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New Transfer Ribonucleic Acid Species During Sporulation of *Bacillus subtilis*

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The transfer ribonucleic acid (tRNA) populations from log-phase cells, sporulating cells (stage III), and dormant spores were compared by tRNA-deoxyribonucleic acid hybridization techniques. New tRNA species not found in log-phase cells were observed in stage III cells. Some of the tRNA made during sporulation were also present in dormant spores. Although the role and function of these new tRNA species cannot be ascribed directly to the sporulation process, their presence indicates that new tRNA genes can be transcribed during sporulation and suggests that translational control may be exerted during sporulation by tRNA.

The aminoacyl-transfer ribonucleic acid (tRNA) patterns of sporulating cells and spores of *Bacillus subtilis* have been investigated by use of various comparative chromatographic methods (4, 8, 10, 19). These studies have indicated that the amount of various isoaccepting tRNA species is either increased, reduced, or unchanged during the sporulation process. It has been proposed that those tRNA's whose relative levels are changed during spore formation may exert regulation at the translational level during spore formation (8).

The previous studies have been interpreted as showing primarily quantitative changes of tRNA species present in vegetative cells during the sporulation process. The present studies were undertaken to determine whether new species of tRNA were synthesized during sporulation. The approach has been to use tRNA-deoxyribonucleic acid (DNA) hybrid competition techniques to compare the tRNA species present in log-phase and sporulating cells. The results indicate that additional tRNA species are made during sporulation.

MATERIALS AND METHODS

Organism and media. *B. subtilis* WB746 was used in all experiments. A modified Schaeffer (2× SG) medium which contained nutrient broth (Difco), 16 g/liter; $MgSO_4 \cdot 7\ H_2O$, 0.5 g/liter; KCl, 2 g/liter; $Ca(NO_3)_2$, 10^{-3} M; $MnCl_2$, 10^{-4} M; $FeSO_4$, 10^{-6} M; and glucose, 1 g/liter, was used for growth and sporulation. A very rich medium containing tryptose (Difco), 25 g/liter; yeast extract (Difco), 20 g/liter; K_2HPO_4, 3 g/liter; and glucose, 30 g/liter, was used to grow cells to a high density for the preparation of DNA. The cells were grown at 37 C with vigorous aeration.

To obtain maximal and synchronous sporulation, cells were transferred three times during log stage into fresh 2× SG medium. Under these conditions, 90% sporulation was routinely obtained. Some batches of nutrient broth gave poor growth rates and yields and also poor sporulation (about 50%). Each new lot of nutrient broth was checked for sporulation frequency. Spores were prepared and cleaned as described previously (7).

Isolation and purification of tRNA. For the preparation of log-phase bulk tRNA, cells were grown to mid-log phase in 2× SG medium; $MgCl_2$ was added to the growth medium to a final concentration of 1 mM to prevent lysis, and the cells were chilled by the addition of ice and harvested with a Sharples centrifuge. They were washed once with TMM buffer [0.002 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, 0.01 M magnesium acetate, 0.003 M 2-mercaptoethanol (pH 7.2)] containing 15% sucrose and then suspended in TMM buffer. To the cell suspension was added 1.1 volumes of phenol previously saturated with TMM buffer. The mixture was gently shaken for 2 h at room temperature. The mixture was fractionated by centrifugation into an upper aqueous layer and a lower phenol layer. The upper layer was saved, and the lower phenol layer was reextracted with 0.5 volume of TMM. After a second centrifugation, the two upper aqueous fractions were pooled and reextracted with 0.5 volume of TMM-saturated phenol for four to five times, until no visible, white precipitate appeared at the interface. To the final aqueous supernatant fraction were added 0.1 volume of 20% potassium acetate and 2 volumes of cold 95% ethanol. After standing at −20 C for 6 to 8 h, the tRNA precipitate was collected by centrifugation at 4,500 × *g* for 20 min.

To remove any acylated amino acids, the tRNA precipitate was dissolved in 0.5 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.8, and incubated at 35 C for 1 h. The pH was then reduced to 7.0, and ribosomal RNA was precipitated

by the addition of NaCl to a final concentration of 1 M for 1 h at 4 C. After removal of ribosomal RNA by centrifugation, the tRNA was precipitated by the addition of potassium acetate and ethanol as described above. The precipitate was dissolved in TMM buffer and dialyzed against TMM buffer for 6 h.

The tRNA was then passed through a diethylaminoethyl-cellulose column. The column was equilibrated with TMM buffer. After the tRNA was applied, the column was washed with TMM buffer containing 0.15 M KCl until the absorbancy of the eluant at 260 nm was equal to the buffer blank. Then 0.5 M KCl was applied to elute the tRNA. The tRNA fractions were pooled and dialyzed against 1× SSC (0.15 M NaCl, 0.015 M sodium citrate).

