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Thermus thermophilus Nucleoside Phosphorylases Active in the Synthesis of Nucleoside Analogues

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Cells extracts from *Thermus thermophilus* HB27 express phosphorolytic activities on purines and pyrimidine nucleosides. Five putative encoding genes were cloned and expressed in *Escherichia coli*, and the corresponding recombinant proteins were purified and studied. Two of these showed phosphorolytic activities against purine nucleosides, and third one showed phosphorolytic activity against pyrimidine nucleosides *in vitro*, and the three were named TtPNPI, TtPNPII, and TtPyNP, respectively. The optimal temperature for the activity of the three enzymes was beyond the water boiling point and could not be measured accurately, whereas all of them exhibited a wide plateau of optimal pHs that ranged from 5.0 to 7.0. Analytical ultracentrifugation experiments revealed that TtPNPI was a homohexamer, TtPNPII was a monomer, and TtPyNP was a homodimer. Kinetic constants were determined for the phosphorolysis of the natural substrates of each enzyme. Reaction tests with nucleoside analogues revealed critical positions in the nucleoside for its recognition. Activities with synthetic nucleobase analogues, such as 5-iodouracil or 2,6-diaminopurine, and arabinosides were detected, supporting that these enzymes could be applied for the synthesis of new nucleoside analogs with pharmacological activities.

Nucleoside phosphorylases (NPs) constitute a family of ubiquitous enzymes involved in the salvage pathway of nucleosides in prokaryotic and eukaryotic organisms, in which they catalyze the reversible phosphorolysis of nucleosides (Fig. 1A). They are generally classified attending to their substrate specificity as purine NPs (PNPs; EC 2.4.2.1) or pyrimidine NPs (PyNPs; EC 2.4.2.2). Other classifications regarding their molecular mass and structure have also been made (4), although several NPs have been reported to deviate from these proposed classes (6–8, 14). It is not possible to establish a clear relationship between a given sequence or structure and its nucleoside specificity.

Human NPs are therapeutic targets in tumoral cells, where they are overproduced. Treatment of these types of cancer involve strategies, such as the design of inhibitors of human NPs that hinder the ability of tumoral cells to replicate their DNA by limiting the available pool of nucleotides, and the administration of nucleobase analogues, which are innocuous when conjugated with a pentose but cytotoxic once phosphorolyzed by an NP (20, 21, 29). Aside from these applications, there are a number of other examples of the use of nucleoside analogues with antirheumatic, antiviral, or antimicrobial properties (27, 34).

The synthesis of nucleoside analogues can be achieved by either chemical or enzymatic methods or by a combination of both (33). The enzymatic methods have several advantages, such as a high regio- and stereoselectivity, mild reaction conditions, and limited use of contaminant reagents and organic solvents. NPs are one of the most widely spread tools for the synthesis of these compounds since they catalyze the specific combination of nucleobases and sugars (5, 17, 22).

To date, NPs from different mesophiles, such as *Escherichia coli* (15–16, 31) or *Bacillus subtilis* (5), have been used in the synthesis of pharmacologically active compounds (16, 31). However, mesophilic enzymes exhibit a limited ability to withstand aggressive conditions with respect to pH, organic solvents, or elevated temperatures, which are frequently required to reduce the viscosity of the medium or to increase the solubility and concentration of some substrates (32). In these cases, enzymes from thermophilic microorganisms (or thermozymes) are ideal substitutes for mesophilic enzymes. The adaptive features found in thermozymes, such as a highly polar surface, a more compact hydrophobic core, and a reduced number of cavities and external loops, correlate not only with an increased thermostability but also with a wider tolerance to other stresses, such as low pH, organic solvents, and denaturing agents. Another important side effect of their natural thermostability of interest in an industrial process is that many of them are easily purified from the soluble fraction after a heat shock treatment that denatures most of the host proteins of the mesophilic host where they are overproduced. To date, thermophilic NPs from the moderate thermophile *Geobacillus stearothermophilus* are the only examples that have been applied in the synthesis of nucleosides (11, 30).

We reported previously the existence of nucleoside synthase activities in the whole cells of several *T. thermophilus* strains, including the sequenced strain HB27 (2). Here, we search for the genes encoding these activities in the genome of *T. thermophilus* HB27 (9) and identify three NP activities with a complementary set of specificities for substrates. We show that these enzymes are active in the synthesis of both natural and synthetic nucleosides at high temperatures.

