NOTES

Histidinol Phosphate Phosphatase, Catalyzing the Penultimate Step of the Histidine Biosynthesis Pathway, Is Encoded by *ytvP* (*hisJ*) in *Bacillus subtilis*

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The deduced product of the *Bacillus subtilis ytvP* gene is similar to that of *ORF13*, a gene of unknown function in the *Lactococcus lactis* histidine biosynthesis operon. A *B. subtilis ytvP* mutant was auxotrophic for histidine. The only enzyme of the histidine biosynthesis pathway that remained uncharacterized in *B. subtilis* was histidinol phosphate phosphatase (HolPase), catalyzing the penultimate step of this pathway. HolPase activity could not be detected in crude extracts of the *ytvP* mutant, while purified glutathione *S*-transferase-YtvP fusion protein exhibited strong HolPase activity. These observations demonstrated that HolPase is encoded by *ytvP* in *B. subtilis* and led us to rename this gene *hisJ*. Together with the HolPase of *Saccharomyces cerevisiae* and the presumed HolPases of *L. lactis* and *Schizosaccharomyces pombe*, HisJ constitutes a family of related enzymes that are not homologous to the HolPases of *Escherichia coli*, *Salmonella typhimurium*, and *Haemophilus influenzae*.

In *Bacillus subtilis*, all enzymes involved in the histidine biosynthesis pathway have been characterized at the genetic level, except histidinol phosphate phosphatase (HolPase) (10, 18). This enzyme (L-histidinol-phosphate phosphohydrolase; EC 3.1.3.15) catalyzes dephosphorylation of histidinol phosphate to histidinol, the direct precursor of histidine. In the well-studied prokaryotes *Escherichia coli*, *Salmonella typhimurium*, and *Haemophilus influenzae*, HolPase activity is associated with the N-terminal domain of the HisB bifunctional enzyme. The C-terminal domain of HisB exhibits imidazoleglycerol phosphate dehydratase activity (4, 5, 22). However, this does not appear to be a common feature of these enzymes, as searches for similarities with the *E. coli* HisB bifunctional enzyme revealed the existence, in many organisms across all three kingdoms (eubacteria, archaea, and eukaryotes), of imidazoleglycerol phosphate dehydratases not fused to any other protein domain. For most of these organisms, nothing is known about the HolPase. A search for homologues to the N-terminal domain of the *E. coli* enzyme does not reveal any well-conserved domain, except in the *S. typhimurium* and *H. influenzae* bifunctional enzymes, and only the more distantly related *Streptomyces lincolnensis* LmbK protein has been associated with this family as a putative HolPase (19). A HolPase has been characterized at the genetic, physiological, and biochemical levels in the yeast *Saccharomyces cerevisiae* (8, 14), but its sequence has no similarity with the corresponding *E. coli* enzyme (12).

The complete genome of *B. subtilis* has been sequenced; among the 4,100 open reading frames identified, about 70% (termed *y* genes) correspond to putative genes for which the

function has not been ascertained (10). The goal of the *B. subtilis* systematic functional analysis program is to assign a function to these genes. The applied strategy consists of constructing a collection of mutants that correspond to each of these genes and looking for a phenotype. We are currently testing whether the utilization of various carbon sources is affected in these mutants.

In the work presented here, we demonstrate that the *ytvP* gene encodes the *B. subtilis* HolPase, thus completing our knowledge of the histidine biosynthesis pathway in this organism.

The *B. subtilis ytvP* mutant BFA1037 is devoid of HolPase activity. *B. subtilis* BFA1037 [*trpC2 ytvP'*::pBFA1037 (Ery^r)] is a *ytvP* mutant, obtained by single crossing-over integration of plasmid pBFA1037 into the chromosome of the reference strain 168 Marburg (*trpC2*). The integrative plasmid pBFA1037 was derived from pMUTIN2mcs (21) by insertion, between its unique *Hind*III and *Bam*HI sites, of a 218-bp *Hind*III/*Bam*HI *ytvP* internal fragment amplified by PCR with *B. subtilis* 168 chromosomal DNA as a template and primers ytvP-H (5'-GCCGAAGCTTGGACAGCTTAGCTTAGCAT ACGG-3') and ytvP-B (5'-CGCGGATCCACCGCTTCAAT GCTTCC-3'). Correct integration of a single copy of plasmid pBFA1037 into the *ytvP* gene, confirmed by Southern blot analysis (17), led to disruption of *ytvP* and created a *ytvp'-lacZ* transcriptional fusion (Fig. 1).

