Regulation of levels of purine biosynthetic enzymes in *Bacillus subtilis*: effects of changing purine nucleotide pools

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The genes encoding the enzymes of IMP biosynthesis in *Bacillus subtilis* constitute the *pur* operon, whereas the genes encoding GMP biosynthetic enzymes, *guaA* (GMP synthetase) and *guaB* (IMP dehydrogenase), and the *purA* gene encoding adenylosuccinate (sAMP) synthetase all occur as single units. The *purB* gene encodes an enzyme involved in both IMP and AMP biosynthesis and is located in the *pur* operon. The levels of purine biosynthetic enzymes (except for GMP synthetase) were repressed in cells grown in the presence of purine compounds. Transcription of the *pur* operon is regulated negatively by adenine and guanine compounds. Our results suggest that ATP and guanine (or hypoxanthine) act as low molecular mass repressors. The level of IMP dehydrogenase was repressed by guanosine, but not in the presence of adenine, and was negatively correlated with the GTP/ATP pools ratio. The level of sAMP synthetase was repressed by adenine and increased by guanosine, and was positively correlated with the GTP/ATP pools ratio. It appears that the mode of regulating purine biosynthetic enzyme levels coincides with the cellular need for the individual enzymes.

Introduction

The Gram-positive bacterium *Bacillus subtilis* directs the formation of inosine monophosphate (IMP) via a ten-step pathway. IMP is then converted to AMP and GMP via two separate pathways, both comprising steps catalysed by two enzymes (Fig. 1). The purine gene-enzyme relationship has been established in *B. subtilis* (Fig. 1; Ebbole & Zalkin, 1987; Saxild & Nygaard, 1988; Kanzaki & Miyagawa, 1990). The purine genes are located at four different loci on the *B. subtilis* chromosome (Piggot & Hoch, 1985). The genes encoding the enzymes of IMP biosynthesis comprise the *pur* operon, which consists of clusters of overlapping genes, *purEKB-purCQLF-purMNH-purD*. Transcription starts from a sigmaA-dependent promoter 242 bp upstream of *purE* (Ebbole & Zalkin, 1987). The *guaA*, *guaB* and *purA* genes occur as single units. The purine gene organization differs from that known in other micro-organisms. In *Escherichia coli* and *Salmonella typhimurium* the genes occur either as single units or small operons, which are

dispersed on the chromosome (Neuhard & Nygaard, 1987).

In both *B. subtilis* (Momose *et al.*, 1966; Nygaard *et al.*, 1988) and enteric bacteria (Neuhard & Nygaard, 1987) repressed levels of the purine biosynthetic enzymes are found when purine bases or nucleosides are present in the growth medium. Under these conditions purine synthesis *de novo* is reduced, and the preformed purines added to the growth medium are utilized for purine nucleotide synthesis. Purine auxotrophic mutants of *B. subtilis* show repressed levels of three of the enzymes of IMP biosynthesis, when grown with adenosine, guanosine or inosine. Furthermore, adenosine decreases the level of sAMP synthetase and guanosine decreases the level of IMP dehydrogenase (Nishikawa *et al.*, 1967). Similar results, using *pur* gene fusions integrated in the *pur* operon, and representing genes of each of the clusters of the *pur* operon, were obtained by Ebbole & Zalkin (1989a). These authors also provided evidence for the absence of internal promoters in the *pur* operon. Transcription of the *pur* operon is suggested to be regulated independently by adenine and guanine nucleotides (Ebbole & Zalkin, 1987). The rate of transcription initiation is regulated by the interaction of a putative repressor protein, with a 116 nucleotide control region overlapping the -35 region of the *pur* promoter; the co-

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Abbreviations: PRPP, 5-phosphoribosyl- α -1-pyrophosphate; sAMP, adenylosuccinate.

