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Two Genes Encoding Uracil Phosphoribosyltransferase Are Present in *Bacillus subtilis*

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Uracil phosphoribosyltransferase (UPRTase) catalyzes the key reaction in the salvage of uracil in many microorganisms. Surprisingly, two genes encoding UPRTase activity were cloned from *Bacillus subtilis* by complementation of an *Escherichia coli* mutant. The genes were sequenced, and the putative amino acid sequences were deduced. One gene showed a high level of homology to UPRTases from other organisms, whereas the other gene with a low level of homology to other UPRTases turned out to be the *pyrR* gene—the repressor of the *pyr* operon. The role of these genes in uracil metabolism was established by an analysis of the phenotypes of *upp* and *pyrR* mutants.

Cloning of genes encoding UPRTase activity. *Bacillus subtilis* is able to utilize different exogenous pyrimidine sources, including uracil (8). The key reaction in uracil salvage is the reaction of a uracil molecule with a 5′-phosphoribosyl-α-1-pyrophosphate (PRPP) molecule, resulting in the formation of UMP, catalyzed by uracil phosphoribosyltransferase (UPRTase) (6). Gene complementation in *Escherichia coli* was used as a strategy for the cloning of genes from *B. subtilis* encoding UPRTase activity. The *E. coli* strain BM604 has a pyrimidine requirement, since it is mutated in the *pyrF* gene, and is furthermore unable to utilize uracil as pyrimidine source because of a *upp* mutation. To clone *upp* from *B. subtilis*, different genomic libraries constructed in pUN121 (7) or pLNA2 (1) were used to transform BM604 (Table 1). Transformed cells were plated on selective minimal plates (3) supplied with uracil (20 μg/ml) and tetracycline (8 μg/ml). All transformants were screened for the presence of a pyrimidine requirement. Clones were isolated from a *Bcl*I library in pLNA2 and a *Hin*dIII library in pUN121. Detailed restriction endonuclease mapping of plasmid DNA extracted from the *Hin*dIII clone (pJAM100) and *Bcl*I clone (pJAM200) revealed that the cloned fragments did not contain identical DNA segments.

To confirm that the cloned DNA in pJAM100 and pJAM200 is indeed from *B. subtilis*, Southern blot experiments were conducted (12). The *B. subtilis* DNA fragments on the two plasmids could be identified on the chromosome. Furthermore, no cross-hybridization was seen, confirming that the two genes are present on the chromosome at different locations (not shown). *E. coli* BM604 harboring pJAM100 and pJAM200 was analyzed for the presence of UPRTase activity as previously described (11). The UPRTase activities linked to pJAM100 and pJAM200 were determined to be 56 and 1.0 U/mg, respectively. No measurable activity (less than 0.02 U/mg) was found in strains harboring the vectors; 1 U/mg is defined as the amount of enzyme able to form 1 nmol of UMP per min per mg of total protein at 37°C. These results demonstrate that the UPRTase activity is linked to both the *upp*-complementing plasmids.

Deletion subclones of pJAM100 and pJAM200 were constructed in order to define the DNA sequences responsible for the *upp*-complementing activity and to use as templates for DNA sequencing (13). pJAM200 was shown to contain a sequence identical to the 181-amino-acid open reading frame (PyrR) in the *pyr* operon shown to be the regulator of the structural genes responsible for the de novo synthesis of UMP (17). PyrR is thus an example of a regulatory protein having catalytic activity. The open reading frame of pJAM100 encoding UPRTase activity was identical to an open reading frame of 209 amino acid residues found during the course of the *B. subtilis* genome sequencing program around 320°. The deduced amino acid sequences of the two open reading frames were aligned with those of known UPRTases (Fig. 1). By comparing the sequences it can be concluded that the UPRTase cloned on pJM100 belongs to the same family as the UPRTases identified in the other organisms, whereas PyrR has very poor homology to this family. Therefore, the UPRTase gene cloned on pJAM100 is termed *upp*. The alignment of the *upp* open reading frames reveals that the number of conserved amino acid residues is between 45 and 80% (Fig. 1). This enzyme seems to be highly conserved throughout the bacterial world.