Any contaminating polysaccharides were removed by treatment with the KH_2PO_4-K_2HPO_4-methoxyethanol system as described by Kirby (9). To the pooled tRNA which had been dialyzed against 1× SSC, an equal volume of cold 2.5 M potassium phosphate buffer, pH 7.2, and of 2-methoxyethanol was added. This mixture was shaken vigorously at 0 C. The upper phase was obtained by centrifugation at 10,000 × *g* for 5 min. To the upper phase, an equal volume of 0.2 M sodium acetate and a 0.5 volume of 1% cetyltrimethyl ammonium bromide were added. After standing in the cold for 5 min, the cetyltrimethyl ammonium bromide-RNA pellet was obtained by centrifugation at 5,000 × *g* for 10 min (15). The pellet was washed two times with cold 0.1 N sodium acetate in 70% ethanol and then was dissolved in and dialyzed against TMM buffer.

For the preparation of bulk tRNA from stage III cells, which have a tougher cell wall, these forms were disrupted with the Vibrogen (RHO Scientific, Inc.) at 4 C in the presence of TMM buffer, glass beads, and phenol (0.1 ml/g of cell). Phenol was used to prevent ribonuclease activity and any breakdown of tRNA. Spores were disrupted in a Braun homogenizer with glass beads and phenol. The disrupted cell or spore mixture was centrifuged at 6,000 × *g* for 15 min to remove glass beads and some cell debris. The supernatant fraction was decanted and centrifuged at 12,000 × *g* for 20 min to sediment the remaining cell debris. The brownish-looking supernatant liquid was centrifuged once more at 105,000 × *g* for 4 h to remove ribosomes. The upper one-half to two-thirds of the clear supernatant liquid after ultracentrifugation was carefully removed and used for the preparation of tRNA by the phenol method as described above.

In vitro labeling of tRNA. Methylation of tRNA was carried out by use of a modification (6) of the method described by Smith et al. (16). Five microcuries of [^{3}H]dimethylsulfate (375 mCi/mmol) dissolved in ether was added to 1.1 mg of tRNA in 0.05 ml of 0.3 M phosphate buffer, pH 7.5, and the mixture was allowed to incubate for 4 h at room temperature. A plateau of methylation was reached between 3 and 4 h of incubation (7). The labeled [^{3}H]tRNA was precipitated with 95% ethanol, dissolved in 1× SSC, and precipitated twice more with ethanol. The final precipitate was dissolved in 1× SSC, and the specific activity of the tRNA was determined. All the experiments were done with [^{3}H]tRNA of the following specific activities: log tRNA, 54,000 counts/min per μg; stage III tRNA, 30,000 counts/min per μg; and spore tRNA, 91,000 counts/min per μg.

Separation of heavy and light strands of DNA. The methods for the isolation of DNA (13, 21) and for the separation of the complementary strand fragments of *B. subtilis* DNA by methylated albumin-kieselguhr (MAK) column chromatography (12, 18) were described previously.

Hybridization conditions. Hybridization of tRNA to denatured DNA was carried out by modification (11) of the filter technique of Gillespie and Spiegelman (5). The alkali-denatured DNA in 5× SSC was poured on the membrane filters (Millipore filters, 47 mm in diameter, with a pore size of 0.22 μm) which had been presoaked in 5× SSC. The DNA-containing filters were washed with 100 ml of 5× SSC and dried at room temperature for 4 h and in an 80 C vacuum oven for 2 h. Hybridization was carried out in triplicate by incubation of the 6-mm DNA filters, which were punched from the large filter, with radioactive tRNA in a solution containing 50% formamide, 5× SSC, and 1 mg of yeast RNA per ml for 12 h at 47 C in a total volume of 0.5 ml. The hybridization filters were washed four times in 2 ml of the hybridization mixture without the labeled tRNA at 47 C and four times in 2 ml of 5× SSC. The filters were treated with 15 μg each of ribonuclease T_1 and of pancreatic ribonuclease per ml for 1 h at room temperature to digest away unpaired tRNA. Finally, the filters were washed four times in 2 ml of 5× SSC, dried, and counted for radioactivity.

For hybridization of heavy (H) and light (L) DNA strands with [^{3}H]tRNA, the H and L fractions from the MAK column were pooled and dialyzed against 1/10× SSC buffer at 4 C and then adjusted to 5× SSC buffer. A 47-mm membrane filter (0.22-μm pore size; type GS, Millipore Corp.) was placed into a filtration apparatus (filtering diameter of 33 mm) and saturated with 5× SSC buffer. Then 400 μg of either H or L DNA strands was poured onto the filter. Retention of DNA was monitored by analyzing the flow-through buffer for absorption at 260 nm. Each 6-mm filter punched from the area of filtration contained 12.5 μg of DNA. Depending on the experiment, one to four DNA-containing filters were used in the reaction mixture. Hybridization conditions and filter treatment after hybridization were identical to that described in the previous paragraph.

