

Cloning and Characterization of the *str* Operon and Elongation Factor Tu Expression in *Bacillus stearothermophilus*

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The complete primary structure of the *str* operon of *Bacillus stearothermophilus* was determined. It was established that the operon is a five-gene transcriptional unit: 5'-*ybxF* (unknown function; homology to eukaryotic ribosomal protein L30)-*rpsL* (S12)-*rpsG* (S7)-*fus* (elongation factor G [EF-G])-*tuf* (elongation factor Tu [EF-Tu])-3'. The main operon promoter (*strp*) was mapped upstream of *ybxF*, and its strength was compared with the strength of the *tuf*-specific promoter (*tufp*) located in the *fus-tuf* intergenic region. The strength of the *tufp* region to initiate transcription is about 20-fold higher than that of the *strp* region, as determined in chloramphenicol acetyltransferase assays. Deletion mapping experiments revealed that the different strengths of the promoters are the consequence of a combined effect of oppositely acting *cis* elements, identified upstream of *strp* (an inhibitory region) and *tufp* (a stimulatory A/T-rich block). Our results suggest that the oppositely adjusted core promoters significantly contribute to the differential expression of the *str* operon genes, as monitored by the expression of EF-Tu and EF-G.

The streptomycin operon (*str*) belongs among the most-conserved operons in prokaryotic evolution (20, 27). The *str* operon of *Escherichia coli*, from which most of our knowledge about this operon is derived, is composed of four genes: *rpsL* (coding for ribosomal protein S12), *rpsG* (ribosomal protein S7), *fus* (elongation factor G [EF-G]), and *tufA* (elongation factor Tu [EF-Tu]). It is transcribed from its main promoter situated upstream of the *rpsL* gene (43). Besides the main promoter, two additional promoters, located within the *fus* gene, direct the transcription of the *tufA* gene (1, 57, 59). Zengel and Lindahl estimated the combined activity of these additional promoters to be about 30% of the activity of the main promoter (58, 59). These promoters, as well as the second copy of the *tuf* gene (*tufB*) present in the *E. coli* chromosome (21), contribute to the increased expression of EF-Tu relative to the expression of other genes of the *str* operon.

Approximately 50 *str* operons have already been sequenced. However, only a few of them were also characterized functionally by their transcriptional products and transcriptional starts. In our previous work, we cloned and characterized the *tuf* gene of *Bacillus stearothermophilus*. A *tuf*-specific promoter (*tufp*) was detected in the *fus-tuf* intergenic region. Based on Northern blot experiments, the ratio between the *tuf*-specific and the polycistronic transcript was estimated to be at least 10:1 (28).

In the present work, we extended our studies to the rest of the *str* operon of *B. stearothermophilus* with a special focus on the characterization of the promoters of this operon. As a prerequisite, the complete primary structure of the *str* operon of *B. stearothermophilus* was determined, and the main operon promoter (*strp*) was mapped. By using the chloramphenicol acetyltransferase (CAT) assay technique, the strength of the *strp* promoter was compared with the strength of the previously identified *tufp* promoter. The proposed stimulatory function of an A/T-rich block preceding *tufp* was experimentally confirmed

and an inhibitory *cis* element acting on *strp* was discovered. Based on the comparison of EF-G and EF-Tu expression, we suggest that the promoter for the *tuf* gene plus the *cis* elements acting on *tufp* and on *strp* significantly contribute to the differential expression of the *str* operon genes in gram-positive *B. stearothermophilus*. An “additional” gene, one preceding the *rpsL* gene and designated *ybxF*, was found to belong to the *str* operon in *B. stearothermophilus* and *B. subtilis*. The implications derived from the presence of the *ybxF* gene in this highly conserved operon are outlined.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. stearothermophilus* (CCM 2184) and *B. subtilis* 168 were used as the sources of DNA and RNA, and *B. subtilis* MI 115 (*leu* r_m⁻ m_m⁻ *recA4*) as a host in CAT assay experiments. *E. coli* DH5 α served as a host in cloning and subcloning experiments. Plasmids pUC18 and pUC19 (54) were used in cloning and subcloning experiments. Promoter probe *E. coli*-*B. subtilis* shuttle vector pCPP-3, obtained upon request from Band et al. (3), was modified by insertion of a multiple cloning site (MCS). The resulting constructs were named pCPP-31 and pCPP-32, depending on the orientation of the MCS (see Fig. 6). Plasmids are available upon request. Shuttle vector pCPP-31 was used as a promoter probe vector in CAT assay experiments. Plasmids pCPP-31.X1-7 and pCPP-31.Y1-3, derivatives of pCPP-31, were constructed to evaluate the activity of *strp* and *tufp* (see Fig. 8). pCPP-31 was digested with *EcoRI* and *PstI*, and appropriate, PCR-amplified, inserts were ligated into it. Precise endpoints of cloned fragments are indicated in Fig. 2 and 7. Primer sequences used to amplify the promoter regions that were cloned into these plasmids are available upon request. The plasmid copy number of the constructs (in *B. subtilis*) was assessed regularly, according to the method of Labes et al. (29). The plasmid copy number was approximately 20, and it varied <10% among the individual constructs. Sequence inspection of the region upstream of the MCS did not reveal any known motif (secondary structures; presence of core promoter-like sequences in both the plasmid and the inserted DNA that could upon ligation create an artificial promoter; and UP element-like sequence adjacent to the MCS) that could, in the context of the inserted DNA, influence the results of the CAT assays.

Media and transformation. *B. stearothermophilus* and *B. subtilis* 168 were grown as described by Krásný et al. (28). *E. coli* competent cells were prepared, and consecutive transformations were carried out as described by Hanahan (17). Transformants were selected by plating onto RMK (composition per liter: Bacto-tryptone, 20 g; Bacto-yeast extract, 5 g; 1 M KCl, 10 ml; 20% glucose, 16.7 ml; 80% [wt/vol] MgSO₄ · 7H₂O, 10 ml) agar plates supplemented either with ampicillin (80 μ g/ml) or neomycin (20 μ g/ml). *B. subtilis* MI 115 cells were made competent, and transformation experiments were carried out according to the method of Dubnau and Davidoff-Abelson (12). Transformants were selected by plating them onto agar plates prepared from the enriched Spizizen medium (composition per liter: (NH₄)₂SO₄, 2 g; KH₂PO₄, 6 g; sodium citrate · 2H₂O, 1 g;

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MgSO₄ · 7H₂O, 0.2 g; glucose, 0.5%; K₂HPO₄ · 3H₂O, 18.3 g; tryptone, 20 g; yeast extract, 5 g) supplemented with neomycin (20 µg/ml).

