Expression of a New Operon from *Bacillus subtilis*, *ykzB-ykoL*, under the Control of the TnrA and PhoP-PhoR Global Regulators

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The *ykzB* and *ykoL* genes encode two peptides, of 51 and 60 amino acids, the functions of which are unknown. The *ykzB* and *tnrA* genes are contiguous and transcribed divergently. Expression of *ykzB* and *ykoL* is induced by glutamate and is under the control of the TnrA global regulator of nitrogen utilization. TnrA regulated its own synthesis in glutamate minimal medium. Two DNA sequences (TnrAB1 and TnrAB2) homologous to the TnrA binding site are present in the region between *tnrA* and *ykzB*. Deletion mapping indicated that the TnrAB2 binding site was involved in activation of the *ykzB* promoter. In addition, transcription of *tnrA* depends on the presence of the TnrAB1 binding site. The *ykzB* and *ykoL* genes are probably in the same transcriptional unit. A single promoter involved in transcription in the presence of glutamate was mapped by primer extension. *ykoL* expression was induced by phosphate limitation and depended on the PhoP-PhoR two-component regulatory system. Its promoter was mapped to the region between *ykoL* and *ykzB*. Four boxes similar to the PhoP binding site are present upstream from the *ykoL* promoter. These boxes are probably recognized by PhoP~P during the activation of transcription in phosphate limitation conditions.

In *Bacillus subtilis*, the expression of several genes, such as *glnA* (glutamine synthetase), *ansA* (asparaginase), *ansB* (aspartase), *ureABC* (urease), *gabP* (γ-aminobutyrate permease), *nasABCDEF* (nitrate assimilatory enzymes), and *nrgAB* (ammonium permease and nitrogen-regulated PII-like protein), that are involved in the transport and catabolism of nitrogen-containing compounds is controlled by nitrogen availability (2, 8, 9, 19, 20, 28). Three regulatory proteins, GlnR, TnrA, and CodY, and a sigma factor called SigL are involved in the control of nitrogen metabolism in response to nitrogen supply. GlnR and TnrA are involved in the regulation of genes with nitrogen source-dependent expression. GlnR and TnrA are very similar and belong to the MerR family of regulators. GlnR is a negative regulator of *glnRA* operon expression and represses several TnrA-regulated genes (24). It has been shown in vitro that GlnR binds simultaneously to two operators in the *glnRA* promoter region. TnrA activates transcription of the *gabP*, *ureABC*, *nrgAB*, and *nas* genes in response to nitrogen limitation. TnrA is also a negative regulator of the ammonium assimilatory enzymes, glutamine synthetase and glutamate synthase, during nitrogen-limited growth (29). The TnrA protein is active only during nitrogen limitation, whereas GlnR is active in conditions of nitrogen excess. All of the promoters positively regulated by TnrA contain a GlnR/TnrA binding site (TGT-NAN$_7$-TNACA) located upstream from the −35 region (9). The CodY protein controls several genes in nitrogen metabolism, acetate metabolism, and competence. CodY represses

sion occurs in cells growing in a medium rich in amino acids. CodY is thought to bind to a DNA structure containing AT-rich DNA sequences (25). The *sigL* gene in *B. subtilis* encodes a sigma factor equivalent to the sigma 54 of gram-negative bacteria (4). SigL is required for the transcription of several operons involved in the catabolism of arginine, ornithine (*roc* operons), isoleucine, and valine (*bkd* operon) (5). Specific transcription factors controlling these operons have been identified (3, 5). However, there is no global nitrogen regulatory system equivalent to the NtrB/NtrC two-component system of gram-negative bacteria.

Under phosphate starvation conditions, several genes in *B. subtilis*, including *phoA* and *phoB* (alkaline phosphatases), *pstS* (inorganic phosphate transport system), *tuaA* (teichuronic acid biosynthesis), are activated by the two-component (PhoP-PhoR) regulatory system response regulator and histidine kinase. All *pho*-regulated promoters require a minimum of four consensus PhoP binding sequence repeats (TT(A/T/C)A(C/T)A) for activation (7). During the *B. subtilis* function analysis program, 1,200 mutants were constructed using the pMUTIN4 vector to study the function and regulation of the unknown "*y*" genes (27). This plasmid can be used to construct *lacZ* fusions. The capacity of various amino acids to act as nitrogen sources or inducers of gene expression and induction by phosphate starvation have been studied. Upon screening of this collection of mutants, we found that the expression of 30 *y* genes was increased by the presence of glutamate in the growth medium. In one of these mutant strains, *lacZ* expression was strongly induced by glutamate as well as by phosphate starvation. This strain contains pMUTIN4 inserted into the *ykoL* gene. In this work, we describe the regulation of the *ykzB-ykoL* operon and of the divergent gene *tnrA*. We found that this divergon was positively controlled by TnrA and by the two-component system, PhoP-PhoR.

