Nucleosides as a carbon source in *Bacillus subtilis*: characterization of the *drm*–*pupG* operon

Raymond Schuch,¹ Araik Garibian,² Hans H. Saxild,³
Patrick J. Piggot⁴ and Per Nygaard²

Author for correspondence: Per Nygaard. Tel: +45 353 22005. Fax: +45 353 22040.
e-mail: Nygaard@mermaid.molbio.ku.dk

¹ Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences Bethesda, MD 20814, USA

² Department of Biological Chemistry, Institute of Molecular Biology, University of Copenhagen, Sølvgade 83, 1307 Copenhagen K, Denmark

³ Department of Microbiology, Technical University of Denmark, 2800 Lyngby, Denmark

⁴ Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, PA 19140, USA

In *Bacillus subtilis*, nucleosides are readily taken up from the growth medium and metabolized. The key enzymes in nucleoside catabolism are nucleoside phosphorylases, phosphopentomutase, and deoxyriboaldolase. The characterization of two closely linked loci, *drm* and *pupG*, which encode phosphopentomutase (Drm) and guanosine (inosine) phosphorylase (PupG), respectively, is reported here. When expressed in *Escherichia coli* mutant backgrounds, *drm* and *pupG* confer phosphopentomutase and purine-nucleoside phosphorylase activity. Northern blot and enzyme analyses showed that *drm* and *pupG* form a dicistronic operon. Both enzymes are induced when nucleosides are present in the growth medium. Using mutants deficient in nucleoside catabolism, it was demonstrated that the low-molecular-mass effectors of this induction most likely were deoxyribose 5-phosphate and ribose 5-phosphate. Both Drm and PupG activity levels were higher when succinate rather than glucose served as the carbon source, indicating that the expression of the operon is subject to catabolite repression. Primer extension analysis identified two transcription initiation signals upstream of *drm*; both were utilized in induced and non-induced cells. The nucleoside-catabolizing system in *B. subtilis* serves to utilize the base for nucleotide synthesis while the pentose moiety serves as the carbon source. When added alone, inosine barely supports growth of *B. subtilis*. This slow nucleoside catabolism contrasts with that of *E. coli*, which grows rapidly on a nucleoside as a carbon source. When inosine was added with succinate or deoxyribose, however, a significant increase in growth was observed in *B. subtilis*. The findings of this study therefore indicate that the *B. subtilis* system for nucleoside catabolism differs greatly from the well-studied system in *E. coli*.

Keywords: purine-nucleoside phosphorylase, phosphopentomutase, ribonucleoside, deoxyribonucleoside, catabolism

INTRODUCTION

Nucleoside phosphorylases are involved in the intracellular metabolism of nucleosides obtained from the external environment or arising from the intracellular breakdown of nucleotides. However, the regulation and the components of this system are not well characterized in Gram-positive bacteria. Two purine-nucleoside

phosphorylases exist in *Bacillus subtilis* (Jensen, 1978): one specific for adenosine (and deoxyadenosine), PupA, and another specific for guanosine (and deoxyguanosine) and inosine (and deoxyinosine), PupG. Based on uptake studies, an adenosine-specific transport system and a transport system for guanosine and inosine have been suggested for *B. subtilis* (Beaman *et al.*, 1983). The nucleoside phosphorylases catalyse the cleavage of ribonucleosides and deoxyribonucleosides to the free base plus ribose 1-phosphate or deoxyribose 1-phosphate, respectively (Fig. 1). The bases serve anabolic (re-utilization in nucleotide synthesis) or catabolic (use as

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Fig. 1. Nucleoside catabolic pathways in *B. subtilis*. The enzymes are indicated by their gene symbols: *cdd*, cytidine deaminase; *pupA*, adenosine phosphorylase; *pupG*, guanosine (inosine) phosphorylase; *pdp*, pyrimidine nucleoside phosphorylase; *drm*, phosphopentomutase; *dra*, deoxyriboaldolase; *drsK*, deoxyribokinase and *rbsK*, ribokinase. Purine base indicates adenine, guanine or hypoxanthine.

