGTCCACCA    - 6 -
Ile (NAU)          *                     **             ***    ***     *     *** *
              GGACCCTTAGCTCAGTTGGTTAGAGCATCCGGCTCATAACCGGATGGTCACTGGTTCAAGTCCAGTAGGGTCCACCA    - 35 -

Ser (UGA)     GGAGGAATACCCAAGTCTGGCTGAAGGGATCGGTCTTGAAAACCGACAGGGTGTCAAAGCCCGCGGGGGTTCGAATCCCTCTTCCTCCGCCA  - 17 -
                 *  *             *
              GGAAGATTACCCAAGTCTGGTTGAAGGGATC . . .

3'-flanking                       →    9 tRNA genes
  region                          →    unsequenced
```

FIG. 3. Comparison of a tRNA gene region from *B. subtilis* and *Spiroplasma* sp. strain BC3. Sequences for *Spiroplasma* are from M. J. Rogers, A. Steinmetz, and R. T. Walker (41a). Sequences for *B. subtilis* are from Green and Vold (16).

There is greater divergence in the intergenic spaces, with longer spacer segments in *Spiroplasma*, even though the *Spiroplasma* genome is only about 10^9 daltons. An interesting difference is that the $tRNA^{Ala}$ gene in *Spiroplasma* encodes the CCA end, whereas the $tRNA^{Ala}$ gene in *B. subtilis* lacks the CCA end. *Spiroplasma* sp. strain BC3 is a cell wall-less organism in the *Mycoplasmataceae* family (56). *Mycoplasma*, *Lactobacillus*, and *Bacillus* have been grouped together as related gram-positive organisms in procaryotic descent (13). Therefore, it seems possible that there may be features of tRNA gene organization in common among gram-positive organisms, and these may differ significantly in the case of *E. coli* and perhaps in other gram-negative organisms.

SEQUENCE ANALYSIS OF tRNA STRUCTURAL GENES

Sequences of Mature tRNAs

Before discussing what is known about the sequences of tRNA genes, it might be informative to digress to review studies with mature tRNAs of bacilli. Sequences for mature tRNAs have been compiled by Sprinzl and Gauss (49). This compilation includes 10 tRNAs from *B. subtilis* and 3 from *B. stearothermophilus*. The tRNAs represented, giving the amino acid acceptor class followed by the modified anticodon sequence, are as follows: Ala(mo^5U G C), Arg(ICG), Gly($cmnm^5U$ C C), Lys(UUU or $cmnm^5s^2U$ U U), Met(CAU), fMet(CAU), Phe(GmAA), Thr(mo^5U G U), Tyr(QUA), and Val(mo^5UAC) from *B. subtilis* and Phe(GmAA), Tyr(QUA), and Val(GAC) from *B. stearothermophilus*. (The abbreviations for the modified nucleosides are: mo^5U, 5-methoxyuridine; I, inosine; $cmnm^5U$, 5-carboxymethylaminomethyluridine; $cmnm^5s^2U$, 5-carboxymethylaminomethyl-2-thiouridine; Gm, 2′-*O*-methylguanosine; Q, quenosine.)

