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Nucleotide Sequence of the *outB* Locus of *Bacillus subtilis* and Regulation of Its Expression

ALESSANDRA M. ALBERTINI,[1] TIZIANA CARAMORI,[1] DENNIS HENNER,[2] EUGENIO FERRARI,[2]† AND ALESSANDRO GALIZZI[1]*

Dipartimento di Genetica e Microbiologia, Università di Pavia, Pavia 27100, Italy,[1] and Department of Cell Genetics, Genentech Inc., South San Francisco, California 94080[2]

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The *outB* gene is one of the genes involved in the process of spore outgrowth in *Bacillus subtilis*. The gene has been cloned in bacteriophage lambda and subcloned in plasmids. We have determined the sequence of 2,553 base pairs around the *outB* locus. The locus was found to code for a protein of about 30,000 daltons. Analysis of the in vivo transcripts from this region by RNase protection experiments revealed the presence of two start sites for transcription. Two potential promoters for these transcripts can be tentatively assigned from the sequence data. The amount of one transcript is highest during outgrowth and vegetative growth and absent during the stationary phase. The second transcript is present at a low level throughout the cell cycle.

Temporal control of gene expression in *Bacillus subtilis* is well documented for a number of genes involved in sporulation or sporulation controlled. In most instances control is exerted at the transcriptional level, and temporally regulated promoters often utilize alternative forms of RNA polymerase, characterized by different sigma subunits (8, 14, 15, 21, 23, 26). Presently five different factors have been described (σ^{43}, σ^{37}, σ^{32}, σ^{28}, σ^{29}), each endowing the core enzyme with the ability to recognize different promoters (9–11, 13, 28). Additional regulatory factors have been proposed to explain the pleiotropic effect of mutations in several sporulation genes, in particular *spo0A*, *B*, *E*, *F*, *H*, and *K* (7, 22, 30).

While considerable data have been collected toward understanding the regulatory mechanisms acting during sporulation, very little is known about the devices which regulate gene expression during spore germination and outgrowth. The isolation and molecular cloning of genes whose products appear to be specific to the outgrowth phase of spore germination should provide a tool to study the timing of expression of genes during this phase of the bacterial cell cycle.

The *outB* gene has been identified through a mutation (*outB81*) which confers the phenotype of producing spores which are unable to complete outgrowth at 47°C (1). From a *B. subtilis* DNA library in Charon 4A, we have isolated the wild-type allele of *outB* (4). From the results of dot blot hybridization experiments of the cloned DNA to RNA extracted from *B. subtilis* cells at different developmental stages, the gene appears to be transcribed only during spore outgrowth (6).

We have determined the nucleotide sequence of the gene *outB*, and by RNase protection experiments we show two transcripts which extend into the *outB* gene. One transcriptional initiation site is preceded by a potential promoter sequence which is homologous to sequences recognized by *B. subtilis* σ^{43}-type RNA polymerase. Transcription from this site is turned on during outgrowth. The second transcript is 53 nucleotides shorter than the first and could originate from a downstream promoter. This transcript is present at low levels throughout the cell cycle. The data suggest that gene *outB* is under the control of two promoters.

MATERIALS AND METHODS

Bacterial strains and growth. The *B. subtilis* strains used were PB1424 (*hisB2 trpC2 metD4*) and PB2427 (*hisB2 trpC2 outB81*). Transformation of competent cells was performed by the method of Hoch et al. (12).

Escherichia coli HB101 (F⁻ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20* [Sm^r] *xyl-5 mtl-1 supE44*), JM83 [*ara*Δ(*lac-proAB*) *rpsL20 thi* φ*80* d*lacZ*Δ*M15*], and JM101 [supE Δ(*lac-proAB*) *thi* (F′ *traD36 proAB lacI*^{q} *Z*Δ*M15*)] were used for transformation and plasmid propagation, and the medium used was LB medium (tryptone [Difco], 10 g; yeast extract, 5 g; NaCl, 10 g; water to 1 liter; pH 7.1). To detect expression of *lacZ* in colonies, strains were plated on LB agar containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) per ml.

