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Protein Synthesis In Vitro by *Micrococcus luteus*

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***Bacillus subtilis* and related gram-positive bacteria which have low to moderate genomic G+C contents are unable to efficiently translate mRNA derived from gram-negative bacteria, whereas *Escherichia coli* and other gram-negative bacteria are able to translate mRNA from both types of organisms. This phenomenon has been termed translational species specificity. Ribosomes from the low-G+C-content group (low-G+C group) of gram-positive organisms (*B. subtilis* and relatives) lack an equivalent to *Escherichia* ribosomal protein S1. The requirement for S1 for translation in *E. coli* (G. van Dieijen, P. H. van Knippenberg, J. van Duin, B. Koekman, and P. H. Pouwels, Mol. Gen. Genet. 153:75–80, 1977) and its specific role (A. R. Subramanian, Trends Biochem. Sci. 9:491–494, 1984) have been proposed. The group of gram-positive bacteria characterized by high genomic G+C content (formerly *Actinomyces* species and relatives) contain S1, in contrast to the low-G+C group (K. Mikulik, J. Smardova, A. Jiranova, and P. Branny, Eur. J. Biochem. 155:557–563, 1986). It is not known whether members of the high-G+C group are translationally specific, although there is evidence that one genus, *Streptomyces*, can express *Escherichia* genes in vivo (M. J. Bibb and S. N. Cohen, Mol. Gen. Genet. 187:265–277, 1982; J. L. Schottel, M. J. Bibb, and S. N. Cohen, J. Bacteriol. 146:360–368, 1981). In order to determine whether the organisms of this group are translationally specific, we examined the in vitro translational characteristics of a member of the high-G+C group, *Micrococcus luteus*, whose genomic G+C content is 73%. A semipurified coupled transcription-translation system of *M. luteus* translates *Escherichia* mRNA as well as *Bacillus* and *Micrococcus* mRNA. Therefore, *M. luteus* is translationally nonspecific and resembles *E. coli* rather than *B. subtilis* in its translational characteristics.**

The phenomenon of translational species specificity, demonstrated by the inability of ribosomes from *Bacillus subtilis* to translate mRNA derived from *Escherichia coli*, has been observed in several gram-positive species. Besides *B. subtilis*, these include *Clostridium pasteurianum*, *Streptococcus faecalis*, and *Peptococcus asacharolyticus* (29). Translational specificity has not been observed in any of the several gram-negative genera that have been tested, including *Azotobacter* and *Pseudomonas*. These results led to the conclusion that gram-positive bacteria are translationally specific, while gram-negative bacteria are translationally nonspecific (29). An evolutionary tree including several genera of interest is shown in Fig. 1. This tree, based on 16S rRNA sequence data, illustrates several points. All the genera that are translationally specific have a genomic G+C content of under 50%, with the exception of *Acetobacter*. The translationally nonspecific genera have G+C contents of 50% or more and, in addition, the ribosomes of these genera contain a protein equivalent to ribosomal protein S1, whereas none of the translationally specific genera contain S1. S1 has been shown to be necessary for translation in *E. coli* (33).

There is a distinct evolutionary group of gram-positive bacteria whose translational characteristics have not been determined. This high-G+C-content group (high-G+C group) (formerly *Actinomyces* species and relatives) (6) is characterized by high genomic G+C content and the presence of ribosomal protein S1 (20). A crude transcription-translation system from one member, the genus *Streptomyces*, had very low activity (32).

In this report, we examine the translational characteristics of a member of this group, *Micrococcus luteus*, in order to determine whether it is translationally specific. Because the group is relatively close-knit, we predict that all members of the high-G+C group share the same translational characteristics.

MATERIALS AND METHODS

Reagents. *Escherichia* tRNA, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and all restriction enzymes were purchased from Boehringer-Mannheim Biochemicals, Indianapolis, Ind.; ultrapure cesium chloride was from Bethesda Research Laboratories, Gaithersburg, Md.; amino acids, cyclic AMP, ATP, CTP, phosphoenolpyruvate (monocyclohexylammonium salt), lysozyme (chicken egg white), pyruvate kinase (rabbit muscle), bovine serum albumin, rifampin, Tris, dithiothreitol, and diethylpyrocarbonate were from Sigma Chemical Corporation, St. Louis, Mo.; GTP and UTP were from P-L Biochemicals, Milwaukee, Wis.; calcium leucovorin was from American Cyanamid Co., Pearl River, N.Y.; ultrapure phenol was from Clontech Laboratories, Inc., Palo Alto, Calif.; ultrapure sucrose and ultrapure acrylamide were from Schwartz/Mann Biotech, Inc., Cleveland, Ohio; other electrophoresis materials were from Bio-Rad Laboratories, Richmond, Calif.; 2-mercaptoethanol was from Eastman Kodak, Rochester, N.Y.; EDTA was from Fisher Scientific, Fair Lawn, N.J.; trichloroacetic acid (TCA) was from Mallinckrodt, Inc., Paris, Ky.; [α-^{32}P]CTP (400 Ci/mmol) and [^{35}S]methionine (1,500 Ci/mmol) were from Amersham Corp., Arlington Heights, Ill. [^{35}S]cysteine (1,000 Ci/mmol) was from ICN Biochemicals, Inc., Cleveland, Ohio. T7 phage, φ29 DNA, T7 RNA polymerase, and T7 mutant DNA transcripts were the gifts of Paul Hager of this department. *Escherichia* and *Bacillus* RNA polymerases were the gifts of Michael Chamberlin of this department. All other chemicals were reagent grade or better.

