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Transcription Analysis of the *Bacillus subtilis* PucR Regulon and Identification of a *cis*-Acting Sequence Required for PucR-Regulated Expression of Genes Involved in Purine Catabolism

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The PucR protein of *Bacillus subtilis* has previously been suggested to regulate the expression of 15 genes, *pucABCDE*, *pucFG*, *pucH*, *pucI*, *pucJKLM*, *pucR*, and *gde*, all of which encode proteins involved in purine catabolism. When cells are grown under nitrogen-limiting conditions, the expression of these genes is induced and intermediary compounds of the purine catabolic pathway affect this expression. By using *pucR* deletion mutants, we have found that PucR induces the expression of *pucFG*, *pucH*, *pucI*, *pucJKLM*, and *gde* while it represses the expression of *pucR* and *pucABCDE*. Deletions in the promoters of the five induced operons and genes combined with bioinformatic analysis suggested a conserved upstream activating sequence, 5′-WWWC NTTGGTTAA-3′, now named the PucR box. Potential PucR boxes overlapping the −35 and −10 regions of the *pucABCDE* promoter and located downstream of the *pucR* transcription start point were also found. The positions of these PucR boxes are consistent with PucR acting as a negative regulator of *pucABCDE* and *pucR* expression. Site-directed mutations in the PucR box upstream of *pucH* and *pucI* identified positions that are essential for the induction of *pucH* and *pucI* expression, respectively. Mutants with decreased *pucH* or increased *pucR* expression obtained from a library of clones containing random mutations in the *pucH*-to-*pucR* intercistronic region all contained mutations in or near the PucR box. The induction of *pucR* expression under nitrogen-limiting conditions was found to be mediated by the global nitrogen-regulatory protein TnrA. In other gram-positive bacteria, we have found open reading frames that encode proteins similar to PucR located next to other open reading frames encoding proteins with similarity to purine catabolic enzymes. Hence, the PucR homologues are likely to exert the same function in other gram-positive bacteria as PucR does in *B. subtilis*.

The soil bacilli can utilize different nitrogen-containing compounds as their sole source of nitrogen. *Bacillus subtilis* prefers ammonia or glutamine as its nitrogen source, and it assimilates all of its nitrogen by the glutamine synthetase (GS)-catalyzed reaction NH_3 + glutamate + ATP → glutamine + ADP + P_i. When *B. subtilis* is grown in media with a less preferred nitrogen source, a number of enzymes and permeases involved in the assimilation of nitrogen from alternative nitrogen-containing compounds are induced. Among them are asparaginase (1), *gabP*-encoded γ-aminobutyric acid permease (6), *ureABC*-encoded urease (4, 20), *amtB-glnK*-encoded ammonium transport proteins (19), and *nasABCDEF*-encoded nitrate assimilatory enzymes (9). The induction of these genes is dependent on the global nitrogen-regulatory protein TnrA. A second global nitrogen-regulatory protein, GlnR, is active during nitrogen excess conditions and negatively regulates the expression of the *glnRA* operon encoding GlnR and GS. TnrA and GlnR are homologous and bind to the same DNA sequence, known as the TnrA/GlnR box (5′-TGTNAN$_7$TNACA-3′). During growth with excess nitrogen (glutamine or ammonia plus glutamate), feedback-inhibited GS stimulates GlnR binding activity but inhibits the action of TnrA through a direct protein-protein interaction (24), thereby preventing the expression of alternative nitrogen-assimilatory pathways. When cells are grown with limited nitrogen, the binding of GS to TnrA disappears and TnrA becomes active while GlnR binding activity is inhibited. This leads to the induction of alternative nitrogen-assimilatory pathways (7, 21, 23).

B. subtilis is capable of taking up purine bases and using them for nucleotide synthesis and as a source of nitrogen. To enter the catabolic pathway, guanine, adenine, and hypoxanthine must be converted to xanthine (10). The genes encoding the enzymes and permeases necessary for a complete degradation of purine bases to ammonia and for the transport of uric acid and allantoin have recently been identified (15). They are *pucABCDE* (xanthine dehydrogenase), *pucF* (allantoate amidohydrolase), *pucH* (allantoinase), *pucI* (allantoin permease), *pucJK* (uric acid transport), and *pucLM* (uricase). Inactivation of the *pucR* gene prevents the expression of *gde* (guanine deaminase) and all of the *puc* genes (except *pucA*). The PucR protein (purine catabolism regulator) shows limited sequence homology to hypothetical proteins from various organisms and was suggested to be a transcriptional regulator responsible for the regulation of expression of the genes of the purine catabolic pathway (15).

This work presents genetic evidence that the PucR protein controls the expression of the genes involved in the purine catabolic pathway and that induction requires a *cis*-acting regulatory element. Furthermore, we have shown that TnrA induces transcription of *pucR* under nitrogen-limiting conditions

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
B. subtilis		
168	*trpC2*	C. Anagnostopoulos[a]
HØR18	*trpC2 pucR*::pBOER	15
HØR26	*trpC2 pucR*::P_{spac}-*pucR*	Transformation of 168 by pIMut4 selecting for Er^r
LCC26	*trpC2 tnrA62*::*Tn*917	Transformation of 168 with DNA from SF416R selecting for Er^r (2)
E. coli		
MC1061	F^- *araD139* Δ(*ara-leu*)*7697 galE15 galK16* Δ(*lac*)*74 rpsL* (Str^r) *hsdR2* ($r^- m^+$) *mcrA mcrB1*	Laboratory stock
Plasmids		
pDG268neo	Ap^r (*E. coli*) Neo^r (*B. subtilis*); vector used for construction of transcriptional *lacZ* fusion designed to integrate into the *amyE* gene of *B. subtilis*	11
pIMut4	Ln^r, Er^r, P_{spac}-*pucR*, $LacZ^-$; pMUTIN4 digested with *Eco*RI-*Sac*I, ligated with PCR-generated fragment containing RBS and 211 bp of the 5′-end of *pucR*; integration into the *pucR* locus results in a 3′ truncated PucR copy expressed from the *pucR* promoter and an intact PucR copy expressed from P_{spac}	This work
pMUTIN4	Integrational plasmid used for gene inactivation by recombination and for generation of transcriptional *lacZ* fusions	17
pBOE335	Ap^r (*E. coli*) Cm^r (*B. subtilis*); integration vector, pUC19 containing *cat* gene cloned into *Kas*I site	11
pBOER	Cm^r; pBOE335 containing an internal part of the *pucR* transcriptional unit; integration of pBOER results in *pucR* inactivation	15

