JOURNAL OF BACTERIOLOGY, Apr. 1996, p. 2459–2461 Vol. 178, No. 8
0021-9193/96/$04.00+0
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Lactobacillus bulgaricus Asparagine Synthetase and Asparaginyl-tRNA Synthetase: Coregulation by Transcription Antitermination?

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Received 11 December 1995/Accepted 15 February 1996

Genes encoding the ammonia-dependent asparagine synthetase (*asnA*) and asparaginyl-tRNA synthetase (*asnS*) have been cloned from *Lactobacillus bulgaricus* ATCC 11842. The nucleotide sequence suggests that *asnA* and *asnS* are organized as one operon and regulated by the tRNA-directed transcription antitermination mechanism (T. M. Henkin, Mol. Microbiol. 13:381–387, 1994).

Two different and structurally unrelated enzymes which are involved in the biosynthesis of asparagine from aspartic acid exist. In *Escherichia coli* these enzymes are the ammonia-dependent asparagine synthetase (AS-A) encoded by *asnA* (3) and the glutamine-dependent asparagine synthetase (AS-B) encoded by *asnB* (16). AS-A is found only in prokaryotes, whereas AS-B is found in both prokaryotes and eukaryotes (10). While the AS-A enzyme uses only ammonia as the amino group donor, the AS-B enzyme can use either glutamine or ammonia. Genes encoding these enzymes have been cloned from a variety of different organisms including *E. coli* (14, 16), mammals (1, 2), and plants (12). Comparison of amino acid sequences of *E. coli* AS-A and AS-B revealed that the proteins are genetically unrelated, although these enzymes have the same properties since they use aspartate and ammonia as substrates and hydrolyze ATP (15). AS-B is a member of the *purF* family of glutamine-dependent amidotransferases (16). Ammonia-dependent asparagine synthetase, on the other hand, contains a short region with high homology to the ATP binding motif of aspartyl-tRNA synthetase, a class II aminoacyl-tRNA synthetase. Thus, it was proposed that the ammonia-dependent asparagine synthetase evolved from aspartyl-tRNA synthetase (10).

Recently, a novel mechanism of transcription regulation was described for gram-positive eubacteria (reviewed in references 8 and 9). The transcription of at least 18 amino acid biosynthesis enzymes and aminoacyl-tRNA synthetases in gram-positive eubacteria is regulated by a common transcription antitermination process. Each gene is induced by limitation for the cognate amino acid. The mRNA leader regions of these genes can be folded into putative terminator and antiterminator structures and include a conserved T-box sequence. It was shown for the *Bacillus subtilis* tyrosyl-tRNA synthetase gene that $tRNA^{Tyr}$ stimulates transcription and that the tRNA anticodon interacts with a cognate codon in the leader region of the mRNA. As aminoacyl-tRNA synthetases are dependent for their reaction on the supply of amino acids, the coregulation of aminoacyl-tRNA synthetases and the enzyme(s) responsible for the biosynthesis of the cognate amino acid is desirable. Recently, such a case was described for *B. subtilis*, in which *cysE*, the gene encoding the first enzyme in cysteine biosynthesis, and *cysS*, which encodes cysteinyl-tRNA synthetase, are overlapping and regulated together by a T-box-type mechanism (7). Here, we characterize the *Lactobacillus bulgaricus asnA* and *asnS* genes and propose the utilization of the tRNA-directed transcription antitermination mechanism. This would represent the second case of a regulatory linkage of aminoacyl-tRNA synthetase and cognate amino acid biosynthesis.

***L. bulgaricus* ammonia-dependent asparagine synthetase.** In the course of our studies on lactose metabolism in lactobacilli (13) a 3.6-kb DNA fragment of genomic DNA from *L. bulgaricus* ATCC 11842 was isolated. Sequence analysis (GenBank accession no. X89438) revealed that the DNA fragment contained two open reading frames (ORF1 and ORF2). They are presumably arranged as an operon, as there are no sequences upstream of ORF2 resembling a promoter. The deduced amino acid sequence of ORF1 (336 amino acids) shows 50 or 54% identity with that of the *E. coli* or *Haemophilus influenzae asnA* gene product, the ammonia-dependent asparagine synthetase (Fig. 1). The ATG codon of the putative *L. bulgaricus asnA* gene was assigned by finding a potential ribosome binding site (gcagAGGCAAGag) in the proper position relative to ORF1, even though this sequence is not highly matched with the consensus sequence (aaAGGAGG) of lactobacilli (4). Presently, there are only three asparagine synthetase A genes known. As Fig. 1 shows, there is extensive homology between the proteins from gram-negative and gram-positive eubacteria.

