

# Structure–function relationships of UMP kinases from *pyrH* mutants of Gram-negative bacteria

Hiroshi Sakamoto,<sup>1</sup> Stéphanie Landais,<sup>1</sup> Cécile Evrin,<sup>1</sup>  
Christine Laurent-Winter,<sup>2</sup> Octavian Bârză<sup>1</sup> and Rod A. Kelln<sup>3</sup>

## Correspondence

Hiroshi Sakamoto  
hiroshi@pasteur.fr

<sup>1,2</sup>Laboratoire de Chimie Structurale des Macromolécules<sup>1</sup> and Plate-forme 3 Protéomique<sup>2</sup>, Institut Pasteur, 75724 Paris Cedex 15, France

<sup>3</sup>Department of Chemistry and Biochemistry, University of Regina, Regina, Saskatchewan, Canada S4S 0A2

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Bacterial uridine monophosphate (UMP) kinases are essential enzymes encoded by *pyrH* genes, and conditional-lethal or other *pyrH* mutants were analysed with respect to structure–function relationships. A set of thermosensitive *pyrH* mutants from *Escherichia coli* was generated and studied, along with already described *pyrH* mutants from *Salmonella enterica* serovar Typhimurium. It is shown that Arg-11 and Gly-232 are key residues for thermodynamic stability of the enzyme, and that Asp-201 is important for both catalysis and allosteric regulation. A comparison of the amino acid sequence of UMP kinases from several prokaryotes showed that these were conserved residues. Discussion on the enzyme activity level in relation to bacterial viability is also presented.

## INTRODUCTION

Nucleoside monophosphate (NMP) kinases are ubiquitous enzymes involved in the biosynthesis of nucleotides. Each enzyme catalyses the synthesis of a nucleoside diphosphate that is, in turn, converted to a nucleoside triphosphate by a non-specific nucleoside diphosphate kinase. In prokaryotes, there are five NMP kinases, one for the phosphorylation of each NMP, whereas in eukaryotic organisms, the phosphorylation of both uridine monophosphate (UMP) and cytidine monophosphate (CMP) is carried out by a bifunctional UMP/CMP kinase (Liljelund & Lacroute, 1986).

For several organisms, the genes encoding NMP kinases have been cloned and sequenced, and the corresponding enzyme studied with respect to biochemical properties and structural features. Recent studies indicate that these enzymes are potential targets for drug design leading to new therapeutic compounds active against pathogens (Munier-Lehmann *et al.*, 2001). Adenosine monophosphate kinases and UMP/CMP kinases have been the subjects of study for some time (Dreusicke *et al.*, 1988; Müller-Dieckmann & Schultz, 1994) and more recently, the scope has been expanded to include CMP and TMP kinases (Briozzo *et al.*, 1998; Munier-Lehmann *et al.*, 2001). However, much less is known about GMP kinases and bacterial UMP kinases. To date, UMP kinase or UMP/CMP kinase has been found in most organisms investigated and, in many cases, the corresponding gene has been defined through genomic

sequencing programs. The latest members to be characterized were the UMP kinase from *Lactobacillus lactis* (Wadskov-Hansen *et al.*, 2000) and the human UMP/CMP kinase (Liou *et al.*, 2002).

Among the bacterial NMP kinases, UMP kinase deserves special attention as the enzyme shows a number of distinctive features. First, UMP kinase is the only NMP kinase known to be endowed with a second biological role besides its catalytic activity, namely, the participation of UMP kinase in regulating the transcription of the *carAB* operon (Kholti *et al.*, 1998). Second, UMP kinase is a homohexamer, and its catalytic activity is allosterically regulated by GTP/UTP (Serina *et al.*, 1996). A third feature is that UMP kinase displays very low sequence similarity to other NMP kinases, which should allow for the design of highly specific inhibitors of its catalytic activity (Serina *et al.*, 1995).