Genetic analysis of the genomic sequences contained in pJAM100 and pJAM200. Sequence analysis indicated that the pJAM100 and pJAM200 plasmids contained DNA fragments from the 320° region and the *pyr* operon of the *B. subtilis* genome, respectively. To confirm this, the following genetic analysis was made. A 186-bp *upp* internal *Eco*RV-*Hin*dIII fragment was isolated from pJAM100 and cloned into the integrational plasmid vector pBOE335 digested with *Sma*I and *Hin*dIII, creating pHH1006. pHH1006 was transformed into *B. subtilis* 168 by selecting for Cm^r-creating strain HH222 (*upp-11*). Since pBOE335 derivatives cannot replicate in *B. subtilis*, pHH1006 will integrate into the chromosome in the region of homology (the *upp* gene), thus creating a disrupted *upp* gene. Southern blot analysis confirmed that a single-copy integration had occurred in the expected region (data not shown). Strain HH212, carrying a defective *glyC* allele mapping at 320°, was transformed with DNA isolated from strain HH222 selecting for Cm^r. Among 200 Cm^r transformants, 199 had become Gly^+, indicating a close linkage between the *upp* and *glyC*

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TABLE 1. Bacterial strains and plasmids[a]

Strain or plasmid	Genotype or description	Source
B. subtilis		
1A300	*trpC2 thyA thyB glyC*	BGSC
1A610	*trpC2 pyrC*::Tn*917*	BGSC
DB104	*hisA1*	R. L. Switzer
DB104ΔpyrR	*hisA1* Δ*pyrR*	R. L. Switzer
HH123	*trpC2 hisA1*	ED361, P. Nygaard
HH206	*hisA1 pyrR*::pJAM200	Tf of DB104 by pJAM200; Cm^r
HH207	*hisA1 upp-11*	Td of DB104 by HH222; Cm^r
HH211	*hisA1* Δ*pyrR upp-11*	Td of DB104ΔpyrR by HH222; Cm^r
HH212	*trpC2 glyC*	Tf of HH123 by 1A300; His^+ and Gly^-
HH222	*trpC2 upp-11*	Tf of 168 by pHH1006; Cm^r
E. coli		
BM604	HfrH *thi galE*, Δ(*att*λ-*bio*) *deoA deoC argA cytR upp udp pyrF30*	B. Mygind
SØ106	*lacZ rpsL strA thi pyrF30*	B. Mygind
Plasmids		
pC194	Cm^r	L. Boe
pUC19	Ap^r	Laboratory stock
pUN121	Ap^r Tc^r	Laboratory stock
pLNA2	Ap^r Tc^r Cm^r; pUN121 partially digested with *Pst*I, blunt ended with T4 DNA polymerase, and ligated with 1.6-kb *Cla*I fragment of pC194 blunt ended with Klenow polymerase containing the *cat* gene	L. N. Andersen
pBOE335	Cm^r Ap^r; 1.6-kb *Cla*I fragment from pC194 containing the *cat* gene ligated to *Kas*I-digested pUC19	L. Boe
pJAM100	Ap^r Tc^r; *upp*-complementing plasmid	This work
pJAM200	Ap^r Tc^r Cm^r; *upp*-complementing plasmid	This work
pHH1006	Ap^r Cm^r; 186-bp *Eco*V-*Hin*dIII internal *upp* fragment from pJAM100 ligated to *Sma*I and *Hin*dIII-digested pBOE335	This work
pACYC184	Cm^r	F. Hansen
pJM370	Ap^r; *upp* from *L. lactis*	J. Martinussen

[a] Ap, ampicillin; Tc, tetracycline; Cm, chloramphenicol; BGSC, Bacillus Genetic Stock Center; Tf, transformation of the first strain either with the noted plasmid or with DNA from the second strain; Td, transduction of the first strain with AR9 phages propagated on the second strain.