Plasmid constructions. The isolation of the *outB* locus from a *B. subtilis* DNA library in lambda phage Charon 4A and the subcloning of a 3.6-kilobase (kb) *Pst*I fragment in pBR322 to give rise to pO.P have been previously described (4). Plasmids were prepared by the procedure of Birnboim and Doly (3). They were constructed by ligation of approximately equimolar amounts of gel-purified fragments. The extent of the *B. subtilis* DNA insert in each plasmid is shown in Fig. 1.

Plasmid pJH101-1.1 contains a 1.7-kb fragment of *B. subtilis* DNA inserted into pJH101 (5), digested with *Hin*dIII and treated with alkaline phosphatase from calf intestine (Boehringer GmbH, Mannheim, Federal Republic of Germany). The *B. subtilis* fragment was obtained by digestion of pO.P with *Hin*dIII, and the 1.7-kb fragment was purified by polyacrylamide gel electrophoresis before ligation. Transformants of HB101 resistant to ampicillin (100 μg/ml) were selected and screened for sensitivity to tetracycline (12.5 μg/ml).

Plasmids pUC13-i, pUC13-g, and pUC13-h are derivatives of pUC13 (19). Their *B. subtilis* DNA inserts are 0.77, 0.51, and 1.14 kb, respectively, and were obtained from pO.P by digestion with *Pvu*II. The fragments were ligated to the pUC13 vector digested with *Sma*I. Transformants of JM83 resistant to ampicillin were selected, and plasmid DNA from white (on X-gal plates) colonies was analyzed by acrylamide

* Corresponding author.
† Present address: Genencor, Inc., South San Francisco, CA 94080.

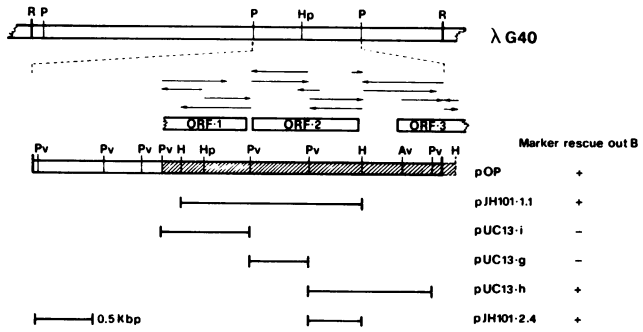

FIG. 1. Restriction map of the region surrounding the *outB* locus. The upper part of the figure shows a simplified restriction map of C4BsG40, from which fragments were subcloned for marker rescue experiments and sequencing. The dashed portion of the insert of plasmid pO.P represents the 2,533 bp whose sequence was determined. Arrows show the sequencing strategy. The three possible ORFs are indicated, reading left to right. The results of marker rescue experiments involving the *outB81* mutation are summarized to the right of each clone. The plasmid DNA was used to transform competent cells of the mutant strain PB2427, which is temperature sensitive during spore outgrowth. After 30 min of exposure to DNA at 35°C the cells were centrifuged, suspended in an equal volume of nutrient broth containing 10^{-5} M $MnCl_2$, and incubated at 35°C until sporulation. The cells were then collected by centrifugation, washed with distilled water, suspended in an equal volume of water, and heated at 80°C for 15 min to inactivate the vegetative cells. The spores were checked for transformation, selecting for the ability to yield colonies at 47°C (Ts^+). Restriction site abbreviations: R, *Eco*RI; P, *Pst*I; Hp, *Hpa*I; Pv, *Pvu*II; H, *Hin*dIII; Av, *Ava*I.

gel electrophoresis after digestion with suitable restriction enzymes.

Plasmid pJH101-2.4 was constructed by subcloning the *Pvu*II-*Hin*dIII fragment of 488 base pairs (bp) from pO.P into plasmid pJH101, digestion with *Eco*RV and *Hin*dIII, and purification by agarose gel electrophoresis. Transformants of HB101 resistant to ampicillin were screened for the presence of the appropriate size plasmid.

Plasmids pSP64.2 and pSP65.1 are derivatives of pSP64 and pSP65 (18), respectively. The insert of 240 bp, containing the transcription start region of *outB*, was derived by digestion of pUC13-g with *Eco*RI and *Dra*I. The gel-purified fragment was ligated to pSP64 and pSP65 digested with *Eco*RI and *Sma*I. The ligation mixture was used to transform competent *E. coli* cells, selecting for ampicillin resistance. The structure of each plasmid was confirmed by restriction analysis.

