

# Characterization of pyrimidine nucleoside phosphorylase of *Mycoplasma hyorhina*: implications for the clinical efficacy of nucleoside analogues

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In the present paper we demonstrate that the cytostatic and antiviral activity of pyrimidine nucleoside analogues is markedly decreased by a *Mycoplasma hyorhina* infection and show that the phosphorolytic activity of the mycoplasmas is responsible for this. Since mycoplasmas are (i) an important cause of secondary infections in immunocompromised (e.g. HIV infected) patients and (ii) known to preferentially colonize tumour tissue in cancer patients, catabolic mycoplasma enzymes may compromise efficient chemotherapy of virus infections and cancer. In the genome of *M. hyorhina*, a TP (thymidine phosphorylase) gene has been annotated. This gene was cloned, expressed in *Escherichia coli* and kinetically characterized. Whereas the mycoplasma TP efficiently catalyses the phosphorolysis of thymidine ( $K_m = 473 \mu\text{M}$ ) and deoxyuridine ( $K_m = 578 \mu\text{M}$ ), it

prefers uridine ( $K_m = 92 \mu\text{M}$ ) as a substrate. Our kinetic data and sequence analysis revealed that the annotated *M. hyorhina* TP belongs to the NP (nucleoside phosphorylase)-II class PyNPs (pyrimidine NPs), and is distinct from the NP-II class TP and NP-I class UPs (uridine phosphorylases). *M. hyorhina* PyNP also markedly differs from TP and UP in its substrate specificity towards therapeutic nucleoside analogues and susceptibility to clinically relevant drugs. Several kinetic properties of mycoplasma PyNP were explained by *in silico* analyses.

**Key words:** antiviral/anticancer activity, *Mycoplasma hyorhina*, nucleoside analogue, pyrimidine nucleoside phosphorylase (PyNP), thymidine phosphorylase (TP), uridine phosphorylase (UP).

## INTRODUCTION

The treatment of cancer and many viral infections [caused by e.g. HIV, HSV (herpes simplex virus), Varicella Zoster virus, cytomegalovirus, hepatitis C virus or hepatitis B virus] is largely based on the use of nucleoside-derived therapeutics [1,2]. These molecules mimic the nucleic acid building blocks and may therefore act as antimetabolites in DNA/RNA synthesis or as fraudulent substrates for enzymes involved in nucleoside metabolism. Thus, after enzymatic activation (usually phosphorylation), they directly or indirectly interfere with the cellular or viral DNA/RNA synthesis. Owing to the nature of these drugs they may also be subject to enzymatic inactivation (e.g. deamination, phosphorolysis or dephosphorylation) by enzymes involved in nucleoside(s) catabolism. It has previously been demonstrated that mycoplasma-derived TP (thymidine phosphorylase) activity compromises the cytostatic action of several nucleoside-based chemotherapeutics in cancer cell cultures [3–6].

Mycoplasmas are the smallest autonomously replicating organisms and are characterized by the lack of a cell wall and a strongly reduced genome (600–1200 kb). Many of these bacteria, belonging to the class of the Mollicutes, have a parasitic lifestyle and reside in the human body causing asymptomatic infections [7]. In particular immunocompromised patients (e.g. patients suffering from AIDS or hypogammaglobulinaemia) are known to be prone to mycoplasma infections [8,9]. Despite their high tissue specificity, mycoplasmas are now regularly being isolated from organs different from their usual habitats owing to the

increasing number of patients suffering such immunodeficiencies [7]. Furthermore, it was shown that some of these prokaryotes, in particular *Mycoplasma hyorhina*, tend to preferentially colonize tumour tissue in cancer patients [10–17]. Taken together, these studies indicate that a mycoplasma infection may not only affect the health of cancer patients or immunocompromised individuals, but may also compromise the efficacy of chemotherapeutic treatment.

Previously, we hypothesized that the treatment of patients using purine and pyrimidine antimetabolite drugs may be optimized by (i) the elimination of an underlying mycoplasma infection by antibiotics, (ii) suppression of mycoplasma-encoded enzymes in human tumour tissue and/or (iii) the development of mycoplasma-insensitive nucleoside analogue prodrugs [5,6,18]. In the absence of such approaches, cancer patients may receive suboptimal chemotherapeutic treatment. In the present paper, we demonstrate that the *M. hyorhina*-derived phosphorolytic activity in cell cultures is not only responsible for the decreased cytostatic activity of certain nucleoside analogues, but also for their decreased antiviral activity towards different human viruses. To gain further insight into the molecular basis of these observations, we cloned, expressed and characterized the putative *M. hyorhina*-encoded TP. The kinetic properties of this enzyme shed new light on pyrimidine nucleoside metabolism in Mollicutes and reveal a distinct substrate specificity when compared with human or *Escherichia coli* TP and UP [Urd (uridine) phosphorylase]. Structural and functional studies revealed two distinct families of NPs (nucleoside phosphorylases): the NP-I family (containing purine NP and UP) and the NP-II family [containing TP and

Abbreviations used: ara-T, thymine arabinoside; ara-U, uracil arabinoside; AZT, azidothymidine; BAU, 5-benzylacyclouridine; BVDU, (E)-5-(2-bromovinyl)-deoxyuridine; BVU, (E)-5-(2-bromovinyl)-uracil; 2-dRib-1-P, 2-deoxyribose-1-phosphate; D4T, stavudine; dThd, thymidine; dUrd, 2'-deoxyuridine; 7-DX, 7-deazaxanthine; 5-FU, 5-fluorouracil; GST, glutathione transferase; HSV, herpes simplex virus; NP, nucleoside phosphorylase; PyNP, pyrimidine nucleoside phosphorylase; Rib-1-P, ribose-1-phosphate; TFT, 5-trifluoromethyl-dUrd; Thy, thymine; TP, thymidine phosphorylase; TPI, TP inhibitor; UP, uridine phosphorylase; UPP1, human UP1; Ura, uracil; Urd, uridine; VV, vaccinia virus.

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PyNPs (pyrimidine NP) [19]. Our kinetic and computational sequence analysis data demonstrate that the mycoplasma-encoded TP activity is due to the presence of an NP-II class PyNP in the cell cultures that is distinct from the NP-II class TP and NP-I class UP enzymes. Our findings explain the dramatic effect mycoplasmas may have on the antiviral and cytostatic efficiency of several chemotherapeutics in infected human cell cultures.

## EXPERIMENTAL

### Chemicals

Nucleosides, nucleobases, nucleoside analogues and all of the inorganic compounds were purchased from Sigma–Aldrich unless stated otherwise. TPI {a potent TP inhibitor/5-chloro-6-(1-[2-iminopyrrolidinyl]methyl)Ura hydrochloride, where Ura is uracil} [20] was kindly provided by Professor Vern Schramm (Albert Einstein College of Medicine, New York, NY, U.S.A.). BAU (5-benzylacetyluridine) was purchased from RNDCHEM. 7-DX (7-deazaxanthine) was synthesized as described previously [21].

### Cell cultures

MCF-7 human breast carcinoma cells were kindly provided by Professor Godefridus Peters (VU University Medical Center, Amsterdam, The Netherlands). MCF-7 cells were infected with *M. hyorhinis* (strain number A.T.C.C. 17981) resulting in a chronically infected cell line henceforth referred to as MCF-7.Hyor. All of the cells were maintained in DMEM (Dulbecco's modified Eagle's medium; Invitrogen) with 10% foetal bovine serum (Biocrom), 10 mM Hepes and 1 mM sodium pyruvate (Invitrogen). Cells were grown at 37°C in a humidified incubator with a gas phase of 5% CO<sub>2</sub>.

### Biological assays

The antiviral assays were based on inhibition of virus-induced cytopathicity in MCF-7 and MCF-7.Hyor cell cultures. Cells were seeded in 96-well plates (Thermo Fisher Scientific) at 20000 cells/well and were allowed to proliferate for 24 h at 37°C. The cells were then exposed to fresh medium containing 100 CCID<sub>50</sub> (50% cell culture infectious dose) of virus [VV (vaccinia virus), HSV-1 (strain KOS), or HSV-2 (strain G)] and different concentrations of the test compound in the presence or absence of TPI (10 μM). The cells were incubated at 37°C and viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds. In addition, the antiviral activity of 5-iodo-dUrd (where dUrd is 2'-deoxyuridine) against VV was compared in MCF-7 and MCF-7.Hyor cells that were pretreated for 4 days with 1 μg/ml tetracycline, an antibiotic targeting mycoplasmas.

To compare the cytostatic activity of nucleoside analogues in mycoplasma-infected and control cancer cell lines, MCF-7 and MCF-7.Hyor cells were seeded in 48-well plates (Thermo Fisher Scientific) at 10000 cells/well. After 24 h, an equal volume of fresh medium containing the test compounds was added. On day 5, cells were trypsinized and counted in a Coulter counter. The IC<sub>50</sub> value was defined as the compound concentration required to reduce cell proliferation by 50%.

