Thiolation and 2-Methylthio- Modification of *Bacillus subtilis* Transfer Ribonucleic Acids

BARBARA S. VOLD,* MARIA E. LONGMIRE,† AND DUANE E. KEITH, JR.

Biomedical Research Laboratory, SRI International, Menlo Park, California 94025

Six thionucleosides found in *Bacillus subtilis* transfer ribonucleic acids were investigated: N^6-(Δ^2-isopentenyl)-2-methylthioadenosine, 5-carboxymethylaminomethyl-2-thiouridine, 4-thiouridine, 2-methylthioadenosine, N-[(9-β-D-ribofuranosyl-2-methylthiopurin-6-yl)carbamoyl]threonine, and one unknown (X_1). The presence of N-[(9-β-D-ribofuranosyl-2-methylthiopurin-6-yl)carbamoyl]threonine was demonstrated based on the affinity of the transfer ribonucleic acid containing it for an immunoadsorbent made with the antibody directed toward N-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl]-L-threonine. The existance of N-[(9-β-D-ribofuranosyl-2-methylthiopurin-6-yl)carbamoyl]threonine in two species of lysine transfer ribonucleic acids was also confirmed by high-resolution mass spectrometry. Four of these thionucleosides—N^6-(Δ^2-isopenenyl)-2-methylthioadenosine, 2-methylthioadenosine, 5-carboxymethylaminomethyl-2-thiouridine, and the unknown designated X_1—occurred only in specific areas in the elution profile of an RPC-5 column and probably affect the chromatographic properties of the transfer ribonucleic acids containing them. In contrast with *Escherichia coli*, where 4-thiouridine is the most frequent type of sulfur-containing modification, approximately one-third of the sulfur groups in *B. subtilis* transfer ribonucleic acid are present as thiomethyl groups on the 2 position of an adenosine or modified adenosine residue.

The modified nucleosides of *Bacillus subtilis* tRNA's show some differences from those of *Escherichia coli*, mainly in the types and amounts of modifications involving thiolation. Previous work by Goehler and Doi (11) had shown that *B. subtilis* tRNA's had at least three thionucleotides, one of which was s⁴U; the others were not identified. Cerutti et al. (8) reported a content for s⁴U in *B. subtilis* tRNA's which was much lower than that in *E. coli* tRNA's. The presence of other thionucleosides has been noted in certain tRNA's that have been sequenced. Yamada et al. (30) found cmnm⁵s²U in *B. subtilis* tRNA₁ᴸʸˢ; ms²i⁶A has been found in tRNAᴾʰᵉ (3, 19); ms²i⁶A and s⁴U have been found in tRNA₂ᵀʸʳ (15, 19); and ms²t⁶A has been found in tRNA₁ᴸʸˢ (29). We have previously reported the presence of s⁴U and two other thionucleosides in tRNA's from *B. subtilis* (24) and estimated the overall level of thiolation to be between 0.36 and 0.44 mol of total thionucleoside per mol of tRNA. The content of thionucleosides is 3 to 4 times greater in *E. coli* tRNA's than in *B. subtilis* tRNA's. Nevertheless, there are at least five

thiolated nucleosides in *B. subtilis*, as described in this paper. Thus, the lower degree of thiolation is not reflected by fewer thiolated nucleosides, but rather by what seems to be a more complex pattern of thiolation, with about one-third of the sulfur groups being present as thiomethyl groups on the 2 position of an adenosine or modified adenosine residue.

MATERIALS AND METHODS

Abbreviations. Nucleoside abbreviations used are: s⁴U, 4-thiouridine; ms²i⁶A, N^6-(Δ^2-isopentenyl)-2-methylthioadenosine; ms²A, 2-methylthioadenosine; i⁶A, N^6-(Δ^2-isopentenyl)adenosine; s²C, 2-thiocytidine; mam⁵s²U, 2-thio-5-(N-methylaminomethyl)uridine; cmnm⁵s²U, 5-carboxymethylaminomethyl-2-thiouridine; ms²t⁶A, N-[(9-β-D-ribofuranosyl-2-methylthiopurin-6-yl)carbamoyl]threonine; and t⁶A, N-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl]-L-threonine.

