Functional analysis of the *outB* gene of *Bacillus subtilis*

TIZIANA CARAMORI, SABINA CALOGERO, ALESSANDRA M. ALBERTINI and ALESSANDRO GALIZZI*

Dipartimento di Genetica e Microbiologia 'A. Buzzati-Traverso', Università di Pavia, Via Abbiategrasso 207, 27100 Pavia, Italy

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Three additional alleles of the *outB* gene of *Bacillus subtilis*, whose activity is required for spore outgrowth, were identified. The nucleotide sequence of three mutant genes was determined. Analyses of dominance–recessivity showed that the wild-type allele is dominant over the mutant ones. When the *outB* gene was placed under the control of the inducible *spac-I* promoter, the presence of IPTG was necessary to obtain normal growth. The results suggested that the *outB* gene is required for growth of *B. subtilis*. Expression of *outB* from the sporulation promoter *spoIID* negatively affected subsequent spore outgrowth, without altering vegetative growth and sporulation.

Introduction

Genetic techniques have been used extensively to analyse the regulation of gene expression during the process of sporulation of the Gram-positive bacterium *Bacillus subtilis*. The genetic dissection of the process, coupled to molecular investigations, has led to the elucidation of at least some of the elements involved in the temporal control of gene expression; e.g. the presence of a phosphorelay responsive to environmental stimuli and controlling the initiation of sporulation (Burbulys *et al.*, 1991) and the sequential activation of specific RNA polymerase sigma factors (Stragier & Losick, 1990).

Less is known about the process of spore germination and outgrowth (Setlow, 1981; Moir *et al.*, 1991). We have described one gene, *outB*, whose activity is necessary for spore outgrowth; the gene was identified through a mutation (*outB81*) conferring a temperature-sensitive phenotype during spore outgrowth (Albertini & Galizzi, 1975). At the non-permissive temperature (47 °C) outgrowth is blocked and the most apparent defect is an early block in RNA synthesis, with only a small amount of protein synthesis taking place. No DNA synthesis could be detected. The product of the *outB* gene resembles an *Escherichia coli* protein apparently involved in the utilization of nitrogen sources, and *outB81* mutants are affected in the utilization of a number of amino acids as sole sources of nitrogen (Albertini & Galizzi, 1990). The possible connection between spore outgrowth and assimilation of nitrogen sources has made it desirable to

obtain a better understanding of the genetics and physiology of *outB*. In this work we investigated the dominance–recessivity relationships between the wild-type and four mutant alleles of *outB*, determined the nucleotide sequence of three of the mutant alleles and studied the effect of the unscheduled expression of *outB* upon spore outgrowth.

Methods

Strains and plasmids. The *B. subtilis* strains and plasmids used are listed in Tables 1 and 2. The *E. coli* strain used for plasmid transformation and amplification was DH5 α [F^- (ϕ 80*lacZ* Δ M15)]

Table 1. *B. subtilis* strains

Strain	Genotype	Reference or derivation*
PB1424	<i>hisB2 trpC2 metD4</i>	Laboratory stock
PB2427	<i>hisB2 trpC2 outB81</i>	Albertini & Galizzi (1975)
PB2353	<i>thyA thyB arg outB47 (tscG47)</i>	Galizzi <i>et al.</i> (1976)
PB2354	<i>thyA thyB arg outB48 (tscH48)</i>	Galizzi <i>et al.</i> (1976)
PB2355	<i>thyA thyB arg outB49 (tscB49)</i>	Galizzi <i>et al.</i> (1976)
PB5051	<i>hisB1 trpC2 outB47</i>	PB2353 $\rightarrow$ PB1424
PB5052	<i>hisB2 trpC2 outB48</i>	PB2354 $\rightarrow$ PB1424
PB5053	<i>hisB2 trpC2 outB49</i>	PB2355 $\rightarrow$ PB1424
PB2474	<i>hisB2 trpC2 metD4 outB⁺::pJH1.1 (outB)</i>	This work
PB2475	<i>hisB2 trpC2 metD4 nrdA-polC::pJH81 (outB81)</i>	This work
PB2473	<i>hisB2 trpC2 metD4 P_{out}⁻outB⁺::cat::P_{spac}-outB</i>	This work
PB2476	<i>hisB2 trpC2 metD4 outB⁺::pJIID (P_{spoIID}-outB)</i>	This work
PB2477	<i>hisB2 trpC2 outB81::pJH1.1 (outB)</i>	This work

* Arrows indicate construction by transformation.