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and that PucR is not fully active unless purine degradation products are also present.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains used in this work are listed in Tables 1 and 2 and Fig. 3 through 6. All strains are isogenic derivatives of *B. subtilis* strain 168. Under nitrogen excess conditions, *B. subtilis* was grown in Spizizen minimal salt medium containing ammonia and glutamate (11). Under nitrogen-limiting conditions, cells were grown in Spizizen minimal salt medium in which disodium sulfate (final concentration, 0.2%) was substituted for ammonium sulfate. The minimal media were supplemented with 0.4% glucose as a carbon source and 40 mg of L-tryptophan/liter. Allantoin (250 mg/liter) was added where indicated. Cells were cultured at 37°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added in a 0.1 mM concentration. Antibiotics were used for selection in the following final concentrations: ampicillin (Ap), 100 mg/liter; erythromycin (Er), 1 mg/liter; lincomycin (Ln), 25 mg/liter; neomycin (Neo), 5 mg/liter; chloramphenicol (Cm), 6 mg/liter.

DNA manipulations and genetic techniques. Isolation of DNA and RNA and basic molecular techniques, including primer extension analysis, were performed as described previously (12, 26).

Construction of transcriptional *lacZ* fusions. DNA fragments containing native promoters, promoters with deletions, or site-directed mutagenized sequences generated by PCR amplification were used. Random mutagenized sequences were generated by a modified PCR procedure (26). Specific DNA primers fitted with appropriate 5′-positioned restriction sites were used in a PCR to amplify the DNA fragments in question. PCR products were purified and digested with the restriction enzymes specific to the restriction sites incorporated at the 5′ ends of the PCR primers. The digested DNA fragments were ligated to plasmid pDG268neo, which had been digested with the same enzymes. The ligation mixtures were transformed into *Escherichia coli* MC1061 by selection for Ap^r. Clones containing the promoter DNA fragments fused in front of *lacZ* in pDG268neo were identified, and the plasmid construct was isolated. The purified plasmids were linearized by digestion with *Kpn*I and transformed into *B. subtilis* 168 by selection for Neo^r. Linearized pDG268neo derivatives were integrated by double homologous recombination into the *amyE* gene, resulting in Neo^r and amylase-negative transformants. The promoter containing fragments cloned in front of *lacZ* was amplified by flanking *neo*- and *lacZ*-specific primers, and the nucleotide sequence was determined to identify clones with the correct DNA sequence.

Strains carrying both a *lacZ* fusion and either the *pucR*, P_{spac}-*pucR*, or *tnrA* genotype were obtained by transforming the *lacZ* fusion strains with DNA isolated from HØR18, HØR26, or LCC26, respectively.

Enzyme assay. Cells were harvested in the exponential growth phase and homogenized by sonication in Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$; pH 7). β-Galactosidase activity was measured as previously described (2). One unit of activity is defined as 1 nmol of substrate converted per min per mg of protein. The total protein content was determined by the Lowry method. All enzyme levels reported are the means of results of at least three experiments. The variation was less than 25%.

Homology searches. Preliminary sequence data were obtained from The Institute for Genomic Research website (http://www.tigr.org).

RESULTS

Mapping of the transcription start site of the *pucABCDE*, *pucFG*, *pucH*, *pucI*, *pucJKLM*, and *pucR* transcriptional units. Based on inspection of the nucleotide sequence and on the pattern of gene expression described by Schultz et al. (15), six potential transcriptional units, *pucABCDE*, *pucFG*, *pucH*, *pucI*, *pucJKLM*, and *pucR*, were suggested. For all six potential promoter-containing regions, the transcription start site (+1 position) was determined by primer extension analysis (Fig. 1). No cDNA was produced in the reactions with RNA extracted from cells grown with excess nitrogen, whereas the presence of RNA from cells grown under induced conditions (glutamate plus allantoin) resulted in detectable amounts of specific cDNA products. The pattern of five specific cDNA products generated in the reaction containing the *pucH*-specific DNA primer was repeatedly obtained in three independent experi-

