The *yexA* gene product is required for phosphoribosylformylglycinamide synthetase activity in *Bacillus subtilis*

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The *yexA* gene encodes an 84 amino acid reading frame; in *Bacillus subtilis* it is positioned between the *purC* and *purQ* genes of the purine biosynthetic operon. Disruption of *yexA* resulted in a purine-auxotrophic phenotype. When *yexA* was expressed *in trans* it was able to complement a *yexA* mutation. Growth experiments and enzyme analysis of *yexA* mutant strains revealed a defective phosphoribosylformylglycinamide synthetase (FGAM synthetase). In the organisms in which FGAM synthetase has been studied a single polypeptide is responsible for activity. In some organisms two separate genes – in *B. subtilis* the *purL* and *purQ* genes – encode polypeptides with similarity to the N-terminal and the C-terminal region, respectively, of the single-polypeptide FGAM synthetase. Thus, active FGAM synthetase in *B. subtilis* requires the *yexA* gene product in addition to the *purL* and *purQ* gene products. Open reading frames with sequence similarity to *yexA* are found in other Gram-positive organisms, in a cyanobacterium and in methanogenic archaea. The designation *purS* is proposed for this novel function in purine biosynthesis in *B. subtilis*.

Keywords: *yexA*, purine biosynthesis, *purS*, *Bacillus subtilis*, FGAM synthetase

INTRODUCTION

The formation of inosine monophosphate (IMP) from 5'-phosphoribosyl 1'-pyrophosphate (PRPP) proceeds through an 11-step enzyme-catalysed pathway known as the *de novo* purine biosynthetic pathway. The fourth step in biosynthesis of purines is catalysed by the enzyme phosphoribosylformylglycinamide (FGAM) synthetase (EC 6.3.5.3), which catalyses the reaction 5'-phosphoribosylformylglycinamide (FGAR) + glutamine + ATP → FGAM + glutamate + ADP + P_i. Functional FGAM synthetases purified from chicken liver, *Salmonella typhimurium* and *Escherichia coli* consist of monomeric polypeptides of 133 kDa, 135 kDa and 141 kDa, respectively (Schendel *et al.*, 1989). Antibodies raised against FGAM synthetase from Chinese hamster ovary cells cross-react with a human protein with a molecular mass of 150 kDa (Barnes *et al.*, 1994).

The *E. coli* FGAM synthetase is encoded by the *purL*

gene. The PurL polypeptide contains two domains, an N-terminal ATPase domain and a C-terminal glutamine-binding domain (Schendel *et al.*, 1989). Nucleotide sequence analysis of the *pur* operon of *Bacillus subtilis* indicated that FGAM synthetase in this organism is encoded by two genes, *purQ* and *purL* (Ebbøle & Zalkin, 1987). Alignment of the primary structure of the *E. coli* FGAM synthetase and the structure of the *B. subtilis* Q and L subunits showed that the PurQ sequence is homologous to the C-terminal part and the *purL* sequence is homologous to the N-terminal part of the *E. coli* PurL sequence. This indicated that the N- and C-terminal domains of FGAM synthetase in *B. subtilis* are located on separate proteins.

The *B. subtilis* *pur* operon has the gene order *purE*, *K*, *B*, *C*, *yexA*, *Q*, *L*, *F*, *M*, *N*, *H* and *D* (Ebbøle & Zalkin, 1987). The different genes are organized in groups with overlapping coding regions: *purE*–*K*–*B*; *purC*–*yexA*–*purQ*–*L*–*F*; *purM*–*N*–*H*; and *purD*. The open reading frame designated *yexA* located immediately upstream of *purQ* encodes an unknown function. *yexA* has a reading frame that overlaps with both *purC* and *purQ*. In this report we present evidence that FGAM synthetase in *B.*

Abbreviations: FGAM, phosphoribosylformylglycinamide; FGAR, 5'-phosphoribosylformylglycinamide; GAR, phosphoribosylglycinamide; sAMP, adenylosuccinate.