Plasmids. Plasmid pNM2-21 containing the *Micrococcus*

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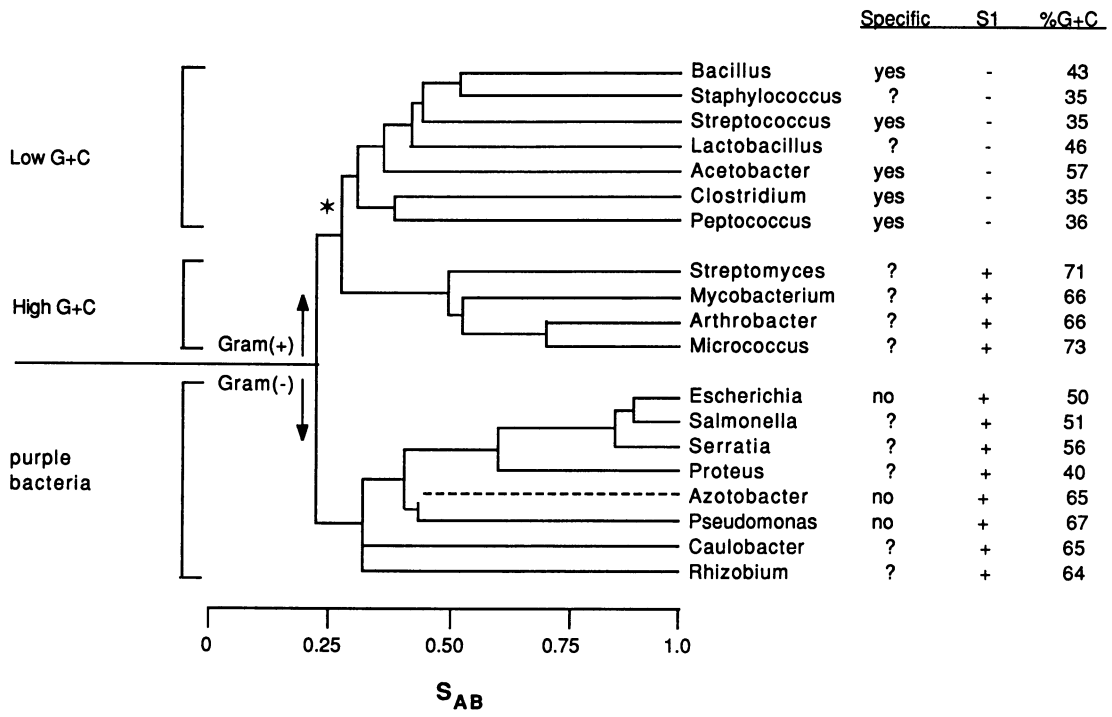

FIG. 1. Phylogenetic tree of some bacteria. This tree (24) was based on 16S rRNA S_{AB} values of Fox et al. and Woese (6, 36). "Specific" refers to whether the organism has been found to be translationally specific, as described in the text. S1 data are from references found in the text, and % G+C values are from Bergey's Manual of Determinative Bacteriology (3). Asterisk indicates origin of translational specificity (see Discussion).

streptomycin (*str*) operon was the gift of T. Ohama, Department of Biology, Nagoya University, Nagoya, Japan (22). Plasmid pTTS12-7 was constructed by ligation of the 900-bp *Xma*I fragment containing the genes coding for ribosomal proteins S12 and S7 into *Xma*I-digested pTTQ18r (Amersham Corp.). These proteins will be referred to as S12-*M* and S7-*M*. Correct orientation of the insert was determined by restriction digestion. Plasmid pEϕ6 was the gift of Robert Vellanoweth of this department (34). Plasmid pTTgene6 was constructed by ligation of the 500-bp *Eco*RI-*Hin*dIII fragment of pEϕ6 containing ϕ29 gene 6 into pTTQ19r (Amersham Corp.). Plasmid pKK60 containing the *Escherichia cheZ* gene was the gift of Scot Kuo and Daniel Koshland of this department (13). Plasmid pTTcheZ was constructed by LaWanda Jones in this laboratory by ligating the 2.9-kb *Pst*I-*Hin*dIII fragment containing *cheZ* into pTTQ18r.

Preparation of ribosomes. *Escherichia* vacant couples (which are ribosomes freed of exogenous RNA) were made as previously described (26) except the zonal centrifugation was for 2.5 h.

Micrococcus ribosomal subunits were made with cells grown in PYC (10 g of Bacto-Peptone, 5 g of Bacto yeast extract, 1 g of Casamino Acids, and 5 g of NaCl per liter, pH 7.3) to A_{600} = 1.0. They were slowly cooled and then frozen. The cells were washed in 10 volumes of standard buffer consisting of 20 mM Tris-acetate (Tris-OAc) (pH 7.8), 100 mM NH_4OAc, 10 mM $Mg(OAc)_2$, 0.5 mM EDTA, and 10 mM 2-mercaptoethanol and then resuspended in 2 volumes of the same buffer. The S-30 was prepared by adding lysozyme to 2.5 mg/ml, incubating at 15°C for 2 min, and sonicating at 50 W for 6- to 10-s pulses. The extract was centrifuged for 30 min at 30,000 × *g*, and the supernatant was recentrifuged for 15 min. The S-30 was made in 2% sucrose, and EDTA was added to 9 mM to chelate Mg^{2+}. The S-30 (20 ml) was then centrifuged through a 550-ml, 10 to 20% linear sucrose gradient in a Beckman Ti14 zonal rotor as previously described to make subunits (24). The 30S and 50S peaks were collected and pooled separately as described, and standard buffer with 30 mM $Mg(OAc)_2$ was added to raise the concentration to 10 mM $Mg(OAc)_2$. They were pelleted and resuspended in standard buffer containing 10 mM $Mg(OAc)_2$ and 1 mM dithiothreitol instead of 2-mercaptoethanol. They were stored at −70°C in small aliquots.

Micrococcus vacant couples were made from an S-30 preparation by a modification of the method of Sharrock (26). Two *Micrococcus* subunit preparations obtained as described above were pooled and pelleted and then recentrifuged through a 10 to 35% biphasic gradient at 35,000 rpm for 3.5 h to produce vacant couples.