Labeling of tRNA's with ³⁵S. Cells of *B. subtilis* 168 *trpC2* were labeled with ³⁵S in vivo by adding 0.5 mCi of L-[³⁵S]cysteine (Amersham Corp.; 48 mCi/mmol) to 20 ml of medium consisting of 10 g of tryptone (Difco Laboratories), 5 g of yeast extract (Difco), 5 g of NaCl, 10^{-3} mol of $CaCl_2$, 10^{-5} mol of $MnCl_2$, 10^{-6} mol of $FeCl_3$, and 40 mg of L-tryptophan per liter. The use of L-[³⁵S]cysteine enabled the labeling of tRNA's with sulfur in a rich medium that would support sporulation, although L-cysteine may not be

the ultimate sulfur donor for *B. subtilis* (28). Specific activities of 4×10^4 to 6×10^4 cpm per unit of absorbancy at 260 nm of tRNA were obtained. The use of sodium [^{35}S]sulfate did not result in any detectable incorporation under the same conditions. Since the alteration in chromatographic elution properties of the tyrosine tRNA isoacceptors has been shown to be due to a change in the thiomethylation of i^6A during development (15), it seemed desirable to do the labeling under conditions in which sporulation and normal development were possible. The tRNA's were extracted with phenol after the cells were broken with lysozyme, and the tRNA fraction was isolated after chromatography of the extract on a column of Sephadex G-100 (24).

Cells of *E. coli* K-12, strain W3110 (*lacZ U118 trpR Azi^r/Val^r*), were labeled with 0.5 mCi of L-[^{35}S]cysteine in 50 ml of medium containing 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter. The tRNA was extracted as previously described (27).

Digestion of tRNA to nucleosides and analysis by two-dimensional TLC. tRNA's were digested to nucleosides by incubating approximately 1 unit of absorbancy at 260 nm of tRNA in 20 μl of 0.05 M ammonium acetate (pH 4.5) plus 1 mM EDTA with 4 U of RNase T_2, 20 U of RNase T_1, and 0.06 mg of pancreatic RNase, 37°C, 6 h. Nucleosides were obtained by further digestion after adding Tris to 5 mM, adjusting the pH to 7.5, adding 1 U of alkaline phosphatase, and continuing the incubation at 37°C for 6 h. Two-dimensional thin-layer chromatography (TLC) analyses were performed on cellulose-coated, 20- by 20-cm TLC plates backed with aluminum by using 1-butanol–isobutyric acid–concentrated ammonium hydroxide–water (75:37.5:2.5:25, vol/vol/vol/vol) in the first dimension and saturated ammonium sulfate–0.1 N sodium acetate (pH 6.0)–isopropanol (79:19:2, vol/vol/vol) in the second dimension (21).

RPC-5 column chromatography. tRNA's were fractionated on an RPC-5 column (0.6 by 19 cm) at 25°C with a gradient of 0.3 to 1.2 M NaCl in 10 mM sodium acetate (pH 4.5), 10 mM $MgCl_2$, 10 mM EDTA, and 20 mM 2-mercaptoethanol. Fractions of 2 ml were collected at a flow rate of 0.5 ml/min.

Preparation of antibody and immunoadsorbent gel. Antibodies to t^6A were elicited in rabbits by using a bovine serum albumin-nucleoside conjugate. The preparation and specificity of the antibodies have been described previously (26). The immunoadsorbent was made by coupling partially purified immunoglobulin fraction to CNBr-activated Sepharose 4B as described previously (27).