genes. Plasmid pJAM200 was transformed into strain DB104 by selection for Cm^r-creating strain HH206[*pyrR*::pJAM200 (Cm^r)]. As in the case of plasmid pHH1006, pJAM200 will integrate into the chromosome at the site of homology. Then strain 1A610 [*pyrC*::Tn*917* (Er^r)] was transduced with AR9 phages propagated on strain HH206. The selection was for Cm^r. Among 100 Cm^r transductants, 98 had become Er^s, indicating a close linkage between the pJAM200 insertion point and the *pyrC* gene, which is the fourth gene in the *pyr* operon (10).

Analysis of the two UPRTase activities. An isogenic series of strains containing either the Δ*pyrR* mutation, the *upp-11* mutation, or both mutations was constructed in the following way. Strains DB104 (*hisA1*) and DB104ΔpyrR (*hisA1* Δ*pyrR*) (17) were transduced with AR9 phages propagated on strain HH222, thus producing the strains HH207 (*hisA1 upp-11*) and HH211 (*hisA1* Δ*pyrR upp-11*), respectively. The effect of the *upp* and Δ*pyrR* mutations and the *upp* Δ*pyrR* double mutation was analyzed in four ways: (i) assay of UPRTase activity in crude extract, (ii) pyrimidine excretion, (iii) effect of the pyrimidine analog 5-fluorouracil on growth, and (iv) incorporation of uracil and uridine. The results are presented in Table 2. In these experiments, the different strains were grown in Spizizen salts (16)-buffered glucose-glutamate minimal medium supplemented with thiamine-HCl (1 μg/ml) and L-histidine (40 μg/ml) as described previously (14). Strains HH207 and HH211 were grown in the presence of 5 μg of chloramphenicol per ml.

To measure the content of UPRTase activity originating from *upp* and *pyrR*, crude extracts from exponentially growing cells were assayed as previously described (11). A disruption of the *upp* gene on the chromosome leads to a 99% reduction of UPRTase activity, whereas a *upp pyrR* double mutant lacks any measurable activity. Furthermore, no drop in UPRTase activity due to a *pyrR* mutation is seen. This result suggests that the UPRTase activity responsible for the phosphoribosylation of uracil is encoded by the *upp* gene and that the enzymatic activity encoded by the *pyrR* gene product plays no significant role in this reaction.

It has been reported that an *E. coli* strain carrying a *upp* mutation excretes uracil to the medium (9). To test whether the different strains excrete pyrimidines, the amount of pyrimidines present in the medium after exponential growth was measured by using a bioassay. Strains to be tested were grown in glucose minimal medium to an optical density at 450 nm (OD_{450}) of 1, 2, or 3. To prevent excision of the internal *upp* fragment, resulting in a regeneration of the *upp* gene, strains HH207 and HH211 were grown in the presence of chloramphenicol. The growth medium was cleared by centrifugation, the pH was adjusted to 7.0, and subsequently the medium was sterilized by filtration through a 0.22-μm-pore-size Millipore filter. The medium was supplied with ampicillin (100 μg/ml) to prevent growth of contaminants and inoculated with 10^6 cells of the indicator strain BM604 (*pyrF30 upp*) harboring pJM370 (*upp*$^+$ Ap^r) and pACYC184 (Cm^r). The pyrimidine requirement of this strain can be satisfied by uracil, cytosine, uridine, and cytidine. To correlate the growth yield and uracil concentration, fresh medium supplied with ampicillin (100 μg/ml), chloramphenicol (20 μg/ml), and increasing amounts of uracil (0 to 10 μg/ml) was inoculated with the indicator strain. All