nitrogen sources) functions. However, the degradation of the purine bases in *B. subtilis* occurs only when cells are growing on poor nitrogen sources (Christiansen *et al.*, 1997). When nucleosides are added as the sole nitrogen source, the growth rate is reduced sixfold compared with that seen when glutamate plus ammonia serve as the nitrogen source (Nygaard *et al.*, 1996). The pathways for the degradation of nucleosides in *B. subtilis* are shown in Fig. 1. The ribose 1-phosphate formed from ribonucleosides can be converted to ribose 5-phosphate by phosphopentomutase and then further catabolized or converted to phosphoribosylpyrophosphate which is utilized in the synthesis of nucleotides. Deoxyribose 1-phosphate, formed from deoxyribonucleosides, is also a substrate for phosphopentomutase and is converted to deoxyribose 5-phosphate. Deoxyribose 5-phosphate cannot be rescued for deoxy nucleotide synthesis and is degraded by deoxyriboaldolase encoded by the *dra* gene. The *dra* gene is located in the *dra-nupC-pdp* operon. The *nupC* locus encodes a pyrimidine-nucleoside transporter that mediates the transport of uridine, thymidine and deoxyuridine. The *pdp* gene encodes the only pyrimidine-nucleoside phosphorylase in *B. subtilis* and can phosphorylate uridine, thymidine and deoxyuridine to the free base. Uracil can be rescued for nucleotide synthesis, but cannot serve as a nitrogen source. The *dra-nupC-pdp* operon is negatively regulated by the *deoR* gene product (Saxild *et al.*, 1996; Zeng & Saxild, 1999).

In *Escherichia coli* and *Salmonella typhimurium*, a single enzyme catalyses the phosphorolytic cleavage of adenosine, guanosine, inosine and the corresponding deoxyribonucleosides (Jensen & Nygaard, 1975). *E. coli*, in addition, contains a purine-nucleoside phosphorylase with specificity towards xanthosine, guanosine and inosine (Seeger *et al.*, 1995). The synthesis of

these phosphorylases and other proteins involved in nucleoside catabolism and transport in enterobacteria is induced several-fold by nucleosides added to the growth medium. As a result of this induction, the nucleosides are rapidly catabolized and serve as excellent carbon sources in *E. coli* (Hammer-Jespersen, 1983; Munch-Petersen & Mygind, 1983).

Previously, we cloned and sequenced two adjacent *B. subtilis* genes, encoding products similar to phosphopentomutase and purine-nucleoside phosphorylase. These loci were designated *drm* and *pnp* (now referred to as *pupG*), and their sequences were submitted to GenBank in 1995 (accession number U32685). Here, we report the characterization of the products of these loci with respect to the regulation of their expression and their functions. These studies identify distinct differences between the *B. subtilis* nucleoside-catabolizing pathway and that of *E. coli*.

METHODS

Bacterial strains. The bacterial strains and plasmids used are described in Table 1. Strains constructed by transformations with plasmids as donors were confirmed by Southern-blot analysis (Sambrook *et al.*, 1989). Strains SL6250 and SL6550 contain single-copy chromosomal insertions of plasmid pPP400 and pPP424, in the *pupG* and *drm* gene, respectively. Strain SL6479 bears a single copy *drm-lacZ* transcriptional fusion inserted by double crossover recombination at the *amyE* locus (Table 1).

Growth and media. Cells were grown at 37 °C as described previously (Jochimsen *et al.*, 1975; Saxild *et al.*, 1995; Schuch & Piggot, 1994). As minimal medium for *B. subtilis* the Spizizen salt-buffered medium was used; L-broth was used as rich medium. All plasmids were maintained in *E. coli* DH5 α . When appropriate, bacteria were grown in the presence of antibiotics at the following concentrations: chloramphenicol,

Table 1. Bacterial strains and plasmids

Tf., transformed; Er, erythromycin; Cm, chloramphenicol; Ap, ampicillin.