Table 1. *Bacillus subtilis* strains used

Strain	Genotype	Source or reference
168	<i>trpC2</i>	C. Anagnostopoulos*
QB-917	<i>trpC2 hisA1 thr-5</i>	R. A. Dedonder*
PB3394	<i>metB5 purB26</i>	M. Chilton*
60077	<i>trpC2 hisB purM2</i>	Freese <i>et al.</i> (1979)
61857	<i>trpC2 metB4 guaA2</i>	Freese <i>et al.</i> (1979)
ED95	<i>ilvA1 sacA78 upp pupA3</i>	J. Neuhardt†
ED157	<i>trpC2 hisA1 purM2</i>	‡Transformation of QB-917 by 60077, Thr ⁺ and Pur ⁻
ED160	<i>trpC2 hisA1 purB26</i>	‡Transformation of QB-917 by PB3394, Thr ⁺ and Pur ⁻
ED234	<i>trpC2 hisA1 guaA2</i>	‡Transformation of ED160 by 61857, Ade ⁺
ED250	<i>trpC2 hisA1 thr-5 purA2</i>	P. Duckert†
ED252	<i>trpC2 hisA1 purH1 ade1</i>	P. Duckert†
ED265	<i>trpC2 ade1</i>	‡Transformation of ED252 by 168, Pur ⁺ and Thr ⁺
ED279	<i>trpC2 ade1 apt-7</i>	P. Nygaard
ED287	<i>trpC2 hisA1 apt-7</i>	‡Transformation of QB-917 by ED279, Thr ⁺

* Obtained through the Bacillus Genetic Stock Center, Ohio, USA.

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‡ Transformation of the first strain with DNA from the second as described in Methods.

Methods

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. For enzyme analysis and nucleoside triphosphate and PRPP determinations cells were grown at 37 °C in MOPS-buffered minimal medium (Freese *et al.*, 1979), prepared with 2 mM-phosphate (Saxild & Nygaard, 1987). The medium was supplemented with 50 µg L-tryptophan, L-histidine and L-threonine ml⁻¹, 1 µg thiamin ml⁻¹ and with 1 mM of the stated purine compounds. Cells were grown exponentially, monitored by determining doubling time, for several generations to OD₄₃₆ = 0.8 corresponding to 0.16 mg dry wt bacteria ml⁻¹ (Saxild & Nygaard, 1987). Purine auxotrophic strains were starved for purine by transferring exponentially growing cells to purine free medium. At OD₄₃₆ = 0.8 cells were washed and resuspended in pre-warmed purine-free medium and incubated for 90 min at 37 °C.

Transformation. Chromosomal DNA was isolated according to Young & Wilson (1974). Transformation of *B. subtilis* strains was done according to the method of Boylan *et al.* (1972) as described previously (Saxild & Nygaard, 1988).

Chemicals. Fine chemicals were purchased from Sigma. [³²P]Orthophosphate was from AEC Risø, Denmark, and ¹⁴C-labelled compounds were from Amersham.

Enzyme assays. Cells from 80 ml of culture (OD₄₃₆ = 0.8) were harvested by centrifugation, washed in 0.9% NaCl, and stored overnight at -20 °C. Preparation of cell-free extracts and determination of PRPP amidotransferase, glycinamide ribonucleotide synthetase, sAMP lyase, sAMP synthetase, IMP dehydrogenase and GMP synthetase activities were done as described previously (Saxild & Nygaard, 1988). One enzyme unit (nkat) is defined as the amount of enzyme that converts 1 nmol substrate to product s⁻¹. Enzyme levels are averages from at least three independent experiments, and are given as nkat (mg protein)⁻¹.

Determination of nucleoside triphosphate and PRPP pools. [³²P]Orthophosphate (0.8 MBq) was added to 2 ml of exponentially growing cultures at OD₄₃₆ = 0.1. At OD₄₃₆ = 0.8, cells were extracted with formic acid, and the content of ATP, GTP and PRPP was determined by a two-dimensional chromatography method (Jensen *et al.*, 1979). Pool levels are averages from at least three independent experiments.

Results

Effects of addition of purine compounds to wild-type cells

The effects of purines in the growth medium were monitored by determining levels of purine enzymes and purine nucleotide and PRPP pools. The enzymes which catalyse the branching from IMP to AMP and GMP (Fig. 1), and three enzymes representing different gene clusters within the *pur* operon (*purB*, *purF*, *purD*) were studied. The technique used to measure nucleotide pools allows the determination of the eight nucleoside triphosphates and PRPP (Jensen *et al.*, 1979).

Table 2 shows the effect of purine addition. Addition of adenine led to increased ATP pool size and diminished PRPP pool size, increased growth rate and decreased levels of sAMP synthetase (*purA*) and of the *pur* operon encoded enzymes. Guanosine addition led to increased GTP pool size and decreased levels of the enzymes corresponding to the *pur* operon and the *guaB* gene, while the level of sAMP synthetase (*purA*) was increased. Adenine and guanosine added together led to a more pronounced repression of the level of the *pur* operon encoded enzymes and decreased levels of sAMP synthetase (*purA*) and had no effect on the level of IMP dehydrogenase (*guaB*). Hypoxanthine stimulated growth and decreased the levels of the *pur* operon and *guaB* encoded enzymes, while xanthine had little effect on purine enzymes. The GMP synthetase (*guaA*) level was not influenced by purines.

