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Definition of the *Bacillus subtilis* PurR Operator Using Genetic and Bioinformatic Tools and Expansion of the PurR Regulon with *glyA*, *guaC*, *pbuG*, *xpt-pbuX*, *yqhZ-folD*, and *pbuO*

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The expression of the *pur* operon, which encodes enzymes of the purine biosynthetic pathway in *Bacillus subtilis*, is subject to control by the *purR* gene product (PurR) and phosphoribosylpyrophosphate. This control is also exerted on the *purA* and *purR* genes. A consensus sequence for the binding of PurR, named the PurBox, has been suggested (M. Kilstrup, S. G. Jessing, S. B. Wichmand-Jørgensen, M. Madsen, and D. Nilsson, J. Bacteriol. 180:3900–3906, 1998). To determine whether the expression of other genes might be regulated by PurR, we performed a search for PurBox sequences in the *B. subtilis* genome sequence and found several candidate PurBoxes. By the use of transcriptional *lacZ* fusions, five selected genes or operons (*glyA*, *yumD*, *yebB*, *xpt-pbuX*, and *yqhZ-folD*), all having a putative PurBox in their upstream regulatory regions, were found to be regulated by PurR. Using a machine-learning algorithm developed for sequence pattern finding, we found that all of the genes identified as being PurR regulated have two PurBoxes in their upstream control regions. The two boxes are divergently oriented, forming a palindromic sequence with the inverted repeats separated by 16 or 17 nucleotides. A computerized search revealed one additional PurR-regulated gene, *ytiP*. The significance of the tandem PurBox motifs was demonstrated in vivo by deletion analysis and site-directed mutagenesis of the two PurBox sequences located upstream of *glyA*. All six genes or operons encode enzymes or transporters playing a role in purine nucleotide metabolism. Functional analysis showed that *yebB* encodes the previously characterized hypoxanthine-guanine permease PbuG and that *ytiP* encodes another guanine-hypoxanthine permease and is now named *pbuO*. *yumD* encodes a GMP reductase and is now named *guaC*.

We have detailed knowledge about the regulation of expression of the *pur* genes, which encode enzymes of the purine biosynthetic pathway in bacteria (24, 25). In *Escherichia coli*, the *pur* genes are scattered on the chromosome and are found as single genes or small operons. A regulatory protein, the PurR repressor, of the LacI type of regulatory proteins, regulates the expression of the *pur* genes or operons. When *E. coli* grows in the presence of guanine or hypoxanthine, these compounds are taken up and salvaged and at the same time they bind to the PurR repressor. PurR binds to a 16-bp palindromic sequence that overlaps the −35 promoter region of the *pur* genes (11) and inhibits transcription of the *pur* genes.

In *B. subtilis*, the genes encoding the biosynthesis of IMP are located in the *pur* operon. Three other genes (*purA*, *guaA*, and *guaB*) required for AMP and GMP synthesis are located as single genes. Expression of the *pur* operon is subject to dual regulation of transcription termination and transcription initiation. Termination of transcription is regulated by a termination-antitermination mechanism in a 242-nucleotide mRNA leader region preceding the first gene of the *pur* operon (5). The termination mechanism is triggered by guanine or hypoxanthine; however, the molecular mechanism has not been clarified. Initiation of transcription of the *pur* operon, and also of the *purA* and *purR* genes, is repressed in response to the presence of adenine in the culture medium (5). Addition of adenine results in lowering of the cellular pool of the low-molecular-weight effector molecule phosphoribosylpyrophosphate (PRPP) (15). Two regulatory elements are required for this regulation, the PurR repressor and a DNA operator site for repressor binding. PurR binding to the operator site is blocked by PRPP. The PurR protein is a 62-kDa homodimer (17, 21) that—as judged by footprinting analysis—interacts with a region between −149 and −29 relative to the transcriptional start site of the *pur* operon (17). A second protein encoded by *yabJ*, which is located in an operon with *purR*, has been suggested to act together with the PurR repressor (10).

Recently, a regulatory protein, also named PurR, that activates *pur* gene transcription was identified in *Lactococcus lactis*. *L. lactis* PurR shows extensive amino acid sequence identity with the *B. subtilis* PurR repressor (7). The *L. lactis* PurR protein binds upstream of the promoter region of *pur* genes (6). Based on genetic analysis and sequence comparison between the nucleotide sequences upstream of genes in *B. subtilis* and *L. lactis*, Kilstrup and coworkers were able to suggest a possible *cis*-acting sequence (5′-AWWWCCGAACWWTH-3′), named the PurBox, which is required for PurR-mediated control of gene expression (6).

In the present work, we provide evidence that an operator site comprised of two PurBoxes is required for PurR control in *B. subtilis* and that other genes of importance for purine synthesis are also regulated by PurR.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this work are listed in Table 1. DNA primers used for PCR amplification and primer extension are listed in Table 2. *B. subtilis* was grown in Spizizen minimal salt medium supplemented with 0.2% L-glutamate, 40 mg of L-tryptophan per liter, and 1 mg of thiamine per liter and with 0.4% glucose as a carbon source. Purine compounds (adenine and guanosine) were added to a final concentration of 1 mM. For selection of antibiotic resistance, antibiotics were used at the following concentrations: ampicillin, 100 mg/liter; neomycin, 5 mg/liter; erythromycin, 1 mg/liter; lincomycin, 25 mg/liter; chloramphenicol, 6 mg/liter.

Nucleic acid manipulation and genetic techniques. Isolation of DNA and RNA and basic molecular biology techniques were performed as previously described (12, 13, 26).

Construction of transcriptional *lacZ* fusions. Different promoter-containing PCR products were generated by using the primer combinations listed in Tables 1 and 2. The various DNA fragments were digested with restriction enzymes and ligated into pDG268cat or pDG268neo digested with the same enzymes and transformed into *E. coli* MC1061 selecting for Ap^r. Plasmids extracted from *E. coli* were integrated into the *B. subtilis* chromosome as described before (12). The *yumD′-lacZ* fusion was constructed by amplifying an internal segment of the *yumD* gene and cloning it in front of the *lacZ* gene of pMutin4 (20). The resulting plasmid was transformed into the *B. subtilis yumD* locus by selecting for Er^r as described by Vagner and coworkers (20).