Isolation of *B. subtilis* RNA. *B. subtilis* spores were prepared on nutrient agar supplemented with $MnCl_2$ (10^{-5} M) and chloramphenicol (5 μg/ml). The spores were collected, treated with lysozyme, washed with distilled water, and purified by centrifugation in 70% Urografin (Schering, SpA; Milan) (25). Following heat activation at 70°C for 15 min, the spores were inoculated in 200 ml of nutrient broth (Difco) supplemented with 0.5% glucose, and growth was monitored by measuring the optical density at 560 nm. After 12 and 24 min of incubation, 20-ml samples were withdrawn, and at 30, 60, 90, and 120 min of incubation the sample volume was 10 ml. Samples (10 ml) at log phase (1.0 optical density unit) and transition phase (T_0) and at 2 and 4 h after exponential growth were obtained from cultures in nutrient broth supplemented with $MnCl_2$ (10^{-5} M), inoculated with a 1:100 dilution of an overnight culture in nutrient broth. Further growth was stopped by adding sodium azide (50 μg/ml) and rapid chilling. The cells were collected by centrifugation. The cell pellets were frozen at −20°C and then thawed in 2 ml of lysis buffer (20 mM Tris hydrochloride, pH 7.6, 50 mM NaCl, 10 mM $MgCl_2$) containing lysozyme (200 μg/ml). To the suspension 0.4 ml of macaloid suspension (National Lead Co., Houston, Tex.) prepared as described previously (17), and 50 U of RNase inhibitor from human placenta (Boehringer) were added. The cells were ruptured by sonication in the presence of glass beads (0.1 mm diameter). The samples were kept in ice during treatment, and sonication was done in six pulses of 1 min each, with a 2-min rest between pulses. After sonication the samples were incubated with 80 μg of DNase (Worthington, Freehold, N.J.) per ml for 30 min at room temperature and then extracted with water-saturated phenol at 65°C for 5 min, and the aqueous phase was extracted with phenol-chloroform. After ethanol precipitation the RNA was dissolved in diethylpyrocarbonate-treated water (17) containing the RNase inhibitor (100 U/ml).

RNase mapping. In vitro synthesis of ^{32}P-labeled RNA was performed following the Promega Biotec Riboprobe system recommendations, with SP6 grade [α-^{32}P]UTP (800 Ci/mmol) from Amersham International. Plasmid pSP64.2 linearized with *Eco*RI was the template for the synthesis of the antisense probe, and pSP65.1 linearized with *Pst*I was the template for the synthesis of the sense probe. The labeled probes were precipitated with ethanol, and the pellet was dissolved in 50 μl of hybridization buffer (40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], pH 6.8, 400 mM NaCl, 1 mM EDTA, 80% [vol/vol] formamide). Hybridization mixtures were prepared in 30 μl of hybridization buffer containing 1 μl of labeled probe, 2 μg of in vivo RNA, and 8 μg of yeast tRNA. The samples were incubated at 90°C for 5 min and then at 36°C for 18 h. Hybridization was terminated by addition of 300 μl of RNase digestion buffer (10 mM Tris hydrochloride, pH 7.5, 5 mM EDTA, 200 mM NaCl, 100 mM LiCl) containing 15 μg of RNase A (Boehringer). After incubation at 25°C for 1 h digestion was terminated by addition of 20 μl of 10% sodium dodecyl sulfate and 50 μg of proteinase K (Boehringer) and incubation at 37°C for 20 min. The samples were extracted twice with water-saturated phenol–chloroform (1:1), and the RNA-RNA duplexes were recovered by ethanol precipitation in the presence of 15 μg of carrier yeast tRNA, rinsed, and redissolved in loading buffer (80% formamide, 2 M urea, and dyes). Samples were denatured for 5 min at 90°C and analyzed on 5% acrylamide gels containing 8 M urea. The gels were exposed to X-ray films for 12 to 24 h. For each time point, three independent RNA preparations were tested.

DNA sequence analysis. Fragments of DNA, originally from plasmid pO.P, were subcloned in M13mp18 and M13mp19 for sequencing by the chain termination method of Sanger et al. (24). For certain regions dITP was substituted for dGTP to reduce "compression" artifacts (20). The sequence was determined by sequencing both strands.