Strain/plasmid	Characteristics	Source/reference/ construction
<i>B. subtilis</i> strains		
168	<i>trpC2</i>	C. Anagnostopoulos*
HH232	<i>trpC2 deoR::erm</i> , Er ^R	Saxild <i>et al.</i> (1996)
HH234	<i>trpC2 dra::erm</i> , Er ^R	Saxild <i>et al.</i> (1996)
HH244	<i>metB10 lys-3 trpC2 drm::cat::erm</i> , Er ^R Cm ^S	SL6550 Tf. with <i>Bam</i> HI-digested pPH46, Er ^R
HH245	<i>metB10 lys-3 trpC2 amyE::drm-lacZ drm::cat::erm</i> , Er ^R Cm ^R	HH244 Tf. with SL6479, Cm ^R
MB24	<i>trpC2 metC3</i>	Schuch & Piggot (1994)
SL4	<i>trpC2 lys-3 metB10</i>	Schuch & Piggot (1994)
SL5651	SL4 <i>amyE::erm</i> , Er ^R	Zhang <i>et al.</i> (1996)
SL6250	SL4/pPP400 (<i>pupG::cat</i>), Cm ^R	This work
SL6479	SL5651/pPP420 (<i>amyE::drm-lacZ</i>)	This work
SL6550	SL4/pPP424 (<i>drm::cat</i>), Cm ^R	This work
<i>E. coli</i> strains		
DH5α	φ80 <i>dlacZ</i> Δ <i>M15 recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 deoR</i> Δ(<i>lacZYA-argF</i>)U169	GibcoBRL
SØ199	<i>metB rpsL relA spoT supF lamB purE</i>	Jochimsen <i>et al.</i> (1975)
SØ446	<i>metB rpsL relA spoT supF lamB purE deoD apt</i>	Jochimsen <i>et al.</i> (1975)
HO1077	<i>metB rpsL relA spoT supF lamB guaA deoD deoB thyA zjj-202::Tn10</i>	B. Hove-Jensen†
Plasmids		
pBOE335	Ap ^R Cm ^R	Saxild <i>et al.</i> (1996)
pDH32	Cm ^R transcriptional <i>lacZ</i> fusion vector designed to integrate at the <i>amyE</i> locus	Shimotsu & Henner (1986)
pHM2	Vector bearing a 3.3 kb insert of <i>B. subtilis</i> DNA extending from <i>drm</i> to <i>spoIIA</i>	Liu <i>et al.</i> (1982)
pJH101	Cm ^R integrative vector	Ferrari <i>et al.</i> (1983)
pKE5	Vector bearing a 3.5 kb insert of <i>B. subtilis</i> DNA extending from <i>pupG</i> to <i>spoIIM</i>	Smith <i>et al.</i> (1993)
pPH46	Ap ^R Er ^R , pBOE335 digested with <i>StyI</i> (blunt-ended with Klenow polymerase) and ligated with a 1 kb <i>erm</i> -containing <i>EcoRI</i> fragment (blunt-ended with Klenow polymerase) from pUC7 <i>erm</i>	P. Hubrecht‡
pUC7 <i>erm</i>	Ap ^R Er ^R	W. de Vos§
pPP400	pJH101 digested with <i>EcoRI</i> , treated with calf intestinal phosphatase and ligated with a 364 bp <i>EcoRI</i> internal fragment of <i>pupG</i> (nt 2445764–2446128)	This work
pPP420	pDH32 digested with <i>EcoRI</i> (blunt-ended with Klenow polymerase) and <i>Bam</i> HI, and ligated with a 1.6 kb <i>PvuII</i> – <i>Bgl</i> II fragment containing the <i>ripX</i> – <i>drm</i> intergenic region and the 5' end of <i>drm</i> (nt 2448926–2447300)	This work
pPP424	pJH101 digested with <i>EcoRV</i> and <i>Bam</i> HI, treated with calf intestinal phosphatase and ligated with the 180 bp <i>EcoRV</i> – <i>Bgl</i> II fragment of <i>drm</i> (nt 2447480–2447300)	This work

*INRA, Jouy en Josas, France.

†University of Copenhagen.

‡Technical University of Lyngby.

§The Netherlands Institute of Dairy Research.

5 µg ml⁻¹; erythromycin, 1 µg ml⁻¹; ampicillin, 50 µg ml⁻¹. Spore germination was assessed by the method of Nicholson & Setlow (1990).

Sporulation. Sporulation was induced as described by Piggot & Curtis (1987). Time is indicated in hours after the end of

exponential growth. At t_{20} – t_{24} , the extent of heat-resistant-spore formation was determined by plating appropriate diluted aliquots of the cultures on L-broth; the remainder was heated at 90 °C for 20 min, and aliquots were plated on L-broth. Colonies arising from heat-treated and non-heat-

treated samples were enumerated to determine the proportion of the starting population of cells that were heat-resistant spores.

Enzyme assays. β -Galactosidase was assayed at 30 °C as described by Nicholson & Setlow (1990). Activities of nucleoside-catabolizing enzymes were determined at 37 °C as described previously (Hammer-Jespersen *et al.*, 1971; Jensen, 1978). Enzyme activity is given as nmol product formed min⁻¹ (= 1 unit).

DNA preparation and sequencing. Methods used for transformation and for chromosomal and plasmid DNA isolation have been described previously (Wu *et al.*, 1989; Saxild *et al.*, 1995). DNA sequencing was using a Sequenase kit (US Biochemical). All sequencing analysis was done on double-stranded plasmid DNA templates. Using plasmids pHM2 and pKE5, we determined the nucleotide sequence of the *drm*-*pupG* operon.

RNA analysis. RNA preparation, Northern-blot analysis and primer extensions were performed as described previously (Penn *et al.*, 1984; Wu *et al.*, 1989; Nygaard *et al.*, 1996). For Northern analyses, RNA samples (20 µg per sample) and size markers (Promega) were separated on 1.2% agarose gels, transferred to nylon membranes (Stratagene) and probed as described by Sambrook *et al.* (1989). The *pupG*-specific probe was the 364 bp *EcoRI* fragment (nt 2445764–2446128), and the *drm*-*pupG*-specific probe was the 823 bp *HindIII* fragment (nt 2445693–2446516) (Kunst *et al.*, 1997).