Enzyme assays and measurement of purine base uptake. Cell extracts were made as described before (15). Serine hydroxymethyltransferase (SHMT) activity was determined in a coupled assay using L-*allo*-threonine as the substrate (16). GMP reductase activity was determined by measuring the formation of [^{14}C]IMP from [^{14}C]GMP. A 50-μl volume of assay buffer contained 0.2 mM NADPH, 0.1 mM [^{14}C]GMP (50 mCi/mmol), and glucose-6-phosphate dehydrogenase (10 U), as well as 2 mM glucose-6-phosphate to regenerate NADPH. Cell extract was added, and after 1, 2, 4, and 8 min, 5-μl samples were removed and spotted on a polyethyleneimine-impregnated thin-layer chromatography plate (Merck, Darmstadt, Germany). The chromatogram was dried and developed in 0.4 M phosphate buffer (pH 3.4) to separate IMP from GMP. The plate was dried, and radioactivity was measured in an InstantImager (Packard). β-Galactosidase activity was determined as described previously (2). All enzyme determinations were repeated at least three times. Enzyme activity is given as nanomoles of product formed per minute (equals 1 U). Total protein was determined by the method of Lowry et al. Uptake of purine bases was performed as described by Saxild and Nygaard (14).

Bioinformatic tools. Searches for specific nucleotide sequences in the *B. subtilis* genome were performed by using the WinSeq computer software developed by Flemming Hansen (unpublished). The machine-learning algorithm ANN-Spec, which was designed to discover ungapped patterns in DNA sequences (23), was used to analyze the *B. subtilis* genome sequence for the presence of the tandem-PurBox sequence. The computer program for UNIX systems is available from us.

RESULTS

Initial search for potential PurR binding sites on the *B. subtilis* chromosome. The PurR binding sequence (PurBox) 5′-AWWWCCGAACWWTH-3′ (6) was used as the query sequence in a computerized search of the *B. subtilis* genome using the WinSeq computer software. An alignment of the *B. subtilis* PurBox sequences upstream of the *pur* operon, *purA* and *purR*, revealed that the *purR* and *pur* operon PurBoxes diverge from the consensus sequence at one and two positions, respectively. These three positions were therefore considered less important and were assigned a low-importance weight, whereas all other positions were assigned a high-importance weight. Using these search parameters, we found 249 potential PurBoxes with zero, one, or two low-weight mismatches or one high-weight mismatch. Because PurR is reported to bind to a regulatory region upstream of the affected genes, the locations of the 249 potential PurBoxes were examined. Those PurBoxes that are located 0 to 350 nucleotides upstream of the start codon of the downstream open reading frame (ORF) were selected. This assortment resulted in 46 ORFs.

Test for possible PurR control of the expression of six selected operons. Among the 46 ORFs, six operons or genes, including *xpt-pbuX*, *yebB*, *glyA*, *yumD*, *yqhZ-folD*, and *rapB*, were selected for further analysis. The upstream control regions containing the putative PurBox sequence were cloned in front of *lacZ* in plasmid pDG268(*cat*) or pMutin4 and inserted into the chromosome. A fusion of the *pur* operon promoter was also constructed and used as a positive control (*amyE*::*purE′-lacZ*). An isogenic series of strains was constructed that contains the respective *lacZ* fusions in a *purR* genetic background. All strains were grown in minimal medium with or without adenine, and the β-galactosidase level was determined. The expression of all of the genes, except *rapB*, was repressed two- to threefold in the presence of adenine, and the levels were increased in a *purR* genetic background (Table 3). When the DNA sequences of the upstream regulatory regions of the identified PurR-controlled genes were compared, it became evident that they all contain two divergently oriented PurBox-like sequences separated by 16 or 17 nucleotides (Fig. 1). From the alignment of putative PurBoxes upstream of the six selected operons or genes, it appears that of all the PurR-regulated genes are preceded by one PurBox sequence with relatively high sequence similarity to the consensus sequence and by another PurBox sequence that has a more degenerated sequence. The upstream region of *rapB* contains only one PurBox sequence.

Computerized search for regulatory regions containing the tandem PurBox motif. We then wanted to test whether a refined computer search using the novel information about the PurR binding motif could identify the expected PurR-regulated genes and perhaps predict new genes that did not appear in the initial search. Potential promoter-containing DNA sequences in the *B. subtilis* genome were organized in a list of 4,222 entries, each containing a 400-nucleotide sequence upstream of one of the 4,222 predicted ORFs in the *B. subtilis* genome. This list was searched for sequences having the tandem PurBox motif. Using the ANN-Spec bioinformatics software (23), a weight matrix for the PurBox sequence was calculated on the basis of the sequence of a total of 16 PurBoxes located pairwise upstream of *purR*, *purA*, *yqhZ-folD*, *yumD*, *purE*, *glyA*, *xpt-pbuX*, and *yebB*, respectively. The program calculates an arbitrary score for each of the potential PurBox sequences. The file containing the 4,222 potential promoter regions was searched for sequences having two potential PurBoxes separated by no less than 11 and no more than 21 nucleotides. A total of 129 sequences were found, and Table 4 lists the 10 top-ranked loci for which the upstream 400-bp sequence contains the tandem PurBox motif having one or two PurBox sequences with a high score. For the remaining 116 loci, the scores for one or both potential PurBoxes were below the level of significance. As expected, the program identified all of the genes that were used to calculate the weight matrix. The upstream region of *ytiP* and *ytjP* was also found to contain a potential tandem PurBox motif with the correct spacing of 16 or 17 nucleotides between the PurBox sequences. *ytiP* and *ytjP* are divergently oriented on the chromosome and are separated by a 96-bp intercistronic region. The two potential PurBoxes are located closest to the *ytiP* reading frame. The 432-amino-

TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Genotype or characteristics	Source or reference
B. subtilis		
168	*trpC2*	C. Anagnostopoulos[a]
168/pMAP65	*trpC2*/pMAP65	168 transformed with pMAP65 selecting for Neo^r
HH355	*trpC2 yumD*::[pTM007 *yumD′-lacZ* (erm)]	168 transformed with pTM007 selecting for Er^r
HH413	*trpC2 glyA*::[pHH1101 (*cat*)]	168 transformed with pHH1101 selecting for Cm^r
HH417	*trpC2 amyE*::[pHH1108 *pbuG′-lacZ* (*cat*)]	168 transformed with pHH1108 selecting for Cm^r
HH418	*trpC2 amyE*::[pHH1109 *xpt′-lacZ* (*cat*)]	168 transformed with pHH1109 selecting for Cm^r
HH419	*trpC2 amyE*::[pHH1108 *pbuG′-lacZ* (*cat*)] *purR*::*neo*	HH417 transformed with LCC28 selecting for Neo^r
HH420	*trpC2 amyE*::[pHH1109 *xpt′-lacZ* (*cat*)] *puR*::*neo*	HH418 transformed with LCC28 selecting for Neo^r
ED448	*trpC2 yqhZ*::pMutin4 pMAP65	168/pMAP65 transformed with YQHZd selecting for Er^r
ED449	*trpC2 ywoE*::pMutin1 pMAP65	168/pMAP65 transformed with BFA2232 selecting for Er^r
ED453	*trpC2 ytiP*::pMutin1 *purR*::*neo*	BFA2025 transformed with LCC28 selecting for Neo^r
KB-3A	*trpC2 amyE*::[pKB3-1 *purE′-lacZ* (*cat*)]	168 transformed with pKB3-1 selecting for Cm^r
KB-3Am	*trpC2 amyE*::[pKB3-1 *purE′-lacZ* (*cat*)] *purR*::*neo*	KB-3A transformed with LCC28 selecting for Neo^r
KB-4C	*trpC2 amyE*::[pKB4-4 *rapB′-lacZ* (*cat*)]	168 transformed with pKB4-4 selecting for Cm^r
KB-4Cm	*trpC2 amyE*::[pKB4-4 *rapB′-lacZ* (*cat*)] *purR*::*neo*	KB-4C transformed with LCC28 selecting for Neo^r
KB-5D	*trpC2 amyE*::[pKB5-4 *yqhZ′-lacZ* (*cat*)]	168 transformed with pKB5-4 selecting for Cm^r
KB-5Dm	*trpC2 amyE*::[pKB5-4 *yqhZ′-lacZ* (*cat*)] *purR*::*neo*	KB-5D transformed with LCC28 selecting for Neo^r
PEH03	*trpC2 amyE*::[pPEH04 *glyA′-lacZ* (*cat*)] *purR*::*neo*	PEH06 transformed with LCC28 selecting for Neo^r
PEH06	*trpC2 amyE*::[pPEH04 *glyA′-lacZ* (*cat*)]	168 transformed with pPEH04 selecting for Cm^r
PEH07	*trpC2 amyE*::[pPEH06 *glyA′* (G_{-110}→C)-*lacZ* (*cat*)]	168 transformed with pPEH06 selecting for Cm^r
KN05n	*trpC2 amyE*::[pKN05n *glyA′-lacZ* (*neo*)]	168 transformed with pKN05n selecting for Neo^r
PEH08	*trpC2 amyE*::[pPEH05 *glyA′* Δ(A_{-120}–T_{-99})-*lacZ* (*cat*)]	168 transformed with pPEH05 selecting for Cm^r
KN07c	*trpC2 amyE*::[pKN07c *glyA′* (C_{-78}→G)-*lacZ* (*cat*)]	168 transformed with pKN07c selecting for Cm^r
KN08c	*trpC2 amyE*::[pKN08c *glyA′* (T_{-76}→G)-*lacZ* (*cat*)]	168 transformed with pKN08c selecting for Cm^r
KN09c	*trpC2 yabJ*::[pKN09-2 (*cat*)]	168 transformed with pKN09-2 selecting for Cm^r
KN015cn	*trpC2 amyE*::[pKN05n *glyA′-lacZ* (*neo*)] *yabJ*::[pKN09-2 (*cat*)]	KN05c transformed with pKN09-2 selecting for Cm^r
LCC28	*purR*::*neo*	2
TM307	*trpC2 yumD*::[pTM007 *yumD′-lacZ* (*erm*)] *purR*::*neo*	HH355 transformed with LCC28 selecting for Neo^r
YQHZd	*trpC2 yqhZ*::pMutin4	K. Kobayashi[b]
BFA2025	*trpC2 ytiP*::pMutin1	Micado database[c]
BFA2026	*trpC2 ytjP*::pMutin1	Micado database
BFA2232	*trpC2 ywoE*::pMutin1	Micado database
BFA2255	*trpC2 yebB*::pMutin1	Micado database
E. coli MC1061	F$^-$ *araD139* Δ(*ara-leu*)*7696 galE15 galK16* Δ(*lac*)X74 *rpsL* (Str^r) *hsdR2* (r$^-$ m$^-$) *mcrA mcrB*	Laboratory stock
Plasmids		
pMutin4	Ap^r (*E. coli*) or Er^r (*B. subtilis*); integrational vector for knockout mutations and formation of transcriptional *lacZ* fusions; IPTG-inducible P_{spac} promoter introduced to ensure expression of downstream genes	20
pMAP65	Neo^r (*B. subtilis*); plasmid overexpressing LacI	9
pDG268neo	Ap^r (*E. coli*) or Neo^r (*B. subtilis*); vector used for integration of transcriptional *lacZ* fusions into *amyE* gene of *B. subtilis*	12
pDG268cat	Ap^r (*E. coli*) or Cm^r (*B. subtilis*); vector used for integration of transcriptional *lacZ* fusions into *amyE* gene of *B. subtilis*	12
pBOE335	Ap^r (*E. coli*) or Cm^r (*B. subtilis*); integrational vector, pUC19 containing *cat* gene cloned into *KasI* site	12
pTM007	pMutin4 digested with *Hin*dIII and *Bam*HI and ligated to a PCR fragment (primers 18 and 19) digested with the same enzymes	This work
pHH1101	pBOE335 digested with *Eco*RI and *Bam*HI and ligated to a *glyA* internal PCR fragment (primers 8 and 9) digested with the same enzymes	This work
pHH1108	pDG268cat digested with *Hin*dIII and *Bam*HI and ligated to a PCR fragment (primers 14 and 15) digested with the same enzymes	This work
pHH1109	pDG268cat digested with *Eco*RI and *Bam*HI and ligated to a PCR fragment (primers 12 and 13) digested with the same enzymes	This work
pKB3-1	pDG268cat digested with *Eco*RI and *Bam*HI and ligated to a PCR fragment (primers 16 and 17) digested with the same enzymes	This work
pKB4-4	pDG268cat digested with *Eco*RI and *Bam*HI and ligated to a PCR fragment (primers 10 and 11) digested with the same enzymes	This work
pKB5-4	pDG268cat digested with *Eco*RI and *Bam*HI and ligated to a PCR fragment (primers 20 and 21) digested with the same enzymes	This work
pPEH04	pDG268cat digested with *Eco*RI and *Bam*HI and ligated to a PCR fragment (primers 1 and 6) digested with the same enzymes	This work