Table 1. Bacterial strains, plasmids and PCR primers

Strain/plasmid/ PCR primer	Characteristics	Reference, source or coordinates*
<i>B. subtilis</i> 168	<i>trpC2</i>	C. Anagnostopoulos, CNRS, Jouy-en-Josas, France
BFA2257	<i>trpC2 yexA::pMutin1 (erm)</i>	Transformation of 168 by pHH1053, Er ^R
HH306	<i>trpC2 yexA::pHH1059 (cat)</i>	Transformation of 168 by pHH1059, Cm ^R
HH309	<i>trpC2 yexA6</i>	HH306 screened for Cm ^S
HH330	<i>trpC2 yexA6 amyE::pDG268 (neo)</i>	Transformation of HH309 by pDG268neo digested with <i>KpnI</i> , Neo ^R
HH332	<i>trpC2 yexA6 amyE::pDG268 (neo)::pHH1091 (yexA⁺) (erm)</i>	Transformation of HH330 by pHH1091, Er ^R
ED148	<i>trpC2 purM</i>	CU869 (Saxild & Nygaard, 1988)
ED249	<i>trpC2 hisA1 thr-5 purL1</i>	Saxild & Nygaard (1988)
<i>E. coli</i> MC1061	F ⁻ <i>araD139 Δ(ara-leu)7696 galE15 galK16 Δ(lac)X74 rpsL</i> (Str ^R) <i>hsdR2 (r⁻ m⁻) mcrA mcrB</i>	Laboratory stock
Plasmids pMutin1	Ap ^R (<i>E. coli</i>), Er ^R (<i>B. subtilis</i>); integrational vector for knockout mutations and formation of transcriptional <i>lacZ</i> fusion; the IPTG-inducible P _{spac} promoter is introduced to ensure expression of downstream genes	Vagner <i>et al.</i> (1998)
pDG268neo	Ap ^R (<i>E. coli</i>), Neo ^R (<i>B. subtilis</i>); vector used for integration of transcriptional <i>lacZ</i> fusions into the <i>amyE</i> gene of <i>B. subtilis</i>	Saxild <i>et al.</i> (1996)
pBOE335	Ap ^R (<i>E. coli</i>) Cm ^R (<i>B. subtilis</i>); integrational vector, pUC19 containing the <i>cat</i> gene cloned into the <i>KasI</i> site	Saxild <i>et al.</i> (1996)
pHH1053	pMutin1 digested with <i>Bam</i> HI and <i>Hind</i> III ligated to a 156 bp PCR fragment (primers 07 + 08) digested with the same enzymes	This work
pHH1059	pBOE335 digested with <i>Eco</i> RI and <i>Pst</i> I and ligated to a 399 bp PCR fragment (primers 01 + 02) digested with <i>Pst</i> I and <i>Bam</i> HI and to a PCR fragment (primers 03 + 04) digested with <i>Eco</i> RI and <i>Bam</i> HI	This work
pHH1091	pMutin1 digested with <i>Hind</i> III and <i>Bam</i> HI and ligated to a 1040 bp PCR fragment (primers 05 + 06) digested with the same enzymes; this cloning brings <i>yexA</i> expression under the control of the IPTG-inducible P _{spac} promoter	This work
PCR primers†		
Primer 01	5'-GCCCTGCAGAAGGCACGGAGCTTGAG-3†	701444–701460
Primer 02	5'-GCGGGATCCTTACTTTATACATGATGAATGCC-3'	701843–701821
Primer 03	5'-GCGGGATCCAGGTTGAGGAGGTAGTCG-3'	702060–702077
Primer 04	5'-CCGGAATTCATTCTCCCTTTTCGTAGG-3'	702484–702468
Primer 05	5'-GCCGAAGCTTAAGGCACGGAGCTTGAG-3'	701444–701460
Primer 06	5'-GCGGGATCCATTCTCCCTTTTCGTAGG-3'	702484–702468
Primer 07	5'-GCCGAAGCTTCTAGATCCACAAGGGAG-3'	701873–701891
Primer 08	5'-GCGGGATCCCAAGAAGTTTTTCGCAC-3'	702029–702013

* Coordinates were taken from the *B. subtilis* genome sequence (Kunst *et al.*, 1997).