RESULTS

Two main experimental approaches were taken to determine whether new tRNA species were transcribed during sporulation. The tRNA populations isolated from log-phase cells, stage III cells, and dormant spores were each labeled in vitro and hybridized to DNA to saturation levels to determine the number of different tRNA species present in each population. Secondly, hybrid competition studies were performed to show the molecular differences between the three tRNA populations.

In vitro labeling of tRNA. The tRNA isolated from the three stages were labeled in vitro with the use of [^{3}H]dimethylsulfate as described previously (7). To test the purity and labeling of the tRNA, the labeled tRNA preparations were applied to and eluted from MAK columns. Figure 1 shows a typical elution pattern for the labeled tRNA. Most of the radioactivity eluted at the position of the marker tRNA (fractions 30 to 65). Very little radioactivity eluted with the ribosomal-messenger RNA (mRNA) (fractions 90 to 150). However, for the hybridization experiments, unlabeled RNA from the ribosome fraction was added to the hybridization reaction mixture at 40 times the amount of the corresponding labeled tRNA. This was done to prevent any hybridization of ribosomal RNA or RNA fragments which may have been contaminating the labeled tRNA fraction.

Saturation of DNA sites by tRNA during hybridization. Since the specific activity of each in vitro labeled tRNA preparation was known, it was possible to determine the number of sites in the DNA genome which were filled by tRNA during hybrid saturation studies. Figure 2 shows the pattern of saturation of DNA sites by tRNA taken from cells at the three stages. With all three populations of labeled tRNA, 20 to 25 μg of tRNA was sufficient to saturate 50 μg of DNA, and maximum hybrid formation was reached after incubation of the reaction mixture for 12 h at 47 C. The results of these experiments are summarized in Table 1, which shows that tRNA from stage III cells saturated 33 to 41 sites, compared to 23 to 28 sites for tRNA from log-phase cells and 26 to 31 sites for tRNA from dormant spores. These are minimal values, because the hybridization method probably is not completely efficient. However, these results clearly indicate that more species of tRNA are present during sporulation than during log

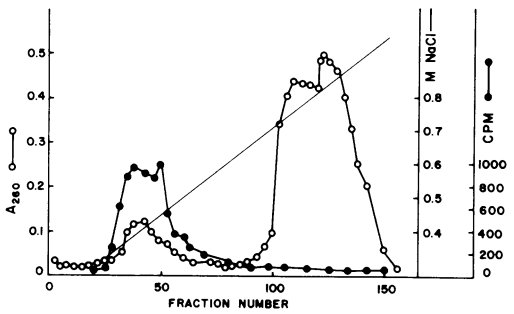

FIG. 1. *Elution profile of in vitro labeled log-phase cell [^{3}H]tRNA from a MAK column. Unlabeled bulk RNA (3 mg) from log-phase cells was mixed with in vitro ^{3}H-labeled log-phase tRNA (3 μg; 27,000 counts/min) before loading onto a MAK column. The column size was 1.9 by 12 cm. A linear gradient of 0.3 M NaCl (175 ml) to 1.0 M NaCl (175 ml) in 0.05 M phosphate buffer, pH 6.8, was used. The flow rate was 150 ml/h and each fraction contained 2 ml. Radioactivity was determined by precipitation of the whole 2-ml fraction with 10% trichloroacetic acid, by collection on glass-fiber filters, and counting with a Packard Tri-Carb spectrometer. Symbols: O, absorbancy at 260 nm; ●, radioactivity; ——, molarity of NaCl in 0.05 M phosphate buffer, pH 6.8.*