RESULTS

Fractionation of ^{35}S-labeled tRNA's on RPC-5 and TLC analyses of thionucleosides. In *E. coli* tRNA's, four nucleosides have been identified—s^2C, s^4U, ms^2i^6A, and mam^5s^2U, the most commonly occurring thionucleoside being s^4U (7, 16, 23). Similar to *E. coli*, *Pseudomonas aeruginosa* tRNA's contain s^4U, s^2C, mam^5s^2U, and one unidentified thionucleoside, with 65% of the thionucleosides being present as

s^4U (23). When we compared the thionucleosides isolated from *B. subtilis* or *E. coli* labeled in vivo with ^{35}S in very similar medium, chosen for *B. subtilis* because it supports normal sporulation, we found s^2C, s^4U, ms^2i^6A, and mam^5s^2U in *E. coli* tRNA's, with s^4U being predominant, as would be expected. *B. subtilis* tRNA's also contained s^4U and ms^2i^6A; however, s^4U was not the predominant nucleoside, and there was no evidence for s^2C or mam^5s^2U. One nucleoside (designated X_1 in Fig. 2) chromatographed near the position of mam^5s^2U as a nucleoside; however, a comparison of nucleotide patterns showed no radioactivity in the area characteristic of mam^5s^2Up. We have no suggestion as to the identity of this nucleoside at present.

Instead of using total [^{35}S]tRNA's, we attempted to achieve some purification by chromatographing the [^{35}S]tRNA's on a column of RPC-5 (Fig. 1). The majority of the radioactivity chromatographed in five areas, the fractions from which were pooled, and the recovered tRNA's were digested to nucleosides and analyzed by two-dimensional TLC. Since the major areas absorbing in the UV had little or no radioactivity from ^{35}S, it is possible that several *B. subtilis* tRNA's contain no thionucleosides at all. This is compatible with the previously determined value of 0.36 to 0.44 mol of thionucleoside per mol of *B. subtilis* tRNA (24).

The TLC analyses of the thionucleosides from each of the RPC-5 fractions are presented in Fig. 2. The identity of s^4U, ms^2A, and ms^2i^6A was confirmed by co-chromatography with known standards. As mentioned above, we do not know the identity of the nucleoside designated X_1; however, it does not seem to be mam^5s^2U. X_2 is probably $cmnm^5s^2$U based on its chromatographic behavior in this particular solvent system (30) and its known occurrence in *B. subtilis* tRNA. We think the area labeled a_1 is an artifact because its presence is highly variable from preparation to preparation. Since it occurs in the same RPC-5 fractions as X_2 ($cmnm^5s^2$U), it may be a degradation product of X_2. Also the areas near the origin, O_{1-3}, are not always reproducible, although one of them is a real nucleoside which we have subsequently identified as ms^2t^6A.