† Underlined sequence indicates the position of the restriction site.

subtilis (and most likely also in several other organisms), in addition to the PurQ and PurL subunits, also requires the presence of the *yexA* gene product for activity.

METHODS

Bacterial strains, growth media and growth conditions. Bacterial strains and plasmids used in this work are listed in

Fig. 1. Creation of the *yexA6* in-frame deletion mutation and the strategy used to recombine the mutation into the *pur* locus of the *B. subtilis* chromosome. The relevant part of the *pur* operon is shown. The genes are not drawn to scale. Symbols: small numbered horizontal arrows indicate the position and orientation of the DNA primers used in the PCR reactions; B, *Bam*HI; P, *Pst*I; E, *Eco*RI; *P_{pur}*, *pur* operon promoter; T, transcription terminator; *purC*, phosphoribosylaminoimidazole-succinocarboxamide synthetase; *purQ*, FGAM synthetase subunit; *bla*, β -lactamase; *cat*, chloramphenicol acetyltransferase.

Table 1. As defined medium for *B. subtilis* strains, Spizizen's salts buffered minimal medium (Saxild & Nygaard, 1987) supplemented with 40 mg L-tryptophan l⁻¹, 1 mg thiamin. HCl l⁻¹, 0.2% L-glutamate and 0.4% glucose was used. Where indicated, MOPS-buffered minimal medium was used (Saxild & Nygaard, 1991). L-broth was used as rich medium. Bacterial strains were cultivated at 37 °C and growth was measured by determining the OD₄₅₀. Antibiotics were used at the following final concentrations: ampicillin (Ap), 100 µg ml⁻¹; chloramphenicol (Cm), 5 µg ml⁻¹; neomycin (Neo), 5 µg ml⁻¹; erythromycin (Er), 1 µg ml⁻¹; lincomycin, 25 µg ml⁻¹. IPTG and X-Gal were added to a final concentration of 1 mM and 40 µg ml⁻¹, respectively.

Enzyme assays and labelling of phosphorylated intermediates of the purine biosynthetic pathway. Preparation of cell-free extracts and measurement of the activities of adenylosuccinate (sAMP) lyase (Saxild & Nygaard, 1991), phosphoribosylglycinamide (GAR) synthetase (Houlberg *et al.*, 1983) and β -galactosidase (Miller, 1972) were carried out as pre-

viously described. Activity of FGAM synthetase using ¹⁴C-labelled FGAR was measured essentially as described by Houlberg *et al.* (1983). Protein was determined by the Lowry method. The intracellular accumulation of intermediates of the purine biosynthetic pathway in purine-starved cells was determined as described before (Saxild & Nygaard, 1988). Cells were grown in the presence of 0.1 mM xanthine. At OD₄₅₀ 0.5, cells were collected by filtration and resuspended in purine-free medium containing [³²P]orthophosphate. ³²P-labelled compounds from cells grown for 2 h in 1 mM phosphate MOPS-buffered minimal medium containing 0.4 MBq [³²P]orthophosphate ml⁻¹ were extracted with formic acid and separated on polyethyleneimine-impregnated cellulose on plastic sheets as described by Jensen *et al.* (1979).