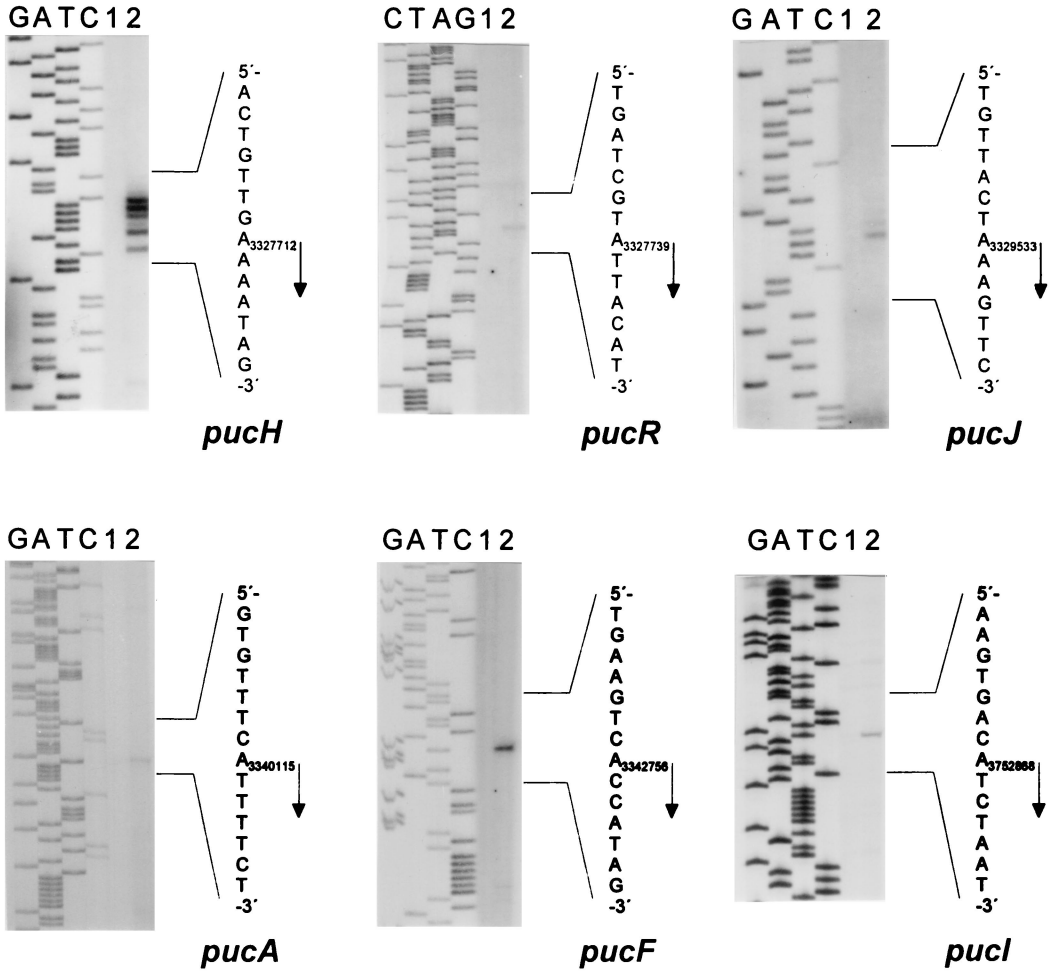


FIG. 1. Mapping of the transcription start sites for *pucA*, *pucF*, *pucH*, *pucI*, *pucJ*, and *pucR* by primer extension. Total RNA was isolated from strain 168 grown with ammonia and glutamate (lanes 1) or glutamate and allantoin (lanes 2) as the nitrogen source. The most likely transcription start point for each gene is indicated by an arrow.

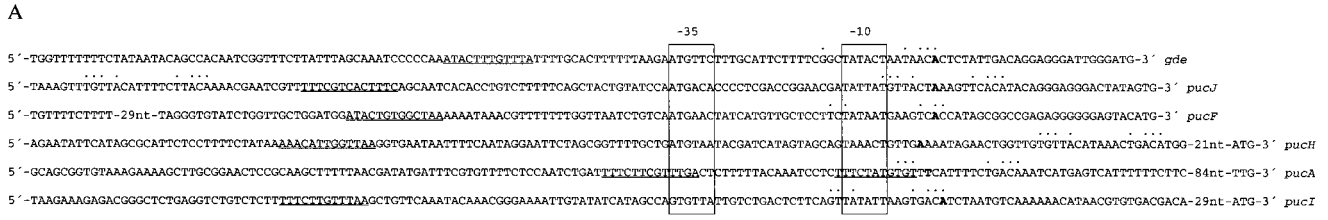
ments and may be due to transcriptional slippage (25). However, the position corresponding to the 5′ end of the longest cDNA product was chosen as the +1 position for *pucH* transcription.

The nucleotide sequences of five of the promoter regions together with that of *gde* (10) were aligned with respect to the deduced −10 and −35 promoter elements (Fig. 2A). A sequence resembling the consensus TnrA/GlnR box (5′-TGTNA N_7TNACA-3′) was detected overlapping the +1 position of all of the genes except for *pucH*, for which it was located downstream of the +1 position (Fig. 2A). In the case of *pucJ*, a second TnrA/GlnR box was located upstream of the −35.

Transcriptional control of *pucA*, *pucF*, *pucH*, *pucI*, *pucJ*, *pucR*, and *gde* expression requires PucR. DNA fragments containing the regulatory regions upstream of *pucA*, *pucF*, *pucH*, *pucI*, *pucJ*, *pucR*, and *gde* were fused to the *lacZ* reporter gene and integrated into the *amyE* locus. The β-galactosidase activity in cultures of the various fusion strains grown under different nitrogen conditions was determined. All *lacZ* fusions were induced during growth with limited nitrogen (glutamate). The addition of allantoin to glutamate cultures resulted in a further 2- to 49-fold increase of expression of all *lacZ* fusions, except for *pucA′-lacZ* and *pucR′-lacZ* expression, which were repressed 28- and 4-fold, respectively (Table 2). These results are in agreement with the results described by Schultz et al. (15).

To demonstrate the role of PucR in the expression of the *puc* gene *lacZ* fusions, a *pucR* disruption mutation (*pucR*::pBOER) was introduced into the *lacZ* fusion strains. Compared to the expression in wild-type fusion strains grown under nitrogen-limiting conditions (glutamate), the expression of the *lacZ* fusions—except for that of *pucR*—was decreased in the respective *pucR* derivatives grown under the same conditions (Table 2). The decrease in expression varied between 2-fold for *pucI* and 334-fold for *pucJ*. The β-galactosidase level in the *pucR* derivatives grown with allantoin was in general reduced to approximately less than 1% of the levels in the wild-type strains, except for *pucI*, in which it was significantly higher. This may suggest that additional regulatory factors have an influence on *pucI* expression. Again, *pucA′-lacZ* and *pucR′-lacZ* expression were derepressed in the *pucR* strain compared to those in the wild type (Table 2). We therefore conclude that *pucR* acts as a positive factor in relation to the pathway-specific induction of *pucH*, *pucJ*, *pucF*, *pucI*, and *gde* expression and as a negative factor in relation to *pucR* and *pucA* expression.

A



B

PucR box consensus sequence	**WWWCNTTGGTTAA**
pucF PucR box	**ATAC**TG**TGG**C**TAA**
pucH PucR box	**AAAC**A**TTGGTTAA**
pucI PucR box	**TTTT**C**TTG**T**TAA**
pucJ PucR box	**TTTC**GTCAC**TT**TC
gde PucR box	**ATACTTTGTTTA**T

FIG. 2. (A) Alignment of the PucR-regulated promoters of *pucA*, *pucF*, *pucH*, *pucI*, *pucJ*, and *gde* with respect to the −10 and −35 sequences. Boldface letters indicate transcription start sites (as determined from results shown in Fig. 1), −10 and −35 sequences are boxed, PucR boxes (5′-WWWCNTTGGTTAA-3′) are underlined, and matches to TnrA/GlnR boxes (5′-TGTNAN$_7$TNACA-3′) are indicated by dots above the nucleotides. nt, nucleotides. (B) Alignment of the potential PucR boxes in the PucR-induced genes. Boldface indicates a match to the consensus sequence.

We introduced the *lacZ* fusions into a *pucD* disruption strain to test whether the PucR-dependent induction also would occur under nitrogen-limiting conditions (glutamate) in a strain unable to produce purine degradation products, as a *pucD* disruption strain cannot degrade guanine or hypoxanthine to uric acid. The *lacZ* fusion strains carrying the *pucD* disruption displayed the same expression pattern (data not shown) as the wild-type strains. Thus, we can conclude that PucR is able to induce transcription without purine degradation products but that the induction is much more effective when purine degradation products are available.