The occurrence of ms^2A in the nucleoside digest is worth noting. The presence of the nucleoside ms^2A was confirmed by co-chromatography of the labeled nucleoside with an internal standard kindly provided by Nelson Leonard. The nucleotide ms^2Ap has been reported to occur in the tRNA's of *Bacillus stearothermophilus* (2). However, the biosynthesis of the 2-methylthio derivative of i^6A in *E. coli* is thought to occur in a sequential manner by which the i^6A
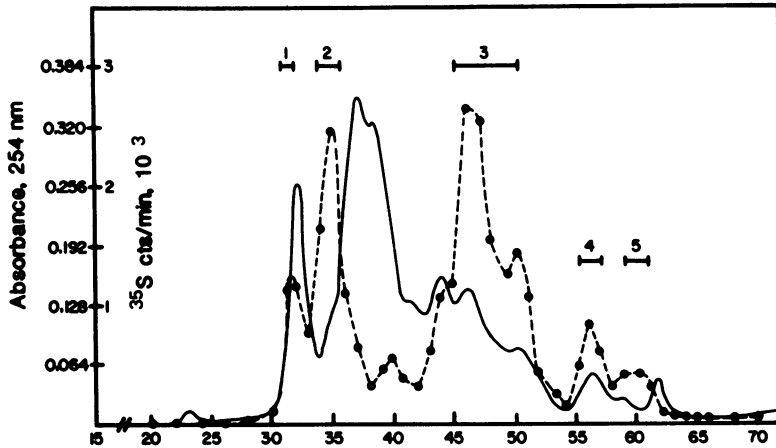
FIG. 1. *Chromatography of tRNA's labeled in vivo with ^{35}S and fractionated on a column of RPC-5. Absorbance at 254 nm was monitored by a UV detector with an analytical flow cell and is represented by a solid line. Fractions containing ^{35}S were located by liquid scintillation spectroscopy of samples. Radioactivity is indicated by a dashed line. Fractions marked by numbered bars were collected and further analyzed as shown in Fig. 2.*
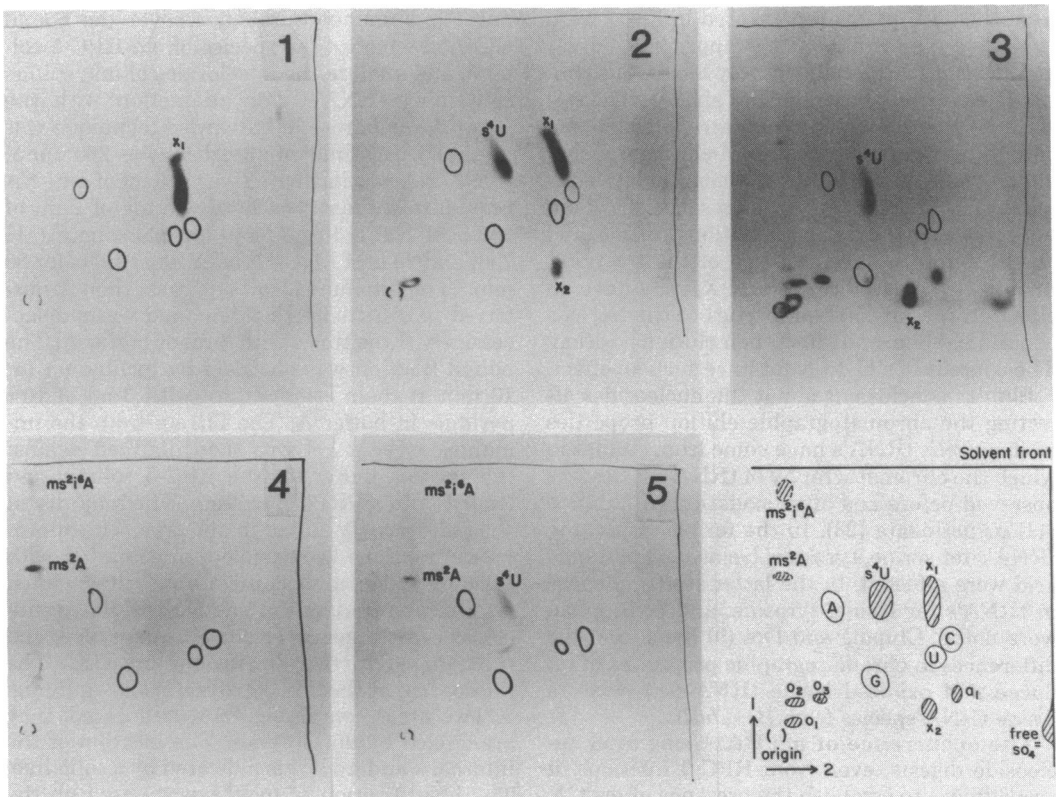

FIG. 2. *TLC analyses of the thionucleosides present in the various tRNA fractions eluted from RPC-5. Fractions designated by numbered bars in Fig. 1 were collected, digested to nucleosides, and analysed by TLC as described in the text. Positions for the major nucleosides were located by UV and shown in the figure as circled areas. Areas containing radioactivity were located by fluorography. The figure is a composite of fluorograms of TLC analyses of fractions 1 through 5 (Fig. 1) plus a schematic diagram summarizing all areas.*

must be formed before the thiomethyl group is added (1). Therefore, ms^2A would not be expected to be a normal biosynthetic precursor. Whether ms^2A arose by degradation of ms^2i^6A, however, is open to question. Although i^6A is susceptible to decomposition, the complete removal of the isopentenyl side chain requires rather drastic chemical treatment (12). Nevertheless, an enzyme activity has been detected in *Lactobacillus acidophilus* that can remove the isopentenyl group at the macromolecular level (17), and it is possible that such an enzyme activity exists in *B. subtilis*. The ms^2A group could also arise from degradation from ms^2t^6A; however, there is so little ms^2t^6A present that it seems very unlikely.