### Purification of TP<sub>Hyor</sub> (*M. hyorhinis* TP)

In the recently published *M. hyorhinis* HUB-1 genome [22], a TP gene, but no UP gene, was found. Therefore a codon-optimized DNA sequence encoding the TP<sub>Hyor</sub> was synthetically

assembled between the EcoRI and NotI restriction sites of a pIDTsmart vector (Integrated DNA technologies). The fragment was subsequently subcloned between the EcoRI and NotI sites of the pGEX-5X-1 bacterial expression vector (Amersham Pharmacia) and expressed in *E. coli* BL21(DE3)pLysS as a GST (glutathione transferase) fusion protein according to the procedure described previously [23]. Bacteria were grown for 8 h at 37°C in LB (Luria–Bertani) medium containing ampicillin (100 μg/ml) and chloramphenicol (40 μg/ml), diluted 1:30 in fresh medium and grown overnight under the same conditions. Next, the culture was diluted 1:10 in fresh medium and incubated for 2 h at 37°C. Then cultures were placed at 27°C for 4 h after which IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.1 mM to induce the production of the GST–TP fusion protein. After 15 h of further growth at 27°C, cells were pelleted (6000 g for 10 min at 4°C) and resuspended in lysis buffer [50 mM Tris/HCl (pH 7.5), 1 mM dithiothreitol, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 0.1 mM PMSF and 0.15 mg of lysozyme]. Bacterial suspensions were homogenized and lysed by means of a French Pressure cell press and ultracentrifuged (20000 rev./min for 15 min at 4°C using a Beckman Coulter Type 70 T; fixed angle rotor). GST–TP<sub>Hyor</sub> (henceforth referred to as TP<sub>Hyor</sub>) was purified from the supernatant using glutathione–Sepharose 4B (Amersham Pharmacia) following the manufacturer's instructions. Briefly, a 50% slurry of glutathione–Sepharose was added to the bacterial supernatant (2 ml/750 ml of broth), incubated at 4°C and then washed three times with 10 bed volumes of lysis buffer without lysozyme and PMSF. Bound proteins were eluted in 50 mM Tris/HCl (pH 7.5) containing 0.1% Triton X-100, 10 mM glutathione and 20% glycerol. SDS/PAGE (10% gel) revealed a GST-fusion protein of ~75 kDa (48 kDa for TP<sub>Hyor</sub> and 25 kDa for GST) (Supplementary Figure S1 at <http://www.BiochemJ.org/bj/445/bj4450113add.htm>).

### Enzyme assays

Determination of the pH and temperature optima

The TP<sub>Hyor</sub>-mediated phosphorolysis of dThd (thymidine) was assayed under different pH and temperature conditions. dThd (100 μM) was incubated in the presence of the enzyme (9 nM) at 37°C with varying pH buffer conditions (pH = 5.5–8.5). Reactions were carried out in a total volume of 500 μl of phosphorolysis buffer (10 mM Tris/HCl, 1 mM EDTA, 150 mM NaCl and 200 mM potassium phosphate). At different time points (0, 20, 40 and 60 min), 100 μl fractions were withdrawn, transferred to an Eppendorf tube and heated at 95°C for 3 min to inactivate the enzyme. Next, the samples were rapidly cooled on ice for 15 min and cleared by centrifugation at 16000 g for 15 min. Thy (thymine) was separated from dThd on a reverse phase RP-8 column (Merck) and quantified by HPLC analysis (Alliance 2690, Waters). The separation was performed by a gradient from 100% buffer B [50 mM NaH<sub>2</sub>PO<sub>4</sub> (Acros Organics) and 5 mM heptane sulfonic acid (pH 3.2)] to 75% buffer B and 25% acetonitrile (BioSolve) (10 min linear gradient of 100% buffer B to 98% buffer B and 2% acetonitrile; 10 min linear gradient to 90% buffer B and 10% acetonitrile; 5 min linear gradient to 75% buffer B and 25% acetonitrile; and 5 min linear gradient to 100% buffer B followed by equilibration at 100% buffer B for 10 min). UV-based detection of dThd was performed at 266 nm. The TP<sub>Hyor</sub>-mediated phosphorolysis of dThd was also compared after incubation at 20°C and at 37°C in a similar assay carried out in TP buffer [10 mM Tris/HCl, 1 mM EDTA, 150 mM NaCl and 200 mM potassium phosphate (pH 7.6)].

### Determination of TP<sub>Hyor</sub> substrate specificity

To study the phosphorolysis of different nucleosides and nucleoside analogues by TP<sub>Hyor</sub>, different potential substrates (100  $\mu$ M) were exposed to the enzyme (45 nM) and incubated at 37 °C in TP buffer in a total volume of 300  $\mu$ l. At different time points (0, 10, 30 and 60 min), 65  $\mu$ l fractions were withdrawn, transferred and processed as described above. Nucleobases and nucleosides were separated by HPLC analysis as described above and for each product UV-based detection was performed at the specific wavelength of optimal absorption. Separation of BVDU [(*E*)-5-(2-bromovinyl)-dUrd] from its respective base BVU [(*E*)-5-(2-bromovinyl)-Ura] was performed by a linear gradient from 98 % buffer C [1 mM potassium phosphate buffer, (pH 5.5)] and 2 % buffer D [1 mM potassium phosphate buffer (pH 5.5) and 80 % methanol] to 20 % buffer C and 80 % buffer D. After injection of the samples, 98 % buffer C and 2 % buffer D was run for 10 min before the start of the gradient (10 min linear gradient from 2 % to 80 % buffer D). After 5 min running at 80 % buffer D, a 5 min linear gradient to 98 % buffer C and 2 % buffer D was performed followed by equilibration at 98 % buffer C for 5 min.

### Kinetic assays

The enzymatic activity of TP<sub>Hyor</sub> towards different substrates (dThd, dUrd, Urd, 5-fluoro-dUrd, 5-iodo-dUrd, 5-fluoro-Urd and 5-iodo-Urd) was evaluated. The nucleoside-to-nucleobase conversion at varying concentrations of substrate was studied in a reaction containing 9 nM enzyme incubated in TP buffer at 37 °C for 20 min. For each substrate, at least 10 different concentrations in the following range were assayed: dThd and dUrd, 100–6000  $\mu$ M; Urd, 25–2000  $\mu$ M; 5-fluoro-dUrd, 25–1000  $\mu$ M; and 5-iodo-dUrd, 5-fluoro-Urd and 5-iodo-Urd, 25–500  $\mu$ M. In the kinetic assays where the enzymatic activity of TP<sub>Hyor</sub> was evaluated at varying concentrations of P<sub>i</sub> (10 different concentrations in the range of 0.1–50 mM), the nucleoside substrate was kept fixed at a concentration of 10 $\times$   $K_m$ . After incubation, samples were processed and analysed by HPLC as described above. The Michaelis constant ( $K_m$ ) and turnover number ( $k_{cat}$ ) were determined by means of non-linear regression analysis (using GraphPad Prism5).

### Competition experiments

To determine the substrate preference of TP<sub>Hyor</sub>, dThd and Urd (each at 100  $\mu$ M) were exposed in one reaction to 9 nM TP<sub>Hyor</sub>. After incubation at 37 °C in TP buffer, fractions were collected at 0, 20, 40 and 60 min and processed as described above.

To study whether both substrates are mutually exclusive, TP<sub>Hyor</sub>-mediated phosphorolysis of a fixed concentration of dThd (100  $\mu$ M) was studied in the presence of different concentrations of Urd (1 mM, 0.5 mM, 0.25 mM, 0.1 mM and 0 mM) and vice versa. After a 30 min incubation of the substrates with the enzyme (9 nM) at 37 °C in TP buffer, samples were processed as described above.

### Anabolic activity of TP<sub>Hyor</sub>

The *M. hyorhinis* TP-mediated coupling of 2-dRib-1-P (2-deoxyribose-1-phosphate) to Thy and Ura and the coupling of Rib-1-P (ribose-1-phosphate) to Ura was studied. Sugars (1 mM) and nucleobases (100  $\mu$ M) were incubated at 37 °C for 20 min in the presence of 9 nM TP<sub>Hyor</sub> in TP buffer with varying concentrations of inorganic phosphate (P<sub>i</sub> = 50 mM; 5 mM; 0.5 mM and 0 mM) in a total volume of 200  $\mu$ l. The

formation of dThd and (d)Urd was quantified by HPLC analysis as described above.

### Inhibition assays

In the assays where the inhibitory effect of the TP inhibitors 7-DX and TPI and the UP inhibitor BAU was evaluated, a variety of inhibitor concentrations, including 500  $\mu$ M, 250  $\mu$ M, 100  $\mu$ M, 25  $\mu$ M and 0  $\mu$ M (control), were added to a reaction mixture that contained 100  $\mu$ M substrate (dThd or Urd) in TP buffer containing 2 mM P<sub>i</sub>. Next, the reaction mixture was exposed to different phosphorolytic enzymes [TP<sub>Hyor</sub>, human TP, *E. coli* TP or UPP1 (human UPI; derived from tumour tissue)] and, after a 20 min incubation at 37 °C, the substrate degradation was determined by HPLC as described above.