DNA manipulations. Isolation of chromosomal DNA (Saxild & Nygaard, 1987) and plasmid DNA (Birnboim & Doly, 1972), and transformation of *B. subtilis* (Boylan *et al.*, 1972) and *E. coli* (Hanahan, 1983), were carried out by standard methods. Restriction enzyme digestions and alkaline phos-

Fig. 2. Recombination of a $P_{\text{spac}}\text{-yexA}^+$ construction into the *lacZ* gene located in the *amyE* locus in strain HH330 (*amyE::pDG268neo yexA6*). Symbols: white-headed black arrows indicate plasmid-encoded genes; thin arrows indicate the direction of transcription of the gene in question; *erm*, erythromycin-resistance gene; *neo*, neomycin-resistance gene; *lacZ*, β -galactosidase; *amyE*f, α -amylase (5' part); *amyE*b, α -amylase (3' part); *lacI*, *E. coli* lactose operon repressor. The rest of the symbols are defined in the legend to Fig. 1.

phatase treatment were done as recommended by the supplier (Gibco-BRL). For Southern blot analysis, DNA was blotted onto a nitrocellulose membrane (GeneScreen, NEN Research Products). The chemiluminescence labelling and detection kit Southern Light (Tropix, PE Applied Biosystems) was used for light detection of the hybridization products. The recipe supplied by the manufacturer was followed. PCR was done by incubating template DNA with forward and reverse primers ($8 \mu\text{g ml}^{-1}$ each), 0.5 mM dNTPs and 1 unit *Taq* polymerase (Amersham Pharmacia Biotech) in 10 mM Tris pH 7.0 and 0.5 mM Mg^{2+} . The reactions were run in a Biometra Trio 48 Thermocycler for 30 cycles consisting of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. The reaction product was purified by centrifugation through a Pharmacia GFX Spin column.

DNA sequencing. Sequencing of double-stranded DNA templates was performed by using the Amersham thermocycle chain-termination sequencing kit containing ^{33}P -labelled dideoxynucleotides (Amersham Pharmacia Biotech). Reaction products were separated on a 6% polyacrylamide gel and visualized by autoradiography.

Construction of an in-frame deletion mutation in *yexA*. The method used to construct strain HH309 (*yexA6*) is illustrated in Fig. 1. Two fragments containing the 5' and the 3' end of the *yexA* reading frame, respectively, were generated by PCR. The fragment containing the 5' end was digested with *Pst*I and *Bam*HI and the other fragment was digested with *Eco*RI and *Bam*HI. The fragments were simultaneously ligated into plasmid pBOE335 digested with *Pst*I and *Eco*RI. The resulting plasmid, pHH1059, was transformed into *B. subtilis* strain 168 selecting for Cm^R . Since pBOE335 cannot replicate in *B. subtilis* the plasmid integrates into the chromosome in the *pur* locus as illustrated in Fig. 1. The resulting strain, HH306, was grown for several generations in the absence of Cm and plated on L-broth agar plates without Cm . The resulting colonies were then tested on Cm -containing agar plates and Cm^S clones were isolated. One of the clones (HH309) was chosen, and

primers 01 and 04 were used to generate a PCR fragment that covers the *yexA* region. Sequence analysis of the PCR fragment revealed that the replacement of the wild-type *yexA* allele with the *yexA6* in-frame deletion was successful.

Cloning and expression of *yexA* from an IPTG-inducible promoter. Primers 05 and 06 (Table 1) were used to generate a 1040 bp PCR fragment containing the 3' end of the *purC* gene, the entire *yexA* reading frame and the 5' end of the *purQ* gene. The fragment was ligated into plasmid pMutin1. The resulting plasmid, pHH1091, contains the *yexA* gene placed after the IPTG-inducible P_{spac} promoter and in front of the *lacZ* reporter gene (Vagner *et al.*, 1998) as illustrated in Fig. 2. Plasmid pHH1091 was transformed into strain HH330, which contains the plasmid pDG268neo integrated into the *amyE* gene. Since no promoter is present in front of the *lacZ* gene of pDG268, strain HH330 is β -galactosidase-negative. Because pHH1091 cannot replicate in *B. subtilis*, Er^R transformants of strain HH330 will only be formed if recombination between the two identical *lacZ* sequences or the two identical *yexA* sequences occurs. Five Er^R transformants were analysed by Southern blotting and one clone (HH332, Fig. 2) with pHH1091 recombined into the *amyE* locus was isolated.

