The genes *lepA* and *hemN* form a bicistronic operon in *Bacillus subtilis*

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The *lepA* operon of *Bacillus subtilis* was found to be bicistronic and to consist of the two genes *lepA* and *hemN*, which encode a putative GTP-binding protein and an oxygen-independent coproporphyrinogen III oxidase, respectively. The *lepA* operon is located immediately upstream of the *dnaK* operon. Both operons are transcribed in the same direction and are not separated by an obvious transcription-terminator-like structure. The *lepA* operon is preceded by a potential vegetative promoter, and there is a putative strong intergenic terminator between *lepA* and *hemN*. Northern blot experiments revealed only a transcript corresponding to *lepA*, but expression of *hemN* was demonstrated in slot-blot and immunoblot experiments using antibodies raised against His-tagged HemN. The data suggest that most of the transcripts originating at the potential vegetative promoter are terminated at the intergenic terminator. Readthrough transcription into the downstream *dnaK* operon was not found.

Keywords: *lepA*, *hemN*, *Bacillus subtilis*, bicistronic operon, riboprobe

INTRODUCTION

We have described previously the cloning and sequencing of the *dnaK* operon of *Bacillus subtilis*, which consists of the four genes *brcA*, *grpE*, *dnaK* and *dnaJ* (Wetzstein *et al.*, 1992). A careful inspection of the DNA sequence up- and downstream of the *dnaK* operon failed to detect any DNA sequence resembling a transcription-terminator-like sequence. This finding prompted us to ask whether additional gene(s) up- and/or downstream of the *dnaK* operon might also be part of this operon. Transcriptional analysis of the *dnaK* operon using a riboprobe complementary to the *dnaJ* mRNA led to the detection of a novel transcript, suggesting the existence of additional genes downstream of *dnaJ* (G. Homuth, unpublished). This result has been confirmed by DNA sequencing of the region downstream of *dnaJ* and led to the detection of three additional open reading frames (ORFs) which are followed by a potential rho-independent terminator (Y. Kobayashi, personal communication). Cloning and sequencing of the upstream region led to the detection of two ORFs preceded by a putative rho-independent terminator and a potential vegetative promoter. An alignment of the amino acid sequences deduced from the two ORFs with proteins in the EMBL database revealed

significant homology of the promoter-proximal ORF to LepA and of the promoter-distal ORF to HemN of *Escherichia coli* (March & Inouye, 1985; Troup *et al.*, 1995). Transcriptional and immunoblot analysis indicated that both genes form a single operon which is not induced on temperature upshift.

METHODS

Bacterial strains and media. The bacterial strains and plasmids used are listed in Table 1. *E. coli* and *B. subtilis* strains were grown aerobically at 37 °C in L broth (LB). When necessary, LB was supplemented with ampicillin (Ap, 200 µg ml⁻¹), neomycin (Nm, 10 µg ml⁻¹) or chloramphenicol (Cm, 5 µg ml⁻¹). If not indicated otherwise, *E. coli* strain DH10B was used as recipient in the cloning experiments.

DNA isolation and cloning of the *lepA* operon. Total DNA was extracted from overnight cultures of *B. subtilis* as described by Dubnau & Davidoff-Abelson (1971). Plasmid DNA was isolated from *E. coli* by the alkaline lysis method (Birnboim & Doly, 1979) and all DNA manipulations were performed as described by Sambrook *et al.* (1989). For cloning of the complete *lepA* gene, 1 mg chromosomal DNA was digested completely with *EagI* and the restriction fragments were separated in a 10–40% (w/v) sucrose density gradient. Fractions containing the desired 2.8 kb *EagI* fragment were identified by hybridization using the oligonucleotide ON1 [5'-d(GAGGGGACATCTCGCG)-3'], complementary to a sequence near the 3' end of *lepA*. The fractions were pooled, ethanol-precipitated, ligated to *EagI*-digested and