RESULTS

Organization and sequence of the *drm*-*pupG* gene cluster

Sequence analysis of the region immediately upstream of the *dacF*-*spolIA* operon was performed using plasmids pHM2 and pKE5 (Table 1). The ORF immediately upstream of *dacF* encoded a putative product of 271 amino acids with extensive similarity to guanosine (inosine) nucleoside phosphorylases of a broad range of organisms. On this basis, we refer to the ORF as *pupG* (formerly called *pnp*). The second ORF was identified immediately upstream of the *pupG* RBS, and found to encode a putative protein of 396 residues. A BLAST search (Altschul *et al.*, 1990) revealed that this product was 47% identical over its entire sequence to phosphopentomutase (phosphodeoxyribomutase) of *E. coli* (Hammer-Jespersen, 1983; Valentin-Hansen *et al.*, 1984). We refer to this ORF herein as *drm*. Upstream of the *drm* RBS was a 161 bp intergenic region, followed by the 3' end of another ORF, now called *ripX* (Kunst *et al.*, 1997). In the *ripX*-*drm* intergenic region we identified a putative rho-independent transcription terminator. The energy of binding for this stem-loop structure was calculated to be $-19.8 \text{ kcal mol}^{-1}$ ($-82.8 \text{ kJ mol}^{-1}$) using the rules of Tinoco *et al.* (1973). These sequences may serve to stop transcription from *ripX* into *drm*. The remaining region between the stem-loop and *drm* (roughly 130 bp) could serve as a promoter region directing the transcription of *drm* and possibly *pupG*. That *drm* and *pupG* may form an operon is supported by the proximity of the *drm* and *pupG* translation stop and start codons, respectively.

Fig. 2. Northern-blot analysis of the *drm*-*pupG* region of the *B. subtilis* chromosome. RNA samples were obtained from L-broth cultures at t_{-1} (lane 1), t_0 (lane 2) and t_2 (lane 3). The numbers are hours relative to the end of exponential growth (t_0). The arrow indicates the major message. Probes used were either specific to *pupG* (a) or to both *drm* and *pupG* (b).

Complementation of *E. coli* purine-nucleoside catabolism defects

To confirm the identity of *drm* and *pupG*, plasmids pKE5 (expressing *drm* from its native promoter) and pHM2 (expressing *pupG* from a vector-encoded promoter) were transformed into *E. coli* strains HO1077 (*deoB*) and SØ446 (*purE deoD*), respectively, and scored for complementation. Strain HO1077 is a phosphopentomutase-deficient mutant and cannot use thymidine as its sole carbon source (Hammer-Jespersen, 1983). Introduction of pKE5 into HO1077 restored the ability of this strain to grow on minimal medium containing inosine as a carbon source. Additionally, phosphopentomutase activity increased from $<1 \text{ unit (mg protein)}^{-1}$ in HO1077 to 8 units $(\text{mg protein})^{-1}$ in HO1077/pKE5. Strain SØ446 is purine auxotrophic and is unable to use thymidine as its sole purine source. The transformed strain SØ446/pHM2 did grow with inosine as the sole purine source, indicating that the *B. subtilis* *pupG* gene could complement the salvage-pathway defect arising from mutation of the *E. coli* *deoD* gene. Inosine phosphorylase activity increased from $<1 \text{ unit (mg protein)}^{-1}$ in SØ446 to 85 units $(\text{mg protein})^{-1}$ in SØ446/pHM2. The *B. subtilis* *drm* and *pupG* loci, therefore, do encode phosphopentomutase and purine-nucleoside phosphorylase activity, respectively.

Transcription of the *drm*-*pupG* locus

Transcription of *drm* and *pupG* during both late-exponential and early-stationary-phase L-broth cultures of strain SL4 was examined by Northern-blot analysis. Using a *pupG*-specific probe, we detected a predominant 2.1 kb transcript (Fig. 2a, lanes 1 and 2). With a probe containing both *drm* and *pupG* sequences, a similar 2.1 kb message was visualized (Fig. 2b, lanes 1 and 2).

Table 2. Effects of mutations in genes encoding enzymes involved in the catabolism of nucleosides on the expression of the *pupG* gene, the *drm* gene and the transcriptional LacZ fusion (*drm-lacZ*) in *B. subtilis*

Cells were grown in glucose and succinate minimal medium and were harvested in the exponential growth phase. Enzymes were assayed as described in the Methods section. Data are means of three or more experiments that differed by less than 15 %. Nucleosides were added at a concentration of 1 mg ml⁻¹.

