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The *Bacillus subtilis outB* Gene Is Highly Homologous to an *Escherichia coli ntr*-Like Gene

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The *Bacillus subtilis outB* gene was found to have strong similarities to an *Escherichia coli* gene complementing *ntr*-like mutations in *Rhodobacter capsulatus*. The deduced gene products had 52% identical amino acids (65% similar residues). The phenotype of strains affected in the OutB function indicates that this *B. subtilis* gene may be involved in nitrogen utilization.

The identification, cloning, and sequencing of genes specifically expressed during defined stages of the *Bacillus subtilis* cell cycle constitute one of the main tools in understanding the regulation of the differentiation processes typical of the life cycle of this organism: sporulation and spore germination.

The spore germination process is traditionally divided into three steps: activation, germination, and outgrowth. The last step, outgrowth, is characterized by the cessation of degradative processes and by the onset of anabolic activities, punctuated by the onset of RNA, protein, and DNA synthesis. Several mutants of *B. subtilis* with an altered phenotype during spore outgrowth have been isolated (9).

One such mutant identified the *outB* locus, which has been the subject of more in-depth analysis because of the possible involvement of this gene in the regulation of spore outgrowth (3). The *outB* gene was identified by means of a temperature-sensitive mutant (*outB81*), whose spores were unable to outgrow at 46°C in rich medium. The first function blocked at the nonpermissive conditions during outgrowth of the mutant spores was RNA synthesis. Even though the main defect due to the *outB81* allele was a block of spore outgrowth at the nonpermissive temperature, an altered phenotype was detectable during vegetative growth at the permissive temperature (35°C) as well. The mutant cells grew more slowly than the parental cells in rich medium but with an efficiency of plating of 1. In addition, colony size and morphology of the *outB* mutant strain on minimal agar plates were distinct: colonies were small, smooth, and with clear-cut outlines (Fig. 1).

Sequencing and cloning of the *outB* locus (1) have unambiguously identified the coding sequence of the *outB* gene as an open reading frame corresponding to a polypeptide of 265 to 272 amino acid residues with an expected molecular mass of 29.5 to 30.6 kilodaltons and an estimated pI of 5.78. The OutB product is probably essential at all stages of the cell cycle: no viable transformants were obtained from transformation experiments using an integrative plasmid carrying a fragment internal to the coding sequence of the *outB* gene, suggesting that interruption of the *outB* gene is lethal to *B. subtilis*. Transcription of the gene was directed by two promoters, P1 and P2. P1 (σ^A type) promoted transcription from the onset of outgrowth to the stationary phase (t_0). P2 drove a low level of *outB* transcription at all stages of the bacterial cell cycle. Transcription from the P1 promoter was autogenously regulated, and the repression was exerted mostly in the *cis* configuration (2).

Similarity with other gene products. A computer-aided similarity search (carried out with the Wordsearch program of the Genetics Computer Group sequence analysis software of the University of Wisconsin, using the NBRF-PIR protein data base) revealed strong similarities between the hypothetical *outB* gene product (GenBank accession number M15811) and the product of an *Escherichia coli* gene (GenBank accession number M15328) complementing *ntr*-like mutations in *Rhodobacter capsulatus* (4). This gene was fortuitously detected on an R′ plasmid in the course of experiments designed to isolate from *R. capsulatus* sequences able to complement *ntr*-like mutations of this organism. The sequence had no homology with the *R. capsulatus* DNA sequence; in fact, it was derived from *E. coli*. Antibodies raised against the product of this *E. coli* gene were unable to detect similar products in *R. capsulatus*. Recently, Willison and co-workers (J. C. Willison, G. Ahombo, and P. M. Vignais, *in* Ullrich et al., ed., *Inorganic Nitrogen Metabolism*, in press) isolated the *R. capsulatus ntr-like* gene (*adgA*), which showed limited homology (about 25% for the predicted translation product) with the *E. coli* counterpart. The *E. coli* gene was unrelated to other known *E. coli* or *Klebsiella pneumoniae ntr*-like genes. The finding that *ntr*-like mutations in *R. capsulatus* could be complemented by an *E. coli* gene suggested to Allibert and co-workers (4) the hypothesis that nitrogen metabolism in *R. capsulatus* and *E. coli* may have regulatory elements in common.