Effects of purine starvation on purine enzyme levels

Mutants defective in the purine biosynthetic pathway either have a general or a specific purine requirement.

Table 2. Effects of purines on pools of ATP, GTP and PRPP, and levels of purine biosynthetic enzymes in wild-type cells of *B. subtilis*

Strain QB-917 was grown and pools and enzyme levels analysed as described in Methods. Pools and enzyme levels are given as a percentage of values obtained in minimal medium (doubling time 50 ± 4 min). These values are averages $\pm$ SD of eight independent experiments. Pool sizes [nmol (mg dry wt) $^{-1}$] were as follows: ATP, 3.3 ± 0.10 ; GTP, 0.98 ± 0.10 ; PRPP, 1.13 ± 0.10 . Enzyme activities [nkat (mg protein) $^{-1}$] were as follows: *purA*, 30.0 ± 4.2 ; *purB*, 6180 ± 480 ; *purF*, 696 ± 66 ; *purD*, 1212 ± 66 ; *guaA*, 468 ± 84 ; *guaB*, 606 ± 102 .

Purine added* (1 mM)	Relative pool size			Relative enzyme level†					
	ATP	GTP	PRPP	<i>purA</i>	<i>purB</i>	<i>purF</i>	<i>purD</i>	<i>guaA</i>	<i>guaB</i>
Adenine (49 min)	169	136	58	12	43	20	41	106	100
Guanosine (54 min)	109	130	168	328	25	14	30	90	50
Adenine + guanosine (47 min)	170	126	59	10	6	6	6	82	114
Hypoxanthine (48 min)	100	104	101	86	32	22	34	94	73
Xanthine (52 min)	106	107	97	130	86	66	66	115	73

* Times in parentheses after the name of the purine are doubling times.

† Enzymes are represented by their gene symbol (cf. Fig. 1).

Table 3. Effects of purine starvation on pools of ATP, GTP and PRPP, and levels of purine biosynthetic enzymes in strains of *B. subtilis* defective in purine biosynthesis

Cells were grown and pools and enzyme levels analysed as described in Methods. Pools and enzyme levels are given as a percentage of values from wild-type cells grown in minimal medium (doubling time 54 min); cf. Table 2.

Strain	Relevant genotype	Purine added* (1 mM)	Relative pool size			Relative enzyme level†					
			ATP	GTP	PRPP	<i>purA</i>	<i>purB</i>	<i>purF</i>	<i>purD</i>	<i>guaA</i>	<i>guaB</i>
ED250	<i>purA</i>	Adenine (60 min)	150	120	60	<1	33	19	40	94	106
		None	1	110	66	<1	45	64	88	85	95
ED234	<i>guaA</i>	Guanosine (52 min)	104	121	157	349	20	16	38	<1	63
		None	131	41	101	305	39	16	42	<1	318
ED157	<i>purM</i>	Adenine + guanosine (54 min)	160	121	57	18	6	4	4	72	118
		None	5	23	323	168	169	126	403	85	144

* Times in parentheses after the name of the purine are doubling times.

† Enzymes are represented by their gene symbol (cf. Fig. 1).

Here we studied three mutants: strain ED157 (*purM*), which is defective in IMP biosynthesis (Fig. 1); a *purA* mutant (ED250), which requires adenine; and a *guaA* mutant (ED234), requiring guanosine for growth. In the *purA* mutant, enzyme activities corresponding to the *pur* operon were repressed and the pool sizes of ATP and GTP were increased while the PRPP pool was diminished (Table 3). The major effect of adenine starvation

was a diminished ATP pool size and some derepression of the level of the *pur* operon encoded enzymes. In the *guaA* mutant we found repressed levels of the *pur* operon and *guaB* encoded enzymes, increased levels of sAMP synthetase (*purA*) and increased pool sizes of GTP and PRPP. Guanosine starvation led to increased levels of IMP dehydrogenase (*guaB*) and diminished GTP pool size. To study the effect of starving the cells for both

Table 4. *Effects of adenine and adenosine on pools of ATP, GTP and PRPP, and levels of purine biosynthetic enzymes in strains of B. subtilis defective in purine salvage and interconversion*

Cells were grown and pools and enzyme levels analysed as described in Methods. Pools and enzyme levels are given as percentage of values from wild-type cells grown in minimal medium (doubling time 54 min); cf. Table 2.