The TLC analyses from RPC-5 fractions were helpful in characterizing the types of thionucleosides present in *B. subtilis* tRNA's; however, they also showed that the presence of four of the thionucleosides in tRNA's could be correlated with the elution position of the tRNA from the RPC-5 column. The amounts of radioactivity from the TLC plates represented in Fig. 2 were evaluated by cutting out the appropriate areas and measuring the radioactivity in a liquid scintillation spectrophotometer (Table 1). The nucleoside X_1 predominated in early-eluting species; X_2 predominated in mid-eluting species; and ms^2A and ms^2i^6A predominated in late-eluting species. Interestingly, the presence of s^4U did not show a correlation with elution position, and it was found in at least three of the fractions. Like ms^2i^6A, therefore, X_1 and X_2 may have an effect on the physical properties of the tRNA's containing them and affect their elution position. The content of s^4U does not have such an effect.

Similar conclusions about thionucleosides affecting the chromatographic elution properties of *B. subtilis* tRNA's have come from studies in which the chromatography of tRNA species was observed before and after oxidation with iodine (11) or periodate (23). In the former study, the tRNA's for serine, tyrosine, lysine, and glutamic acid were affected. In the latter study, changes in tRNA's for lysine, tyrosine, and tryptophan were noted. Chuang and Doi (9) have used the differences in chromatographic properties of reduced and oxidized lysine tRNA's to purify a lysine tRNA species from *B. subtilis*.

The occurrence of ms^2t^6A. Using total nucleoside digests, even from RPC-5 fractions, it was difficult to establish the presence of ms^2t^6A, although Yamada and Ishikura (29) have reported the presence of that nucleoside in a purified species of *B. subtilis* tRNA. Since we had made an antibody to t^6A, we decided to employ that antibody to try to enrich for ms^2t^6A. Thus, an experiment was designed to label cells with

TABLE 1. *Distribution of thionucleosides in fractions from an RPC-5 column* [a]

Radioactive area [b]	RPC-5 fraction				
	1	2	3	4	5
X_1	96	43	1	2	2
X_2 ($cmnm^5s^2U$)	1	10	28	2	1
ms^2i^6A	0	1	1	22	9
ms^2A	0	0	2	54	23
s^4U	3	38	25	12	61

[a] The amount of each thionucleoside is expressed as the percentage of radioactivity in each radioactive area compared with the total for that particular TLC plate.

[b] ms^2t^6A is not included in this table because of the lack of definition of the areas near the origin. Also, the area called a_1 was omitted since, as discussed in the text, it is probably an artifact generated by X_2. Both areas near the origin and area a_1 were found predominantly in fraction 3 in which they represented 32 and 11% of the total radioactivity on the TLC plate.

