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Transfer Ribonucleic Acid Synthesis During Sporulation and Spore Outgrowth in *Bacillus subtilis* Studied by Two-Dimensional Polyacrylamide Gel Electrophoresis

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The synthesis of transfer ribonucleic acid (tRNA) was examined during spore formation and spore outgrowth in *Bacillus subtilis* by two-dimensional polyacrylamide gel electrophoresis of in vivo ^{32}P-labeled RNA. The two-dimensional gel system separated the *B. subtilis* tRNA's into 32 well-resolved spots, with the relative abundances ranging from 0.9 to 17% of the total. There were several spots (five to six) resolved which were not quantitated due to their low abundance. All of the tRNA species resolved by this gel system were synthesized at every stage examined, including vegetative growth, different stages of sporulation, and different stages of outgrowth. Quantitation of the separated tRNA's showed that in general the tRNA species were present in approximately the same relative abundances at the different developmental periods. tRNA turnover and compartmentation occurring during sporulation were examined by labeling during vegetative growth followed by the addition of excess phosphate to block further ^{32}P incorporation. The two-dimensional gels of these samples showed the same tRNA's seen during vegetative growth, and they were in approximately the same relative abundances, indicating minimal differences in the rates of turnover of individual tRNA's. Vegetatively labeled samples, chased with excess phosphate into mature spores, also showed all of the tRNA species seen during vegetative growth, but an additional five to six minor spots were also observed. These are hypothesized to arise from the loss of 3′-terminal residues from preexisting tRNA's.

Investigations of mechanisms controlling bacterial spore development have involved an intense examination of the regulatory role that tRNA may play in this system (18, 20, 21, 35). Sporulation in *Bacillus subtilis* is accompanied by significant qualitative and quantitative changes in at least eight iso-accepting tRNA species (37). The chromatographic changes for at least two tRNA species ($tRNA^{Tyr}$ and $tRNA^{Lys}$) are clearly the result of post-transcriptional tRNA modification (15, 37). Hybridization experiments using in vitro labeled bulk tRNA extracted from cultures at different growth stages have suggested that there are some tRNA species which are unique to either vegetative growth or sporulation (14). There is compelling evidence that transcription of new RNA species occurs during sporulation, and novel tRNA genes could be among those transcribed (7, 14, 19, 26). Additionally, during sporulation a large fraction of tRNA species loses its 3′-terminal adenylate and sometimes one or both neighboring cytidylates (32, 36).

During germination total tRNA synthesis begins within a few minutes and is continuous during outgrowth (1, 8). It is not known whether the same tRNA species are synthesized then as during vegetative growth or whether individual tRNA's are synthesized continuously or periodically during the cell cycle.

tRNA species labeled in vivo with ^{32}P can be resolved reproducibly by two-dimensional (2-D) polyacrylamide gel electrophoresis (9, 11, 12). The use of in vivo ^{32}P labeling and separation of the resulting labeled tRNA's on 2-D gels offer several advantages over methods previously used to study tRNA synthesis during bacterial spore development. First, only those tRNA's that are actually synthesized during the labeling period are examined, rather than any accumulation of tRNA's from earlier stages of growth. Second, a broad spectrum of tRNA's can be studied within a single gel, and preferential degradation of tRNA's can be monitored. Third, potential artifacts associated with the in vitro

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labeling of tRNA via methylation or acylation are avoided, and the uniform labeling of the tRNA species with ^{32}P allows subsequent analysis (by hybridization or fingerprinting) of individual tRNA molecules of interest. The primary drawbacks of the method are the inability to presently correlate the tRNA's displayed on gels with previous chromatographic studies and the fact that individual tRNA spots resolved in a gel could be composed of several tRNA species.

The present study employed 2-D gel electrophoresis to examine the synthesis and degradation of tRNA during the life cycle of *B. subtilis*. Given the limitations of this method (see above), our results indicate that there is no preferential transcription, degradation, or compartmentation of unique tRNA species during spore formation. The tRNA's made during spore outgrowth were also apparently the same as those synthesized at other stages of the life cycle. Quantitation of the separated tRNA's isolated from all stages (vegetative growth, sporulation, outgrowth) also showed that an overwhelming proportion of the tRNA's were synthesized in approximately the same relative abundances.

MATERIALS AND METHODS

Bacterial strains and media. The GSY266 strain of *B. subtilis* is a *metB3* derivative of strain 168M originally from the laboratory of C. Anagnostopoulos (38). The sporulation medium (LDSM) and nutrient agar plating medium (NAT) have been described previously (31). Low-phosphate (low-P) LDSM medium was prepared by treating the nutrient broth component of LDSM with NH_4OH and $MgCl_2$ as described by Pieczenik et al. (28). The inorganic phosphate concentration in low-P LDSM was less than 0.1 mM (6). Germination medium (GM-12) was modified from a synthetic medium (GM-11) used previously (16, 38). The major changes were to remove the phosphate components to allow high specific activity for $^{32}P_i$ labeling and to replace the buffering and ionic strength of the removed phosphate with Tris-hydrochloride and KCl, respectively. GM-12 salts contained (per liter): Tris base, 6 g; trisodium citrate, 1.1 g; $(NH_4)_2SO_4$, 2.2 g; and KCl (pH 7.1), 7.5 g. GM-12 amino acids contained (per liter): L-alanine, 0.5 g; L-arginine, 0.2 g; L-asparagine, 0.5 g; L-glutamate, 1 g; L-glutamine, 1 g; L-histidine, 0.5 g; L-isoleucine, 0.1 g; L-leucine, 0.05 g; L-methionine, 0.5 g; L-serine, 0.05 g; L-threonine, 0.2 g; and L-valine, 0.2 g. Before use, 1 part of amino acids was added to 9 parts of salts. Glucose (0.5%), 2.5 mM $MgSO_4$, 4 μM $FeCl_3$, and 1 mM potassium phosphate were added to the indicated final concentrations. Minimal salts solution contained (per liter): K_2HPO_4, 14 g; KH_2PO_4, 6 g; $(NH_4)_2SO_4$, 2 g; and trisodium citrate, 1 g.

Growth of cells and conditions for sporulation. Cultures were incubated with vigorous aeration on a New Brunswick rotary shaker (37°C). Growth was measured as the increase in optical density at 525 nm (OD_{525nm}) with a Gilford 240 spectrophotometer (2×10^8 colony-forming units per ml at 1 OD unit). For $^{32}P_i$ labeling, cells were grown in low-P LDSM. The end of logarithmic growth was designated as t_0, and sporulation events were monitored by phase-contrast microscopy and timed as hours after t_0. Sporulating cultures were established as follows. Strain GSY266 was streaked onto NAT plates and incubated overnight at 37°C. The cells were suspended in low-P LDSM at an OD_{525nm} of 0.2, incubated at 37°C until the OD_{525nm} reached 2.0, and then diluted 10-fold into warmed low-P LDSM. Samples were removed for labeling, measurement of sporulation events, or isolation of spores.