The assertion that UMP kinase activity is essential for life is based in part on knowledge of the UTP biosynthetic pathway (Neuhard & Kelln, 1996). Genetic studies with Gram-negative bacteria have provided direct evidence that UMP kinase is an essential enzyme, as conditional-lethal mutants of *pyrH* (the gene encoding UMP kinase) have been isolated (Ingraham & Neuhard, 1972; Smallshaw & Kelln, 1992). The thermosensitive *Escherichia coli* mutant KUR1244, harbouring the *pyrH88(ts)* allele, was used in the first report on the cloning of the wild-type *pyrH* allele by complementation of the thermosensitive phenotype (Smallshaw & Kelln, 1992).

In the present study, we report the characterization of the

Abbreviations: ATCase, aspartate transcarbamoylase; 5-FOA, 5-fluoro-orotic acid.

molecular basis leading to the thermosensitivity of the KUR1244 *pyrH* mutant, as well as the characterization of other *pyrH*(ts) mutants obtained in a new set of mutagenesis and selection experiments. Furthermore, three additional *pyrH* alleles from available Gram-negative pathogens were also characterized for purposes of comparison with existing data on *E. coli*. Implications in terms of using UMP kinase as a target for drug design or attenuation procedures are discussed.

## METHODS

**Bacteriology, mutant isolation and molecular cloning techniques.** Strains and vectors used in this work are shown in Table 1. Minimal medium A was as described by Sambrook *et al.* (1989) and contained 0.2% glucose as carbon source (AG medium) unless stated otherwise. Individual amino acids, as required, were added at 50 µg ml<sup>-1</sup>. All thermosensitive strains were maintained on AG medium containing 0.1% Casamino acids and thiamine (2 µg ml<sup>-1</sup>) and were cultured at 30 °C. Thermosensitivity and complementation

**Table 1.** Strains and plasmids

Strain/plasmid	Relevant genotype/features	Reference
<b><i>E. coli</i></b>		
NM554	F <sup>-</sup> <i>araD139</i> Δ( <i>ara-leu</i> )7696 <i>galE15 galK16</i> Δ <i>lacX74 rpsL hsdR2 mcrA mcrB1 recA13</i>	Raleigh <i>et al.</i> (1988)
W2915	<i>thr1 leuB6 lacY1 proA2 thi1 galK2 mtl1 xyl5 ara14 supE44</i>	Smallshaw & Kelln (1992)
KUR1244	W2915 <i>pyrH88</i>	Smallshaw & Kelln (1992)
KUR1393	W2915 <i>pyrH132</i>	This work
KUR1397	W2915 <i>pyrH412</i>	This work
KUR1398	W2915 <i>pyrH432</i>	This work
MC4100-42-14:40	<i>araD139</i> Δ <i>lacU169 rpsL thi pyrH42</i>	Roovers <i>et al.</i> (1988)
<b><i>S. enterica</i> serovar Typhimurium</b>		
KR1497	<i>pyrH1609</i>	Ingraham & Neuhard (1972)
KR1550	<i>pyrH1631</i>	Jensen (1989)
KR1633	<i>dum-1 (pyrH)</i>	Krogan <i>et al.</i> (1998)
KR1639	Wild-type LT2	Krogan <i>et al.</i> (1998)
<b>Plasmids</b>		
pET22b	Low-copy-number complementation plasmid, recombinant protein overproduction vector	Novagen
pET24ma	High-copy-number complementation plasmid, recombinant protein overproduction vector	D. Sourdive, Collectis, Romainville, France
mK12 UMK	High-copy-number complementation, wild-type UMP kinase from <i>E. coli</i>	This work
mBs UMK	High-copy-number complementation, wild-type UMP kinase from <i>B. subtilis</i>	This work
mKUR1244	High-copy-number complementation, <i>pyrH88</i> allele from <i>E. coli</i>	This work
mK12 CMK	High-copy-number complementation, wild-type CMP kinase from <i>E. coli</i>	This work
mBs CMK	High-copy-number complementation, wild-type CMP kinase from <i>B. subtilis</i>	This work
bK12 UMK	Low-copy-number complementation, wild-type UMP kinase from <i>E. coli</i>	This work
bKUR1244	Low-copy-number complementation, R11H variant from <i>E. coli</i>	This work
bKUR1393	Low-copy-number complementation, R11H variant from <i>E. coli</i>	This work
bKUR1397	Low-copy-number complementation, G232D variant from <i>E. coli</i>	This work
bKUR1398	Low-copy-number complementation, R11H/S106F/E196K variant from <i>E. coli</i>	This work
bSt UMK	Low-copy-number complementation, wild-type UMP kinase from <i>S. enterica</i> serovar Typhimurium	This work
bKR1633	Low-copy-number complementation, A122T variant from <i>S. enterica</i> serovar Typhimurium	This work
bKR1497	Low-copy-number complementation, D201N/E241D variant from <i>S. enterica</i> serovar Typhimurium	This work
bKR1550	Low-copy-number complementation, D201G variant from <i>S. enterica</i> serovar Typhimurium	This work