Bacillus vacant couples were made as described previously (26) except standard buffer contained 20 mM, instead of 10 mM, $Mg(OAc)_2$. The separating gradients were in standard buffer with 4 mM $Mg(OAc)_2$, and the final gradient was in standard buffer with 13 mM $Mg(OAc)_2$.

Ribosomal high-salt wash (HSW) and high-speed supernatant (S-150) fractions from all organisms were prepared as previously described (28) by using S-30 fractions described above. *Micrococcus* tRNA was prepared according to the method of Vold and Minatogawa (35) from *Micrococcus* cells grown in PYC media and harvested at log phase.

DNA and RNA isolation and transcription reactions. T7 DNA was isolated by Jack McGill in this laboratory as previously described (8). Plasmid DNA was isolated by the alkaline lysis method and purified with cesium chloride. In vitro transcription reactions were done as previously described (8). For quantitation, [^{32}P]CTP was added to 20 to 50

cpm/pmol, and an aliquot of the reaction was TCA precipitated and counted.

Translation assays. All translation assays were carried out in a final volume of 30 μl. The reactions were incubated for 20 min at 37°C, unless *Micrococcus* ribosomes or S-150 was used, in which case the incubation was at 30°C.

Unless otherwise indicated, coupled assays were carried out in a semipurified system which contained 0.72 A_{260} U ribosomes, S-150, and HSW optimized for each system, 17 μg of pyruvate kinase per ml, 10 μM unlabeled methionine, 0.5 μl of [^{35}S]methionine (5 pmol) at a specific activity of 30 to 50 cpm/fmol, and coupled mix (5, 23, 32), containing the following: 55 mM Tris-OAc (pH 8.2), 1.7 mM dithiothreitol, 1.19 mM ATP (pH 7), 0.71 mM CTP (pH 7), 0.71 mM UTP (pH 7), 0.71 mM GTP (pH 7), 26.4 mM phosphoenolpyruvate (pH 7), 0.63 mM cyclic AMP, 0.34 mM each of 19 amino acids (no methionine), 3.4 μg of leucovorin per ml, and 667 μg of *Escherichia* tRNA per ml. NH_4OAc, KOAc, and $Mg(OAc)_2$ were added to optimal concentrations, which unless indicated were 150, 37, and 14 mM, respectively. Reactions were started by the addition of 0.1 mg of DNA per ml. For quantitation, two 5-μl aliquots were taken and precipitated with 1 ml each of 5% TCA containing 0.5% methionine. The aliquots were stored on ice for 5 min and were then incubated at 95°C for 10 min to deacylate tRNA. They were cooled and filtered on 24-mm GF/C filters (Whatman) and washed with 6 ml of cold 5% TCA containing 0.5% methionine and then 6 ml of 100% ethanol. The filters were dried under a heat lamp and then added to 5 ml of ScintA (Beckman) or Universol ES (ICN) and counted in a Beckman LS8100 scintillation counter at a tritium efficiency of 54%. To visualize the products by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), one volume of SDS sample buffer (5 μl) consisting of 100 mM Tris-Cl (pH 6.8), 20% glycerol, 2% SDS, 0.1% bromophenol blue, and 2 M 2-mercaptoethanol was added to a 5-μl aliquot of the reaction mixture, and the sample was heated at 95°C for 3 min. When [^{35}S]cysteine was used as label, samples for electrophoresis were TCA precipitated as recommended by ICN prior to loading. Samples for electrophoresis were kept for up to one month at 4°C with no degradation.

Uncoupled assays were carried out as coupled assays, except uncoupled mix contained no CTP or UTP, and rifampin was added to 20 μg/ml. Unless noted, reactions were started by the addition of 7 μl of a transcription reaction mixture.

Electrophoresis and analysis. PAGE was carried out by the method of Laemmli (14) with 12.5 or 15% gels with an acrylamide/bisacrylamide ratio of 37.5:1. The stacking gel was 5% acrylamide. Gels were run at 35 mA for 2 h and fixed in 25% isopropanol–10% acetic acid (Destain Solution) for 30 min. For fluorography, gels were soaked in water for 10 min and then in 1 M sodium salicylate (4) for 30 min. Gels were dried in a Hoeffler apparatus at 80°C and then exposed to film (Kodak XAR) for various lengths of time. To get a linear densitometric response, film was preflashed to an A_{540} of 0.1 to 0.2 (15). Densitometry was performed on an EC910 instrument from EC Apparatus equipped with a 3390A integrator from Hewlett-Packard.

RESULTS

The *Micrococcus* translation system consisted of vacant couple ribosomes containing little or no associated mRNA (26), HSW containing initiation factors, and S-150 containing tRNA, enzymes, and other factors necessary for translation.

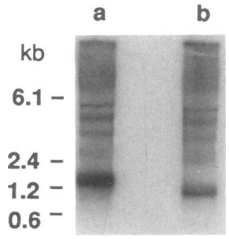

FIG. 2. Transcription of pTTS12-7 and pTTgene6. Transcription in the presence of [^{32}P]CTP was carried out as described in Materials and Methods. Samples (5 μl) were electrophoresed through a 1.5% TGE (Tris-glycine-EDTA)-SDS agarose gel, dried under vacuum, and autoradiographed for 21 h. Lane a, pTTS12-7; lane b, pTTgene6.

We chose two genes from the *Micrococcus str* operon as templates for in vitro transcription (22). This operon comprises four genes homologous to *Escherichia* ribosomal proteins S7 and S12 and elongation factors EfTu and EfG. The plasmid containing the operon was obtained from T. Ohama, and the two ribosomal protein genes were subcloned together into pTTQ18, which contains an *Escherichia* promoter and terminator, to make pTTS12-7. Transcripts from this plasmid would be 1.3 kb if the *tac* promoter and *rrnB* terminator were used. Gene 6 from ϕ29 was subcloned into pTTQ18 to serve as a positive control, and transcripts from this plasmid, pTTgene6, should be 0.8 kb.