Measurement of sporulation events. The final level of sporulation (at t_{24}) and development of heat resistance was measured by diluting 0.1 ml of culture into 0.9 ml of minimal salts solution at 80°C. After 10 min, dilutions were plated on NAT medium. Development of toluene resistance was measured by diluting 0.5 ml of culture into 2.0 ml of minimal salts solution containing 0.5 ml of toluene and vigorously mixing the sample for 10 min at room temperature (22). After the solution had stood for 15 min, dilutions were made in minimal salts solution and plated on NAT medium. The appearance of dipicolinic acid was measured by a colorimetric assay (13).

Isolation of spores. Culture samples (10 to 20 ml) were harvested by centrifugation (12,000 × *g* for 10 min) and washed twice with W buffer (0.15 M KCl in 0.05 M Tris-hydrochloride, pH 7.6). Mature spores were isolated as previously described (34).

Germination and outgrowth of spores. Spores for the germination and outgrowth studies were grown in a synthetic sporulation medium (34). They were lyophilized and stored at −20°C. Spores were heat activated and germinated as previously described (38). Germination was monitored by measuring the decrease in OD_{525nm}. In the low-P GM-12 medium, DNA synthesis began at 110 to 120 min after the onset of germination; this was the same as observed in GM-11 medium, which contains 45 mM phosphate (38).

Labeling with $^{32}P_i$. Incorporation of ^{32}P into cold trichloroacetic acid-precipitable materials was determined by diluting the cell samples 10-fold into cold 5% trichloroacetic acid containing 50 mM phosphate. Samples were stored overnight on ice, and the precipitates were collected on Reeve-Angel glass fiber ultrafine (984H) filters. The filters were washed with cold 5% trichloroacetic acid and then with cold 95% ethanol, and they were then dried and counted in Liquifluor scintillation fluid (New England Nuclear Corp.).

For ^{32}P labeling and extraction of tRNA, culture samples (10 to 20 ml) were transferred to warmed flasks containing $^{32}P_i$. Labeling during vegetative growth was from an OD_{525nm} of 0.2 until the end of logarithmic growth. Cultures were labeled for hourly intervals during the course of sporulation. Labeling periods were followed by a 15-min chase period with excess unlabeled phosphate (final concentration, 20 mM). The cells were then chilled on ice, harvested, and washed twice by centrifugation (12,000 × *g* for 10 min) in W buffer. Samples harvested at t_{24} were treated for spore isolation as described previously (34). The washed cell pellet (or spore pellet) was suspended

in 1 to 2 ml of cold W buffer and transferred to Braun-MSK homogenization flasks. The cell samples were frozen at −20°C and lyophilized.

RNA extraction. All buffers and glassware used for RNA extraction and subsequent handling of the RNA samples were autoclaved. The following components were added to the lyophilized cell samples: 4 g of acid-washed (6 N HCl) glass beads (diameter, 470 μm; type 070; 3M Co., St. Paul, Minn.), 2.5 ml of buffer E (40 mM EDTA, 0.2 M NaCl, 20 mM Tris-hydrochloride, pH 7.4) containing 1% sodium dodecyl sulfate, and 2.5 ml of phenol saturated with buffer E (4, 10). Samples were agitated in a Braun-MSK homogenizer (Quigley-Rochester, Inc., Rochester, N.Y.) for 3 min with CO_2 purging. Microscopic examination showed that this treatment was sufficient to break more than 90% of the spores in the sample. The recovery of ^{32}P label after phenol extraction and two ethanol precipitations (see below) was approximately 70% of the total counts in labeled spores. The broken cells in the phenol emulsion were centrifuged, and the aqueous and phenol phases were recovered and reextracted. RNA was precipitated from the combined aqueous phase by the addition of 2 volumes of −20°C absolute ethanol. After 2 to 4 h at −20°C, the precipitate was washed to remove phenol, dissolved in 0.2 M sodium acetate (pH 5), and precipitated again with ethanol. The RNA sample was dissolved in 0.1 M NaCl–10 mM Tris (pH 7.9) and loaded onto a DEAE-cellulose column (DE 32; Whatman; 6 mm by 2.5 cm). The column was washed with 0.3 M NaCl–10 mM Tris (pH 7.9), and the 4S and 5S RNAs were preferentially eluted with the same buffer containing 1 M NaCl (5). Yeast carrier RNA (20 μg/ml) was added, and the sample was precipitated with ethanol.

Polyacrylamide gel electrophoresis and autoradiography. 2-D gel electrophoresis was conducted by the procedures of Ikemura et al. (11, 12), with the modification that urea was added to the gel system (9). The first-dimension gel contained 10% polyacrylamide, 0.4% bisacrylamide, and 5 M urea prepared in TB buffer (80 mM Tris base, 80 mM boric acid, and 1 mM disodium EDTA, pH 8.3). For polymerization, 1.35 ml of a fresh 10% ammonium persulfate solution and 0.3 ml of *N,N,N',N'*-tetramethylethylenediamine were added to 300 ml of gel solution. The RNA samples (approximately 10^7 cpm) were electrophoresed in an E-C 480 vertical long path gel system (40 cm by 12 cm by 3 mm; E-C Apparatus Inc., St. Petersburg, Fla.) at 18°C. Electrophoresis (usually at 500 V) was terminated when the xylene cyanol dye had migrated 35 cm.

A gel strip containing the 4S and 5S RNA regions (located by autoradiography) was cut from the gel and cast into a second-dimension gel between glass plates. This gel (22 cm by 23 cm by 3 mm) consisted of 20% acrylamide, 0.8% bisacrylamide, and 5 M urea in TB buffer. The top and bottom of the gel were sealed with a 1- to 2-cm plug of 1.2% agarose in TB buffer. This second-dimension gel was run in a cold room (4°C) at 600 V. The progress of the xylene cyanol dye was measured, and the electrophoresis was continued until the dye front would have migrated 50 cm. After electrophoresis, the gel was autoradiographed by using Kodak XRP film and a Dupont intensifying screen (at 2°C). Sections (spots) containing the [^{32}P]RNA were cut from the gels by using a tracing of the autoradiography to monitor location. ^{32}P-labeled samples were counted in 10 ml of water by Cerenkov radiation (3). Doubly labeled samples (^{32}P and ^{3}H) were counted in a toluene–Triton X-100 mixture (2:1) containing 0.1 g of dimethyl-POPOP {1,4-bis-[2]-(4-methyl-5-phenyloxazolyl)benzene} and 4 g of PPO (2,5-diphenyloxazole) per liter. Spillover of ^{32}P was subtracted from the ^{3}H channel.