Enzymes and chemicals. Restriction enzymes, T4 DNA ligase, shrimp alkaline phosphatase, DNA Megaprime Labeling Kit, T7 Sequenase DNA Sequencing Kit, QUAN-T-CAT kit, [α -³²P]dCTP, and [γ -³²P]ATP were purchased from Amersham (Germany); RNase A, T4 polynucleotide kinase, RNasin, and avian myeloblastosis virus reverse transcriptase were purchased from Promega. The Expand High-Fidelity PCR System was purchased from Boehringer Mannheim. Deep Vent DNA polymerase was from New England Biolabs. All enzymes and kits were used according to the manufacturers' recommendations. Oligonucleotide primers were purchased from GeneriBiotech (Czech Republic).

Nucleic acids preparation and manipulation. *B. stearothermophilus* and *B. subtilis* genomic DNAs were extracted and purified as described earlier (33) with some modifications. Plasmid DNAs were prepared with the Wizard System purchased from Promega. Gel extractions were carried out with the QiaexII kit from Qiagen. Restriction mapping, Southern hybridization analysis, agarose gel electrophoresis, and subcloning of DNA fragments were performed by standard procedures (47). *B. stearothermophilus* and *B. subtilis* RNA was prepared from cells harvested in the mid-log-growth phase with the RNeasy kit purchased from Qiagen. Formaldehyde agarose electrophoresis, blotting, and Northern blot hybridization were carried out as described previously (47). RNA marker from Gibco-BRL was used as a molecular weight marker in Northern blot hybridization experiments.

Cloning of the *fus* gene. A pair of primers was designed to amplify the region containing the entire *fus* gene: PIII from residues 1053 to 1073 (25) (5'-GCGT GCGCGATCATTCTCTG-3') and PIV from residues 16 to 36 (28) (5'-GAG CGCAGAAACCGCACGTG-3'). The Expand High-Fidelity PCR System was used to ensure the fidelity of the PCR. The 2.3-kb product was cloned into pUC18. Three recombinant clones were selected for further analysis.

Cloning of the region upstream of *rpsL*. Ligation-mediated PCR (LM-PCR) (44) was used to clone the region upstream of the *rpsL* gene with the following modifications. Primers PV (5'-TCTCCGTTGACCAAGTTTGCC-3') and PVI (5'-GTTTTTCGGCGTCATG-3') were used to amplify a part of the *rpsL* gene, which was used as a probe in Southern hybridization experiments. A *Bcl*I digest of chromosomal DNA was established to contain a 1.4-kb fragment containing at its 3' end the 5'-end region of the *rpsL* gene. Primers PVII (5'-TAGCTTATT CCTCAAGGCACGACG-3') and PVIII (5'-GATCCGTCGTGC-3') were assembled into a linker. The linker was ligated to the *Bcl*I digest of chromosomal DNA. Subsequently, the ligation mixture was digested with *Bcl*I. Primers PVII and PIX (5'-CAAAGTGGTCAACGGAGAATCGCC-3'; from positions -105 to -83 [25]) from the region adjacent to the *rpsL* gene were used to amplify the 1.4-kb fragment. The Expand High-Fidelity PCR System was used to ensure the fidelity of the PCR. The 1.4-kb fragment was cloned into pUC18, and three clones were selected for further analysis.

DNA sequence determination. DNA sequencing was done in both directions with either T7 Sequenase DNA Sequencing Kit or fluorescent AutoRead Sequencing Kit purchased from Pharmacia. At least three clones from independent PCR reactions of each DNA fragment were sequenced. The *fus* gene sequence was obtained by sequencing of subcloned DNA fragments. The 1.4-kb fragment from the region upstream of the *rpsL* gene was first sequenced from both sides to verify its identity. It contained the 3' end of the *rpoC* gene at its 5' end, and it ended upstream of the *rpsL* gene. The 3'-end half of the fragment reaching far into the *rpoC* gene was then separately cloned and sequenced.

PCR: Northern blot hybridization probes. The following pairs of primers were used to amplify the respective regions of the *str* operon: for *B. stearothermophilus*, *fus* probe, PAN (5'-GCACAATGGAAGGCCACCGC-3') and VOK (5'-GC CCAGCCGGAGAAGACCGGG-3'), and *ybxF* probe (containing the entire *ybxF* gene), *B. subtilis*, *fus* probe, nucleotides (nt) 3772 to 3791 (forward primer) and nt 4019 to 4038 (reverse primer), and *ybxF* probe, nt 2429 to 2428 (forward primer) and nt 2661 to 2680 (reverse primer) (numbering is according to Yasumoto and coworkers [55]).

Primer extension experiments. The primer extension experiments were carried out as described previously (28) with the following modifications. A total of 25 µg of *B. stearothermophilus* total RNA was hybridized to the primer SMICH (5'-C GATCACTCCGTTGCTTCC-3'), complementary to the 5' end of the *ybxF* gene. The hybridization temperature was set at 30°C.

2D electrophoresis. *B. stearothermophilus* EF-Tu and EF-G were isolated essentially as described earlier (23, 28). *B. stearothermophilus* S30 fractions from the mid-exponential and stationary (overnight culture) phases were prepared from bacterial cells resuspended in the following buffer: 20 mM cacodylic acid sodium salt (pH 6.8), 60 mM KCl, 5 mM MgCl₂, 5 mM β -mercaptoethanol, 10 µM phenylmethylsulfonyl fluoride, and 15% glycerol. Estimation of the protein concentration was performed by the method of Bradford (6). A total of 0.5 µg of the S30 fraction was resolved on Mini 2D electrophoresis gels (Bio-Rad) according to the manufacturer's recommendations. The density of the second-dimension gel was 12%. EF-Tu and EF-G were analyzed in a parallel experiment to allow the identification of EF-Tu and EF-G spots in the *B. stearothermophilus* proteome map. Proteins were visualized either by staining with Coomassie blue R-250 or by silver staining (4). The relative staining efficiencies of identical amounts of isolated EF-Tu and EF-G were about equal. The relative amounts of EF-Tu and EF-G in the two-dimensional (2D) map were determined by densitometric measurements, normalized to the molecular weights of these proteins.