### Bioinformatics and computational *in silico* analysis

Protein alignments and pairwise alignment scores were calculated using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The one-to-one threading method implemented in the Phyre2 server [24] provided different TP<sub>Hyor</sub> models using as templates the three-dimensional structures of several TPs and PyNPs that have been solved by X-ray crystallography and are deposited in the PDB. The computer graphics program PyMOL (<http://www.pymol.org/>) was used for molecular visualization and superimposition.

## RESULTS

### *M. hyorhinis* infection compromises the biological activity of therapeutic nucleoside analogues

Human breast carcinoma MCF-7 cells were infected with *M. hyorhinis* (designated MCF-7.Hyor) and used to study the effect of the mycoplasma infection on the antiviral/antiproliferative activity of various nucleoside analogues.

The antiviral activity of 5-halogenated dThd analogues, including the clinically approved antiherpetic agents 5-iodo-dUrd (idoxuridine) and BVDU (Brivudin) against different viruses was determined in MCF-7 and MCF-7.Hyor cell cultures. As shown in Table 1, the inhibitory activity of the drugs against VV, HSV-1 and HSV-2 infection was decreased by 6–55 fold in MCF-7.Hyor cell cultures when compared with mycoplasma-free control MCF-7 cells. The antiviral activity of the compounds could be rescued by pretreating the cells for 4 days with tetracycline (1  $\mu$ g/ml), an antibiotic targeting mycoplasmas (results not shown). The antiviral activity could also be fully restored upon administration of 10  $\mu$ M TPI, a powerful TP inhibitor (Table 1). Since MCF-7 cells show a very low level of endogenous TP expression (undetectable by Western blot analysis) [25], the data indicate that mycoplasma-encoded TP, expressed in the MCF-7.Hyor cell cultures, is responsible for the decreased antiviral activity of the drugs. In contrast, the activity of BVDU, an antiherpetic agent that is used to treat Varicella Zoster virus infections and known to be an excellent substrate for both human and *E. coli*-derived TP [26–28], was not compromised in the mycoplasma-infected cell cultures. Instead, BVDU became 5- and 3-fold more inhibitory against VV and HSV-2 respectively in mycoplasma-infected cell cultures, and this increased activity was lost upon addition of TPI.

We also investigated the cytostatic activity of fluoropyrimidines in mycoplasma-infected and -free cell cultures. We previously demonstrated that the decreased cytostatic activity of halogenated dThd analogues could be rescued by elimination of the mycoplasmas using an antibiotic or by the addition of TPI to

**Table 1** Inhibitory activity of dThd analogues against viral infection in MCF-7 and MCF-7.Hyor cellsResults are the means  $\pm$  S.D. of two independent experiments.

## (a) VV infection

Compound	$EC_{50}$ ( $\mu$ M)			
	MCF-7		MCF-7.Hyor	
	Alone	+ TPI	Alone	+ TPI
5-Chloro-dUrd	2.8 $\pm$ 1.1	2.2 $\pm$ 0.7	47.6 $\pm$ 4.0	1.6 $\pm$ 0.7
5-Bromo-dUrd	1.6 $\pm$ 0.3	1.6 $\pm$ 0.2	48.5 $\pm$ 3.3	1.9 $\pm$ 0.1
5-Iodo-dUrd	1.8 $\pm$ 0.6	2.9 $\pm$ 1.1	$\geq$ 100	2.7 $\pm$ 1.2
BVDU	47.4 $\pm$ 3.7	$\geq$ 44.7	10.2 $\pm$ 1.4	$\geq$ 100

## (b) HSV-1 (KOS) infection

Compound	$EC_{50}$ ( $\mu$ M)			
	MCF-7		MCF-7.Hyor	
	Alone	+ TPI	Alone	+ TPI
5-Chloro-dUrd	7.7 $\pm$ 3.2	8.2 $\pm$ 1.7	44.7 $\pm$ 0	3.5 $\pm$ 1.1
5-Bromo-dUrd	1.5 $\pm$ 0.6	1.5 $\pm$ 0.6	44.7 $\pm$ 0	1.1 $\pm$ 0.4
5-Iodo-dUrd	1.8 $\pm$ 0.5	1.9 $\pm$ 0.1	47.6 $\pm$ 4.0	1.3 $\pm$ 0.2
BVDU	0.04 $\pm$ 0.02	0.02 $\pm$ 0.006	0.04 $\pm$ 0.03	0.02 $\pm$ 0.01

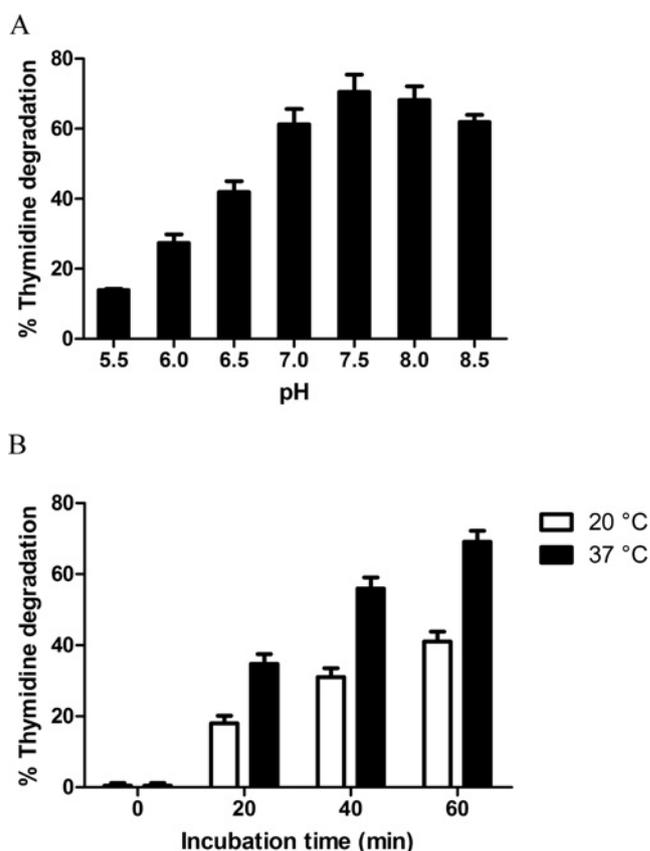
## (c) HSV-2 (G) infection

Compound	$EC_{50}$ ( $\mu$ M)			
	MCF-7		MCF-7.Hyor	
	Alone	+ TPI	Alone	+ TPI
5-Chloro-dUrd	8.7 $\pm$ 1.4	8.5 $\pm$ 1.9	51.0 $\pm$ 5.7	1.6 $\pm$ 0.7
5-Bromo-dUrd	2.0 $\pm$ 0	1.9 $\pm$ 0.1	46.6 $\pm$ 3.3	1.9 $\pm$ 0.1
5-Iodo-dUrd	2.1 $\pm$ 0.2	2.0 $\pm$ 0	63.1 $\pm$ 31.9	2.0 $\pm$ 0.3
BVDU	1.5 $\pm$ 0.6	5.0 $\pm$ 1.7	0.5 $\pm$ 0.3	4.0 $\pm$ 0

the infected cell cultures [3,6]. The antiproliferative activity of 5-fluorouridine, being a poor substrate for human- or *E. coli*-derived TP and human UPP1 (results not shown), was decreased by 20-fold in mycoplasma-infected MCF-7.Hyor cells ( $IC_{50} = 0.226 \pm 0.126 \mu$ M) when compared with control cells ( $IC_{50} = 0.011 \pm 0.003 \mu$ M). However, the cytostatic activity of this compound could also be fully restored upon administration of TPI (10  $\mu$ M), suggesting that mycoplasma-encoded TP has unique characteristics, distinct from its mammalian and *E. coli* counterparts, and would deserve further investigation and kinetic characterization.

**Determination of substrate specificity of TP<sub>Hyor</sub>**Substrate specificity of TP<sub>Hyor</sub> for natural pyrimidine nucleosides

The *M. hyorhinis* gene responsible for the TP activity was cloned and the enzyme was expressed and purified as described (see the Experimental section). The phosphorolysis of dThd catalysed by the purified TP<sub>Hyor</sub> was found to be optimal at pH 7.5 (Figure 1A) and at a temperature of 37 °C (Figure 1B). To determine the substrate specificity of TP<sub>Hyor</sub> towards natural pyrimidine nucleosides, different nucleosides were exposed to the enzyme and phosphorolysis was monitored. TP<sub>Hyor</sub> catalyses the conversion of dThd and dUrd, but also, surprisingly, of Urd into their respective bases (Thy and Ura) and phosphorylated sugars (2-dRib-1-P and Rib-1-P). Neither cytidine nor 2'-deoxycytidine were substrates for the enzyme (Table 2). The Michaelis constant ( $K_m$ ) and the turnover number ( $k_{cat}$ ) were determined for different substrates using non-linear regression

**Figure 1** pH and temperature dependence of TP<sub>Hyor</sub>-mediated dThd phosphorolysis

(A) pH-dependent dThd (100  $\mu$ M) degradation after a 60 min incubation in the presence of TP<sub>Hyor</sub>. (B) Temperature-dependent dThd (100  $\mu$ M) degradation after a 60 min incubation in the presence of TP<sub>Hyor</sub>. Results are the means  $\pm$  S.D. of two independent experiments.