Chromatographic comparisons on RPC-5 columns of isoaccepting tRNA species from *B. subtilis* resolved 42 species of mature tRNA (53). Two-dimensional polyacrylamide gel electrophoresis separated about 40 species (21). The relative amounts of certain isoaccepting tRNA species change during differentiation of *B. subtilis* (12, 30, 53). In the only two cases that the primary sequence of sets of isoaccepting species which change during growth has been determined, this change was due to a posttranscriptional modification which in both cases occurred in the anticodon loop (34, 54). Experiments with separation by gel electrophoresis of tRNAs labeled in vivo at different growth stages also supported the conclusion that posttranscriptional controls rather than synthesis of new tRNA species were likely to be responsible for changes in the complement of tRNAs (21). On the other hand, the hybridization experiments of Jeng and Doi suggested that several tRNAs with new primary sequences appeared during sporulation (24), so the question of whether some tRNA species may be unique to a specific phase of development in the bacilli is still open. To answer it, it will be necessary to analyze primary sequences and modified

TABLE 2. Summary of tRNA gene sequences from *B. subtilis*, indicating anticodons represented and genes not encoding the CCA 3′ terminus

Amino acid acceptor group[a]	Unmodified anticodon	tRNA gene region in which sequence occurs[b]				
		2	4	6	16	21
Ala	UGC	++		+		−
Arg	ACG			+		+
Asn[c]	GUU					+
Asn[c]	GUU			−	+	
Asp	GUC		+		+	+
Cys	GCA				−	
Gln	UUG				−	
Glu	UUC		−		−	−
Gly	UCC					+
Gly	GCC			+	+	+
His[d]	GUG					+
His[d]	GUG				+	
Ile[e]	GAU	++				
Ile[e]	GAU					+
Ile/Met[f]	CAU					+
Leu	CAA				−	
Leu	CAG					+
Leu	UAA				+	−
Lys	UUU		+			+
Met	CAU					+
fMet	CAU				+	+
Phe	GAA		−		+	+
Pro	UGG			+		+
Ser	UGA					+
Ser	GCU					+
Ser	GGA				+	
Thr	UGU				−	+
Thr	GGU			−		
Trp	CCA				+	
Tyr	GUA				+	
Val	UAC				+	+

[a] Amino acid acceptor groups are based on unmodified anticodon assignments except in the case of Ile(NAU) mentioned below. Each listing represents a different primary sequence. The number of nucleotide differences between genes with the same anticodon is given below.

[b] Numbers refer to the number of tRNA genes found in a particular region. References for the first publication in which the sequence appeared are as follows: 2-gene group (33, 37); 4-gene group (61); 6- gene group (57); 16-gene group (58); 21-gene group (16, 58). The 2-gene group appears at two locations in the genome (37). +, 3′ CCA terminus encoded in the gene; −, sequence is missing.

[c] There are four base changes between these Asn(GUU) sequences.

[d] There is one base change between these His(GUG) sequences.

[e] There are two base changes between these Ile(GAU) sequences.

[f] This tRNA gene sequence has been considered a methionine isoacceptor by one group (58) and an isoleucine acceptor by another group (16), as discussed in the text.

nucleosides of all the tRNA species which vary in relative amounts with growth stage. Alternatively, transcriptional analysis of promoters for tRNA genes could suggest that a particular tRNA gene is expressed only at a specific growth stage. For instance, the last tRNA gene in the cluster of 16 tRNA genes is preceded by its own promoter and thus may be transcriptionally unique (58). However, this and all other promoters suggested to date preceding or within tRNA gene regions are sigma-55 type promoters (refer to Table 3), which presumably could be read at any growth stage. Other promoters may exist, however, and transcriptional analysis of these regions in vivo and in vitro will be important areas for further investigation.

Sequences of tRNA Structural Genes

Sequences for the tRNA genes have been compiled by Sprinzl and Gauss (48). The May 1984 compilation did not include the 16 tRNA gene sequences reported by Wawrousek et al. (58). These gene sequences will not be repeated here, but a summary of the unmodified anticodons which are represented is given in Table 2. Whether the CCA terminus is encoded in the gene sequence is also indicated. As noted earlier, an estimate of 10 unsequenced tRNA genes is based on hybridization experiments with restriction fragments of total genomic DNA. Along with the 51 sequences in Table 2, this would lead to an expected total of about 60 tRNA genes for *B. subtilis*.