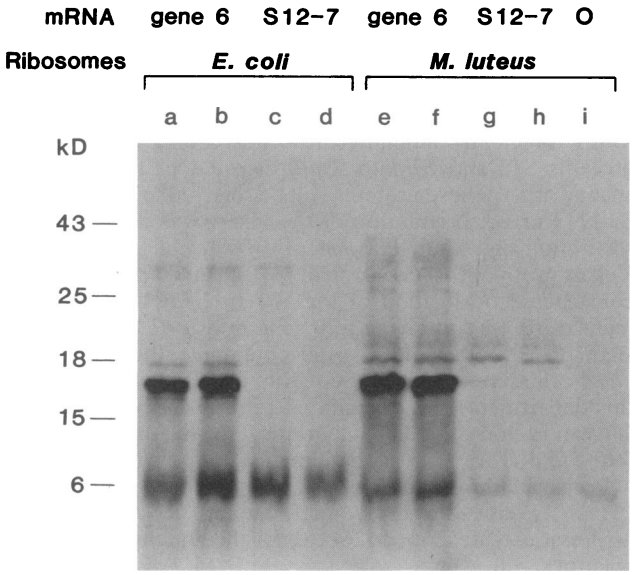

FIG. 3. Uncoupled translation of pTTgene6 and pTTS12-7 by *M. luteus* and *E. coli*. Reactions were carried out and samples were prepared as described under "Translation assays" in Materials and Methods, except in this experiment, tubes contained 125 mM NH_4OAc. Reaction mixtures also contained 15 μg of *Escherichia* HSW. *Escherichia* reaction mixtures (lanes a through d) contained 90 μg of *Escherichia* S-150 and 20 μg *Escherichia* tRNA. *Micrococcus* reaction mixtures (lanes e through f) contained 154 μg of *Micrococcus* S-150 and 11.4 μg of *Micrococcus* tRNA. Samples were electrophoresed through a 15% PAG and fluorographed for 4 days. Lane i contained no RNA with *Micrococcus* ribosomes. Lanes a and b, c and d, e and f, and g and h were duplicate reactions. Lanes a and b and e and f contained pTTgene6 transcripts. Lanes c and d and g and h contained pTTS12-7 transcripts.

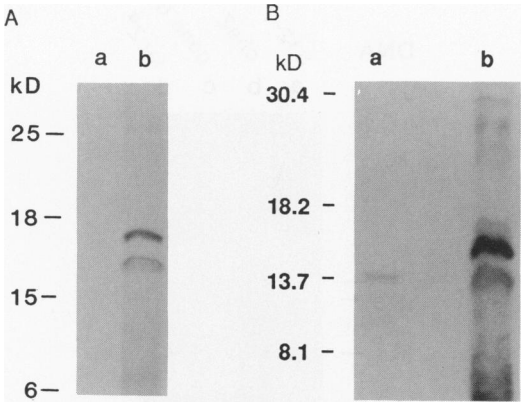

FIG. 4. Coupled translation of pTTS12-7 by the *Micrococcus* semipurified system. (A) Reactions were carried out and samples were prepared as described. Reaction mixtures contained no added RNA polymerase. Samples were electrophoresed through a 15% PAG and fluorographed for 48 h. Lane a, no DNA; b, pTTS12-7 DNA. (B) Reactions were carried out as described with [^{35}S]Cys and [^{35}S]Met. Samples containing [^{35}S]Cys were prepared for electrophoresis as described. Samples were electrophoresed through a 15% PAG and fluorographed for 5 days. Lane a, reaction with [^{35}S]Cys as label; lane b, reaction with [^{35}S]Met as label.

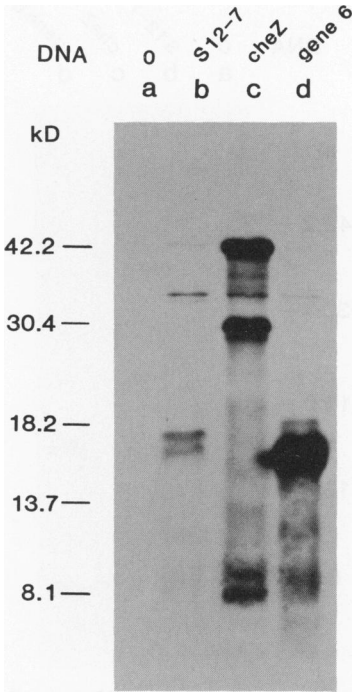

FIG. 5. Coupled translation of three DNAs by *E. coli*. Reactions were carried out and samples were prepared as described. Reaction mixtures contained 120 μg of *Escherichia* S-150 and 7.5 μg of *Escherichia* HSW. Samples were electrophoresed through a 15% PAG and fluorographed for 15 h. Lanes: a, no DNA; b, pTTS12-7 DNA; c, pTTcheZ DNA; d, pTTgene6 DNA.

The transcription of circular pTTS12-7 and pTTgene6 by *Escherichia* RNA polymerase is shown in Fig. 2. Both plasmids produce major specific transcripts corresponding to 1.3 kb for pTTS12-7 and 0.8 kb for pTTgene6. The minor products (6 kb and smaller) most likely represent transcripts which did not terminate at the terminator but continued around the plasmid.

Transcripts from pTTS12-7 and pTTgene6 were added to translation reaction mixtures containing either *Escherichia* or *Micrococcus* ribosomes. A major product of 17 kDa, corresponding to the gene 6 product, is made by both types of ribosomes (Fig. 3, lanes a and b and e and f), but the transcripts from pTTS12-7 are not translated by either *Micrococcus* or *Escherichia* ribosomes (Fig. 3, lanes c and d and g and h). The band at approximately 18 kDa in lanes a and b and e through h is probably an artifact, since it appears when either type of ribosome is used. The lower-molecular-weight bands are background products which are most likely products of premature translational termination.

