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

LIBOR KRÁSNÝ, TOMÁŠ VACÍK, VLADIMÍR FUČÍK, AND ŽIŽI JONÁK*
Department of Protein Biosynthesis, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, 166 37 Prague 6, Czech Republic

Received 22 May 2000/Accepted 9 August 2000

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'-ybfF (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 ybfF, 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 two promoters. The operon in E. coli chromosomal (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 the present 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 characteristics 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 ybfF, was found to belong to the str operon in B. stearothermophilus and B. subtilis. The implications derived from the presence of the ybfF 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 (letu- mna- recA4) as a host in CAT assay experiments. E. coli DH5α 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 were 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 PvuI, 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.

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

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'-ybfF (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 ybfF, 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.
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 of 0.6, 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 QUANT-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 M Cs of pCP31 and pCPP32 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 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 44 (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 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). Dithyrrin upstream of the rpsL gene is the fragment (reading frame ORF) of 2,249 nt that is 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 guest

str OPERON OF B. STEAROTHERMOPHILUS

MgSO₄, 7H₂O, 0.2 g; glucose, 0.5%; K₂HPO₄, 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, and DNA labeling kit, were purchased from Gibco-BRL. All enzymes and kits were used according to the manufacturer’s recommendations. Oligonucleotide primers were purchased from GeneriBiotech (Czech Republic).

Northern blot hybridization 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 Qiaex II 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 RNAeasy 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: PI11I from residues 1053 to 1073 (5′-GGCGT GGGCCGATTTCCTGCTTGTG-3′) and PIV from residues 16 to 36 (28) (5′-GAG CGAGGAGAGGACGACGC-3′). The Expand High-Fidelity PCR System was used to carry out the PCR. The 1.4-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′-TCCTCTGGGTACGAGCTGCG-3′) and PVII (5′-GGTTTTTTGCACGCTGATC-3′) were used as a part of the rpsL gene, which was used as a probe in Southern hybridization experiments. A Bgl II 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 and PV were used in 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 PCRs of each DNA fragment were sequenced. The fus gene 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 rpsL gene at its 3′ end, and it ended in the fus gene. The 2.3-kb 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 primers were 5′-GAGCGAGGAGAGGACGACGC-3′ and 5′-GGTTTTTTGCACGCTGATC-3′ (PVII); for B. subtilis, fus primers were 5′-GGTTTTTTGCACGCTGATC-3′ and 5′-GGTTTTTTGCACGCTGATC-3′ (PVII). GCACCGGAGAGGACGACGC-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 4229 to 4248 (forward primer) and nt 2661 to 2680 (reverse primer) (numbering is according to Yasuo-moto 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′-GCTACATCCTTGTTGCTTGC-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%, and EF-Tu and EF-G were resolved 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 amino acid sequences isolated from EF-Tu and EF-G were analyzed by EF-Tu and EF-G in the two-dimensional (2D) map were determined by densitometric measurements, normalized to the molecular weights of these proteins.
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. stearothermophilus and B. subtilis.

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

FIG. 1. str operon of B. stearothermophilus. 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 Krášny 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.

FIG. 2. Nucleotide sequence of the region upstream of the rpsL gene, and deduced amino acid sequences of the ybxF gene and the 3'9 end of the rpoC gene of B. stearothermophilus. The Shine-Dalgarno sequences are in underlined italics. The −35 and −10 regions of the strp 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 stability of this structure was calculated to be −49.3 kJ mol−1 at 65°C (RNAdraw V1.0 [34]). The IR extends from the 3' end of the rpoC gene (from −159) to the 5' end of the A tract. 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).
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 σ70 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 carried 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.
sequence upstream of the MCS:

```
GAATTTCGACGCTTCGGCCGGCACGTTTATCTCTTACTTGGTGAATCG
```

pCPP-31:

```
EcoRI SacI SmaI PstI
GAATTTCGACGCTTCGGCCGGCACGTTTATCTCTTACTTGGTGAATCG
```

pCPP-32:

```
PstI SmaI SacI EcoRI
GAATTTCGACGCTTCGGCCGGCACGTTTATCTCTTACTTGGTGAATCG
```

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 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.X2). (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 ~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 150 bp (pCPP-31.X6) slightly relieved the suppression bringing 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

![A-T-rich block](image_url)

FIG. 7. Sequence alignment of B. steareothermophilus and B. subtilis tuf promoter regions. The 3′ ends of the sequences end with start codons of the fus gene. 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.
the 3′-terminal part of the rpoC gene fragment, controlled by the inserted tuf 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 archaeabacterium Methanococcus vannielli (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 tuf/p of B. stearothermophilus revealed that the transcription proceeds about 20 times more efficiently from tuf/p 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-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 unbound 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 tuf/p 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 (αCTD) of RNA polymerase (9, 22, 46). Therefore, a contact between the A/T-rich block and the αCTD RNAP may be the mechanism that contributes to the efficient transcription of the tuf gene in B. stearothermophilus 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. stearothermophilus, 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. stearothermophilus 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. stearothermophilus 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 (35) 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 tuf/p 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 ybxF function, homologous to eukaryotic ribosomal protein L30, is characteristic for other gram-positive bacteria. The relative activities of tuf/p and strp in streptomycetes, however, were not assessed.

The ybxF gene has a paralogous counterpart on the 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. stearothermophilus and B. subtilis chromosomes, the ymcC gene. The ymcC gene is organized in the nusA-inB region and codes for a protein of unknown function (7, 48). In most of the known archaeabacterial genomes, the nusA gene and the ymcC orthologue are organized upstream of the rpsL gene (see, for example, references 8, 24, and 30). FCA of B. subtilis genes positions ymsL and ybxF close to each other (data not shown). Thus, the
yhfF gene in bacilli may have originated by duplication of the ymcC gene or vice versa. It is also interesting to note that the gene adjacent to ymcC, 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 yhfF gene in the str operon bears further phylogenetic implications. A database search revealed that also some other gram-positive bacteria have the yhfF 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 yhfF gene. Thus, our results support the hypothesis that archaeabacteria 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).

ACKNOWLEDGMENTS

We thank D. Draper for comments on the structure of the fus gene of B. steareothermophilus and L. Výborová and K. Zemanová for skillful technical assistance.

This work was supported by grant 75195-540305 from the Howard Hughes Medical Institute (to J.J.) and by grant 204/98/063 from the Grant Agency of the Czech Republic (to J.J.).

REFERENCES

13. Gupta, R. S. 1997. Protein phylogenies and signature sequences: evolutionary relationships within prokaryotes and between prokaryotes and eu-


ERRATA

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

LIBOR KRÁSNÝ, TOMÁŠ VACÍK, VLADIMÍR FUČÍK, AND JIRÍ JONÁK

Department of Protein Biosynthesis, Institute of Molecular Genetics, Academy of Sciences
of the Czech Republic, 166 37 Prague 6, Czech Republic

Volume 182, no. 21, p. 6114–6122, 2000. Page 6119, Fig. 8A: The 5’ endpoint of the cloned DNA fragment in pCPP-31.X5 should read “G-159*” instead of “T-159*.”

Volume 182, no. 22, 2000, Table of Contents (print version) and Author Index

Table of Contents and Author Index: “Astrid Müller” was inadvertently omitted from the list of authors for the article “Characterization of SepL of Enterohemorrhagic Escherichia coli,” p. 6490–6498.