CAT assay experiments. *B. subtilis* MI 115 transformed with respective plasmids (see Fig. 8) was grown in enriched Spizizen medium at 37°C. The cells were harvested at an optical density at 600 nm of ~0.6. Then, 1 ml of the cell suspension was centrifuged at 12,000 × g at 4°C. The supernatant was discarded, and the pellet was resuspended in 1 ml of ice-cold 0.1 M Tris-Cl (pH 7.8). Next, 100 µl of this suspension was transferred into a fresh tube, and the cells were disrupted by sonication at 0°C (twice, for 10 s each time; amplitude, 0.6 µm; Soniprep 150). The sonicated cells were pelleted, and the supernatant (S30) was used in subsequent steps. Then, 0.04 µl of the supernatant, diluted in 40 µl of 0.1 M Tris-Cl (pH 7.8), was used to conduct CAT assays with the QUAN-T-CAT kit (Amersham). The CAT assays were conducted according to the manufacturer's recommendations. Estimation of protein concentration was performed by the method of Bradford (6).

FCA. Factorial correspondence analysis (FCA) was performed with the assistance of the database Indigo, accessible through the World-Wide Web at <http://indigo.genetique.uvsq.fr>.

Accession numbers. The *fus* gene sequence and the sequence of the region upstream of the *rpsL* gene have been submitted to the EMBL database under accession numbers AJ249559 and AJ249558, respectively. The MCSs of pCPP-31 and pCPP-32 have been submitted to the EMBL database under accession numbers AJ133759 and AJ133760, respectively.

RESULTS

Structure and genomic organization of the *str* operon. Sequences of two fragments of the *str* operon of *B. stearothermophilus* have already been reported: (i) the *rpsL* (S12) and *rpsG* (S7) genes (25) and (ii) the *tuf* gene (28). To determine the primary structure of the remaining parts of the *str* operon, the *fus* gene located between the *rpsG* and *tuf* genes was cloned and sequenced (Fig. 1). The *fus* gene consists of 2,079 nt. It codes for EF-G, a protein of 692 amino acid (aa) residues with a calculated 77,038-Da molecular mass (excluding possible posttranslational modification) and a pI of 5.10. EF-G is a monomeric GTP-binding protein involved in translocation of peptidyl-tRNA from the A to the P site on the ribosome. Starting from the aa 46 (Gly), the amino acid sequence of the N terminus of EF-G deduced here differs from that proposed by Kimura (25). This difference is due to a missing G nucleotide at this position in his published DNA sequence. The nucleotide identity of the *B. stearothermophilus fus* gene to the *fus* gene of *B. subtilis* is 72%.

Subsequently, the LM-PCR cloning strategy (44) was used to clone the unknown region upstream of the *rpsL* gene (Fig. 2; see also Materials and Methods). Directly upstream of the *rpsL* gene is an open reading frame (ORF) of 249 nt separated from the 5' end of the *rpsL* gene by 90 nt. It shares 61% nucleotide identity with an analogous ORF of *B. subtilis*. It was therefore assigned to *ybxF*. The *ybxF* gene codes for a protein of 82-aa residues with a calculated molecular mass of 8,591 Da and a pI of 8.91. The region upstream of the *ybxF* gene is occupied by the 3' end of another ORF. Since it is 72% identical to the *rpoC* gene of *B. subtilis* that codes for the β' subunit of RNA polymerase (55), it was assigned to *rpoC*. The intergenic region between *rpoC* and *ybxF* is 136 nt long and contains a palindromic structure that most likely serves as a transcription termination signal for the *rpoC* gene (Fig. 2). At the 3' end, the *str* operon is followed by the S10 operon (28).

The *str* operon is a five-gene transcriptional unit. Based on the cloning and sequencing experiments, a question emerged: does the *ybxF* gene of *B. stearothermophilus* belong to the *str* operon? Even in *B. subtilis* the answer to this question was unknown. So far, it has been assumed that the *str* operon of *B. subtilis* is a four-gene transcriptional unit (20). To address this question experimentally, Northern blot hybridization experiments were carried out. In Fig. 3A, lane 1, total RNA of *B. stearothermophilus* was hybridized with a DNA fragment from the *fus* gene. A single band corresponding in size to 4.9 kb was detected. In parallel, the *ybxF* was used as a probe. A signal appeared in precisely the same position as in the experiment

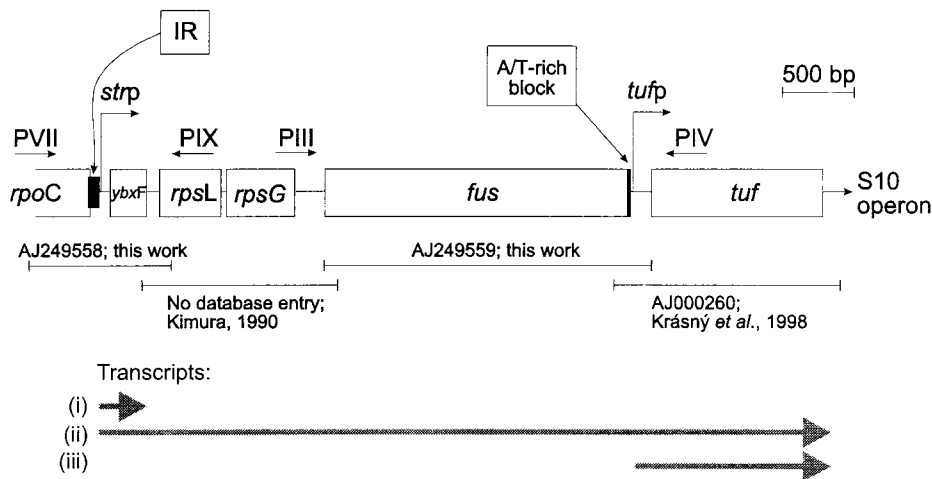


FIG. 1. *str* operon of *B. stearothersophilus*. Horizontal arrows represent PCR primers used in cloning experiments (for details, see Materials and Methods). The EMBL accession numbers assigned to nucleotide sequences are indicated below the scheme in combination with the sequences of Kimura (25) and Krásný et al. (28). The positions of the functional promoters *strp* and *tufp* and their transcriptional products are shown. Transcripts: i, *ybxF* transcript terminated in the *ybxF-rpsL* intergenic region; ii, full-length transcript of the *str* operon; and iii, *tuf* gene transcript transcribed from the *tuf*-specific promoter (see reference 28). The A/T-rich block and the IR are indicated.

with the *fus* gene fragment as a probe (Fig. 3A, lane 2). This experiment was repeated with *B. subtilis* RNA and corresponding probes. The result was identical: the *ybxF* probe hybridized in the same position as the *fus* probe (Fig. 3B). The conclusion

drawn from these experiments is that the *ybxF* gene is a part of the *str* operon in both *B. stearothersophilus* and *B. subtilis*.