The uncoupled system from *M. luteus* was modified by the addition of nucleotides and RNA polymerase to function as a coupled transcription-translation system. pTTS12-7 DNA was added directly to this system as a template. The results are shown in Fig. 4A. Lane a is the zero DNA control, and lane b shows the formation of the two products at 14 and 17 kDa which correspond to the molecular weights of proteins S12-*M* and S7-*M*, respectively. Because S7-*M* has five methionine residues, its signal is expected to be stronger than that of S12-*M*, which has only two methionine residues.

To demonstrate that the products seen in Fig. 4A, lane b were truly S12-*M* and S7-*M*, [^{35}S]cysteine was substituted for [^{35}S]methionine in the translation reaction mixture. The deduced amino acid sequence of the DNA reveals that there is one cysteine residue in S12-*M* and none in S7-*M* (22). Only the smaller protein at 14 kDa is visible on a gel when cysteine is labeled, which is consistent with the identification of S12-*M* as the band at 14 and S7-*M* at 17 kDa (Fig. 4B).

Assays to optimize conditions for coupled transcription-translation of pTTS12-7 showed that the optimal salt concentrations were 37 mM KOAc, 150 mM NH_4OAc, and 14 mM $Mg(OAc)_2$ (data not shown). In addition, neither tRNA nor RNA polymerase is necessary for expression of pTTS12-7, and both were slightly inhibitory to translation (data not shown). tRNA additional to what is present in the S-150 may cause nonspecific inhibition. There was measurable RNA polymerase activity in all of the S-150 preparations, and they all produced transcripts of approximately 800 bp from pTTgene6 DNA (data not shown). Addition of *Escherichia* RNA polymerase to the system did increase transcription (data not shown), but that RNA may be in excess, which somehow interferes with translation.

Genes from each of the two other genera, *Bacillus* and *Escherichia*, were chosen to serve as test DNAs. Gene 6 from ϕ29 and *cheZ* from *E. coli*, like genes S12-*M* and S7-*M* from *M. luteus*, were cloned into pTTQ to yield pTTgene6 and pTTcheZ. All three constructs contained identical transcriptional signals. Only the sequences 3′ to the promoter region, including the translation initiation region and the genes themselves, differed among the three constructs.

Homologous coupled transcription-translation systems from both *B. subtilis* and *E. coli* were made. Transcription of each plasmid was quantitated by using TCA precipitation with labeled CTP. Within each system, all constructs yielded similar amounts of mRNA (data not shown). The addition of RNA polymerase to the *Escherichia* and *Bacillus* coupled systems did not increase expression (data not shown).

As shown in Fig. 5, *E. coli* can transcribe and translate the *Escherichia* and *Bacillus* genes, a result which has been demonstrated previously (18). The *cheZ* product is a protein of 29 kDa (Fig. 5, lane c) (13). The other bands seen in lane

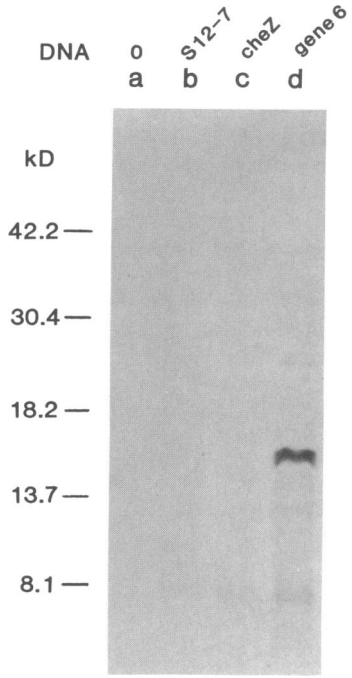

FIG. 6. Coupled translation of three DNAs by *B. subtilis*. Reactions were carried out and samples were prepared as described, except tubes contained 100 mM NH_4OAc. Reactions also contained 144 μg of *Bacillus* S-150, no HSW, and no tRNA. Samples were electrophoresed through a 15% PAG and autoradiographed for 8.5 h. Lane contents were identical to those in Fig. 5.

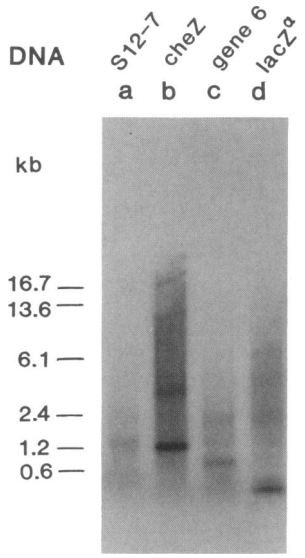

FIG. 7. Transcription of three DNAs by *B. subtilis*. Translation reactions were carried out and samples were prepared as described, except that [^{32}P]CTP was substituted for [^{35}S]Met at a specific activity of 30 cpm/pmol. Reaction mixtures contained 144 μg of *Bacillus* S-150, 48 μg of *Bacillus* HSW, and no tRNA. Samples (5 μl) were electrophoresed through a 1.5% TGE-SDS agarose gel and autoradiographed for 4 h. Lanes: a, pTTS12-7 DNA; b, pTTcheZ DNA; c, pTTgene6 DNA; d, pTTQ18 DNA.

c are products of other genes on the plasmid, including the β-lactamase at 32 and the *lac* repressor at 38 kDa. Gene 6 mRNA is very well translated by *E. coli* (Fig. 5, lane d). The expression of gene 6 is salt sensitive in *E. coli* (34), but these reactions were done at relatively low salt concentrations (see Materials and Methods). *E. coli* can also transcribe and translate the *Micrococcus* genes (Fig. 5, lane b). In lane b, S7-*M* at 17 kDa is seen as a doublet, and S12-*M* is not seen. Since S12-*M* has only two methionine residues, it was not always visible on gels. The reason for the doublet at S7-*M* is unknown. The amount of product from the *Micrococcus* gene is lower than that from either the *Escherichia* or the *Bacillus* gene. This may result from the high G+C content of the *Micrococcus* DNA. The fact that *E. coli* can translate all three mRNAs is consistent with results that show that *E. coli* can translate mRNA from a wide variety of organisms (28).