Deletion analysis of *puc* gene promoter regions. Since PucR induces transcription from the promoters of *pucF*, *pucH*, *pucI*, *pucJ*, and *gde* and the PucR C-terminal sequence shows some similarity to a LysR-like DNA binding domain (15), we looked for the presence of a conserved sequence upstream of the promoters that might function as a recognition site for PucR. As we could not identify any conserved sequence by visual inspection, we made a systematic deletion analysis of all five promoter regions. In all five analyses, transcription ceased when the deletions reached a certain point (Fig. 3), but no obviously common DNA sequence could be immediately identified around these points. The Ann-spec (18) bioinformatics computer program was used to search for conserved sequences within a region spanning 100 bp upstream of the −35 sequence of the promoters of *pucF*, *pucH*, *pucI*, *pucJ*, and *gde*. Since LysR-like regulators normally recognize the sequence T-N$_{11}$-A (8, 13), the request was for a 13-bp sequence. The five 100-bp sequences were searched for a conserved sequence and compared to approximately 4,100 other upstream gene sequences from *B. subtilis*. Several 13-bp sequences were suggested. However, only one of the suggested sequences was supported by the experimental data presented in Fig. 3. This sequence was 5′-WWWCNTTGGTTAA-3′, tentatively called the PucR box. The potential PucR box sequences in front of the *puc* genes and *gde* are aligned in Fig. 2B. Partial or total deletion of the

TABLE 2. β-Galactosidase produced from transcriptional fusions[a]

Strain	Relevant genotype	β-Galactosidase activity (U/mg of protein)		
		Glutamate plus NH_4^+	Glutamate	Glutamate plus allantoin
HH422	*amyE*::*pucA′-lacZ*	1	335	12
CJ13	*amyE*::*pucA′-lacZ pucR*::pBOER	1	145	117
LB032	*amyE*::*pucF′-lacZ*	1	37	1,818
CJ12	*amyE*::*pucF′-lacZ pucR*::pBOER	1	2	19
CO7	*amyE*::*pucH′-lacZ*	2	17	354
CJ10	*amyE*::*pucH′-lacZ pucR*::pBOER	2	2	3
HØR19	*amyE*::*pucI′-lacZ*	1	23	400
HØR29	*amyE*::*pucI′-lacZ pucR*::pBOER	1	10	60
LB035	*amyE*::*pucJ′-lacZ*	2	1,002	2,336
CJ14	*amyE*::*pucJ′-lacZ pucR*::pBOER	1	3	8
CO3	*amyE*::*pucR′-lacZ*	1	27	6
LB175	*amyE*::*pucR′-lacZ pucR*::pBOER	1	508	420
LB031	*amyE*::*gde′-lacZ*	4	12	160
ED441	*amyE*::*gde′-lacZ pucR*::pBOER	5	5	3

[a] Fusions were between *lacZ* and the regulatory region upstream of *pucA, pucF, pucH, pucI, pucJ, pucR*, or *gde* integrated at the *amyE* locus of the wild-type *B. subtilis* strain and a *pucR* derivative grown under different nitrogen conditions. Cells were grown in glucose minimal medium supplemented with the indicated nitrogen source.

pucF'-lacZ fusions	DNA sequence	β-Galactosidase activity (U/mg of protein)	
		- allantoin	+ allantoin
	WWWCNTTGGTTAA -35		
LB052	-99 GTGTATCTGGTTGCTGGATGG**ATACTGTGGCTAA**AAAATAAACGTTTTTTTGGTTAATCTGTCAATGAAC	28	1846
LB128	-81 TGG**ATACTGTGGCTAA**AAAATAAACGTTTTTTTGGTTAATCTGTCAATGAAC	34	1704
LB116	-77 **TACTGTGGCTAA**AAAATAAACGTTTTTTTGGTTAATCTGTCAATGAAC	2	36
LB034	-45 TAATCTGTCAATGAAC	4	3
pucH'-lacZ fusions			
	WWWCNTTGGTTAA -35		
CO7	-112 CATAGCGCATTCTCCTTTTCTATAA**AAACATTGGTTAA**GGTGAATAATTTTCAATAGGAATTCTAGCGGTTTTGCTGATGTAA	17	354
CO8	-89 ATAA**AAACATTGGTTAA**GGTGAATAATTTTCAATAGGAATTCTAGCGGTTTTGCTGATGTAA	18	342
LB086	-76 **TTAA**GGTGAATAATTTTCAATAGGAATTCTAGCGGTTTTGCTGATGTAA	3	1
pucI'-lacZ fusions			
	WWWCNTTGGTTAA -35		
HØR39	-93 TCT**TTTTCTTGTTTAA**GCTGTTCAAATACAAACGGGAAAATTGTATATCATAGCCAGTGTTA	15	350
HH345	-80 **TAA**GCTGTTCAAATACAAACGGGAAAATTGTATATCATAGCCAGTGTTA	3	2
pucJ'-lacZ fusions			
	TnrA/GlnR box		
	TGT-A-------T-ACA **WWWCNTTGGTTAA** -35		
LB035	-113 **TGTTA**CATTTTC**TTACA**AAACGAATCGTT**TTTCGT**CAC**TTT**CAGCAATCACACCTGTCTTTTTCAGCTACTGTATCCAATGACA	1002	2336
LB056	-96 AAACGAATCGTT**TTTCGT**CAC**TTT**CAGCAATCACACCTGTCTTTTTCAGCTACTGTATCCAATGACA	1012	5900
LB085	-67 ATCACACCTGTCTTTTTCAGCTACTGTATCCAATGACA	8	14
gde'-lacZ fusions			
	WWWCNTTGGTTAA -35		
LB031	-119 (26nt)-TCGGTTTCTTATTTAGCAAATCCCCCAA**ATACTTTGTTTA**TTTTGCACTTTTTTAAGAATGTTC	12	160
LB053	-80 TAGCAAATCCCCCAA**ATACTTTGTTTA**TTTTGCACTTTTTTAAGAATGTTC	39	131
LB087	-60 **TTGTTTA**TTTTGCACTTTTTTAAGAATGTTC	1	1

FIG. 3. Deletion analysis of PucR-induced promoters. The sequences upstream of the −35 sequence of the indicated gene-*lacZ* fusions, including the PucR box, are shown. The downstream sequence fusion points are +97 for *pucH'-lacZ*, +74 for *pucI'-lacZ*, +137 for *pucJ'-lacZ*, +94 for *pucF'-lacZ*, and +78 for *gde'-lacZ*. In the case of *pucJ*, the upstream TnrA/GlnR box (Fig. 2B) is also indicated. Cells containing the various *lacZ* fusions were grown under nitrogen-limiting conditions (glutamate) either with or without allantoin. nt, nucleotides. Boldface indicates a match to the consensus sequence.

