Functional analysis of the *Bacillus subtilis* *purT* gene encoding formate-dependent glycinamide ribonucleotide transformylase

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The *purT* gene from *Bacillus subtilis* encoding the formate-dependent glycinamide ribonucleotide transformylase T was cloned by functional complementation of an *Escherichia coli* *purN purT* double mutant. The nucleotide sequence revealed an open reading frame of 384 amino acids. The *purT* amino acid sequence showed similarity to the enzyme phosphoribosylaminoimidazole carboxylase encoded by the *purK* gene but not to the *N*¹⁰-formyltetrahydrofolate-dependent glycinamide ribonucleotide transformylase N enzyme encoded by the *purN* gene. The glycinamide ribonucleotide transformylase T level was repressed in cells grown in rich medium compared to minimal-medium-grown cells. However, when the culture entered the stationary-growth phase the enzyme level increased in rich medium and decreased in minimal medium. By comparing the deduced amino acid sequence of the *B. subtilis* *purT* gene product with translated nucleotide sequences in various databanks, evidence for the existence of putative *purT* genes in the Gram-negative bacteria *Pasteurella haemolytica* and *Pseudomonas aeruginosa* was obtained.

Keywords: 5'-phosphoribosylglycinamide transformylase, growth phase, *purT* gene sequence, *purT* gene expression, *purT-lacZ* gene fusion

INTRODUCTION

The *de novo* synthesis of inosine monophosphate (IMP) proceeds by a 11-step pathway. The third step is the formylation of glycinamide ribonucleotide (GAR) to formylglycinamide ribonucleotide (formyl-GAR). In *Escherichia coli* and *Bacillus subtilis* this reaction is catalysed by two different enzymes; the *N*¹⁰-formyltetrahydrofolate (*N*¹⁰-formyl-THFA)-dependent GAR transformylase N and the formate-dependent GAR transformylase T, which are encoded by the *purN* and *purT* genes, respectively (Smith & Daum, 1987; Ebbole & Zalkin, 1987; Nygaard & Smith, 1993; Saxild *et al.*, 1994a). In *B. subtilis* the *purN* gene is located in the 12-gene *pur* operon, which encodes all enzymes necessary for the biosynthesis of IMP. The

level of the purine biosynthetic enzymes is decreased when purine compounds are added to the growth medium (Saxild & Nygaard, 1991). This repression is achieved by a transcription termination-antitermination mechanism (Ebbole & Zalkin, 1989). The *purT* gene maps between the *cysA* and *aroI* markers at 20° on the linkage map. No significant repression of the *purT* gene expression by purines has been observed (Saxild *et al.*, 1994a). In contrast, the expression of the *E. coli* *purT* gene is transcriptionally regulated by the PurR repressor protein and hypoxanthine/guanine (Meng & Nygaard, 1990; Nygaard & Smith, 1993). In *purN* mutants, in which growth is dependent on PurT activity, growth in purine-free medium is reduced and is inhibited by glycine (Nygaard & Smith, 1993; Saxild *et al.*, 1994a). Recently, a gene, *purU*, which encodes a *N*¹⁰-formyltetrahydrofolate hydrolase that cleaves *N*¹⁰-formyltetrahydrofolate into formate and tetrahydrofolate, was identified in *E. coli* (Nagy *et al.*, 1993). It appears that PurU provides the major source of formate for the PurT-catalysed reaction under aerobic conditions, and that this reaction is inhibited by glycine. This explains why growth of *purN* mutants is inhibited by glycine.

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Abbreviations: GAR, glycinamide ribonucleotide; IMP, inosine monophosphate.

The EMBL accession number for the sequence data reported in this paper is X78962.

In this report we describe the molecular cloning and nucleotide sequence of the *B. subtilis purT* gene. Also the identification of the transcriptional start site and of the *purT* promoter and studies of *purT* gene expression are reported.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used are listed in Table 1. Growth in liquid medium at 37 °C was followed by measuring OD₄₅₀. As minimal medium for *B. subtilis*, the Spizizen salt-buffered medium was used (Saxild & Nygaard, 1987); for *E. coli*, a phosphate-buffered salt medium was used (Monod *et al.*, 1951). Both media were supplemented with thiamin-HCl (1 µg ml⁻¹), and amino acids (50 µg ml⁻¹) and hypoxanthine (45 µg ml⁻¹) were added to auxotrophic strains when required. L-broth was used as a rich medium. Antibiotics were used at the following concentrations: ampicillin (Ap), 100 µg ml⁻¹; tetracycline (Tc), 8 µg ml⁻¹; chloramphenicol (Cm), 5 µg ml⁻¹; and neomycin (Neo), 5 µg ml⁻¹.

DNA manipulations and genetic techniques. Chromosomal DNA from *B. subtilis* was isolated according to the method of Saxild & Nygaard (1987). Small-scale plasmid preparations from *E. coli* were prepared as described by Birnboim & Doly (1979). Large-scale plasmid preparations from *E. coli* were isolated from 200 ml L-broth cultures. Transformation (Boylan *et al.*, 1972) and transduction (Love *et al.*, 1976) of *B. subtilis* and transformation of *E. coli* (Hanahan, 1983) have previously been described by Saxild & Nygaard (1987, 1988). For transduction the generalized transducing phage AR9 was used, which is related to the phage PBS1 (Love *et al.*, 1976). Treatment of DNA with restriction enzymes (Boehringer Mannheim), T4 DNA ligase and Klenow polymerase (Gibco-BRL) was performed as recommended by the suppliers.