In contrast to the results with *E. coli*, *B. subtilis* is able to transcribe and translate only its own gene (Fig. 6, lane d), and neither the *Escherichia* (Fig. 6, lane c) nor the *Micrococcus* DNA (Fig. 6, lane b) is expressed. An overexposure of the gel reveals a higher zero DNA background in lane a but no products corresponding to those expected from pTTcheZ or pTTS12-7 (data not shown). However, all three of the genes are transcribed (Fig. 7). Each lane contains as its major product a specific transcript corresponding approximately to that predicted from the size of the cloned genes. The signal from pTTS12-7 (lane a) is weak, but other experiments confirm that a product of approximately 1.3 kb is produced from pTTS12-7 in a *Bacillus* coupled system (data not shown). The *cheZ* transcript is approximately 1.2 kb (Fig. 7, lane b), and the gene 6 transcript is 0.8 kb (Fig. 7, lane c). Figure 7, lane d shows the transcript made from the vector alone, which contains the *lacZ*$^{\alpha}$ gene.

Because we were using a coupled system, it was important to show that translation, and not transcription, was the barrier to expression of the *Micrococcus* gene in the *Bacillus* system. One way to prove this point was to transcribe the genes in the *Escherichia* system, which does transcribe all three genes, and then add the *Bacillus* ribosomes. Accordingly, we carried out transcription using *Escherichia* S-150, which contains *Escherichia* RNA polymerase in addition to other enzymes and factors, in the presence of *Bacillus* ribosomes. Only the *Bacillus* DNA, pTTgene6 and φ29, is well expressed in this heterologous system (Fig. 8, lanes h

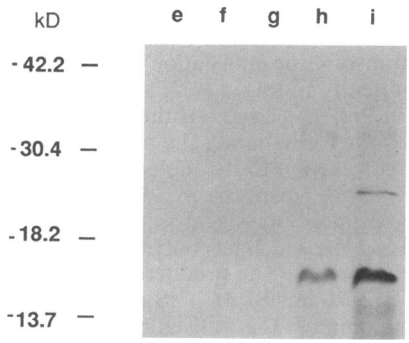

FIG. 8. Coupled translation by *Bacillus* ribosomes with *Escherichia* S-150. Reactions were carried out and samples were prepared as described. Samples were electrophoresed through a 15% PAG and autoradiographed for 8 h. Reaction mixtures contained 150 μg of *Escherichia* S-150 and 48 μg of *Bacillus* HSW. Lanes: e, no DNA; f, pTTS12-7 DNA; g, pTTcheZ DNA; h, pTTgene6 DNA; i, φ29 DNA.

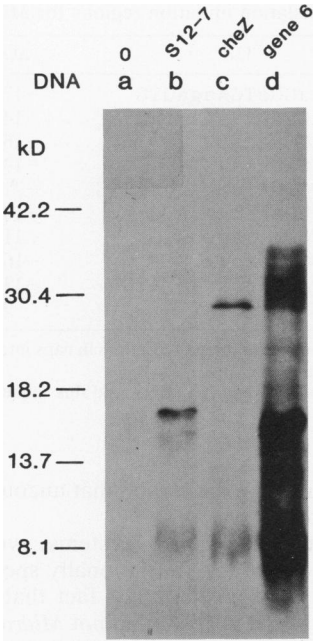

FIG. 9. Coupled translation of three DNAs by *M. luteus*. Reactions were carried out and samples were prepared as described. Reaction mixtures contained 225 μg of *Micrococcus* S-150 and 4.4 μg of *Micrococcus* HSW. Samples were electrophoresed through a 15% PAG and fluorographed for 15 h. Lanes: a, no DNA; b, pTTS12-7 DNA; c, pTTcheZ DNA; d, pTTgene6 DNA.

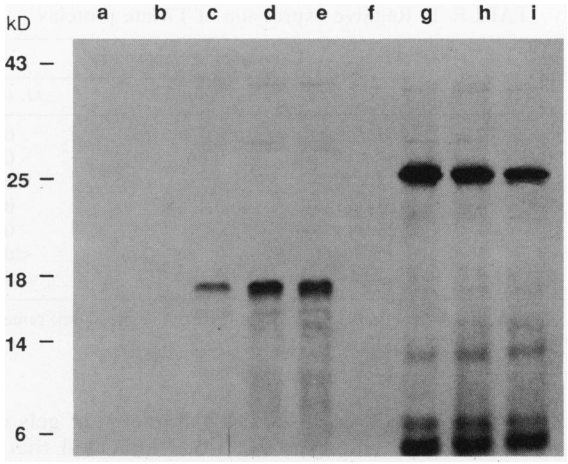

FIG. 10. Coupled translation by *Micrococcus* 30S and *Bacillus* 50S subunits. Reactions were carried out and samples were prepared as described. Reaction mixtures contained 120 μg of *Escherichia* S-150, 7.5 μg of *Escherichia* HSW, and 10 pmol of *Micrococcus* 30S subunits. Samples were electrophoresed through a 15% PAG and fluorographed for 48 h. Lanes: a, no DNA; b and f, no 50S subunits; c and g, 23 pmol of *Micrococcus* 50S subunits; d and h, 12 pmol *Bacillus* 50S subunits; e and i, 23 pmol of *Bacillus* 50S subunits. Lanes b through e, pTTS12-7 DNA; lanes f through i, pTTcheZ DNA.

and i). In Fig. 8, lane g, a faint band corresponding to CheZ can be seen. Overexposure of this gel confirms the presence of this band (data not shown). Therefore, transcripts of *cheZ* made by *Escherichia* RNA polymerase are weakly translated by *Bacillus* ribosomes with *Escherichia* factors. However, the *Micrococcus* gene is not expressed at all (Fig. 8, lane f). Since in this system the DNA was transcribed, we conclude that the inability of *Bacillus subtilis* to express the *Micrococcus* gene is most certainly because of translational, not transcriptional, barriers.