During growth tests performed in chemically defined media, we observed that strain BFA1037 was unable to grow in liquid MM medium (1) containing succinate (15 mM) and glutamate (30 mM) as carbon sources and supplemented with tryptophan (25 mg/liter), whereas the wild-type strain 168 could grow under these conditions (Table 1). The same observation was made with either glucose, glucitol, glycerol, gluconate, asparagine, proline, or arginine as a carbon source, and in all cases, growth of the *ytvP* mutant could be restored to the wild-type level by adding Casamino Acids (data not shown). This suggest-

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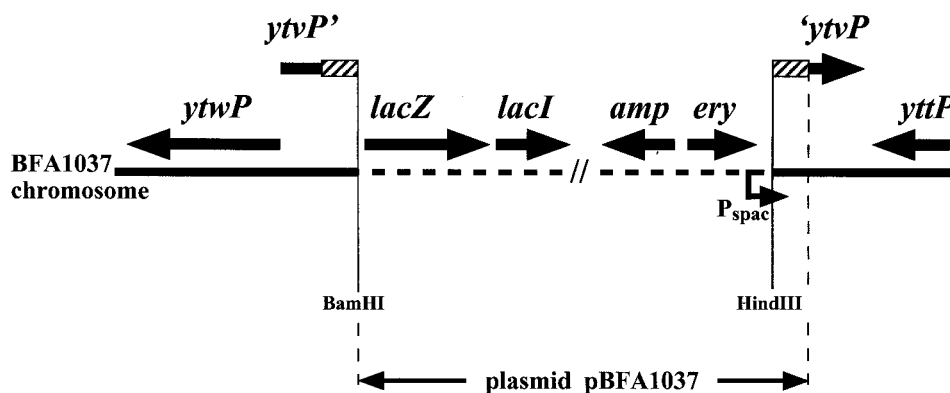
FIG. 1. Structure of the *ytvP* chromosomal region of mutant BFA1037. Integration of plasmid pBFA1037 led to disruption of *ytvP* and created a *ytvP′-lacZ* transcriptional fusion. The 218-bp *ytvP* internal fragment (symbolized by hatching) used as integration platform is duplicated. In addition to genes conferring resistance to ampicillin (*amp*) and erythromycin (*ery*), plasmid pMUTIN2mcs and its pBFA derivatives also carry a *lacI* gene and a P_{spac} isopropyl-β-D-thiogalactopyranoside-inducible promoter (broken arrow), which minimizes potential polar effects on expression of genes downstream of the integration site (21). Genes are symbolized by arrows indicating their orientation (not drawn to scale).

ed that the mutant was auxotrophic for one or several amino acids.

The *ytvP* gene (position 3030690 to 3031496 of the *B. subtilis* genome [10]) seems to be monocistronic. It encodes a 268-amino-acid protein, which exhibits weak but significant similarity (28% identity) to the product of *ORF13*, the last gene of the *Lactococcus lactis* histidine biosynthesis operon (7) (Fig. 2). Although the function of this *L. lactis* protein remains unknown, location of its structural gene suggested that in *B. subtilis* YtvP could be implicated in histidine biosynthesis. We observed that addition of histidine could indeed restore the growth of strain BFA1037 to wild-type level (Table 1). Growth could also be significantly improved by the addition of histidinol, although not as efficiently as with histidine (Table 1). These results showed that strain BFA1037 is auxotrophic for histidine and suggested that it is affected in HolPase activity.

A 1,054-bp *Eco*RI/*Bam*HI fragment carrying the entire wild-type *ytvP* coding sequence and promoter region was amplified by PCR with primers ptvpE (5′-CCTGAATTCCAATGACA AACTCCATAATG-3′) and ftvpB (5′-CGCGGATCCGGA CAGGAGACGTGG-3′) from *B. subtilis* strain 168 chromosomal DNA. This fragment was cloned into plasmid pDG1662 and integrated into the *amyE* locus of strain MO1099, following previously described methods (9). The *amyE′::ytvP* chromosomal region of the resulting strain was then transferred into BFA1037, which led to full complementation of the growth defect of this strain (Table 1). This showed that the phenotype of BFA1037 was due to the *ytvP* mutation.