We found strong similarity values between this *E. coli* gene and the *B. subtilis outB* gene. The alignment of the two deduced gene products showed scores of 52% amino acid identity and 65% amino acid similarity (Fig. 2). These are values of similarity previously obtained from comparison of the primary sequences of proteins involved in basic functions of *E. coli* and *B. subtilis*, such as proteins of the *spc* ribosomal operon (40 to 60% identity with 80% similarity in the case of the L5 protein [11]) and RecA of *E. coli* and RecE of *B. subtilis* (63% identity and 72% similarity [R. E. Yasbin, personal communication]). Scores of 43% identity and 65% similarity with the *E. coli* gene product have been found by Strauch et al. in the case of glutamine synthetase of *B. subtilis* (15). The products of *outB* and of the *ntr*-complementing gene also showed a remarkable correspondence in length (272 versus 273 deduced amino acid residues). At the nucleotide level, the *outB* gene and its *E. coli* counterpart

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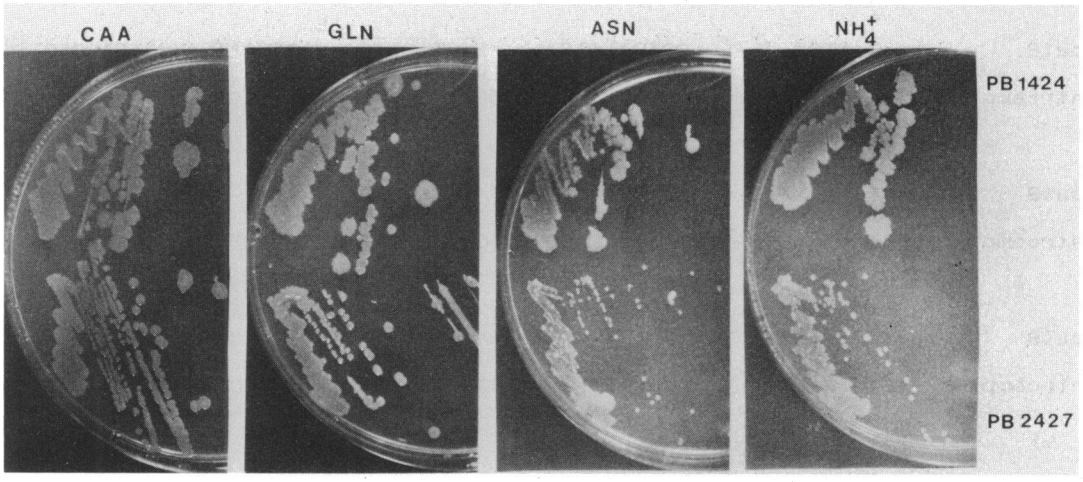

FIG. 1. Colony size and morphology of the PB1424 parental strain (*hisB2 trpC2 metD4*) and of the mutant strain PB2427 [*outB81*(Ts) *hisB2 trpC2*]. The medium used was Spizizen (12) minimal agar with the indicated nitrogen sources, supplied at a final concentration of 0.2%. The plates were incubated at 37°C for 98 h.

had 57% identity in the coding region and 62% identity in the sequence upstream of the putative ribosome-binding site (Fig. 3). The similarity was particularly high (84.6%) around the −35 box; the sequence TCTTGTT(T/C)A(T/C), tandemly repeated in the *ntr*-complementing gene, was also found in *outB*.