^{35}S, extract the tRNA, expose the tRNA to an anti-t^6A immunoadsorbent, recover the bound tRNA's, separate the species on an RPC-5 column, and analyze the nucelosides of any sulfur-containing tRNA's. For interaction with the anti-t^6A Sepharose, a batchwise technique was used. About 9 units of absorbancy at 260 nm of $[^{35}S]$tRNA was incubated with 0.5 ml of anti-t^6A immunoadsorbent in a total volume of 2 ml of 150 mM NaCl, 10 mM sodium phosphate (pH 7.4), and 20 mM $MgCl_2$ (buffer A) at 19°C for 30 min. The immunoadsorbent was then transferred to a fritted disk funnel and washed successively three times with 2 ml of buffer A. The bound fraction was obtained by incubation for 10 min at room temperature with 3 ml of 10% pyridine in buffer A. The filtrate from the immunoadsorbent gel was then dialyzed against the starting buffer for the RPC-5 column and loaded onto an RPC-5 column. The majority of the radioactivity eluted in one area. The appropriate fractions were pooled, precipitated with ethanol, collected on a membrane filter, eluted into an appropriate buffer for digestion to nucleosides by a gentle enzymatic procedure, and fractionated in two dimensions by TLC. The fluorogram of the TLC analysis is shown in Fig. 3. Two areas containing ^{35}S were located; they are circled by dotted lines. The location of the internal standard s^4U is indicated by a solid line. The identification of ms^2t^6A was based on the co-chromatography of a standard obtained from *B. subtilis* $tRNA_1^{Lys}$ provided by Y. Yamada and H. Ishikura. The TLC plate also seemed to contain a small amount of X_2, but it is essentially invisible in Fig. 3. It is important to consider that *B. subtilis* also contains a species, threonine
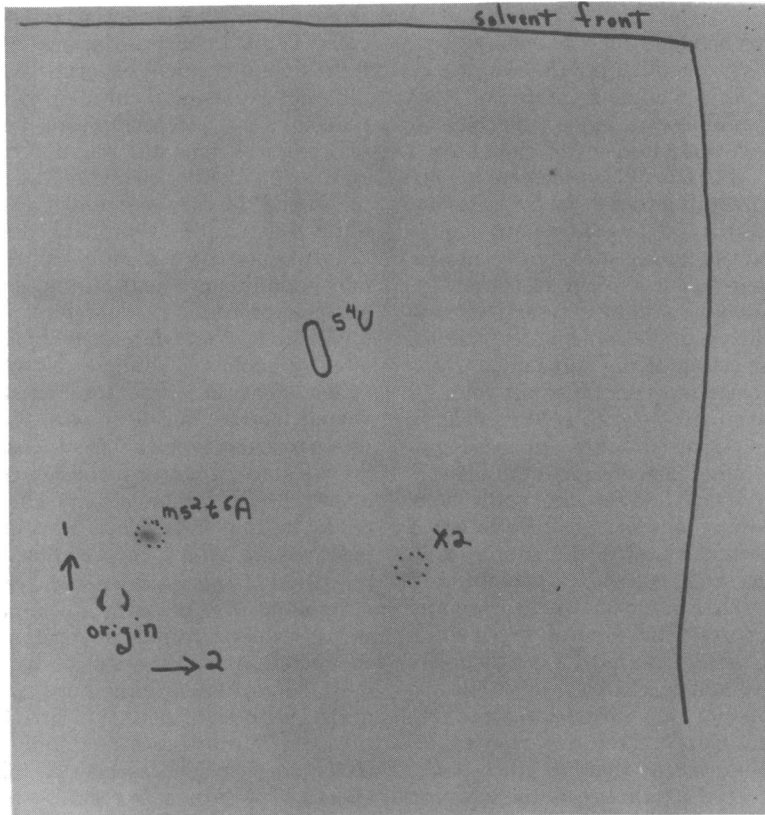
FIG. 3. *Fluorogram of the [³⁵S]nucleosides obtained by digesting the [³⁵S]tRNA fraction from RPC-5 chromatography of tRNA's that had been bound to anti-t⁶A immunoadsorbent. The solvent system is described in the text. s⁴U was included as an internal standard.*

tRNA, which has the t^6A modification without the thiomethyl modification (13). So apparently the thiomethylating enzyme discriminates between t^6A in lysine tRNA and t^6A in threonine tRNA.

Identification of ms^2t^6A in *B. subtilis* lysine tRNA's by analysis by mass spectrometry. Yamada and Ishikura reported the presence of ms^2t^6A in lysine $tRNA_1$ from *B. subtilis* based on its ultraviolet absorption spectra and co-chromatography with ms^2t^6A obtained from rabbit liver $tRNA^{Lys}$ (29). We wished to confirm the presence of this nucleoside by mass spectrometry and also see whether its presence could be detected in the other of the two major lysine isoacceptors, $tRNA_3^{Lys}$. Purified species of $tRNA_1^{Lys}$ and $tRNA_3^{Lys}$ made in our laboratory were analyzed by H. Pang and J. A. McCloskey (unpublished results). The two tRNA samples were hydrolyzed and trimethylsilylated, and their high-resolution mass spectra were photographically recorded (H. Pang and J. A. Mc-Closkey, 28th Annual Conference on Mass Spec-

trometry, and Allied Topics, New York, N.Y., 1980, p. 384–385, abstr. WAMOB1). A compound was identified on both tRNA samples having the same properties as the ms^2t^6A previously isolated from rabbit liver tRNA and characterized by high-resolution mass spectrometry (31).