Southern blot analysis. DNA was blotted onto a nitrocellulose membrane (GeneScreen, NEN Research Products). The DIG DNA Labelling and Detection kit (Boehringer Mannheim) was used for colorimetric detection of the hybridization products. The recipe supplied by the manufacturer was followed.

DNA sequencing. DNA sequence was obtained by the chain-termination reaction method using dideoxynucleotides as described by Sanger *et al.* (1977). All sequencing analysis was done on double-stranded plasmid DNA templates.

Primer extension analysis. RNA from *B. subtilis* was isolated according to the method of Resnekov *et al.* (1990) with some modifications. Cells were grown in minimal medium to an OD₄₅₀ of 1.0. The culture (20 ml) was poured into crushed ice and centrifuged for 5 min at 7000 g. Cells were washed three times in 1 ml ice-cold buffer (50 mM Tris/HCl, pH 7.5; 50 mM NaCl; 5 mM EDTA) and transferred to an Eppendorf micro-centrifuge tube. The cells were treated with lysozyme by resuspension in 200 µl buffer (20 mM sodium maleate, pH 6.5; 0.5 M sucrose; 20 mM MgCl₂) containing 3.5 mg lysozyme ml⁻¹ followed by incubation on ice for 30 min. The following reagents were then added: 200 µl 0.1 M sodium acetate, pH 4.0, containing 2% (w/v) SDS; 10 µl 0.5 M EDTA, pH 8.0; and RNase inhibitors (4 µl 1%, w/v, heparin; 1 µl 1 M phenanthroline). The cells were lysed by heating to 65 °C for 3 min. Proteinase K (20 mg ml⁻¹; 40 µl) was added and incubation continued for 5 min on ice. The lysate was treated with 400 µl hot (65 °C) phenol for 3 min and the two phases were separated by centrifugation for 5 min. The upper phase containing the

nucleic acids was transferred to a new tube and the phenol extraction was repeated. The aqueous phase was then extracted with buffered (10 mM Tris/HCl, pH 8.0; 1 mM EDTA) phenol/chloroform (1:1, v/v) at room temperature. Nucleic acid was precipitated by adding 3 M sodium acetate, pH 4.8 (0.1 vol.), and 96% (v/v) ethanol (2 vols) followed by centrifugation for 10 min. The pellet was washed with 70% (v/v) ethanol and dried and resuspended in 90 µl H₂O. The solution was heated to 80 °C for 2 min and then placed on ice. DNA was removed by adding the following reagents: 10 µl buffer (250 mM KCl; 25 mM MgCl₂; 500 mM Tris/HCl, pH 6.7), 40 units RNasin (Promega) and 30 units DNase I (RNase-free) from Boehringer Mannheim. After 10 min incubation at room temperature, the solution was extracted twice with phenol/chloroform and once with chloroform, and RNA was precipitated with sodium acetate and ethanol as described above. The dried pellet was resuspended in 100 µl 10 mM potassium phosphate, pH 6.5, and stored at -20 °C. This procedure normally gives approximately 100 µg RNA. The single-stranded DNA primer used for primer extension was labelled with [³²P]phosphate at the 5' terminus using T4 polynucleotide kinase and [γ-³²P]ATP. The primer extension reaction was performed as described by Sambrook *et al.* (1989) with the following modifications. RNA (20–40 µg) in phosphate storage buffer (10 µl) was mixed with approximately 1 pmol (1 µl) radiolabelled DNA primer. The mixture was heated to 70 °C for 10 min and placed on ice, then 4 µl reaction buffer (250 mM Tris/HCl, pH 8.3; 375 mM KCl; 15 mM MgCl₂), 2 µl 0.1 M DTT, 1 µl dNTP (10 mM concentration each) and 2 µl H₂O were added. After incubation for 2 min at 37 °C, 200 units of reverse transcriptase was added and the incubation continued for 1 h. The radiolabelled cDNA products were separated on a 6% (w/v) polyacrylamide sequencing gel and visualized by autoradiography.

Enzyme assays. Preparation of cell-free extracts for enzyme measurements has previously been described by Saxild & Nygaard (1988). Detection of α-amylase activity in *B. subtilis* cells was performed by spraying colonies grown on L-broth agar plates containing 1% (w/v) starch with a solution of 0.5% iodine and 1% (w/v) potassium iodide. The α-amylase-producing colonies formed a clear halo, whereas the α-amylase-negative colonies did not. The catechol-2,3-dioxygenase (XylE) activity in *B. subtilis* cells was screened by spraying colonies grown on minimal medium agar plates with a solution of 0.5 M catechol. XylE⁺ colonies became yellow, whereas XylE⁻ colonies did not. Formate-dependent GAR transformylase T activity was determined according to Nygaard & Smith (1993). GAR synthetase and hypoxanthine phosphoribosyltransferase activities were determined as described before (Saxild & Nygaard, 1991, 1988). One unit is defined as 1 nmol substrate converted min⁻¹ at 37 °C. Determination of β-galactosidase activity (Miller, 1972) was performed according to Saxild *et al.* (1994b). Protein was determined by the Lowry method.