Strain	Relevant genotype	Addition to medium	PupG*	Drm*	Drm-LacZ*
Glucose-grown cultures					
SL6497	<i>drm-lacZ</i>	None	32	10	32
		Thymidine	63	20	68
		Inosine	53	18	73
		Adenosine	52	17	67
SL6250	<i>pupG</i>	None	<2	9	–
		Inosine	<2	10	–
		Adenosine	<2	17	–
HH245	<i>drm</i> <i>drm-lacZ</i>	None	9	<0.5	38
		Thymidine	8	<0.5	32
		Inosine	8	<0.5	32
HH234	<i>dra</i>	None	30	10	–
		Thymidine	55	16	–
HH232	<i>deoR</i>	None	68	13	–
		Thymidine	117	22	–
		Inosine	107	21	–
Succinate-grown cultures					
SL6497	<i>drm-lacZ</i>	None	85	29	69
		Deoxyribose	109	–	95
		Ribose	103	–	109
HH245	<i>drm</i> <i>drm-lacZ</i>	None	–	–	81
		Thymidine	–	–	82
		Inosine	–	–	62

* Units (mg protein)⁻¹.

The transcript size observed in each case strongly suggests that *drm* (a 1.2 kb gene) and *pupG* (a 0.8 kb gene) are cotranscribed as an operon. A minor 0.9 kb band was also noted. This band may reflect a low level of expression of a specific *pupG* transcript or a processing/breakdown product of the 2.1 kb transcript. Expression of the *drm-pupG* transcript was barely detectable in mRNA samples isolated at *t*₂ (Fig. 2, lanes 3), suggesting that it may be repressed in later stationary phase. To confirm the operon structure of *drm-pupG*, we also analysed expression of single-copy *drm-pupG* transcriptional fusions to *lacZ* integrated at the chromosomal *amyE* locus. Fusion to *lacZ* of several distinct DNA fragments extending from within *pupG* to at least 300 bp upstream of it yielded background levels of β -galactosidase activity (data not shown), indicating that *pupG* is not likely to be transcribed from its own promoter. In contrast, a 1.4 kb *PvuII*-*BglII* fragment containing the *ripX-drm* intergenic region and the 5' end of *drm* fused to *lacZ* in SL6479 does support high-level β -galactosidase activity in L-broth cultures: 135

units (mg protein)⁻¹ at *t*₋₁, 176 at *t*₀ and 141 at *t*₂. *pupG* is, therefore, probably cotranscribed with *drm* from a promoter in the *ripX-drm* intergenic region. The lower enzyme level observed at *t*₂ reflects the fact that the *drm-pupG* operon is no longer transcribed at this stage of growth (Fig. 2).

Role of Drm and PupG in sporulation and spore germination

Several observations show that the intracellular levels of a purine compound are important for the initiation of sporulation (Beaman *et al.*, 1983) and for the germination of spores (Gardner & Kornberg, 1967; Engelbrecht, 1972; Senesi *et al.*, 1991). Based on these findings, we investigated the requirements for *drm-pupG* in spore formation. Strains SL6250 (*pupG*) and SL6550 (*drm*) displayed 65 % and 61 % sporulation, respectively, after overnight growth in liquid sporulation medium (a time at which the wild-type parental strain SL4 yielded 70 % sporulation). Drm and PupG are,

therefore, not required for efficient spore formation. Similarly, Drm and PupG are also dispensable for spore germination (data not shown).

Identification of the low-molecular-mass effector of induction

Preliminary analysis had indicated that the levels of Drm and PupG, but not that of PupA, were increased by the addition of ribonucleosides and deoxyribonucleosides to the growth medium. The level of PupA was uninducible and remained between 27 and 34 units (mg protein)⁻¹. The activities of Drm and PupG in wild-type cells grown in the presence of thymidine, inosine and adenosine are shown in Table 2. No induction was observed in the presence of any of the naturally occurring purine and pyrimidine bases (data not shown). To investigate whether a nucleoside must be degraded to induce, experiments were conducted with mutants defective in nucleoside catabolism. In a *pupG* mutant defective in inosine degradation, inosine no longer induced Drm synthesis, while increased Drm activity was seen when adenosine was added (Table 2). This indicates that a nucleoside must be phosphorylated to act as an inducer. To determine whether the first products of nucleoside degradation, ribose 1-phosphate or deoxyribose 1-phosphate, were the low-molecular-mass effector molecules, induction experiments were performed in a *drm* mutant strain. This mutant is unable to convert ribose 1-phosphate and deoxyribose 1-phosphate, formed from inosine and thymidine, respectively, to ribose 5-phosphate and deoxyribose 5-phosphate. The level of PupG and β -galactosidase activity from a *drm-lacZ* transcriptional fusion was measured in the *drm* knock-out mutant HH245 grown in the presence of either inosine or thymidine. The PupG level was low due to the interruption of the *drm* gene and this level was not affected by addition of nucleosides to the growth medium. However, the β -galactosidase activity, driven by the *drm* promoter, was also unaffected. This indicates that ribose 1-phosphate and deoxyribose 1-phosphate must be further metabolized to affect the enzyme levels. In the *dra* mutant HH234, which can degrade deoxyribose 1-phosphate one step more to deoxyribose 5-phosphate and no further, addition of thymidine results in increased levels of Drm and PupG, indicating that the deoxyribose 5-phosphate formed may act as a low-molecular-mass effector molecule. Since no induction is observed in the *drm* mutant unable to convert ribose 1-phosphate to ribose 5-phosphate, ribose 5-phosphate is a likely low-molecular-mass effector molecule too. An alternative way of forming ribose 5-phosphate and deoxyribose 5-phosphate is through phosphorylation of ribose and deoxyribose (Fig. 1). Induction experiments with ribose and deoxyribose were performed with cultures grown on succinate as the carbon source, because neither ribokinase (O'Reilly *et al.*, 1994) nor deoxyribokinase (X. Zeng, personal communication) is synthesized when glucose serves as carbon source. When added to cultures growing on succinate both ribose and deoxyribose