The EMBL accession number for the sequence reported in this paper is X91655.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Source or reference
<i>E. coli</i>		
M15	F ⁻ Sm ^r <i>lacZ</i> Δ	Hochuli <i>et al.</i> (1988)
DH10B	F ⁻ <i>mcrA</i> Δ(<i>mrr</i> - <i>hsdRMS</i> - <i>mcrBC</i>) φ80d <i>lacZ</i> ΔM15 Δ <i>lacX</i> 174 <i>deoR</i> <i>recA1</i> <i>araD</i> 139 Δ(<i>ara</i> <i>leu</i>)7697 <i>galU</i> <i>galK</i> λ ⁻ <i>rpsL</i> <i>endA1</i>	Bethesda Research Laboratories
C600 <i>penB</i>	<i>leuB6 thi-1 lacY1 supE44</i> transduced to <i>pncB80</i> using P1 and donor strain	This study
MRi93	MRi93 <i>zad::Tn10 pncB80</i>	Lopilato <i>et al.</i> (1986)
<i>B. subtilis</i>		
1012	<i>leuA8 metB5 trpC2 hsrM1</i>	Saito <i>et al.</i> (1979)
<i>lepA::cat</i>	Strain 1012 with <i>lepA::cat</i>	This study
<i>lepAΔ</i>	Strain <i>lepA::cat</i> with <i>lepA::cat</i> replaced by <i>lepAΔ</i>	This study
<i>hemN::cat</i>	Strain 1012 with <i>hemN::cat</i>	This study
<i>hemNΔ</i>	Strain <i>hemN::cat</i> with <i>hemN::cat</i> replaced by <i>hemNΔ</i>	This study
Plasmids		
pBluescript KS ⁺ /II SK ⁺	Ampicillin resistance	Stratagene
pDS56	Ap ^r Cm ^r (His6)-expression vector	Hochuli <i>et al.</i> (1988)
pDMI.1	Km ^r <i>lacI</i> ^q	Hochuli <i>et al.</i> (1988)
pUC18cm	pUC18 with 1.3 kb <i>cat</i> cassette	Beall & Lutkenhaus (1989)
pUC18cm-S	pUC18cm with <i>Sma</i> I site replaced by <i>Sal</i> I site allowing the recovery of <i>cat</i> cassette as <i>Sal</i> I fragment	This study
pNEXT33A	pBR322 with <i>metD::neo</i> of <i>B. subtilis</i>	Itaya & Tanaka (1990)
pSED157	740 bp <i>Hpa</i> I- <i>Eco</i> RV fragment of <i>brcA</i> cloned into <i>Hind</i> II of pSPT18	S. Engelmann, unpublished
pGH01	2856 bp <i>Eag</i> I fragment of chromosomal <i>B. subtilis</i> DNA cloned into <i>Eag</i> I site of pBluescript KS ⁺	This study
pGH01b	2856 bp <i>Eag</i> I fragment of chromosomal <i>B. subtilis</i> DNA cloned into <i>Cla</i> I site of pBluescript KS ⁺	This study
pGH02/03	1256 bp <i>Cla</i> I fragment of pGH01 cloned into <i>Cla</i> I site of pBluescript KS ⁺ ; both possible orientations	This study
pGH04/05	878 bp <i>Cla</i> I fragment of pGH01 cloned into <i>Cla</i> I site of pBluescript KS ⁺ ; both possible orientations	This study
pGHEE	1293 bp <i>Eag</i> I- <i>Eco</i> RI fragment of pGH01 cloned into <i>Cla</i> I site of pBluescript KS ⁺	This study
pGHEECAT	1300 bp <i>Hind</i> III- <i>Bam</i> HI fragment of pUC18cm cloned into <i>Cla</i> I site of pGHEE	This study
pGHΔ <i>Cla</i>	Deletion of the internal 1.3 kb <i>Cla</i> I fragment of <i>lepA</i>	This study
pGH06	<i>hemN</i> amplified by PCR and flanked by <i>Hind</i> III and <i>Bam</i> HI sites and cloned into pBluescript II SK ⁺	This study
pBT01	pUC18 carrying <i>lepA hemN brcA grpE dnaK</i>	Schulz <i>et al.</i> (1995)
pYA07	1.3 kb <i>Sal</i> I fragment of pUC18cm-S cloned into unique <i>Sal</i> I site of pBT01	This study
pmH01	pBT01 cut with <i>Eag</i> I and religation of the larger fragment; carries an in-frame deletion in <i>hemN</i>	This study
pmH04	Coding sequence of <i>hemN</i> amplified by PCR and cloned in pDS56	This study

dephosphorylated pBluescript KS⁺ and used to transform *E. coli* C600 *penB*. The resulting transformants were screened by colony hybridization using ON1.

Hybridization. For Southern hybridizations, chromosomal DNA was digested to completion with the appropriate restriction enzymes and separated in 0.8% (w/v) agarose gels. DNA was transferred onto a nylon membrane (Quiagen) by vacuum blotting and hybridized with oligonucleotides labelled at their 5'-termini using DIG-[11]-ddUTP as described previously (Zuber & Schumann, 1991). For colony hybridization, colonies were picked onto duplicate plates. Nylon membranes

were used for colony lifts and hybridization was performed as described by Grunstein & Wallis (1979).

Analysis of transcription. Preparation of total RNA, Northern blotting, hybridization and slot-blot analysis were performed as described previously (Wetzstein *et al.*, 1992). In all slot-blot experiments, 1 µg total RNA per slot was used. As hybridization probes, we used either the DIG-labelled oligonucleotide ON2 (5'-d(CTTGCTCTTCTGTTTCG)-3', complementary to *grpE*-mRNA) or DIG-labelled RNA probes. These had been synthesized *in vitro* with T7 RNA polymerase (Boehringer Mannheim, DIG-RNA Labeling kit) from linearized plasmids

pGH04, pGH06 (see Fig. 1) or pSED157. Plasmid pGH04 contains the internal *Cla*I fragment of *lepA* inserted in pBluescript KS⁺; pGH06 contains the complete *hemN* gene amplified by the polymerase chain reaction (PCR), flanked by *Bam*HI and *Hind*III sites and inserted into pBluescript II SK⁺; and pSED157 contains the internal *Hpa*I/*Eco*RV fragment of *brcA* cloned into the *Hind*II site of pSPT18 (S. Engelmann, unpublished). By using the T7 promoter, DIG-labelled RNA probes (riboprobes) complementary to the transcripts of *lepA*, *hemN* and *brcA* were synthesized.

DNA sequencing. DNA was sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977) using deoxyadenosine [³⁵S]dATPαs [8 μCi μl⁻¹, specific activity 650 Ci mmol⁻¹ (24.1 TBq mmol⁻¹) Amersham] and a Sequenase kit (USB). Sequencing of the complete *lepA* gene was achieved using plasmids pGH02/03 and pGH04/05 (Fig. 1).