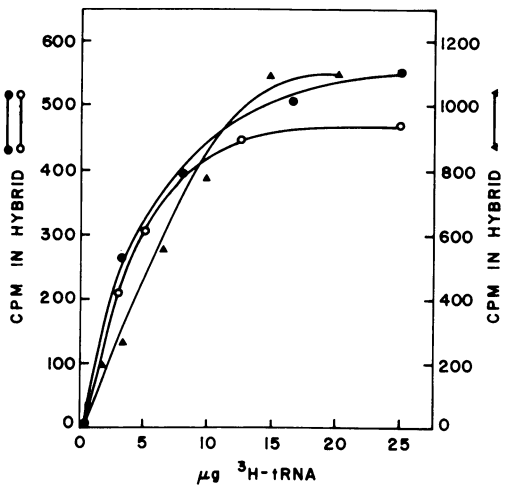

FIG. 2. *Saturation of DNA sites by [^{3}H]tRNA from various stages of growth. The hybrid reaction mixtures contained 51 μg of alkali-denatured DNA and increasing amounts of [^{3}H]tRNA from log-phase cells (54,000 counts/min per μg), stage III cells (30,000 counts/min per μg), and spores (91,000 counts/min per μg). Each point is the average of results from three hybrid reaction mixtures. Symbols: ●, log-phase [^{3}H]tRNA; O, stage III [^{3}H]tRNA; ▲, spore [^{3}H]tRNA.*

TABLE 1. *Saturation of DNA sites by [^{3}H]tRNA from various stages of growth*

tRNA species	% of DNA hybridized to tRNA[a]		No. of tRNA sites/genome[b]	
	Expt 1	Expt 2	Expt 1	Expt 2
Log phase	0.019	0.023	23	28
Stage III	0.028	0.034	33	41
Spore	0.022	0.026	26	31

[a] The value for each experiment is the average of results from three reaction mixtures. These data were calculated from the results obtained in hybrid saturation studies (Fig. 2).

[b] Calculated on the basis of a genome with a molecular weight of 3×10^9 and tRNA with a molecular weight of 2.5×10^4.

phase. The dormant spore contains an intermediate number of tRNA species.

Distribution of tRNA sites on the H and L strands of DNA. Since previous studies with mRNA populations from these stages of growth had shown that new mRNA's were transcribed from the H and L strands of DNA during sporulation (3, 18, 21), and since the current saturation studies suggested that new tRNA species were present in sporulating cells, the hybridization of tRNA to separated complementary strands of DNA was analyzed.

In Fig. 3 is shown the elution pattern of single stranded L (fractions 18 to 47) and H (fractions 59 to 71) DNA from a MAK column. Several fractions from the MAK column were tested for their ability to hybridize with log-phase tRNA. In confirmation of previous studies (12, 14), most of the tRNA hybridized with the H strand; however, a small but significant and consistent amount of tRNA hybridized with the L strands. The relative amounts of tRNA from the various growth phases hybridizing at saturation to the pooled H and L DNA strands are shown in Table 2. Approximately 90% of the tRNA hybridized to the H strands at all growth phases.

Hybrid competition experiments. A series of experiments was performed to determine the amount of competition which occurred between the tRNA populations for DNA sites. Since the saturation data above had indicated that the stage III tRNA population contained more tRNA species than the log-phase tRNA population, hybrid competition experiments were devised to show more definitively that new tRNA genes were being expressed during sporulation. The competition experiments were done at saturating concentrations of labeled tRNA with the complementary strands of DNA to obtain information concerning possible differential transcription of tRNA genes located on the complementary strands.

In these experiments the tRNA population from one of the three growth phases was labeled with ^3H, and unlabeled tRNA from all three phases was used to compete against the labeled tRNA for DNA sites. The homologous unlabeled

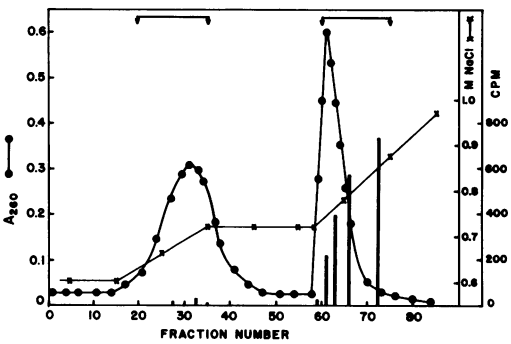

FIG. 3. *Separation of the complementary strands of B. subtilis DNA by MAK column chromatography. Alkali-denatured B. subtilis DNA (2.8 mg) was applied to a MAK column (1.9 by 20 cm) and eluted by an intermittent salt gradient. Fractions of 3.2 ml were collected, and the flow rate was 50 ml/h. The recovery of DNA was about 80%. Individual L and H fractions were dialyzed against 2× SSC, and then 0.02 mg of DNA was immobilized on filters and hybridized with [^3H]tRNA from log-phase cells with a specific activity of 18,000 counts/min per mg. The bars represent the counts hybridized. The arrows above the two absorption peaks indicate those fractions which were collected for the L and H strands, respectively.*

TABLE 2. *Hybridization of H- and L-DNA strands with [^3H]tRNA from various growth stages*

tRNA species	Relative hybridization to H and L strands of DNA[a]								No. of tRNA sites per genome[b]			
	Counts/min hybridized				% Hybridized				H		L	
	H		L		H		L					
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
Log	438	496	82	93	91	91	9	9	39	44	4	4
Stage III	375	406	82	90	90	90	10	10	60	69	4	4
Spore	815	912	163	172	91	91	9	9	43	48	4	4