caused slightly increased enzyme levels in both wild-type and the *drm* mutant strain, supporting the suggestion that both ribose 5-phosphate and deoxyribose 5-phosphate are low-molecular-mass effector molecules. Addition of thymidine or inosine to a *deoR* mutant (HH232) resulted in the same level of induction as in wild-type cells (Table 2). This indicates that the *B. subtilis* deoxynucleoside regulator protein DeoR, which negatively regulates the expression of the *dra-nupC-pdp* operon, does not appear to be involved in the regulation of expression of the *drm-pupG* operon.

Expression of the *drm-pupG* operon during growth in minimal medium

The apparent difference in enzyme levels observed when nucleosides were added to glucose- and succinate-grown cells was studied in more detail. Expression of the *drm-pupG* operon was followed by determining both Drm and PupG activity after addition of thymidine and inosine. We also investigated whether the expression was co-regulated with the *dra-nupC-pdp* operon by measuring pyrimidine nucleoside phosphorylase (Pdp) activity. The level of all three enzymes was increased in thymidine-supplemented cultures; however, a more dramatic increase was observed when succinate served as the carbon source (Table 3). Addition of inosine only affected the level of Drm and PupG. There was no additive effect when both thymidine and inosine were added to the growth medium. The addition of nucleosides to the cultures in most situations resulted in an increased growth rate, most significantly in succinate-grown cultures. From these experiments, we conclude that the expression of the *drm-pupG* operon appears to be subject to catabolite repression.

Identification of two *drm-pupG* transcription start sites

From the Northern blot and *lacZ* fusion studies, *drm-pupG* appeared to be co-transcribed from a promoter in the *ripX-drm* intergenic region. The induction experiments indicated that transcription was increased when either a ribonucleoside or a deoxyribonucleoside was present in the growth medium. The transcription start points were identified by primer-extension mapping (Fig. 3). Total RNA was prepared from strain 168 grown in minimal succinate medium (Fig. 3, lanes 1 and 2) and in the same medium to which inosine (Fig. 3, lanes 3 and 4) or thymidine (Fig. 3, lanes 5 and 6) was added. Primers complementary to the nucleotide sequence from position 2447596–2447613 or 2447512–2447529 were used for both primer extension and to generate a sequence ladder. Only results obtained with the upstream primer 2447596–2447613 are shown. Two transcriptional-start sites are inferred from the 5' end of the mRNA and are positioned at nucleotide numbers 2447716 and 2447687 (53 and 24 nt upstream of the putative translational-start signal of *drm*); they were detected with both primers. The same two trans-

Table 3. Effects of nucleosides on growth and induction of the synthesis of phosphopentomutase (Drm), guanosine (inosine) phosphorylase (PupG) and pyrimidine nucleoside phosphorylase (Pdp) in *B. subtilis* (168)

Cells were grown in glucose and succinate minimal medium and were harvested in the exponential growth phase. Enzymes were assayed as described in the Methods section. Nucleosides were added at a concentration of 1 mg ml⁻¹. Data are means of three experiments that differed by less than 15%. Numbers in parentheses indicate induction (-fold).

Addition to growth medium	Drm*	PupG*	Pdp*	Doubling time (min)
Glucose-grown cultures				
None	12	85	33	46
Thymidine	20 (1.7)	113 (1.3)	99 (3.0)	48
Inosine	20 (1.7)	103 (1.2)	28 (0.8)	42
Thymidine + inosine	19 (1.6)	108 (1.3)	78 (2.4)	40
Succinate-grown cultures				
None	23	105	89	108
Thymidine	70 (3.0)	265 (2.5)	1318 (14.8)	83
Inosine	40 (1.7)	178 (1.7)	86 (1.0)	83
Thymidine + inosine	61 (2.7)	219 (2.1)	928 (10.4)	71

* Units (mg protein)⁻¹.