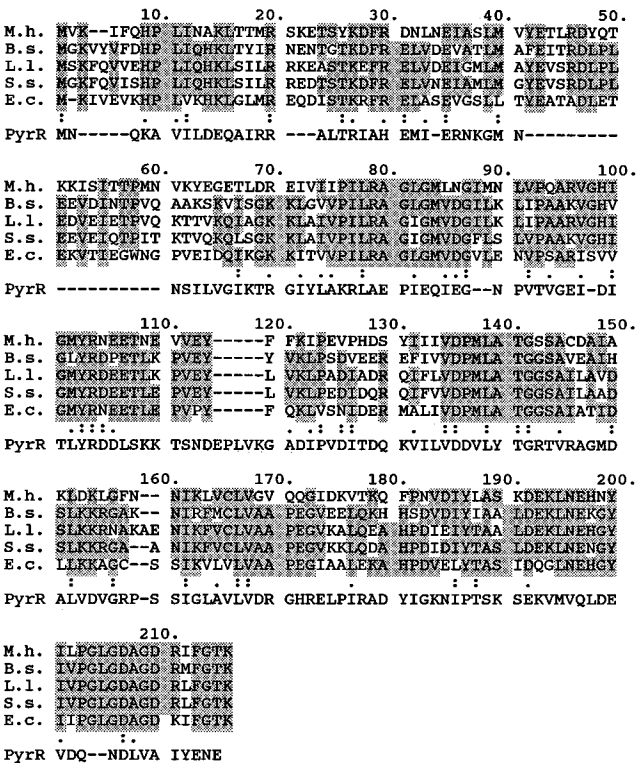

FIG. 1. Amino acid sequence alignment of UPRTases from *Mycoplasma hominis* (M.h.) (15), *Bacillus subtilis* (B.s.) (this work), *Lactococcus lactis* (L.l.) (5), *Streptococcus salivarius* (S.s.) (4), *Escherichia coli* (E.c.) (2), and *pyrR-Bacillus subtilis* (PyrR) (17). Shaded residues represent amino acids which are identical among at least three of the five UPRTases. Dots above the PyrR sequence indicate identical amino acid residues (:) or conservative replacements (.).

tubes were incubated at 37°C for 20 h. Growth yield was established by measuring OD_{450}. The cell densities in the cultures with known amounts of uracil were used to quantify the uracil excretion of the different strains. The presence of 1 μg of uracil per ml facilitates the growth of the *E. coli* strain to a density of 0.17 OD_{450} units. This value was also obtained by using SØ106 (*pyrF30*) in the absence of antibiotics. As seen in Table 2, even a *B. subtilis* wild-type strain excreted pyrimidines to the growth medium. The introduction of a *upp* mutation had only a minor effect on excretion. Only at high densities was the excretion of pyrimidines significantly increased, suggesting a tight control of the de novo pyrimidine synthesis. The effect of the *pyrR* mutation was more dramatic: pyrimidines were excreted in high amounts to the medium, reflecting high pyrimidine pool sizes in the cell.

A characteristic of *upp* mutants is their resistance towards FU. UPRTase catalyzes the conversion of FU into 5-fluoro-UMP (FUMP), the first step in the pathway to the very toxic compound 5-fluorodeoxy-UMP (dFUMP) (6). The *upp* and *pyrR* derivatives were streaked on minimal agar plates supplied with FU in various concentrations in order to test their sensitivity to this analog. The numbers in Table 2 represent the FU concentrations resulting in a 50% reduction of colony size for a given strain after 24 h of incubation at 37°C. The resistance of the *upp* mutant towards FU is expected since the formation of UMP from PRPP and uracil is the main metabolic pathway of uracil (6). The *pyrR* mutation confers resistance towards FU. This can be explained by an increased concentration of UMP in these cells, due to overexpression of the pyrimidine biosynthetic genes, thus resulting in FUMP being inhibited by UMP. Furthermore, the FU uptake is probably significantly reduced because of the high concentrations of pyrimidines both external and internal. At concentrations above 2 μg/ml the growth of the *upp* mutant was significantly inhibited, and beyond 5 μg/ml no colonies were formed. This finding indicates that despite the loss of the majority of UPRTase activity, FU is still metabolized either by the remaining UPRTase activity encoded by *pyrR* or by an alternative pathway. Since the *upp pyrR* double mutant is sensitive to FU concentrations above 1 μg/ml, it can be concluded that uracil can be metabolized by a pathway other than through the UPRTase-catalyzed step. Recently it has been shown that *Lactococcus lactis* is metabolizing uracil through deoxyuridine to dUMP in a *upp* strain by the action of thymidine phosphorylase and thymidine kinase (5). Surprisingly, the *upp* mutant is resistant to higher concentrations of FU than the *upp pyrR* double mutant. There is no obvious explanation for this finding, but it may be a consequence of an increased activity of the enzymes catalyzing the steps in the alternative pathway in the double mutant as discussed below.