Overexpression and purification of HemN and antibody production. To facilitate the overproduction and purification of HemN, the gene was first amplified by PCR using pBT01; both primers had *Bam*HI recognition sites at their termini. The amplicon was cleaved with *Bam*HI and cloned into *Bam*HI-linearized pDS56 (Hochuli *et al.*, 1988), resulting in pMH04. This plasmid was then introduced by electroporation into *E. coli* M15 (pDML1) for overproduction of His6-HemN. The His6-tagged protein was purified from IPTG-induced cells (2 mM IPTG for 3 h) as described by Hochuli *et al.* (1988) and was used to raise polyclonal antibodies in a rabbit.

Western immunoblot analysis. SDS-PAGE was performed as described by Laemmli (1970). For immunoblot analysis, the gels were electroblotted for 1 h at 0.8 mA cm⁻² onto nitrocellulose membranes (45 μm, Schleicher & Schuell) with a Trans Blot Semi-Dry Transfer Cell (Bio-Rad) according to the manufacturer's recommendations. To block nonspecific binding-sites, the filters were incubated in 10% (w/v) milk powder in AP-T buffer (1 M Tris/HCl, pH 7.4, 1 M NaCl, 25 mM MgCl₂) for 1 h at room temperature. After blocking, filters were washed once for 15 min and twice for 5 min at room temperature with AP-T. Subsequently, filters were incubated for 1 h in AP-T containing a 1:500 dilution of the polyclonal primary antibodies and were further washed once for 15 min and twice for 5 min in AP-T. After washing, filters were incubated for 1 h in AP-T containing a 1:5000 dilution of secondary antibodies (donkey anti-rabbit antibodies) conjugated with horseradish peroxidase (Amersham). Excess antibodies were removed by washing for 1 × 15 min and 4 × 5 min in AP-T. Immunoreactive bands were visualized by a chemiluminescence reaction according to the manufacturer's instructions (Amersham, ECL System).

Construction of chromosomal insertion and deletion mutants. To obtain a *lepA::cat* insertion, the 1293 bp *Eag*I-*Eco*RI fragment was recovered from pGH01 and cloned into the *Cla*I site of pBluescript KS⁺ after filling in the 5' protruding ends of both the vector and the donor fragment resulting in pGHEE. The *cat* cassette of pUC18Cm was then recovered as a 1348 bp *Hind*III-*Bam*HI fragment, blunt-ended and inserted into *Cla*I-linearized and blunt-ended pGHEE resulting in pGHEECAT. In this plasmid, transcription of *lepA* and *cat* occurs in the same direction. To recombine the *cat* cassette into the chromosomal *lepA* gene, *B. subtilis* 1012 was transformed with pGHEECAT. Transformants were selected on plates containing Cm, and chromosomal DNA of recombinants was screened by Southern blotting for replacement recombination at the *lepA* locus (data not shown). One of these recombinants (*lepA::cat*) was kept for further studies. To facilitate deletion of the internal *Cla*I fragment of *lepA*, pBluescript KS⁺ was linearized with *Cla*I, end-filled with Klenow fragment and

ligated to the blunt-ended 2.8 kb *Eag*I fragment resulting in pGH01b. For the construction of an out-of-frame deletion mutant, pGH01 was digested with *Cla*I and the protruding ends were filled-in before self-ligation (pGHΔ*Cla*). The out-of-frame deletion was confirmed by DNA sequencing. This deletion was then crossed into the *B. subtilis* chromosome using strain *lepA::cat* as recipient and the method described by Itaya *et al.* (1990) with pNEXT33A as the cotransforming plasmid. Replacement of *lepA::cat* by *lepA*Δ was verified by Southern blotting. The resulting strain was denoted *B. subtilis* *lepA*Δ (data not shown). A *hemN::cat* insertion mutant was constructed by inserting the 1.3 kb *cat* cassette recovered from pU18Cm-S into the unique *Sal*I site of pBT01 resulting in pYA07. The *Sal*I site within *hemN* is situated near its 5' end (Fig. 1). Subsequently, this insertion mutation was crossed into the chromosome of *B. subtilis* 1012, and transformants were analysed by Southern blotting (data not shown); strain *B. subtilis* *hemN::cat* was retained for further studies. To obtain a *hemN* deletion mutant, pBT01 was digested with *Eag*I, and the larger fragment was self-ligated resulting in pMH01. By this treatment, a 0.6 kb internal fragment of *hemN* was removed resulting in an in-frame deletion. This deletion reduced the size of the potential HemN protein from 363 to 167 amino acid residues. Plasmid pMH01 was then used to replace the *hemN::cat* insertion mutation in strain *hemN::cat* by *hemN*Δ as described above for *lepA* resulting in strain *hemN*Δ.

Computer programs. Sequence analysis was performed by using the PC/GENE package (IntelliGenetics) for IBM personal computers and HUSAR (Heidelberg Unix Sequence Analysis Resources).