Unusual Features of Certain tRNA Structural Genes

tRNA genes containing an A at position 32. The $tRNA^{Leu}{}_{CAG}$ gene (found in the 21-gene cluster) contains an A at the 5′ end of the anticodon loop (position 32). This was confirmed by sequencing both strands of the DNA fragment containing this gene (16); an A at this position was found independently by two different laboratory groups (16, 58). The $tRNA^{Thr}{}_{GGU}$ gene found in the 6-gene cluster (57) also contains an A at position 32. All other procaryotic tRNAs and tRNA gene sequences so far reported contain a pyrimidine at this site. However, a purine has been found in this position only in tRNAs of chloroplasts and mitochondria (48, 49).

Extra G at the 5′ end of the gene for histidine tRNA. All known mature $tRNA^{His}$ sequences have an extra nucleotide at the 5′ end of the molecule, usually a G (49). In *Drosophila melanogaster* and *Schizosaccharomyces pombe*, this nucleotide is added to the $tRNA^{His}$ posttranscriptionally and is not encoded in the gene (6). In *B. subtilis*, the two histidine tRNA genes, one found in the 16- and one in the 21-gene cluster, both have a G already at the 5′ terminus of the gene sequence. G in this position (position 0 for the mature tRNA) is very unusual and suggests that the G encoded in the sequence may survive as the 5′ terminus. It remains to be seen whether the 5′-end-processing enzyme, ribonuclease (RNase) P, can retain the additional 5′ G or whether the G is removed after transcription and another G is added back.

tRNA with a methionine anticodon might accept isoleucine. Amino acid-accepting categories for the tRNA gene sequences in Table 2 are based on the unmodified anticodon sequence, except for one tRNA with a methionine anticodon. This tRNA gene occurs in the 21-gene cluster; it was given the assignment of a methionine acceptor by one group (58) and of an isoleucine acceptor by another (16). No direct data are yet available on which amino acid the product of this gene will accept. The rationale for considering it an isoleucine acceptor (16) is based on the following points. (i) There are three genes with a CAU anticodon in the 21-tRNA-gene cluster, but only two mature methionine-accepting tRNAs have been identified (55). Two of the CAU acceptors have sequences corresponding to the sequences of mature fMet and Met tRNA (59, 60); the third gene has a different sequence showing 58 and 66% homology to fMET and Met tRNAs, respectively, but 76 to 77% homology to other *B. subtilis* isoleucine tRNAs. (ii) The third tRNA gene with the anticodon CAU has an A-U pair in the third position of the aminoacyl stem, which is unusual for tRNAs which act as substrates for methionyl tRNA synthetase (45). (iii) There is precedent for a tRNA with a CAU anticodon modified in the wobble position recognizing the isoleucine codon AUA (14, 27, 28).