Levels of thionucleosides in total tRNA's isolated from cells in different stages of growth. Sequence studies on the two tyrosine isoacceptors from *B. subtilis* showed that they had the same primary sequence but differed in that the species which was prominent in exponentially growing cells contained i^6A in the anticodon loop, whereas the isoacceptor prominent in the stationary phase contained ms^2i^6A (18). Studies on isolated nucleosides from mixed tRNA's indicated that 29% of the i^6A population was thiomethylated at midexponential growth and 67% was thiomethylated at the stationary phase (25). This change in degree of thiomethylation causes a chromatographic alteration in the tyrosine isoacceptors and has been suggested to be associated with the initiation of sporulation

(6). An increase in the thiomethylation of i⁶A may also be responsible for chromatographic alterations observed in other tRNA's which contain the i⁶A moiety, such as tryptophan tRNA and some species of leucine and serine tRNA's.

To see whether there were any major changes in the levels of other thionucleosides, we labeled cells with ^{35}S under different growth conditions, extracted the tRNA's, digested them to nucleosides, separated the nucleosides by two-dimensional TLC, made an autoradiogram of the TLC plate, cut out areas containing radioactivity, and counted them in a scintillation counter. Table 2 presents a comparison of the percentage which each thionucleoside represented of the total for [^{35}S]tRNA's extracted from *B. subtilis* cells in exponential growth or stationary phase. Exponential cells were harvested at 1 unit of absorbancy at 660 nm per ml, and the radioactive label was present throughout growth. Stationary-1 cells were harvested 2 h after the end of exponential growth, and the label was present throughout growth. Stationary-2 cells were also harvested 2 h after the end of exponential growth, but the radioactive label was added only after the culture had reached 1 unit of absorbancy at 660 nm per ml. Stationary-2 cultures plus glucose cells were treated the same as stationary-2 cultures, except that 1% glucose was added to the culture 15 min before the addition of the label. The addition of 1% glucose in midexponential growth suppressed the efficiency of sporulation after 10 h of growth from 80 to 0.003%. The culture with glucose was added to the protocol in the hope of amplifying any dif-

TABLE 2. *Levels of thionucleosides under different growth conditions*[a]

Thionucleoside[b]	Growth stage[c]			
	Exponential	Stationary-1	Stationary-2	Stationary-2 plus glucose
ms²i⁶A	21 (±9)	27	29	32
ms²A	9 (±5)	14	15	19
s⁴U	11 (±7)	12	18	4
X₁	24 (±8)	22	19	17
X₂ (cmnm⁵s²U)	21 (±4)	14	12	15
Areas near origin (partly ms²t⁶A)	7 (±6)	8	5	6

[a] Values are expressed as the percentage which each radioactive area represents of the total for a TLC analysis. Standard deviations are given in the first column. Each number represents the mean for six independent experiments.

[b] Areas containing radioactivity are as designated in Fig. 2 for *B. subtilis*.

[c] Growth conditions are explained in the text.

ference between sporulating and nonsporulating cells. The standard deviations were calculated from six independent experiments starting with different exponential cultures, so the variability reflects what one might expect repeating the entire process on a different day with a different culture. As mentioned before, the areas near the origin and the area designated a₁ on Fig. 2 were quite variable. This is probably due to the instability of some thionucleosides and the generation of degradation products during the isolation and analysis procedures. Therefore, it is probably unwise to evaluate differences of less than about 50% by such a technique. Nevertheless, it is evident that there were no dramatic changes in thionucleosides during growth, such as the lack of a particular type of thionucleoside at a given growth stage, which could be detected by looking at mixed tRNA populations. Therefore, differences in the thionucleoside content between isoaccepting tRNA species that occur during growth in *B. subtilis* are not the result of loss of enzyme activity or substrate limitation due to shifts in the nutritional environment or biosynthetic pathways, but are selective changes that must be regulated with more specific mechanisms. With the use of mixed tRNA's, thionucleosides that are present in only one or a few tRNA species, such as ms²t⁶A, might be overlooked. Therefore, it is desirable to look at purified tRNA species in addition to getting a general picture from mixed tRNA's.