Besides the polycistronic transcripts of 4.9 kb (*B. stearothersophilus*) and 4.7 kb (*B. subtilis*), the *ybxF* probes of both

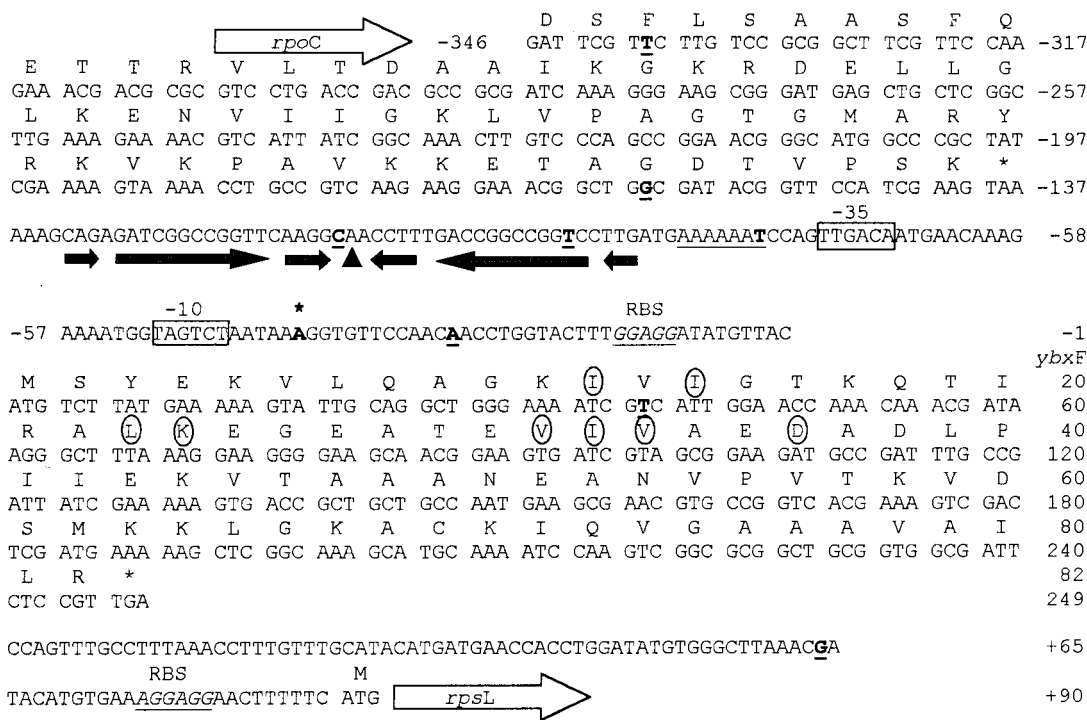


FIG. 2. Nucleotide sequence of the region upstream of the *rpsL* gene, and deduced amino acid sequences of the *ybxF* gene and the 3' end of the *rpoC* gene of *B. stearothersophilus*. The Shine-Dalgarno sequences are in underlined italics. The -35 and -10 regions of the *strp* promoter are boxed. The +1 nucleotide of the *str* operon transcript is in boldface, indicated by an asterisk. The A-tract upstream of *strp* is underlined. The transcription termination signal of the *rpoC* gene is underlined with shaded arrows. The top of the palindrome is indicated by a triangle. The circled amino acids denote the RNA-binding motif (26). The underlined boldface letters denote the 5' and 3' ends of the cloned DNA fragments (see also Fig. 8).

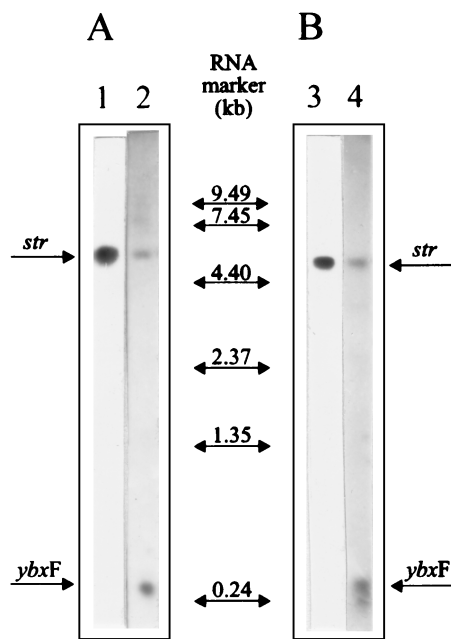


FIG. 3. Northern blot hybridization analysis of *B. stearothermophilus* (A) and *B. subtilis* (B) RNA. Each lane contains 5 μ g of total RNA. Hybridization was carried out with *fus* probes (lanes 1 and 3) or *ybxF* probes (lanes 2 and 4). The polycistronic (*str*) and *ybxF* (*ybxF*) transcripts are indicated. The RNA molecular weight marker was from Gibco-BRL.

bacteria hybridized to a small transcript of approximately 0.3 kb (Fig. 3, lanes 2 and 4). Its length suggests that it comprises the entire *ybxF* gene and terminates in the *ybxF-rpsL* intergenic region. This transcript is most likely not a processing product because both the *ybxF* and *fus* probes detected no other transcripts indicative of processing of the *str* mRNA.

Mapping of the *str* promoter. S1 nuclease experiments mapped the 5' end of the *str* mRNA transcript upstream of the *ybxF* gene (data not shown). The +1 nucleotide, determined by primer extension experiments (Fig. 4), was mapped 6 bp downstream of the TAGTCT sequence that has 5 nt identical to the *B. stearothermophilus* -10 promoter consensus sequence (TA TTA/CT [52]; Fig. 2). Further upstream, separated by a spacer of 17 nt, is a hexanucleotide TTGACA sequence that in 5 out of 6 nt matches the *B. stearothermophilus* -35 promoter consensus sequence (TTGACT/C). Inspection of the corresponding region of *B. subtilis* revealed a similar promoter-like structure. The -10 region (GATAAT, consensus TATAAT) and the -35 region (TTGACA, same as consensus) are separated by 16 nt. The promoter sequences of both organisms comply with all requirements on *Bacillus* promoter sequences necessary for efficient recognition by the σ^A subunit of the RNA polymerase involved in transcription of housekeeping genes (16).