* Author for correspondence. Tel. (39) 382 391548; fax (39) 382 528496.

Table 2. *Plasmids*

Plasmid	Description	Source
pJH101	Integrative	Ferrari <i>et al.</i> (1983)
pBS19	Replicative	G. Gray, Genentech, USA
pDH88	Integrative	Henner (1990)
pSR5	<i>spoIID</i> promoter fused to <i>lacZ</i>	Rong <i>et al.</i> (1986)
p999	pBS19 with 999 bp fragment carrying <i>outB</i> sequence	Albertini <i>et al.</i> (1988)
pO.P	pBR322 carrying the <i>outB</i> region	Ferrari <i>et al.</i> (1985)
pJH1.1	pJH101 with 1.7 kb fragment carrying <i>outB</i> sequence	Albertini <i>et al.</i> (1987)
pC10	pBS19 with an 859 bp <i>SphI</i> – <i>HindIII</i> fragment carrying the coding sequence of <i>outB</i> , without promoter	This work
pGEM81	pGEM-7Z (Promega) with an 859 bp <i>SphI</i> – <i>HindIII</i> fragment, carrying the <i>outB81</i> mutation	This work
p981	859 bp <i>SphI</i> – <i>HindIII</i> fragment from pGEM81 cloned into p999 in place of the wild-type allele	This work
pJH81	pJH101 with 1.0 kb <i>EcoRI</i> – <i>HindIII</i> fragment from p981 and a 1.7 kb <i>PstI</i> fragment from pUG2-15 (Gianni & Galizzi, 1986)	This work
poutIID	pC10 with a 280 bp fragment from pSR5, placing the <i>spoIID</i> promoter upstream of <i>outB</i>	This work
pJIID	1.1 kb <i>EcoRI</i> – <i>HindIII</i> fragment from poutIID cloned in pJH101	This work
pS109	pDH88 carrying a 532 bp <i>RsaI</i> fragment of <i>outB</i> downstream of the <i>spac-1</i> promoter	This work

recA1 endA1 gyrA96 thi-1 hsdR17 ($r_k^- m_k^+$) *supE44 relA1 deoR* Δ (*lacZYA-argF*)U169]. Plasmid pC10 is a derivative of pBS19 into which an 859 bp *SphI*–*HindIII* fragment derived from pO.P was ligated. The integrative plasmid pJIID, with the *outB* gene placed downstream of the *spoIID* promoter, was constructed via several stages. A *HindIII*–*PvuII* fragment of 280 bp, containing the *spoIID* promoter and ribosome-binding sequence, was isolated from plasmid pSR5 (Rong *et al.*, 1986), treated with the Klenow fragment of DNA polymerase to make blunt the *HindIII* end, and then ligated to a 100-fold excess of phosphorylated *SphI* linker (Promega). Following restriction with *SphI* the fragment was ligated into *SphI*-cut, dephosphorylated pC10. The ligation mixture was used to transform *E. coli* DH5 α to chloramphenicol resistance and the colonies were screened by plasmid DNA extraction and restriction analysis. One plasmid clone, with the insert in the opposite orientation to that required, was digested with *SphI*, and the purified band relegated into pC10. A clone with the fragment in the correct orientation (poutIID) was isolated, as verified by double digestion with *HindIII* and *AvaI* and by nucleotide sequencing with custom-made primers. A fragment containing the *spoIID*–*outB* fusion was obtained by digestion of poutIID with *EcoRI* and *HindIII*, and the purified fragment was ligated into pJH101 cut with *EcoRI* and *HindIII*.

Plasmid pGEM81 was constructed by cloning a *SphI*–*HindIII* fragment from PB2427, containing the mutant *outB81* allele, into pGEM-7Z. Ligated DNA was transformed into DH5 α and putative *outB81* clones were screened by hybridization with the 859 bp *SphI*–*HindIII* fragment of p999. The insert was subsequently sequenced. The *outB81* allele was recloned into p999 and the insertional

vector pUG2-15 (Gianni & Galizzi, 1986). Plasmid pS109, containing the wild-type *outB* gene under the control of the *spac-1* promoter, was constructed by cloning a 532 bp *RsaI* fragment from plasmid p999 into *SmaI*-cut pDH88 (Henner, 1990). Recombinants were selected in *E. coli* DH5 α and clones with the insert correctly oriented were identified by double digestion with *HindIII*/*PvuII*, *BglII*/*PvuII* and *BglII*/*DraI*. Plasmids were prepared by the procedure of Birnboim & Doly (1979).