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TABLE 1. *B. subtilis* strains used in this study

Strain	Relevant genotype	Source or reference
168	*trpC2*	Laboratory stock
FA5	*trpC2 amyE*::(*ykoL600'-'lacZ cat*)	This study
FA6	*trpC2 amyE*::(*ykoL600'-'lacZ cat*) *tnrA*::Tn*917*	This study
FA18	*trpC2 amyE*::(*ykoL600'-'lacZ cat*) *phoPR*::*tet*	This study
FA19	*trpC2 amyE*::(*ykoL600'-'lacZ cat*) *tnrA*::Tn*917 phoPR*::*tet*	This study
FA20	*trpC2 amyE*::(*ykoL200'-'lacZ cat*)	This study
FA21	*trpC2 amyE*::(*ykzB'-'lacZ cat*)	This study
FA22	*trpC2 amyE*::(*tnrA'-'lacZ cat*)	This study
FA23	*trpC2 amyE*::(*ykoL200'-'lacZ cat*) *tnrA*::Tn*917*	This study
FA24	*trpC2 amyE*::(*ykzB'-'lacZ cat*) *tnrA*::Tn*917*	This study
FA25	*trpC2 amyE*::(*tnrA'-'lacZ cat*) *tnrA*::Tn*917*	This study
FA26	*trpC2 amyE*::(*ykoL200'-'lacZ cat*) *phoPR*::*tet*	This study
FA27	*trpC2 amyE*::(*ykzB'-'lacZ cat*) *phoPR*::*tet*	This study
FA28	*trpC2 amyE*::(*tnrA'-'lacZ cat*) *phoPR*::*tet*	This study
FA29	*trpC2 amyE*::(*tnrA'-'lacZ cat*) Δ*tnrAB2*	This study
FA30	*trpC2 amyE*::(*ykzB'-'lacZ cat*) Δ*tnrAB1*	This study
FA31	*trpC2 amyE*::(*tnrA'-'lacZ cat*) Δ*tnrAB1B2*	This study
FA32	*trpC2 amyE*::(*ykzB'-'lacZ cat*) Δ*tnrAB1B2*	This study
FA33	pMUTIN4MCS::*ykoM erm*	This study
SF706T	*tnrA*::Tn*917 amyE*::*neo* ϕ(*gabP-lacZ*)706 *trpC2*	S. H. Fisher
MH5913	*pheA1 trpC2 phoPR*::*tet*	F. M. Hulett

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *B. subtilis* strains used in this work are described in Table 1. *Escherichia coli* DH5α [*supE44* Δ*lac* U169 (ϕ80 *lacZ*Δ*M15*) *hsdR17 secA1 endA1 gyrA96 thi-1 relA1*] was grown at 37°C in Luria-Bertani (LB) medium and used for cloning experiments. *B. subtilis* was grown in LB medium and transformed with plasmid or chromosomal DNA as previously described (12). Transformants were selected on SP plates (4) supplemented with erythromycin (1 μg/ml), lincomycin (25 μg/ml), chloramphenicol (5 μg/ml), or kanamycin (5 μg/ml). *B. subtilis* 168 derivative strains were grown in minimal medium [11.4 mM K$_2$SO$_4$, 62 mM K$_2$HPO$_4$ · 5H$_2$O, 44 mM KH$_2$PO$_4$, 3.4 mM sodium citrate · 7H$_2$O, 0.8 mM MgSO$_4$ · 7H$_2$O, 0.4% glucose, 0.005% L-tryptophan, 4 mg of FeCl$_3$/liter, 0.2 mg of MnSO$_4$/liter, 5.5 mg of CaCl$_2$/liter 1.7 mg of ZnCl$_2$/liter, 0.43 mg of CuCl$_2$ · 2H$_2$O/liter, 0.6 mg of CaCl$_2$ · 6H$_2$O/liter 0.6 mg of Na$_2$MoO$_4$ · 2H$_2$O/liter, 10 mM (NH4)$_2$SO$_4$ or potassium glutamate adjusted to pH 7 with 10 N NaOH] or in low-phosphate defined medium (LPDM) or high-phosphate defined medium (HPDM) (11), each supplemented with 50 μg of L-tryptophan/ml. *B. subtilis tnrA* mutants were constructed using *tnrA*::Tn*917* chromosomal DNA (strain SF706T provided by S. H. Fischer) transferred by transformation with selection for transposon-encoded erythromycin resistance. *B. subtilis phoP-phoR* mutants were constructed using chromosomal DNA from the strain MH5913 (provided by F. M. Hulett) and selected for tetracycline resistance.