were tested at 42 °C and viability was confirmed by scoring resulting colonies at 30 and 42 °C. Where viability was reduced below 1%, this was considered as non-complementation.

Chemical mutagenesis was carried out by treating a growing culture of strain W2915 at a density of  $1 \times 10^8$ – $2 \times 10^8$  cells ml<sup>-1</sup> with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at 0.1 mg ml<sup>-1</sup> (Miller, 1992). Following mutagenesis, the cells were phenotypically expressed for 24–48 h in minimal medium plus requirements with 0.2% succinate as sole carbon source to select against *dctA* mutants in the population (Baker *et al.*, 1996). Appropriate dilutions were then plated to a selective and differential medium containing the following: 5 µg 5-fluoroorotic acid (5-FOA) ml<sup>-1</sup>, 0.3% glycerol as carbon source and required amino acids, to which cells of strain KR1650 were incorporated to serve as an indicator bacterium (Kelln *et al.*, 1975; O'Donovan & Gerhart, 1972) for the detection of uracil excretion. Plates were incubated at 30 °C for 48–72 h and those 5-FOA-resistant colonies exhibiting uracil excretion were purified on the selective medium. The isolates were then tested for growth at 42 °C and mutants showing temperature sensitivity were screened for elevated expression of *pyrBI* by measuring the level of aspartate transcarbamoylase (ATCase) activity (Krogan *et al.*, 1998). Any isolates exhibiting the four-part phenotype of 5-FOA resistance, uracil excretion, temperature sensitivity and elevated ATCase activity were retained for further characterization.

General cloning methods were essentially as described by Sambrook *et al.* (1989). Genes encoding UMP or CMP kinases from various origins were amplified by thermocycling and cloned into vectors pET22b or pET24ma (Table 1), providing plasmids for both complementation analysis and for enzyme overproduction. Cloned DNA was sequenced using the dideoxy chain-termination method.

**Biochemical analytical procedures.** Recombinant enzymes were produced and purified as follows. Strain BL21(DE3)/pDIA17 was used as the recipient for all recombinant plasmids used for overexpression. Cultures were grown at 30 °C in 2YT medium containing appropriate antibiotics until a value of 1 at OD<sub>600</sub> was reached. IPTG was then added to a final concentration of 1 mM and incubation was continued for 3 h. Cells were harvested by centrifugation, resuspended in 20 mM Tris/HCl, pH 7.5, and disrupted by sonication (Landais *et al.*, 1999). The recombinant enzyme was partially purified by several cycles of washing/centrifugation as described by Serina *et al.* (1995). Samples from non-overproducing strains were prepared as follows: strains were grown in 2YT medium at 30 °C to an OD<sub>600</sub> of 4, then harvested, sonicated in 20 mM Tris/HCl, pH 7.5, and then the extract was heated for 10 min at 70 °C in the presence of 1 mM UTP. Bacterial debris was removed by centrifugation and UMP kinase activity was assayed in the supernatant as described below.

UMP kinase activity was assayed at 30 °C in a coupled system (Serina *et al.*, 1995), with 1 mM ATP, 0.3 mM UMP, pH 7.4. Regulation by GTP/UTP was assayed in the presence of 0.5 mM effectors. One unit corresponds to 1 µmol UDP formed min<sup>-1</sup>. Thermosensitivity of the enzymes was assayed after a 10 min heat denaturation treatment in a thermocycler, with the wild-type enzyme used as an internal control in each series. Indicated *T<sub>m</sub>* values represent the denaturing temperature where 50% of the UMP kinase activity was lost. Protein concentrations were determined by the method of Bradford (1976). All data come from triplicate experiments.