Materials. [^{32}P]orthophosphoric acid (HCl-free, carrier-free) was obtained from New England Nuclear Corp., Boston, Mass. Rifampin came from Calbiochem-Behring. Liquid phenol was purchased from Mallinckrodt and was distilled and stored at −20°C. Urea (ultrapure) was purchased from Schwartz/Mann. Acrylamide was either Bio-Rad (Richmond, Calif.) electrophoresis grade or Fisher reagent grade, recrystallized from chloroform. Yeast carrier RNA (Sigma Chemical Co.) was extracted three times with phenol and ethanol precipitated three times from 0.2 M sodium acetate, pH 5. The preparation was dissolved at 10 mg/ml in sterile distilled water and stored at −20°C.

RESULTS

Growth, sporulation, and $^{32}P_i$ labeling in low-P LDSM medium. Fig. 1A shows a comparison of the growth and sporulation of strain GSY266 in LDSM and low-P LDSM media. The doubling times are nearly identical (approximately 25 min), but cells growing in low-P LDSM left exponential growth at one-half the cell density attained by cells growing in LDSM. The low-P culture remained at 50% of the LDSM cell density throughout the course of sporulation. Dipicolinic acid accumulation began at t_6 to t_7, and the final levels of sporulation (90%) and the final amounts of dipicolinic acid (0.19 pmol/spore) were the same in the two media. Toluene resistance, associated with stage III of sporulation (22), began to develop at about t_5 in low-P LDSM (Fig. 1B), and 10% of the cells were toluene resistant by t_7. Heat resistance, one of the final events in spore maturation, began to develop at about $t_{6.5}$.

Pulse-chase experiments can be difficult to interpret due to preferential re-utilization of prelabeled intermediates and RNA turnover products. Therefore, we examined the effects of rifampin and of excess phosphate on the kinetics of $^{32}P_i$ incorporation in this system. Figure 2 shows that rifampin reduced the incorporation of ^{32}P more than 50% within 11 min of addition. The addition of excess phosphate alone even more markedly reduced the ^{32}P incorporation, but it is apparent that the unlabeled phosphate (more than 200-fold excess) did not equilibrate with the labeled phosphate pool over the interval examined. Similar results have been reported for *Escherichia coli* for short-term $^{32}P_i$ labeling

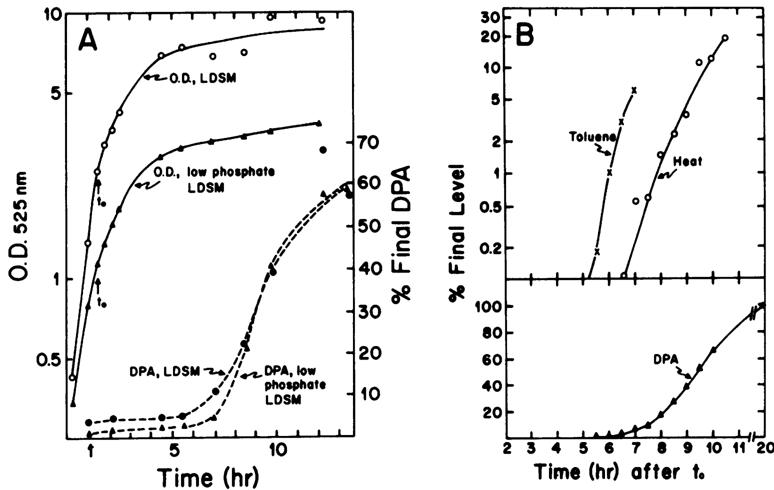

FIG. 1. *Sporulation of strain GSY266. Strain GSY266 was grown in LDSM or low-P LDSM at 37°C. At the indicated intervals samples were removed for measuring absorbancy, dipicolinic acid (DPA) level, and appearance of resistance to toluene and heat. (A) Symbols: solid lines, absorbancy of the culture (OD_{525nm}); dashed lines, dipicolinic acid level; △ and ▲, low-p LDSM; ○ and ●, LDSM. t_0 designates the end of logarithmic growth. (B) Sporulation in low-P LDSM. Symbols: ×, toluene resistance; ○, heat resistance; ▲, dipicolinic acid level.*

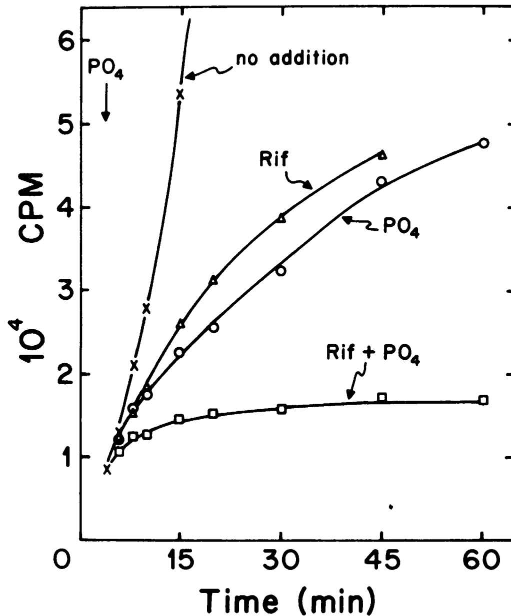

FIG. 2. *Effect of excess phosphate and rifampin (Rif) on $^{32}P_i$ incorporation during vegetative growth. Strain GSY266 was grown in low-P LDSM at 37°C until the OD_{525nm} was 0.6. Cells were labeled for 4 min with $^{32}P_i$ (10 μCi/ml), after which samples of the labeled culture were added to flasks containing either rifampin (5 μg/ml), excess phosphate (20 mM), or both rifampin and phosphate. At intervals, 0.1-ml samples were removed and cold trichloroacetic acid-precipitable counts were determined. Symbols: ×, no addition; △, rifampin (5 μg/ml); ○, phosphate (20 mM); □, rifampin and phosphate.*

(25). Simultaneous addition of rifampin and excess phosphate terminated $^{32}P_i$ incorporation, but no turnover of short-term ^{32}P-labeled material was observed under these conditions.