**Table 2** Substrate specificity of TP<sub>Hyor</sub> for pyrimidine nucleosides and nucleoside analogues

Substrate for TP <sub>Hyor</sub>	No substrate for TP <sub>Hyor</sub>
Natural pyrimidine nucleosides	
dThd	Cytidine
dUrd	Deoxycytidine
Urd	
Pyrimidine nucleoside analogues	
5-Trifluoromethyl-dUrd	2',2'-Difluoro-2'-deoxycytidine
5-Fluoro-dUrd	2',2'-Difluoro-2'-deoxyuridine
5-Chloro-dUrd	Ara-U
5-Bromo-dUrd	Ara-T
5-Iodo-dUrd	BVDU*
5-Fluoro-Urd	AZT
5-Iodo-Urd	D4T
	6-Azauridine

\*Very poor phosphorolysis of BVDU was observed at the highest enzyme concentration.

analysis and are displayed in Table 3. The specificity constant ( $k_{cat}/K_m$ ) was calculated as an estimate for the catalytic efficiency of the enzyme. Remarkably, the TP<sub>Hyor</sub>-mediated phosphorolysis of Urd ( $K_m = 92 \mu$ M,  $k_{cat}/K_m = 0.092$ ) was found to be almost twice as efficient when compared with dThd ( $K_m = 473 \mu$ M,  $k_{cat}/K_m = 0.046$ ) and dUrd ( $K_m = 578 \mu$ M,  $k_{cat}/K_m = 0.043$ ).

**Table 3** Kinetic parameters of TP<sub>H<sub>yor</sub></sub>

The  $K_m$  and  $k_{cat}$  values for the natural substrates ( $\pm$  S.E.M.) were computationally determined using non-linear regression analysis (using GraphPad Prism 5) from data obtained in four (dThd and dUrd) or three (Urd) independent experiments. The kinetic parameters for the other compounds were derived from data obtained in two independent experiments.

Substrate	$K_m$ ( $\mu$ M)	$k_{cat}$ ( $s^{-1}$ )	$k_{cat}/K_m$ [( $s \cdot \mu$ M) $^{-1}$ ]
Natural substrates			
dThd	473 $\pm$ 25	21.6 $\pm$ 0.5	0.046
dUrd	578 $\pm$ 29	24.6 $\pm$ 0.5	0.043
Urd	92 $\pm$ 8	8.5 $\pm$ 0.2	0.092
Nucleoside analogues			
5-Fluoro-dUrd	169 $\pm$ 9	11.9 $\pm$ 0.2	0.070
5-Iodo-dUrd	144 $\pm$ 25	9.5 $\pm$ 0.6	0.066
5-Fluoro-Urd	47 $\pm$ 4	6.1 $\pm$ 0.1	0.130
5-Iodo-Urd	69 $\pm$ 7	6.4 $\pm$ 0.2	0.093
P <sub>i</sub>			
Co-substrate dThd	797 $\pm$ 107	17.5 $\pm$ 0.6	0.022
Co-substrate Urd	388 $\pm$ 43	8.6 $\pm$ 0.2	0.022

When dThd or Urd were added at fixed saturating substrate concentrations and the inorganic phosphate concentration was varied in the reaction mixture, an identical phosphorolytic efficacy ( $k_{cat}/K_m = 0.022$ ) was found for inorganic phosphate in the presence of either nucleoside substrate (Table 3).

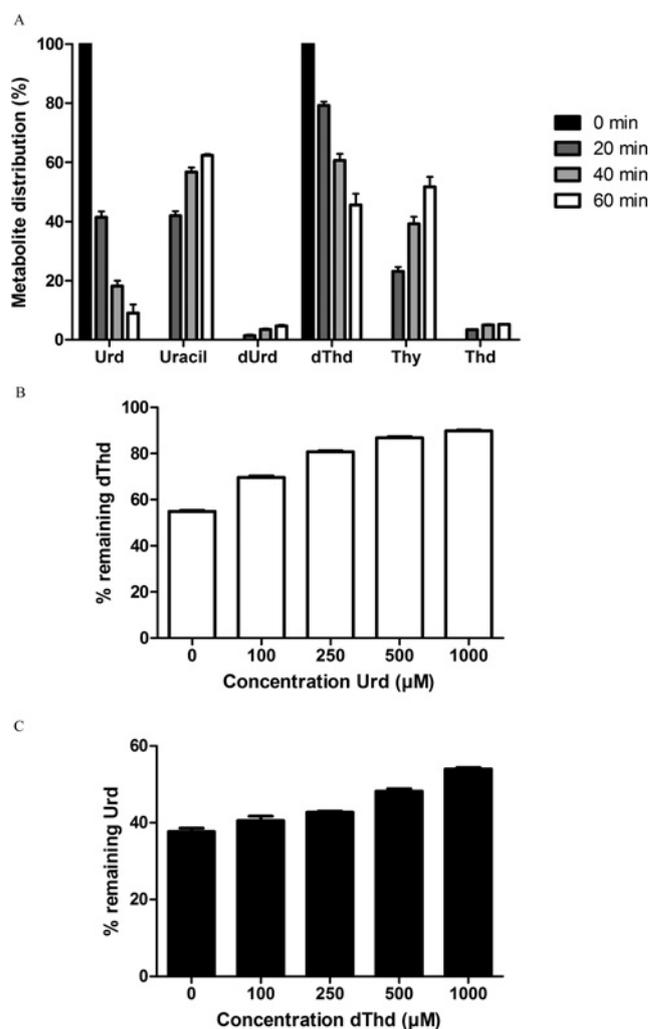
#### Substrate specificity of TP<sub>H<sub>yor</sub></sub> for nucleoside analogues

Next, the substrate specificity and kinetic parameters for a variety of antiviral and antitumour nucleoside analogues were determined (Tables 2 and 3). TFT (5-trifluoromethyl-dUrd) and the 5-halogenated dThd and Urd analogues were found to be efficient substrates for TP<sub>H<sub>yor</sub></sub>-mediated phosphorolysis. In contrast, BVDU, an excellent substrate for human and *E. coli* TP, was only poorly recognized by the mycoplasma-derived enzyme (<5% BVU formation from BVDU compared with ~66% Thy formation from dThd after a 10 min incubation under identical reaction conditions). Also ara-T (Thy arabinoside) and ara-U (Ura arabinoside), and the anti-HIV agents AZT (azidothymidine/zidovudine) and D4T (stavudine) which are not a substrate for human and *E. coli* TP, were not converted by TP<sub>H<sub>yor</sub></sub>. Whereas the glycosidic bond of the riboside analogue 5-fluoro-Urd was efficiently cleaved by this enzyme, the cytostatic antimetabolite drug 6-azauridine was not susceptible to phosphorolysis by TP<sub>H<sub>yor</sub></sub>. As expected, the difluorinated cytidine analogue gemcitabine (2',2'-difluoro-2'-deoxycytidine) as well as its deaminated metabolite 2',2'-difluoro-2'-deoxyuridine were not cleaved either (Table 2).

Overall, the 5-halogenated thymidine analogues 5-fluoro-dUrd (floxuridine) and 5-iodo-dUrd (idoxuridine), which represent clinically approved drugs for the treatment of cancer and herpesvirus infections respectively, were found to be better substrates for TP<sub>H<sub>yor</sub></sub> than dThd. Likewise, TP<sub>H<sub>yor</sub></sub>-catalysed phosphorolysis of the Urd analogues 5-fluoro-Urd and 5-iodo-Urd was found to proceed more efficiently when compared with Urd. In general, Urd and its analogues were found to be preferred substrates over dThd and its analogues (Table 3).

#### dThd and Urd compete for phosphorolysis by TP<sub>H<sub>yor</sub></sub>

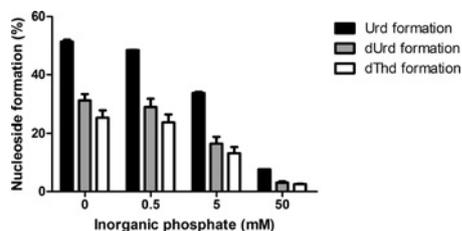
Since the enzymatic parameters demonstrated a more efficient TP<sub>H<sub>yor</sub></sub>-mediated phosphorolysis of Urd when compared with dThd, we next investigated whether both natural substrates are

**Figure 2** Competition for TP<sub>H<sub>yor</sub></sub>-mediated phosphorolysis

(A) TP<sub>H<sub>yor</sub></sub>-mediated metabolite formation from an equimolar mixture of Urd and dThd. (B) TP<sub>H<sub>yor</sub></sub>-mediated phosphorolysis of a fixed dThd (100  $\mu$ M) concentration in the presence of varying Urd concentrations after a 30 min incubation. (C) TP<sub>H<sub>yor</sub></sub>-mediated phosphorolysis of a fixed Urd (100  $\mu$ M) concentration in the presence of varying dThd concentrations after a 30 min incubation. Results are the means  $\pm$  S.D. of two independent experiments.

mutually exclusive or can be concomitantly used as a substrate by the enzyme. In a first set of experiments, dThd and Urd were mixed at equimolar concentrations (100  $\mu$ M) and simultaneously incubated with the enzyme. Metabolite formation was then monitored as a function of time. As shown in Figure 2(A), both dThd and Urd were time-dependently converted into their respective base with Urd being more efficiently cleaved than dThd. After a 60 min concomitant incubation of both substrates with the enzyme, ~90% of the supplied Urd and only ~50% of the supplied dThd were converted. In addition, Ura and Thy were found to be coupled again with the (deoxy)ribose moieties (derived from Urd and dThd) in an anabolic reaction mediated by TP<sub>H<sub>yor</sub></sub>, resulting in the formation of 2'-deoxyuridine (2-dRib-1-P and Ura) and thymine riboside (Rib-1-P and Thy).