RESULTS

Cloning and genetic characterization of the *B. subtilis purT* gene

The *B. subtilis purT* gene was cloned by functional complementation in *E. coli*. The *E. coli* purine auxotrophic strain TX680 (*purN purT*) was transformed with a genome library of *EcoRI* restriction fragments from *B. subtilis* strain HH124 (*purN*) cloned into plasmid pLNA2, which confers Tc^R in *E. coli*. By using DNA from strain HH124, cloning of the *purN* gene was avoided. In one

Table 1. Bacterial strains and plasmids

BGSC, Bacillus Genetic Stock Center, Columbus, Ohio, USA; Ap, ampicillin; Tc, tetracycline; Cm, chloramphenicol; Neo, neomycin.

Strain/plasmid	Characteristics	Source or reference
<i>B. subtilis</i>		
168	<i>trpC2</i>	BGSC
QB928	<i>trpC2 aroI96 dal-1 purB33</i>	BGSC
QB944	<i>trpC2 cysA14 purA16</i>	BGSC
HH123	<i>trpC2 hisA1</i>	Saxild <i>et al.</i> (1994a)
HH124	<i>trpC2 hisA1 purN-602</i>	Saxild <i>et al.</i> (1994a)
HH179	<i>trpC2 hisA1 purN-602 purT-46</i>	Saxild <i>et al.</i> (1994a)
HH202	<i>trpC2 hisA1 purN-602 purT-46 :: pHJ3 (purT⁺) (cat)</i>	Transformation of HH179 by pHJ3, Cm ^R
HH208	<i>trpC2 amyE :: purT-lacZ (neo)</i>	Transformation of 168 by pHH1009 digested with <i>Xba</i> I, Neo ^R
<i>E. coli</i>		
MT102	F ⁻ <i>araD139 Δ(argF-leu)7696 Δ(lac)X74 galU galK hsdR2 (r⁻m⁺)mcrB1 rpsL (Str^R)</i>	Laboratory stock
TX680	<i>ara Δ(gpt-pro-lac) thi rbs-221 ilvB2102 ilvHI2202 purN'-lacZ⁺Y⁺ :: Kan^R purT</i>	Smith & Nygaard (1993)
Plasmid		
pKS+	Ap	Stratagene
pAMB22	Tc Cm	Zukowski & Miller (1986)
pC194	Cm	BGSC
pUN121	Ap Tc	Nilsson <i>et al.</i> (1983)
pDG268neo	Ap Neo. pBR322 derivative containing the <i>B. subtilis</i> <i>amyE</i> gene in which a promoterless <i>lacZ</i> gene and a DNA cassette containing the <i>neo</i> gene have been inserted. Transcriptional fusions can be obtained by inserting DNA fragments into a unique <i>Hind</i> III site in front of the <i>lacZ</i> gene.	C. W. Price*
pLNA2	pUN121 partially digested with <i>Pst</i> I, blunt-ended with Klenow polymerase and ligated with a 1.6 kb blunt-ended <i>Cla</i> I fragment of pC194 containing the <i>cat</i> gene	L. N. Nonboe†
pHJ3	pLNA2 containing the <i>B. subtilis</i> <i>purT</i> gene on a 6 kb <i>Eco</i> RI fragment	This work
pHJ4	pKS+ digested with <i>Pst</i> I ligated to a 3.4 kb <i>Pst</i> I fragment from pHJ3 containing the <i>purT</i> gene	This work
pHJ8	Deletion of a 500 bp <i>Hind</i> III fragment from pHJ4	This work
pHJ14	pAMB22 digested with <i>Sma</i> I + <i>Eco</i> RI ligated with a 355 bp <i>Eco</i> RI- <i>Hinc</i> II fragment from pHJ4 containing the <i>purT</i> promoter	This work
pHJ16	pAMB22 digested with <i>Sma</i> I + <i>Eco</i> RI ligated with a 260 bp <i>Eco</i> RI- <i>Ssp</i> I fragment from pHJ4 containing the <i>purT</i> promoter lacking the -10 region	This work
pHH1009	pDG268neo digested with <i>Hind</i> III ligated with a 500 bp <i>Hind</i> III fragment from pHJ4 containing the <i>purT</i> promoter	This work

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experiment six Tc^R transformants appeared on purine-free minimal medium agar plates. Transformation of strain TX680 with the same amount of DNA resulted in approximately 7000 Tc^R transformants on minimal medium agar plates. The plasmids from the TX680 Pur⁺ clones were isolated and all contained a 6 kb *Eco*RI fragment cloned in pLNA2. One of the plasmids was chosen and named pHJ3. The ability to complement the

purine deficiency of strain TX680 was preserved when the orientation of the *Eco*RI insert in pHJ3 was reversed (data not shown). This indicated that the *purT* gene was most likely expressed from an internal promoter. Cell-free extracts of TX680 harbouring pHJ3 grown in LB medium contained 22 units (mg protein)⁻¹ GAR transformylase T activity. The *purT* gene was subcloned into plasmid pBluescript KS+ on a 3.4 kb *Pst*I fragment (pHJ4).