Intracellular level of EF-G and EF-Tu and quantitative evaluation of the *tufp* and *strp* promoters. Prior to quantitative evaluation of *strp* and *tufp*, the relative intracellular concentrations of EF-G and EF-Tu were determined. Direct densitometric measurements within the proteome, the 2D profile map of *B. stearothermophilus* proteins, indicated that the ratio of molar concentrations of EF-Tu and EF-G is approximately 9:1 (Fig. 5). The experiments were performed with cells from the exponential and stationary phases (see Materials and Methods) with identical results. This experiment was also car-

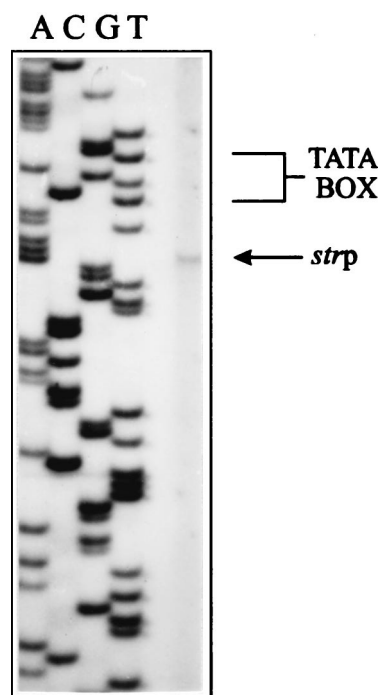


FIG. 4. Primer extension analysis of the polycistronic transcript of *B. stearothermophilus* *str* operon. The 5' end of the transcript, initiated at *strp*, is indicated by an arrow. Lanes A, C, G, and T contain sequencing ladder of the respective genomic region generated with the same primer. The -10 region is indicated by a bracket.

ried out with *B. subtilis*. The EF-Tu/EF-G ratio was almost the same as in *B. stearothermophilus*: 10:1 (data not shown).

Since both our previous experiments (28) and the experiments presented here suggested that transcription of the *str* operon of *B. stearothermophilus* is controlled from two promoters, various portions of the putative promoter regions were cloned into a newly constructed promoter probe *E. coli*-B.

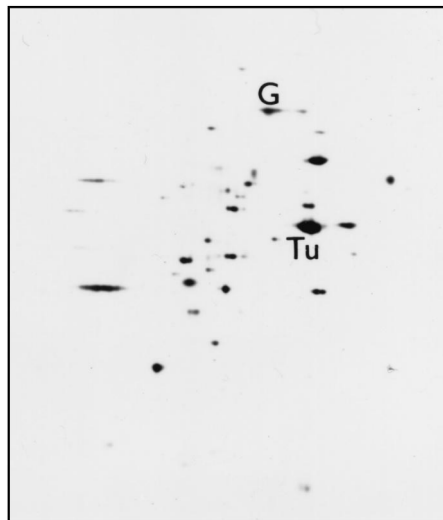


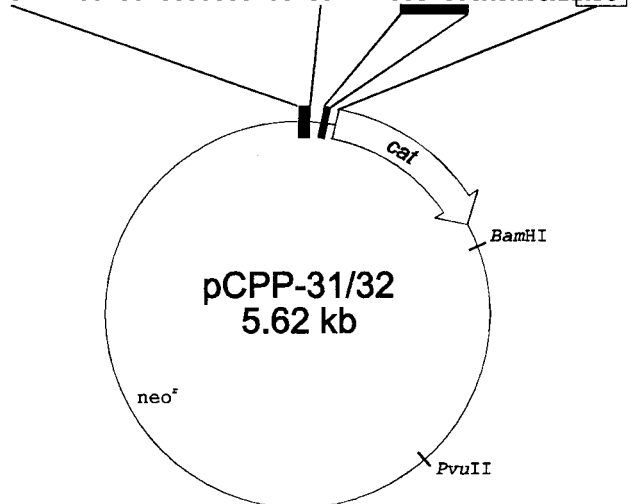
FIG. 5. 2D electrophoresis of *B. stearothermophilus* S30 fraction from the mid-exponential phase. The first dimension was run from left to right, with the more acidic proteins migrating further to the right. The second dimension was run from the top down. The positions of EF-Tu and EF-G are indicated.

sequence upstream of the MCS:

GAATTGTGCTGTTTTATCCTTTACCTTGCTACAAACCCCTTAA
AACGTTTTTAAAGGCTTTTAAAGCCGCTGTACGTTCTTAAAGGAA

pCPP-31:

EcoRI SacI SmaI PstI RBS start
GAATTCGAGCTCCCGGGCTGCAGCAATTCGGAGGCATATCAAATG



pCPP-32:

PstI SmaI SacI EcoRI RBS start
GAATTGCTGCAGCCCGGGAGCTCGAATTCGGAGGCATATCAAATG

FIG. 6. Map of pCPP-31 and pCPP-32. The ribosome binding site (RBS) is underlined with a rectangle shaded in gray. The *cat* gene accession number (GenBank) is K00544 (18). The MCS sequences and their flanking regions are deposited in the EMBL database under accession numbers AJ133759 (pCPP-31) and AJ133760 (pCPP-32). For details, see Materials and Methods.

subtilis shuttle vector pCPP-31 (Fig. 6) to assess promoter strengths of the cloned DNA fragments. The cloning experiments were conducted in gram-negative *E. coli*, and the constructs were used to transform gram-positive *B. subtilis* to provide for a homologous environment in subsequent CAT assay experiments. The nucleotide sequences of the cloned DNA fragments are shown in Fig. 2 (the *strp* region) and 7 (the *tufp* region).