DNA manipulations. Standard procedures were used to extract plasmids from *E. coli* (1, 22). Restriction enzymes were used as recommended by the manufacturers. DNA fragments were amplified by PCR (18, 21) using the *Taq* DNA polymerase (Stratagene). DNA sequences were determined by the dideoxy-chain termination method (23) using the modified T7 DNA polymerase (26) (Pharmacia).

β-Galactosidase assays. β-Galactosidase activity was estimated on plates by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) hydrolysis. β-Galactosidase specific activities were determined as described previously (16, 17) and expressed as nanomoles of *o*-nitrophenol produced per minute per milligram of protein. Induction by glutamate was measured in samples taken in exponential growth phase, while induction by phosphate starvation was measured at the beginning of the stationary phase.

Plasmid constructs. pMUTIN4MCS (27) was used for gene disruption and for the construction of transcriptional fusions with the *E. coli lacZ* gene. The BFS1843 (*ykoL*) mutant was constructed in K. Devine's laboratory by insertion of pMUTIN4MCS between bases 1397629 and 1397747 of the *B. subtilis* genome (13). The *ykoM'-lacZ* transcriptional fusion was constructed using a *Hin*dIII/*Bam*HI DNA fragment corresponding to the *ykoM* central region generated by PCR with oligonucleotides YKOMH (5'-GCCGAAGCTTCCGTGA TAGCAAAGAGCACGGTTT-3') and YKOMB (5'-CGCGGATCCGCTCCC TTACTAAAAACCCGTTTC-3'). This fragment was inserted between the *Hin*dIII and *Bam*HI sites of pMUTIN4MCS, and the recombinant plasmid was used to transform *B. subtilis* 168. Integration of the construct into the *ykoM* gene was selected by erythromycin resistance, giving strain FA33.

The *ykzB*, *ykoL*, and *tnrA* promoter regions were obtained by PCR amplification using *B. subtilis* chromosomal DNA as the template. The corresponding PCR-amplified DNA fragments were introduced into the *Eco*RI and *Bam*HI sites of pAC5 (15) to create *lacZ* translational fusions (Fig. 1). These constructs were used to transform *B. subtilis*. Recombinant strains were selected using chloramphenicol resistance. In these strains, the *lacZ* fusions were integrated as single copies at the *amyE* locus. The synthetic primers used for PCR amplification were as follows: YKOL1 (5'-CGGAATTCCCTGATCTGTCTTACGG-3') or YKOLE (5'-CGGAATTCGATCGGTTCAAAACGGAC-3') and YKOL2 (5'-CGGGATCCCGAAGCTCAGAAATCGT-3'); YKZBE (5'-CGGAATTCG AATGATCTTCTGTGGTC-3'), YKZBE2 (5'-CGGAATTCCAAATAGAAG ATTTTTGAAAAAATAC-3'), or YKZBE3 (5'-CGGAATTCATAATGCGTA ACACCCAA-3') and YKZBB (5'-CGGGATCCGCCAGTGAGACTGCTTT-3'); TNRAE (5'-CGGAATTCGCCAGTGAGACTGCTTT-3'), TNRAE2 (5'-C GGAATTCCAAAAATCTTCTATTTGATGTTAG-3'), or TNRAE3 (5'-CGG AATTCGCGTCAGGTTTTTTCTCT-3') and TNRAB (5'-CGGGATCCGAA TGATCTTCTGTGGTC-3').

RNA extraction and primer extension. RNA extraction, primer extension, and oligonucleotide labeling reactions were performed as described previously (6). *B. subtilis* 168 was grown in minimal medium with ammonium sulfate or potassium glutamate as the sole nitrogen source to activate glutamate-inducible promoters and in LPDM or HPDM to activate phosphate starvation-inducible promoters. RNA was extracted, and primer extension was performed using the YKZBPE primer (5'-AACGTCTCCAGCATTTCATCACGC-3') for the *ykzB* transcript and the YKOLPE primer (5'-GGCGTCGTTCTTTTTCTAAAGCGG-3') for the *ykoL* transcript.

RESULTS

Characterization of glutamate-induced genes. Within the framework of the European systematic function analysis of *B. subtilis* genes, 1,200 mutants were constructed. Genes to be interrupted by the pMUTIN4MCS (27) vector were selected from the 3,000 unknown open reading frames identified during the complete *B. subtilis* genome sequencing project (13). The corresponding proteins show no significant similarities to known proteins in databases. The unknown genes were interrupted by single or double crossover, leading to (i) inactivation of the target gene and (ii) transcriptional fusions between the promoters and the reporter gene, *lacZ*. We were interested in nitrogen metabolism and amino acid utilization. To investigate the potential function of the unknown genes, we tested the growth of the various mutant strains on minimal medium