<i>Pst</i> I	
CTGCAGTGTGCGGGTTTCGTTTTCATGCGATGCTCCTGCGGTGTGGTGGTCTTGGAT	-177
TAAGTGTAGCATGATTTCGGGGGATTCTTGTATGATGTTGCTGTTTAAAGAAATAGAGAAA	-117
GCAGACCGTTATTGTCTGCTTTCGTTTAAAGAAATTTAAACCTCAATAATA	-57
ATTTTATCTTTTAAATAAAATTTATTTTCAAATATTTTCAATATATGTTTGGTGGTGGAC	4
RBS	
AGATAGGAGGGGCAAGATGATCAATCAAGAAAGGTTTATGTTAGGTTTCAAGTGAAT	64
M Y Q S K K V L L L G S G E	15
TAAGTAAAGAGGTAGTGTGAAAGCCACGCTCTGGGGTACAGACGGTGGCAGTTGACA	124
L G K E V V I E A Q R L G V Q T V A V D	35
GTTACGAGCATGCTCCGGCGATGAGGTGCGCATACAGTATGCTGTTGATATGCTGG	184
S Y E E A P A M Q V A H N S Y V V D M L	55
ACCCAGAGCAGATCAGAACCATCATGAGAAAGAAATCCGATTGATTGCTGCGTGAAG	244
D P E Q I R T I I E K E N P D L I V P E	75
TTGAGGCGATTCCGACGGATGAATTCCTGAAGCTTGAAGAGGAAGGATTTCACGTTATCC	304
V E A I A T D E L L K L E E E G F E V I	95
CGAATGCCCTGCGCCAGTGCATGATGGATCGGGAAGGCATCAGACGACTTCCGCGCG	364
P N A R A A K L T M D R E G I R R L A A	115
AAACGCTGGAGTTCGACAGCTGGCTATGAATTCGGAATACATATGATGAATTTATAC	424
E T L G L A T A G Y E F A N T Y D E F I	135
AAAGCGCAGCTCAGATCGGTTTCTTGTGTCGTTAAACCATGATGATGTTCTTCTGGAA	484
Q A A A Q I G F P C V V K P L M S S S G	155
AAAGCCAAAGTGTGTGCGCTCTGAAGCGGATTAGAGAGCTGCTGGAGACGGCGATGG	544
K G Q S V C R S E A D L E S C W E T A M	175
AAAGCGGACGGGTGAAAGACGGCCGCGTGCATGCTGAGGAGTTTATCCCTTTCGAATCAG	604
E G G R V K N G R V I V E E F I P F E S	195
AAATCACACTCTTAACCGTACGTCGGTTAACGGTACGGCATTTTGTGAGCCGATCGGT	664
E I T L L T V R A V N T A F C E P I G	215
ATGTCCAAAGGACGGGATTATATCGAATCGTGGCCGCGATGATATGACAGACGAC	724
E V Q K D G D Y I E S W Q C H D M T E Q	235
AAATAGAAGAAGCAAGCATATTGCGAAAGACATCAGGATGAGCTTGGCGGATACGGCC	784
Q I E E A M D I A K T I T D E L G G Y G	255
TGTTGGTGTGAGCTGTTCTTTCGCAAGATAGGGTATATTTCAAGTGAAGTATCTCTCC	844
L F G V E L F L A K D R V Y F S E V S P	275
GTCCGATGATACGGGTCTTGTGACGCTGGTGACGCAAAATTTGTGCAAAATTCGCGTGC	904
R P H D T G L V T L V T Q N L S E F A L	295
ATGTCCGGGCGATTCTCGGCTTTCGATTACAGAAATCACACAGCTTTCCTCGGCCCA	964
E V R A I L G F P I T E I T Q L S P G A	315
GCAGGCCCTCAAAGCGCCGGAAGAATAGCGGATACACTTGTGAAGGATTGGAAGACG	1024
S R P L K A P E E L A D Y T V E G L E N	335
CACTGGCAGTCCCAAGACTCAAGTCCGCTCTTGGAAAGCCGATAACAAAGCCGGAC	1084
A L A V P K T Q V R V F G K P I T K A G	355
GTGATGAGCAGTTGCGCTTCTGCTGCTGATTGAGTTGAAACGGCAAGAGAGAAATCAA	1144
R R M A V A L S A A D S V E T A R E M A	375
AGAAAGCGTTGGACAGCTAATTTTAAATAGAGTTTGAACAGGCTTGTGATGGGACAA	1204
K K A L D Q L I L K	384
GGCCTGTTTTTCTTCTCCGTAAGTATTTATCATAAGAAATCAGAAACCTGATTATAA	1264
CGAT	
TGTAAAGTCTTCCTGATACGGGTGGTGGACACTAAAGGAGGAGATGACAAATGAA	1324
M K	2
ATTAGTCCCAAGATTCAAGAAACAA	1349
L V P R F R K Q	10

Fig. 1. Nucleotide and deduced amino acid sequences of the *purT* gene encoding GAR transformylase T. The *purT* promoter elements are indicated by lines above the sequence. The putative ribosome-binding site (RBS) is underlined. A stem and loop structure specifying a potential rho-independent transcription terminator is indicated by arrows above the sequence. The nucleotide beneath the plus sign is the first nucleotide of the *purT* messenger. *mpr* encodes an extracellular metalloprotease (Sloma *et al.*, 1990).