RESULTS

Integration of pMutin1 into the *yexA* locus and phenotype of a *yexA* mutant strain

A 156 bp PCR product internal to the *yexA* reading frame was amplified by the use of primers 07 and 08 (Table 1). The DNA fragment was digested with *Hind*III and *Bam*HI and cloned into the *Hind*III and *Bam*HI sites of plasmid pMutin1, resulting in pHH1053. Derivatives of plasmid pMutin1 contain – in addition to a part of the gene to be knocked out, here *yexA* – the P_{spac} promoter under the control of the *lacI* gene product. The

promoter drives the transcription of the region downstream of the integration point and can be induced by IPTG (Vagner *et al.*, 1998). Plasmid pHH1053 was integrated into the *yexA* gene by a single homologous recombination event, which was confirmed by Southern blot analysis (data not presented). When tested for growth on minimal agar plates the resulting strain (BFA2257) had a purine-auxotrophic phenotype both with and without added IPTG. BFA2257 could use hypoxanthine as purine source, which indicates that the purine-negative phenotype was due to a defect in the formation of IMP (Saxild & Nygaard, 1988).

Isolation and phenotype of a mutant strain containing an in-frame deletion in *yexA*

The five genes *purC*, *yexA*, *purQ*, *purL* and *purF* have overlapping coding sequences, which might indicate translational coupling (Ebböle & Zalkin, 1987). Translational coupling means that translation of a certain gene requires the translation of the overlapping upstream gene. In *B. subtilis* translational coupling has been shown to be important for the expression of the *pbuX* gene (Christiansen *et al.*, 1997), but has not been demonstrated to function in the expression of genes in the *pur* operon (Ebböle & Zalkin, 1989). However, it could not be excluded that the plasmid integration into *yexA* in BFA2257 affects the translation of the downstream gene *purQ* and that the purine-negative phenotype was due to *purQ* deficiency and not caused by the disruption of *yexA*. To test this possibility, an in-frame deletion of 73 codons of the *yexA* reading frame was constructed and recombined into the chromosome as described in Methods (Fig. 1). The resulting strain, HH309, also showed a purine-negative phenotype. Like BFA2257, HH309 was able to use hypoxanthine as a purine source, indicating a similar defect in both mutant strains.

Identification of the metabolic block in the *yexA* mutants

To narrow down the biochemical defect in the *yexA* mutants we determined their purine enzyme levels, growth on different purine sources, thiamin requirement, and accumulation of intermediary compounds of the purine biosynthetic pathway under conditions of purine starvation as described before (Saxild & Nygaard, 1988).

The accumulation studies showed an accumulation of FGAR in the *yexA* mutant, which indicates a block in the reaction catalysed by FGAM synthetase. The key experiment is shown in Fig. 3. Strain ED249 (*purL*) accumulated FGAR and ED148 (*purM*) accumulated both FGAR and FGAM. Strain HH309 (*yexA6*) accumulated large amounts of FGAR, thus resembling the *purL* strain ED249. The phenotypic similarity of HH309 to ED249 indicates that FGAM synthetase activity in HH309 is impaired. Strain BFA2257 was subjected to purine starvation in both the presence and absence of

Fig. 3. Autoradiogram of ^{32}P -labelled extracts of *B. subtilis* mutant strains starved for purine and outline of the first five steps in *B. subtilis* purine biosynthesis. (a) The ^{32}P -labelled intermediates of the purine biosynthetic pathway were separated by thin-layer chromatography as described in Methods. Lane 1, HH309 (*yexA6*); lane 2, ED249 (*purL*); lane 3, BFA2257 (*yexA::pMutin1*) + IPTG; lane 4, BFA2257 – IPTG; lane 5, 168 (wild-type); lane 6, ED148 (*purM*). (b) Gene–enzyme relationships of the initial five steps of purine biosynthesis. PRPP, phosphoribosylpyrophosphate; PRA, phosphoribosylamine; GAR, phosphoribosylglycinamide; FGAR, phosphoribosyl-formylglycinamide; FGAM, phosphoribosylformylglycinamide; AIR, phosphoribosylaminoimidazole; *purF*, phosphoribosylamidotransferase; *purD*, phosphoribosylglycinamide synthetase; *purN*, (N^{10} -formyl-THFA)-dependent phosphoribosylglycinamide formyltransferase; *purT*, formate-dependent phosphoribosylglycinamide formyltransferase; *purQL*, FGAM synthetase subunits; *purM*, phosphoribosylaminoimidazole synthetase.