We compared HolPase activity in extracts of the wild-type strain 168 and of the mutant strain BFA1037. For this purpose, *B. subtilis* cells were grown at 37°C with shaking in liquid medium (MM containing succinate and glutamate plus tryptophan, supplemented with histidine [50 mg/liter] when required), harvested by centrifugation when the culture reached an A_{600} of 0.5, and concentrated 10-fold in triethanolamine-HCl buffer (0.1 M triethanolamine-HCl, 10 mM Na$_2$EDTA [pH 7.5]). Glass beads (diameter, 0.10 to 0.11 mm; Braun Sciencetec) were added to the suspension (about 0.5 g/ml), and the cells were broken by vigorous vortexing followed by sonication. After centrifugation, the supernatant was applied to a prepacked Sephadex G-50 column (Pharmacia, Uppsala, Sweden) pre-equilibrated with triethanolamine-HCl buffer. The phosphate concentration of each fraction was estimated with an ascorbate/ammonium molybdate color-developing reagent (13). Fractions containing phosphate-free proteins were col-

lected, and protein concentration was determined by the Bradford method (3). HolPase activity was assayed as previously described (13). No significant HolPase activity (<0.4 U/mg of protein) could be detected in extracts of the *ytvP* mutant strain BFA1037 grown in the presence of histidine, whereas extracts of the wild-type strain grown with and without histidine exhibited in both cases significant and roughly equal levels of HolPase activity (6.4 and 6.9 U/mg of protein, respectively).

From these results, we concluded that the *ytvP* gene encodes either the *B. subtilis* HolPase or a positive regulator of it.

ytvP encodes HolPase activity. An 812-bp *Bam*HI/*Eco*RI fragment carrying the entire *ytvP* sequence was amplified by PCR with primers 5′ytvP-Bam (5′-CGCGGATCCATGCAAA AGCGAGACGGAC-3′) and 3′ytvP-Eco (5′-CCGGAATTCT AGCACCGTTCCAAAAACG-3′) from *B. subtilis* 168 chromosomal DNA. This fragment was cloned between the *Bam*HI and *Eco*RI sites of plasmid pGEX-2T (Pharmacia), generating pGST::YtvP, a plasmid allowing overproduction of a glutathione *S*-transferase (GST)-YtvP fusion protein. Purification of GST and GST-YtvP proteins from *E. coli* TG1 [*supE thi hsdD5* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q} *Z*Δ*M15*)] transformed respectively with pGEX-2T and pGST::YtvP was performed, following previously described methods (20). As estimated by polyacrylamide gel electrophoresis (11), the purity of both proteins was over 95%, and the apparent molecular mass of each purified product corresponded to that expected (26 and 57 kDa for GST and GST-YtvP, respectively) (results not

TABLE 1. Growth of the *B. subtilis* wild-type strain and the *ytvP* mutant

Growth medium supplement[a]	Doubling time (min)[b]		
	168 (wild type)	BFA1037 (*ytvP* mutant)	Complemented BFA1037 (*ytvP* mutant with *amyE′::ytvP*)
None	136	>750	138
Histidine (50 mg/liter)	132	135	127
Histidinol (50 mg/liter)	128	408	126
Histidinol (250 mg/liter)	124	301	110

[a] The growth medium was MM containing succinate and glutamate plus tryptophan.
[b] Values correspond to a typical experiment. Similar results were reproducibly obtained in several experiments.

FIG. 2. Alignment of the *B. subtilis* YtvP (HisJ) protein (BSUB; accession no. PIR:D70002), the *S. cerevisiae* His9p HolPase (SCER; accession no. PIR:S56280), and the presumed HolPases *L. lactis* ORF13 gene product (LLAC; accession no. PIR:D47754) and *S. pombe* His9p homologue (SPOM; accession no. SPTREM BLNEW:E1316727). Residues conserved (identity and conservative change) in at least two of these proteins are against a black background (residues conserved in YtvP) or a grey background (residues not conserved in YtvP); groups of conservative change considered are G and A; D and E; F and Y; I, L, V, and M; K and R; N and Q; and S and T. Dashes indicate gaps introduced to maximize alignment.

shown). In vitro assays established tha the GST-YtvP fusion protein possesses strong HolPase activity (840.0 U/mg of protein versus <0.1 U/mg of protein for GST), thus demonstrating that in *B. subtilis* this activity is encoded by *ytvP*. We therefore propose to rename this gene *hisJ*.