RESULTS

Detection of the *lepA* homologue in *B. subtilis*

We have reported previously the cloning of a 5.2 kb *Hind*III fragment of chromosomal DNA of *B. subtilis* (Wetzstein *et al.*, 1992). This fragment contained the genes *brcA*, *grpE* and a large region of *dnaK* near one end. Since no obvious transcription termination site could be

Fig. 1. Genetic and restriction map of the *lepA* operon and location of different mutations. The 1.3 kb *cat* cassette was inserted near the 5' end of either *lepA* (*lepA::cat*) or *hemN* (*hemN::cat*); internal deletions inactivated either *lepA* (*lepA*Δ) or *hemN* (*hemN*Δ). Inserts in various plasmids described in the text are shown. C, *Cla*I; E, *Eag*I; Ec, *Eco*RI; H, *Hind*III; S, *Sal*I; P_{A1} and P_{A2}, potential vegetative promoters preceding the *lepA* and *dnaK* operons, respectively; T, putative rho-independent transcription terminator; CIRCE, repressor-binding site.

TAAAAGAAGCAGGCGTAAATCGCTTTAGCTTTGGTGTCACAAAGCTTGAAGACGACTGTG	2400
K E A G V N R L S F G V Q T F E D D L L	
TAGAAAAAATCGGCCAGTGACACAAGCAAAAAGACTATTACCCTCTTTTTGAAAGAGCCG	2460
E K I G R V H K K Q D V F T S F F E R A C R	
GCGAAATCGGTTTTAGATAATCATGCTCTGATCTCATGTTCCGGGCTCCGGGGCAAACCG	2520
E I G F E N I S L D L M F G L P G Q T L	
TCAAGCATCTGGAAACATCCAGTAACAACCTGCTTTATCATTTGAGCGCTGAGCAATTATCCG	2580
K H L E H S S N N T A L S L D A E H Y S V	
TTTACTCCCTTTATTGTAGAACCGAAACGGCTATTTTATAATTTGATGCAAAAAGGCGCGC	2640
Y S L I V E P K T V F Y N L M K Q K R L	
TTTCATCTCCGCCCGCAGGAGCAGGAAGCTGAAATGATGAAATAGTGATGAGCAAGATGG	2700
H L P F P Q E Q E A E M Y E I V M S K M E	
AAGCTCACGCCTTCATCAATATGAAATCAGCAACTTTGCTAAGCGACTGGAATCTTA	2760
A H G I H T T G Y E I S N F A K A G M E S K	
AGCAACAATTCACGATTTGGGAGCAAGCAATATTTTGGGTTTCGGAGCTGGCGCTCAG	2820
H N L T Y W S N E Q Y F G F G A G A H	
GATATATTGGCGGCACACGAAACAGTGAACGCTGGAGCTGTTAAGCATTACATTGACCTGA	2880
Y I G G G T R T V N V G P V K H Y I D L I	
TAGCTGAAAAGGGTTTTCCGTACAGADATACATCAAGTGACACAGAAAGACAGATTG	2940
A E K G F P Y R G T T H E V T T E E Q I E	
AGGAAGAAATGTTTTTAGGTTTGAGCAAAAACAGCCGGCTCAGCAAAAAACGATTTCGAC	3000
E E M F L G L R K T A G V S K K R F A E	
AAAAATACGGCCGGTTCCCTGATGACTATTTCCCAAGCGGTGTTAAAGACCTCGCTGAAA	3060
K Y G R L D L D G L F P S V L K D L A E K	
AAGGGCTGATTCACAATTCGGAGTCTGCCGTTTATTTGACTCATCAAGGAAATATTAGG	3120
G L I H N S E A V Y L T G G G N Y	
-35	17 bp
CAATGAAGTTTTTGGCGCTTTTTTTTTGGGTGAGTTATAATTGACATTTTCTTGTGTTGTG	3180
-10 S >>>>>>>> CIRCE <<<<<<<< *****	
ATACCTTTGTTTATAGAAATTAGCACTCGCTTATTAGAGAGTGCTACAGAGCTGATGATGAT	3240
hrcA	
GTTTAACAATCTGCTGCTGCTGATCCCTCAGGTTATAATCAAGGTAATTTTATAATCGG	3300
L T N R O L L L I L O V I N D F F I K S A	

detected upstream of *brcA*, we decided to sequence the unknown region of the 5.2 kb *Hind*III fragment to unravel its genomic organization. DNA sequencing revealed a complete ORF (exhibiting significant homology to *hemN* of several bacterial species; see below) immediately upstream of *brcA* and the 3' end of a second ORF whose deduced amino acid sequence (123 amino acids residues) exhibited significant similarity to the LepA protein of *E. coli*. This finding prompted us to clone and sequence the complete *lepA* homologue.

Southern blotting of chromosomal DNA of *B. subtilis* digested with different restriction enzymes was used to identify an appropriate fragment for cloning and sequencing (data not shown). A 2.8 kb *EagI* fragment was chosen and cloned as described in Methods. An *E. coli* *pcnB* strain was used as recipient to keep the copy-number of the vector plasmid low (Lopilato *et al.*, 1986). Screening of 100 individual transformants by colony hybridization using the DIG-labelled oligonucleotide ON1 comple-

Fig. 3. Northern blot analyses. Total RNA was isolated from *B. subtilis* 1012 at 37 °C (0 min) and at 5, 10, 15 and 30 min after a temperature shift to 52 °C. The filter was hybridized with DIG-labelled riboprobes complementary to *lepA* (a) or *hrcA* (b). Each lane contains 10 µg RNA.

mentary to a sequence near the 3' end of *lepA* led to the detection of one clone that gave a clear hybridization signal (pGH01). Restriction enzyme analysis of the insert revealed two *Cla*I sites which were found later to be situated within *lepA* (Fig. 1). To facilitate sequencing of the remaining region of *lepA*, the internal *Cla*I fragment was cloned in both orientations into pBluescript KS⁺ resulting in pGH04/05. The upstream region containing the 5' end of *lepA* was also recovered as a *Cla*I fragment (one site is within *lepA*, the other within the polylinker of the vector) and yielded pGH02/03. When sequencing of the 1256 bp inserts within pGH04/05 was finished, the DNA sequence of the 5' end of *lepA* was found in the EMBL database (Takemura *et al.*, 1993). The complete DNA sequence of *lepA* and *hemN* is presented in Fig. 2.