Deletion of 500 bp from one end of the 3.4 kb *Pst*I fragment (pHJ8; Table 1) resulted in the loss of *purT* complementation ability, indicating that either the start or the end of the *purT* gene was located in this region. Plasmid pHJ3 was transformed into the purine auxotrophic *B. subtilis* strain HH179 (*purN purT*) selecting for Cm^R . Since plasmids derived from pLNA2 cannot replicate in *B. subtilis*, Cm^R transformants could only be formed if the plasmid integrated into the chromosome by a single crossing-over event in the *purT* region. All the tested Cm^R transformants had a purine prototrophic phenotype. A transducing AR9 lysate was made from one of the Cm^R transformants (HH202) and was used for transduction in two of the Dedonder mapping kit strains, QB944 (*cysA*) and QB928 (*aroI*) (Dedonder *et al.*, 1977), by selecting Cm^R transductants. Twenty-seven CysA^+ clones were found among 138 Cm^R QB944 transductants and 59 AroI^+ clones found among 138 Cm^R QB928 transductants. These frequencies are in good agreement with the previously reported location of the *purT* gene between *cysA* and *aroI* on the linkage map (Saxild *et al.*, 1994a).

Nucleotide and derived amino acid sequence

Deletion analysis of the cloned DNA led to the identification of the *purT* gene on a 1.5 kb *Pst*I-*Cla*I fragment. The nucleotide sequence of both strands of the 1.5 kb *Pst*I-*Cla*I fragment was determined and the nucleotide sequence of the *purT* gene is shown in Fig. 1. The *purT* gene contained an open reading frame of 1152 bp which gives a protein of 384 amino acids. Three potential translational start codons in the N-terminal part of the *purT* reading frame were found, one 126 nucleotides and another 159 nucleotides downstream from the ATG start codon shown in Fig. 1. The ATG at position +22 is the most likely translation start site (i) because of the position of a potential Shine-Dalgarno sequence, and (ii) from comparison with the *E. coli* and *Pasteurella haemolytica* *purT* sequences (see Discussion). Immediately downstream of the *purT* gene we located the *mpr* gene encoding an extracellular metalloprotease (Sloma *et al.*, 1990).

Mapping of the *purT* promoter region and the 5' end of the *purT* transcript

Putative σA -35 and -10 regions preceded the *purT* structural gene. The upstream DNA sequence was screened for promoter activity by inserting the *Pst*I-*Hinc*II fragment (nucleotides -237 to +118 in Fig. 1) from pHJ4 in front of the promoterless *xylE* gene of pAMB22 (pHJ14). A similar plasmid (pHJ16) containing the *Pst*I-*Ssp*I fragment (nucleotides -237 to -23) was also constructed (Table 1). Strain HH123 was XylE^+ when it contained pHJ14, and XylE^- when it contained pHJ16. These results showed that a promoter was situated immediately upstream of the *purT* structural gene.

Primer extension using reverse transcriptase enzyme was used to map the 5' end of the *purT* mRNA isolated from strain HH123. A primer complementary to the nucleotide

Fig. 2. Primer extension analysis of the *purT* transcription start site. The primer annealed to nucleotides +67 to +95. The dideoxy-sequencing ladder was obtained with the same primer used for cDNA synthesis. RNA was isolated from wild-type strain HH123. The position corresponding to the start of transcription is marked by an arrow.

sequence from +67 to +95 was used. Fig. 2 shows the result of the primer extension experiment. The same primer was used to generate the sequencing ladder. The start site of transcription, which is numbered +1, was

located 21 nucleotides upstream of the putative translational start codon.

Expression of the *purT* gene

purT gene expression was determined by measuring GAR transformylase T activity and β -galactosidase activity from a transcriptional fusion. The construction of a transcriptional fusion between the *purT* promoter region and the *E. coli lacZ* reporter gene in the plasmid vector pDG268neo is described in Table 1. The resulting plasmid, pHH1009, was linearized with *Xho*I, which cuts the DNA outside of the *amyE-neo-lacZ* region, and transformed into *B. subtilis* strain 168 selecting for Neo^R. Since the *neo* gene and the *purT-lacZ* fusion gene were flanked by sequences of the *amyE* gene (encoding α -amylase), Neo^R transformants resulted from a double crossing-over event in the *amyE* gene. This produced a disrupted *amyE* gene which now contained the *neo* gene and the *purT-lacZ* fusion. Two α -amylase-deficient Neo^R transformants were purified and their chromosomal DNA subjected to a Southern blot analysis. They both contained a single copy integration of the *purT-lacZ* fusion into the *amyE* gene (data not shown). One of the strains was named HH208 [*purT-lacZ* :: *amyE* (Neo^R)]. Strain HH208 was grown in minimal medium and in L-broth medium. The expression of the *purT* gene was determined by measuring GAR transformylase T activity and by measuring β -galactosidase from the transcriptional fusion (Table 2). For comparison we included the determination of levels of two other purine enzymes involved in IMP synthesis; GAR synthetase, encoded by the *purD* gene located within the *pur* operon, and hypoxanthine phosphoribosyltransferase, encoded by the *hpt* gene. In cells grown in minimal medium a reduced level of GAR transformylase T activity was seen when cells entered the stationary growth phase but transcription, as judged from the level of β -galactosidase activity, was not reduced, perhaps indicating some kind of inactivation of the enzyme. The addition of hypoxanthine to the minimal medium had no effect on *purT* gene expression. The level of GAR synthetase paralleled that of GAR transformylase