To determine the effectiveness of an excess phosphate chase under the conditions used for labeling of tRNA, the effect of excess phosphate on the incorporation of $^{32}P_i$ was examined after a longer labeling period (two generations) (Fig. 3). In the absence of excess phosphate, cells continued to incorporate the ^{32}P label after leaving the exponential growth phase at t_0 (Fig. 3A). A culture containing added phosphate stopped accumulating label by 60 min after phosphate addition (Fig. 3A). The turnover of the phosphate-labeled material made during vegetative growth was followed over the course of sporulation (Fig. 3B). Again, $^{32}P_i$ incorporation appeared to stop within 60 min after the addition of excess phosphate. Beginning at t_3, the amount of cold trichloroacetic acid-precipitable counts began to drop at a rate of approximately 6%/h. This suggests that there is turnover of prelabeled components during sporulation and that the incorporation of label into newly synthesized RNA is diluted by excess phosphate. Because the amount of ^{32}P incorporated specifically into RNA is not known (<50% of the total incorporation as suggested by rifampin inhibition), the rate of RNA turnover cannot be calculated from these experiments. However, in similar experiments we found that 85% of the ^{32}P label in the 1 M NaCl-DEAE eluate (primarily 4S and 5S RNA) had turned over by t_{10} (D. J. Henner,

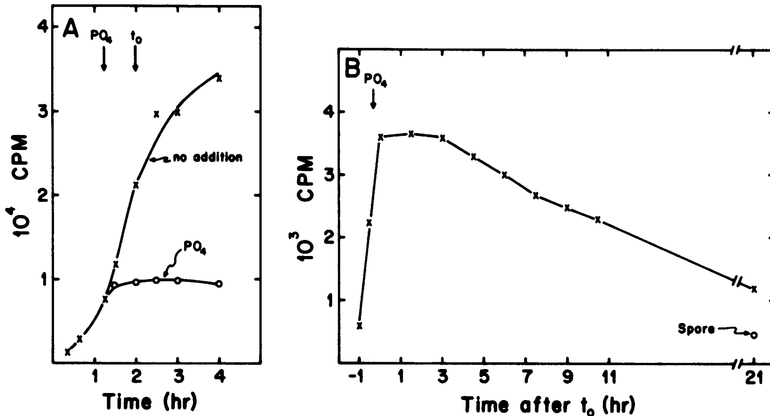

FIG. 3. *Effect of excess phosphate on incorporation of* $^{32}P_i$ *during vegetative growth and sporulation. Strain GSY266 was grown in low-P LDSM at 37°C until early stationary phase and then diluted to an* OD_{525nm} *of 0.1 in low-P LDSM containing* $^{32}P_i$ *(1 μCi/ml). At the point indicated, excess phosphate (20 mM) was added, 0.1-ml samples were removed, and the counts incorporated into cold trichloroacetic acid-precipitable material were determined. (A) Incorporation and chase during vegetative growth. Symbols: ×, no addition; ○, 20 mM phosphate.* t_0 *is indicated. (B) Excess phosphate chase during sporulation. Symbols: ×, whole cells; ○, counts recovered in spores isolated at* t_{21}.

Ph.D. thesis, University of Virginia, Charlottesville, 1979).

Synthesis of tRNA species during vegetative growth and sporulation. Figure 4 shows the 2-D gel separation of tRNA labeled either during vegetative growth (1.5 generations) or at different intervals during the course of sporulation in low-P LDSM. The 2-D gel system resolved the tRNA's into nearly 40 spots, 32 of which were present in sufficient levels for quantitation (>5,000 cpm). As judged by the gel autoradiographs, all of the tRNA's resolved by this system are synthesized during both vegetative growth and sporulation. Although there are some intensity differences for the same spot on different gels, photoreproductions of the original autoradiograph can sometimes give misleading visual impressions. Therefore, areas on the gels corresponding to the spots on the autoradiograph were cut out and counted for Cerenkov radiation (3). The values for each spot are shown in Table 1 as percentages of the total counts recovered from the gel. In a separate series of gels (data not shown) the tRNA abundance was quantitated by a double-labeling technique, using a tritiated tRNA standard (Table 1). The data in Table 1 confirm the visual impression obtained with the autoradiographs (Fig. 4); there is no evidence for the synthesis of new tRNA's during the course of sporulation, although a few spots did show changes in abundance (Table 1).

Turnover of tRNA's during sporulation. An examination of the relative turnover of tRNA species during sporulation and their compartmentation into spores was carried out by labeling a culture during vegetative growth and then extracting the RNA after a phosphate chase until t_{10} or until mature spore formation (Fig. 5). The 2-D gels of these samples showed that all of the tRNA species present during vegetative growth were still present in cells at t_{10} and in mature spores. The quantitation data (Table 2) show no striking differences in the relative abundances of most of the tRNA species. However, several spots (e.g., spots 14, 21, 39, and 43) do show at least twofold variations. This is not too surprising, as compartmentation, turnover (possibly differing between the two compartments), and modification could all be playing a role in the final complement of tRNA's. The turnover rates of the different tRNA's probably only vary slightly, for the long time course of the chase (10 h) would tend to magnify slight differences in the rates of turnover of individual tRNA's into large differences in final abundance. The RNA samples extracted from mature spores display spots not observed in the vegetative sample (Fig. 5, arrows). These spots appeared consistently on gels of samples extracted from mature spores. They were also observed on gels of RNA samples obtained from isolated spores labeled late in sporulation (t_9 to t_{10}) and chased with excess phosphate until t_{24}, but were not seen in cells labeled from t_9 to t_{10} (Fig. 4) or in cultures labeled from t_9 to t_{10} and chased to t_{11} (Henner, Ph.D. thesis). This indicates that their appearance is a very late event in spore formation.

Synthesis of tRNA during spore outgrowth. The synthesis of tRNA's during the outgrowth period after spore germination was examined to determine whether the same tRNA species were made as during vegetative growth.