Western blots with polyclonal anti-UMP kinase were performed as previously described (Landais *et al.*, 1999). Strains were grown in 2YT medium at 30 °C and harvested at an OD<sub>600</sub> of 4. Strain KUR1244 was also grown at 30 °C to an OD<sub>600</sub> of 0.3, then up-shifted to 42 °C until growth stopped. All samples were collected by centrifugation,

sonicated, heated in the presence of 1 mM UTP and then centrifuged. Protein concentration was measured in each sample, then serial dilutions of the supernatants were subjected to SDS-PAGE (Laemmli, 1970), transferred to a nitrocellulose membrane and immunodetected using polyclonal anti-UMP kinase antibodies. Immunoblots were scanned and the abundance of UMP kinase in the wild-type strain was considered as 100%.

## RESULTS AND DISCUSSION

### KUR1244 *pyrH88(ts)* strain

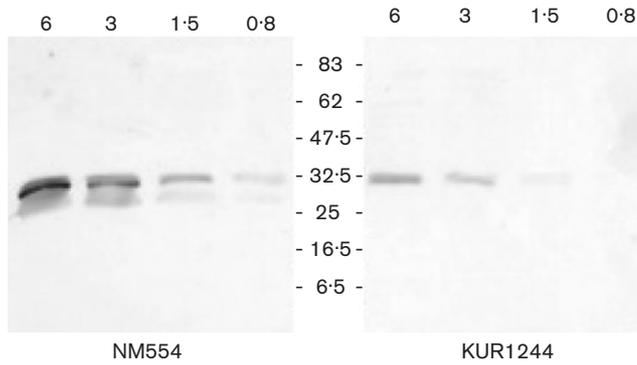
In the present study, the *pyrH88(ts)* mutation in KUR1244, which was obtained by chemical mutagenesis and selection for low *in vivo* UMP kinase activity, was complemented with *pyrH* genes harboured in multicopy plasmids. The various *pyrH* genes originated from bacteria (*E. coli*, *Bacillus subtilis*, *Salmonella enterica* serovar Typhimurium). The wild-type *pyrH* allele from *E. coli* was cloned in to both low- and high-copy-number vectors, mediating either threefold or 70-fold elevated levels of UMP kinase activity, respectively (Tables 1 and 2). Either plasmid type complemented the thermosensitivity of KUR1244. Complementation also occurred at low activity levels of the UMP kinase from *B. subtilis*. Also, *B. subtilis* CMP kinase at high levels complemented due to the cross-over UMP kinase activity of the CMP kinase. High gene dosage of the *pyrH88(ts)* allele per se never led to a complete restoration of the wild-type phenotype.

UMP kinase activity and its abundance were investigated *in vivo* in wild-type *E. coli* and KUR1244. At the permissive temperature, KUR1244 showed a lowered *in vivo* UMP kinase activity (Table 2). By Western blot analysis, we also observed a significantly reduced amount of enzyme when the strain was grown at the permissive temperature (Fig. 1). A temperature shift to 42 °C resulted in a further drop in the level of UMP kinase activity to below the detection threshold of the enzyme assay and this drop corresponded with a halt in growth. At this stage, the UMP kinase abundance was 5% of the nominal amount observed in the

**Table 2.** *In vivo* UMP kinase activity and abundance in *E. coli* strains

Assay of UMP kinase activity was performed as described in Methods for non-overproducing strains. ND, Not done.

<i>E. coli</i> strain	UMP kinase activity (U mg <sup>-1</sup> )	UMP kinase abundance at 30 °C (%)
Wild-type	0.034	100
KUR1244	0.003	30 (5 after 42 °C shift)
KUR1244/K12 <i>pyrH</i> (low dose)	0.100	ND
KUR1244/K12 <i>pyrH</i> (high dose)	2.400	ND



**Fig. 1.** UMP kinase abundance in strain KUR1244. Determination of the abundance of UMP kinase was carried out by Western blotting as described in the Methods. Samples containing 0.8, 1.5, 3 or 6 µg protein were submitted to SDS-PAGE (12.5%), transferred onto a nitrocellulose membrane and immunodetected using polyclonal anti-UMP kinase antibodies. The abundance of UMP kinase in the wild-type NM554 strain is considered as 100%. Molecular masses are indicated.

wild-type *E. coli* strain. The effect of the temperature up-shift was reversible to some extent, as down-shifting to 30 °C restored growth, albeit with a loss of viability (data not presented).