tRNA Genes That Do Not Encode the CCA 3′ Terminus

It is a common generalization that the tRNA genes of procaryotes encode the CCA end, whereas those of eucary-

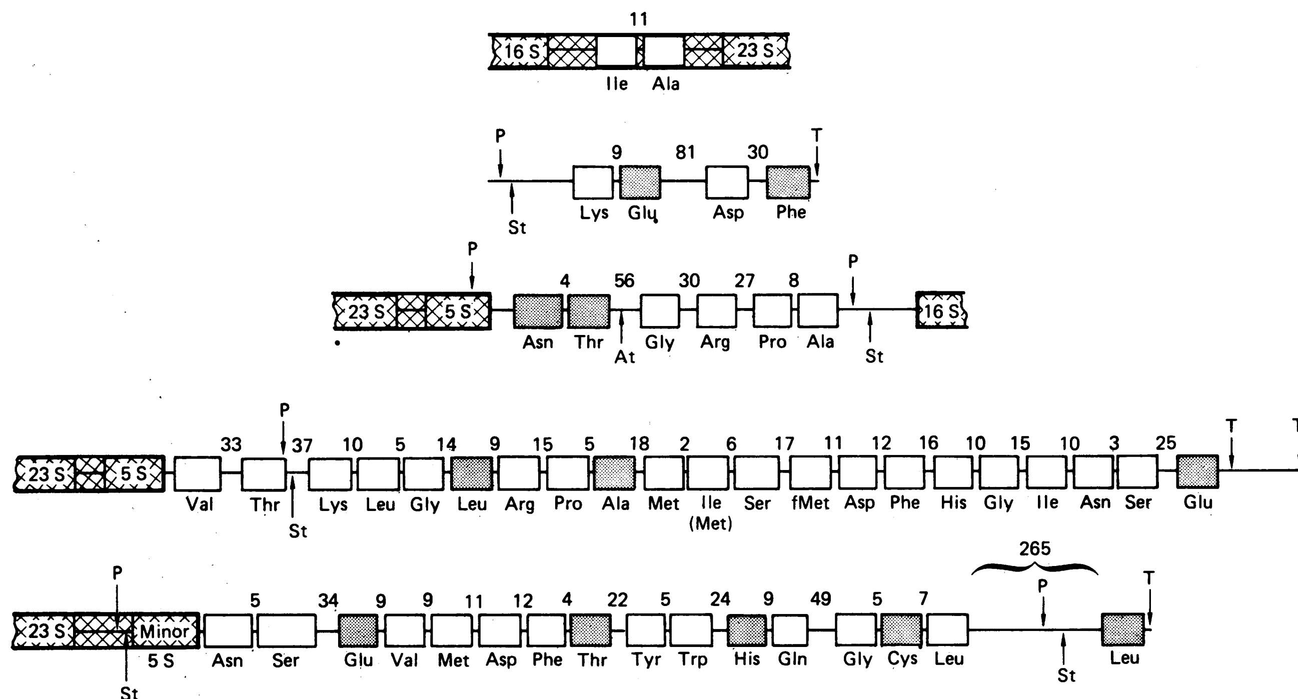

FIG. 4. Putative transcriptional control elements for *B. subtilis* tRNA gene regions. Abbreviations: P, promoter; T, terminator; At, attenuator; St, stringent sequence. Hatched areas indicate regions within the 16S-23S-5S rRNA gene set. Solid boxes indicate tRNA genes in which the 3'-terminal CCA end is not encoded. Numbers above the lines indicate the number of nucleotides in the spacer regions.

otes, including tRNA genes of mitochondria (1) and chloroplasts (29), do not. All of the tRNA genes so far studied from *E. coli* encode the CCA terminus in the gene. However, the following cases are interesting exceptions. In the archaebacterium *Sulfolobus solfataricus*, the two tRNA genes which have been sequenced do not encode the CCA end (26), and in the *E. coli* phage T4, some tRNA genes encode the CCA end whereas others do not (14).

In *B. subtilis*, tRNA genes both with and without the CCA end can be found. Of the 51 tRNA gene sequences, 12 (24%) do not have the CCA sequence at the 3' terminus encoded in the gene (Table 2). Genes lacking the CCA end occur in every tRNA gene cluster except for two tRNA genes in the 16S to 23S rRNA spacer region (Fig. 4). They occur without obvious order in the clusters. The same gene lacking a CCA end in one cluster may contain it when it occurs in another cluster (refer to Table 2). The following genes have only been found once and lack the CCA end: $tRNA^{Cys}$(GCA); $tRNA^{Gln}$(UUG), $tRNA^{Leu}$(CAA), and $tRNA^{Thr}$(GGU). No data are yet available to test whether genes lacking the CCA end can be expressed as functional tRNAs. *B. subtilis* and *E. coli* contain enzymes which can add the CCA end, so the question may be whether these genes can be processed efficiently. It has been suggested that the lack of the CCA end makes tRNA processing less efficient in the *E. coli* system (10, 18, 44); however, processing does occur. It is curious that *B. subtilis* has incorporated a type of gene organization including tRNA genes that lack or encode the CCA end. This type of mixed arrangement has only been reported in one other system, the tRNA genes of the *E. coli* phage T4 (14).