Features of the *lepA* operon

The *lepA* gene has a size of 1839 bp (Fig. 2, nucleotides 128–1966) and encodes a putative polypeptide of 612 amino acids with a predicted molecular mass of 68.6 kDa. The LepA protein shows strong homology to LepA of *E. coli* (57% identical and 21% similar amino acid residues) and contains four highly conserved sequence motifs (G1, G2, G3 and G4; see Fig. 2), which are characteristic for elongation-factor-like GTPases (March, 1992). The second gene of this locus, *hemN*, with a size of 1098 bp (Fig. 2, nucleotides 2019–3119), encodes a potential polypeptide of 366 amino acids with a predicted molecular mass of 40 kDa.

Features of the non-coding regions

Putative ribosome-binding sites were identified in front of both genes (Fig. 2, marked by asterisks), located five nucleotides upstream from the start codons of both *lepA* and *hemN*. These sites consist of nine (*lepA*) and ten (*hemN*) nucleotides complementary to the 3' end of *B. subtilis* 16S rRNA (Moran *et al.*, 1982). A promoter region was identified which resembles the consensus sequence of promoters recognized by the vegetative sigma factor of *B. subtilis* (Haldenwang, 1995) upstream of *lepA* (nucleotides 56–84) with a –35 sequence, 5'-TTGAAT-3', and a –10

sequence, 5'-TATAAT-3', separated by 17 bp (consensus sequence underlined). An inverted repeat is situated 5' to the promoter with the sequence GAAAGAGAAAAG-CAACCCA-N₇-TAGGGAACCTTTCTCTTTC and a free energy of –21.2 kJ mol⁻¹. Between *lepA* and *hemN*, a second inverted repeat has the sequence ATAGAAG-CCGCCGCA-N₆-TACTGCGGCTTCTT and a free energy of –19.1 kJ mol⁻¹. Both inverted repeats are probably rho-independent transcription terminators. While the first one separates the *lepA* operon from the upstream operon, the second could constitute an intergenic terminator (see below). No known promoter sequence was found downstream of the putative intergenic terminator.

Next, we analysed expression of the *lepA*–*hemN* genes at the level of transcription. Since the downstream *dnaK* operon is heat-inducible and *lepA*–*hemN* are not separated from the *dnaK* operon by an obvious transcription-terminator-like structure, we decided to test for heat-inducibility of the *lepA* and *hemN* genes.

Synthesis of mRNA

The results of the Northern blot analyses presented in Fig. 3(a) revealed a transcript of about 1.8 kb, the size of the *lepA* gene, the amount of which did not increase after temperature upshift. As a positive control, we included the heat-shock gene *hrcA*. Here, a transient increase in the amount of *hrcA*-specific transcript is clearly visible (Fig. 3b). From this result, we conclude that the 1.8 kb transcript corresponds to the *lepA* gene which is not subject to heat induction. Several attempts to demonstrate a larger transcript corresponding to both *lepA* and *hemN* failed. Even when using a *hemN* riboprobe we were unable to detect this hypothetical bicistronic transcript (data not shown). This failure was not due to technical difficulties, because in other experiments dealing with the expression of the *dnaK* operon we succeeded in demonstrating a transcript of about 8 kb (G. Homuth, unpublished).

Since we failed to detect a bicistronic transcript encompassing *lepA* and *hemN*, we decided to test for a *hemN*

Fig. 4. Relative amount of *hemN* and *grpE* mRNA in *B. subtilis* 1012 (□) and *B. subtilis lepA::cat* (▨) before (0 min) and after temperature upshift from 37 °C to 52 °C as determined by slot-blot analysis. Total RNA isolated at 37 °C and at different times after heat shock was hybridized either with a *hemN*-specific riboprobe (a) or with ON2 complementary to the *grpE* transcript (b). Luminographs were quantified with the WinCam-System (Cybertech). The amount of transcript present in *B. subtilis* 1012 at 37 °C was assigned an arbitrary value of 1 unit for both *hemN* (a) and *grpE* (b).

transcript by slot-blotting. Total RNA was isolated from *B. subtilis* 1012 and hybridized to a *hemN* riboprobe. In this case, *hemN*-specific signals were obtained which did not increase after temperature upshift (Fig. 4a). In contrast, the amount of *grpE* transcript included as control for a heat-shock gene exhibited enhanced synthesis after temperature upshift (Fig. 4b); *grpE* is the second gene of the *dnaK* operon located immediately downstream of *brcA*. We conclude from these results that *hemN* is expressed under our experimental conditions, but at a level too low to be detectable by Northern blots.