The *pyrH88(ts)* allele was further characterized with respect to biochemical features of the enzyme it encodes, as well as the nature of the mutation. The *pyrH88(ts)* allele was cloned by first amplifying the allele using KUR1244 genomic DNA as template. The resulting amplified fragment was introduced into vectors pET22b and pET24ma for sequence analysis and recombinant protein overproduction. The *pyrH* mutation in KUR1244 was shown to cause an R11H substitution in the polypeptide. The corresponding recombinant enzyme was purified and characterized for its main biochemical features. The enzyme displayed a lowered catalytic activity corresponding to 45% of wild-type enzyme activity, but regulation of activity by GTP/UTP was retained. Resistance to heat denaturation was impaired, as the  $T_m$  value was reduced to 55 °C compared to 63 °C for the wild-type enzyme. Protein synthesis/folding appeared defective since overproduction at a temperature higher than 30 °C resulted in irreversible denaturation of the mutant enzyme.

#### Other *E. coli pyrH(ts)* mutant strains

To broaden the information base regarding the *E. coli* enzyme, a collection of temperature-sensitive *E. coli pyrH* mutant strains was generated and characterized. As detailed in Methods, chemical mutagenesis was performed on strain W2915 coupled with selective and corresponding screening steps. Ultimately, three new *pyrH(ts)* strains were obtained: KUR1393, KUR1397 and KUR1398.

As described above for KUR1244, each new thermosensitive

strain was tested for complementation by *pyrH*<sup>+</sup>. Complementation was observed in all cases with the wild-type *pyrH* allele from *E. coli* in either high or low copy number. Complementation with a low copy number of *pyrH*, or a high copy number of *cmk* from *B. subtilis* occurred as well.

All *pyrH* alleles were cloned by utilizing thermocycling amplification, then sequenced and their corresponding recombinant protein produced. The amino acid change in the enzyme from KUR1393 was the same as for KUR1244, namely R11H. The same change, along with two additional alterations (S106F, E196K) occurred in the enzyme from KUR1398. The enzyme from strain KUR1397 harboured a single distinct substitution at position 232, namely, a glycine to aspartate change.

Not unexpectedly, the properties of the recombinant UMP kinase variant from KUR1393 mimicked those observed with the enzyme from KUR1244. In the case of the UMP kinase variant from KUR1397, the G232D modification also led to biochemical features comparable to the R11H variant. Resistance to heat denaturation was altered, as the  $T_m$  value was lowered to 57 °C and catalytic activity was reduced to 17% of the wild-type enzyme activity. Despite numerous attempts, the recombinant enzyme from KUR1398 could not be obtained in a soluble form.

The data provided herein show that the thermosensitive phenotype of KUR1244 is indeed linked to a defect of UMP kinase catalytic activity, an intrinsic property of a variant enzyme. Although the ability to affect transcription regulation of *carAB* was retained at the permissive temperature, the mutant enzyme displayed both a reduced catalytic activity and an increased thermosensitivity. The enzyme abundance was also found to be lowered *in vivo*. Therefore, the tenfold decrease in activity of the mutant strain *in vivo* as compared to the wild-type *E. coli* is a combination of two effects: a decrease in specific activity and a decrease of the protein abundance. Since the *in vitro*-measured  $T_m$  of the R11H mutant is still far from the non-permissive temperature of 42 °C, we infer that the low *in vivo* abundance of this variant reflects a folding and/or oligomerization defect. Hence KUR1244 may be more appropriately described as a mutant that is temperature-sensitive for synthesis, i.e., a *tss* mutant. In conjunction with the expanded study involving three newly isolated *pyrH(ts)* mutants of *E. coli*, the accumulated findings showed that the R11 residue is a key target for replacement when selection is based on 5-FOA resistance, uracil excretion, elevated ATCase activity and thermosensitivity. Another target residue is found at position G232, near the C terminus of the polypeptide.