(a)

C T A G 1 2 3 4 5 6

(b)

2 4 4 7 7 7 TTTCTCATTATTTCCAAATACATTGTCAACGGGAA TGAAAACGGTTTAT ACTTGAGT
 -35 CRE -10 -35

2 4 4 7 7 1 7 TGTCAGACCTCTTAGCGATACAATAGACGTATAGAAAAAAGGAGGCTTTCAAGATG
 +1 -10 +1 *drm* Start codon

Fig. 3. Primer extension analysis of *drm-pupG* mRNA from cells grown in minimal succinate medium and organization of the *drm-pupG* regulatory region. (a) The primer used corresponds to nt 2447596–2447613. The sequence ladder (C, T, A, G) was obtained with the same primer used for cDNA synthesis. The positions that correspond to the start of transcription are marked with arrows. Lanes 1 and 2 contain RNA from cells grown in the absence of inducer. Lanes 3 and 4 contain RNA from cells grown with 1 mg inosine ml⁻¹ and lanes 5 and 6 contain RNA from cells grown with 1 mg thymidine ml⁻¹. Lanes 1, 3 and 5 contain 3 µl samples and lanes 2, 4 and 6 contain 4 µl samples. (b) Lines above the sequence indicate the location of the putative –35 and –10 regions of the *drmP1* promoter and underlined sequences indicate the *drmP2* promoter elements. The boxed sequence indicates the location of the putative *cre* sequence. The transcription-start points for *drmP1* and *drmP2* are indicated with a +1, above or under the sequence, respectively. Letters in bold indicate the putative translational start of the *drm* reading frame.

criptional-start sites were seen when strain MB24 was grown in minimal glucose medium (data not shown). The distal promoter is referred to as *drmP1* and the

proximal as *drmP2*. The –10 and –35 sequences for both transcription-start sites are very similar to the σ^A consensus recognition sequences (TATAAT for the –10

site and TTGACA for the -35 site). As judged by band intensity, both promoters were active under all conditions, with the highest activities in the inosine- and thymidine-supplemented cultures. The increase in transcription resulting from inosine or thymidine addition was reflected by the increased levels of Drm and PupG in these cultures (Table 3). Furthermore, the *drm* proximal promoter (*drmP2*) appears to be favoured over the distal promoter (*drmP1*) by a ratio of approximately 2:1.

Catabolism of nucleosides

The ability to grow on inosine (0.1%) or thymidine (0.1%) as the carbon source was compared with growth on ribose (0.1%) and deoxyribose (0.1%). Except for the initial steps, the degradation of inosine and thymidine follow the same pathways as that of ribose and deoxyribose, respectively (Fig. 1). When wild-type cells were incubated in liquid media with ribose, deoxyribose, inosine or thymidine as the sole carbon source and ammonium ions as the nitrogen source, no growth on inosine or deoxyribose was observed after 36 h, while cells grew with a doubling time of 280 min on ribose and 210 min on thymidine. The doubling time on inosine plus deoxyribose was 8 h. For comparison, we found that *E. coli* (strain SØ199) grew on thymidine and inosine as the sole carbon source with a doubling time of 87 min and 115 min, respectively. When *B. subtilis* strains were tested on agar plates containing inosine or deoxyribose as carbon sources and ammonium ions as nitrogen source, colonies were visible after 2 d incubation; they were visible after 1 d when thymidine served as the sole carbon source. The colonies were significantly larger than those observed when no carbon source was added to the plate. To test whether Drm or PupG activities were required for growth on nucleosides and pentoses as the carbon source, strain SL6550 (*drm*) and strain SL6250 (*pupG*) were incubated with a mixture of inosine and deoxyribose, thymidine or ribose as carbon sources. Both strains grew on ribose, and strain SL6250 also on thymidine. These results indicate that Drm activity is required for the utilization of thymidine and inosine as a carbon source and that PupG also was required for inosine utilization.

DISCUSSION

E. coli has served as a paradigm for the analysis and understanding of metabolic pathways in bacteria. However, often there are major differences with respect to how pathways from different bacteria are organized and regulated. The anabolic pathways responsible for purine-nucleoside salvage in *E. coli* and *B. subtilis* are quite different. Only *E. coli* is able to phosphorylate guanosine and inosine to GMP and IMP, and only *B. subtilis* can phosphorylate deoxyadenosine and deoxyguanosine, to dAMP and dGMP (Neuhard & Nygaard, 1987; Nygaard, 1993).