Figure 8 schematically represents all of the recombinant constructs used in this study and summarizes the results of the CAT assay experiments obtained for bacteria from the mid-exponential phase. (i) The region upstream of the *rpsL* gene,

where the main promoter of *str* operons is regularly found, e.g., in *E. coli* (43), did not possess any promoter activity (pCPP-31.X1). Its effect on the expression of CAT remained at a level comparable to the background expression from the insertless pCPP-31. (ii) In contrast, insertion of *strp* and *tufp* core promoters (fragments containing -35 and -10 hexamers) upstream of the otherwise promoterless *cat* gene of pCPP-31 induced a strong expression of CAT (constructs pCPP-31.X2 and pCPP-31.Y1, respectively). Both core promoters were equally active in vivo. (iii) The previously identified A/T-rich block preceding the -35 region of *tufp* (Fig. 7) (28) stimulated transcription from the *tufp* core promoter (-10 and -35) by a factor of about three (pCPP-31.Y2). (iv) The DNA sequence upstream of the A/T-rich block was not found to significantly influence the transcriptional activity of *tufp*. The DNA fragment containing *tufp* and the A/T-rich block, extended by about 300 bp further upstream of the A/T-rich block (pCPP-31.Y3), was approximately as active in CAT assays as the nonextended fragment (pCPP-31.Y2). The $\sim 10\%$ difference in activities between pCPP-31.Y2 and pCPP-31.Y3 may rather be attributed to the overall nucleotide composition of the region upstream of the A/T-rich block than to a particular structural element. (v) In contrast to *tufp*, the activity of the *strp* promoter was not influenced by an A-tract (construct pCPP-31.X3) situated directly upstream of the -35 hexamer, i.e., in a position analogous to the A/T-rich block of *tufp* (Fig. 2). (vi) However, further upstream extension of the *strp* region for approximately 70 bp (pCPP-31.X5) resulted in a sharp, ~ 10 -fold drop in the promoter activity. This unexpected finding indicated the presence of an inhibitory element in the intergenic region partially suppressing the core promoter activity. (vii) Further upstream extension of the promoter region for 180 bp (pCPP-31.X6) slightly relieved the suppression brought about by the 70-bp inhibitory region (IR). Nevertheless, ~ 7 -fold inhibition of the core promoter activity was retained. (viii) The IR contains the termination palindrome for the transcription of the *rpoC* gene. To test whether the palindromic structure could be responsible for the inhibition by extruding into a cruciform, pCPP-31.X4 was constructed. The insert comprised only the 3'-end half of the palindrome. Despite this substantial modification, which abolishes the formation of any cruciform based on the palindrome, the inhibitory activity of the insert was retained, although 2.4-fold decreased with respect to pCPP-31.X6, indicating that the palindrome is not essential for the inhibition. (ix) Since the IR is in vivo most likely transcribed, the pCPP-31.X7 was constructed to check whether the simultaneous transcription of the *rpoC* gene influences transcription initiation from *strp*. The simultaneous transcription of

	-250																			-168	
Bst	ggttatgogacatcgctccgcttogaacacgcaaggcgoggaacattctcaatggtatattgatcactacgaagaagt	ccgaaaaac																			
Bsu	ggttacgctactgcaacttcggttctaatacgcgaaggctcgogtaactgctcactatgcacatggatcactacggagaagtgcgcaagagc																				
	-167	A/T-rich block	-35		-10	*															-81
Bst	atgcgcatgaaattatcaaaaaaaaaataaaggogaataa	ctgatttctcttgctagttccgctataaataacttat	g	aaagtaagact																	
Bsu	gtcgcagaagaattatcaaaaaaaaaataaaggogaataa	ctgatttctcgcgcttaactcaagtataactactatt	g	aaagatgagga																	
	-80	stop <i>fus</i>			RBS																-1 \rightarrow <i>tuf</i>
Bst	tgggaacattttgttcccaagcctctataccttacctaactatacaaattcat	at	tttacttttaaggagatctttctc	ATG																	
Bsu	agtgaagctttcttccactctctatcactctatacattactaataaagctcttaaggagatatttaga	ATG																			

FIG. 7. Sequence alignment of *B. stearothermophilus* and *B. subtilis* *tuf* promoter regions. The 3' ends of the sequences end with start codons of *tuf* genes. The black rectangle below the sequence denotes the stop codon of the *fus* gene. The -35 and -10 hexamers are indicated by thick lines above the sequence. The asterisk denotes the transcription-initiation site as determined previously (28). The ribosome binding site (RBS) is italicized. The shaded box contains the A/T-rich block. The thick underlined letters mark the endpoints of cloned DNA fragments as described in Fig. 8.