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Genotype or characteristics	Source or reference
pPEH06	pDG268cat digested with *Eco*RI and *Bam*HI and ligated to a PCR fragment (primers 2 and 6) digested with the same enzymes	This work
pPEH05	pDG268cat digested with *Eco*RI and *Bam*HI and ligated to a PCR fragment (primers 3 and 6) digested with the same enzymes	This work
pKN07c	pDG268cat digested with *Eco*RI and *Bam*HI and ligated to a PCR fragment (primers 5 and 6) digested with the same enzymes	This work
pKN08c	pDG268cat digested with *Eco*RI and *Bam*HI and ligated to a PCR fragment (primers 4 and 6) digested with the same enzymes	This work
pKN05n	pDG268neo digested with *Eco*RI and *Bam*HI and ligated to a PCR fragment (primers 1 and 6) digested with the same enzymes	This work
pKN09-2	pBOE335 digested with *Eco*RI and ligated to a *yabJ* internal PCR fragment (primers 22 and 23) digested with the same enzyme	This work

[a] Centre National de la Recherche Scientifique, Jouy-en-Josas, France.
[b] http://bacillus.genome.ad.jp/.
[c] http://locus.jouy.inra.fr/cgi-bin/genmic/madbase_home.pl.

TABLE 2. DNA primers used in this work

Gene and primer no.	5′-linked restriction site sequence	Nucleotide sequence[a]	Coordinates[b]
glyA			
1	*Eco*RI	5′-GCCGGAATTCAATAAATTCCGAACTTTAAATTA-3′	(−120)–(−98)
2	*Eco*RI	5′-GCCGGAATTCAATAAATTCC**C**AACTTTAAATTA-3′	(−120)–(−98)
3	*Eco*RI	5′-GCCGGAATTCAAATTTAAGTTATTAATATTCG-3′	(−98)–(−77)
4	*Eco*RI	5′-GCCGGAATTCAATAAATTCCGAACTTTAAATTAAATTTAAGTTA TTAATATTCG**G**TTTTA-3′	(−120)–(−72)
5	*Eco*RI	5′-GCCGGAATTCAATAAATTCCGAACTTTAAATTAAATTTAAGTTA TTAATATT**G**GTTTTTA-3′	(−120)–(−72)
6	*Bam*HI	5′-GCGGGATCCTTAGTCTGTTGGCGTTC-3′	(+118)–(+102)
7	None	5′-TTAGTCTGTTGGCGTTC-3′	(+118)–(+102)
8	*Eco*RI	5′-GCCGGAATTCTTTGCTTGTGCGCC-3′	3789200–3789213
9	*Bam*HI	5′-GCGGGATCCCAAGTGTTTAACGCC-3′	3789465–3789451
rapB			
10	*Eco*RI	5′-GCCGGAATTCATACGTAGAAAAACCG-3′	3771315–3771300
11	*Bam*HI	5′-GCGGGATCCGGCCATGCCCCTCC-3′	3771183–3771196
xpt			
12	*Eco*RI	5′-GCCGGAATTCCGAATCCCCTTGAAATACG-3′	2319557–2319539
13	*Bam*HI	5′-GCGGGATCCCGCGATTATATGAGTG-3′	2319423–2319438
pbuG			
14	*Hin*dIII	5′-GCCGAAGCTTGTTTATTACGAACAAAATCCG-3′	693858–693878
15	*Bam*HI	5′-GCGGGATCCTTCCCGCGATTATACG-3′	693980–693956
purE			
16	*Eco*RI	5′-GCCGGAATTCAGATCGTTCCGTGCGGG-3′	697749–697765
17	*Bam*HI	5′-GCGGGATCCGATTATATGAGGTCGTG-3′	697907–693956
yumD			
18	*Hin*dIII	5′-GCCGAAGCTTTCCGTACGTTTACCGCC-3′	3302180–3302196
19	*Bam*HI	5′-GCGGGATCCACAGCATTTGAGTGCCC-3′	3302478–3302462
yqhZ-folD			
20	*Eco*RI	5′-GCCGGAATTCTTTCTTCATGAACGTG-3′	2529102–2529087
21	*Bam*HI	5′-GCGGGATCCCATTTTCTTTCTCCTTTG-3′	2528935–2529952
yabJ			
22	*Eco*RI	5′-GCCGGAATTCGGCCAAATCCCTTTGACTCC-3′	55413–55432
23	*Eco*RI	5′-GCCGGAATTCAACTGTTCCATATCCGCG-3′	55544–55527

[a] Each underlined sequence indicates the position of the 5′-linked restriction site. Letters in boldface (in primers 2, 4, and 5) indicate mutational changes.
[b] Numbers in parentheses indicate nucleotide positions relative to the +1 transcriptional start site of *glyA*. Other numbers indicate the genome sequence coordinates (8) of the primer 5′ and 3′ ends, respectively.