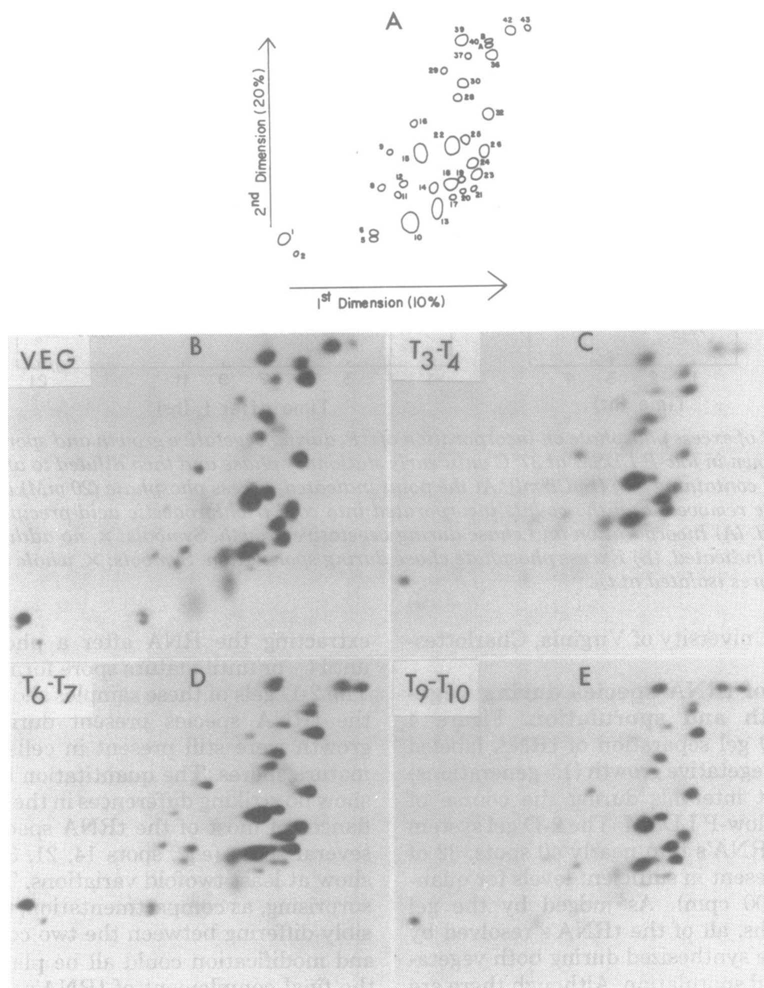

FIG. 4. *Comparison of tRNA's labeled at intervals during vegetative growth and sporulation. Strain GSY266 was grown in low-P LDSM. Vegetative cells (5 ml) were labeled with $^{32}P_i$ during growth from 0.5 to 1.5 OD_{525nm} units (B). Sporulating cells (10 ml) were labeled for 1-h periods from t_3 to t_4 (C), t_6 to t_7 (D), and t_9 to t_{10} (E). ^{32}P (200 μCi/ml) incorporation was terminated by a 20-min chase of excess unlabeled phosphate (20 mM) before harvest. RNA was extracted and subjected to 2-D gel electrophoresis. A schematic representation of the 2-D gel autoradiograph is shown (A). Quantitation of the gel tRNA spots is presented in Table 1.*

In addition, an examination of tRNA synthesis by 2-D gel electrophoresis could distinguish whether different tRNA's are expressed at different times, in a manner analogous to the periodic synthesis of enzymes observed during outgrowth (16, 38). Three time points were chosen in relation to DNA synthesis for labeling tRNA. One sample was labeled well before the onset of DNA synthesis (60 to 90 min), another was labeled from 110 to 150 min (at the onset of DNA synthesis), and a third was labeled from 210 to 240 min, when the amount of DNA had increased by 60% (Henner, Ph.D. thesis). A control culture was labeled for two generations during vegetative growth in the same medium. Figure 6 shows the series of gels obtained from samples labeled during outgrowth. The pattern of spots displayed appeared identical on all of the gels, indicating that the same tRNA's were synthesized during each particular labeling interval in outgrowth. The quantitation shows some variation in the relative abundances of the tRNA's synthesized during outgrowth (Table 3). The tRNA's from vegetative cells labeled in GM-12 (1 mM phosphate) and the tRNA's from vegetative cells labeled in low-P LDSM show wide variations in abundance (e.g., spots 5, 6, 16, 18, 19, 25, and 26), indicating that there might

TABLE 1. *Quantitation of tRNA spots labeled at intervals during sporulation*[a]

Spot	Relative amt			
	Vegetative	t_3-t_4	t_6-t_7	t_9-t_{10}
1[b]	1.0 (5.46)[c]	1.0 (2.66)	0.9 (4.40)	0.9 (4.25)
2[b]	1.0 (0.97)	1.2 (0.88)	1.4 (0.78)	1.5 (0.62)
5	1.0 (0.75)	0.8 (0.77)	0.9 (0.83)	0.8 (1.33)
6	1.0 (1.02)	1.5 (0.99)	1.3 (1.17)	1.3 (1.44)
8	1.0 (0.85)	1.5 (1.44)	1.8 (1.22)	ND (1.28)[d]
9	1.0 (0.55)	1.2 (1.41)	ND (0.93)	2.0 (0.85)
10	1.0 (3.67)	0.8 (3.85)	0.6 (3.62)	0.6 (3.20)
11	1.0 (1.11)	1.0 (1.12)	0.9 (1.38)	0.8 (1.30)
12	1.0 (1.26)	0.8 (1.74)	1.1 (1.79)	0.8 (1.87)
13	1.0 (3.17)	0.9 (3.39)	0.7 (3.16)	0.8 (2.95)
14	1.0 (2.31)	0.9 (3.79)	1.0 (2.92)	0.8 (2.30)
15	1.0 (2.09)	1.0 (4.73)	1.4 (2.20)	1.3 (1.99)
16	1.0 (1.29)	1.1 (2.51)	1.6 (2.19)	1.7 (1.69)
17	1.0 (0.54)	1.9 (1.35)	1.8 (0.99)	1.6 (0.77)
18	1.0 (7.89)	0.8 (7.49)	1.1 (8.79)	0.8 (7.24)
19	1.0 (1.33)	1.3 (1.51)	1.1 (1.43)	0.9 (2.11)
20	1.0 (0.66)	1.4 (0.98)	1.0 (0.73)	0.9 (0.74)
21	1.0 (0.51)	2.5 (0.84)	1.5 (0.83)	2.2 (1.18)
22	1.0 (15.70)	0.8 (14.25)	0.8 (13.90)	0.9 (12.29)
23	1.0 (4.49)	1.1 (3.45)	1.2 (5.09)	1.1 (4.96)
24	1.0 (4.72)	0.8 (4.59)	0.6 (5.62)	0.5 (6.91)
25	1.0 (3.97)	0.8 (2.10)	0.6 (2.63)	0.7 (3.30)
26	1.0 (4.39)	0.9 (2.45)	0.8 (3.99)	0.6 (3.62)
28	1.0 (1.69)	1.5 (3.37)	1.5 (2.28)	1.4 (1.34)
29	1.0 (0.72)	1.6 (1.64)	2.4 (0.91)	2.1 (0.78)
30	1.0 (3.41)	1.6 (3.80)	1.3 (2.91)	1.2 (3.92)
32	1.0 (5.24)	0.6 (3.01)	0.6 (4.53)	0.6 (5.23)
36	1.0 (6.25)	0.6 (3.52)	0.7 (5.19)	0.7 (5.30)
37	1.0 (1.21)	1.5 (2.55)	2.0 (1.62)	1.8 (1.56)
39	1.0 (5.52)	0.6 (4.58)	0.6 (3.72)	0.7 (4.63)
40A	1.0 (1.27)	0.7 (2.29)	1.1 (2.20)	1.4 (2.03)
40B	ND (1.68)	ND (1.53)	ND (1.29)	ND (2.25)
42	1.0 (3.71)	0.8 (3.53)	1.0 (2.51)	1.1 (2.48)
43	1.0 (0.59)	1.1 (1.88)	1.5 (2.19)	1.7 (2.30)

[a] Strain GSY266 was labeled with $^{32}P_i$ during vegetative growth and for 1-h periods (from t_3 to t_4, t_6 to t_7, and t_9 to t_{10}) as described in the legend to Fig. 4. RNA was extracted from vegetative and sporulating cells disrupted in the presence of phenol and lysis buffer. Each ^{32}P sample was co-electrophoresed with a standard preparation of [^3H]uracil-labeled tRNA from vegetative *B. subtilis*. Each RNA spot revealed by autoradiography was cut out, eluted from the gel, and counted as described in the text. The data are presented as the $^{32}P/^3H$ value divided by the $^{32}P/^3H$ value for the corresponding spot on the vegetative gel. Values were normalized for counts per minute applied to the gel.