RESULTS

Sequence of the *outB* gene. The source of DNA for sequencing was a 3.5-kbp *Eco*RI fragment contained in plasmid pO.P, capable of transforming to Ts^+ strains with the *outB81* mutation, whose presence makes the spores temperature sensitive during outgrowth (4). The 3.5-kbp fragment was further subcloned into M13 phage derivatives mp18 and mp19, and the sequence of 2,533 contiguous nucleotides was determined by the Sanger technique (24). The analysis of the nucleotide sequence revealed three possible large open

reading frames (ORFs), all three on the same DNA strand (Fig. 1). Only one of the ORFs (ORF2) was entirely within the determined sequence. This ORF was bracketed by a strong ribosomal binding site (AGGAGG) and a potential terminator sequence with a ΔG of -29.9 kcal/mol (Fig. 2). The coding capacity of ORF2 was for a polypeptide of 29,514 to 30,662 daltons, depending on which methionine codon is used as the start for initiation of translation. The deduced polypeptides were rich in charged residues (30%), with an excess of acid residues (46, versus 36 basic ones). Computer comparison has shown no significant homology to any known sequence at either the DNA or the protein level. Two potential promoter sequences lay upstream of this ORF (Fig. 3). One potential promoter (P1) was highly homologous to the consensus sequence recognized by the major form of RNA polymerase (E σ^{43}), with two base variations, a C in the -35 region replacing the more common G and a T instead of an A in the -10 region. The spacing between the -35 and -10 region was 18 bp, a length commonly found in *B. subtilis* promoters. The second potential promoter (P2) resembled the *ctc* gene promoter recognized by E σ^{37} (27). The homology was higher for the -35 region (identity to the consensus sequence in five of six bases) and less prominent for the -10 region (identity of seven bases out of nine). The two potential promoters were 51 bp apart. A last feature of the sequence deserves mention, i.e., a region of dyad symmetry overlapping the hypothetical σ^{37} promoter (Fig. 3). A dyad symmetry around a σ^{37} promoter has also been noted in the subtilisin gene (29).

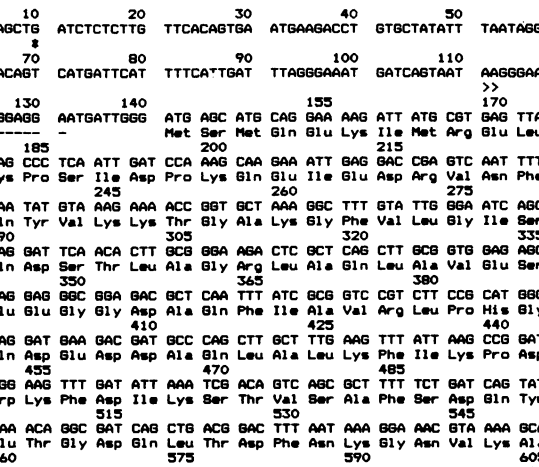

FIG. 2. Sequence of the *outB* gene. The sequence is shown in the 5′-to-3′ direction. The putative Shine-Dalgarno sequence is underlined; an inverted repeat sequence following the peptide-coding region is doubly underlined. The two starts of transcription are indicated (>>) below the sequence. Asterisks indicate stop codons.

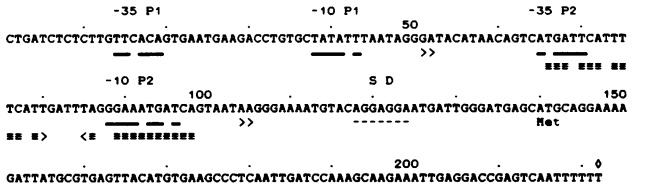

FIG. 3. Promoter region of the *outB* gene. The two putative promoter sequences are indicated. Residues identical to the consensus sequences are underlined. The approximate start sites of transcription are indicated (>>), and the region of dyad symmetry is shown underlined three times. The ribosome-binding site (SD) is underlined. The diamond at position 222 indicates the limits of the region of homology to *outB* (relevant for the RNase protection experiments).