A

Strains FA	β-Galactosidase specific activity (U/mg protein)					
	MMN	MME	HPDM	LPDM	HPDME	LPDME
5	1	1140	8	405	3240	3160
6 (tnrA::Tn917)	1	1	13	360	3	
18 (phoPR::tet)	1	655	12	17	1460	675
19 (tnrA::Tn917 phoPR::tet)	1	1	2	4	4	7
20	1	6	4	535	7	640
26 (phoPR::tet)	1	6	4	10		
21	2	4930	28	25	9160	9275
24 (tnrA::Tn917)	1	3	6			

B

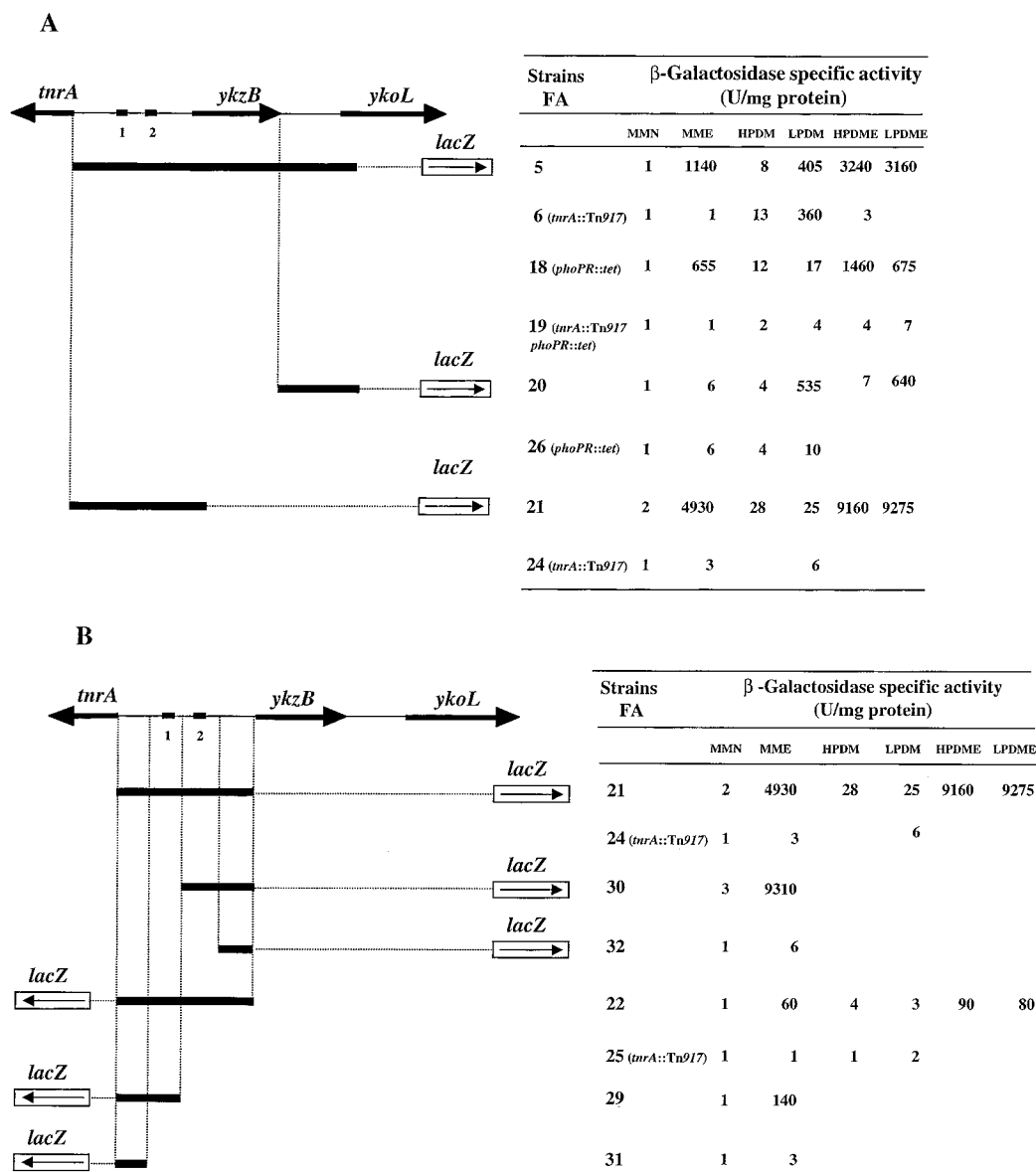| Strains FA	β-Galactosidase specific activity (U/mg protein)					
	MMN	MME	HPDM	LPDM	HPDME	LPDME
21	2	4930	28	25	9160	9275
24 (tnrA::Tn917)	1	3		6		
30	3	9310				
32	1	6				
22	1	60	4	3	90	80
25 (tnrA::Tn917)	1	1	1	2		
29	1	140				
31	1	3				