Growth conditions and preparation of spores. For growth of *E. coli*, LB medium [tryptone (Difco), 10 g; yeast extract, 5 g; NaCl, 10 g; water to 1 litre; pH 7.1] was used. Expression of *lacZ* in colonies was determined by plating on LB agar containing 40 μ g X-Gal ml⁻¹.

B. subtilis was grown in nutrient broth (NB, Difco) containing 0.5% glucose. The utilization of various nitrogen sources was evaluated by using Spizizen (1958) medium, to which the nitrogen source, sterilized by filtration, was added at a final concentration of 0.2%.

B. subtilis spores were prepared on nutrient agar supplemented with MnCl₂ (10⁻⁵ M). The spores were collected, treated with lysozyme, washed with distilled water, and purified by centrifugation in 70% (v/v) Urografin (Schering) (Siccardi *et al.*, 1975). Following heat activation at 70 °C for 15 min, the spores were inoculated into nutrient broth supplemented with 0.5% glucose and growth was monitored by measuring the optical density at 560 nm.

Transformation experiments. Competent *E. coli* cells were prepared by the method of Hanahan (1985). Transformation of competent *B. subtilis* cells was performed by the method of Hoch *et al.* (1967).

DNA sequence analysis. The *outB81* mutant allele was cloned directly from chromosomal DNA prepared according to Saito & Miura (1963), as described above. To clone the *outB47*, 48 and 49 mutations the polymerase chain reaction (PCR) was used. Chromosomal DNA extracted from the mutant strain was amplified by use of two synthetic oligonucleotides, a forward primer (21-mer, 5'CGACAGTGTGACATCCATACG3') corresponding to the nucleotide sequence just upstream of the published *outB* sequence (Albertini *et al.*, 1987), and a backward primer (21-mer; 5'GATTAATGGTAAAGCTGAATC3') corresponding to the complementary strand, downstream of the *HindIII* site. The PCR was run for 40 cycles with the following parameters: denaturation, 1.5 min at 94.5 °C for the first cycle and 30 s thereafter; annealing, 1 min at 51 °C for the first seven cycles, at 46 °C for the next seven cycles and at 41 °C for the last 26 cycles; and elongation, 1.5 min at 72 °C. The reagents and the thermocycler (Perkin-Elmer Cetus Corp.) were used in accordance with the manufacturer's instructions. The PCR product was purified from an agarose gel, digested with *SphI* and *HindIII*, and cloned in pGEM-4Z. For each mutant strain two independent clones were obtained and sequenced. DNA sequence determination was by the chain-termination method (Sanger *et al.*, 1977) using T7 DNA polymerase (Pharmacia LKB), and SP6 and T7 promoter or custom-made primers. Sequence analysis of both strands was accomplished by the supercoil sequencing method of Chen & Seeburg (1985). Oligonucleotides were synthesized by the phosphoramidite method by means of a Cyclone plus DNA synthesizer (Milligen Biosearch).

Results and Discussion

Three new alleles of *outB*

We have previously shown that three mutations (*tscB49*, *tscG47* and *tscH48*) conferring a temperature-sensitive phenotype upon *B. subtilis* vegetative cells could be rescued by plasmid pO.P, isolated by virtue of its ability to transform strains with the *outB81* mutation to a Ts⁺ phenotype (Ferrari *et al.*, 1985). Subsequently we

Table 3. Growth of wild-type *B. subtilis* and three *outB* mutants on various nitrogen sources

+++ , Colonies appear after 24 h and colony diameter is 2–3 mm after 98 h incubation; ++ , colonies appear after 48 h and colony diameter is 1 mm after 98 h of incubation; ± , colonies appear very late and colony diameter is 0.1 mm after 98 h; – , no growth observed after 98 h.

Nitrogen source	PB1424 (<i>outB</i> ⁺)	PB5051 (<i>outB47</i>)	PB5052 (<i>outB48</i>)	PB5053 (<i>outB49</i>)
Casamino acids	+++	+++	+++	+++
Glutamine	+++	+++	+	+++
Asparagine	+++	+++	±	+++
Glutamate	++	+	+	++
(NH ₄) ₂ SO ₄	++	++	–	++
KNO ₃	++	++	–	++

subcloned and sequenced a region of this plasmid corresponding to the *outB* gene (Albertini *et al.*, 1987). Marker rescue, following transformation with DNA fragments obtained from pO.P, indicated that the three mutations might also be located in *outB*. This was shown unequivocally by transformation of the three mutant strains PB5051 (*tscG47*), PB5052 (*tscH48*) and PB5053 (*tscB49*) to Ts⁺ with plasmid p999 (Fig. 1), which carries the entire *outB* gene (Albertini *et al.*, 1988). Since the recipient strains were recombination proficient, the outcome of the transformation experiments could be due to complementation, to marker rescue or to both. As a result of these experiments we have renamed mutations *tscG47*, *tscH48* and *tscB49* as *outB47*, *outB48* and *outB49*, respectively.