Do *lepA* and *hemN* form a bicistronic operon or is *hemN* expressed from its own promoter? If they form a bicistronic operon, a *lepA::cat* mutant should largely prevent expression of *hemN* due to a strong transcription terminator located immediately downstream of the *cat*

Fig. 5. Immunoblot analysis of HemN protein in extracts from different *B. subtilis* strains. Lanes: A, *B. subtilis* 1012; B, *lepA::cat*; C, *lepAΔ*; D, *hemN::cat*; E, *hemNΔ*. Each lane contains 10 µg protein.

gene (Horinouchi & Weisblum, 1982). Slot-blot experiments detected only small amounts of *hemN* transcript in the *lepA::cat* strain (Fig. 4a), thereby strongly suggesting that both genes form a single operon (see also below). Does the *lepA* insertion mutation influence expression of the downstream *dnaK* operon? To answer this question total RNA isolated from the *lepA::cat* mutant was hybridized to the *grpE*-specific oligonucleotide ON2. The results presented in Fig. 4b clearly demonstrate that expression of *grpE* before and after temperature upshift is not significantly influenced in the *lepA::cat* mutant. We conclude from these results that expression of the *lepA* operon does not interfere with the expression of the downstream *dnaK* operon under the experimental conditions described.

Immunoblot analysis of *lepA* mutants strongly suggest that *lepA* and *hemN* form a bicistronic operon

The transcriptional analysis of the *lepA* locus suggested that *lepA* and *hemN* form a bicistronic operon. To obtain further support for this idea, wild-type *B. subtilis* and several isogenic mutant strains were analysed for the presence or absence of the HemN protein (Fig. 5). Whereas HemN was synthesized in the wild-type strain (lane A) and not in the *hemN::cat* and *hemNΔ* strains (lanes D and E), it was present in the *lepAΔ* strain (lane C), but not in the *lepA::cat* strain (lane B). These data convincingly confirm our conclusion from the foregoing section that the two genes form a bicistronic operon. The failure of strain *lepA::cat* to produce HemN is probably due to the strong transcription terminator immediately downstream of the *cat* gene as already discussed (Horinouchi & Weisblum, 1982).

Mutations in *lepA* do not impair growth of the cells after heat shock

To further test the possibility that the *lepA* operon might interfere with the expression of the downstream *dnaK* operon, we analysed growth properties of the different *lepA* operon mutants at high temperature. We have already shown that insertion of the *cat* cassette within *dnaK* (Schulz *et al.*, 1995), *grpE* (L. Kim, unpublished results) or *dnaJ* (G. Homuth, unpublished results) prevents growth of the mutant strains at temperatures above 50 °C. If *lepA* is involved either directly or indirectly in the regulation of the heat-shock response, insertion of a *cat* cassette should impair growth of that

Fig. 6. Growth of different *B. subtilis* strains in LB medium (100 ml). Bacteria were first grown at 37 °C to mid-exponential phase and then shifted to 52 °C. The time of the temperature shift is indicated by the arrow. (●) *B. subtilis* 1012; (▲) *lepA::cat*; (■) *lepAΔ*.

strain after temperature upshock. *B. subtilis* strains *lepA::cat* and *lepAΔ*, and 1012 as a control, were grown in LB at 37 °C till mid-exponential phase and then shifted to 52 °C. Growth of the three strains was followed and, as shown in Fig. 6, neither the insertion nor the deletion mutant differed in its growth behaviour from that of the wild-type strain. Similar results were obtained with the *hemN* insertion and deletion strains (data not shown). These results demonstrate that the *lepA* operon is not involved in the expression of the downstream *dnaK* operon.

DISCUSSION

The present publication reports on the cloning, sequencing, and transcriptional and translational analysis of the bicistronic *lepA* operon of *B. subtilis*, which consists of the promoter-proximal *lepA* and the promoter-distal *hemN* genes. This is the first report that *lepA* and *hemN* form a single operon. Both genes have already been described four times. In three cases [in *E. coli*, *Salmonella typhimurium* and *Pseudomonas fluorescens* (March & Inouye, 1985; van Dijk *et al.*, 1990; Black *et al.*, 1992)], *lepA* forms a bicistronic operon with *lepB*. The *lepA* gene encodes a protein which belongs to the superfamily of GTPases (March, 1992), and cells carrying a *lepA* null mutation in *E. coli* do not give rise to any phenotype (Dibb & Wolfe, 1986). The *hemN* gene has been identified in *E. coli*, *S. typhimurium*, *Rhodobacter sphaeroides* and *Haemophilus influenzae* (Troup *et al.*, 1995; Xu & Elliott, 1994; Coomber *et al.*, 1992; Fleischman *et al.*, 1995). In all four cases, *hemN*, which encodes an oxygen-independent coproporphyrinogen III oxidase, forms a monocistronic operon.

The *hemN* gene of *B. subtilis* was inactivated with no change in phenotype under the conditions used. This could be due to a second oxygen-dependent copy of a *hemN*-like gene. Such a gene, named *hemF*, has already been described for several other bacterial species (Xu &

Elliott, 1993; Troup *et al.*, 1994). Experiments are in progress to test for increased expression of *hemN* under strictly anaerobic conditions. If *hemN* is turned on under these conditions, it will be interesting to analyse for enhanced expression of *lepA* also. Since no role has yet been found for the LepA protein, it is tempting to speculate that it might fulfil a function under anaerobic conditions.