IPTG. As mentioned above, IPTG induces the P_{spac} promoter, which results in the transcription of genes located downstream of the pMutin1 insertion point (Vagner *et al.*, 1998). In the *B. subtilis* *pur* operon the *purQ*, *L*, *F*, *M*, *N*, *H* and *D* genes are located downstream of *yexA* (Ebböle & Zalkin, 1987). When BFA2257 was starved for purine in the absence of IPTG a very low amount of FGAR was produced. Purine starvation in the presence of IPTG resulted in the accumulation of large amounts of FGAR. So in the presence of IPTG BFA2257 is phenotypically FGAM synthetase negative. It can therefore be concluded that

Table 2. Level of purine biosynthetic enzymes in the wild-type and *yexA* mutant strains grown in the presence of different purine compounds

Cells were grown in glucose minimal medium supplemented with the stated purine compound (xanthine, 0.1 mM; adenine, 1 mM; guanosine, 1 mM) and with or without IPTG. Enzyme activities were determined as described in Methods. The listed values are means of three experiments.

Strain	Relevant genotype	Addition to medium	t_d^* (min)	Gene order and gene expression†	Enzyme activity [nmol min ⁻¹ (mg protein) ⁻¹]		
					β -Galactosidase (<i>lacZ</i>)	sAMP lyase (<i>purB</i>)	GAR synthetase (<i>purD</i>)
168	Wild-type	None	46	$P_{pur}-purB-yexA-purD-T$	ND	118	13
		Xanthine	48	→	ND	117	18
		Adenine + Guanosine	46	→	ND	9	4
BFA2257	<i>yexA::pMutin1</i>	Xanthine	52	$P_{pur}-purB-yexA'-lacZ-T-P_{spac}-purD-T$	1578	239	<1
		Xanthine + IPTG	50	→	940	194	7
		Adenine + Guanosine	48	→	55	16	<1
HH309	<i>yexA6</i>	Xanthine	52	$P_{pur}-purB-\Delta yexA-purD-T$	ND	221	42
		Adenine + Guanosine	48	→	ND	12	4
HH332	<i>yexA6 P_{spac}-yexA⁺</i>	Xanthine	52	$P_{pur}-purB-\Delta yexA-purD-T//amyE::P_{spac}-yexA-lacZ-T$	7	235	49
		Xanthine + IPTG	48	→	152	125	21

ND, Not determined.

* t_d , doubling time.

† Only relevant genes are noted. The gene order of the *pur* operon is $P_{pur}-E-K-B-C-yexA-Q-L-F-M-N-H-D-T$. Arrows indicate the various transcripts formed in the cell under the growth condition in question. P, promoter; T, terminator.

the downstream-located *purF*, *purD* and *purN* genes, which encode the first three steps of the pathway (Fig. 3), are only weakly expressed in the absence of IPTG, but efficiently expressed in the presence of IPTG. If these genes are expressed, *purQ* and *purL* are most likely also expressed. However, it could not be exclusively concluded that the accumulation of FGAR in strain HH309 and in BFA2257 grown in the presence of IPTG was due to the lack of the *yexA* gene product. The possibility that the mutational changes could result in some kind of polar effect on the expression of the downstream-located *purQ* and *purL* genes could not be ruled out.