The four mutant alleles of *outB* are responsible for somewhat different phenotypes. Strains with the *outB81* mutation are temperature-sensitive in spore outgrowth with an early block in the synthesis of RNA (Albertini & Galizzi, 1975). The strains with the *outB47*, 48 and 49 mutations are all temperature-sensitive for growth (both outgrowth and vegetative), but have slightly different phenotypes. Following a shift from permissive to non-permissive temperature, during vegetative growth, the mutations *outB47* and *outB48* have no apparent effect on the synthesis of DNA and proteins, whereas *outB49* produces a delayed stop in the synthesis of both types of macromolecules (Galizzi *et al.*, 1976).

Strains with the *outB81* mutation, in addition to being impaired in spore outgrowth at the non-permissive temperature, are defective in the utilization of a number of nitrogen sources irrespective of temperature. In particular, they are unable to utilize asparagine, proline, glutamic acid, ammonium or nitrate as sole nitrogen source (Albertini & Galizzi, 1990). Strains with the three new *outB* mutations were tested for their capacity to grow at 35 °C on different nitrogen sources (Table 3). The *outB49* mutation had little or no effect on vegetative

growth on any of the nitrogen sources, while the *outB47* mutation strongly reduced growth on glutamic acid and the *outB48* mutation impaired the utilization of asparagine and glutamic acid and completely abolished growth on ammonium and nitrate. Thus, with the exception of *outB49*, all mutations in the *outB* gene affected the ability of the vegetative cells to utilize various nitrogen sources, albeit to different extents.

Nucleotide sequence of the *outB* alleles

The four mutant genes were cloned directly from chromosomal DNA or following amplification by PCR. The nucleotide sequences of the *outB47*, *outB49* and *outB81* alleles were determined and in each case a single base substitution was observed. Despite repeated attempts, we did not observe any change in the sequence obtained from *outB48*. Two of the mutations were transitions: CG to TA for *outB49*, changing histidine 82 to tyrosine; and GC to AT for *outB81*, changing glycine 157 to glutamate. The *outB81* mutation is located downstream of the internal *PvuII* site of *outB*. This is in agreement with previous genetic data showing that a plasmid (pJH101-2.4) carrying the *PvuII*–*HindIII* fragment, corresponding to the right half of the gene, could transform strains with the *outB81* mutation to Ts⁺ (Albertini *et al.*, 1987). The third mutation (*outB47*) was a CG to AT transversion, changing aspartate 159 to glutamate. The latter is the most conservative substitution, and strains with this mutation have the mildest phenotype. The vegetative cells of this mutant are unable to give visible colonies at 47 °C. Despite this, when a culture is shifted from permissive to non-permissive temperature, the synthesis of DNA and proteins proceeds normally for at least 90 min (Galizzi *et al.*, 1976) and only utilization of glutamic acid is affected (Table 3).

The sequence data unequivocally demonstrate that the three mutations affect the same gene. An earlier indication that the two mutations are tightly linked was provided by the fact that the recombination index between *outB47* and *outB49* (*tscG47* and *tscB49* respectively in the previous denomination) was 0.02 (Galizzi *et al.*, 1976).

The wild-type allele is dominant

The relationships of dominance and recessivity among the various *outB* alleles were analysed by the use of integrational vectors. Plasmid pJH1.1 (Fig. 1), a derivative of pJH101 (Ferrari *et al.*, 1983), contains a 1.7 kb *HindIII* fragment which includes the entire wild-type *outB* gene (Albertini *et al.*, 1987). Transformants from the parental strain PB1424 and from the *outB81* mutant were obtained after selection for chloramphenicol.

Fig. 1. Restriction map of the region surrounding the *outB* locus. The arrow indicates the origin and direction of transcription of *outB*. Fragments subcloned in various plasmids are shown. The results of marker rescue experiments involving the *outB*47, 48 and 49 mutations are summarized to the right of the clones. Restriction site abbreviations: H, *Hind*III; P, *Pst*I; Pv, *Pvu*II; S, *Sph*I. ND, Not determined.