TABLE 3. Effect of purine repressor PurR on expression of selected *B. subtilis* genes having putative PurBox sequences in their regulatory regions

Strain	Relevant genotype	β-Galatosidase activity (U/mg of protein)[a]	
		MM[b]	MM + Adenine
KB-3A	*amyE*::*purE'-lacZ*	145	49
KB-3Am	*amyE*::*purE'-lacZ purR*	341	228
HH418	*amyE*::*xpt'-lacZ*	92	56
HH420	*amyE*::*xpt'-lacZ purR*	338	292
HH417	*amyE*::*yebB'-lacZ*	164	62
HH419	*amyE*::*yebB'-lacZ purR*	635	507
PEH06	*amyE*::*glyA'-lacZ*	402	158
PEH03	*amyE*::*glyA'-lacZ purR*	1,815	1,790
HH355	*yumD*::[pTM007 *yumD'-lacZ*]	23	7
TM307	*yumD*::[pTM007 *yumD'-lacZ*] *purR*	422	391
KB-5D	*amyE*::*yqhZ'-lacZ*	251	113
KB-5Dm	*amyE*::*yqhZ'-lacZ purR*	682	670
KB-4C	*amyE*::*rapB'-lacZ*	15	10
KB-4Cm	*amyE*::*rapB'-lacZ purR*	7	9

[a] Values are means of three experiments. The variation was less than 20%.
[b] MM, glucose minimal medium.

acid primary sequence of YtiP is 47% identical to *yebB* of *B. subtilis.* The 463-amino-acid primary sequence of YtjP is 40% identical to a dipeptidase from *L. lactis* (472 amino acids, accession no. AAC45369). In order to analyze whether the genes are subject to purine control, BFA2025 (*ytiP*) and BFA2026 (*ytjP*) were grown in minimal medium supplemented with adenine or guanosine. The basal level in BFA2025 was 22

FIG. 1. Alignment of the tandem-PurBox motif located upstream of nine PurR-regulated genes or operons. Only one DNA strand is shown. Boxed sequences are individual PurBox sequences. Shaded positions indicate nucleotides (nt) that diverge from the 5′-AWWWC CGAACWWTH-3′ consensus sequence defined by Kilstrup and coworkers (6). Letters in the two bottom boxes show nucleotides that are conserved in the tandem PurBox motif. Lightface letters indicate nucleotides that are conserved in eight of the nine PurBoxes, and boldface letters indicate nucleotides that are conserved in all nine PurBoxes.

TABLE 4. The 10 genes and reading frames (out of 129 candidates) in the *B. subtilis* genome showing the highest scores for the upstream twin PurBox sequences identified by the ANN-Spec bioinformatic software[a]

Locus	PurBox score		Distance (nt)
	Gene distal	Gene proximal	
purA	6.44	12.78	17
glyA	10.23	12.22	17
purR	9.95	11.92	16
yqhZ	10.49	11.81	16
yumD	9.31	11.75	16
yebB	9.21	11.35	16
xpt	11.30	10.01	16
ytiP	8.95	9.57	17
purE	10.98	8.38	16
ytjP	10.32	7.89	17

[a] The ANN-Spec bioinformatic software was described by Workman and Stormo (23).
[b] nt, nucleotides.

U/mg of protein, and the expression was repressed by adenine (to 9 U/mg of protein) and induced by guanosine (to 49 U/mg of protein). *ytjP* (BFA2026) expression did not respond to addition of purines (data not shown). Inactivation of *purR* in BFA2025 (strain ED453) resulted in derepression of *ytiP* expression both in the presence of adenine (66 U/mg of protein) and in the absence of adenine (81 U/mg of protein). We therefore concluded that *ytiP*, but not *ytjP*, belongs to the PurR regulon.

***cis*-acting elements involved in PurR repression of *glyA* expression.** The *cis*-acting requirements for PurR control of *glyA* expression was studied in more detail. The *glyA* transcriptional start site was determined in a primer extension experiment (data not shown), and the site is indicated in Fig. 2. Putative σ^A −10 and −35 regions are located at suitable distances upstream of the +1 position. The DNA fragment covering the region from −120 to +118 (Fig. 2) directed PurR-regulated transcription when fused to *lacZ* in a wild-type genetic background (PEH06, Table 3). The same fusion was constitutively expressed in a *purR* genetic background (PEH03, Table 3). A fusion with a DNA fragment with nucleotides −120 to −99

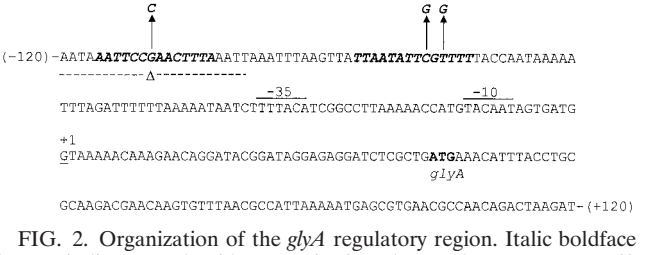

FIG. 2. Organization of the *glyA* regulatory region. Italic boldface letters indicate nucleotides constituting the tandem-PurBox motif. Boldface roman letters indicate the translational start codon of the *glyA* reading frame. Arrows and letters above the PurBox sequences indicate base pair substitutions in the various strains described in Table 5. The Δ symbol surrounded by dashed lines indicates the extent of the PurBox deletion in strain PEH08 (Table 5). Lines above the sequence indicate the locations of the putative −10 and −35 regions of the *glyA* promoter. The designation +1 indicates the transcriptional start site determined by primer extension analysis of *glyA* mRNA from cells grown in glucose minimal medium using primer 7 (Table 2).