Strain	Relevant genotype	Purine or nucleoside added* (1 mM)	Relative pool size			Relative enzyme level†			
			ATP	GTP	PRPP	<i>purA</i>	<i>purB</i>	<i>purF</i>	<i>purD</i>
ED265	<i>ade</i>	None (52 min)	101	101	89	152	103	99	119
		Adenine (66 min)	123	82	56	13	50	55	66
ED287	<i>apt</i>	None (54 min)	99	95	97	142	107	103	112
		Adenine (54 min)	96	92	104	151	80	87	97
ED279	<i>ade apt</i>	None (54 min)	99	102	80	243	96	73	97
		Adenine (52 min)	98	105	107	253	83	74	94
ED95	<i>pupA</i>	None (54 min)	70	80	107	88	96	90	103
		Adenine (60 min)	138	112	72	16	28	9	28
		Adenosine (49 min)	101	94	68	50	47	33	55
QB-917	Wild-type	Adenosine (48 min)	120	110	63	8	34	12	13

* Times in parentheses after the name of the purine or nucleoside are doubling times.

† Enzymes are represented by their gene symbol (cf. Fig. 1).

adenine and guanine nucleotides, we shifted the *purM* mutant to purine-free medium. This led to increased levels of the enzymes encoded by the *pur* operon, the *purA* gene and the *guaB* gene. The pool sizes of ATP and GTP were reduced and that of PRPP increased (Table 3).

Effects of adenine on purine enzyme levels in salvage mutants

To determine whether adenine must be metabolized to affect purine gene expression, we studied the effects of adenine and adenosine in mutants defective in adenine phosphoribosylation (*apt*), adenine deamination (*ade*) and adenosine phosphorylase (*pupA*). Adenine had no significant effect on enzyme levels and pool sizes in the *apt* mutant. In the *ade* mutant, the sAMP synthetase (*purA*) level was markedly reduced and the *pur* operon expression was reduced to 50%. The ATP pool was increased and the GTP and PRPP pools were decreased (Table 4). In the *ade apt* mutant elevated levels of sAMP synthetase, unaffected by adenine, were seen. Analysis of the *pupA* mutant, with reduced capacity for adenosine metabolism, revealed that the repressing effect of

adenosine, but not the adenine effect, was significantly reduced (Table 4). This indicates that phosphorolysis of adenosine to adenine is required for maximal effect of adenosine.

Discussion

To understand the interaction between *de novo* synthesis of purines and purine salvage it is pertinent to recall that the two processes occur simultaneously, because purine bases and purine nucleosides are continuously formed and re-utilized (Nygaard, 1983). Exogenous purines are efficiently utilized for purine nucleotide synthesis. This has a sparing effect on purine synthesis *de novo*, and often stimulates growth (Table 2). During growth a balanced supply of adenine and guanine nucleotides is required. The mode of regulation of the purine biosynthetic pathway is mediated through feed-back inhibition mechanisms on central enzymes, and on the synthesis of purine biosynthetic enzymes (Neuhard & Nygaard, 1987). Regulation of purine enzyme synthesis seems to be of significance only at higher concentrations of purine compounds in the growth medium.

Fig. 2. Levels of sAMP synthetase and IMP dehydrogenase activity as a function of the GTP/ATP pools ratio. (a) Specific activity of sAMP synthetase (*purA*); (b) IMP dehydrogenase (*guaB*). Data from Tables 2, 3 and 4.

In the present study we used purines that, most likely, are present in the natural habitat of *B. subtilis*. Adenine and guanine compounds in a given habitat reflect cellular degradation, while hypoxanthine and xanthine compounds are excretion products. *B. subtilis* can utilize bases, nucleosides and nucleotides as sole purine source; adenine and hypoxanthine compounds serve equally well, guanine less well, and xanthine is a poor purine source (Nygaard, 1983). Because of the poor solubility of guanine in the culture medium, we have used only guanosine, which is converted to guanine inside the cell (Saxild & Nygaard, 1987).