ORF2 shows 41% identity with the *E. coli asnS* gene product, asparaginyl-tRNA synthetase, and represents the *L. bulgaricus* AsnRS gene (10a).

ORF1 complements asparagine auxotrophy in *E. coli* and encodes active asparagine synthetase. To examine whether ORF1 is the gene encoding ammonia-dependent asparagine synthetase, a complementation of asparagine auxotrophy in *E. coli* JF448 (6) was carried out. The structural gene of ORF1 was amplified by PCR and subcloned into pSK^- at the *Sma*I site. Two different recombinant plasmids were obtained; in pSKlacAS the *asnA* ORF is behind the β-galactosidase promoter, whereas in pSKT7AS the ORF is cloned behind the T7

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         1                                                                                                 90
L. bulg  maklvipsdy dpkmt-akrk rq-atsgkps sefgtamnle risa-mfvkk ss-ln----- w--p-sftlh dgnege-q-- --------wa
E. coli             mktay-akqr qi-fvkshfs rqleerlgli evqa-ilsrv gd-tq----- c--a-qvkvk alpdaq-e-- --------qt
H. infl             mkktf-lqqq ei-fvkntft qnlieqlgii evqg-ilsqv gn-mq----- i--a-qvnvk cipnav-e-- --------ht
Consens             •••••I•••• ••S••••••• •••••••••• •V••P••••• ••G••DNLSG •EK•V••••• ••••••F•VV HSLAKWKR••

         91                                                                                               180
L. bulg  -khyg-shg- ---tn-n-i- k---v-dnl- ---------- k-idkse-te at-rq--qr- -et-kgm-yh -ral-pqaay h--ee-s--t
E. coli  -gqhd-sag- ---th-k-l- p---r-spl- ---------- r-mgdge-qf st-ks--ea- -ag-kat-aa -see-glapf .--dq-h--h
H. infl  -arfn-ked- ---vh-k-l- p---s-dpt- ---------- k-ipegr-nf ay-ke--ns- -ra-rlt-la -ear-dipsi .--kq-t--h
Consens  L••••F•••E GLF••M•A•R •DED•L•••H SVYVDQWDWE •V•••••R•• ••L••TV••I •••I•••E•• V•••F••••• •LP••I•FV•

         181                                                                                              270
L. bulg  -e--ea---s -tpse--dk- cq-k-----e h---a-plsk kp--l----- ---t...... ..----l-f -yep-qr--- v---s----e
E. coli  -q--ls---d -dakg--ra- ak-l-----v g---k-s.dg hr--v----- ---stpselg hag----i-v -npv-ed--- l---g----a
H. infl  -e--vk---d -sske--na- ck-y-----i g---k-s.dg kp--g----- ---tteseng ykg----i-v -ndq-gk--- l---g----e
Consens  S•EL••RYP• L••••RE••I ••E•GAVFL• •IGG•L•••• ••HD•RAPDY DDW••••••• •••LNGD•L• W•••L••AFE •SSM•IRVD•

         271                                                                      346
L. bulg  dr-qe-lk-a -a--r-d-p- --a--k-dl- ys-------- --c----qgp -.-e--as-- -deiv-kcqa akiqll
E. coli  dt-kh-la-t -d--r-e-e- --a--r-ei- qt-------- --t----qlp -i-q--cg-- -aavr-svps ll
H. infl  sa-rl-vg-t -d--h-k-d- --d--n-kl- lt-------- --a----rkk -i-e--ss-- -keml-efsn il
Consens  ••L••Q••L• G•ED•L•L•W HQ•LL•G••P ••IGGGIGQS RL•MLLL••• H•G•VQ••VW P••••E•••• ••
```

FIG. 1. Sequence comparison of ORF1 (*L. bulgaricus* [*L. bulg*]) and ammonia-dependent asparagine synthetases from *E. coli* and *H. influenzae* (*H. infl*) The consensus sequence indicates fully conserved positions in all three proteins.

A

```
                                            -35                       -10
TTTGCCAGCA GTGCCTACCA GCAAGTGGTC CAGGAATTTG AAGGCCTTAG CTTGATCGTG