#### *S. enterica* serovar Typhimurium *pyrH* mutants

In *E. coli*, a comprehensive set of *pyrH* mutant strains was characterized, both for recombinant UMP kinase catalytic activities and phenotypic features. We therefore investigated *pyrH* mutants from other related Gram-negative

organisms to gain comparative insights on UMP kinases from one bacterial species to another.

For *S. enterica* serovar Typhimurium, strains defective in UMP kinase activity have been described since the early 1970s. Mutant strain *pyrH1609* was shown to be cryo-sensitive and endowed with a reduced UMP kinase activity *in vivo* (Ingraham & Neuhard, 1972). Mutant strain *pyrH1631* was described in a study of *pyr* gene expression in response to nucleotide pool shifts and was obtained by selection for the ability to use 2,6-diaminopurine as an exogenous purine source (Jensen, 1989). Recently, the *dum-1* mutation was demonstrated as a mutant *pyrH* allele, involved in establishing thymidine auxotrophy in a *dcd cdd thyA*<sup>+</sup> background (Krogan *et al.*, 1998). The nature of the three corresponding mutations was not determined, nor were the biochemical properties of the respective UMP kinase variant enzymes assessed.

We undertook the cloning and sequencing of *pyrH* alleles from strains KR1633 (*dum-1*), KR1497 (*pyrH1609*) and KR1550 (*pyrH1631*), along with that of the wild-type LT2 strain, KR1639. The data in Table 3 include the UMP kinase activity of each strain and a summary of the amino acid differences in the variants, determined to be A122T (*dum-1*), D201N and E241D (*pyrH1609*) and D201G (*pyrH1631*).

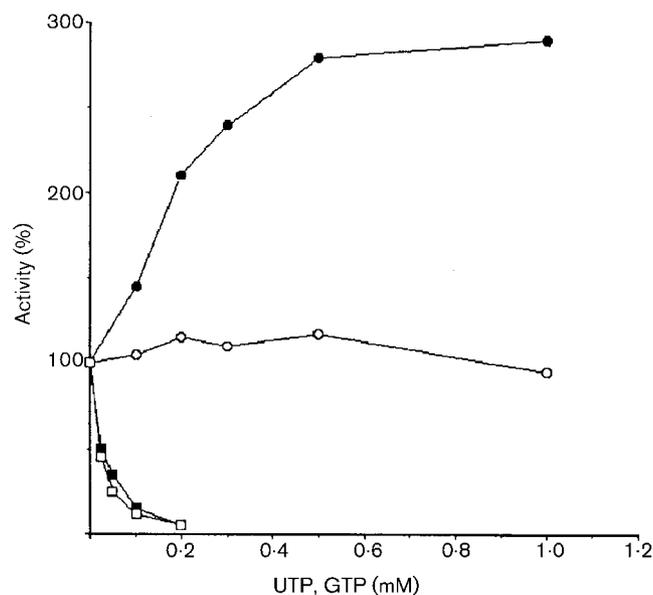
The mutant and wild-type UMP kinases were overproduced and purified. With respect to wild-type UMP kinase, the catalytic activities of the variants were 26 (*dum-1*), 1 (*pyrH1609*) and 16% (*pyrH1631*). Comparative biochemical analysis of the purified D201G and wild-type recombinant enzymes indicated identical *T<sub>m</sub>* values (63 °C) but a sevenfold reduced activity of the modified UMP kinase. Moreover, the D201G mutant showed a significant loss of sensitivity to activation by GTP, whereas the sensitivity to inhibition by UTP was retained (Fig. 2).

Data for three *S. enterica* serovar Typhimurium *pyrH* mutants confirmed that all strains have lowered catalytic activities. Catalytic activity of the recombinant A122T

**Table 3.** Properties of *S. enterica* serovar Typhimurium *pyrH* mutants

Assay of UMP kinase activity was performed on partially purified enzyme preparations as described in Methods. The wild-type enzyme activity was approximately 50 U mg<sup>-1</sup>, and was taken as 100%.