Table 2. Effects of growth stage on the expression of purine genes

Strain HH208 was grown in minimal medium with and without hypoxanthine (Hx; 45 μ g ml⁻¹), and in L-broth. Cells were harvested in the exponential phase, and in the stationary phase 2–3 h after they had left the exponential-growth phase. Enzyme activities ($\pm$ SD) were determined as described in Methods. PurT, GAR transformylase T; PurT-LacZ, β -galactosidase produced by the transcriptional *purT-lacZ* fusion; PurD, GAR synthetase; Hpt, hypoxanthine phosphoribosyltransferase.

Growth medium	Growth phase	Enzyme activity [U (mg protein) ⁻¹]			
		PurT	PurT-LacZ	PurD	Hpt
Minimal	Exponential	37 $\pm$ 12	48 $\pm$ 3	42 $\pm$ 8	95 $\pm$ 11
	Stationary	16 $\pm$ 7	55 $\pm$ 8	16 $\pm$ 4	102 $\pm$ 33
Minimal + Hx	Exponential	33 $\pm$ 8	48 $\pm$ 8	15 $\pm$ 8	154 $\pm$ 4
	Stationary	10 $\pm$ 2	49 $\pm$ 6	3 $\pm$ 1	168 $\pm$ 15
L-broth	Exponential	9 $\pm$ 5	6 $\pm$ 2	3 $\pm$ 2	220 $\pm$ 4
	Stationary	20 $\pm$ 5	21 $\pm$ 4	18 $\pm$ 1	143 $\pm$ 45

T except for the overall reduction in enzyme level in hypoxanthine-supplemented medium. The level of hypoxanthine phosphoribosyltransferase, in contrast, was not affected by the growth phase but was slightly elevated in the hypoxanthine-supplemented growth medium. When cells grown in rich medium (L-broth) entered the stationary phase a reversed pattern was seen; both GAR transformylase T and GAR synthetase activity increased as did *purT* gene transcription, while the level of hypoxanthine phosphoribosyltransferase, which was higher in rich medium, was hardly affected by the change in growth phase. Several attempts to find the compounds that are responsible for the repression of *purT* expression in rich medium failed; this included the addition of various combinations of amino acids dependent on one-carbon metabolism and of adenine, guanine and formate to the growth medium.

DISCUSSION

The *B. subtilis purT* gene was cloned and its nucleotide sequence determined. The *purT* gene is most likely a monocistronic transcriptional unit, based on the following. (i) A start site for transcription was located to a position 21 nucleotides upstream of the start site for translational initiation of a 384 amino acid open reading frame. (ii) The +1 position is preceded by a potential σA promoter sequence: (−34)TTTATT-N₁₈-TATAAT (−7). (iii) Fusion to a promoterless *xyIE* gene at a *SspI* restriction site situated between the −35 and −10 regions resulted in the loss of promoter activity whereas fusions to a distal point gave a *xyIE*⁺ phenotype. (iv) A potential secondary structure for a rho-independent transcription termination was found immediately downstream from the translational stop site. The ΔG value for this stem-loop structure was calculated to be −18 kcal mol^{−1} (−75.6 kJ mol^{−1}) (Tinoco *et al.*, 1973).

The expression of the *purT* gene in *B. subtilis* is not affected by purine bases in the growth medium (Saxild *et al.*, 1994a) as are the other *pur* genes (Saxild & Nygaard, 1991), and the *purT* control region has no sequence similarity or structural features (operator sites or secondary structures) in common with the promoter regions of the *B. subtilis guaA*, *guaB* or *purA* genes or the *pur* operon. However, we have now provided evidence for the regulation of the cellular level of GAR transformylase T. The expression of the *purT* gene and the synthesis of GAR transformylase T, like that of the GAR synthetase, is reduced in rich medium and is affected by the growth phase, resulting in low enzyme levels in the exponential phase and higher levels in the stationary phase. The reversed pattern is seen in minimal medium, in which the GAR transformylase T level decreases in stationary phase but the transcription is not altered. A major difference between *purT* and *purD* gene expression is the repressive effect of hypoxanthine on *purD* expression only (Table 2). Under all the above growth conditions the level of the purine salvage enzyme hypoxanthine phosphoribosyltransferase only varied twofold. The disappearance of GAR transformylase T and GAR synthetase activity in