TTAATCGCTT TCTGCGCCAT CGCAGCCGCT GTCTACCTGC TCAAGAAGAA GGGCCTAATC

                                  -35                   -10
TTTAAAAAAT TTTTTTGCCT TGATCTTGA CAAAAGTTAA ATCCGTATTA GAATTTTACT

                                                             *1
TAATCAAATA AGTCAAAAGA TGAGAAAGAC GAGTAGGAAA GCACAATCTT GCAGAGAGTT

       *2                                   Asn codon
GCCAGCTGGT GAAAAGGCCA ACAGACCCTG GCTTTCTGAA TAACACTTTT TCCAGATCGC

                                        *3
CGGCTGAAGT CACAGTAAGC CGTGCCGCCT GCAGGACGTT ACCCTGCTAG AGCATGTTGG

                                    T-Box
TGCTCGAAGT GGTTCGTTTA GAACAAGGTG GGTGGTACCG CGGAAAGATG CCTTTCGTCC

CTGTAGTTTT TGGGGATGAA AGGCTCTTTT TATGCCCTTT TGACAAATAT TAGATTTTTA

   SD        fMet
GCAGAGGCAA GAGATATGGC TAAATTAGTT ATTCCAAGTG ATTACGATCC GAAAATGACC

ATCGCGAAAC GGAAAAGGCA ATCCGCTACA TCCGGAAAAC CTTCCAGCGA
```

B

Terminator

Antiterminator

FIG. 2. Nucleotide sequence of the leader region of the *L. bulgaricus asnA* gene and proposed transcription antiterminator structure. The conserved sequences are indicated (numbered asterisks [A] and open arrows [B]). The nucleotides in the AAC asparagine codon which may act as specifier sequence are circled (B). Half-barbed arrows, T box. The putative ribosome binding site (Shine-Dalgarno [SD] sequence) and initiator methionine (fMet) are indicated.

TABLE 1. Asparagine synthetase activities of transformants

Strain[a]	Relative activity (U)[b]
JF448/pSK$^-$	0.0
JF448/pSKlacAS	9,192
JF448/pSKT7AS/pGP1-2	1,036
JF448/pET15b	0.8
JF448/pET15b/pGP1-2	0.5
JF448/pET15bAS/pGP1-2	789

[a] Grown at 37°C for 16 h in Luria-Bertani broth supplemented with ampicillin (50 μg/ml) or ampicillin plus kanamycin (25 μg/ml). Strain JF448 is AsnA$^-$ AsnB$^-$ and can be complemented by *asnA* (6).

[b] Measured in S-100 preparations (15). One unit was defined as the amount catalyzing the formation of 1 nmol of β-aspartyl hydroxamate per min.

promoter. These plasmids were introduced into strain JF448; in the case of pSKT7AS the cells also carried plasmid pGP1-2 (17) expressing T7 RNA polymerase. Asparagine synthetase assays were performed with the S-100 preparations of transformed and untransformed cells; the data clearly show that the heterologously expressed gene gives rise to active enzyme (Table 1). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the S-100 extract showed the overexpressed protein band with a molecular mass of 39 kDa, which is in good agreement with the calculated molecular mass of ORF1 (38.2 kDa). Both plasmids allowed effective complementation of the asparagine auxotrophy in strain JF448, as evidenced by growth on minimal medium plates without asparagine (data not shown). Thus, ORF1 encodes active asparagine synthetase and presumably is the *asnA* gene of *L. bulgaricus.*

Transcriptional regulation of the *asnA* gene. The putative −35 and −10 consensus sequences for transcription promotion were found approximately 340 nucleotides upstream of the start codon of *asnA* (Fig. 2). The mRNA leader region contains not only an asparagine codon (actually two adjacent ones) as specifier sequence but also the particular conserved sequences (e.g., the T box) which are known to be involved in the common transcription antitermination mechanism found for many gram-positive aminoacyl-tRNA synthetase and amino acid biosynthesis genes (reviewed in reference 9). Therefore, it seems likely that the transcription of *L. bulgaricus asnA* and also of *asnS* is regulated by the common antitermination mechanism which relies on the presence of uncharged $tRNA^{Asn}$ in the cell. The increase of the uncharged $tRNA^{Asn}$ caused by insufficient endogenous asparagine would trigger transcription antitermination, which would result in transcription read-through of the *asnA* leader region. This case is reminiscent of the regulation of *cysE* and *cysS* in *B. subtilis*, in which genes encoding an enzyme in amino acid biosynthesis and an aminoacyl-tRNA synthetase are regulated together by transcription antitermination (7). This is different from the situation in *E. coli*, in which *asnA* transcription is regulated by a 17-kDa protein, encoded by *asnC*, and endogenous asparagine (5, 11).

We are grateful for Andrea Pfeifer's encouragement and thank Tina Henkin for useful discussions.

This work was supported by a grant from NIH.

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