[b] Spots 1 and 2 represent the two forms of 5S RNA.

[c] From a separate series of gels (Fig. 4) each RNA spot revealed by autoradiography was cut out and counted for Cerenkov radiation (3). The values for each RNA spot are presented in parentheses and are percentages of the total counts per minute found in all of the RNA spots. No spot quantitated contained less than 5,000 cpm.

[d] ND, Not determined.

be some control of the relative amounts of tRNA's resulting from medium composition or growth rate. The proportion of counts in vegetative 5S rRNA increased more than twofold when cells were grown in GM-12 compared with low-P LDSM medium.

DISCUSSION

Identity and resolution of the 2-D gel spots. The identifications of the ^{32}P-labeled spots visualized on the 2-D gels as tRNA and 5S rRNA are made on the following basis. The long labeling periods followed by an excess phosphate chase insure that the extracted labeled material consists of stable macromolecules. *B. subtilis* mRNA, with a half-life of a few minutes in both vegetative and sporulating cells, should not contribute significantly to the labeled material (17). The purification procedure included a standard DEAE-cellulose chromatography step for enrichment of 4S and 5S RNA. Additionally, the position of the labeled species agrees with pub-

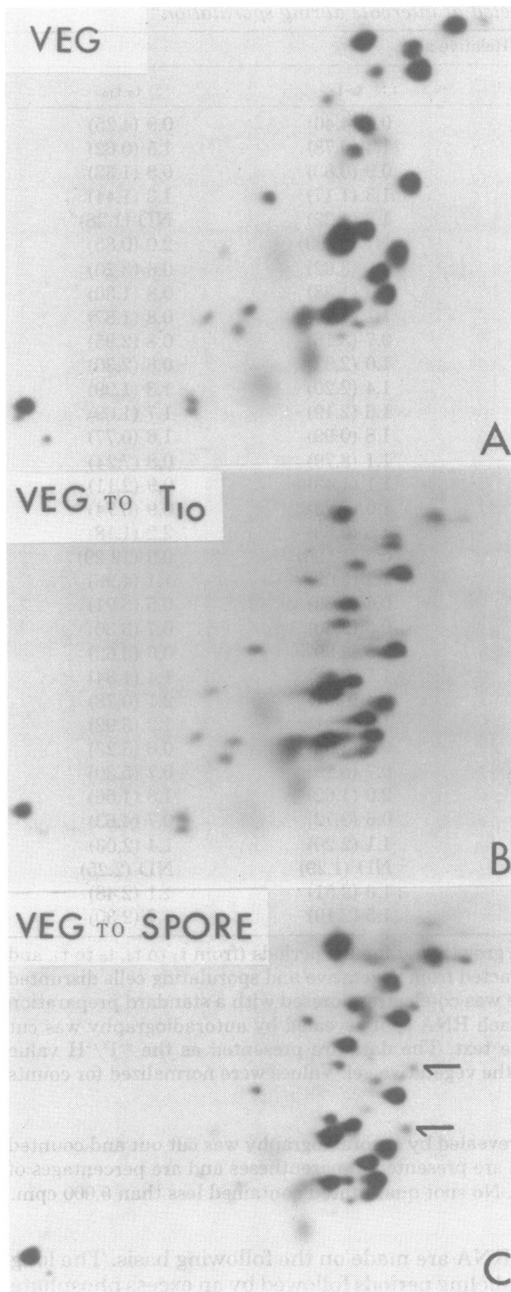

FIG. 5. Comparison of tRNA's synthesized during vegetative growth and chased into spores. Strain GSY266 growing in low-P LDSM was labeled with $^{32}P_i$ (200 µCi/ml) for two generations during vegetative growth. Unlabeled phosphate (20 mM) was added, and samples of the culture were harvested at three different times. The RNA was extracted and subjected to 2-D gel electrophoresis. (A) Cells harvested after a 15-min chase. (B) Cells harvested at t_{10}. (C) Spores harvested at t_{24}. Quantitation of the gel tRNA spots is shown in Table 2. Arrows show two of the new spots seen in spores.

TABLE 2. *Quantitation of vegetatively labeled tRNA chased until t_{10} or into isolated spores*[a]

Spot[b]	Relative amt after the following period of ^{32}P incorporation and chase:			
	Vegetative[c]	Vegetative to t_{10}	Vegetative to spore	t_9-t_{10}[c]
1	5.46	5.14	7.12	4.25
2	0.97	0.67	1.02	0.62
5	0.75	1.37	0.73	1.33
6	1.02	1.66	1.05	1.44
8	0.85	1.13	0.76	1.28
9	0.55	0.94	0.73	0.85
10	3.67	3.48	2.59	3.20
11	1.11	1.17	0.63	1.30
12	1.26	1.28	0.81	1.87
13	3.17	3.10	3.30	2.95
14	2.31	4.94	2.63	2.30
15	2.09	3.52	2.30	1.99
16	1.29	1.40	1.22	1.69
17	0.54	0.55	0.59	0.77
18	7.89	6.53	4.55	7.24
19	1.33	1.56	1.35	2.11
20	0.66	1.01	0.82	0.74
21	0.51	2.00	2.21	1.18
22	15.70	12.39	10.06	12.29
23	4.49	4.23	4.67	4.96
24	4.72	6.21	5.69	6.91
25	3.97	3.86	3.67	3.30
26	4.39	4.02	4.77	3.62
28	1.69	1.79	1.38	1.34
29	0.72	0.98	1.21	0.78
30	3.41	3.59	4.30	3.92
32	5.24	6.42	5.25	5.23
36	6.25	5.67	4.92	5.30
37	1.21	1.48	2.27	1.56
39	5.52	3.25	8.33	4.63
40A	1.27	1.36	2.64	2.03
40B	1.68	1.15	2.15	2.25
42	3.71	1.74	3.24	2.48
43	0.59	0.43	1.02	2.30

[a] Strain GSY266 was labeled with $^{32}P_i$ during vegetative growth (Fig. 5). Excess phosphate (20 mM) was added to the culture, and samples were removed after 15 min (vegetative) or at t_{10} (vegetative to t_{10}) or t_{24} (vegetative to spore). Isolated spores were prepared from the culture harvested at t_{24}. RNA was extracted and subjected to 2-D gel electrophoresis, and the RNA spots revealed by autoradiography were cut out and counted for Cerenkov radiation (3). The values for each RNA spot are presented as percentages of the total counts per minute found in all of the RNA spots.

[b] Spots 1 and 2 represent the two forms of 5S rRNA.

[c] Values for vegetative cells and for cells labeled at t_9 to t_{10} are from Table 1.