	V	QGGELGKEV	IE	Q	LGV	AVD	Y	APA			
<i>B. subtilis</i>	MYGSKVLLGSGELGKEVVEIAQRLGVQTVAVDSYCHAPAM								42		
<i>E. coli</i>	MTLLGTLALPAATRVMLGSGELGKEVAIECORLGVVEIVADRYADAPAM								50		
<i>P. haemolytica</i>	MTTIGTPLRTNATKVMMLGSGELGKEVIEIQPLGVVEIVADRYADAPAM								50		
<i>P. aeruginosa</i>	MTRIGTPLSPATRVLLCGSGELGKEVAIEIQRLGCEVIVADRYGNAPAM								50		
	VAN	MLD	R	E	E	P	IVPE	HAIAI	L	LE	DO
<i>B. subtilis</i>	QVANNSTVVDMLDPEQIRTIIEKENPDLIVPEVEAIAITDELLKLEEGPH										92
<i>E. coli</i>	HVAHRSHVINMLDGAALRRVVELEKPHYIVPEIEAIAITDMLIQLEEGNL										100
<i>P. haemolytica</i>	QVAHRAYTISMLDGNALRLDVEKEKPDFIVPEVEAIAITATLVELEQEGYN										100
<i>P. aeruginosa</i>	QVAHRSHVINMLDGAALRAVIEQEKPHYIVPEIEAIAITATLVELEAEGYT										100
	V	P	A	A	L	T	N	R	E	E	P
<i>B. subtilis</i>	VIPNARAALTLMDRGTIRRLAAETLGLATAGYEFANTYDFEQAAQIGP										142
<i>E. coli</i>	VVPCARATKLTMDREGIRRLAAEELQLPTSTYRFADSPSPFEAVADIGY										150
<i>P. haemolytica</i>	VIPTRAKATQLTMNREGIRRLAAEELGLKTSYRFVDFVDFEQAAQIGI										150
<i>P. aeruginosa</i>	VVPTARAAQLTMNREGIRRLAAEEL-										125
	PC	VKP	MSSSG	QG	S	W	QGR	GRVIVE	F		
<i>B. subtilis</i>	PCVVKPLMSSSGQGQSVCRSEADLSCWETAMEGGRVGRVIVEEPIFP										192
<i>E. coli</i>	PCIVKPMSSSGQGQTFIRSAEQLAQAMKIAQGGRAAGRVIVEGVVFP										200
<i>P. haemolytica</i>	PCVVKPMSSSGHGQSVIKSEADLQAWDYQQGGRAAGRVIVEGVVFP										200
	EIT	LT	V	G	F	H	Q	DGDY	ESWQP	M	A
<i>B. subtilis</i>	ESEITLLTVRAVNGTAFCEPIGHVQKDDGYIESWQPHDMTEQQIEANDI										242
<i>E. coli</i>	DFEITLLTVSAVDGVHFCAPVGHRRQEDGDYRESWQPMQMSPLALERAQEI										250
<i>P. haemolytica</i>	DYEITQLTVRHINGIVFSSHR-HIQVDGDYRESWQPMQMSDIALKAQET										249
	A	LOG	G	NOVELF	D	F	EVSPRPHDTG	VT	Q	LS	F
<i>B. subtilis</i>	AKTITDELGGYGLFGVELFLAKDRVYFSEVSPRPHDTGMVTLVTQNLSEF										292
<i>E. coli</i>	ARKVVLALGGYGLFGVELFVCGDEVIFSEVSPRPHDTGMVTLISQDLSEF										300
<i>P. haemolytica</i>	AEKITSALGGRGIFGVLEFVCGDEIFNEVSPRPHDTGIVTMASQELSQF										299
	ALH	RA	LO	P	I	P	AS				
<i>B. subtilis</i>	ALHVRALGFPITEITQLSPGASRPLKAPEELADYTVGLEGNALVAPKTO										342
<i>E. coli</i>	ALHVRALGFLPGVGIQVGAASAVILPQLTSQNTVFQNVQNAV-ADLQ										349
<i>P. haemolytica</i>	ALHARAILGLPIEYIRISPAASKAIVVECKSDNVRFQGVKDLAEIGTN										349
	R	FGK	RR	V	L	A	E	A			
<i>B. subtilis</i>	VRVFGKPIITKAGRRMAVALSAADSVETARENKALDQLILK										384
<i>E. coli</i>	IRLFGKPEIDSGRRIGVALATAEASVDATERAKHAAGQVVKVQ										392
<i>P. haemolytica</i>	IRLFGKGEVNGHRRRLGVILARDENTVRALETSSRAYDKLDILQ										392

Fig. 3. Alignment of the amino acid sequences for various identified and putative GAR transformylase T enzymes. The *B. subtilis* PurT sequence was taken from Fig. 1. The *E. coli* sequence (accession number L20897), the *P. haemolytica* sequence (accession number M93021) and the *P. aeruginosa* sequence (accession number M82832) were obtained from GenBank. Residues written above the sequences represent amino acids that are identical in the *B. subtilis*, *E. coli*, *P. aeruginosa* and *P. haemolytica* sequences.

the stationary phase in cells growing in minimal medium is most likely due to enzyme inactivation, as reported for the *purF* gene-encoded glutamine phosphoribosylpyrophosphate amidotransferase (Ruppen & Switzer, 1983). In contrast to this, increased synthesis of both GAR transformylase T and GAR synthetase is observed in cells grown in rich medium when entering the stationary-growth phase. We cannot at present explain why the coupling of *purT* gene expression and that of the *pur* operon, here represented by *purD*, is not observed when free purine bases or nucleosides are present in the growth medium.