Location of the *outB* gene. That the above mentioned ORF (ORF2) corresponds to the *outB* locus was demonstrated by marker rescue in transformation experiments with plasmid subclones. Plasmid pJH101-1.1, carrying a 1,672-bp *Hin*dIII fragment, transformed to Ts^+ strains with the *outB81* mutation. The fragment embraces all of ORF2 and a portion of ORF1 (Fig. 1). On the other hand plasmid pUC13-i, carrying a slightly larger portion of ORF1, failed to transform *outB*. Plasmid pJH101-2.4, with only the right half of ORF2, did transform to Ts^+ strains carrying *outB*. In addition to marker rescue experiments, plasmid pJH101-2.4 can be used as an integrative plasmid, selecting for chloramphenicol-resistant (Cam^r) transformants. Among 44 such transformants of a Ts^- strain, 20 were Ts^+, whereas 24 were still Ts^-. The presence of Ts^+ among the transformants with the plasmid inserted into the chromosome further demonstrates the position of the *outB81* mutation in the fragment homologous to that cloned into the plasmid. Therefore *outB81* must lie in the portion of the sequence corresponding to ORF2.

Transcriptional start sites of *outB*. The transcriptional start sites of the *outB* gene were mapped by RNase protection experiments. RNA extracted from wild-type cells of *B. subtilis* was hybridized to RNA probes prepared in vitro with the SP6 system (18). For this purpose a DNA fragment of 222 bp, from the *Pvu*II site at position 7 (Fig. 2) to the *Dra*I site at position 229, was recloned in plasmids pSP64 and pSP65. From the sequence data, the fragment should comprise a portion of the coding sequence of ORF2 and approximately 130 bp upstream of it. The pSP64 and pSP65 derivatives carrying the 222-bp insert in both orientations were cut at the *Eco*RI (pSP64) or *Pst*I (pSP65) site downstream of the cloned fragment and used in vitro as templates for the SP6 RNA polymerase. Both sense and antisense ^{32}P-labeled RNAs were used to probe for the presence of complementary transcripts in vivo. Following hybridization the duplex mixtures were treated with pancreatic RNase, and the protected fragments were analyzed by urea-acrylamide gel electrophoresis. The RNA samples to be probed were obtained from *B. subtilis* cells at various times of growth to also estimate the time of expression of the *outB* gene. Only probes obtained from pSP64-2 gave hybrids, whereas the ones derived from pSP65-1 did not. From the position of the restriction sites used for cloning and the nucleotide sequence of *outB*, it can be deduced that transcripts from pSP64-2 should be antisense and thus complementary to the mRNA from ORF2, whereas the transcripts obtained with pSP65-1 should be the sense strand. Figure 4 shows the results of the mapping experiment with the pSP64-2 probe: two transcripts

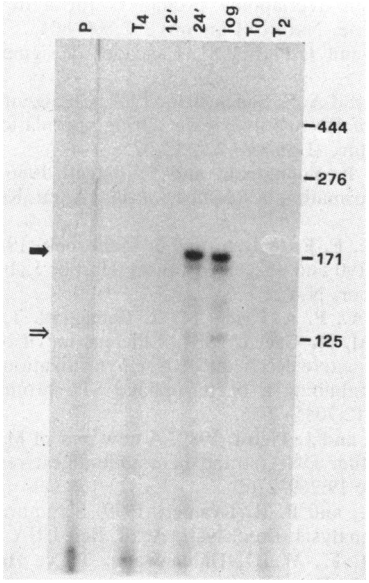

FIG. 4. mRNA mapping of the *outB* gene. These RNase protection experiments used as a probe RNA synthesized in vitro with plasmid pSP64-2. This RNA is complementary to the sequence reported in Fig. 3. RNA was derived from outgrowing spores (12 and 24 min) and from cells in log phase and at T_0, T_2, and T_4 of a wild-type strain. Lane P, Control experiment performed with the probe without added RNA. The numbers refer to length (in bases) of RNA standards obtained from the pSP64 template cut at various positions before in vitro transcription. The closed and open arrows point to the longer and shorter transcripts, respectively.

of different length were present. The length of the transcripts was estimated by comparison with single-stranded RNA molecules synthesized from the pSP64-2 template, cut at specific sites prior to the in vitro transcription. The longer transcript was 170 to 175 bases long; this could represent a start site at adenine 52 (or guanine 51) (Fig. 3). The amount of this transcript was highest during outgrowth and vegetative growth and absent during the stationary phase (T_0, T_2, and T_4; i.e., at the end of exponential growth and 2 and 4 h, respectively, from the end of exponential growth). In some experiments the transcript was more abundant during outgrowth than vegetative growth. At different time points during outgrowth (30, 60, 90, and 120 min after inoculation) the transcripts were present at the same level found at 24 min (data not shown).