Fig. 2. Germination and outgrowth curves of four *B. subtilis* strains. (a) Parental strain PB1424, (b) PB2427, (c) PB2475, (d) PB2477. ○, Growth at 35 °C; ▲, growth at 47 °C.

Transformants (PB2474 and PB2477 respectively) were grown in the presence of 2.5 µg chloramphenicol ml⁻¹ and, following sporulation, replica-plated at 35 °C and 47 °C. Both strains were able to outgrow at the non-permissive temperature. Outgrowth of the heterogenote PB2477 transformants in liquid culture is shown in Fig. 2(d). The capacity of the spores to outgrow was only slightly affected compared to the parental (PB1424) strain with or without the inserted plasmid pJH1.1 (Fig. 2a and data not shown). These results indicate that the *outB*81 allele is recessive to the wild-type gene.

We also constructed an integrative plasmid (pJH81) carrying the mutant allele *outB*81. This plasmid was again a derivative of pJH101 which, in addition to the *outB*81 fragment, contained a fragment of *B. subtilis* DNA derived from a region of the chromosome between *nrdA* and *polC* (Table 2), to provide a site for a 'Campbell-type' integration and thereby avoid the possibility of gene conversion of the incoming plasmid. Transformants of *outB*81 in the parental background (PB2575) produced spores that could outgrow at both the permissive and non-permissive temperatures. The outgrowth at 47 °C was slower than that obtained at 35 °C (Fig. 2c), in contrast to the faster outgrowth observed at 47 °C for the parental strain PB1424 (Fig. 2a). The presence of one or more copies of the mutant allele therefore had some effect on growth. We cannot exclude the possibility that a limited amount of amplification of the *outB*81 allele carried by the transforming plasmid accounts for the different behaviours of PB2475 and PB2477 (Fig. 2c and d, respectively). Nevertheless the heterogenote strain PB2475 could outgrow at the non-permissive temperature, confirming that the wild-type allele is dominant over *outB*81.

Both the wild-type gene and the *outB*81 mutation were introduced by the same procedures into strains PB5051 (*outB*47), PB5052 (*outB*48) and PB5053 (*outB*49). The three mutant alleles were recessive to the wild-type, i.e. growth at the non-permissive temperature was normal (data not shown). However, the merodiploids with *outB*81 failed to grow at 47 °C, indicating a lack of complementation between the mutants affected in vegetative growth and the mutant altered in spore outgrowth.

The dominance of the wild-type *outB* allele over the mutant alleles is a strong indication of the loss of function at the non-permissive temperature in the mutant strains. The dominance is not complete; spores of the heterogenote strain PB2475, with copies of the *outB*81 and wild-type alleles, are delayed in outgrowth at the non-permissive temperature. This could be easily explained if the native OutB protein is an homodimer or multimer, so that even a single mutant subunit could affect the functionality of the protein. An example of such an interaction is the *i*^{-D} mutations of the *lac* operon. It may be relevant that the *E. coli* protein homologous to OutB has been shown to be a dimer of *M_r* 60000 (Allibert *et al.*, 1987).

The outB gene is essential for normal growth

We have previously noted our inability to obtain *outB* insertion mutants by transformation with pJH101 derivatives carrying different DNA fragments internal to the transcription unit (Albertini & Galizzi, 1990). This led us

Fig. 3. Construction and integration of pS109 by homologous recombination. The plasmid is indicated by solid lines, and the chromosomal DNA by dashed lines. The box indicates the *outB* gene. The position of the promoters P_{spac} and P_{out} is also indicated. The figure is not to scale.

Fig. 4. Effect of IPTG on growth of *B. subtilis* PB2473, bearing the P_{spac} -*outB* fusion. An overnight culture of PB2473, grown in NB without inducer, was diluted in fresh NB in the presence of 10^{-4} M IPTG (●) or in the absence of inducer (○). At the time point indicated by the arrow, IPTG (10^{-4} M) was added to a parallel culture (△).