Willison et al. (in press) suggested for the *adgA* gene a regulatory role in the nitrogen metabolism of *R. capsulatus* as a result of the pleiotropic effect of its mutations. Unfortunately, evidence for a similar role of the *E. coli ntr*-complementing gene is at the moment only indirect. The *outB* gene, as can be deduced from the phenotype associated with the *outB81* mutation, plays a role during spore outgrowth but is also involved in vegetative growth, since it is actively transcribed during this phase. The *outB* gene is important during exponential growth, as suggested by the altered morphology on minimal agar plates of the *outB81* mutant colonies.

The *outB81* mutant phenotype on different nitrogen sources. The strong similarity with the *E. coli ntr*-complementing gene prompted us to reevaluate the phenotype of the *outB81* mutant in the presence of different nitrogen sources. The test was performed by using Spizizen medium or agar (14) in which the nitrogen source was added at a final concentration of 0.2%. We tested both vegetative cells and spore inocula at 35 and 46°C.

Vegetative cells of the *outB81* mutant strain grew at 35°C on all nitrogen sources tested but with different growth rates (Table 1). The growth rate of the mutant cells was comparable to the growth rate of the parental cells only on medium

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OutB          1 MSMQEKIMRELHVKPSIDPKQEIEDRVNFLKQYVKKTG.AKGFVLGISGG 49
                ! ::: ::: !     :  !       :     !:       :  !!!!!!!
Ntrcompl      1 MTLQQQIIKALGENRRLMLKRKFVV.VSISESYLQTYPFIKSLVLGISGG 49

OutB         50 QDSTLAGRLAQLAVESIREEGGDA..QFIAVRLPHGTQQDEDDAQLALKF 97
                !!!!!!!! !:!:: :! ! !:   !!!!!!! ! ! !!:! ! !: !
Ntrcompl     50 QDSTLAGKLCQMAINELRLETGNESLQFIAVRLPYGVQADEQDCQDAIAF 99

OutB         98 IKPDKSWKFDIKSTVSAFSDQYQQETGDQLTDFNKGNVKARTRMIAQYAI 147
                ! :::    :!!  ! ! !:!   ! ! :! :! ::! !!! !! !!! !
Ntrcompl    100 IQPDRVLTVNIKGAVLA.SEQALREAGIELSDFVRGNEKARERMKAQYSI 148

OutB        148 GGQEGLLVLGTDHAAEAVTGFFTKYGDGGADLLPLTGLTKRQGRTLLKEL 197
                :!    :!:!!!!!!!!:!!!!!!!!!!!! !: !!  ! !!!!: !!  !
Ntrcompl    149 AGMTSGVVVGTDHAAEAITGFFTKYGDGGTDINPLYRLNKRQGKQLLAAL 198

OutB        198 GAPERLYLKEPTADLLDEKPQQSDETELGISYDEIDDYLEGKEVSAKVSE 247
                : !!:!! ! !!!!! !:!!   !!  !!: !!!!!!!!!!!!   !
Ntrcompl    199 ACPEHLYKKAPTADLEDDRPSLPDEVALGVTYDNIDDYLEGKNVPQQVAR 248

OutB        248 ALEKRYSMTEHKRQVPASMFDDWWK 272
                 :!  !  !!!!!  !  :!!!!!!
Ntrcompl    249 TIENWYLKTEHKRRPPITVFDDFWK 273

 !=IDENTICAL RESIDUE  :=CONSERVATIVE SUBSTITUTION
 IDENTITY SCORE: 52%   SIMILARITY SCORE: 65%
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FIG. 2. Optimal alignment of the products deduced from the sequences of the *outB B. subtilis* gene (NBRF EC number A26936) (1) and of the *ntr*-complementing *E. coli* gene (Ntrcompl; NBRF EC number A26928) (4). Conservative amino acid changes are defined as any within the following groups: (I, L, M, V), (H, K, R), (D, E, N, Q), (A, G), (F, Y, W), (S, T), (P), and (C).