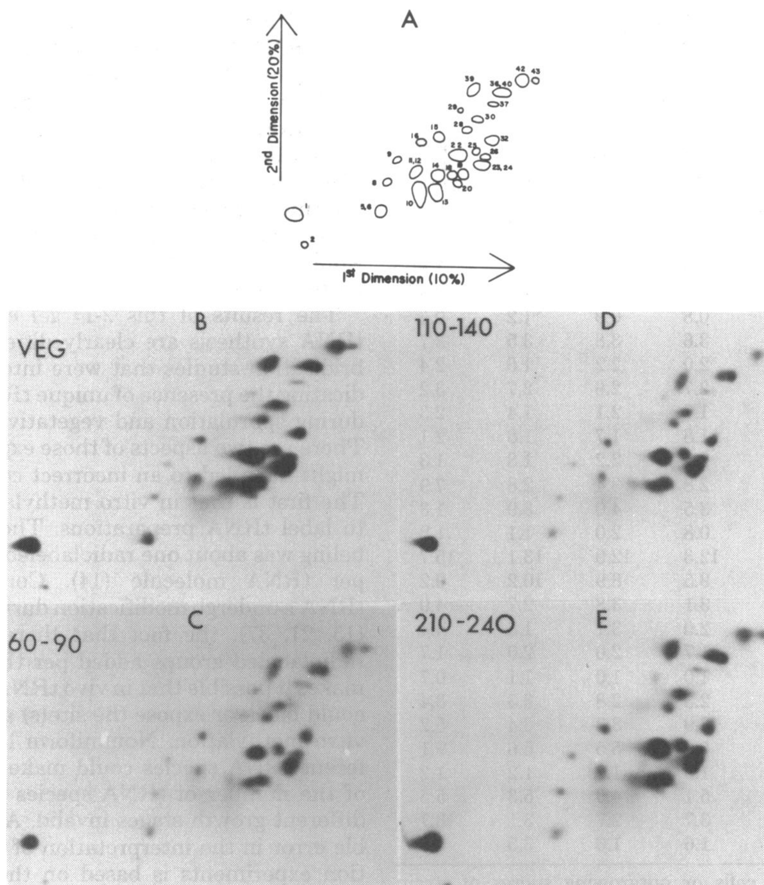

FIG. 6. *Synthesis of tRNA during spore outgrowth. Spores of strain GSY266 were heat activated, washed, and suspended at 200 μg/ml in GM-12 medium at 30°C. At 60, 110, and 210 min after germination was initiated, 20-ml samples of the culture were added to flasks containing 2 mCi of $^{32}P_i$ and incubated for 30 min. An exponential culture (20 ml) of strain GSY266 growing in the same medium was labeled at 100 μCi/ml during growth from an OD_{525nm} of 0.2 to 1.0. RNA was extracted from all samples and subjected to 2-D gel electrophoresis. The numbering system used in the schematic diagram (A) is as consistent as possible with that shown in Fig. 2. (B) Exponential growth (vegetative). (C) Outgrowth (60 to 90 min). (D) Outgrowth (110 to 140 min). (E) Outgrowth (210 to 240 min). Quantitation of the tRNA spots is presented in Table 3.*

lished reports of the migration of 4S and 5S RNA on similar gel systems (9, 11, 12). The identification of spots 1 and 2 as the major and minor 5S rRNA species is based on the work of Raue et al. (29). The proportion of label in the minor species, 15% in our system compared with 16% reported by Raue et al. (29), is consistent with this interpretation. We are convinced that certainly the majority of these spots represent tRNA (and 5S rRNA), although the presence of a few other small stable RNAs cannot be ruled out. However, their presence would not affect our general conclusions regarding tRNA synthesis.

The ability of the 2-D gel system to resolve individual tRNA's is crucial to our interpretation of the results. Estimates of the number of tRNA genes in *B. subtilis* by hybridization studies vary between 40 and 70 (14, 24, 33), although tRNA gene duplications could make these figures overestimates of the number of unique tRNA sequences. The number of spots resolved on the 2-D gels in this study (excluding the two 5S rRNA spots) is approximately 40. This number correlates with the lower estimates of the number of tRNA genes. Similar 2-D gel studies of tRNA in *E. coli* and *Saccharomyces cerevisiae* showed 30 and 40 spots, respectively, most of which were pure tRNA species (9, 11). Although it is possible that some of the spots contain more than one tRNA species, we feel that the resolution of the system makes it unlikely that tRNA species

TABLE 3. *Quantitation of tRNA labeled during outgrowth of spores*[a]

Spot[b]	Relative amt after the following period of ^{32}P incorporation:				
	Vegetative (GM-12)	60–90 min	110–140 min	210–240 min	Vegetative (LDSM)[c]
1	13.3	18.9	12.0	19.4	5.5
2	1.6	1.4	1.1	1.3	1.0
5, 6	4.3	2.9	2.2	2.1	1.8
8	1.4	1.1	1.1	1.3	0.9
9	1.1	0.8	0.9	1.2	0.6
10	4.3	3.6	3.8	3.5	3.7
11, 12	3.2	2.0	2.2	1.6	2.4
13	3.1	2.7	2.6	2.7	3.2
14	3.6	1.7	2.1	1.4	2.3
15	1.4	1.8	1.7	1.8	2.1
16	2.0	2.1	2.2	1.8	1.3
18	3.6	2.8	3.4	2.8	7.9
19	4.7	3.5	4.0	3.9	1.3
20, 21	1.9	0.8	2.0	1.1	1.2
22	11.3	12.3	12.9	13.1	15.7
23, 24	8.1	9.5	8.9	10.2	9.2
25	2.1	3.1	3.8	2.7	4.0
26	2.4	2.0	3.1	1.8	4.4
28	1.9	1.7	2.0	2.0	1.7
29	0.7	1.0	1.0	1.1	0.7
30	3.4	2.3	2.8	3.3	3.4
32	4.2	3.9	3.3	3.4	5.2
36, 40	4.9	5.6	5.5	5.6	9.1
37	1.3	1.9	1.2	1.2	1.2
39	4.7	5.1	4.9	5.3	5.5
42	4.5	3.7	2.7	3.1	3.7
43	1.1	1.6	1.6	3.5	0.6

[a] Vegetative cells or outgrowing spores of strain GSY266 were labeled with $^{32}P_i$ in GM-12 medium as described in the legend to Fig. 6. RNA was extracted, and the polyacrylamide gel samples were analyzed as described in Table 2, footnote *a*. The values for each RNA spot are presented as percentages of the total counts per minute found in all of the RNA spots.

[b] Spots 1 and 2 represent the two forms of 5S rRNA.