TABLE 5. Effects of mutational changes in the *glyA* regulatory region on the expression of a *glyA-lacZ* fusion[a]

Strain	Relevant genotype	Change in *glyA* regulatory region	Adenine added	β-Galactosidase activity (U/mg of protein)
PEH06[b]	*amyE*::*glyA′-lacZ*	None	−	402 ± 47
			+	158 ± 8
PEH08	*amyE*::*glyA′* Δ(A_{-120}–T_{-99})-*lacZ*	A_{-120} to T_{-99} deletion	−	2,176 ± 66
			+	2,182 ± 79
PEH07	*amyE*::*glyA* (G_{-110}→C)-*lacZ*	G_{-110}→C substitution	−	2,017 ± 123
			+	1,803 ± 93
KN08c	*amyE*::*glyA* (T_{-76}→G)-*lacZ*	T_{-76}→G substitution	−	428 ± 60
			+	82 ± 10
KN07c	*amyE*::*glyA* (C_{-78}→G)-*lacZ*	C_{-78}→G substitution	−	2,310 ± 346
			+	1,550 ± 387

[a] Strains were grown in glucose minimal medium plus and minus adenine (1 mM).
[b] Values are from Table 3.

deleted was also constitutively expressed (PEH08, Table 5), indicating that the deduced PurBox (nucleotides −116 to −103 in Fig. 2) is required for PurR control. A G_{+110}→C substitution was introduced into the −120 to +118 fragment, and this also leads to constitutive expression. This observation demonstrates the essential role of the central CG pair of the promoter-distal PurBox in mediating the negative control of gene expression by PurR. The promoter-proximal PurBox sequence was altered in two ways. T_{+76} was replaced with a G, and in theory, this should create a more consensus-like PurBox sequence. C_{+78} was replaced with a G, and in theory, this should result in a less consensus-like PurBox sequence. When fused to *lacZ*, the fragment containing the T_{+76}→G mutation mediated a stronger repression by PurR in medium with adenine present whereas a fusion with the fragment containing the C_{+78}→G mutation reduced repression by adenine to 1.3-fold, compared to 2.7-fold repression in the wild type.

Is the *yabJ* gene product involved in the regulation of expression of PurR-controlled genes? The *yabJ* gene located downstream of the *purR* gene has been suggested to encode a protein involved in the adenine-mediated repression of *purA* gene expression (10), although this was not observed when the *purR-yabJ* operon was first identified (21). To investigate whether the expression of the *glyA* gene was altered in a *yabJ* mutant, we determined the effects of adenine and guanosine on *glyA* expression in both the wild type and a *yabJ* mutant strain. As a control, we determined *purA* gene expression (Table 6). However, we found that adenine repression and guanosine induction of both genes were similar in wild-type strains and *yabJ* mutant strains. This finding favors the view that the *yabJ* gene product has no effect on *glyA* and *purA* gene expression.

TABLE 6. Effect of *yabJ* disruption on expression of *glyA* and *purA*[a]

Relevant genotype	Enzyme activity (U/mg of protein)[b]					
	MM		MM + adenine		MM + guanosine	
	glyA	*purA*	*glyA*	*purA*	*glyA*	*purA*
Wild type	576	2.1	226	0.7	1,272	4.2
yabJ	572	2.5	265	1.0	1,121	5.9

[a] Cells were grown in minimal medium (MM) with purines added at 1 mM.
[b] *glyA* expression was determined in strains KNO5n and KN015cn (*yabJ*), both containing a *glyA-lacZ* transcriptional fusion in the *amyE* gene; *purA* expression was determined in strains 168 and KNO9c (*yabJ*) by measuring adenylosuccinate synthetase activity. Values are means of three experiments. The variation was less than 20%.

Function of PurR-controlled genes *glyA*, *yumD*, *yqhZ-folD*, *yebB*, and *ytiP*. The derived amino acid sequence of *glyA* has high amino acid sequence similarity to SHMT from *E. coli* (accession no. P00477). In agreement with this, *glyA* mutant strain HH413 required glycine for growth. The SHMT levels were determined in cultures grown in the presence of 1 mM guanosine to induce the expression of the enzyme. The SHMT activity was found to be 3.2 U/mg of protein in strain 168 and <0.2 U/mg of protein in strain HH413. This indicates that the *glyA* gene actually encodes SHMT activity.

The derived amino acid sequence of *yumD* shows high amino acid sequence similarity to GMP reductase from *E. coli* (accession no. AAC73215) and to other putative GMP reductases and IMP dehydrogenases. The levels of GMP reductase were <0.03 U/mg of protein in HH355 (*yumD*) and 4.9 U/mg of protein in strain 168 grown in the presence of the inducer guanosine. This indicates that *yumD* encodes GMP reductase, and we suggest the new designation *guaC*.

The YqhZ primary structure has 40% amino acid sequence identity with the protein encoded by *E. coli nusB* (accession no. X00681). NusB has been shown to be involved in factor-dependent transcription termination in *E. coli*. An *E. coli nusB* mutant shows a reduced growth rate (19); however, this was not observed in a *B. subtilis yqhZ* mutant (see below). The derived amino acid sequence of *folD* has 52% amino acid sequence identity with the *E. coli folD* gene product (accession no. P24186), which encodes the bifunctional enzyme methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase (3), which forms N^{10}-formyl-tetrahydrofolate (N^{10}-formyl-THFA), which is essential for de novo synthesis of IMP (Fig. 3). Another reaction in which N^{10}-formyl-THFA is used is the synthesis of formylmethionyl tRNA, a reaction that is not essential for the growth of *B. subtilis* (1). To show that

