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

HANS HENRIK SAXILD† and PER NYGAARD*

University Institute of Biological Chemistry B, Sølvgade 83, 1307 Copenhagen K, Denmark

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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-

† Present address: Department of Microbiology, Technical University, DK-2800 Lyngby, Denmark.

Abbreviations: PRPP, 5-phosphoribosyl-α-1-pyrophosphate; sAMP, adenylosuccinate.
Fig. 1. Pathway of purine biosynthesis and interconversion in *B. subtilis*. Enzymes of relevance for the present study are identified by their gene symbols: *purF*, PRPP amidotransferase; *purD*, glycaminamide ribonucleotide (GAR) synthetase; *purM*, aminoimidazole ribonucleotide (AIR) synthetase; *purB*, adenylosuccinate (sAMP) lyase; *purA*, sAMP synthetase; *guaB*, IMP dehydrogenase; *guaA*, GMP synthetase; *apt*, adenine phosphoribosyltransferase; *ade*, adenine deaminase; and *pupA*, adenosine phosphorylase. PRA, phosphoribosylamine; FGAR, formylglycinamide ribonucleotide; FGAM, formylglycinamidine ribonucleotide; CAIR, carboxyaminoimidazole ribonucleotide; sAICAR, aminoimidazolesuccinocarboxamide ribonucleotide; AICAR, aminoimidazolecarboxamide ribonucleotide.

repressor is probably an adenine compound. However, *in vitro* data could not show that the protein–DNA interaction depends on an adenine compound (Ebbole & Zalkin, 1989b). The *pur* transcript contains a 242 nucleotide untranslated leader mRNA capable of forming a transcriptional terminator structure (Ebbole & Zalkin, 1988). The transcriptional termination is regulated by a guanine compound and is believed to be mediated by a trans-acting factor involved in the formation of an antiterminator stem-loop structure in the mRNA leader region. No small molecular effector molecule has been identified.

In *E. coli* all purine genes, except for *purA*, comprise a regulon that is regulated at the level of transcription initiation by the *purR* encoded repressor protein (PurR) (Meng et al., 1990). *In vivo* studies in *S. typhimurium* (Houlberg & Jensen, 1983) and *E. coli* (Meng & Nygaard, 1990) have provided evidence for guanine and hypoxanthine as small molecule repressors. *In vitro* analyses have demonstrated that the PurR protein, in the presence of guanine or hypoxanthine, is capable of binding to a specific nucleotide sequence (Meng & Nygaard, 1990; Rolfes & Zalkin, 1990). This sequence is found in the −35 region of purine biosynthetic genes (He et al., 1990). The PurR protein has been shown also to repress the *pyrC* gene (dihydroorotase) of pyrimidine biosynthesis in *S. typhimurium* (Neuhard et al., 1990) and *E. coli* (Choi & Zalkin, 1990; Wilson & Turnbough, 1990). Involvement of GTP in expression of other genes of the pyrimidine biosynthesis pathway has also been observed (Jensen, 1989).

In the present paper, we describe how the levels of the enzymes of purine biosynthesis in *B. subtilis* are regulated to ensure a balanced supply of AMP and GMP. To explore the effect of various purine compounds on growth, enzyme levels and nucleotide pool size we have used wild-type cells, mutants defective in purine salvage and interconversion and purine auxotrophic mutants.
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 OD_{436} = 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 OD_{436} = 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 Rise, Denmark, and ¹⁴C-labelled compounds were from Amersham.

Enzyme assays. Cells from 80 ml of culture (OD_{436} = 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, glycaminide 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 triphosphates and PRPP pools. [³²P]Orthophosphate (0.8 MBq) was added to 2 ml of exponentially growing cultures at OD_{436} = 0.1. At OD_{436} = 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 *gua* 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 ± 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.

<table>
<thead>
<tr>
<th>Purine added* (1 mM)</th>
<th>Relative pool size</th>
<th>Relative enzyme level†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP</td>
<td>GTP</td>
</tr>
<tr>
<td>Adenine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(49 min)</td>
<td>169</td>
<td>136</td>
</tr>
<tr>
<td>Guanosine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(54 min)</td>
<td>109</td>
<td>130</td>
</tr>
<tr>
<td>Adenine + guanosine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(47 min)</td>
<td>170</td>
<td>126</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(48 min)</td>
<td>100</td>
<td>104</td>
</tr>
<tr>
<td>Xanthine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(52 min)</td>
<td>106</td>
<td>107</td>
</tr>
</tbody>
</table>

* 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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Purine added* (1 mM)</th>
<th>Relative pool size</th>
<th>Relative enzyme level†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ATP</td>
<td>GTP</td>
</tr>
<tr>
<td>ED250</td>
<td>purA</td>
<td>Adenine (60 min)</td>
<td>150</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>1</td>
<td>110</td>
</tr>
<tr>
<td>ED234</td>
<td>guaA</td>
<td>Guanosine (52 min)</td>
<td>104</td>
<td>121</td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>131</td>
<td>41</td>
</tr>
<tr>
<td>ED157</td>
<td>purM</td>
<td>Adenine + guanosine</td>
<td>160</td>
<td>121</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(54 min)</td>
<td>5</td>
<td>23</td>
</tr>
</tbody>
</table>

* 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
Purine enzyme levels in Bacillus subtilis

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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Purine or nucleoside added* (1 mM)</th>
<th>Relative pool size</th>
<th>Relative enzyme level†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ATP</td>
<td>GTP</td>
<td>PRPP</td>
</tr>
<tr>
<td>ED265</td>
<td>ade</td>
<td>None</td>
<td>(52 min)</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adenine</td>
<td>(66 min)</td>
<td>123</td>
</tr>
<tr>
<td>ED287</td>
<td>apt</td>
<td>None</td>
<td>(54 min)</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adenine</td>
<td>(54 min)</td>
<td>96</td>
</tr>
<tr>
<td>ED279</td>
<td>ade apt</td>
<td>None</td>
<td>(54 min)</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adenine</td>
<td>(54 min)</td>
<td>98</td>
</tr>
<tr>
<td>ED95</td>
<td>pupA</td>
<td>None</td>
<td>(54 min)</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adenine</td>
<td>(60 min)</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adenosine</td>
<td>(49 min)</td>
<td>101</td>
</tr>
<tr>
<td>QB-917</td>
<td>Wild-type</td>
<td>Adenosine</td>
<td>(48 min)</td>
<td>120</td>
</tr>
</tbody>
</table>

* 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.
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 (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...
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 (Ebbole & 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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References