Addition of purines to cultures causes repressed levels of the *purA* and *guaB* encoded enzymes and the enzymes encoded by the *pur* operon (Tables 2, 3 and 4). Little is known, however, about regulation of the expression of the *purA* and *guaB* encoded enzymes at the transcriptional level. We could not find any clear correlation between enzyme levels and nucleotide pool sizes. However, when the levels of sAMP synthetase and IMP dehydrogenase were plotted versus the GTP/ATP pools ratio (Fig. 2), a positive correlation between increased GTP/ATP pools ratio and increased level of sAMP synthetase and decreased level of IMP dehydrogenase was observed. Accordingly, GTP increases and ATP decreases the level of sAMP synthetase while the reverse is true for IMP dehydrogenase. These changes in pools and enzyme levels clearly respond to the cellular need for AMP and GMP. The nucleoside triphosphates (ATP and GTP) are the major components of each nucleotide pool under exponential growth conditions, and they vary in parallel with the total nucleotide pools of adenine and guanine, respectively (Chapman & Atkinson, 1977). Following growth on adenine the cells contained low concentrations of PRPP and increased ATP pools, while purine starvation led to increased PRPP pools and low ATP pools. Guanosine addition resulted in increased PRPP pool size. The PRPP pool size correlated positively with the GTP/ATP pools ratio. Whether PRPP is directly involved in the regulation of the expression of the *purA* and *guaB* genes remains to be established. The reduced PRPP pool size caused by adenine addition is caused, most likely, by the strong

inhibiting effect of ADP on PRPP synthetase activity (Arnvig *et al.*, 1990). By computer analysis, we could not find sequences in the upstream region of the *guaB* structural gene similar to those involved in the adenine and guanine control regions of the *pur* operon, suggesting a different mechanism of regulation of expression.

The expression of the *pur* operon has been studied in detail, and the current model (Zalkin & Ebbole, 1989) suggests that transcription initiation is controlled by an adenine compound and that a guanine compound promotes early transcription termination. In agreement with this, we found throughout that the enzymes encoded by genes of the *pur* operon are affected by addition of adenine and guanosine to the growth medium, but we also observed that hypoxanthine addition represses these genes (Tables 2 and 3). No low molecular mass effector of purine gene expression in *B. subtilis* has been identified. In *E. coli* hypoxanthine and guanine appear to be low molecular mass repressors of purine gene expression (Meng & Nygaard, 1990). We found that the expression of the *pur* operon was significantly influenced by addition of purines to the growth medium. Under most conditions the expression of the *purB* and the *purD* genes were co-ordinately regulated, except in purine-starved cells. The expression of the *purF* gene, encoding PRPP amidotransferase, was less well correlated with that of the other *pur* genes. This might be explained by the fact that PRPP amidotransferase is subject to *in vivo* degradation, particularly under starvation conditions (Grandoni *et al.*, 1989). It is also possible that post-transcriptional regulation may affect the enzyme levels, as suggested for a *purC-lacZ* translational fusion in *B. subtilis* (Ebbole & Zalkin, 1989a). Instability during storage and assay could not account for the differences, because enzyme levels were not affected by storage of cells for up to 2 weeks at -20°C , and the extract did not lose activity when kept at 0°C for a number of hours (data not given). Neither individual nucleotide pools, nor any ratio between these correlate satisfactorily with *pur* operon enzyme levels. If we compare the *pur* operon levels exemplified by the *purB* enzyme versus the ATP pool size obtained in cultures that were not supplemented with guanosine or hypoxanthine, a correlation between

Fig. 3. Correlation between sAMP lyase activity and the ATP pool. The specific activity of sAMP lyase (*purB*) is plotted against the ATP pool. Data from Tables 2, 3 and 4; ●, data from experiments with cultures to which hypoxanthine or guanosine were added; ○, data from other cultures.

enzyme and ATP pool size is seen (Fig. 3). Thus, in the absence of hypoxanthine and guanosine, we are determining enzyme levels regulated by the ATP pool size. This regulation, most likely, is mediated via an inhibition of the initiation of transcription, which is controlled by an adenine compound (Ebbolle & Zalkin, 1987). To account for the regulation of the *pur* operon expression under conditions where hypoxanthine or guanosine are present, we still have the ATP effect on initiation of transcription, and on top of that the regulation of transcription attenuation caused by a guanine compound; the highest level of repression is seen under conditions when the ATP pool is high and hypoxanthine or guanosine is present.

We measured purine enzyme levels in strains harbouring mutations that permit manipulation of the metabolism of adenine and adenosine (Table 4). Adenine affects *pur* gene expression (including the *pur* operon, *purA* and *guaB*) only when it can be metabolized, giving rise to an increased ATP pool, and we believe this to be support for the above suggestions that ATP is a low molecular mass effector molecule, but the possibility remains that the effector may be AMP or ADP. Clearly a guanine nucleotide, probably GTP, plays a role in the regulation of the expression of the *purA* and *guaB* genes, but not of the *pur* operon. While it appears that the free bases guanine and hypoxanthine might be low molecular mass repressors of the *pur* operon, we found that the overall regulation of the level of purine biosynthetic enzymes shows a pattern that spares the synthesis of dispensable enzymes, with the exception of GMP synthetase (*guaA*).

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