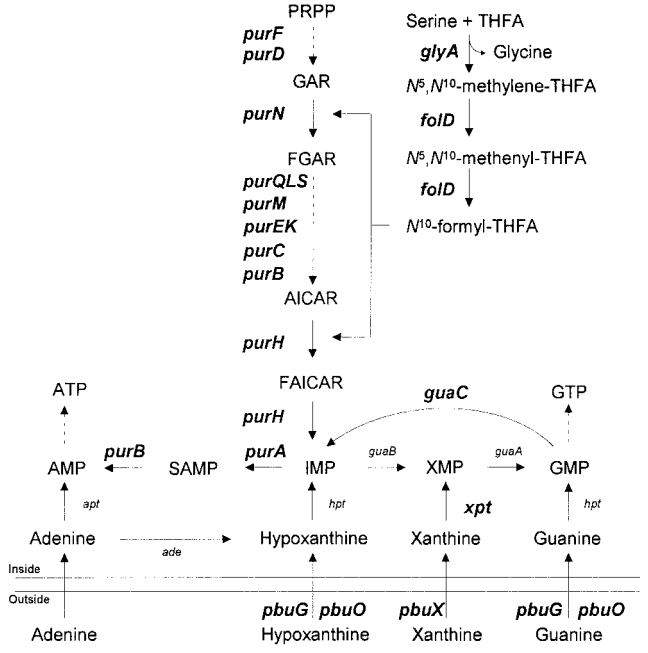

FIG. 3. Map of metabolic pathways in *B. subtilis* that are regulated by PurR. The different enzymatic steps are represented by the corresponding gene designations. Gene designations in a large font and in boldface represent genes that are regulated by PurR, while gene designations in a small font represent genes that are not regulated by PurR. Abbreviations: GAR, phosphoribosylglycinamide; FGAR, phosphoribosylformylglycinamide; AICAR, phosphoribosylaminoimidazole carboxamide; FAICAR, phosphoribosylformamidoimidazole carboxamide; SAMP, adenylosuccinate. Gene designations: *purF*, glutamine PRPP amidotransferase; *purD*, phosphoribosylglycinamide synthetase; *purN*, THFA-dependent phosphoribosylglycinamide transformylases; *purQLS*, phosphoribosylformylglycinamidine synthetases I, II, and III; *purM*, phosphoribosylaminoimidazole synthetase; *purEK*, phosphoribosylaminoimidazole carboxylases I and II; *purC*, phosphoribosylaminoimidazolesuccinocarboxamide synthetase; *purB*, adenylosuccinate lyase; *purH*, phosphoribosylaminoimidazole carboxamide formyltransferase and IMP cyclohydrolase; *purA*, adenylosuccinate synthetase; *guaB*, IMP dehydrogenase; *guaA*, GMP synthetase; *apt*, adenine phosphoribosyltransferase; *hpt*, hypoxanthine-guanine phosphoribosyltransferase; *xpt*, xanthine phosphoribosyltransferase; *guaC*, GMP reductase; *ade*, adenine deaminase; *pbuG*, hypoxanthine-guanine permease; *pbuX*, xanthine permease; *pbuO*, guanine permease; *glyA*, SHMT; *folD*, methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase. Dashed lines indicate multiple enzyme-catalyzed steps.

the *folD* gene actually encodes the enzyme catalyzing the last two steps in the synthesis of N^{10}-formyl-THFA, conditions during which the gene was not expressed were studied. The *folD* gene is located downstream of *yqhZ*. In strain YQHZd, pMutin4 is integrated in *yqhZ* and expression of the downstream gene *folD* is driven by the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible P_{spac} promoter (20). The *yqhZ*::pMutin4 mutation was transformed into strain 168/pMAP65, which overproduces the LacI repressor protein encoded by the pMAP65 plasmid (9). A high level of LacI ensures that the P_{spac} promoter upstream of *folD* is almost completely shut down. The new strain ED448 (*yqhZ*::pMutin4 pMAP65) could grow in rich medium but not in minimal medium unless supplemented with IPTG or hypoxanthine (Table 7), indicating that the function ascribed to *folD* is correct. Growth was further increased when cells were grown in medium supplemented with Casamino Acids, indicating that protein synthesis can be increased despite the presumed lack of formylmethionyl tRNA in strain ED448 (Table 7). A low but significant level of N^{10}-formyl-THFA synthetase activity has been measured in *B. subtilis* (22). N^{10}-formyl-THFA synthetase catalyzes the synthesis of N^{10}-formyl-THFA from THFA and formic acid. However, addition of formic acid to strain ED448 did not stimulate growth, indicating insufficient formation of N^{10}-formyl-THFA from formic acid in the *yqhZ* mutant strain.

TABLE 7. Effect of hypoxanthine and IPTG on growth of a *folD* mutant[a]

Strain	Relevant genotype	Addition	Doubling time (min) in:	
			MM	Casamino Acids medium
ED448	*folD*	None	>600	>600
		IPTG[b]	72	65
		Hypoxanthine	67	37
ED449	Wild type	Hypoxanthine	54	34

[a] Both strains contain plasmid pMAP65. Cells were grown in glucose minimal medium (MM) supplemented with neomycin, erythromycin, and lincomycin plus or minus 0.2% Casamino Acids (Casamino Acids medium).
[b] Expression of *folD* was induced by addition of IPTG (0.2 mM).

The *yebB* gene is located close to the 5′ end of the *pur* operon, within a region that has previously been shown to contain the *pbuG* gene that encodes a hypoxanthine-guanine permease (14). Hypoxanthine uptake was measured in BFA2255 (*yebB*::pMutin1) and was found to be 0.1 U/mg of cell dry weight, compared to 2 U/mg of cell dry weight in strain 168. BFA2255, like a *pbuG* mutant strain (14), was found to be resistant to 0.5 mM azaguanine. These observations indicate that *yebB* and *pbuG* are the same gene, and we therefore suggest the original designation *pbuG* for *yebB*.

The YtiP sequence (432 amino acids) shows 47% amino acid sequence identity with the 440-amino-acid hypoxanthine-guanine permease PbuG. Strain BFA2025 (*ytiP*::pMutin1) was analyzed for its purine base uptake phenotype, and it was found that the mutant strain had a 50% reduction in guanine and hypoxanthine uptake compared to the wild type. This indicates that *ytiP* encodes a guanine-hypoxanthine permease. We suggest that the designation *pbuO* (purine base uptake, 6-oxopurine) replace the designation *ytiP*.

DISCUSSION

Based on the experimental results presented in this work, we were able to expand the *B. subtilis* PurR regulon with six mono- or dicistronic operons. The function and expression of the *xpt-pbuX* operon have been previously reported (2), while the functions of the genes *yumD* (*guaC*), *yebB* (*pbuG*), *glyA*, *yqhZ-folD*, and *ytiP* (*pbuO*) are described in this work. Two genes were shown to encode purine base permeases. *yebB* encodes a high-affinity hypoxanthine-guanine permease already known as *pbuG* (14). A *pbuO* (formerly *ytiP*) mutant was shown to be impaired in guanine and hypoxanthine uptake. The purine base concentration used in the uptake assay was low (1 μM). At this concentration, PbuG has been shown to be the major transport system for guanine because *pbuG* deficiency results

in a low level of guanine uptake (14). The residual guanine uptake at 1 μM guanine in the *pbuG* mutant strain could be due to transport through PbuO. PbuG deficiency has no effect on the growth of a purine-requiring mutant strain when guanine or hypoxanthine is present at a concentration higher than 100 μM (14). Most likely, *pbuO* encodes a guanine-hypoxanthine permease working at purine concentrations higher than 100 μM.