PucR box can explain the loss of promoter activity in deletion mutants of the *pucF'-*, *pucH'-*, *pucI'-*, *pucJ'-*, and *gde'-lacZ* promoter fusions. It appears that the activation of transcription from these five genes requires a *cis*-acting sequence containing the PucR box motif and that this sequence could be part of a recognition site for the putative activator protein encoded by *pucR*.

The effect of the TnrA/GlnR box located upstream of *pucJ* was analyzed in strains LB035 and LB056, in which the TnrA/GlnR box was deleted (Fig. 3). A deletion of the TnrA/GlnR box did not affect expression under nitrogen-limiting conditions (glutamate), but with limited nitrogen plus allantoin the expression was elevated by a factor of 2.5. Hence, the TnrA/GlnR box upstream of the −35 sequence in the *pucJ* promoter exerts a negative regulatory function on *pucJ* expression under induced-growth conditions.

When the potential PucR box in front of *pucR* was deleted, the activity from the *pucR'-lacZ* promoter fusion increased almost 20-fold when cells were grown under nitrogen-limiting conditions (Fig. 4). Surprisingly, the activity was only slightly increased for limited nitrogen supplied with allantoin. This indicates that there might be another sequence important for the repression of the *pucR* promoter when allantoin is present (see below). Two potential PucR boxes overlapping the −35 and −10 sequences in the *pucA* promoter were also found (Fig. 2A and 4). This finding agrees with the data showing that PucR inhibited transcription from the *pucA* promoter under nitrogen-limiting conditions with allantoin present (Table 2).

Site-directed and random mutagenesis analysis of the *pucH* and *pucI* regulatory regions. In order to verify the PucR box motif, site-directed mutagenesis was performed in all of the positions of the PucR box in the *pucH* promoter and β-galactosidase activity was measured in the respective fusion strains (Fig. 5). The three substitutions in LB079, LB094, and LB067 caused an almost complete loss of activity, as observed in some of the promoter deletions (see Fig. 3, strain LB086). Other substitutions had a less dramatic effect. The substitutions in LB076, LB078, and LB081 decreased the activity two- to fourfold compared to that of the wild type. To test whether some of these substitutions had the same effect on the expression from other *puc* promoters, site-directed substitutions were made in the PucR box of the *pucI* regulatory region at the same three positions. Promoter activity in LB129 and LB131 was greatly reduced, as it was in LB079 and LB067, and the promoter activity was impaired in strain LB130, as it was in LB078. Thus, changes in the same positions in the PucR boxes upstream of *pucI* and *pucH* resulted in similar decreases in transcription in the mutants. We therefore conclude that the PucR box suggested by the use of bioinformatics covers some very important bases for PucR activation. Interestingly, the G-to-C mutation in the *pucH'-lacZ* fusion in strain LB094 almost abolished activity; however, in the *pucI* PucR box of the wild type,

pucA'-lacZ fusions	DNA sequence	β-Galactosidase activity (U/mg of protein)	
		- allantoin	+ allantoin
	WWWCNTTGGTTAA **WWWCNTTGGTTAA**		
	-35 +1		
HH422	-217 (147nt)-ATGATTTCGTGTTTTCTCCAATCTGAT**TTTCTT**C**GTTTGA**CTCTTTTTACAAATCCTC**TTTCTATG**TGT**TT**	335	12
LB125	-46 GAT**TTTCTT**C**GTTTGA**CTCTTTTTACAAATCCTC**TTTCTATG**TGT**TT**	151	2
LB126	-36 **GTTTGA**CTCTTTTTACAAATCCTC**TTTCTATG**TGT**TT**	110	3
pucR'-lacZ fusions			
	+1 **AATTGGTTNCWWW**		
CO3	GTATTACATCAGCAAAACCGCTAGAATTCCTATTGAAAATTATTCACC**TTAACCAATGTTT**TTATAGAAAAGGAGAATGCGCTAT +83	27	6
CO9	GTATTACATCAGCAAAACCGCTAGAATTCCTATTGAAAATTATTCACC**TTAACCAATGTTT**TTAT +63	34	16
HH423	GTATTACATCAGCAAAACCGCTAGAATTC +25	475	12
HH424	GTAT +2	615	37

FIG. 4. Deletion analysis of PucR-repressed promoters. The upstream sequences and the positions of the upstream fusion points of the *pucA'-lacZ* fusions are shown (the downstream sequence fusion point is +180), as are the positions of the downstream sequences and the downstream fusion points of the *pucR'-lacZ* fusions (the upstream sequence fusion point is −123). The PucR box is indicated (boldface indicates a match with the consensus sequence), as is the −35 sequence. A complementary PucR box in the *pucR'-lacZ* fusions is located in the noncoding strand. Cells containing the various *lacZ* fusions were grown under nitrogen-limiting conditions (glutamate) either with or without allantoin. nt, nucleotides.

there is a T instead of a G at this position. This indicates that the PucR box might be more degenerate than suggested, as other deviations from the proposed PucR box consensus sequence were observed in the other candidate PucR boxes.

The *pucH* and *pucR* reading frames are divergently oriented and separated by a 174-bp intercistronic sequence that contains a PucR box. To investigate whether there were important positions other than the putative PucR box, random mutagenesis was performed on the upstream promoter sequences of the *pucH* and *pucR* genes fused to *lacZ*. Clones from the *pucH'-lacZ* library in which β-galactosidase activity was decreased under nitrogen-limiting conditions with allantoin were isolated and sequenced. All of the mutations were found in or downstream of the deduced PucR box (Fig. 6). From a similar *pucR'-lacZ* library, clones that showed increased β-galactosidase activity when grown under nitrogen-limiting conditions without allantoin were selected and analyzed. Here the mutations were found in or near the PucR box, corresponding to the same area found in the *pucH'-lacZ* library. The fivefold increase in *pucR'-lacZ* expression in the randomly mutagenized *pucR'-lacZ* mutants was observed only in glutamate-grown cultures (Fig. 6). The same tendency, but more pronounced, was observed in a mutant strain with a deletion of the entire PucR box (475 U/mg of protein; strain HH423, Fig. 4). In both cases, the expression level in glutamate-plus-allantoin-grown cultures was similar to the wild-type level (approximately 6 to 12 U/mg of protein). Only in a *pucR*-defective strain was *pucR'-lacZ* expression constitutively high under both nitrogen conditions (strain LB175, Table 2). Deletion of or mutations in the same PucR box in a *pucH'-lacZ* fusion strain (Fig. 3, 5, and 6)