Gene Copy Number

As discussed above, the expected total number of tRNA genes for *B. subtilis* is approximately 60; gene sequences for 51 of these are known (Table 2). Thirty-one different primary sequences are listed in Table 2. Thus, some tRNA genes are represented more than once. In *E. coli* and *Saccharomyces cerevisiae*, the abundance of mature tRNAs and the occurrence of the respective codons in protein genes have a correlation (22, 23). Codon usage in *B. subtilis* is discussed below.

The number of copies of tRNA genes in the genome also limits the number of possible suppressor tRNAs. Suppressor mutations usually affect the structure of a species of tRNA with several copies, so that one copy is dispensable. Only five suppressor mutations in *B. subtilis* have been identified (9), and these have not been assigned to tRNAs. Suppressors in *B. subtilis* have not received a great deal of attention, and the phenomenon of suppression may be more complicated in *B. subtilis* than in *E. coli*. However, it is possible that some suppressor mutants are not viable because the appropriate tRNA gene exists in a single copy in the genome. Possible candidates are the following *B. subtilis* tRNA genes which could act as nonsense suppressors (15) as a consequence of a single base change but have been found in only one copy so far (and the same anticodon is not duplicated by another gene): Cys(GCA), Gln(UUG), Gly(UCC), Leu(CAA), Trp(CCA), Tyr(GUA), and Ser(UGA).

Which Anticodons Are Represented?

We cannot yet establish codon usage for *B. subtilis*, as has been done for *E. coli* (22) and yeast (23), primarily because we know too few mature tRNA sequences for the bacilli. The sequences of the mature tRNAs are required to know which modifications occur in the anticodon loop since these affect codon recognition. Also, it needs to be established which are major isoacceptors and which are minor. It is interesting that in all cases in which the mature tRNA sequence is known for *B. subtilis* (49), an anticodon has been

TABLE 3. Putative promoters for tRNA genes and sequences potentially associated with the stringent response in *B. subtilis*

Promoter type	−35 region	No. of bp in space	−10 region	No. of bp in space	Sequence associated with stringent response	Cluster (reference)
Directly preceding tRNA gene set	TTGCCA	17	TATTAT	7	GTTGTTA	4-gene cluster (61)
Within a tRNA gene set	TTGCCG	17	TATAAT	6	GTCTATT	21-gene cluster (16)
	TTGACA	17	TATATT	7	GTCGGTT	16-gene cluster (58)
	CCGCCA	17	TACCAT			6-gene cluster (57)
5S preceding tRNA genes in *trrnD*	TTGATT	17	TATAAT	7	GTCTCTG	16-gene cluster (58)

chosen which reduces the number of required primary sequences. This is done by using an anticodon capable of wobble (7). For instance, the lysine tRNAs have U or cmnm^{5}s^{2}U in the first position of the anticodon (the wobble position), which allows one anticodon to recognize both of the known codons (with A or G in the third position of the codon). Similarly, the tyrosine tRNAs have Q at the first position of the anticodon. Q can recognize U or C in the third codon position. Also, if one looks at the first position of the anticodons of the tRNA genes (see Table 2), the unmodified anticodon sequence is compatible with a modified sequence capable of wobble in all cases except for the leucine tRNA. In that case, one leucine tRNA has the non-wobble anticodon CAA. Of course, having more tRNA sequences of mature tRNAs would be more persuasive; however, one can speculate that *B. subtilis* may limit the number of tRNA primary sequences needed by preferring anticodons capable of reading more than one codon.

tRNAs capable of reading the following codons (which cannot be read by an existing wobble anticodon) have not been found: Ala(GGG) or Ala(IGC), Arg(UCU) or Arg(CCG); Leu(GAC), Leu(UAG), or Leu(AAG); Val(GAC); and Pro(GGG). Either *B. subtilis* does not use these codons, simple wobble rules have been modified in some cases, or sequences for these tRNAs will be found in the genome in future studies. Since present studies have probably not described all tRNA genes, it would seem likely that segments containing the above tRNA genes are still to be discovered.