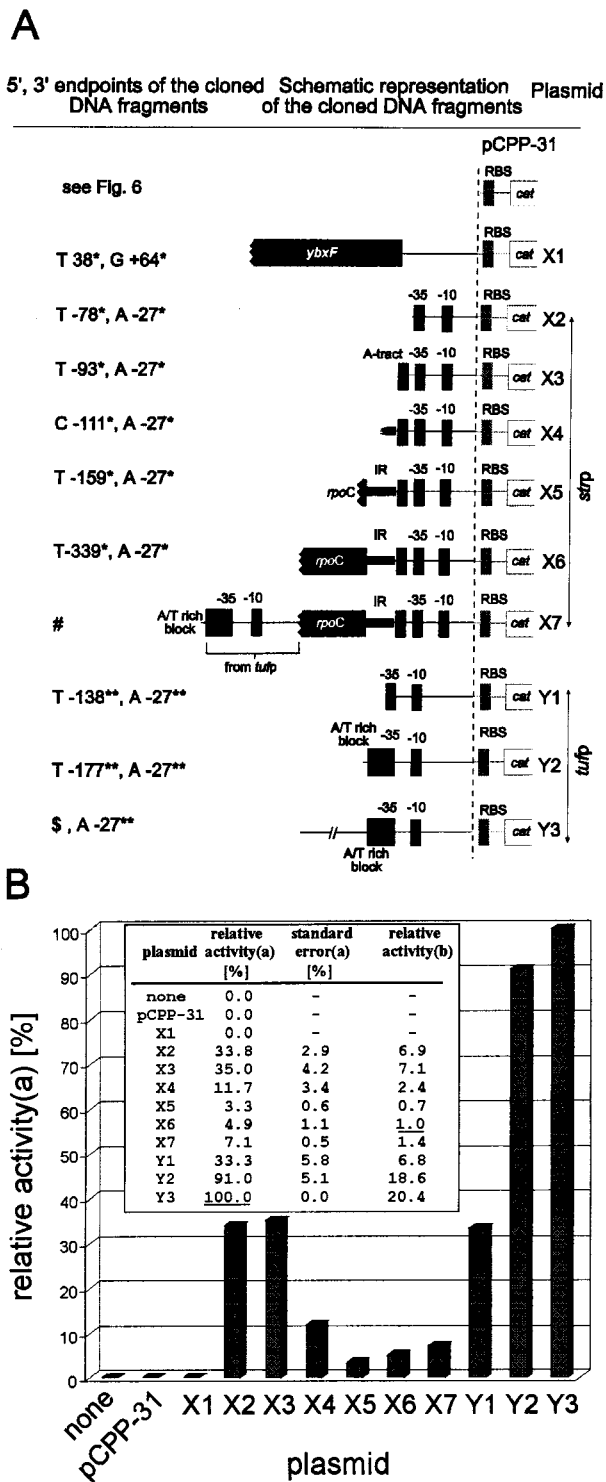


FIG. 8. Schematic structure of pCPP-31 derivatives (A) and their transcriptional activity in CAT assays (B). (A) All DNA inserts were cloned into the *EcoRI-PstI* site of the polylinker upstream of the *cat* gene and its ribosome binding site (RBS) of pCPP-31. The picture is not drawn to scale, and the pCPP-31 part of the plasmid name is omitted. The 'X2'-X7 plasmids carry the *strp* core promoter sequence extended with its upstream DNA regions of increasing size as indicated. The 'Y1'-Y3 plasmids carry the *tufp* core promoter extended in the same way. * and **, see Fig. 2 and 7. #, the DNA insert upstream of the *strp* core promoter is the same as in X6 extended for the *tuf* promoter region specified in 'Y2'; \$, the 5' end of the insert is 499 bp upstream of the *tuf* gene translational start (for nucleotide sequence see EMBL database entry AJ249559). (B) CAT assay results. The background value of the negative control

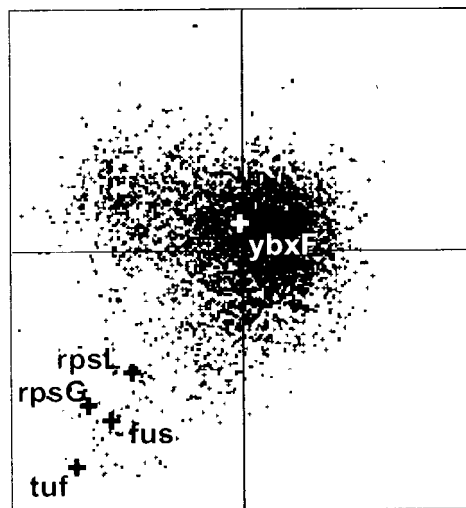


FIG. 9. Factorial correspondence analysis of codon usage in the *B. subtilis* ORFs. The large black crosses represent *rpsL*, *rpsG*, *fus*, and *tuf*. The position of *ybxF* is marked by the large white cross (for details, see the text).

the 3'-terminal part of the *rpoC* gene fragment, controlled by the inserted *tufp* region including the A/T-rich block (pCPP-31.X7), was not found to significantly modify the promoter suppression imposed by the IR.

The deletion mapping experiments provided evidence that the two *cis*-acting regulatory elements, one enhancing (the A/T-rich block) and the other silencing (the *rpoC-ybxF* intergenic region preceding the *strp* core promoter), set the *in vivo* ratio between *strp* and *tufp* transcriptional strength to approximately 1:20.

Codon usage. Our experiments have established a direct positional and transcriptional link between the *rpsL*, *rpsG*, *fus*, and *tuf* genes and the *ybxF* gene within the *B. stearothermophilus* and *B. subtilis* *str* operons. Since most proteins of the translational apparatus have a highly biased codon usage in a particular organism (35), FCA was carried out to address the functional relatedness of *ybxF* with other genes of the *str* operon. This method relates genes according to their usage of synonymous codons (reference 40 and references therein). Genes highly expressed under specific physiological conditions should display a similar codon usage bias (36). Figure 9 shows that the *rpsL*, *rpsG*, *fus*, and *tuf* genes of *B. subtilis* group together, while the *ybxF* gene is positioned at a distance from this cluster (for further interpretation, see Discussion).

DISCUSSION

Transcription of the *str* operon. Our experiments have established that the *str* operons of both *B. stearothermophilus* and *B. subtilis* are transcriptional units composed of five genes: 5'-*ybxF-rpsL* (S12)-*rpsG* (S7)-*fus* (EF-G)-*tuf* (EF-Tu)-3' (Fig. 1). Besides the highly conserved *rpsL*, *rpsG*, *fus*, and *tuf* genes, the *ybxF* gene was found to extend the 5' end of the operon in both organisms. This is a rare observation because until now all

(plasmidless *B. subtilis*) was subtracted. The results are averages of at least two experiments done in duplicates, normalized to the same amounts of protein. a, the activity of the most active promoter region (pCPP-31.Y3) was taken as 100% (underlined); b, relative strengths of promoter regions compared to the activity of the *strp* promoter (pCPP-31.X6). Its activity was taken as 1 (underlined).

genes found to precede the *rpsL* gene of the *str* operon, with the exception of the archaeobacterium *Methanococcus vannielii* (30), have been considered to be genes adjacent to the *str* operon but never as members of the operon (5, 20).

The measurements of the strength of *strp* and *tufp* of *B. stearo-thermophilus* revealed that the transcription proceeds about 20 times more efficiently from *tufp* than from *strp*. The experiments demonstrated that the observed concentration difference between the polycistronic and the *tuf*-specific transcript is based on two differently active promoters. The difference in transcription from these two promoters is brought about by a combined effect of two *cis* elements acting on equally active core promoters (Fig. 1 and 8). The main operon core promoter is downregulated (by the IR), while the *tuf*-specific core promoter is upregulated (by the A/T-rich block).

Sequence inspection of the IR revealed the presence of a palindrome representing the transcription termination signal for the *rpoC* gene. If the palindrome created an unusual DNA structure, such as a cruciform, it might interfere with formation of the transcription-initiation complex (37, 49). We tested this alternative on a plasmid containing only the 3'-end half of the palindrome (pCPP-31.X4). This modification excluded the formation of any palindrome-based cruciform. However, the inhibitory effect of the region was not abolished, only decreased. The promoter activity of the construct was still 2.9-fold lower than that of the *strp* core promoter. Also, *in vitro* S1 nuclease mapping experiments (41) failed to detect any cruciform in pCPP-31.X6 (data not shown). These results suggested an additive, stepwise-like inhibitory effect within the IR sequence. We further tested the *strp* region for the presence of DNA curvature, which could be responsible for the suppression (10, 42, 45). DNA bending manifests itself by anomalous electrophoretic mobility in polyacrylamide gels. DNA fragments containing bends migrate more slowly than unbent DNA fragments (11). The *K* value (the ratio of the apparent length of a fragment to its actual length) of the fragment cloned into pCPP-31.X5 was 1.2, strongly suggesting the presence of a DNA bend (data not shown). Further experiments are required to characterize the detected DNA bend, its position and distribution. Furthermore, the suppression can be aided by protein factors, and we intend to study the topology of the IR to determine its mode of function.