Finally, coupled translation of all three constructs was carried out with the *Micrococcus* system (Fig. 9). S7-*M* (17 kDa) and S12-*M* (14 kDa) are both made by *Micrococcus* ribosomes (Fig. 9, lane b). CheZ (29 kDa) is also made by *Micrococcus* ribosomes (Fig. 9, lane c). The expression of *cheZ*, the gram-negative DNA, contrasts with results which show that gram-positive genera such as *Bacillus* are unable to translate gram-negative mRNAs. The coupled *Micrococcus* system will also transcribe and translate gene 6 DNA (Fig. 9, lane d), as the uncoupled system will translate gene 6 mRNA (Fig. 3). The gene 6 DNA is the best expressed of all the DNAs; the fluorograph has to be overexposed relative to the gene 6 product in order to visualize CheZ. An underexposure of the gel shows only a band at 17 kDa in Fig. 9, lane d (data not shown). Therefore, *M. luteus* translates a wide variety of mRNAs, and it is translationally nonspecific.

Translation experiments using ribosomes composed of mixtures of *Bacillus* and *Escherichia* ribosomal subunits have demonstrated that the *Escherichia* 30S subunit will combine with a *Bacillus* 50S subunit to form a functional 70S ribosome which is not translationally specific (24). It was concluded, therefore, that the 30S subunit defines the translational specificity of the ribosome. We were interested to know whether the same could be said of a *Micrococcus* 30S subunit.

We used *Micrococcus* 30S subunits in combination with *Bacillus* 50S subunits in a coupled reaction with both *Micrococcus* and *Escherichia* DNA, which are not translated by *Bacillus* ribosomes. Figure 10, lanes b and f contained *Micrococcus* 30S subunits only. No products are seen, which confirms that the *Micrococcus* 30S subunits were not contaminated with 50S subunits. Lanes c and g, which contain *Micrococcus* 30S and 50S subunits, show synthesis of the gene 6 product and CheZ, respectively. Lanes d and e, which contain gene 6 DNA and *Micrococcus* 30S subunits, respectively, and different concentrations of *Bacillus* 50S subunits, and lanes h and i, which contain *cheZ* DNA and *Micrococcus* 30S subunits, respectively, and different concentrations of *Bacillus* 50S subunits, show that the *Micrococcus* 30S–*Bacillus* 50S combination produced protein in response to both mRNAs. Therefore, the heterologous ribosome is translationally nonspecific, and the *Micrococcus* 30S subunit determines the translational character of the ribosome.

Although *Micrococcus* ribosomes cannot carry out uncoupled translation of *Micrococcus* mRNA, they can translate other mRNAs in vitro, such as transcripts of the *Bacillus* phage ϕ29. In addition, *Escherichia* phage T7 transcripts of the late region are translated by both *Micrococcus* and *Bacillus* ribosomes, albeit less efficiently than by *Escherichia* ribosomes. Using mutant phage transcripts, Hager and Rabinowitz showed that *B. subtilis* translated individual T7 mRNAs with efficiencies different from those of *E. coli* (8). In general, *B. subtilis* translated transcripts with the strongest Shine-Dalgarno (S/D) sequences, whereas *E. coli* did not distinguish between different mRNAs on the basis of S/D strength.

We used T7 late mRNA to determine the amount of translation of each protein by each of the three uncoupled translation systems. To identify individual proteins, mutant

TABLE 1. Relative expression of T7 late proteins

Protein	Expression by ribosomes[a]		
	E. coli	*B. subtilis*	*M. luteus*
2.5	0.09	0.12	0.14
3.5	0.07	0.30	0.06
5.5	0.24	0	0.20
9	0.10	0.12	0.18
10	0.26	0.14	0.25
11	0	0.18	<0.01
14	0.02	0.11	0.03

[a] Values represent fractions of total synthesis by each system; remaining fractions are minor products.

T7 phage transcripts were used. Densitometry of gels containing each of the wild-type reactions indicated that the pattern of translation of individual proteins by *M. luteus* was similar to the pattern of translation by *E. coli* rather than by *B. subtilis*. The results for *E. coli* and *B. subtilis* agree with Hager and Rabinowitz's results for those bacteria (8). A quantitation of the analysis is shown in Table 1. Protein 5.5, which is well translated by *Escherichia* ribosomes but not translated by *Bacillus* ribosomes, contrasts with protein 11, which is not translated by *Escherichia* ribosomes but is well translated by *Bacillus* ribosomes. In each of these cases and in most of the others, *M. luteus* resembles *E. coli* and not *B. subtilis*. The possible exceptions are proteins 2.5 and 9, which are translated to a similar extent by *E. coli* and *B. subtilis*.

DISCUSSION

We chose to subclone the two ribosomal protein genes from the *Micrococcus str* operon because they were small and therefore were likely to be translated in vitro. The two genes cannot be easily separated because they are translated from overlapping reading frames (22). *M. luteus* translated the pTTS12-7 transcripts only when the purified system was reconstituted as a coupled transcription-translation system. We are uncertain why only coupled translation is active with *Micrococcus* components in vitro. Although translation is coupled to transcription in vivo, active uncoupled in vitro translation systems from different bacterial species have been described previously (10, 16, 17). One explanation for the inability to achieve uncoupled translation in vitro with the high-G+C-group *M. luteus* is that any mRNA produced will immediately form a secondary structure. This structure could prevent ribosomes from attaching to the mRNA and therefore inhibit translation. Secondary structure is known to interfere with translation in *B. subtilis* (34) and *E. coli* (7). A coupled system could prevent this from occurring since the ribosomes can attach directly to the RNA before any secondary structure is able to form.