In addition, the data in Table 2 demonstrate which thionucleosides are most common in occurrence. Assuming that there is 0.44 mol of total thionucleoside per mol of tRNA from *B. subtilis* and that there are 42 different species of tRNA in *B. subtilis*, then one would find the most frequent thionucleoside in about five different tRNA species and the least frequent in about one. Of course, the various tRNA species are present in different amounts. Henner and Steinberg (14) have reported the relative abundance of 32 tRNA species from *B. subtilis* to range from 0.9 to 17% of the total. They also detected five to six species which were not quantitated due to their low abundance. None the less, it is true that thiolated nucleosides are not very common modifications in *B. subtilis* tRNA's, and each type probably occurs in a small subset of tRNA species.

DISCUSSION

The features—determined by ourselves and others—in which the thiolated nucleosides in *B. subtilis* tRNA's differ from the typical procaryotic picture (which is primarily the result of studies with *E. coli*) are the following: (i) s⁴U is

not the major thionucleoside (8, 24, 28); (ii) the overall degree of thiolation is three to four times lower than that of *E. coli* (28); (iii) six thionucleosides have been found in *B. subtilis* tRNA's (ms^{2}i^{6}A, ms^{2}A, ms^{2}t^{6}A, cmnm^{5}s^{2}U, s^{4}U, and one unknown), whereas four thionucleosides have been reported for *E. coli* tRNA's (16, 20), only two of which are also found in *B. subtilis*—s^{4}U and ms^{2}i^{6}A; (iv) in *E. coli* tRNA's 90 to 93% of the i^{6}A moieties were thiomethylated at whatever growth stage was examined (4), whereas in *B. subtilis* the degree of thiomethylation of i^{6}A changes during development (15, 25); and (v) ms^{2}t^{6}A is present in *B. subtilis* tRNA's (29). The ms^{2}t^{6}A modification reported in *B. subtilis* lysine tRNA by Yamada and Ishikura (29) has been found so far in only one other tRNA, the lysine tRNA$_3$ from rabbit liver (31). It has not been found in any of the tRNA's of *E. coli* or any other procaryote.

It is possible that some of the thionucleosides, in particular ms^{2}i^{6}A, ms^{2}A, cmnm^{5}s^{2}U, and X$_1$, exert an influence on the chromatographic properties of the tRNA's containing them since they occur only in specific areas of the elution profile of an RPC-5 column. Although changes in the degree of thiomethylation have been reported in purified tRNA's for tyrosine (15, 18), one cannot detect major changes in the amounts of thiolated nucleosides in general by examining unfractionated tRNA's labeled in vivo with ^{35}S, digested to nucleosides, and chromatographed on two-dimensional TLC. It would seem, therefore, that the changes that are detected are regulated by specific mechanisms and not by a lack of substrate resulting in hypomodified tRNA at a particular growth stage, which has been reported to be the cause of the appearance of hypomodified tRNA's in another system (22).

Evidence for the functional importance of thiolated nucleosides, and particularly thiomethylated nucleosides, has been reported. The increase in the amount of thiomethylation of i^{6}A in the tyrosine isoacceptors described by Buu et al. (6) may be related to the sporulation process. In *E. coli*, Buck and Griffiths have found that certain tRNA's lacking the ms^{2}i^{6}A modification can function as positive regulatory elements of the aromatic acid transport system (5), and the loss of the ms^2 group on i^{6}A decreases the ability of the tRNA to bind to ribosomes in *E. coli* (10) as well as in *B. subtilis* (18). Since *E. coli* tRNA's seem to have only t^{6}A and 90% of the i^{6}A moieties as ms^{2}i^{6}A, it is very interesting that in *B. subtilis* there are tRNA's which contain ms^{2}t^{6}A or t^{6}A and ms^{2}i^{6}A or i^{6}A.

ACKNOWLEDGMENTS

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