Effects of the *yexA* mutation on the expression of the *pur* operon

The levels of sAMP lyase, encoded by the *purB* gene located upstream of *yexA*, and of GAR synthetase, encoded by the *purD* gene located downstream of *yexA*, were determined in wild-type strain 168 and the *yexA* mutant strains BFA2257 and HH332. Two growth conditions were used. In one, xanthine served as the purine source. In this condition purine gene expression is neither derepressed nor repressed. In the other condition adenine and guanosine were added as purine source. In this condition the purine gene expression is maximally repressed (Saxild & Nygaard, 1991). As expected, enzyme levels in the wild-type were not repressed by xanthine, whereas addition of adenine plus guanosine resulted in reduced enzyme levels (Table 2).

In both mutant strains (BFA2257 and HH309) reduced growth rate in the xanthine medium and increased levels of sAMP lyase were observed. In strain BFA2257 β -galactosidase activity driven by the *pur* promoter was assessed and shown to follow the same pattern of expression as *purB*. The increased sAMP lyase level indicates that BFA2257 suffers from a partial purine starvation. The lack of GAR synthetase activity indicates that transcription of the *purD* gene, which is located downstream of the pMutin1 insertion point in *yexA*, was markedly reduced. However, if IPTG was present, significant expression of *purD* was observed. This indicates that genes located downstream of *yexA* were transcribed from the P_{spac} promoter in the presence of IPTG. In strain HH309 the expression of both *purB* and *purD* genes responds as in wild-type cells to the addition of either xanthine or a mixture of adenine and guanosine, indicating normal transcriptional control of genes located downstream of *yexA*. The possibility could not be excluded that translation of the *purQ* gene located downstream of *yexA*, and which has overlapping coding sequence with *yexA*, was affected by the pMutin1 insertion. However, strain HH332, which harbours the *yexA6* allele in-frame deletion, was able to express the *purD* gene. This suggests normal transcription of genes located downstream of *yexA*. Because the reading frame of *yexA6* is continuous and identical to the wild-type reading frame one would expect normal expression of the overlapping downstream *purQ* gene. Most likely both the *purQ* and *purL* genes, which encode subunits of

Table 3. Level of FGAM synthetase activity in wild-type and *yexA* mutant strains as affected by PurQ, PurL and YexA

Cells were grown in glucose minimal medium as described in Table 2. Enzyme activities were determined as described in Methods.

Extract no.	Subunit contents of the resulting extract	FGAM synthetase activity (nmol min ⁻¹ mg ⁻¹)	Source strain	Relevant genotype	Addition to growth medium
1	PurQ PurL YexA	3.8	168	Wild-type	None
2	PurQ PurL	<0.05	HH309	<i>yexA6</i>	Xanthine
3	PurQ PurL YexA	1.6	HH332	<i>yexA6</i> P _{spac} - <i>yexA</i> ⁺	IPTG
4	YexA	<0.05	HH332	<i>yexA6</i> P _{spac} - <i>yexA</i> ⁺	Adenine + Guanosine + IPTG
2+4*	PurQ PurL YexA	1.5			

* Equal amounts of cell extracts were present in the assay mixture.

the FGAM synthetase enzyme, are expressed and what is lacking for FGAM synthetase activity is the *yexA* gene product.

Complementation analysis

To see if the *yexA* gene product could be supplemented *in trans* the *yexA* gene was inserted in another location on the chromosome. Expression of *yexA* was put under the control of the P_{spac}/LacI system by ligation of a PCR fragment containing *yexA* (Table 1) into plasmid pMutin1. The resulting plasmid, pHH1091, was transformed into strain HH330 (*yexA6 amyE::pDG268neo*) as described in Methods. The resulting strain HH332 [*yexA6 amyE::pDG268neo::pHH1091 (yexA⁺)*] (Fig. 2) required either IPTG or purine for growth and expressed both *yexA* and *lacZ* from the P_{spac} promoter. When cells were grown in minimal medium, β -galactosidase activity was increased when IPTG was added (Table 2). The *lacZ* expression reflects the expression of *yexA in trans*, which is built into the construction in front of the *lacZ* gene (see Fig. 2). It therefore was evident that YexA, when produced *in trans*, could complement the *yexA6* deletion mutation.