to conclude that the *outB* function was probably essential for *B. subtilis*. Such a conclusion was based on negative results that could be interpreted in different ways, e.g. non-integration of the transforming plasmid due to some constraint in recombination. In order to test this hypothesis we placed the wild-type *outB* gene under control of the inducible *spac-1* promoter (Fig. 1). This

was achieved by inserting into the chromosome, via homologous recombination, an integrative plasmid carrying the first half of the *outB* gene cloned downstream of a *spac-1* promoter (pS109, Fig. 3). Selection of transformants was carried out in the presence of IPTG and the correct integration was confirmed by Southern hybridization. The strain derived, PB2473, required IPTG to maintain growth at the parental rate; in the absence of the inducer, growth was severely impaired, but could be restored to the parental rate upon addition of IPTG (Fig. 4). The residual growth observed in the absence of IPTG could be due to the reported leakiness of the *spac* promoter (Micka *et al.*, 1991). Both vegetative growth and sporulation occurred normally in the presence of IPTG, but the spores were severely delayed in outgrowth (data not shown). Presumably the continuous presence of the inducer allows expression of *outB* even after its normal switch-off time at the beginning of the stationary phase, and this interferes with normal outgrowth of spores. However, although the process is much slowed, it still occurs, since vegetative cells could be obtained after prolonged incubation. The effect of the unscheduled transcription from the *spac-1* promoter is not easily assigned to any particular time during the growth cycle, since the inducer must always be present to allow normal growth. These results are consistent with the expression of *outB* under the control of a sporulation promoter (see below).

Expression of the outB gene under control of the spoIID promoter impairs spore outgrowth

Transcription of the *outB* gene is initiated from two promoters, P1 and P2. Transcripts originating from P1 are more abundant, are present during outgrowth and vegetative growth, but disappear at the beginning of the stationary phase (Albertini *et al.*, 1987). The switching off of P1 in the stationary phase appears to be regulated, and we wished to determine whether this switch-off was essential for late stages of sporulation or spore outgrowth. We therefore placed the *outB* gene under the control of the *spoIID* promoter. It is well established that the *spoIID* promoter can be transcribed by RNA polymerase with the sigma factor σ^E , and that transcription is absent during vegetative growth and maximal at about 1 to 1.5 h after the end of exponential growth, i.e. at stage II of sporulation (Stragier *et al.*, 1988; Clarke *et al.*, 1986; Rong *et al.*, 1986).

An integrative plasmid derived from pJH101 was constructed with the *outB* coding sequence downstream of the *spoIID* promoter and its ribosome-binding site (pJHIID, Fig. 1). The transformants (PB2476), provided with an extra copy of *outB* under control of the *spoIID* promoter, grew as well as the parental strain and

Fig. 5. Germination and outgrowth of spores of strain PB2476, bearing an additional copy of the *outB* gene under control of the *spoIID* promoter. Heat-activated spores were inoculated in NB containing 0.5% glucose and incubated at 35 °C (○) or 47 °C (▲).

sporulated as efficiently. Spores inoculated into nutrient broth supplemented with glucose showed a loss of refractivity but were unable to outgrow normally (Fig. 5). This phenotype was independent of the temperature of incubation. The block of outgrowth was leaky; after prolonged incubation the culture became turbid and the efficiency of plating of a spore preparation was not less than 0.5. The integration of pJHIID occurred in the chromosome at the site of the *outB* gene, but similar results were obtained when the same construct was inserted in an ectopic position (data not shown).

Transcription of *spoIID* has been reported to occur only in the mother cell (Illing *et al.*, 1990; Driks & Losick, 1991). The observed effect of *spoIID*-promoter-driven transcription of *outB* on spore outgrowth could be accounted for in a number of ways. For example, OutB protein synthesized in the mother cell could be trapped in the spore outer layers and could have deleterious effects upon spore outgrowth. Another explanation is that the OutB protein could reach the forespore compartment, crossing the septum. A simpler explanation, and the one we favour, is that a low level of transcription from the *spoIID* promoter occurs in the forespore compartment and is enough to exert its effect on spore outgrowth. From the analysis of the *outB* transcripts we observed that the level of transcription of the gene is low, even at its maximal level (Albertini *et al.*, 1987).

For the time being we have no explanation for the observation that expression of *outB* after the scheduled shut-off time is detrimental to spore outgrowth. This may be a direct result of the presence of the OutB protein at an inappropriate time, with subsequent accumulation in the forespore. Alternatively, it could be due to an incorrect turning on or off of a number of genes as a consequence of the action of OutB. We favour the latter

explanation, in consideration of the indications that OutB may be involved in its own regulation (Albertini *et al.*, 1988) and in the regulation of expression of different genes.

It has recently been reported (Mitchell *et al.*, 1992) that the OutB protein becomes phosphorylated at the end of exponential growth. Under sporulation conditions the transcription of *outB* is switched off at the beginning of the stationary phase, concomitant with the OutB protein being covalently modified. The allocyclic expression of *outB* in the strain with the *spoIID-outB* fusion may interfere with this modification.

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