The deduced amino acid sequence of the *purT* gene from *B. subtilis* was aligned with the coding sequence of the *purT* gene from *E. coli* and with two sequences from *P. haemolytica* and *Pseudomonas aeruginosa*, respectively, which both encode putative *purT* genes (Fig. 3). The selected initiation codon was chosen among other potential translational start sites for the following reasons: (i) a ribosome-binding site (Larsen *et al.*, 1993), (+5)AGATAGGAGG(+14), is found seven nucleotides upstream of this AUG start codon; (ii) this start site resulted in a reading frame of approximately the same length as found for the *E. coli* and the putative *P. haemolytica* reading frames (Fig. 3). It appears that the *purT* gene products from the various bacterial species are highly conserved (greater than 50% of identical amino

acids). Using the BLAST algorithm (Altschul *et al.*, 1990), the amino acid sequence of the *B. subtilis* GAR transformylase T was compared to sequences available in the Swiss-Prot (release 30.0) and ProSite (release 11.0) sequence databases. The region from amino acid 102 to 117 of the *B. subtilis* PurT amino acid sequence is specific for PurT proteins and might be involved in the binding of GAR. This region could not be found in the N^{10} -formyl-THFA-dependent GAR transformylase N protein sequence of *B. subtilis* or *E. coli*. Several entries for the enzyme phosphoribosylaminoimidazole carboxylase from various organisms were obtained from the Swiss-Prot database. The similarity (27% identity) between the GAR transformylase T and phosphoribosylaminoimidazole carboxylase, encoded by the *purK* gene, has previously been reported (Marolewski *et al.*, 1994). Two regions of *B. subtilis* PurT amino acid sequence, amino acids 5–28 and amino acids 269–283, are conserved in phosphoribosylaminoimidazole carboxylase. The database search did not result in any information that might suggest a possible function of the amino acid residues 269–283. The amino acids 5–28 region is similar to the N-terminal part of the phosphoribosylaminoimidazole carboxylase proteins. When the PurT amino acid sequence was compared to the ProSite sequence database the same region showed similarity to the GTP-binding domain of the α subunit of the heterotrimeric G protein family (Ma *et al.*, 1990) and to the P-loop-type ATP/GTP-binding site (Saraste *et al.*, 1990). The α subunit GTP-binding domain and the P-loop site are located in the N-terminal part of the proteins in which they occur. It is therefore possible that the N-terminal part of GAR transformylase T and perhaps that of phosphoribosylaminoimidazole carboxylase are involved in binding ATP or GTP. Comparison with the ProSite database sequences identified the amino acids 140–157 region as homologous to the ATP-binding catalytic site of the enzyme carbamoyl-phosphate synthetase (CPSase). CPSase catalyses the ATP-dependent synthesis of carbamoyl phosphate from glutamine or ammonia and bicarbonate. Formation of carbamoyl phosphate proceeds through three steps. The first step is the ATP-dependent phosphorylation of bicarbonate to carboxy phosphate. This reaction is believed to take place at one of two homologous ATP-binding domains in the CPSase catalytic subunit. In the second step carboxy phosphate reacts with ammonia or an amino group from glutamine to form carbamate. The other ATP-binding site then catalyses the final phosphorylation of carbamate to carbamoyl phosphate (Simmer *et al.*, 1990). The GAR transformylase T catalysed synthesis of formyl-GAR proceeds through intermediate steps similar to the first two of the CPSase-catalysed reaction. The ATP- and formate-dependent formylation of GAR to formyl-GAR has been shown to proceed through the intermediate formation of formyl phosphate via an ATP-dependent phosphorylation of formate (Marolewski *et al.*, 1994). Formyl phosphate then reacts with the amino group on the GAR molecule to form formyl-GAR. Interestingly, the Swiss-Prot homology search also revealed that the enzyme GAR synthetase contains the same ATP-binding site. GAR synthetase catalyses the ATP-dependent syn-

thesis of GAR by coupling glycine to phosphoribosylamine. While the possible function of the proposed ATP-binding site in the 5–28 region of GAR transformylase T is not obvious, we tend to believe that the 140–157 region is a part of the ATP-binding site in which formation of formyl phosphate takes place because of similarity to sequences in other proteins which catalyse a similar reaction.

GAR transformylase T activities have been found in Gram-positive and Gram-negative bacteria and in the archaea *Sulfolobus shibatae* (P. Nygaard, unpublished data). Since the archaea and eukarya domains share common ancestors (Olsen & Woese, 1993), it is possible that GAR transformylase T activity may also be found among eukaryotic organisms.

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