PUTATIVE TRANSCRIPTIONAL CONTROL ELEMENTS FOR tRNA GENE SETS FROM *B. SUBTILIS*

Primary transcripts from tRNA gene regions have been studied in *E. coli* (see reference 39 and review by Ozeki and Fournier to appear in *Microbiological Reviews*). Little is known at this time about the actual transcriptional units for bacilli; however, we can anticipate new information in the future. Predictions for putative transcriptional control elements for tRNA genes of the bacilli come from comparisons with those used for *E. coli*. Most authors have suggested regions where sequences for promoters or terminators are likely (Fig. 4). Promoter sequences in *B. subtilis* are more diverse than in *E. coli* due to the multiplicity of RNA polymerase activities, each capable of recognizing different promoter sequences, as discussed in the following section. A computer analysis of four tRNA gene regions of *B. subtilis* has been done by Charles Lawrence (C. Lawrence and B. Vold, unpublished data).

Promoters

Initiation of RNA synthesis by bacterial RNA polymerase is a complex process involving interactions between this enzyme, regulatory proteins, and sequences defined as promoters. Four features of DNA that contribute to promoter binding rates are sequences of the −10 and −35 region; the spacer length between these two; and, to some extent, the nonpromoter sequence surrounding these. Most of what we know about transcriptional control signals for procaryotic tRNA genes comes from *E. coli*. *E. coli* has one major RNA polymerase which recognizes one type of promoter sequence (*E. coli* also contains a minor RNA polymerase which reads promoters for heat shock genes. This *E. coli* promoter sequence is recognized in vitro by *B. subtilis* RNA polymerase containing sigma-28 [M. Chamberlain et al., Abstracts of the 9th International Spores Conference, Asilomar, Calif., 1984].) The consensus sequence for the Pribnow box (−10 region) for six tRNA genes plus four rRNA genes of *E. coli* is TATAATG (46), very similar to the consensus proposed by Rosenberg and Court (42). The −35 promoter region shows variations in as many as three positions, but generally corresponds to the TTGACA sequence suggested by Rosenberg and Court (42). The spacer region between the −10 and −35 regions is 16 to 17 nucleotides long.

The situation in *B. subtilis* is more complex. *B. subtilis* contains multiple forms of RNA polymerases which recognize different promoter sequences. The various forms found in *B. subtilis* consist of an RNA polymerase core associated with sigma factors and other polypeptide factors. Although the core polymerase is present throughout development, several core-associated polypeptides occur only in cells at a specific stage of the growth cycle and may represent a mechanism by which *B. subtilis* regulates the differentiation process (11, 32). We do not know whether differences may be related to the complexity of transcription required for spore formation or to the nature of the evolution of gram-positive organisms.

A list of the putative promoter sequences from *B. subtilis* is given by Johnson et al. (25). Tandem promoter sequences are found before the rRNA operons; as far as is known, the tandem promoters for rRNA operons have sequences potentially recognized by the sigma-55-containing RNA polymerase (37, 50). (The molecular weight of sigma-55 was recently reevaluated. Based on the new molecular weight, Doi suggests that it be called sigma-43 in the future [R. Doi et al., Abstracts of the 9th International Spores Conference, Asilomar, Calif., 1984].) Some protein genes in *B. subtilis* also have overlapping promoters. The overlapping promoters may be recognized by the same type of RNA polymerase

(35) or may utilize different types of RNA polymerases. An instance of the latter is the developmentally regulated gene *spoVG*, which has overlapping promoters; the upstream promoter is recognized by the sigma-37-containing RNA polymerase, and the downstream promoter is recognized by sigma-32 (25).

Although transcriptional analysis of these gene sets has not been done, a diagram of putative transcriptional units in which tRNA genes are arranged in *B. subtilis* is presented in Fig. 4, and an evaluation of putative promoters for tRNA genes (including those found promoter-distal to an rRNA gene set) is given in Table 3.