The very efficient nucleoside transport and catabolism found in *E. coli* and the great number of genes encoding regulatory proteins, transport proteins and enzymes

involved in nucleoside catabolism do not have counterparts in *B. subtilis*. In *E. coli*, the genes encoding most of the proteins involved in purine- and pyrimidine-nucleoside catabolism are organized in a regulon composed of several single genes: *nupC*, *nupG*, *tsx*, *cytX* (all encoding transport proteins), *udp*, (encoding uridine phosphorylase), *cdd* (encoding cytidine deaminase) and the *deo* operon *deoABCD* (Valentin-Hansen *et al.*, 1996). This regulon is negatively regulated by the DeoR and CytR proteins. A second operon, *xapAB* (encoding a purine-nucleoside phosphorylase and a nucleoside transport protein), is not part of the regulon, but is positively regulated by the XapR protein (Seeger *et al.*, 1995). The *E. coli* *deoB* and *deoD* genes (equivalent to the *B. subtilis* *drm* and *pupG* genes) are encoded in the *deo* operon, together with *deoA* (encoding thymidine phosphorylase) and *deoC* (encoding deoxyribosomal aldolase). The low-molecular-mass effector that reacts with the regulatory DeoR protein of *E. coli* is deoxyribose 5-phosphate, while ribose 5-phosphate is not active (Hammer-Jespersen, 1983). CytR, on the other hand, reacts with cytidine. Additionally, the CytR-controlled promoters are activated by the cAMP receptor protein (Valentin-Hansen *et al.*, 1996). In *B. subtilis*, the genes identified corresponding to those of the *E. coli* regulon are located in two operons: the *drm-pupG* operon and the *dra-nupC-pdp* operon, in addition to the single genes *cdd*, encoding cytidine deaminase, and *pupA*. Expression of *pupA* and *cdd* has been found not to respond to nucleosides in the growth medium (Nygaard, 1993). The *dra-nupC-pdp* operon is regulated by the DeoR protein (Saxild *et al.*, 1996; Zeng & Saxild, 1999) and the *drm-pupG* operon is not. However, the expression of both operons is increased by deoxyribonucleosides. Additionally, only the expression of *drm-pupG* is affected by the presence of ribonucleosides. Our finding that the transcription of the *drm-pupG* operon started at two different sites could suggest distinct ribose 5-phosphate- and deoxyribose 5-phosphate-controlled start sites. This turned out not to be the case, as similar increases in transcription from both the *drmP1* and *drmP2* promoter were detected in the presence of either thymidine or inosine (Fig. 3). Most likely there is a protein that negatively regulates the initiation of transcription, and which recognizes both ribose 5-phosphate and deoxyribose 5-phosphate. We observed a major difference between growth on inosine or thymidine as the carbon source, compared with growth on free ribose and deoxyribose. Significant growth was only observed when thymidine or ribose served as the sole carbon source. While the level of the nucleoside-catabolizing enzymes is comparable to that of *E. coli*, the nucleoside-transport activity is lower in *B. subtilis*. We therefore suggest that what limits the catabolism of nucleosides in this organism is the transport of nucleosides. In agreement with this is our finding that an *E. coli* strain carrying a plasmid (pHM2) with the cloned *drm* gene grew well on inosine as the carbon source although the Drm level was only 30% of that in wild-type *B. subtilis*, which cannot grow on inosine as the sole carbon source.

Synthesis of the nucleoside-catabolic enzymes in *B. subtilis* is subject to catabolite repression (Saxild *et al.*, 1996). Catabolite repression can occur via a common regulatory mechanism that involves the *cis*-acting *cre* element, 5'-TGWAANCGNTNWCA-3', which is active whether located in the promoter region or within a gene (Miwa *et al.*, 1997). Two such putative elements were found in the *drm-pupG* operon. The first putative *cre* site (nucleotide 2447739–2447726) has 12 out of 14 matches with the common sequence, and overlaps with the putative –10 region of the *drmP1* promoter and extends to within 2 nt of the putative –35 region of the *drmP2* promoter. The second putative *cre* site (nucleotide 2446168–2446155) lies in the *pupG* coding region and matches 11 out of 14 positions. For both *cre* sites the mismatches are located at the 3' end. The presence of these sites offers an explanation for the observed induction pattern (Table 3): that the extent of induction of *Drm* and *PupG* in particular was lower in glucose-grown cultures than in succinate-grown cultures.

A few studies dealing with nucleoside catabolism in other bacilli have shown that the levels of phosphopentomutase and purine-nucleoside phosphorylase in *Bacillus cereus* and *Bacillus thuringiensis* are increased by nucleosides and nucleotides in the growth medium (Gardner & Kornberg, 1967; Ipata *et al.*, 1983; Grigorieva & Sukhodolets, 1979). Another aspect of purine-nucleoside metabolism found in *E. coli* and incidentally also in *B. cereus* is the involvement of purine-nucleoside phosphorylases and adenosine deaminase in the interconversion of adenine compounds to guanine compounds. Such a pathway is not present in *B. subtilis* (Nygaard *et al.*, 1996).

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