To confirm the data obtained in the FU experiment, the ability of the different cells to incorporate uracil and uridine was investigated. Cells were grown in minimal medium at 37°C. At OD_{450}=0.5, [2-^{14}C]uracil or [2-^{14}C]uridine was added to a final concentration of 43 μM (2.0 mCi/mmol). The incorporation rates presented in Table 2 were determined as previously described (5). The uracil incorporation data confirms the re-

TABLE 2. Phenotypes of the different *B. subtilis* strains[a]

Strain	Relevant phenotype or genotype	Doubling time (min)	5-Fluorouracil sensitivity[b] (μg/ml)	UPRTase activity[c] (nmol/(min/mg)	Pyrimidine excretion at different OD_{450}s[d] (μg/ml)			Incorporation of uracil and uridine (nmol/min/mg [dry wt])	
					1	2	3	Uracil	Uridine
DB104	Wild type	42	<0.5	12	0.05	0.3	0.5	0.70	0.83
DB104ΔpyrR	*pyrR*	42	0.5	12	1.0	2.1	3.5	0.03	0.34
HH207	*upp*	42	3.0	0.1	0.1	0.3	1.0	0.09	0.78
HH211	*pyrR upp*	46	1.0	<0.03	1.7	2.8	6.6	0.03	0.32

[a] Cells were grown at 37°C in liquid minimal glucose medium supplied with 40 μg of tryptophan per ml. Cultures of HH207 and HH211 were supplied with 5 μg of chloramphenicol per ml.

[b] Cells were grown at 37°C on solid minimal glucose medium, supplied with the following concentrations of 5-fluorouracil: 0, 0.5, 1, 1.5, 2, 3, 5, and 10 μg/ml. The 5-fluorouracil concentration giving rise to 50% reduction in colony size after 24 h of incubation at 37°C was estimated.

[c] The enzymatic activity of UPRTase in crude extract was determined as described in the text.

[d] Concentrations of uracil in the medium after growth to an OD_{450} of 1, 2, and 3, respectively. The amount of uracil was determined by the bioassay described in the text.

sults obtained by using FU. Both mutations in *upp* and *pyrR* result in a decreased metabolism of exogenous uracil. Furthermore, even in the absence of both UPRTase activities, uracil is still incorporated, thus confirming the existence of an alternative pathway. If this pathway also includes direct formation of the very toxic dFUMP, as shown in *L. lactis* (5), very little FU would have to be metabolized in order to kill the cell, whereas only a limited fraction will be converted into dFUMP if FU is metabolized through FUMP. Assuming that the increased pyrimidine pools in the *pyrR* strains result in an activation of the alternative pathway, either through increased gene expression or by stimulating the enzymatic activities, this would account for the finding that although the *upp* strain is incorporating uracil at three times the rate of the *pyrR* strain, it is more resistant to FU. The inactivation of the *upp* gene has no effect on uridine incorporation. Uridine is directly phosphorylated by the action of the uridine kinase, resulting in the formation of UMP. A *pyrR* mutation leads to a decreased uridine incorporation. This finding is not surprising since it is reasonable to believe that the radioactive-labeled uridine is inhibited by an increased concentration of pyrimidine metabolites, including uridine, because of overexpression of the pyrimidine biosynthetic enzymes.

Nucleotide sequence accession number. The nucleotide sequence of the *upp* gene has been submitted to the EMBL data library and assigned accession number Z38002.

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