The potential promoters which have been suggested for tRNA gene regions, as well as for rRNA genes, all correspond to those potentially recognized by the sigma-55-containing RNA polymerase. This polymerase is the major type of RNA polymerase in vegetative cells and is present at all stages of sporulation. A straightforward interpretation of this result would be that the tRNA genes are not subject to differential regulation of transcription caused by variation in sigma factors of the RNA polymerase. There is no support at present for the transcription of certain tRNA species at a specific stage of sporulation.

The only experiments published to date on cloning and expression of *B. subtilis* tRNA genes concern the segment containing 21 tRNA genes. When these tRNA genes were cloned into pUC8 without the promoter from the rRNA gene set preceding them and in an orientation opposite to that of the beta-galactosidase promoter of the plasmid, elevated expression of genes after the putative internal promoter (refer to Fig. 4) was detected (C. J. Green and B. S. Vold, Fed. Proc. **43**:1637, 1984). This implies that the promoter between the second and third tRNA genes can be recognized by *E. coli* RNA polymerase and probably by the major vegetative *B. subtilis* RNA polymerase containing sigma-55 which recognizes a promoter sequence similar to that recognized by the *E. coli* RNA polymerase.

Sequences Associated with the Stringent Response

In both *E. coli* and *B. subtilis*, starvation for an amino acid results in diminished synthesis of components of the translation system such as tRNAs and rRNAs. *E. coli* promoters subject to stringent control contain a heptanucleotide sequence spanning nucleotide positions -5 to $+2$, the consensus sequence for which is CggC-CC (52). A consensus sequence for promoters subject to stringent control in *B. subtilis*, suggested by Ogasawara et al. (37), is GT(C/T)G(C/T)(T/Pu). Sequences similar to those proposed by Ogasawara et al. (37) are associated with tRNA gene regions, as shown in Table 3 and Fig. 4. It is important to notice, however, that the distance between the -10 region and the sequence associated with the stringent response is different in *B. subtilis* and *E. coli*. In *E. coli*, the relevant sequence begins adjacent to the 5′ end of the Pribnow box, 6 to 7 base pairs upstream from the proposed start site of transcription. In *B. subtilis*, the beginning corresponds to the putative transcription initiation site.

Termination

A comparison of termination signals in *E. coli* and *Salmonella typhimurium* by Rosenberg and Court (42) revealed three common features: a region of hyphenated dyad symmetry, a stretch of T's following the potential loop created by the dyad symmetry, and a G+C-rich region preceding the stop site. Regions such as this are found in *B. subtilis* after tRNA genes (Fig. 4). The fact that a gram-positive organism uses these same types of termination signals helps establish them as typical of procaryotes in general. Whether *B. subtilis* uses other types of termination signals as well has yet to be determined.

It has been observed that the ribosomal operons have double promoters. At least in one case, the *rrnB* operon which contains rRNA and tRNA genes, there is a putative double terminator (C. Green, G. Stewart, M. Hollis, B. Vold, and K. Bott, unpublished data).

A stem-and-loop type structure can also function as an attenuator. Wawrousek and Hansen (57) have suggested that such a signal may be a putative attenuator sequence preventing extensive transcriptional readthrough from the upstream pair of tRNA genes into the downstream set. It will be important for future studies to verify these putative termination or attenuation signals by transcriptional analysis.

Processing Signals

The structural genes for the tRNAs themselves must function at least partly as signals for processing enzymes. There are no structural regions surrounding all tRNA genes which have been identified to date which would serve as universal signals to identify the tRNA genes. The spacer regions between tRNA genes vary greatly, and in some cases tRNA structural genes are separated from one another by only two nucleotides, leaving little room for a nucleotide sequence required as a processing signal. However, some spacer regions contain possible recognition sites for processing enzymes. An example is the spacer regions on either side of the structural genes for $tRNA^{Thr}$ and $tRNA^{Lys}$ in the *trrnD* gene cluster (16). These spacer regions have six-base sequences that could pair, causing the CCA ends of the tRNAs to loop out, creating a type of tRNA precursor structure potentially formed in *E. coli* and *B. subtilis* (33, 36, 43). Palindromic sequences containing double *Pst*I sites may serve as processing signals in the tRNA gene cluster found in the mitochondrial DNA of *Neurospora crassa* (1), but such sequences are not found in any of the *B. subtilis* tRNA gene clusters (C. Lawrence, unpublished data).