[a] In experiments 1 and 2, triplicate hybridization mixtures were made, and the value given is the average of the three reactions. The reaction mixtures contained either 25 μg of H DNA or 50 μg of L DNA. Saturating concentrations of tRNA (25 μg) were used in the reaction mixtures. The specific activities for the tRNA species are: 54,000 counts/min per μg for log tRNA, 30,000 counts/min per μg for stage III tRNA, and 91,000 counts/min per μg for spore tRNA. The background values of 40, 28, and 51 counts/min for log, stage III, and spore tRNA, respectively, have been subtracted.

[b] This value was determined on the basis of a genome size of 3×10^9 daltons and a molecular weight of 2.5×10^4 for tRNA.

tRNA competed efficiently (although not to 100%) and was considered to compete at 100%. The results of these experiments are presented in Fig. 4 through 6.

In Fig. 4a an interesting result is observed with the H DNA strands. The results indicate that stage III tRNA does not compete with 12% of the log-phase tRNA for DNA sites, indicating that the stage III tRNA population is lacking a few tRNA species which are ordinarily found in log-phase cells. The stage III cells do contain all the tRNA species transcribed from the L strand of DNA during log phase (Fig. 4b). The spore tRNA population does not differ from the log-phase tRNA population, since it competes well with log-phase tRNA for both the H and L strands of DNA.

There is definite evidence that stage III cells contain tRNA transcribed from the H DNA which is different from that found in log-phase cells or spores (Fig. 5a). Log-phase and spore tRNA could not compete with 28 and 23%, respectively, of the stage III tRNA for the H strand. A small but significant (9%) is also noted between the tRNA hybridizing to the L strand; again, stage III cells contain tRNA not found in log-phase cells (Fig. 5b). The dormant spore, however, has a very similar pattern of tRNA to that found in stage III cells for the L strand.

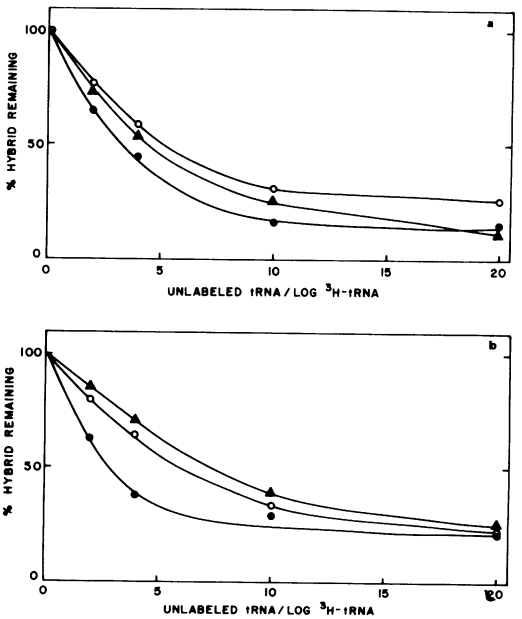

FIG. 4. *Hybrid competition studies between [^{3}H]tRNA from log-phase cells and unlabeled tRNA from various stages of growth for H and L strands of DNA. (a) Each annealing mixture contained 24.8 μg of the H strand of DNA, 25 μg of log-phase [^{3}H]tRNA (54,000 counts/min per μg), and increasing amounts of unlabeled tRNA, as indicated in the abscissa. (b) Each annealing mixture contained 51.2 μg of the L strand of DNA, 25 μg of log-phase [^{3}H]tRNA, and increasing amounts of unlabeled tRNA, as indicated in the abscissa. Symbols: ●, log-phase unlabeled tRNA; ○, stage III unlabeled tRNA; ▲, spore unlabeled tRNA.*