In a second set of experiments, a fixed concentration of dThd (100  $\mu$ M) was incubated for 30 min with the enzyme in the presence of different Urd concentrations. In analogy, 100  $\mu$ M Urd was incubated in the presence of different dThd concentrations for 30 min. Urd dose-dependently decreased the phosphorolysis of dThd (Figure 2B). This inhibition occurred more efficiently



**Figure 3** Anabolic activity of TP<sub>Hyor</sub>

TP<sub>Hyor</sub>-mediated Urd formation from uracil (100 μM) and Rib-1-P (1 mM); dUrd formation from uracil (100 μM) and 2-dRib-1-P (1 mM); and dThd formation from thymine (100 μM) and 2-dRib-1-P (1 mM) in the presence of varying P<sub>i</sub> concentrations after a 20 min incubation at 37 °C. Results are the means ± S.D. of two independent experiments.

than the decreased Urd phosphorolysis in the presence of dThd (Figure 2C). Taken together, these results strongly indicate that dThd and Urd compete for the same substrate-binding site.

#### Urd formation is superior to dThd formation in the anabolic direction of the TP<sub>Hyor</sub> reaction

The TP<sub>Hyor</sub>-mediated coupling of Ura to Rib-1-P and Ura and Thy to 2-dRib-1-P was studied. Urd was more efficiently formed than dUrd and dThd (Figure 3). These findings are in line with the enzymatic parameters determined for the individual substrates and the results obtained in the competition experiments (see above). Nucleoside formation was also found to be highly dependent on the concentration of P<sub>i</sub> present in the reaction mixture, i.e. increasing concentrations of P<sub>i</sub> dose-dependently inhibit the TP-catalysed nucleoside formation (Figure 3).

#### Differential inhibition of TP<sub>Hyor</sub> by known specific TP and UP inhibitors

The human TP inhibitors TPI and 7-DX were examined for their capacity to inhibit the phosphorolysis of dThd (catalysed by TP<sub>Hyor</sub>, human TP or *E. coli* TP) and Urd (catalysed by TP<sub>Hyor</sub> or UPP1). As shown in Table 4, TPI inhibits the phosphorolysis of dThd catalysed by TP of either mycoplasma, human or *E. coli* origin in the lower nanomolar range (IC<sub>50</sub> = 3 nM, 7 nM and 7 nM respectively). TPI also efficiently inhibits the phosphorolysis of Urd catalysed by TP<sub>Hyor</sub> (IC<sub>50</sub> = 5 nM), but does not inhibit the phosphorolysis of Urd by UPP1 (IC<sub>50</sub> ≥ 500 μM) (Table 4). The purine-derived TP inhibitor 7-DX was found to inhibit TP<sub>Hyor</sub>-mediated dThd and Urd phosphorolysis in the micromolar range (IC<sub>50</sub> = 60 μM and 30 μM respectively). A similar inhibitory activity of 7-DX was found against human- and *E. coli*-derived TP (IC<sub>50</sub> = 151 μM and 108 μM respectively, but a 10-fold decreased activity was observed towards UPP1-mediated Urd phosphorolysis (IC<sub>50</sub> = 300 μM) when compared with TP<sub>Hyor</sub>. The well-known UP inhibitor BAU was found to inhibit only the phosphorolysis of Urd catalysed by UPP1 (IC<sub>50</sub> = 0.89 μM), but affected neither the efficiency of mycoplasma-, human- or *E. coli*-catalysed dThd phosphorolysis (IC<sub>50</sub> > 500 μM) nor the phosphorolysis of Urd by TP<sub>Hyor</sub>. These results indicate that even though TP<sub>Hyor</sub> preferably shows Urd phosphorylase activity, the enzyme behaves catalytically more similar to the TPs.

#### Computer-assisted molecular modelling

The complete *M. hyorhinis* genome was recently published for two different strains (strain HUB-1 isolated from swine

**Table 4** Inhibition of different phosphorolytic enzymes by inhibitors of human TP and UP

Results are the means ± S.D. of two independent experiments.

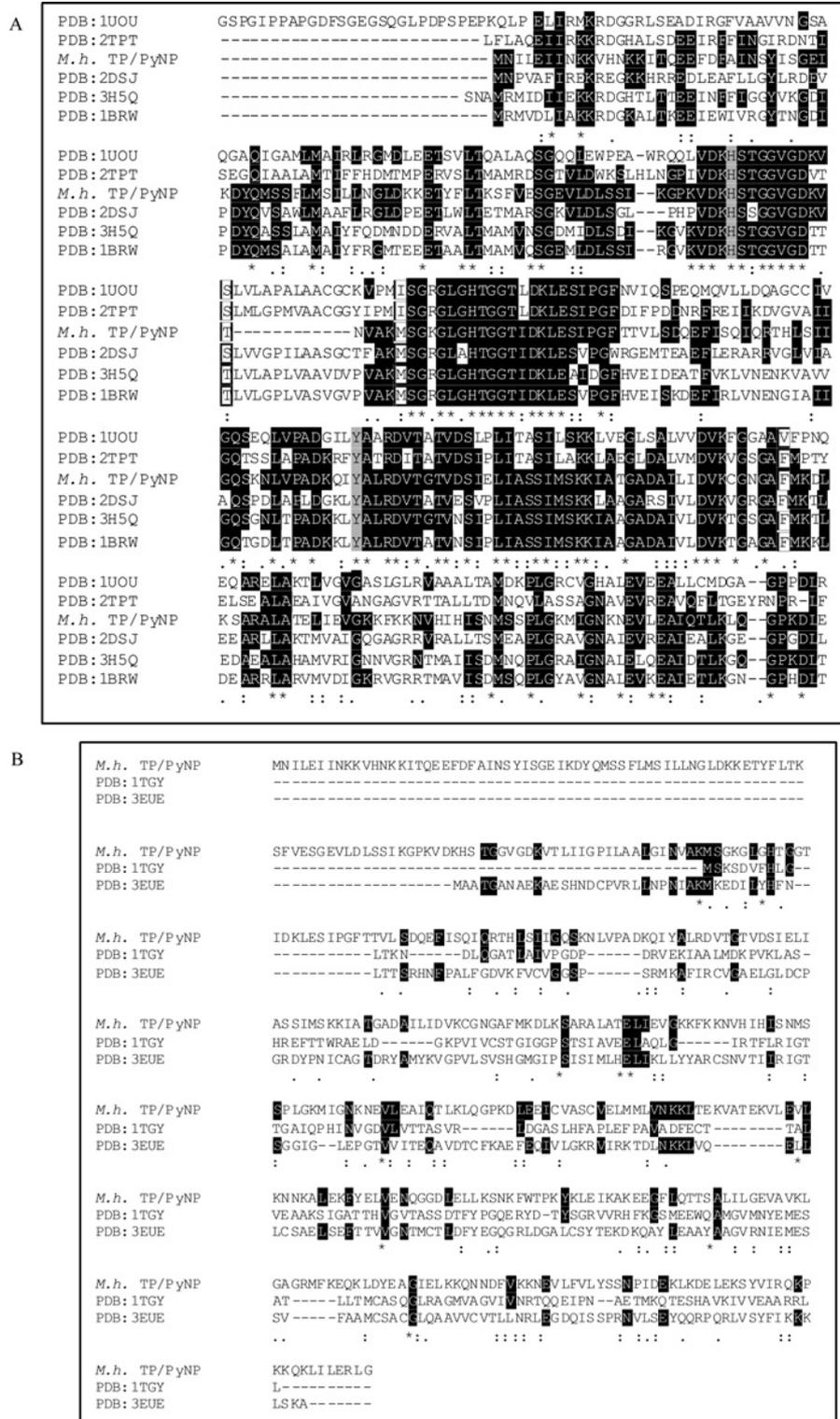
Inhibitor	IC <sub>50</sub> (μM)				
	Substrate = dThd			Substrate = Urd	
	TP <sub>Hyor</sub>	Human TP	<i>E. coli</i> TP	TP <sub>Hyor</sub>	UPP1
TPI	0.003 ± 0.001	0.007 ± 0.001	0.007 ± 0.001	0.005 ± 0.0007	≥ 500
7-DX	60 ± 5	151 ± 33	108 ± 0.7	30 ± 3	301 ± 71
BAU	> 500	> 500	> 500	> 500	0.89 ± 0.02

respiratory tract [22] and strain MCLD derived from a primary human melanoma cell culture [29]). The protein sequence of TP was found to be highly conserved in both *M. hyorhinis* strains (identical sequence, with the exception of Ser<sup>240</sup> in HUB-1 compared with Phe<sup>240</sup> in MCLD). In contrast, no putative UP gene was annotated in these mycoplasma strains.