FIG. 1. Schematic representation of the DNA regions studied and *lacZ* fusions. Black arrows show the orientation of transcription. Black lines in bold type (on thick black lines) represents the PCR-amplified DNA fragments fused to the *lacZ* gene. β-Galactosidase assays were performed using cells harvested at the end of the exponential growth phase. MMN is minimal medium with 10 mM ammonium sulfate; MME is minimal medium with 10 mM potassium glutamate; LPDME and HPDME correspond to LPDM and HPDM in which ammonium sulfate has been replaced by 10 mM potassium glutamate. Black boxes 1 and 2 indicate positions of the TnrAB1 and TnrAB2 sites, respectively. The values presented at the right are the means of two or more assays.

plates supplemented with X-Gal and containing a single amino acid (proline, alanine, aspartate, glutamine, or glutamate) or ammonium sulfate as the sole nitrogen source. The induction or repression of target gene expression on the different plates was estimated by β-galactosidase activity. One of the mutants tested, BFA1843, which showed a strong activity on plates was chosen for further study. In this strain, the expression of the *lacZ* fusion was strongly induced by glutamate, resulting in blue colonies on plates supplemented with this amino acid. β-Galactosidase specific activities were determined to be about 1 U/mg of protein in ammonium sulfate minimal medium and 2,100 U/mg in glutamate minimal medium. It was therefore 2,000 times higher with glutamate (10 mM) than with ammonium sulfate (10 mM) as the sole nitrogen source. The integration of pMUTIN4MCS into BFA1843 interrupts the *ykoL*

gene. The results obtained show that *ykoL* expression is induced by glutamate.

Analysis of the *ykoL* promoter. To identify the promoter of the *ykoL* gene, PCR-amplified DNA fragments containing sequences located upstream from *ykoL* were fused to *lacZ*, creating translational fusions. These fusions were integrated into the *B. subtilis* chromosome at the *amyE* locus of strain 168. β-Galactosidase activity was determined in minimal medium containing 10 mM ammonium sulfate or 10 mM glutamate (Fig. 1A). The fusion present in strain FA5, extending from the *tnrA* NH₂-terminal region to the *ykoL* NH₂-terminal region, gave a high level of β-galactosidase activity in glutamate medium. *lacZ* expression levels were 1,000 times higher with glutamate than with ammonia for this strain. The results also show that the *ykoL* promoter is located between *tnrA* and *ykoL*.
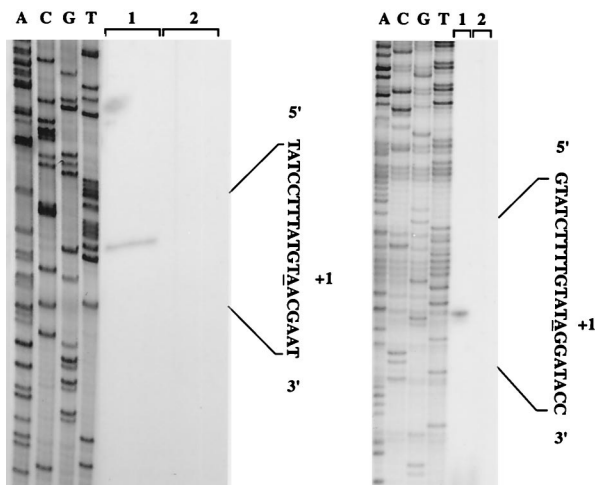
FIG. 2. Primer extension analysis of *ykzB* and *ykoL* mRNAs. (Left) Total RNA was isolated from *B. subtilis* 168 grown in minimal medium containing 10 mM glutamate (lane 1) or 10 mM ammonium sulfate (lane 2) as the sole nitrogen source. (Right): Total RNA was isolated from *B. subtilis* 168 grown in LPDM (lane 1) or HPDM (lane 2). The RNA preparations were used as templates for reverse transcriptase. The oligonucleotide primers used for reverse transcription were also used to prime dideoxy sequencing reactions from the corresponding plasmids (lanes A, C, G, and T). Positions of transcription start sites (+1) are underlined.

FIG. 3. Sequence of the promoter region located between *tnrA* and the *ykzB-ykoL* operon. Potential −10 sequences are indicated by double dashed lines, and the transcriptional start site (+1) is circled. Translational initiation codons are double underlined, and bent arrows indicate the direction of transcription. The TrnA binding sites are boxed, and the binding sites for PhoP are marked with thick black lines.