The A/T-rich block preceding the -35 region was demonstrated to stimulate the *tufp* activity by a factor of three. Generally, A/T-rich blocks, also referred to as upstream (UP) elements, are sequence elements that increase promoter strength (39, 53). The UP elements contribute to promoter recognition via their interaction with the C-terminal domain of the α subunit (α CTD) of RNA polymerase (9, 22, 46). Therefore, a contact between the A/T-rich block and the α CTD RNAP may be the mechanism that contributes to the efficient transcription of the *tuf* gene in *B. stearo-thermophilus* and *B. subtilis*.

It should be emphasized that the experimental design of the CAT assay experiments described here does not rule out the possibility that some unknown regulatory factor may be neutralized by the elevated gene dosage (the plasmid copy number is approximately 20; see Materials and Methods). However, the ratios of EF-Tu to EF-G (this work) and *str* mRNA to *tuf* mRNA (28) are based on measurements of the products of the chromosome-borne genes. The relative strength of the two tested promoters correlates with the relative concentrations of these products. Therefore, we feel it is reasonable to suggest that the results of our experiments reflect the promoter activities in the chromosome.

Our findings are consistent with the idea that the differential

transcription of the *fus* and *tuf* genes significantly contributes to the observed 1:9 EF-G/EF-Tu ratio in *B. stearo-thermophilus*, provided the two classes of the *tuf* mRNAs are not translated with significantly different efficiencies. In *E. coli*, neither the two additional, weak *tufA*-specific promoters nor the second copy of the *tuf* gene, which is transcribed from a promoter approximately as active as the additional *tufA* promoters (1), provide a sufficient excess of the *tuf*-containing mRNAs over the *fus*-containing mRNAs that would explain the 1:10 EF-G/EF-Tu ratio in this organism (56). Thus, while in *E. coli* other mechanisms, such as translational efficiency or mRNA or protein stability, must adjust the final EF-G/EF-Tu ratio by increasing the concentration difference between these two proteins, in *B. stearo-thermophilus* the difference is already created at the transcriptional level. In fact, the difference is more pronounced at the transcriptional than at the translational level, most likely also further accentuated by the partially readthrough termination downstream of the *ybxF* gene, and additional mechanisms must be required to adjust the final EF-G/EF-Tu ratio by reducing the concentration difference created at the transcriptional level. Thus, *E. coli* and *B. stearo-thermophilus* represent two solutions of the same problem.

According to DNA sequence data, it is likely that elements similar to those described here are also active in the *B. subtilis* (55) and *B. halodurans str* operon (50). Therefore, it is tempting to hypothesize that mechanisms of control of the EF-G/EF-Tu ratio have common features among bacilli. The work on streptomycetes conducted by Tieleman and coworkers (51) implies that the presence of a *tuf*-specific promoter may also be characteristic for other gram-positive bacteria. The relative activities of *tufp* and *strp* in streptomycetes, however, were not assessed.

Implications derived from the presence of the *ybxF* gene in the *str* operon. The *ybxF* gene codes for a protein of unknown function, homologous to eukaryotic ribosomal protein L30. The *ybxF* gene product shares 41% amino acid identity and 82% amino acid similarity with a 30-aa conserved region of human-rat liver L30 (38). L30 proteins bind to 28S rRNA (31, 32). A conserved, tentative RNA binding motif (Fig. 2) was identified in these proteins by Koonin and coworkers (26). The deduced amino acid sequences of the *ybxF* genes of both *B. stearo-thermophilus* and *B. subtilis* contain the same RNA binding motif, and their cotranscription with ribosomal proteins and basic pI suggest that they are ribosomal proteins. However, none of the known primary structures of *B. stearo-thermophilus* ribosomal proteins (2, 19) matches the deduced aa sequence of the *ybxF* gene. To resolve whether the product of the *ybxF* gene is a part of the ribosome or possesses a different function, or both, will be the subject of further studies.

The factorial correspondence analysis of *B. subtilis ybxF* gene revealed its codon usage as being significantly different from the remaining four genes of the operon. This result suggests that the *rpsL*, *rpsG*, *fus*, and *tuf* genes have evolved under similar selective constraints, whereas the *ybxF* gene was linked to the rest of the *str* operon at a point in evolution when the aforementioned four genes had already coexisted and participated in translation. This result supports the view that the *ybxF* translation product has a nonribosomal destination.

The *ybxF* gene has a paralogous counterpart on the *B. stearo-thermophilus* and *B. subtilis* chromosomes, the *ymxC* gene. The *ymxC* gene is organized in the *nusA-infB* region and codes for a protein of unknown function (7, 48). In most of the known archaeobacterial genomes, the *nusA* gene and the *ybxF* orthologue are organized upstream of the *rpsL* gene (see, for example, references 8, 24, and 30). FCA of *B. subtilis* genes positions *nusA* and *ybxF* close to each other (data not shown). Thus, the

ybxF gene in bacilli may have originated by duplication of the *ymxC* gene or vice versa. It is also interesting to note that the gene adjacent to *ymxC*, *infB*, codes for a GTP-binding protein, translation initiation factor IF2, that is partially homologous to EF-Tu and EF-G.

The presence of the *ybxF* gene in the *str* operon bears further phylogenetic implications. A database search revealed that also some other gram-positive bacteria have the *ybxF* gene organized in the same genomic context (e.g., *Clostridium acetobutylicum* and *Staphylococcus aureus*), but nothing is known about their transcriptional organization. In contrast, gram-negative bacteria do not have a homologue of the *ybxF* gene. Thus, our results support the hypothesis that archaeobacteria are more closely related to gram-positive bacteria, especially to the low-G+C gram-positive bacteria (e.g., bacilli), than to gram-negative bacteria (13, 14, 15).

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