pucH'-lacZ fusions	DNA sequence	β-Galactosidase activity (U/mg of protein)	
		- allantoin	+ allantoin
	WWWCNTTGGTTAA -35		
CO8 (wt)	-89 ATAA**AAACATTGGTTAA**GGTGAATAATTTTCAATAGGAATTCTAGCGGTTTTGCTGATGTAA	18	342
LB065	**C**----------	19	368
LB075	-**C**---------	14	289
LB076	--**C**--------	10	160
LB093	---**G**-------	10	404
LB077	-----**C**------	8	472
LB078	------**C**-----	10	83
LB079	-------**C**----	2	2
LB094	--------**C**---	4	9
LB080	---------**C**--	7	134
LB066	----------**C**-	8	278
LB067	-----------**C**	4	14
LB081	----------**C**	5	102
pucI'-lacZ fusions			
	WWWCNTTGGTTAA -35		
HØR39 (wt)	-93 TCT**TTTT**C**TTGTTTAA**GCTGTTCAAATACAAACGGGAAAATTGTATATCATAGCCAGTGTTA	15	350
LB130	------**C**------	6	30
LB129	-------**C**-----	2	1
LB131	----------**C**-	5	11

FIG. 5. Site-directed mutational analysis of the proposed PucR box in the *pucH* and *pucI* regulatory regions. The sequences upstream of the −35 region of the *pucH'-lacZ* and *pucI'-lacZ* fusions are listed. The PucR box is indicated (boldface indicates a match to the consensus sequence), and the positions of the upstream fusion points are shown. The downstream sequence fusion points are +97 for *pucH'-lacZ* and +74 for *pucI'-lacZ*. Cells containing the various *lacZ* fusions were grown under nitrogen-limiting conditions (glutamate) either with or without allantoin. wt, wild type.

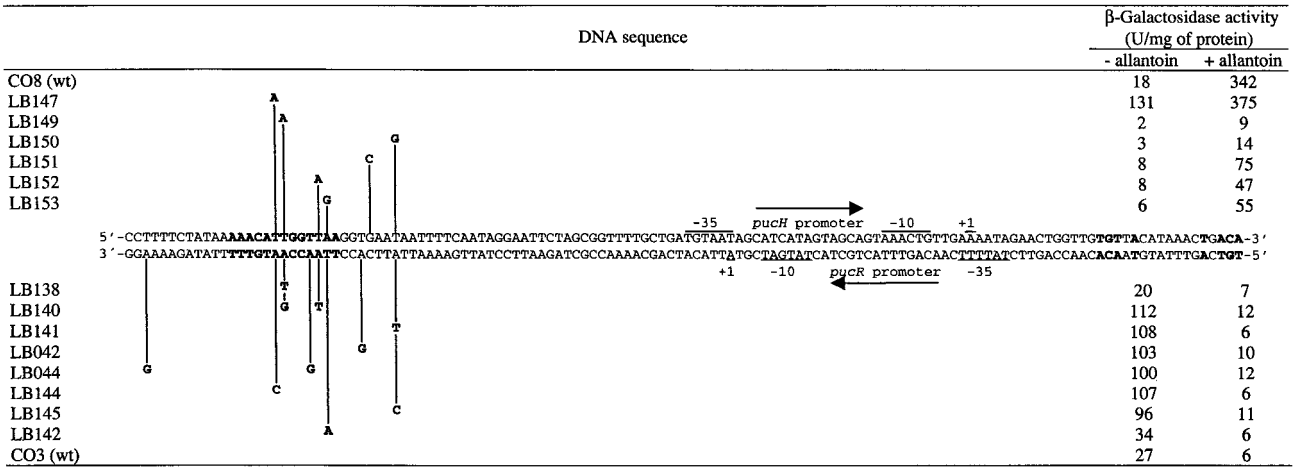


FIG. 6. Random mutational analysis of the proposed PucR box in the *pucH* and *pucR* regulatory region. Part of the *pucH*-to-*pucR* intercistronic region is presented as a double-stranded DNA sequence. The *pucH′-lacZ* fusion strains (the upstream and downstream sequence fusion points are −110 and +97) are listed above the double-stranded DNA sequence, and the *pucR′-lacZ* fusion strains (the upstream and downstream sequence fusion points are −123 and +84) are listed below. The PucR box (5′-WWWCNTTGGTTAA-3′) and TnrA/GlnR box (5′-TGTNAN$_7$TNACA-3′) are listed (boldface indicates a match to the consensus sequence), and the −10 and −35 sequences and the +1 positions are indicated. Cells containing the various *lacZ* fusions were grown under nitrogen-limiting conditions (glutamate) either with or without allantoin. wt, wild type.

resulted in a complete loss of *pucH* expression. This leads us to suggest that the PucR box in the *pucH*-to-*pucR* intercistronic region is strictly required for the induction of *pucH* expression while it is only partially required for the repression of *pucR* expression and that a second *cis*-acting element located upstream of the PucR box is required for complete *pucR* repression. The second *cis*-acting element may be an additional PucR box located upstream of the *pucR* +1 position overlapping the −35 *pucR* sequence. This potential PucR box has less homology to the consensus sequence, and due to the lower homology, it is possible that PucR may bind to it only when an inducer molecule, i.e., allantoin, is present. Hence, PucR binds to this additional PucR box only under nitrogen-limiting conditions with purine degradation products available, and this binding is responsible for the repression of *pucR* expression under these conditions.

The global nitrogen regulator TnrA controls *pucR* expression. A single copy of the palindromic TnrA/GlnR box was identified in the *pucH*-to-*pucR* intercistronic region (Fig. 6). The TnrA/GlnR box is located 10 bp upstream of the deduced −35 region of the *pucR* promoter. TnrA/GlnR boxes were found at a similar distance upstream of other genes that have been shown to be activated by the TnrA activator protein (22). To test whether the induction of *pucR* expression is mediated by TnrA, a *pucR′-lacZ* fusion strain (CO6) with a deletion of the TnrA/GlnR box was constructed and its *pucR′-lacZ* expression was compared to that of strain CO3 (Table 2), which contains a *pucR′-lacZ* fusion including the TnrA/GlnR box. The β-galactosidase level in CO6 grown under nitrogen-limiting conditions (glutamate) was 4 U/mg of protein, compared to 27 U/mg of protein in CO3. A strain (CJ015) was constructed that contained the *pucR′-lacZ* fusion of strain CO3 in a *tnrA* genetic background, and as found in strain CO6, *pucR′-lacZ* expression could no longer be induced (4 U/mg of protein during growth with glutamate). We therefore conclude that TnrA activates the expression of *pucR* during nitrogen-limiting conditions.