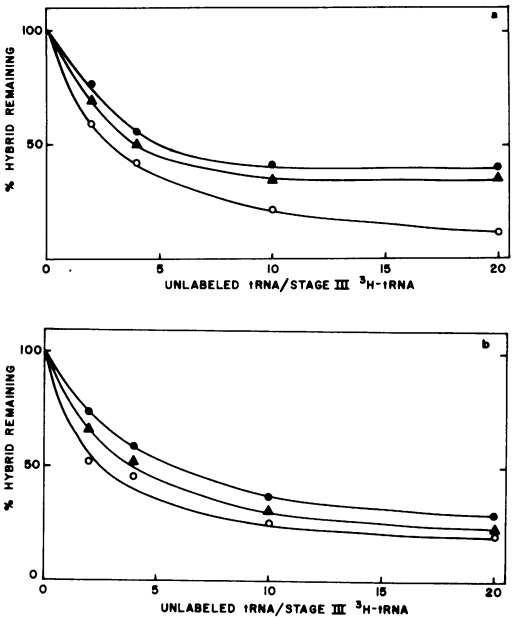

FIG. 5. *Hybrid competition studies between [^{3}H]tRNA from stage III sporulation cells and unlabeled tRNA from various stages of growth for H and L strands of DNA. (a) The conditions were the same as in Fig. 4a except that 25 μg of stage III [^{3}H]tRNA (30,000 counts/min per μg) was present as the labeled tRNA. (b) The conditions were the same as in Fig. 4b except that 25 μg of stage III [^{3}H]tRNA was present as the labeled tRNA. Symbols: ●, log-phase unlabeled tRNA; ○, stage III unlabeled tRNA; ▲, spore unlabeled tRNA.*

The results shown in Fig. 6a support the data described in Fig. 4a, since the log-phase and spore tRNA populations transcribed from the H strand are very similar and contain some tRNA not found in stage III cells. Stage III tRNA could not compete with 10% of the spore tRNA for H DNA. For the L DNA sites the stage III tRNA population contains all species found in log-phase cells (Fig. 4b) plus some additional species (Fig. 6b).

The data in Fig. 4 through 6 were also analyzed by the method of Bishop et al. (2), in which the percentage of hybrid remaining at infinite ratio of unlabeled tRNA to labeled tRNA can be calculated. These calculated results are presented in Table 3 and compared

with the results obtained from the direct plots presented in Fig. 4 through 6. Both the calculated results and the results from the direct plots are very similar.

These results are summarized in Fig. 7 and 8 in terms of the competition between the three populations for DNA sites. The relative number of tRNA species present in different stages of growth has been calculated from the data in Fig. 7 and 8 (see Appendix) and presented in Fig. 9. These data (Fig. 9) illustrate the presence of a greater number of different tRNA species found in stage III cells and in dormant spores as compared to log-phase cells.

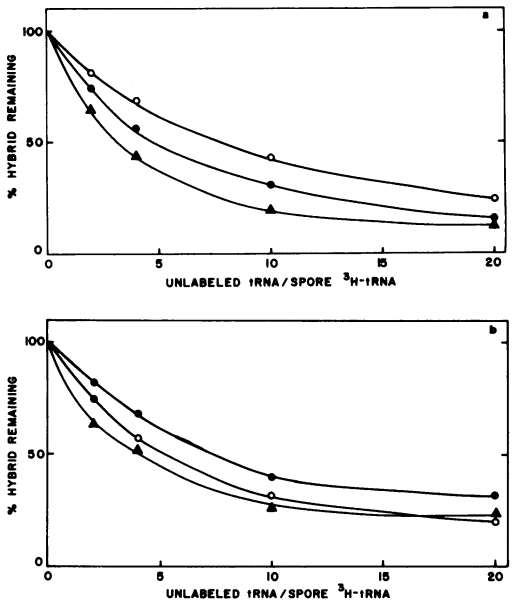

FIG. 6. *Hybrid competition studies between [³H]tRNA from spores and unlabeled tRNA from various stages of growth for H and L strands of DNA. (a) The conditions were the same as in Fig. 4a except that 25 μg of spore [³H]tRNA (90,000 counts/min per μg was present as the labeled tRNA. (b) The conditions were the same as in Fig. 4b except that 25 μg of spore [³H]tRNA was present as the labeled tRNA. Symbols: ●, log-phase unlabeled tRNA; ○, stage III unlabeled tRNA; ▲, spore unlabeled tRNA.*

DISCUSSION

There are several inherent difficulties in doing experiments such as those reported in this paper. To minimize some of the technical problems or variations which might affect the results, the following precautions were taken. (i) The isolation and purification of tRNA fractions from the various stages of growth were done as identically as possible to reduce the possibility of selective loss of certain tRNA species. (ii) In the experiments in which the H and L DNA strands were used, all the experiments were done with the identical H and L preparations to remove the possibility of differences occurring due to the preparations used. (iii) The hybridization experiments were run under identical conditions to eliminate variations in hybridization efficiency. (iv) The same unlabeled tRNA preparations were used for all competition studies. (v) Identical conditions and the same [³H]dimethyl sulfate solution were used to label the tRNA preparations in vitro; all tRNA prep-