Strain FA20, which contains a *lacZ* fusion with a DNA fragment located between the *ykzB* COOH-terminal region and the *ykoL* NH$_2$-terminal region, expressed β-galactosidase at a low level in ammonium sulfate and glutamate minimal media. These results suggest that the glutamate-inducible promoter is not present upstream from the *ykoL* gene but probably resides in the *tnrA-ykzB* intergenic region. To test this hypothesis, strain FA21, containing a DNA fragment extending from the *tnrA* NH$_2$-terminal region to the *ykzB* NH$_2$-terminal region, was constructed and integrated by a double crossover event at the *amyE* locus of strain 168. β-Galactosidase activity in FA21 cells grown in minimal medium containing 10 mM glutamate was 2,500 times higher than that in cells grown in ammonium sulfate. These results indicate that the glutamate-dependent promoter is located upstream from the *ykzB* gene and that the *ykzB* and *ykoL* genes probably form an operon.

Determination of the *ykzB* transcriptional start site. Primer extension experiments were performed to determine the *ykzB* transcriptional initiation start site. Total RNA was extracted from strain 168 grown in minimal medium containing either 10 mM glutamate or 10 mM ammonium sulfate as the nitrogen source. The YKZBPE and YKOLPE primers, which are complementary to the *ykzB* and *ykoL* coding sequences, respectively, were used for the extension reactions. One signal was detected using the YKZBPE primer and RNA extracted from a culture in glutamate medium, whereas no cDNA was detected with cells grown in ammonium sulfate (Fig. 2). The transcription initiation start site was located 29 bp upstream from the translation initiation codon of *ykzB*. Examination of the upstream nucleotide sequence revealed a putative −10 region similar to the Pribnow box (Fig. 3). However, the −35 region differs from the consensus sequence for σ^A-dependent promoters. The same transcription initiation site was detected with the YKOLPE primer (data not shown). These results confirm that *ykzB* expression is induced by glutamate and that *ykzB* and *ykoL* are located in the same transcriptional unit.
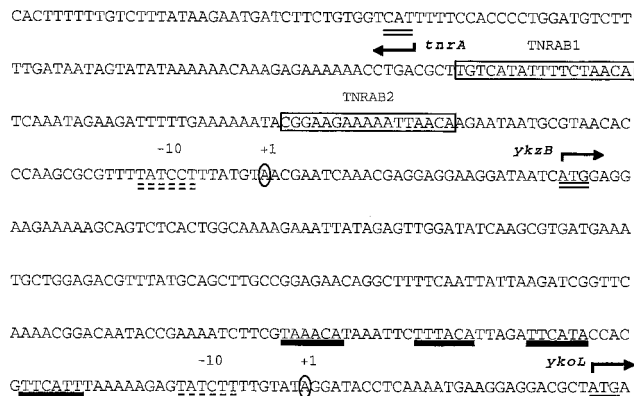
***ykoL* expression is induced by phosphate starvation.** During the systematic function analysis program, it was found that the *ykoL* gene is expressed under phosphate limitation conditions. We analyzed the expression of *ykoL* (FA20) in LPDM and in HPDM (Fig. 1A). As expected, β-galactosidase activity was 100 times higher in cells grown in low-phosphate medium than in cells grown in high-phosphate medium. In parallel, we measured the activity in strain FA21 in low- and high-phosphate media. *ykzB* was not expressed under phosphate limitation, suggesting the existence in the *ykzB-ykoL* operon of an internal promoter allowing *ykoL* expression during phosphate starvation.

Determination of the *ykoL* transcriptional start site under phosphate limitation conditions. To determine the transcriptional start site of the *ykoL* gene, RNA was extracted from strain 168 grown under low- or high-phosphate conditions. Primer extension experiments were performed using the YKOLPE primer. *ykoL* mRNA was absent in high-phosphate medium but was detectable in low-phosphate conditions (Fig. 2). The transcription initiation site was located 28 bp upstream from the translation initiation codon of *ykoL*. The nucleotide sequence of the region preceding position +1 contained a putative −10 region similar to the consensus sequence of σ^A-dependent promoters. In contrast, the potential −35 region diverged greatly from the consensus sequence of the −35 region of promoters recognized by the σ^A-containing RNA polymerase. These results confirm that *ykoL* has its own promoter, activated during phosphate starvation.

Regulation of *ykoL* expression by the two-component PhoP-PhoR system. As the PhoP-PhoR system activates the transcription of genes required for phosphate uptake and utilization during phosphate limitation, we expected that *ykoL*, which is induced in low-phosphate conditions, would be regulated by the PhoP-PhoR system. Evidence in favor of this hypothesis was provided by the presence of four putative PhoP binding sites TT(A/T/C)A(C/T)A with a spacing of 4 to 7 bp between repeats (Fig. 3) (7) and essential for promoter activity as demonstrated for the *tuaA* system (14). We tested whether *ykoL* was indeed regulated by the PhoP-PhoR system using strain FA20, in which the *phoP-phoR* genes were deleted. β-Galactosidase specific activity in the resulting FA26 strain was determined in cells grown in LPDM and HPDM (Fig. 1A). We found that the fusion was not induced by phosphate limitation

in FA26, whereas it was induced in the parental strain FA20. β-Galactosidase activity in FA20 was 100 times higher in LPDM than in HPDM, whereas it was low in both media for the *phoP-phoR* mutant. Therefore, the expression of *ykoL* is controlled by the two-component PhoP-PhoR system in phosphate starvation conditions.