To test whether TnrA also plays a direct role in the PucR-dependent activation of *puc* genes and *gde*, the *pucR* gene expression was put under the control of the IPTG-inducible P$_{spac}$ promoter. This construction (pIMut4, Table 1) was introduced into the *pucA′-*, *pucF′-*, *pucH′-*, *pucI′-*, *pucJ′-*, and *gde′-lacZ* fusion strains. The strains were grown under nitrogen excess conditions (ammonia plus glutamate), in which the TnrA protein is not active. The basal level of *pucR* expression under nitrogen excess conditions for the *pucH′-lacZ* fusion strain under the P$_{spac}$ promoter was 4 U/mg of protein. In the presence of IPTG, which induces *pucR* expression, the level was increased to 11 U/mg of protein, while the addition of both IPTG and allantoin led to a strong induction of expression of the *pucH′-lacZ* fusion (371 U/mg of protein). These levels were similar to the β-galactosidase level of strain CO7, which carries the same *lacZ* fusion in a PucR$^+$ background. The same pattern of expression was observed for *pucF′-*, *pucI′-*, *pucJ′-*, and *gde′-lacZ* fusion strains (data not shown). However, in the case of a *pucA′-lacZ* fusion strain, the β-galactosidase level was low (2 to 3 U/mg of protein) under all three conditions. This experiment demonstrates that synthesis of PucR, even under growth conditions where *pucR* normally is not expressed (excess nitrogen conditions), stimulates transcription from the promoters of *pucF*, *pucH*, *pucI*, *pucJ*, and *gde*. Expression was further stimulated 15- to 42-fold by allantoin, and the PucR protein did not require active TnrA protein to induce expression. PucR induction and the addition of allantoin in the P$_{spac}$-*pucR* strain did not induce *pucA* transcription. This is consistent with the observation that in the presence of allantoin, PucR most likely has a negative effect on *pucA* expression. The induction of *pucA* is PucR independent, since induction is observed in a *pucR* genetic background (Table 2). TnrA may be a candidate for a transcription factor responsible for the

induction of *pucA* under nitrogen-limiting conditions. Evidence for this view comes from the finding that the expression of *pucABCDE* is high in a *glnA* background (10). However, this was not tested experimentally.

DISCUSSION

Similarity searches in the recently fully sequenced genomes and in partially finished sequenced genomes revealed that at least five other gram-positive bacteria, *Enterococcus faecalis* V583, *Bacillus halodurans* C-125, *Mycobacterium smegmatis*, *Listeria monocytogenes* 4-b, and *Clostridium acetobutylicum*, possess proteins with significant amino acid similarity to PucR. In both *E. faecalis* and *B. halodurans*, the *pucR*-like genes are located together with other genes encoding putative purine catabolic functions. The gene homologous to *pucR* in *E. faecalis* (open reading frame [ORF] EF2995) and ORF EF2994, which encodes a putative transaminase similar to *B. subtilis* PucG, are divergently oriented, which suggests that the two genes are divergently transcribed. ORFs encoding a PucF-like allantoate amidohydrolase (EF2997), a PucH-like allantoinase (EF2999), and a PucI-like allantoin permease (EF3000) are located downstream of the putative *pucR* in *E. faecalis*. In *B. halodurans*, the *pucR* (ORF BH0757) is also located at a position that suggests that it is divergently transcribed from three ORFs (BH0758, BH0759, and BH0760) which encode a putative uricase similar to the uricase encoded by the *B. subtilis pucLM* operon. In *C. acetobutylicum*, a PucR homologue (ORF CAC1426) was not found to be linked to other genes recognized as being involved in purine catabolism. In fact, only very limited similarity was found to genes from the purine catabolic pathway in *B. subtilis* in this bacterium. However, ORFs with amino acid sequence similarity to PucR, PucH, and PucJ have been found in the partially sequenced genomes of *Clostridium perfringens*, *L. monocytogenes*, and *M. smegmatis*. Thus, PucR is found not only in *B. subtilis* but also in other gram-positive bacteria and is often linked to other ORFs with similarity to purine catabolic enzymes. Therefore, the PucR homologues in these bacteria are likely to exert the same function as PucR in *B. subtilis*.

Two proteins, SrmR and SdaR, with known functions as transcriptional activators were recorded among the proteins showing similarity to PucR (15). An alignment (15) indicated that PucR, SrmR, and SdaR might contain a LysR-like DNA-binding domain in their C termini. However, the general sequence similarity to the LysR family and other known families of regulatory proteins is not significant, indicating that PucR is not related to any of the other known families. We therefore suggest that PucR, SrmR, SdaR, and the PucR homologues in *E. faecalis*, *B. halodurans*, *M. smegmatis*, *C. perfringens*, and *C. acetobutylicum* may constitute a novel family of transcriptional regulators.

Based on the results presented in this paper and on the work of Schultz et al. (15), we suggest a model for the global nitrogen catabolite repression and pathway-specific regulation of the PucR regulon genes in *B. subtilis*. Purine catabolic genes, together with other genes encoding alternative nitrogen-assimilatory pathways, are not expressed during nitrogen excess conditions due to the inhibition of TnrA activity by GS encoded by *glnA*. The *glnRA* operon repressor GlnR controls the level of *glnA* expression during excess nitrogen conditions (7, 14). Under nitrogen-limiting conditions (glutamate as nitrogen source), TnrA becomes active while GlnR becomes inactive. TnrA activates *pucR* transcription most likely by binding to the TnrA/GlnR box located upstream of the *pucR* promoter (Fig. 6). As shown in Table 2, expression of the *pucABCDE*, *pucFG*, *pucI*, *pucH*, *pucJKLM*, *pucR*, and *gde* operons is induced during growth with glutamate as the nitrogen source and, except in the cases of *pucABCDE* and *pucR*, PucR appears to be required for the expression of all *puc* genes, including *gde* (Table 2). The expression of *pucABCDE*, which encodes the subunits of xanthine dehydrogenase (XDH), which oxidizes hypoxanthine and xanthine to uric acid, is induced to a relatively high level during growth with glutamate as the nitrogen source. Uric acid acts together with allantoin and allantoic acid as the effector molecules for PucR-activated transcription (15).