The shorter transcript was present during all stages examined, albeit at a low level, and its length (115 to 118 bases) corresponds to a start site at adenine 104 or 105 (Fig. 3). The transcriptional initiation site at adenine 52 (or guanine 51) was efficiently utilized by *E. coli* cells harboring a plasmid with the promoter region of the *outB* gene. The shorter transcript was absent in *E. coli* even though the steady-state level of transcripts in this host was higher than in *B. subtilis* (data not shown).

DISCUSSION

The *outB* gene of *B. subtilis* was originally identified by the isolation of a mutation (*outB81*) whose effect was to render the spores temperature sensitive during outgrowth. The earliest observed defect at the nonpermissive temperature was a block in RNA synthesis (1). The unambiguous assignment of ORF2 as the coding sequence of the *outB* gene has allowed more detailed studies of the regulation of its expression. The mRNA mapping experiments show that this gene is expressed from two different transcripts which differ in their temporal regulation. Analysis of the sequence upstream of each transcript allowed assignment of the promoters responsible for these transcripts, although it must be emphasized that these assignments are tentative without further in vivo evidence for which sigma factors are responsible for these transcripts. The major transcript for this gene appears during outgrowth, is present during vegetative growth, and is undetectable during sporulation. The apparent promoter for this transcript (P1) has a sequence typical of σ^{43} promoters. This assignment is partially supported by the observation that transcripts with the same start point are produced in *E. coli*. At present we have no indication of how transcription is regulated. The abrupt appearance of transcripts during outgrowth at 24 min may indicate that transcription at earlier times is impeded by the absence of a positive effector or the presence of a repressor. The repressor hypothesis may be supported by the observation that in stationary phase the transcripts are absent. The putative repressor could very well be accumulated during late log- and stationary-phase growth and be trapped in the spores. During germination and outgrowth the repressor molecules could be inactivated by the proteases that are present at these stages. The region of dyad symmetry in the promoter region of *outB* could represent a site of recognition and binding of a regulatory protein. More complex regulatory mechanisms cannot be excluded.

The promoter (P2) for the second transcript could be recognized by σ^{37}. The transcript is present at a lower level but in constant amount at every time during the cell cycle. The very low amount of this transcript makes its identification difficult in analyses such as those shown in Fig. 4. However, this transcript was consistently present in every such analysis, and similar experiments with strains in which this region of the chromosome is amplified show an identical transcript that is very prominent (A. Galizzi, unpublished data).

If in fact it is transcribed by σ^{37}, gene *outB* offers a unique regulation, since the shorter transcripts are present throughout the cell cycle and not only during the stationary phase, as is the case for other genes apparently transcribed by E σ^{37}, i.e., *spoVG*, *ctc*, and the subtilisin gene (13, 27, 29). More recent data indicate that in vivo *spoVG* transcription is not dependent on the presence of σ^{37}, while transcription of gene *ctc* is completely blocked in a *B. subtilis* strain with an insertion mutation in the structural gene of σ^{37} (2, 16). The same type of analysis should clarify whether the subtilisin gene and the *outB* gene are indeed transcribed in vivo by E σ^{37}. Any generalization about the temporal expression of σ^{37} promoters appears premature.

The transcription pattern of gene *outB* during outgrowth could reflect the relative abundance of different sigma factors in the bacterial spore. Unfortunately our knowledge about the organization of the RNA polymerase present in the spores is very fragmentary and does not allow any firm conclusion.

The fine timing of expression of *outB* by means of two promoters whose utilization follows diverse regulatory mechanisms may indicate an important role of the *outB* gene product in cell metabolism. Transcription from one promoter is regulated, whereas the expression of the gene is constitutively secured at a basal level by the presence of the second promoter. The dual control may have the function of providing a failsafe mechanism, so that gene *outB* is always expressed at a basal level. This should mean that the product

of gene *outB* is not required only during spore outgrowth but is essential for the life of the cell. This is partially supported by the fact that we have been unable to inactivate the gene by insertion into the chromosome of a nonreplicative plasmid carrying a fragment of the coding sequence of the *outB* gene (A. Galizzi, unpublished data).

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