Regulation of *ykzB* and *ykoL* expression by TnrA. During nitrogen-limited growth in *B. subtilis*, TnrA induces the expression of several genes, including *ureABC*, *nasA*, and *gabP*. As TnrA is involved in the regulation of nitrogen metabolism genes, we thought it likely that the expression of *ykzB* would be regulated by this transcriptional factor. To test this, we introduced a null mutation of the *tnrA* structural gene into strain FA21. β-Galactosidase specific activity was measured in the resulting strain, FA24, grown in minimal medium with ammonia or glutamate (Fig. 1A). Activity was much lower in the *tnrA*-defective strain grown in glutamate than in the wild-type strain (1,500 times higher), indicating that *ykzB* expression was positively regulated by TnrA. The *tnrA* mutation was also introduced by transformation into strain FA5, to give strain FA6. β-Galactosidase specific activity was assayed in cultures of FA6 in minimal medium containing 10 mM ammonia or 10 mM glutamate as the sole nitrogen source. The product of *tnrA* was also involved in the induction of *ykoL* expression (Fig. 1A). According to Wray et al. (30), all TnrA-regulated promoters contain a common inverted repeat sequence centered 49 to 51 bp upstream from the transcriptional start site. Two such putative TnrA sites, TnrAB1 and TnrAB2, were found centered 93 and 50 bp upstream from the *ykzB* +1 position (Fig. 3). The first is identical to the proposed TnrA binding site, whereas the second has 15 of a possible 17 matches with the consensus sequence. The role of each site in the regulation of *ykzB* was then assessed. Expression of the fusion in the FA21 strain, covering both TnrA sites, was fully induced by glutamate. Using the same *lacZ* fusion, we sequentially deleted TnrAB1 and TnrAB2 sites to give strains FA30 and FA32, respectively. In strain FA30 (Δ*tnrAB1*), β-galactosidase specific activity was 3,000 times higher in cells grown in the presence of glutamate than in cells grown in ammonia (Fig. 1B). In contrast, β-galactosidase specific activity was very low in strain FA32 (Δ*tnrAB1* Δ*tnrAB2*). These results indicate that the expression of *ykzB* is induced by the transcriptional nitrogen regulatory factor TnrA and that activation occurs through the TnrA site located 50 bp upstream from the transcriptional start site (TnrAB2).

Induction of *ykzB* and *ykoL* by glutamate in phosphate starvation conditions. In strain FA5, a high level of *lacZ* expression was observed in the presence of glutamate in both high- and low-phosphate conditions (Fig. 1A). In the *phoPR* strain FA18, *lacZ* expression was high in the presence of glutamate. As expected, this expression was completely abolished in the *tnrA phoPR* double mutant (FA19). In the FA20 strain containing the *ykzB ykoL* intergenic region, *lacZ* expression was induced in low-phosphate medium, confirming the existence of a PhoPR-dependent promoter. Conversely, in strain FA21, which contains the *ykzB* promoter, *lacZ* expression was induced only by glutamate.

Autoregulation of *tnrA* expression. It has been reported that TnrA positively regulates its own synthesis (9). We investigated the role of the two putative TnrA binding sites in the autoregulation of TnrA, by constructing a *tnrA′-′lacZ* translational fusion using a DNA fragment extending from the *ykzB* NH₂-terminal region to the *tnrA* NH₂-terminal region. This fusion was integrated into the chromosome of strain 168 to give strain FA22 (Fig. 1B). β-Galactosidase specific activity was measured in cultures of cells grown in minimal medium containing 10

mM ammonia or 10 mM glutamate as the sole nitrogen source. Expression of the *tnrA′-′lacZ* fusion was about 60 times stronger in the presence of glutamate than in the presence of ammonia. A *tnrA*::Tn*917* null mutation was introduced by transformation into strain FA22, to give strain FA25. In this strain, β-galactosidase specific activity was very weak, confirming that TnrA is involved in the activation of its own expression. No effect of phosphate limitation was observed on *tnrA* expression.

To study the function of the two putative TnrA binding sites, TnrAB1 and TnrAB2, two DNA fragments, one containing one site (TnrAB1) and the other containing neither, were synthesized and used to construct two translational *lacZ* fusions, giving strains FA29 and FA31, respectively. β-Galactosidase specific activity was assayed in cultures of the FA29 and FA31 strains (Fig. 1B). In cultures of the FA29 strain, β-galactosidase specific activity was strong in minimal medium containing glutamate whereas in strain FA31 (Δ*tnrAB1* Δ*tnrAB2*), it was as weak as that in ammonia, indicating that the TnrAB1 site is essential for *tnrA* expression. Thus, TnrA regulated the induction of its own expression through the specific TnrA binding site TnrAB1.