As mentioned above, the protein most closely related to HisJ is the product of *ORF13* of the *L. lactis his* operon. The structural gene encoding HolPase has not been identified in this bacterium (18). It has been proposed that this activity would be performed by the product of *ORF8*, based on its homology with enzymes catalyzing phosphorylation of a hydroxyl group, which is the opposite reaction to the dephosphorylation of such a group as carried out by HolPase (7). From the work presented here, it seems more likely that *L. lactis* HolPase is encoded by *ORF13*. The *B. subtilis* HisJ protein is also related to the *S. cerevisiae* HolPase and its *Schizosaccharomyces pombe* homologue, although more distantly (24 and 23% identity, respectively) (Fig. 2). These four proteins seem therefore to constitute a family of distantly related HolPases, distinct from the *E. coli*-type HolPases. As recently reported, this HisJ family belongs to a novel enzymatic superfamily designated PHP, which also comprises DNA polymerase domains (2). It has been suggested that these domains possess intrinsic phosphatase activity that hydrolyzes the pyrophosphate released during nucleotide polymerization. Among the PHP HolPases, HolPase activity had been demonstrated only for His9p of *S. cerevisiae*. The PHP superfamily also comprises numerous proteins of unknown function. While several of these seem to be clearly related to DNA polymerases (e.g., the 570-amino-acid *B. subtilis* YshC protein), some, such as the 211-amino-acid *Archaeoglobus fulgidus* AF1233 protein or the 246-amino-acid *Anaerocellum thermophilum* YOR4 protein, might belong to the His9p/HisJ HolPase family or even to other families of phosphatases. Interestingly, the *E. coli*-type HolPases belong to another superfamily of phosphohydrolases. This "DDDD" family also includes several classes of nonspe-

cific acid phosphatases, phosphoglycolate phosphatases, phosphoserine phosphatases, and trehalose-6-phosphatases, but no DNA polymerases (19). In evolutionary terms, the existence of these two superfamilies could reflect the existence of two different ancestral nonspecific phosphatases that have evolved through fusion to other domains having specific properties, such as polymerase or binding of a particular substrate.

Identification of the *hisJ* HolPase structural gene in *B. subtilis* completes our knowledge of the histidine biosynthesis pathway in this bacterium. This also provides strong evidence for the role of the *L. lactis ORF13* gene and will help with the identification of the functions of related gene products in several other organisms.

Transcription of *hisJ* is repressed neither by histidine nor by histidinol. In *L. lactis*, the *ORF13* gene belongs to the *his* operon, the transcription of which is enhanced 15-fold in cultures grown in the absence of histidine (6). The *hisJ* gene characterized in this work does not belong to the *B. subtilis his* operon and seems to be monocistronic. The two neighboring genes (*yttP* and *ytwP*) are oriented in the opposite direction (Fig. 1), and their inactivation does not confer auxotrophy to histidine (data not shown). We did not observe any significant difference in the level of HolPase activity in *B. subtilis* wild type grown in the absence or in the presence of 50 mg of histidine per liter (see above) or even 250 mg of histidine per liter (data not shown). This suggested that neither HisJ enzymatic activity nor *hisJ* transcription is regulated by this amino acid.

We also investigated potential regulation of *hisJ* transcription in the mutant strain BFA1037, in which a *hisJ* (*ytvP*)'-*lacZ* transcriptional fusion is under the control of the *hisJ* promoter (Fig. 1). For these β-galactosidase assays, extracts were prepared and enzymatic activity was determined as previously described (15, 16). The same level of β-galactosidase activity (about 20 to 25 Miller units/mg of protein) was measured in extracts of the mutant grown in liquid MM containing succinate and glutamate plus tryptophan, supplemented with vari-

ous concentrations of histidine or histidinol (β-galactosidase activity in extracts of the wild-type strain 168 grown in the same conditions was <0.5 Miller units/mg of protein). In order to exclude potential effects due to hyperaccumulation of histidinol phosphate (the substrate for HolPase) in the mutant strain BFA1037, this experiment was also performed with the complemented strain. As mentioned above, this strain carries a wild-type *hisJ* gene in the *amyE* ectopic locus and is prototrophic for histidine (Table 1). The same level of β-galactosidase activity (about 15 to 20 Miller units/mg of protein) was measured in extracts of this strain grown in liquid MM containing succinate and glutamate plus tryptophan which was either not supplemented or supplemented with various concentrations of histidine or histidinol (up to 1,250 mg/liter), or even in rich medium (Luria broth). Thus, it appeared that transcription of *hisJ* is not repressed by histidine or by histidinol.

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