Two genes, *glyA* and *folD*, encode enzymes involved in N^{10}-formyl-THFA formation. Based on genetic data and on growth analysis of a *glyA* mutant, Dartois and coworkers suggested that *glyA* encodes SHMT (4). By measuring SHMT activity in a *glyA* knockout mutant, we have finally established the function of this gene in *B. subtilis. folD* was the only gene whose function was only indirectly demonstrated. The gene appears not to be essential as long as IMP can be synthesized from an external purine source. Finally, *yumD* (*guaC*) was identified as the gene encoding GMP reductase activity.

The previously identified PurR-regulated genes (*pur* operon and *purA* [17]) plus the newly identified ones allowed us to construct a map of the PurR-affected pathways in *B. subtilis*. In Fig. 3, it can be seen that the majority of the genes involved in purine base, purine nucleoside, and purine nucleoside monophosphate metabolism are regulated by PurR. Figure 3 also illustrates the three steps of THFA metabolism that are regulated by PurR. In *E. coli*, the formation of N^5,N^{10}-methylene-THFA is regulated by purine levels and PurR through the repression of *glyA* expression (18). However, *folD* in *E. coli* appears not to be controlled by PurR. Among all of the PurR-regulated genes, *yqhZ*, which encodes a potential NusB-like factor involved in transcription termination, is the only gene without an obvious role in purine metabolism.

We have shown that all of the *B. subtilis* genes and operons that have been experimentally demonstrated to be regulated by PurR are preceded by a palindromic sequence composed of two divergently oriented PurBoxes separated by 16 or 17 nucleotides. We have compared our data with previously obtained footprinting data (17) in which purified PurR protein was found to protect an extended region upstream of the *pur* operon, *purA* and the *purR-yabJ* operon. From this comparison, it is evident that the common dyad symmetry 5′-GAAC-$N_{(24–25)}$-GTTC-3′ motif identified by Shin and coworkers (17) is included in the tandem PurBox motif defined in this work (Fig. 1). Characteristic for the footprinting data are the large regions of 80 to 90 nucleotides that were protected by PurR protein. The extended protected regions reported by Shin and coworkers were found to be primarily on the 5′ side in relation to the two PurBoxes and the 5′-GAAC-$N_{(24–25)}$-GTTC-3′ motif. Analysis of the minimal regulatory sequence requirement for full PurR control of *glyA* expression revealed that no extended 5′ region relative to the tandem PurBox motif was required. This leads us to suggest that the binding of PurR to sequences upstream of the twin PurBox sequences, as demonstrated by previous in vitro footprinting experiments, most likely plays no role in vivo. The tandem PurBox motif may be located at various positions both up- or downstream of the transcriptional start site (Fig. 1). In the case of *ytiP* (*pbuO*), *yumD* (*guaC*), and *purR*, the PurBoxes are located close to or overlapping the sequence encoding the potential ribosome binding site. In the case of the *pur* operon, the *xpt-pbuX* operon, and *yebB* (*pbuG*), the PurBoxes are located 230 to 274 nucleotides upstream of the coding region of the first gene of the operon. This long distance is due to the presence of a long untranslated leader sequence that, in the case of the *pur* and *xpt-pbuX* operons, has been shown to be the site for the hypoxanthine-and-guanine-controlled regulatory mechanism. The PurBoxes in front of the *pur* operon, *xpt-pbuX* and *pbuG*, are located 4 (*pur* operon and *pbuG*) and 14 (*xpt-pbuX*) nucleotides upstream of the promoter −35 elements—distances that are consistent with the PurBoxes functioning as repressor binding sites. In the *glyA* regulatory region, the PurBoxes are located 35 nucleotides upstream of the −35 element. This may appear to be a rather long distance. However, as demonstrated by the published footprint analysis (17), PurR protects DNA sequences (approximately 20 nucleotides in length) located downstream of the 5′-GAAC-$N_{(24–25)}$-GTTC-3′ motif, which coincides with the PurBoxes. We speculate that PurR represses *glyA* transcription by first binding to the PurBoxes and then multimerizes along the DNA as suggested previously (7).

Addition of adenine to *B. subtilis* results in a drop in the cellular PRPP pool, thereby increasing the binding of PurR to its operator sequence. This results in an average of 2.5- to 3-fold repression of gene expression (Table 3) (10, 15). In contrast, addition of guanosine increases PRPP pools, resulting in decreased PurR binding and two- to threefold induction of gene expression (Table 7) (10, 15). Rappu and coworkers have suggested that stronger binding of PurR to operator DNA when the PRPP pool is low requires the *yabJ* gene product, and it was suggested that a possible function of YabJ is to interact with PurR to form a multimeric PurR structure. This would result in the binding and protection of the extended operator sequence by PurR observed in footprinting experiments. We investigated the effect of YabJ deficiency on the repression of expression of one of the novel PurR-controlled gene *glyA* and of *purA*, for which the repression was shown by Rappu and coworkers to be YabJ dependent. We were not able to detect any changes in either *glyA* or *purA* expression in a *yabJ* mutant strain compared to that in the wild type (Table 6). The two *yabJ* mutations, however, were not identical. Rappu and coworkers constructed a 39-amino-acid deletion of the YabJ (125 amino acids long) N-terminal end, whereas the mutation analyzed in this report was a 42-amino-acid deletion of the C-terminal end. Even though it appears unlikely that the repressor auxiliary function of YabJ may be dependent on the N-terminal part, this might be a possibility. Until this has been analyzed in more detail, the role of YabJ in the process of PurR-controlled gene expression remains questionable.

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