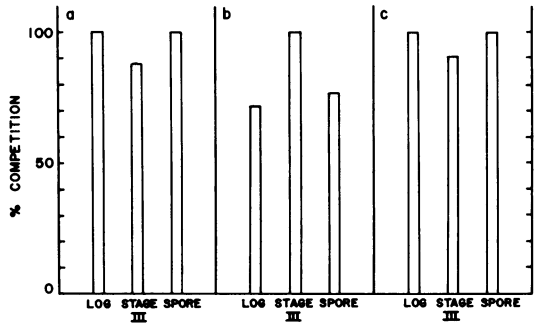

FIG. 7. *Summary of hybrid competition studies with the H strand of DNA. This summary was derived from data presented in Fig. 4a, 5a, and 6a. The percentage of competition is shown between the labeled tRNA population from log-phase cells (a), stage III cells (b), and spores (c) and various unlabeled tRNA, as indicated in the abscissa.*

TABLE 3. *Percent hybrid remaining during hybrid competition studies between homologous and heterologous tRNA species*

Unlabeled tRNA	H DNA/labeled tRNA[a]			L DNA/labeled tRNA[a]		
	Log	Stage III	Spore	Log	Stage III	Spore
Log	0	24.3 (28)	1.1 (0)	0	9.9 (9)	8.8 (11)
Stage III	10.2 (12)	0	9.0 (10)	1.3 (0)	0	0.5 (0)
Spore	0.9 (0)	18.6 (23)	0	5.1 (1)	1.0 (0)	0

[a] The values in parentheses are from the direct plots of the data in Fig. 4 through 6. The other values were calculated by the method of Bishop et al. (2) and represent the percentage of hybrid remaining at infinite ratio of unlabeled tRNA to labeled tRNA. The homologous competition results have been subtracted in each case.

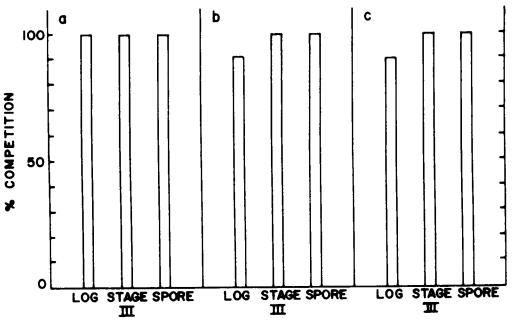

FIG. 8. *Summary of hybrid competition studies with the L strand of DNA. This summary was derived from data presented in Fig. 4b, 5b, and 6b. The percentage of competition is shown between the labeled tRNA population from log-phase cells (a), stage III cells (b), and spores (c) and various unlabeled tRNA, as indicated in the abscissa.*

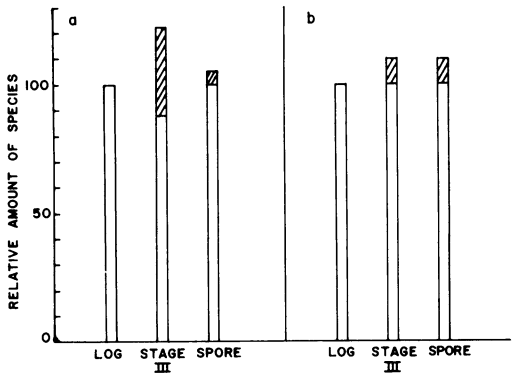

FIG. 9. *Relative amounts of tRNA genes expressed during different stages of growth. This figure represents the relative amounts of tRNA sequences present in log-phase cells, stage III sporulating cells, and dormant spores from the H strand (a) and L strand (b). The clear area represents tRNA species found in log-phase cells, and the hatched area represents sporulation-specific tRNA species. See Appendix for calculations.*

arations were labeled to a saturating level. (vi) Identical growth conditions were used to obtain cells from the various stages of growth. By controlling these factors we have avoided some of the obvious variables which could lead to the results reported above.

The data (Tables 1 and 2) indicate that more tRNA genes are transcribed during sporulation than during the log phase of growth. There is some variation in the results in the actual number of genetic sites filled with tRNA when experiments were compared. A consistently higher value was obtained with the separated strands of DNA (Table 2). However, there was consistency in the quantitative pattern of hybridization, since with either denatured DNA or separated complementary strands of DNA there was a greater number of tRNA's hybridizing to DNA with tRNA obtained from sporulating cells than with tRNA obtained from log-phase cells.