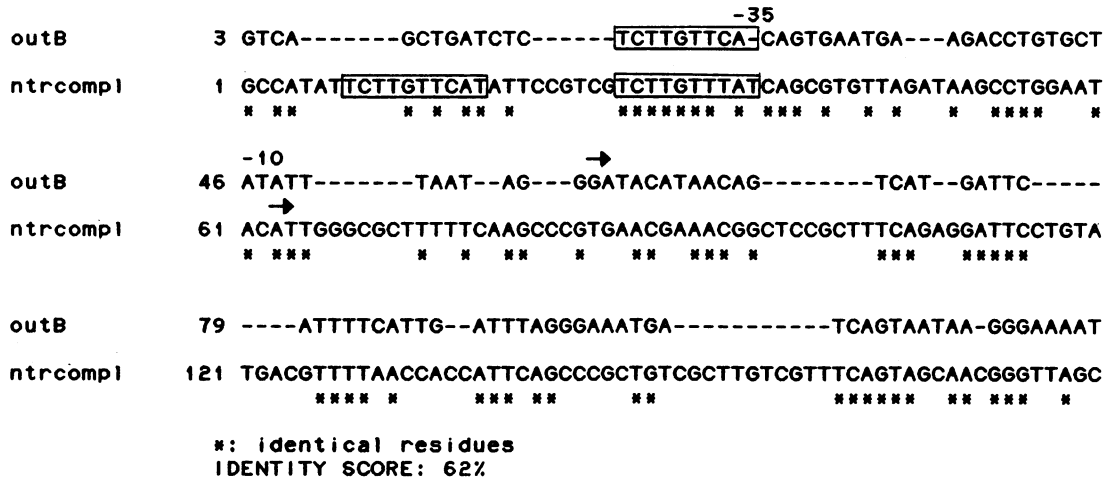

FIG. 3. Optimal alignment of the *outB* and *ntr*-complementing (ntrcompl) genes in the region upstream of the putative ribosome-binding sites. Numbers refer to the published sequence numerations; arrows indicate the transcription start sites. Regions tandemly repeated in the *E. coli* sequence and highly similar to the *outB* sequence are boxed.

supplemented with Casamino Acids (Difco Laboratories) or glutamine (mean generation times relative to those of the parental strain were 1.25 and 1.65, respectively). The mutant strain maintained its typical colony morphology. When asparagine, proline, glutamic acid, or NH_4^+ was the sole nitrogen source, the *outB81* mutant grew slowly, forming colonies only after 4 days of incubation at 35°C. The mean generation time was 4- to 10-fold longer than for the parental cells. At the nonpermissive temperature, the *outB81* mutation prevented growth of the strain on all nitrogen sources tested except Casamino Acids. Unexpectedly, the parental strain was unable to grow at 46°C on glutamine. This temperature-sensitive phenotype was common to all *B. subtilis* strains tested (W168, obtained from J. Hoch; 168, from C. Anagnostopoulos; and the Stanford strain SB19). Despite this fact, the *outB* growth defect on nitrogen sources was manifest, and in some cases stressed, at 46°C as well.

Spore inocula grew to colonies showing the same pattern of nitrogen utilization as the vegetative cells at 35°C, whereas at 46°C they were unable to form colonies in all conditions tested, as expected from their outgrowth-defective phenotype.

TABLE 1. Growth of PB1424 and PB2427 (*outB81*) strains on different nitrogen sources[a]

Nitrogen source	PB1424		PB2427	
	35°C	34°C	35°C	46°C
Casamino Acids	+++	+++	+++ (1.26)	+++
Glutamine ± NH_4^+	+++	±	+++ (1.65)	−
Asparagine ± NH_4^+	+++	+++	± (10.0)	−
Proline ± NH_4^+	+++	+++	± (5.55)	±
Glutamic acid ± NH_4^+	++	+++	± (5.68)	±
$(NH_4)_2SO_4$	++	+++	+ (4.11)	−

[a] Symbols represent growth on Spizizen agar: +++, colonies appeared after 24 h, and colony diameter was 2 to 3 mm after 98 h of incubation; ++, colonies appeared after 48 h, and colony diameter was 1 mm after 98 h of incubation; +, colonies appeared after 48 h, but colony diameter was 0.3 to 0.2 mm after 98 h of incubation; ±, colonies appeared very late, and colony diameter was 0.1 mm after 98 h of incubation; −, no colonies appeared. Numbers in parentheses represent the ratio between mean generation time of the mutant strain relative to that of the parental strain, as estimated from growth in Spizizen medium.