<i>S. enterica</i> serovar Typhimurium LT2 strains	Mutation(s)	UMP kinase activity (%)
KR1639 wild-type	None	100
KR1633 ( <i>dum-1</i> )	A122T	26
KR1497 ( <i>pyrH1609</i> )	D201N, E241D	1
KR1550 ( <i>pyrH1631</i> )	D201G	16



**Fig. 2.** Biochemical features of the D201G variant. Dependence of the activity of recombinant UMP kinase from wild-type (filled symbols) and the D201G mutant (open symbols) from *S. enterica* serovar Typhimurium on GTP (circles) and UTP (squares). A value of 100% corresponds to 50 U protein mg<sup>-1</sup> for the wild-type UMP kinase and 7.2 U protein mg<sup>-1</sup> for the D201G mutant with 1 mM ATP and 1 mM UTP as substrates.

variant matches that found *in vivo* for the *dum-1* mutant strain. The residual catalytic activity of the D201N + E241D variant (*pyrH1609*) corresponds to that of the D201N variant of *E. coli* (Yamanaka *et al.*, 1992). This latter *E. coli* mutant and the *S. enterica* serovar Typhimurium *pyrH1609* mutant display a similar phenotype in that both are cold-sensitive.

### Key residues of bacterial UMP kinases

The R11 and G232 residues are highly conserved among UMP kinases from prokaryotes (Gagyi *et al.*, 2003). This indicates that the R11 residue is at a key position, predisposed to replacement in the selection of *pyrH*(ts) mutants. For catalytic activity, the results obtained with *S. enterica* serovar Typhimurium confirm that the highly conserved D201 residue is important, as also applies to *E. coli*. In this respect it is worth mentioning that the D201N ‘conservative’ substitution first described by Yamanaka *et al.* (1992) and explored enzymically by Bucurenci *et al.* (1998) in *E. coli*, has a more dramatic effect on protein stability and catalysis than that observed with the newly described D201G variant. In the modelled structure of *E. coli* UMP kinase, D201 is situated at the N terminus of helix-7, which is predicted to provide residues to the catalytic centre (Labesse *et al.*, 2002). The severe loss of catalytic activity upon substitution of this residue with asparagine suggests that formation of salt bridges is perhaps critical for stabilization of the active

site. In the case of the D201 to G substitution, an increased flexibility of the polypeptide chain at this level might compensate for the loss of a negative charge at the expense of allosteric activation. Comparative crystallographic analysis of wild-type, D201N and D201G mutants would be able to provide a definitive answer.

What could be the lower limit of UMP kinase activity required to support life? Based on our data of the KUR1244 strain after upshift to 42 °C, and the catalytic activity of the corresponding recombinant protein, one can propose that such a threshold is in the range of an *in vivo* activity of 5 mU mg<sup>-1</sup>. It is tempting to suggest that a conditional mutant of *pyrH* displaying a catalytic activity in the vicinity of this threshold would show a complete loss of viability in the non-permissive conditions.

The essential activity of UMP kinase is catalysis, and bacterial UMP kinases share significant sequence similarities. Based on the current data, we propose that a combination of the low catalytic activity substitutions (i.e. D146N or D201N) (Serina *et al.*, 1995; Bucurenci *et al.*, 1998), along with mutations leading to thermosensitivity of UMP kinase (i.e. R11H or G232D) may provide a general method to construct attenuated bacterial strains, especially from Gram-negative pathogens.

### Concluding remarks

The 3D structure of bacterial UMP kinases is not yet solved, which hampers advances in rational drug design. While numerous variants of UMP kinase are available to decipher its structure–function relationship for catalysis, very few variants address the issue with respect to overall folding and thermal stability. In the last few years impressive advances have been achieved in the design of oligopeptides of therapeutic interest (Gratton *et al.*, 2003). Studies have shown that oligopeptides can be used for intracellular delivery, as well as *in situ* protein–protein interaction. This new technology opens up the field for new antimicrobial compounds based on the disruption of essential catalytic activities. In this respect, the set of *pyrH*(ts) mutants described in this work may also find value for use in rational drug design.

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