A multiple sequence alignment of TP<sub>Hyor</sub> (Figure 4A) with similar enzymes whose three-dimensional structures have been solved and are deposited in the PDB revealed that TP<sub>Hyor</sub> has sequence identities of 35 % and 40 % respectively, with *E. coli* (over 440 amino acids) and human TP (over 273 amino acids) and of 46 % and 42 % respectively, with the PyNP of *Geobacillus stearothermophilus* (over 433 amino acids) and *Thermus thermophilus* (over 423 amino acids). In all of these enzymes the residues making up the phosphate-binding site and the thymidine-binding site are highly conserved. For this reason our homology-built models of TP<sub>Hyor</sub> (Supplementary Figure S2A at <http://www.BiochemJ.org/bj/445/bj4450113add.htm>), which basically differ in the ligand-dependent degree of closure of the active site, are considered to be very reliable (the Ramachandran Z-score of -1.87 obtained from WhatCheck [30] means that phi and psi angles for all residues are within the expected ranges for well refined structures). In contrast, a multiple sequence alignment of TP<sub>Hyor</sub> with human UPP1 and *E. coli* UP (Figure 4B) showed much lower sequence similarity between TP<sub>Hyor</sub> and uridine phosphorylases (pairwise alignment score of 6 % and 3 % respectively), whereas human UPP1 and *E. coli* UP sequences show a similarity score of 21 %.

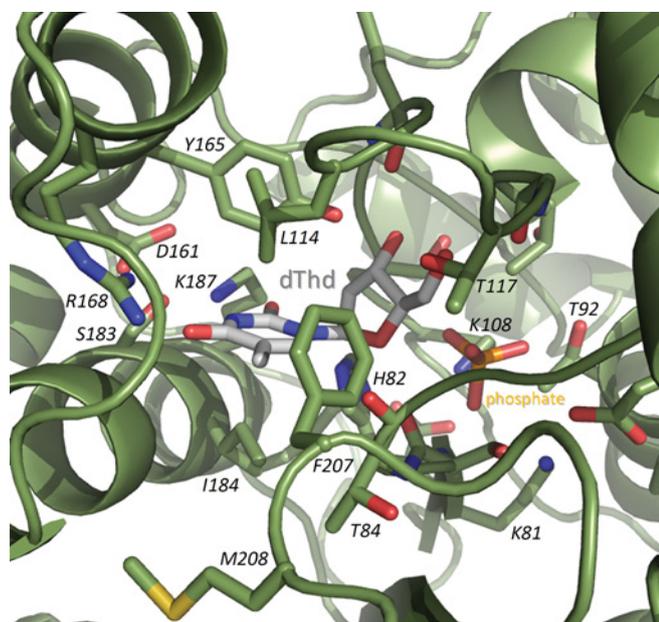
The almost identical amino acid composition and putative architecture of the active site of TP<sub>Hyor</sub> with respect to human TP (as shown in Supplementary Figure S3 at <http://www.BiochemJ.org/bj/445/bj4450113add.htm>) can account for the comparable inhibition of these two enzymes by TPI. In fact, the Arg<sup>202</sup>, Ser<sup>217</sup> and Lys<sup>221</sup> side chains that hydrogen bond to the uracil base of TPI in its crystallographic complex with human TP [31], as well as the hydrophobic side chains of Leu<sup>148</sup> and Ile<sup>218</sup> that stack against the nucleobase ring, are also present in TP<sub>Hyor</sub> (Arg<sup>168</sup>, Ser<sup>183</sup>, Lys<sup>187</sup>, Leu<sup>114</sup> and Ile<sup>184</sup> respectively) (Supplementary Figure S2B). The same can be said about the catalytic His<sup>116</sup> in human TP and His<sup>82</sup> in TP<sub>Hyor</sub>, which are positionally and functionally equivalent to His<sup>85</sup> in *E. coli* TP [32]. These marked similarities, however, cannot explain why TP<sub>Hyor</sub> recognizes Urd as a better substrate than dThd, given that Urd is a very poor substrate, if at all, for human TP.

Interestingly, Lys<sup>108</sup> in the phosphate-binding pocket of TP<sub>Hyor</sub> (Figure 5) is positionally equivalent to Lys<sup>108</sup> in *G. stearothermophilus* PyNP (PDB code 1BRW), Lys<sup>108</sup> in *Staphylococcus aureus* PyNP (PDB code 3H5Q) and Lys<sup>107</sup> in *T. thermophilus* (PDB code 2DSJ), but is replaced by Met<sup>142</sup> and Met<sup>111</sup> respectively, in human (PDB codes 1UOU and 2WK6) and *E. coli* (PDB codes 2TPT and 1AZY) TP. Likewise, the



**Figure 4** Multiple sequence alignment of TP<sub>Hyor</sub> with PDB entries

(A) TP from *E. coli* (PDB code 2TPT) and a human source (PDB code 1UOU) and PyNP from *T. thermophilus* (PDB code 2DSJ), *S. aureus* (PDB code 3H5Q) and *G. stearothermophilus* (PDB code 1BRW). Important catalytic residues are formatted in grey and the most significant differences in the substrate-binding site are boxed. (B) UP from *E. coli* (PDB code 1TGY) and UPP1 (PDB code 3EUE). Black boxes indicate conserved amino acid residues compared with TP<sub>Hyor</sub>. The symbols underneath each block stand for identical amino acids (\*), conserved substitutions (:), and semi-conserved substitutions (.).



**Figure 5** Model of the active site of TP<sub>Hyor</sub>

Detail of the active site of the homology-built model of TP<sub>Hyor</sub> showing the putative positions for the nucleoside substrate (dThd) and the attacking phosphate.

position of the neighbouring Thr<sup>92</sup> in TP<sub>Hyor</sub> is occupied in the latter two enzymes by a serine (Ser<sup>126</sup> in human TP and Ser<sup>95</sup> in *E. coli* TP). It is therefore likely that the nature of the amino acid at these two positions has an influence on transition state formation and/or stabilization of the enzyme–substrate complex during catalysis.

For BAU, there are two X-ray crystal structures available for its complex with *E. coli* UP (PDB code 1U1C) and UPP1 (PDB code 3EUF). The active sites of both enzymes are at the interface between two monomers that, in turn, are part of the ‘trimer of dimers’ that is seen in the crystal structure of the bacterial enzyme. However, many of the residues of *E. coli* UP and UPP1 that interact with BAU are different in TP<sub>Hyor</sub>, and explain why BAU selectively inhibits the *E. coli* UP and UPP1, but not the UP activity of TP<sub>Hyor</sub>. The fact that these two UPs are completely different enzymes from a structural point of view, containing different active site topologies to the TP enzymes, is in agreement with our kinetic observations.

## DISCUSSION

To the best of our knowledge the gene annotated as TP<sub>Hyor</sub> is the first mycoplasma-encoded phosphorylase to be cloned, expressed and characterized. We provide evidence that the presence of TP<sub>Hyor</sub> in cell cultures due to a mycoplasma infection negatively affects the efficiency of nucleoside analogues used in the treatment of virus infections and cancer. The enzyme shows an unusual substrate selectivity since it catalyses not only the phosphorylation of dThd and dUrd, but also Urd, which surprisingly turned out to be the preferred substrate. Thus, although the amino acid alignment of TP<sub>Hyor</sub> with human TP, *E. coli* TP, UPP1 and *E. coli* UP revealed a markedly higher similarity of TP<sub>Hyor</sub> with TP than with UP, the enzyme shows a superior (~2-fold) UP catalytic activity. Extensively studied TP enzymes that display, besides TP activity, also UP activity (i.e. TP from human liver, human placenta and mouse liver) have a much lower (~90–200-fold) UP than TP activity, and vice versa, those UP enzymes that

also possess TP activity display a much lower (~25–160-fold) associated TP than UP activity [33].

However, early studies on pyrimidine nucleoside phosphorylases isolated from e.g. *G. stearotherophilus* and *Haemophilus influenzae* reported comparable TP and UP activities [34,35], as now also shown for TP<sub>Hyor</sub>. Such prokaryotic enzymes indeed do not discriminate at the 2'-position of the ribose, and are considered as a separate and well-defined class of pyrimidine nucleoside cleaving enzymes, referred to as PyNP (EC. 2.4.2.2) and ranked within the NP-II family of phosphorylases. A similar observation was made for the UP/TP activity of the parasite *Giardia lamblia* [36]. PyNP and TP are known to display significant sequence similarity and similar physical properties [19]. X-ray crystallography revealed indeed that the three-dimensional structures of *G. stearotherophilus* PyNP and *E. coli* TP are very similar [37,38].

From our model of TP<sub>Hyor</sub> it is apparent that this enzyme is structurally related to the PyNP subfamily (NP-II) rather than to the UP subfamily (NP-I) of nucleoside phosphorylases (to which human and *E. coli* UP belong) [19] (Figure 4). We therefore believe that TP<sub>Hyor</sub>, due to both its structural nature and catalytic properties, should be annotated as a NP-II class PyNP to distinguish it from the NP-II class TP and NP-I class UP enzymes.