The combination of nitrogen-limiting conditions and excess purine or purine degradation intermediates (e.g., glutamate plus allantoin as the nitrogen source) results in PucR-dependent repression of *pucABCDE* and *pucR* expression and strong induction of *pucFG*, *pucH*, *pucI*, *pucJKLM*, and *gde* expression (Table 2). These observations are in agreement with previous findings (15). Based on the position of the putative PucR recognition site (PucR box) as revealed by genetic and bioinformatic analysis, we are able to explain both the positive and negative roles of PucR in *puc* and *gde* gene expression. The PucR-induced promoters contain PucR boxes located 17 bp (*gde*) to 39 bp (*pucH* and *pucI*) upstream of the −35 elements (Fig. 2 and 3). These upstream promoter positions are consistent with the suggested transcription activator function of PucR. The PucR-repressed promoters in front of *pucR* and *pucABCDE* (Fig. 2 and 4) have PucR boxes overlapping the promoter −35 element (*pucABCDE*) and/or located downstream of the transcription start point (*pucABCDE* and *pucR*). Binding of PucR to these sites in the presence of an inducer represses transcription. An alternative explanation for the repression of *pucR* is that because (i) the *pucR* and *pucH* promoters overlap and (ii) PucR activates *pucH* transcription, the activation of *pucH* transcription (by PucR) reduces *pucR* transcription due to the competition of RNA polymerase for the two overlapping promoters. Since the *pucH* promoter is expressed at low levels in strains containing either a *pucR* mutation or an inactive PucR-binding site, this competition would not occur and the *pucR* promoter would be transcribed at higher levels. By subjecting *pucABCDE* (XDH) expression to uric acid-, allantoin-, or allantoic acid-induced PucR repression, *B. subtilis* is able to modulate the cellular contents of purine catabolic enzymes in response to the available nitrogen source. During nitrogen-limiting conditions in the absence of purine degradation intermediates, XDH (*pucABCDE*) is expressed at higher levels than the other *puc* gene-encoded catabolic enzymes. The presence of uric acid, allantoin, or allantoic acid, which are the inducers of PucR-activated transcription, results in a shift in the relative gene expression towards the expression of *pucLM* (uricase), *pucH* (allantoinase), and *pucF* (allantoic acid amidohydrolase) instead of *pucABCDE* (XDH), which is not required for purine degradation under these growth conditions.

A model for the role of PucR in the regulation of the purine catabolic genes under nitrogen-limiting conditions with purine

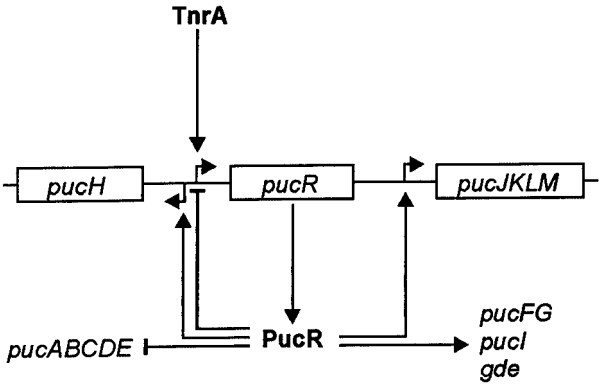


FIG. 7. Model for the role of PucR in the regulation of purine degradation in *B. subtilis* under nitrogen-limiting conditions with purine degradation products (e.g., allantoin) available. TnrA induces transcription from *pucR*, and PucR induces transcription from the purine catabolic genes, as purine degradation products are available. PucR also inhibits its own transcription and transcription from the *pucABCDE* operon. The sizes of and distances between genes and operons are not drawn to scale. Short bent arrows indicate promoters; long arrows indicate induction; lines ending in a bar indicate repression; boxes denote genes or operons.

degradation products available based on the experimental results is presented in Fig. 7. Under nitrogen-limiting conditions, TnrA is activated and induces the expression of PucR. When purine degradation products also are present, PucR induces transcription of *gde* and the *puc* genes, except for *pucABCDE* and *pucR*, which it represses.

The control of purine degradation in the two bacterial species *B. subtilis* and *E. coli* has now been revealed and turns out to be quite different between the species. While purine degradation in *B. subtilis* is subjected to a two-level positive control mechanism involving TnrA (global nitrogen state) and PucR (pathway-specific regulation), the degradation of allantoin in *E. coli* is controlled by a two-level negative control mechanism involving aerobic-anaerobic control (global state) and the *all* operon repressor AllR (pathway-specific induction) (5). The two-level positive control mechanism reported here for *B. subtilis* is similar to those reported for the control of allantoin degradation in *Saccharomyces cerevisiae* (3) and purine degradation in *Aspergillus nidulans* (16). In these two lower eukaryotes, allantoin and purine degradation are also subjected to global nitrogen catabolite control and pathway-specific regulation. The GLN3 protein of *S. cerevisiae* and the AREA protein of *A. nidulans* sense the nitrogen state of the cell and activate transcription under nitrogen-limiting conditions. In concert with the pathway-specific regulatory proteins DAL81 and DAL82, GLN3 activates allantoin-degradative genes in *S. cerevisiae* in the presence of the pathway-specific inducer allophanate. The AREA protein in *A. nidulans* together with the regulatory protein UaY activates purine-degradative genes when uric acid is present. In both *S. cerevisiae* and *A. nidulans*, the global and the pathway-specific regulators act in concert on the relevant promoters, while *B. subtilis* has evolved a hierarchical mechanism by which the global regulator TnrA controls the level of the pathway-specific activator PucR.

The TnrA/GlnR box located upstream of the *pucR* promoter was found to be required for the induction of *pucR* expression. Except in the case of *pucR*, TnrA/GlnR boxes were located around the +1 position of all *puc* genes and *gde*. The presence of TnrA/GlnR boxes in front of *puc* genes and *gde* may indicate that TnrA is required for gene expression. However, this is not likely the case, since the induction of PucR synthesis in strains growing under excess nitrogen conditions—conditions during which TnrA is inactive—resulted in the activation of *puc* and *gde* gene expression. However, in the case of *pucJKLM*, TnrA may play a role in gene expression. The *pucJKLM* operon has two potential TnrA/GlnR boxes, and deletion of the upstream promoter box resulted in derepression of the PucR-dependent *pucJ* expression (Fig. 3). The TnrA/GlnR box and the PucR box are separated by 12 nucleotides (Fig. 2), and binding of TnrA to the TnrA/GlnR box under nitrogen-limiting conditions may reduce the binding efficiency of PucR to the downstream-located PucR box. TnrA may therefore be directly involved in the modulation of functions connected to the uptake and oxidation of uric acid; however, this remains to be shown experimentally.

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