DISCUSSION

The *ykzB* and *ykoL* genes encode two peptides of 51 and 60 amino acids, respectively. This study shows that the expression of *ykzB* and *ykoL* is induced by glutamate. The expression of *ykoL* is also induced by phosphate limitation.

We demonstrated that the product of *tnrA* was involved in the activation of transcription in minimal medium containing glutamate. The induction by glutamate is rather unexpected because the glutamate pool is very large, up to 0.1 M, according to Hu et al. (10). TnrA activity may be modulated by a metabolite produced by the product of *glnA*, as previously suggested (29). Alternatively, the uptake of glutamate may be involved in the induction process. The effect of glutamate on the expression of *tnrA* or the target genes is also not known. In particular, we cannot exclude a direct interaction of glutamate with TnrA. Like other regulatory genes, *tnrA* expression is positively autoregulated, leading to a significant increase in the level of the corresponding protein during induction. This may partly account for the very high level of induction observed for *ykzB*. Indeed, *ykzB* is one of the mostly highly expressed of the 1,200 *y* genes of *B. subtilis* studied during the European functional analysis program (unpublished results). The *tnrA*-regulated genes contain a common inverted repeat (TGT-NAN₇-TNACA) located upstream from the transcriptional start site. This sequence has been shown to be the binding site for the TnrA protein (30). It has been shown that high-level activation of *nrgAB* expression occurs only if the TnrA site is centered 49 to 51 bp upstream from the transcriptional start site, indicating that the relative alignment of the −35 region of the promoter and the TnrA site is critical for activation (30). Two DNA sequences homologous to the TnrA binding site are present in the region between *tnrA* and *ykzB*. Deletion mapping showed that the promoter proximal box (TnrAB2) was involved in the activation of the *ykzB* promoter. The TnrAB2 site is centered 50 bp upstream from the transcriptional start site of the *ykzB* promoter, confirming that the distance between the putative TnrA binding site and the −35 sequence is crucial for promoter activation. The promoters of TnrA-regulated genes contain several mismatches within the −35 and −10 regions. We found that the −35 region of the *ykzB* promoter differed from the consensus sequence for promoters transcribed by the σ^A form of RNA polymerase. Although the *tnrA* promoter was not mapped by primer extension, a similar result was observed for

expression of the *tnrA* gene itself. One putative TnrA binding site, TnrAB1, is necessary for induction of the expression of *tnrA* by glutamate. Removal of the upstream TnrA binding site led to a slight increase in *tnrA* and *ykzB* expression. This may be due to the effects of competition between the proteins bound at the target sites.

As the expression of both *ykzB* and *ykoL* is induced by glutamate, these two genes probably belong to the same transcriptional unit. Moreover, only one promoter involved in transcription in the presence of glutamate was found by primer extension. The *ykzB* and *ykoL* genes are followed by *ykoM* on the chromosomal DNA of *B. subtilis*. However, we have shown that the expression of *ykoM* is not induced by glutamate and that this gene does not belong to the same transcriptional unit (data not shown). *ykoL* gene expression is also induced by phosphate starvation. Activation of *ykoL* transcription depends on the PhoP-PhoR regulatory system. The *ykoL* promoter, which is activated by phosphate limitation, was mapped to the region between *ykoL* and *ykzB*. Four boxes with similarity to the PhoP binding site are present upstream from the *ykoL* promoter. These boxes are probably recognized by PhoP during the activation of transcription in phosphate limitation conditions. Previous work has shown that the predominant binding region in promoters controlled by PhoP is located at approximately the same position (i.e., 21 to 60 bp upstream from the transcription start site). The binding region contained multiple TT(A/T)ACA repeats separated by approximately 5 bp. At least four of these repeats were present in each promoter (7). In the Δ*trnA* Δ*phoPR* double-mutant strain, *ykoL* was not induced by glutamate or phosphate limitation, confirming that the expression of this gene is subject to double regulation.

The function of the YkzB and YkoL peptides is still unknown. However, we have shown that in a strain in which *ykzB* and *ykoL* were deleted and in strains with deletions of *ykzB* alone or *ykoL* alone, there was no difference in the *ykzB* activation of transcription by the *tnrA* gene product in the presence of glutamate (data not shown). Therefore, the products of these two genes are not involved in induction of the operon in the presence of glutamate. The link between glutamate induction and regulation by phosphate limitation is difficult to interpret at the molecular level and needs further investigation.

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