These saturation studies are supported by the information provided by the hybrid competition studies (Fig. 4–6). The competition data show clearly that the log-phase tRNA fraction cannot compete completely with the tRNA fraction of sporulating cells for DNA (Table 3). These results imply that the sporulating cell contains tRNA species which are not transcribed during the log phase of growth. The most interesting aspect of these experiments is the very good agreement in the number of tRNA species involved. The calculated increase in the number of new tRNA species in the sporulating cells (Table 1) of about 10 is matched closely by the calculated number of about nine uncompeted tRNA species present in the sporulating cell (Fig. 5). Furthermore, some of the sporulating cell tRNA species are present in the dormant spore (Fig. 6), since the spore tRNA fraction competes well with the sporulating cell tRNA fraction. This latter fact complements the information obtained about the mRNA population of dormant spores (6), and these results together indicate that the dormant spore cytoplasm contains macromolecules "left over" from the sporulation process. One other fact of significance is the apparent turning off of some tRNA genes during sporulation (Fig. 4 and 6). These results imply that there is negative control of tRNA gene transcription in addition to the positive turning on of some tRNA genes.

One aspect of our experiments does not agree closely with data obtained by others. This concerns the amount of tRNA which hybridizes with the L-strand fragments of DNA. Others have reported little or no hybridization of tRNA to L strands (12, 14); however, on closer inspection of these various published data, there are data which show a low degree of hybridization of tRNA to the L strands. Thus our findings are not completely new. However, another possible reason for our results is that the separation of complementary DNA strands by our method may result in some H strands eluting with the L-strand population. In any case these results do not change the overall conclusions which we have derived from our data.

The results with spore tRNA species indicate that spores contain all the tRNA species found in vegetative cells. This apparent contradiction with the results of Vold and Minatogawa (20),

who reported the absence of two minor log-phase lysyl-tRNA species in spores, suggests that the base sequence of these two minor tRNA species are very similar to the major species and that they could not be distinguished by the hybridization method, whereas the column chromatographic method was able to separate them. Another possibility is that the minor tRNA species are actually precursor molecules to or incompletely modified major tRNA species which are capable of being charged with amino acid. Either of these possibilities would explain the contradiction.

Since several new tRNA genes appear to be transcribed, many questions arise. What regulates the transcription of tRNA genes? Does the overall change in the transcription process (3, 18) during sporulation influence the transcription of tRNA genes? What causes the "turning off" of some tRNA genes? Are specific tRNA species essential for sporulation? What is the function of new tRNA species during sporulation? There are many reports of changes in tRNA species and populations during differentiation processes (see Sueoka and Kano-Sueoka [17] for a review); however, there is no unified theory concerning the possible role or function of tRNA species in the regulation of these processes. One obvious role is the translation of codons of mRNA which are found only or with increased frequency in sporulating cells. The other postulated role is a function of tRNA in regulating the expression of certain biosynthetic pathways. The transcription of new tRNA genes may be linked to the transcription of sporulation-specific genes which may require a change in RNA polymerase structure and/or function. The answer to all these questions will require much further analysis of the transcriptional process and its control. These results do suggest, however, that some type of translational control exerted by tRNA may be significant during sporulation.

APPENDIX

The following are the calculations for Fig. 9.

A. **For H strand DNA (Fig. 9a).**

(i) $x + y$ = tRNA sequences present in log-phase cells.
$y + z$ = tRNA sequences present in stage III cells.
From Fig. 7a, $y = 0.88\ (x + y)$.
From Fig. 7b, $y = 0.72\ (y + z)$.
Assume $x + y = 1.00$
Then $y = 0.88$ and $z = 0.34$.

(ii) From Fig. 7a, spore tRNA could compete with log tRNA 100%.
From Fig. 7c, log tRNA could compete with spore tRNA 100%.
So there is no spore-specific tRNA sequences, and tRNA present in spores with log tRNA sequence = tRNA sequences present in log phase cells = $x + y$.

(iii) $y + z = y + (z_1 + z_2)$ = tRNA sequences present in stage III cells.
$x + y + z_1$ = tRNA sequences present in spores.
From Fig. 7b, $y + z_1 = 0.77\ (y + z_1 + z_2)$
$= 0.77\ (y + z)$.
From Fig. 7c, $y + z_1 = 0.89\ (x + y + z_1)$
since $y = 0.88$ and $z = 0.34$
then $z_1 = 0.05$.

B. **For L strand DNA (Fig. 9b).**

(i) $m + n$ = tRNA sequences present in log-phase cells.
$n + p$ = tRNA sequences present in stage III cells.
From Fig. 8a, $n = m + n$.
From Fig. 8b, $n = 0.91\ (n + p)$.
Assume $m + n = 1.00$
Then $m = 0$, $n = 1.00$, and $p = 0.10$.
So tRNA in sporulation stage III cells contains all tRNA sequences present in log-phases cells and sporulation-specific tRNA.

(ii) From Fig. 8b, spore tRNA could compete with stage III tRNA 100%.
From Fig. 8c, stage III tRNA competed with spore tRNA 100%. So there is no spore-specific tRNA, and tRNA present in spores = tRNA present in stage III cells = $n + p$.

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