Overexpression of *E. coli lepA* in *E. coli* resulted in filamentous growth followed by death of the cells. Interestingly, it was not possible to establish the *B. subtilis lepA*-carrying plasmid pGH01 in *E. coli* DH10B as a high-copy-number plasmid. To prevent a lethal gene dosage effect, the gene was originally cloned in the *E. coli* strain C600 *pcnB*. In this mutant, the copy number of pUC plasmids and their derivatives is strongly reduced (Lopilato *et al.*, 1986). After successful cloning, we tried to use pGH01 for transformation of *E. coli* DH10B. However, all transformants obtained showed deletions within the insert, mainly within the *lepA* promoter region. Consequently, we assumed a lethal gene dosage effect due to overexpression of *B. subtilis lepA* in *E. coli*. This is not surprising in view of the high degree of homology between the LepA proteins of *E. coli* and *B. subtilis*.

An alignment of the five known HemN proteins deduced from their ORFs shows a low overall homology (Fig. 7). On closer examination, two features can be seen. (1) The HemN proteins of *H. influenzae* and *B. subtilis* are considerably shorter than those of the other three species. In particular, they lack about 50 amino acid residues at their N-termini, and there are several gaps near their N-termini. (2) Besides several regions of increased homology, there is a GGGTP motif (around amino acid 120 of *E. coli*) which might be involved in the activity of these proteins. This alignment can be used to specifically mutate regions within the *hemN* genes and to test the mutants for activity under anaerobic conditions.

The genomic organization of the *lepA* operon revealed a strong potential intergenic transcription terminator between *lepA* and *hemN* (Fig. 2). The existence of this terminator was largely confirmed by Northern blot analysis since we failed to demonstrate a bicistronic transcript. This failure could be explained by assuming that the bicistronic transcript is produced, but is highly unstable and is either processed into the *lepA* transcript or degraded. Alternatively, most of the mRNA originating at the potential vegetative promoter could be terminated at the intergenic terminator. Expression of *hemN* was found both at the level of transcription by slot-blot experiments and at the level of translation by immunoblotting. Expression of *hemN* from an independent promoter could be excluded by the analysis of two different *lepA* mutants. Whereas expression of *hemN* was largely prevented in the *lepA::cat* mutant, expression occurred within the *lepA* deletion mutant.

Since the *lepA* operon is located immediately upstream of the *dnaK* heat-shock operon and no obvious transcription-terminator-like structure was found between these two

	10	20	30	40	50	60
Ec	MSVQIQDWDLALIQKYNISOPRITSYPTALEFSEDFGEQAFQAVARY--PERPLSLYVHI					
St	MSVQIQDWDLALIQKYNISOPRITSYPTALEFSEDFGEQAFQAVARY--PERPLSLYVHI					
Rs	MTNIALLSGLGDFAR---VPRITSYPAAPVFSAGVADPQAQIALDPAVVISVYVHV					
Hi	-----MFLP-----PLSLYIHI					
Bs	-----LKSAYIHI					
						*. *.
Ec	PFCHKLICYFCGCKNIVTRQGHKADQYLDALGEIVH--RAPLFAGRH---VSQHLWGGGTF					
St	PFCHKLICYFCGCKNIVTRQGHKADQYLDALGEIVH--RAPLFAGRH---VSQHLWGGGTF					
Rs	PFCHKLICYFCGCKNIVTRQGHKADQYLDALGEIVH--RAPLFAGRH---VSQHLWGGGTF					
Hi	PFCHKLICYFCGCKNIVTRQGHKADQYLDALGEIVH--RAPLFAGRH---VSQHLWGGGTF					
Bs	PFCHKLICYFCGCKNIVTRQGHKADQYLDALGEIVH--RAPLFAGRH---VSQHLWGGGTF					
						*. *.
Ec	TYLNKAQISRLMKLLRENFPQNDAA--EISIEVDPRETELDVLDHLRAEGFNRLSMGVQDF					
St	TYLNKAQISRLMKLLRENFPQNDAA--EISIEVDPRETELDVLDHLRAEGFNRLSMGVQDF					
Rs	TYLNKAQISRLMKLLRENFPQNDAA--EISIEVDPRETELDVLDHLRAEGFNRLSMGVQDF					
Hi	TYLNKAQISRLMKLLRENFPQNDAA--EISIEVDPRETELDVLDHLRAEGFNRLSMGVQDF					
Bs	TYLNKAQISRLMKLLRENFPQNDAA--EISIEVDPRETELDVLDHLRAEGFNRLSMGVQDF					
						*. *.
Ec	NKEVQRLVNRQDEEPIFALLNHAREIGPTSTNIDLIVGLPKQTPESFAFTLKRVAEVPN					
St	NKEVQRLVNRQDEEPIFALLNHAREIGPTSTNIDLIVGLPKQTPESFAFTLKRVAEVPN					
Rs	NKEVQRLVNRQDEEPIFALLNHAREIGPTSTNIDLIVGLPKQTPESFAFTLKRVAEVPN					
Hi	NKEVQRLVNRQDEEPIFALLNHAREIGPTSTNIDLIVGLPKQTPESFAFTLKRVAEVPN					
Bs	NKEVQRLVNRQDEEPIFALLNHAREIGPTSTNIDLIVGLPKQTPESFAFTLKRVAEVPN					
						*. *.
Ec	DRLSVNYAHLPTIFAAQRKIK--DADIPSPQKLDILQETIAFTQSGYQFIMGDHFARP					
St	DRLSVNYAHLPTIFAAQRKIK--DADIPSPQKLDILQETIAFTQSGYQFIMGDHFARP					
Rs	DRLSVNYAHLPTIFAAQRKIK--DADIPSPQKLDILQETIAFTQSGYQFIMGDHFARP					
Hi	DRLSVNYAHLPTIFAAQRKIK--DADIPSPQKLDILQETIAFTQSGYQFIMGDHFARP					
Bs	DRLSVNYAHLPTIFAAQRKIK--DADIPSPQKLDILQETIAFTQSGYQFIMGDHFARP					
						*. *.
Ec	DDELAVAQREGVLHRRNFQGYTTQGDITLLGMGVSAISMGIDCYAQKQELKQYQVDEQ					
St	DDELAVAQREGVLHRRNFQGYTTQGDITLLGMGVSAISMGIDCYAQKQELKQYQVDEQ					
Rs	DDELAVAQREGVLHRRNFQGYTTQGDITLLGMGVSAISMGIDCYAQKQELKQYQVDEQ					
Hi	DDELAVAQREGVLHRRNFQGYTTQGDITLLGMGVSAISMGIDCYAQKQELKQYQVDEQ					
Bs	DDELAVAQREGVLHRRNFQGYTTQGDITLLGMGVSAISMGIDCYAQKQELKQYQVDEQ					
						*. *.
Ec	GNALWRGIALTRDDCIRRDVIKSLICNFRDLDPYIEKQWDLHFAEDYFAEDLKLLAPLAKD					
St	GNALWRGIALTRDDCIRRDVIKSLICNFRDLDPYIEKQWDLHFAEDYFAEDLKLLAPLAKD					
Rs	GNALWRGIALTRDDCIRRDVIKSLICNFRDLDPYIEKQWDLHFAEDYFAEDLKLLAPLAKD					
Hi	GNALWRGIALTRDDCIRRDVIKSLICNFRDLDPYIEKQWDLHFAEDYFAEDLKLLAPLAKD					
Bs	GNALWRGIALTRDDCIRRDVIKSLICNFRDLDPYIEKQWDLHFAEDYFAEDLKLLAPLAKD					
						*. *.
Ec	GLVDVDEKGIQVAKGRLLIRNICMCFDYLQKARMQOFSRVI					
St	GLVDVDEKGIQVAKGRLLIRNICMCFDYLQKARMQOFSRVI					
Rs	GLVDVDEKGIQVAKGRLLIRNICMCFDYLQKARMQOFSRVI					
Hi	GLVDVDEKGIQVAKGRLLIRNICMCFDYLQKARMQOFSRVI					
Bs	GLVDVDEKGIQVAKGRLLIRNICMCFDYLQKARMQOFSRVI					
						*. *.