[c] Vegetative cells labeled in low-P LDSM medium. These data are from Table 1.

unique to a particular stage of development would be hidden by comigration with other tRNA species.

tRNA synthesis during spore development. The 2-D gel patterns of tRNA samples labeled during vegetative growth, sporulation, and outgrowth are strikingly similar. This result and reports that the alterations observed in at least four tRNA's ($tRNA^{Lys}$, $tRNA^{Trp}$, $tRNA^{Phe}$, and $tRNA^{Tyr}$) during sporulation are due to post-transcriptional modifications of vegetative tRNA's (2, 15, 21, 37) strongly suggest that the same tRNA genes are transcribed at all stages of the *B. subtilis* life cycle.

Although the 2-D gel system is sensitive enough to detect a single base difference in the normal form versus the suppressor form of *E. coli* $tRNA^{Trp}$ (base substitution C → U in the anticodon [23]), the effect of tRNA modifications on gel migration has not been analyzed in any rigorous manner and can only be inferred from other data (9, 11, 27, 31). We feel that it is likely that a tRNA base modification such as the thiomethylation reported to occur for some tRNA species during sporulation (2, 15, 21, 37) would not produce an altered tRNA migration pattern in the 2-D gel system that we used.

The results of this 2-D gel examination of tRNA synthesis are clearly different from hybridization studies that were interpreted as indicating the presence of unique tRNA sequences during sporulation and vegetative growth (14). There are two aspects of those experiments that might have led to an incorrect conclusion (14). The first is that in vitro methylation was used to label tRNA preparations. The extent of labeling was about one radiolabeled methyl group per tRNA molecule (14). Considering that tRNA's undergo modification during sporulation (15, 21, 37), the fact that there were so few radiolabeled groups added per tRNA molecule makes it possible that in vivo tRNA modification could block or expose the site(s) available for in vitro methylation. Nonuniform labeling of different tRNA species could make the estimates of the number of tRNA species transcribed at different growth stages invalid. A second possible error in the interpretation of the hybridization experiments is based on the finding that there are new, apparently stable RNA species in the size range of 6S to 8S that appear during the course of sporulation (30; Henner, Ph.D. thesis). These molecules were consistently observed on the first-dimension gels of RNA samples extracted from sporulating cultures. It is possible that the purification procedure (methylated albumin-kieselguhr chromatography) did not separate these molecules from the tRNA molecules, leading to the conclusion that new tRNA species were present (14).

Because of the long labeling periods, the measured abundance is probably a reflection of both the rate of synthesis and the turnover of the individual tRNA's. Overall, we felt that the quantitation of the relative abundances of the tRNA species showed rather unimpressive differences. We feel that the single-label method used as the primary means of quantitation is not accurate enough to evaluate the significance of the changes in abundance that are seen. The use of double-label methods would be beneficial in future investigations of this type.

tRNA synthesis during outgrowth. The complement of tRNA molecules synthesized during spore outgrowth appeared very similar to

that seen during vegetative growth and sporulation. No evidence for periodic synthesis of tRNA's was seen. We felt that it might be possible to see effects of gene dosage on tRNA synthesis during outgrowth when the chromosome is being synchronously replicated. Since the majority of tRNA genes are located near the chromosome origin (24, 33), a gene dosage effect would most likely show up as a decreased abundance for any tRNA encoded near the terminus when the chromosome is partially replicated. In this regard, a few of the spots do show a relative decrease after the initiation of chromosome replication, during the 110- to 140-min and 210- to 240-min intervals (Table 3). These might be candidates for distal tRNA genes, but the caveat noted above regarding quantitation should be kept in mind.

Turnover and compartmentation of tRNA. Turnover of tRNA's during sporulation was examined by one basic experiment in which a culture labeled during vegetative growth was chased with excess phosphate until t_{10} or t_{24}, at which time the culture was harvested and RNA was extracted. A key assumption underlying this procedure is that the excess phosphate chase would markedly reduce the newly synthesized RNA by dilution of the ^{32}P label. Figures 2 and 3 show that the excess phosphate chase considerably reduced $^{32}P_i$ incorporation into cold trichloroacetic acid-precipitable material and eventually blocked further accumulation. During the course of sporulation the number of counts in cold trichloroacetic acid-precipitable material declined, indicating that the addition of excess phosphate was reducing the re-incorporation of the degraded labeled material into newly synthesized macromolecules (Fig. 3b).

A direct extrapolation from cold trichloroacetic acid-precipitable material to a conclusion about RNA cannot be made because the effect of rifampin on RNA synthesis shows that a large proportion of the ^{32}P label is going into cold trichloroacetic acid-precipitable material other than RNA (Fig. 2). However, other experiments showed that a vegetatively labeled culture harvested at t_{10} (after a phosphate chase) had lost 85% of the label that could be recovered in the DEAE-cellulose column eluate, primarily tRNA and 5S RNA (Henner, Ph.D. thesis). This shows that the phosphate chase reduces re-incorporation of ^{32}P label into new RNA molecules.

Insofar as we can conclude that the phosphate chase is effective and that the tRNA molecules seen on the gels after the phosphate chase are ones carried over from vegetative growth (Fig. 5), we can conclude that the turnover rates of the individual tRNA's are very similar, since over a period of 10 h a small difference in turnover rate would be magnified into a large change in relative abundance (Table 2). The presence of a full complement of tRNA molecules synthesized during vetetative growth remaining in sporulating cells in measurable amounts further contradicts the hybridization-competition studies, which concluded that certain tRNA species present in vegetative cells were absent during sporulation (14).

The tRNA that is found in mature spores includes all of the tRNA spots that are seen during vegetative growth (Fig. 5). Quantitation of the tRNA spots in the samples chased into spores also shows relative abundances approximating those of vegetative tRNA, although there are a few exceptions (e.g., spots 21 and 37) (Table 2). Appearing on the gels of ^{32}P-labeled material chased into spores are a number of new spots that do not show up in the gel pattern of samples labeled during vegetative growth or sporulation. These new spots are found whether the tRNA chased into the spore is labeled during vegetative growth or late in sporulation. One possible origin of these spots is tRNA that has lost part of its 3′ CCA terminus, as approximately 30% of the tRNA molecules in mature spores have lost their 3′-terminal A, and a few percent have lost one or both terminal Cs (32, 36). The fraction of molecules losing termini varies among different tRNA species, reaching 80% for $tRNA^{Tyr}$ in *Bacillus megaterium* (32). The possibility that the new spots seen on the 2-D gels of vegetatively labeled cells or cells labeled at t_9 to t_{10} chased into spores are newly synthesized, unique tRNA species is felt to be unlikely primarily because the phosphate chase seems to effectively block re-incorporation of the ^{32}P label (Fig. 3). This would indicate that these new tRNA spots are produced from preexisting molecules. If changes in tRNA play a regulatory role in differentiation as has been postulated (18), it seems most likely in this biological system that posttranscriptional controls are being used to change the complement of tRNA's, rather than synthesis of new tRNA species.

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