Whether the YexA protein was required for FGAM synthetase activity *in vitro* was assessed in wild-type cells, in the *yexA* mutant strain HH309 and in strain HH332, in which the level of PurQ, PurL and YexA could be manipulated (Table 3). FGAM synthetase activity was found in strain 168, but not in strain HH309, which only produces the PurQ and the PurL protein. However, FGAM synthetase activity was found in strain HH332 when grown in the presence of IPTG. This was expected because of the way the construction was made. When adenine and guanosine were added together with IPTG to the growth medium no FGAM synthetase activity was found. Under these conditions the adenine and guanosine repress the expression of the *pur* operon and therefore also the synthesis of PurQ and PurL. However, when extracts of cells synthesizing

PurQ and PurL (extract 2, Table 3) and extracts of cells synthesizing YexA (extract 4, Table 3) were combined, FGAM synthetase activity was restored. These results prove that the *yexA* gene product is required for FGAM synthetase activity in *B. subtilis* and that *yexA* therefore must be considered as a novel purine biosynthetic gene. We suggest that the *yexA* gene is given the designation *purS*.

DISCUSSION

Since 1996 two research consortiums – a European and a Japanese – have performed systematic gene function analysis in *B. subtilis* (Vagner *et al.*, 1998). One of the obligations for each of the research groups involved has been, in an assigned genomic region, to systematically inactivate all genes for which no obvious function could be suggested from the deduced amino acid sequence. The authors of this report were assigned the region sequenced by Borriess *et al.* (1996), which includes the *pur* operon. The function for 11 of the 12 genes of the *pur* operon has been identified from sequence similarity studies (Ebböle & Zalkin, 1987) or by experimental means (Saxild & Nygaard, 1988; Saxild *et al.*, 1994). Only the function of the *yexA* reading frame, which is located between *purC* and *purQ*, has remained unsolved. However, after publication of the nucleotide sequence, possible functions of *yexA* have been suggested. Ebböle & Zalkin (1987) showed that the expression of *yexA* in *E. coli* gave rise to a small protein and they suggested a regulatory function of this protein. More recently, Peltonen & Mäntsälä (1999) observed that reading frames similar to the *yexA* gene exist in other bacteria and they suggested that the *yexA* gene product was required for functional FGAM synthetase activity. However, they provided no experimental evidence to support this suggestion. In this report we demonstrate that a *B. subtilis yexA* mutant lacks FGAM synthetase activity and that it accumulates the substrate FGAR during purine starvation. We suggest that *yexA* is

renamed as *purS*. It appears that an extra peptide, PurS (YexA), is required for FGAM synthetase activity in bacteria like *B. subtilis* in which the enzyme is composed of heteromers. Since FGAM synthetase activity can be restored *in vitro* by adding extracts containing PurQ and PurL to an extract containing only PurS, we suggest that one possible role of PurS is to act as a hinge that links the two catalytic subunits. Another possibility is that PurS plays a role in catalysis. On the other hand we can exclude that PurS is required for stabilizing the PurQ and PurL subunits since we were able to synthesize biologically active PurQ and PurL proteins in the absence of PurS. *yexA* homologues most likely exist in cyanobacteria [*Synechocystis* sp. (accession no. Q55842)], methanogens [*Methanococcus jannaschii* (accession no. Q58988) and *Methanobacterium thermoautotrophicum* (accession no. 026271)] and the Gram-positive bacteria *Mycobacterium tuberculosis* (accession no. AL123456), *Mycobacterium leprae* (accession no. 005755) and *Lactococcus lactis* (Peltonen & Mäntsälä, 1999). These organisms all contain the multi-subunit form of FGAM synthetase encoded by *purQ* and *purL* genes that are similar to those found in *B. subtilis*.

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