We can anticipate an interesting area in the immediate future in which the transcription and processing of tRNA genes of *B. subtilis* will be studied. Transcription can now be readily done with SP6 RNA polymerase by using vectors pSP64 and pSP65 (4, 17).

SUMMARY

At present, data on how tRNA genes are arranged in procaryotes are still not complete enough to allow any firm generalizations. With this caution in mind, the following list summarizes what seems to be common for *B. subtilis* gene regions, mainly in contrast to *E. coli*.

tRNA genes in *B. subtilis* are highly clustered. *B. subtilis* tRNAs have been found in five different cloned gene segments accounting for approximately 90% of all tRNA genes in that organism. Two areas of the genome contain the majority of tRNA genes known so far, one with 16 and the other with 21 tRNA genes. Highly clustered organization in *B. subtilis* may imply a functional role for the cluster. The observation that a gene region from *Spiroplasma* sp. strain BC3 encodes a six-tRNA-gene segment very similar to one found in *B. subtilis* opens the intriguing possibility that a high degree of clustering and other elements of tRNA gene organization may be common to the gram-positive organisms and their evolutionary descendants.

tRNA gene clusters do not contain internally repeated sequences. In *E. coli*, tRNA genes occur singly or in groups of up to seven; tRNA genes within a group are often repeated (39). In *B. subtilis*, even though tRNA gene clusters can have as many as 21 tRNA genes, the same sequence does not occur more than once within a cluster. The same gene may occur in more than one cluster, however.

Spacer tRNA genes are found in only a few rRNA gene sets. Of the 10 rRNA gene sets known to occur in *B. subtilis*, 2 have been shown to have spacer tRNA genes between the 16S and 23S rRNA genes. In both cases, these sequences code for isoleucine and alanine tRNAs.

tRNA genes are usually found in association with rRNA genes. Six tRNA gene regions have been sequenced. Two are spacer tRNA genes within an rRNA gene set, three are found promoter-distal to an rRNA gene set, and only one is not apparently associated with rRNA genes.

Most tRNA genes encode the CCA 3′ terminus. In *B. subtilis*, tRNA genes with and without the CCA terminus can be found, although it is more common for the tRNA genes to contain the CCA sequence. At least one tRNA gene without a CCA end can be found in all tRNA gene clusters so far sequenced (except for the two tRNA genes in the 16S to 23S rRNA spacer region).

Two large clusters of tRNA genes distal to rRNA gene sets may initiate transcription from within the tRNA genes in addition to being part of the larger rRNA transcriptional unit. In addition to the tandem promoters found at the beginning of the rRNA gene sets, other putative promoters can be found within the two large tRNA gene clusters. Suggested promoter sequences for the tRNA gene regions seem to be those recognized by the sigma-55-containing RNA polymerase; however, other recognition sites are possible. The putative internal promoter suggested in the 21-tRNA-gene cluster appears to be recognized by *E. coli* RNA polymerase in vivo. Terminators characterized by a region of hyphenated dyad symmetry and a stretch of T's are found downstream of all tRNA gene regions; however, such structures have not been found within a tRNA gene cluster.

Most tRNA genes have promoter regions including a sequence associated with stringent control. There is a sequence associated with promoters under stringent control which is found in almost all of the putative promoter regions for *B. subtilis* tRNA genes. The position of this sequence in *B. subtilis* promoter regions differs from that of *E. coli*; it is further downstream of the Pribnow box.

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