Fig. 7. Alignment of *B. subtilis* HemN with HemN from other bacterial species. Ec, *E. coli* (Troup *et al.*, 1995); St, *S. typhimurium* (Xu & Elliott, 1994); Rs, *R. sphaeroides* (Coomber *et al.*, 1992); Hi, *H. influenzae* (Fleischmann *et al.*, 1995); Bs, *B. subtilis*. Amino acids are given in the standard one-letter code. Identical amino acids are indicated by an asterisk and conservative replacements (Howe & Margolin, 1986) by a dot below the sequence. Gaps, indicated by dashes, were introduced to obtain maximum alignment.

operons, we envisaged the possibility that the *lepA* operon might be part of the *dnaK* operon (both are transcribed in the same direction). Our data clearly show that neither *lepA* nor *hemN* is transcriptionally activated after temperature upshock. In addition, transcription of the *lepA* operon does not interfere with transcription of the downstream *dnaK* operon at ambient temperatures. A question remains regarding the site at which the *lepA-hemN* transcript is terminated. There are at least three possibilities. (1) The termination of *lepA-hemN* transcription occurs in a rho-dependent manner between *hemN* and the promoter of the *dnaK* operon. (2) There is no termination site downstream of *hemN*, and the *lepA-hemN* transcript is extended into the *dnaK* operon. (3) The CIRCE operator between the *dnaK* promoter and the transcriptional start site of the *dnaK* operon acts as a transcription terminator for the weak *lepA-hemN* transcript. The CIRCE element is a highly conserved inverted repeat structure with the consensus sequence

TTAGCACTC-N₉-GAGTGCTAA, localized between promoters and transcriptional start sites of *dnaK* and *groESL* operons within a broad range of Gram-positive and, particularly, Gram-negative bacteria (Hecker *et al.*, 1996). It represents a repressor-protein-binding site involved in regulation of the heat-shock response (Yuan & Wong, 1995; Schulz & Schumann, 1996). In the case of *B. subtilis* *dnaK*, CIRCE could form a stem-loop structure at the level of RNA with a free energy of 25 kJ mol⁻¹. We cannot exclude the possibility that this hairpin structure is sufficient to terminate the transcription of a weak transcript such as the *lepA-hemN* mRNA.

ACKNOWLEDGEMENTS

We thank S. Engelmann for providing plasmid pSED157. This work was supported by the Deutsche Forschungsgemeinschaft, the CEC Biotech Grant Bio2 CT920254 and the Fonds der Chemischen Industrie.

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Received 8 December 1995; revised 13 February 1996; accepted 19 February 1996.