The data of the present study indicate that one and the same active site in TP<sub>Hyor</sub>/PyNP is responsible for the phosphorylation of both dThd and Urd, which is in agreement with the PyNP properties of the enzyme: (i) increasing dThd concentrations decrease Urd phosphorylase activity and vice versa; (ii) an equimolar mixture of dThd and Urd exposed to TP<sub>Hyor</sub>/PyNP results in the concomitant formation of dUrd (formed from Urd-derived Ura and dThd-derived 2-dRib-1-P) and the thymine riboside (formed from Urd-derived Rib-1-P and dThd-derived thymine); and (iii) the specific TP inhibitor TPI, being inactive against UPP1, is equally effective in inhibiting TP<sub>Hyor</sub>/PyNP-catalysed phosphorylation of dThd and Urd.

Inhibitor studies also reveal that, despite its efficient multifunctional activity on dThd/Urd substrates, the nature of TP<sub>Hyor</sub>/PyNP is much more similar to other TP and PyNP than to UP. TPI is the most potent and selective transition state inhibitor of human TP reported so far. It is currently the subject of clinical trials in combination with TFT to prevent premature breakdown of this anticancer drug to its inactive base [39–41]. We found that TPI is an equally highly potent inhibitor of *E. coli* TP, human TP and TP<sub>Hyor</sub>/PyNP that does not inhibit human UPP1. However, by blocking the TP<sub>Hyor</sub>/PyNP enzyme activity, TPI also annihilates the concomitant UP activity of TP<sub>Hyor</sub>/PyNP. Conversely, BAU, a well-known UP inhibitor that selectively inhibits UPP1 and *E. coli* UP without affecting TP activity [42,43], did not abrogate the UP (and TP) activity of TP<sub>Hyor</sub>/PyNP and thus discriminates between the mycoplasma UP activity of TP<sub>Hyor</sub>/PyNP and the human (and *E. coli*) UP activity.

Although TP<sub>Hyor</sub>/PyNP was expressed as a recombinant GST-fusion protein, we felt that tagging the nucleoside phosphorylase does not compromise its phosphorylase activity. Indeed, the specificity constants ( $k_{cat}/K_m$ ) of dThd phosphorylation by both human TP and human TP-GST were determined using linear regression analysis and were found to be very similar (human TP,  $k_{cat}/K_m = 0.036$ ; human TP-GST,  $k_{cat}/K_m = 0.030$ ). Thus the presence of GST in human TP did not substantially affect the kinetic properties of the enzyme. The catalytic efficiency of GST-tagged TP<sub>Hyor</sub>/PyNP was found to be in the same range when compared with previously characterized orthologue NPs. The turnover number ( $k_{cat}$ ) of TP<sub>Hyor</sub>/PyNP was determined as 21.6, 24.6 and 8.5 s<sup>-1</sup> for dThd, dUrd and Urd respectively. This compares reasonably well with the turnover numbers of human TP

for dThd ( $k_{\text{cat}} = 9.4 \text{ s}^{-1}$ ) and for *H. influenzae* PyNP ( $k_{\text{cat}} = 11.1, 6.4$  and  $52.9 \text{ s}^{-1}$  for dThd, dUrd and Urd respectively) [35,44].

The kinetic characterization of TP<sub>H<sub>yor</sub></sub>/PyNP sheds new light on nucleotide metabolism in mycoplasmas. A potent phosphorolysis of Urd in extracts of mycoplasmas has been described previously [45,46]. Originally this activity was attributed to the assumed presence of mycoplasma-encoded UP. This UP activity (measured, for example, by the increased formation of [<sup>14</sup>C]Ura from [<sup>14</sup>C]Urd or the decreased incorporation of [<sup>3</sup>H]Urd in the RNA of infected cell cultures) was even proposed as a tool for the identification of mycoplasma infections in cells [47,48]. In contrast with the annotation of a TP and a purine nucleoside phosphorylase gene in the genome of different mycoplasma strains [22,49], no UP gene has been annotated in mycoplasmas to the best of our knowledge. This was also pointed out by Bizarro and Schuck [50] who, in the framework of an *in silico* study on the purine and pyrimidine nucleotide metabolism in Mollicutes, suggested a TP to be responsible for UP activity. Indeed, the results obtained in the present study indicate that not a UP, but instead a PyNP is responsible for the release of uracil from Urd in mycoplasma cultures. These findings are also in agreement with our data on the cytostatic activity of 5-fluoro-Urd that is markedly decreased in mycoplasma-infected cell cultures, but efficiently restored in the presence of TPI. The phosphorolytic activity of TP<sub>H<sub>yor</sub></sub>/PyNP against 5-fluoro-Urd could be efficiently blocked by TPI in a cell-free assay (results not shown) pointing again to the UP activity of TP<sub>H<sub>yor</sub></sub>/PyNP being responsible for 5-fluoro-Urd degradation. The occurrence of a multisubstrate enzyme in mycoplasmas fits the minimal cell and genome concept of mycoplasmas, being the smallest autonomously replicating bacteria, rarely exceeding a genome of 1 Mb [51–53].

Surprisingly, the antiherpetic drug BVDU, a well-known and excellent substrate for human and *E. coli* TP [26], was hardly acted upon by TP<sub>H<sub>yor</sub></sub>/PyNP. This finding is in agreement with our observation that mycoplasma infection does not compromise the biological (antiviral) activity of BVDU *in vitro*. Instead, the antiviral activity of BVDU against VV and HSV-2 was found to be slightly (3–5-fold) enhanced in the presence of TP<sub>H<sub>yor</sub></sub>/PyNP. This effect, which could be reversed by administration of the TP inhibitor TPI, may be explained by the TP<sub>H<sub>yor</sub></sub>/PyNP-mediated depletion of intracellular dThd pools, which most likely gives BVDU a competitive advantage for phosphorylation to its biologically active 5'-monophosphate metabolite in mycoplasma-infected cells. Our modelling studies with TP<sub>H<sub>yor</sub></sub>/PyNP provide a partial explanation for the unexpectedly different behaviour of BVDU as a potential substrate for TP<sub>H<sub>yor</sub></sub>/PyNP, human TP and *E. coli* TP in so far as the 2-bromovinyl group at the 5'-position of the uracil ring might be too bulky to fit into the active site of TP<sub>H<sub>yor</sub></sub>/PyNP due to the presence of the phenyl ring of Phe<sup>207</sup>, which is positionally equivalent to the smaller Val<sup>241</sup> in the human TP enzyme. However, the corresponding Phe<sup>210</sup> in the complex of *E. coli* TP with TPI has been shown to change its rotameric state relative to that found in other PyNPs [38]. On the other hand, TPI inhibits TP<sub>H<sub>yor</sub></sub>/PyNP as efficiently as it inhibits human TP despite the fact that it contains a 5-Cl substituent on the uracil base that is bioisosteric with the methyl group of thymine in dThd. The observation that BVDU is not a good substrate for TP<sub>H<sub>yor</sub></sub>/PyNP is important in view of the fact that BVU, the free base of this antiviral agent, is a potent inhibitor of DHP (dihydropyrimidine dehydrogenase) [54–56]. Inhibition of the latter enzyme prevents further catabolism of Ura and Thy and, more importantly, of the anticancer agent 5-FU (5-fluorouracil), leading to the accumulation of 5-FU and its concomitant life-threatening toxicity [57–59]. Accordingly, a mycoplasma infection will not affect DHP activity in cancer

patients treated with BVDU and 5-FU, and thus will not be expected to increase 5-FU toxicity.

BVDU became somewhat more active against VV and HSV-2, but not HSV-1, infection in the presence of mycoplasmas. This can be explained by the markedly different potencies of antiviral activity of BVDU against the particular viruses and/or different targets of inhibition by BVDU. Indeed, BVDU is far more inhibitory to HSV-1 ( $EC_{50} = 0.04 \mu\text{M}$ ) than to HSV-2 and VV ( $EC_{50} = 1.5$  and  $47 \mu\text{M}$  respectively). The molecular mechanisms of action of BVDU against HSV-1 are inhibition of HSV-1 DNA polymerase and incorporation into viral DNA after metabolic conversion into its 5'-mono- and 5'-di-phosphates by HSV-1 thymidine kinase, and to its 5'-triphosphate derivative by cellular enzymes. Instead both VV and HSV-2 encode a thymidine kinase that can only convert BVDU into its 5'-monophosphate derivative, which is known to inhibit thymidylate synthase [60,61]. Therefore it is well possible that thymidylate synthase is the molecular target of BVDU for HSV-2 and VV inhibition. In this case, the dTMP (and dTDP and dTTP) pools might be further decreased, resulting in a more favourable inhibition of the virus infection by the lack of sufficient dTTP for DNA synthesis in the presence of PyNP-expressing mycoplasmas.