The program "zfold," which analyzes potential secondary structure in RNA, was used on the first 300 bases of the cloned region (37). The RNA folds up into a structure with a folding energy of −177.4 kcal/mol (1 cal = 4.184 J). Analysis of the stretch of residues which includes the ribosome binding site (50 bases) shows that there is a hairpin formed with a folding energy of −15.4 kcal/mol, with 60% of the residues paired. In contrast, analysis of the 50 residues including the ribosome binding site of ϕ29 gene 6 gives a hairpin of −1.1 kcal/mol, with only 15% of the residues paired. Within a high-G+C-content organism such as *M. luteus*, secondary structure is very likely to be a factor in translation and may be the reason that uncoupled translation in vitro does not work.

TABLE 2. Translation initiation regions for *Micrococcus* genes

Gene	TIR[a]	ΔG[b]	Reference
S12	AGGAaGGcTGAagaGTG	−17.2	22
S7	AGAAGGAGaaGAagtaATG	−14.4	22
EfTu	AGGAGGaactaGTG	−16.6	22
L14	AGGAGaGacaaGTG	−13.6	21
L24	GGAGGTGAtctgacctcATG	−21	21
L6	AGGcTGAactgacATG	−10.8	21
L30	AGGTGcgtgacgcGTG	−11.6	21
L15	GGAGGTcaacaATG	−16.6	21
adk	AGtGAGGaacacacgATG	−13.6	21
uvrB	AGGGttctgccATG	−9.2	27

[a] TIR, Translation initiation region. Letters in caps interact with 16S rRNA and tRNA anticodon.
[b] ΔG of interaction between 16S rRNA and this sequence.

Using semipurified coupled systems, we were able to confirm that *B. subtilis* is translationally specific and *E. coli* is translationally nonspecific. The fact that *B. subtilis* can translate only its own mRNA, and not *Micrococcus* mRNA, is interesting because previously it was concluded that *B. subtilis* could translate all mRNAs from gram-positive bacteria (29). The barrier to expression is translational and not transcriptional, since even under conditions in which the *Micrococcus* DNA is transcribed (with *Escherichia* S-150), *Bacillus* ribosomes are unable to translate the mRNA produced. Therefore, *Micrococcus* mRNA represents a new type of mRNA, one that is derived from a gram-positive source but is not translated by *Bacillus subtilis*, another gram-positive bacterium.

A characteristic of mRNAs from the low-G+C group is the presence of a strong S/D sequence (9). The translation initiation region sequences for the available *Micrococcus* genes are shown in Table 2. The average ΔG of interaction, −14.5 kcal/mol, is intermediate between the value for *Escherichia* genes (−11.7) and that for low-G+C gram-positive genes (−16.7) (9), although the difference is nonsignificant. More sequences are necessary to generalize about the relative strengths of S/D regions of *Micrococcus* genes, but other observations are worthy of note. First of all, both S12-*M* and S7-*M* have strong S/D sequences, and they were not translated by *B. subtilis*. This result strengthens the hypothesis that a strong S/D sequence is necessary but not sufficient for translation by *B. subtilis*. *B. subtilis* also cannot efficiently translate T7 late genes, which have strong S/D sequences (8). Secondly, there are at least two genes which have S/D sequences with ΔGs weaker than −12 kcal/mol (Table 2). All low-G+C genes have ΔGs that are stronger than −12 kcal/mol (9). These few genes may illustrate that there are differences between S/D sequences of genes in the high- and low-G+C gram-positive bacteria which may account for some of their translational differences.

Several *Streptomyces* genes have been characterized, some of which have very weak or undetectable S/D sequences (1, 11, 31). Surprisingly, two have their transcription and translation starts at the same nucleotide (11, 12). Somehow, without an S/D sequence or any RNA 5′ to the initiation codon, the ribosome must find the correct site on the mRNA to begin translation. We propose that S1 obviates the need for an S/D sequence in *Streptomyces* genes by binding to mRNA (in this case, 3′ to the initiation codon) to bring it into the decoding site on the ribosome for transla-

tion, which is the role that has been proposed for S1 in *E. coli* (30).

M. luteus is the first example of a gram-positive organism that is translationally nonspecific. This trait follows the evolutionary distinction between the low-G+C group and the high-G+C group, corroborating Woese's classification (6). At the same time, the finding is inconsistent with Bergey's classification, which placed *M. luteus* with other coccoid gram-positive species such as *Streptococcus faecalis*, known to be translationally specific (3, 29). On the tree, organisms which are translationally specific are the low-G+C gram-positive bacteria, while others are nonspecific (Fig. 1). The asterisk shows the origin of translational specificity if a minimum of mutational events occurred. This scheme predicts that the ancestral bacterium was translationally nonspecific and that evolution was toward a translationally specific organism.

Because S1 is present in both the high-G+C group and *E. coli* and relatives, the simplest hypothesis on the origin of S1 would be that S1 was present along with a nonspecific ribosome in the ancestral bacterium and that the branch leading to the genus *Bacillus* lost S1 (Fig. 1). Therefore, the S/D sequence would have been a secondary acquisition to ensure correct initiation, with a strong S/D sequence supplanting S1 in the genus *Bacillus*.

In conclusion, translational specificity is not found in all gram-positive bacteria. Unlike *B. subtilis*, *M. luteus* is translationally nonspecific and therefore resembles *E. coli* in its translational characteristics. We predict that all members of the high-G+C gram-positive group will resemble *M. luteus* in this regard and in possessing S1. One member, the genus *Streptomyces*, has some genes which lack an S/D sequence. The lack of requirement for an S/D sequence may indicate that S1 is responsible for bringing the mRNA into the decoding site. Finally, we hypothesize that the ancestral bacterium was translationally nonspecific and that its ribosomes contained S1.

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