In *B. subtilis*, glutamine-requiring mutants have been isolated by several authors (6, 8). All mapped in the *glnA* locus, which identifies the structural gene for glutamine synthetase (GS) (7, 15). The *outB* gene lies near the marker *aroI*, in a completely different map position, and therefore the phenotype of the *outB81* mutant, which required glutamine to grow at a rate comparable to that of the parental strain, was due to a loss of a function only indirectly involved in the GS activity. In addition, the requirement for glutamine at 35°C was not absolute, since the mutant was able to grow, albeit at a reduced rate, on different nitrogen sources, including asparagine, proline, glutamic acid, or NH_4^+.

Effect of the *outB81* mutation on expression of a *glnR'-lacZ* fusion in the presence of different nitrogen sources. To test for a possible effect of the *outB81* mutation on the expression of *glnA*, we measured the activity of the *glnR-A* operon by assaying the β-galactosidase activity of a *glnR'-lacZ* transcriptional fusion (13). The *outB81* mutation severely affected the derepression of the *glnR'-lacZ* fusion in the presence of glutamate and proline (Table 2). In strains with the *outB81* mutation, the level of β-galactosidase was the same as that observed in the parental strain when grown in

TABLE 2. β-Galactosidase assays of *glnR'-lacZ* fusion in *outB*$^+$ or *outB* background[a]

Nitrogen source	β-Galactosidase (U/mg of protein)[b]	
	PB2464 *outB*$^+$	PB2465 *outB*
Glutamine	0.20	0.26 (1.30)
Glutamic acid	71.00	7.20 (0.10)
Proline	131.30	2.95 (0.02)

[a] Strains PB2464 (*hisB2 trpC2 metD4 amyE*::pJHS104E) and PB2465 [*hisB2 trpC2 outB81*(Ts) *amyE*::pHJS104E] were constructed by transformation with DNA derived from strain HJS11 (13). The recipients were strains PB1424 and PB2427, respectively, and selection was for chloramphenicol resistance and screening for Amy$^-$. Liquid cultures in Spizizen minimal medium (14) were tested for β-galactosidase activity. Samples were taken during exponential growth at different optical densities at 525 nm (0.5 to 1.3).

[b] Values represent mean activity of four samples, expressed as modified Miller units in which the optical density of the culture has been changed to milligrams of protein (12). In parentheses are ratios of the activity of the *outB81* strain relative to the activity of the parental strain.

glutamine, whereas the derepression in the presence of glutamate or proline was 10- or 44-fold less, respectively.

The effect on the *glnR-A* operon is only one of the multiple effects exerted by the *outB81* mutation, and some of the other phenotypes cannot be explained by a reduction in GS activity. This is clearly the case for the growth on asparagine or NH_4^+. The mutant strain grew very slowly on such substrates (Table 1), but derepression of GS, as measured by blue color formation on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside plates, did not occur under these conditions in the parental and mutant strains.

In conclusion, the *outB* gene shows strong similarities to an *E. coli* gene apparently involved in the utilization of nitrogen sources, and the phenotype of strains affected in the OutB function indicates that the *B. subtilis* gene may also be involved in nitrogen utilization. *E. coli* and *B. subtilis* regulate their responses to nitrogen availability differently, as can be deduced from data regarding the regulation of GS and glutamate synthase in the two organisms (5, 13). However, similar regulatory elements could be common to these heterologous systems (10), as suggested by the observation that the *B. subtilis* regulatory product GlnR plays some role in control of the *B. subtilis glnA* gene cloned in *E. coli* (13). On the other hand, the observed differences in regulation can be ascribed to the different life-styles of the two bacteria or to the ability of *B. subtilis* to sporulate and germinate.

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