The present study may have important implications for the treatment of cancer patients or patients suffering from viral infections with nucleoside analogues. Mycoplasmas are known to reside in the human body, causing asymptomatic infections. Also, it is widely accepted that particularly immunocompromised patients, such as those suffering from HIV infections, are prone to secondary infections by mycoplasmas, which are then also found in organs different from their usual habitat [7]. Additionally, an increasing number of studies report on the high preferential colonization of human tumours by mycoplasmas [10–17]. Taking these facts into consideration, our findings indicate that the treatment of patients with nucleoside analogues may be compromised by the expression of catabolic enzymes with broad substrate specificity, such as those encoded by mycoplasmas, and could be optimized by the co-administration of a mycoplasma-targeting antibiotic or specific enzyme inhibitors. The unique substrate specificity of TP<sub>H<sub>yor</sub></sub>/PyNP towards natural nucleosides and nucleoside analogues indicates that the design of such a specific mycoplasma PyNP inhibitor could be a feasible goal. We would suggest extending the structure of TPI to allow interaction with the phosphate-binding site in TP<sub>H<sub>yor</sub></sub>/PyNP where Lys<sup>108</sup> and Thr<sup>92</sup> are found instead of Met<sup>142</sup> and Ser<sup>126</sup> in human TP. These conserved amino acid differences may underlie the substrate preferences reported above and could be exploited for specific inhibitor design.

## AUTHOR CONTRIBUTION

Johan Vande Voorde participated in the design of the study, carried out the cell culture experiments, sequence alignments and enzyme experiments and participated in the writing of the paper; Federico Gago performed sequence alignments and modelling; Kristof Vrancken contributed to the cloning of the TP<sub>H<sub>yor</sub></sub>/PyNP gene and to the purification of the enzyme; Sandra Liekens and Jan Balzarini designed and supervised the study and participated in the writing of the paper. All of the authors read and approved the final paper prior to submission.

## ACKNOWLEDGEMENTS

We thank Christiane Callebaut, Eef Meyen, Kristien Minner, Leentje Persoons, Ria Van Berwaer and Peter Vervaeke for their excellent technical assistance, Dr Tarmo Roosild (Nevada Cancer Institute, Las Vegas, NV, U.S.A.) for generously providing human uridine phosphorylase and Professor Vern Schramm (Albert Einstein College of Medicine, New York, NY, U.S.A.) for kindly providing TPI.

## FUNDING

This work was supported by the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen) (to J.V.V.), the 'Fonds voor Wetenschappelijk Onderzoek (FWO) Vlaanderen' [grant number G.0486.08] and the K.U. Leuven [grant number PF 10/18].

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Received 23 December 2011/30 March 2012; accepted 4 April 2012

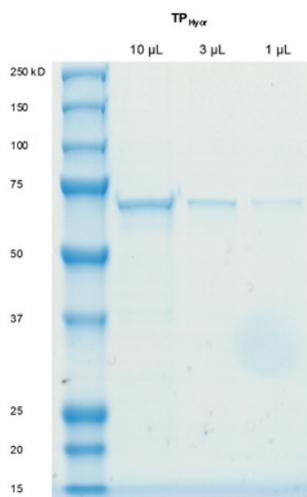
Published as BJ Immediate Publication 4 April 2012, doi:10.1042/BJ20112225

## SUPPLEMENTARY ONLINE DATA

# Characterization of pyrimidine nucleoside phosphorylase of *Mycoplasma hyorhinis*: implications for the clinical efficacy of nucleoside analogues

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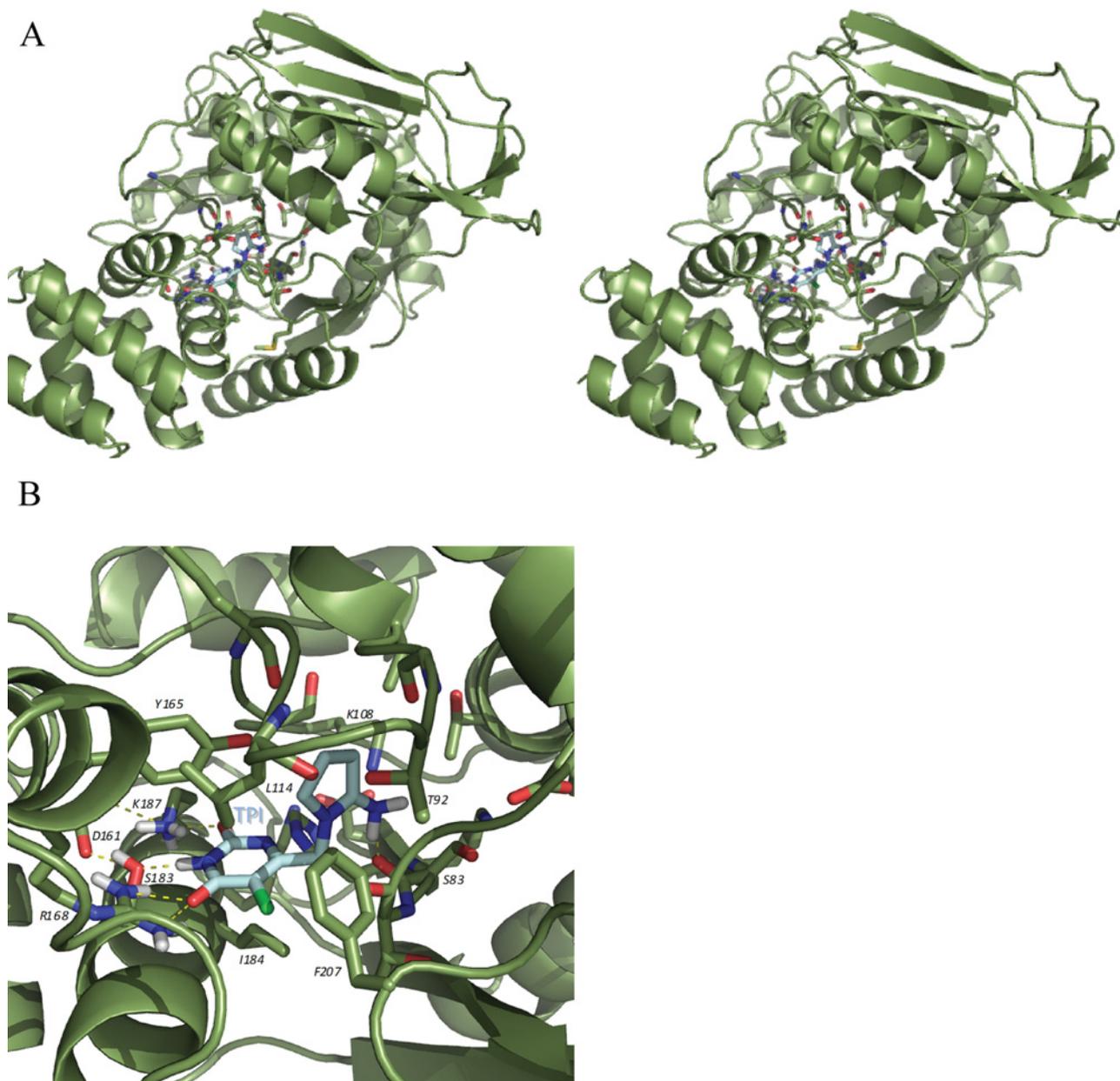
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**Figure S1** Purity evaluation of the *M. hyorhinis* TP–GST fusion protein

Three different volumes of the purified enzyme preparation (10  $\mu$ l, 3  $\mu$ l and 1  $\mu$ l; 0.17  $\mu$ g/ $\mu$ l) were analysed using SDS/PAGE to evaluate the purity of the sample. Proteins were stained using Bio-Safe™ Coomassie G-250 Stain (Bio-Rad Laboratories). Molecular mass is given on the left-hand side in kDa.

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**Figure S2 Homology model of TP<sub>Hyor</sub> in complex with TPI**

(A) Stereoview of a cartoon representation of TP<sub>Hyor</sub> (C atoms in dark green) in complex with TPI (C atoms in blue). (B) Detailed view of (A). The uracil base of TPI (C atoms, cyan; N atoms, blue; and O atoms, red) is sandwiched between the hydrophobic side chains of Leu<sup>114</sup> (above) and Ile<sup>184</sup> (below), and held in place by hydrogen bonds (yellow broken lines) to the side chains of Arg<sup>168</sup>, Ser<sup>183</sup> and Lys<sup>187</sup> (stabilized by the carboxylate of Asp<sup>161</sup>), whereas the amino group on the pyrrolidine ring forms a strong hydrogen bond with the backbone carbonyl of Ser<sup>83</sup>. Phe<sup>207</sup> appears in the foreground, just in front of the 5-chlorine substituent on the uracil that is isosteric with the methyl group of thymine in the dThd substrate (not shown).



**Figure S3** Sequence alignment and secondary structure prediction for TP<sub>Hyor</sub> using human TP as the template

The predicted (Phyre<sup>2</sup> server) secondary structure (green  $\alpha$ -helices and blue  $\beta$ -sheets) of TP<sub>Hyor</sub> closely resembles the known secondary structure of human TP.

Received 23 December 2011/30 March 2012; accepted 4 April 2012

Published as BJ Immediate Publication 4 April 2012, doi:10.1042/BJ20112225