MATERIALS AND METHODS

Strains and culture media. *T. thermophilus* HB27 was used as a source of genes encoding putative NPs (9). The cells were cultured in TB medium (0.8% [wt/vol] peptone, 0.4% [wt/vol] yeast extract, 0.3% [wt/vol] NaCl

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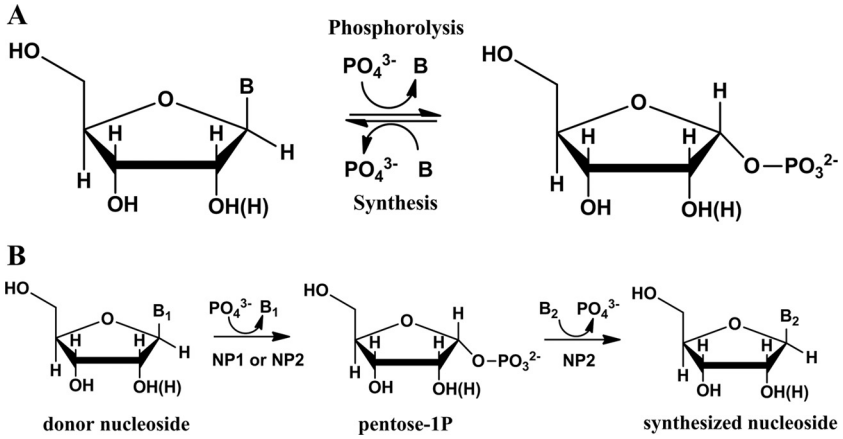

FIG 1 Scheme of the reaction catalyzed by nucleoside phosphorylases. (A) Phosphorolytic and the synthetic pathways. (B) Synthesis of nucleosides by coupled NP activity reactions. A donor nucleoside is cleaved by an NP, yielding a free nucleobase (B_1) and a pentose-1-P. The pentose is then used in a new reaction by either the same or a different NP, along with an acceptor nucleobase (B_2) to synthesize a new nucleoside.

[pH 7.5]) at 65°C with stirring (150 rpm). *E. coli* strains DH5α [*supE44* Δ(*lacZYA-argF*)*U169* (ϕ80d*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thiI relA1*] and BL21(DE3) [*hsdS gal*(ΔcI*ts*857 *ind-1 Sam7 nin-5 lacUV5*-T7 gene 1)] were used for gene cloning and protein expression, respectively. Both strains were grown on Luria-Bertani medium (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 0.5% [wt/vol] NaCl [pH 7.0]) at 37°C with stirring (150 rpm).

Identification of genes encoding putative NPs. The genome of *T. thermophilus* HB27 was examined for putative NPs using the KEGG server. Two putative PNPs with the *T. thermophilus* HB27 locus IDs TTC0194 (UniProt Q72L69) and TTC1070 (UniProt Q72IR2) and a putative thymidine phosphorylase with the locus ID TTC1412 (UniProt Q72HS4) were identified. In addition, a search for paralogues highlighted 11 additional genes, whose products were analyzed by multiple sequence alignments and domain identification. Three of them—loci TTC0188 (UniProt Q72L73), TTC1185 (UniProt Q72IE7), and TTC1491 (UniProt Q72HJ6)—had NP-like domains and were selected for cloning.

Cloning, expression, and purification of NPs. The selected genes were amplified by PCR using the oligonucleotides described in Table 1. In all cases, NdeI and EcoRI restriction sites were included in the forward and reverse primers, respectively. The PCR products were subcloned into pGEM-T and transferred to pET22b. In the resulting expression vectors, the genes were transcribed under the control of a promoter dependent on the T7 phage RNA polymerase. Protein expression was assessed in *E. coli* BL21(DE3) cells by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to exponential-phase cultures (optical density at 600 nm [OD_{600}] of 0.3 to 0.4) growing at 37°C. The cells were harvested by centrifugation when they reached an OD_{600} of 1, washed with 50 mM sodium phosphate buffer (pH 7.0), resuspended in a 1/100 volume of the same buffer, and subjected to sonication (five pulses of 15 s at maximum amplitude; Labsonic M). The crude cell extracts were heated at 80°C for 1 h, and the supernatant containing the produced protein was cleared by ultracentrifugation. The enzyme purity was checked by SDS-PAGE. For analytical ultracentrifugation experiments, the heat-purified extracts were bound to a 1-ml Mini-Macro-Prep High-Q column mounted in a BioLogic LP system (Bio-Rad), equilibrated in the same buffer (50 mM sodium phosphate buffer [pH 7.0]), and eluted with a constant 0 to 0.5 M NaCl gradient. Fractions containing the protein were pooled, desalted, and concentrated using an Amicon Ultra-15 centrifugal filter unit (Millipore). The concentrate was then bound to a 1-ml Mini-Macro-Prep High-S column and eluted with a 0 to 0.5 M NaCl gradient. Protein-containing fractions were pooled, desalted, and concentrated. The protein concentration was measured by the BCA method with the protein assay kit from Bio-Rad.

TABLE 1 Oligonucleotides used in this study

Primer	Sequence (5′–3′)[a]
TTC0188F	TTC*CATATG*GTGTGGCTCCTCCTTTCCCCCA
TTC0188R	TTC*GAATTC*TCAGCCGGGGGGCCTACG
TTC0194F	TTC*CATATG*ATGCCGGATATGGAGCTC
TTC0194R	TTC*GAATTC*TCAGAGCTCGGCGAGGAT
TTC1185F	TTC*CATATG*GTGACCGCCTTCTTCGCCGCCGA
TTC1185R	TTC*GAATTC*TCACTCTACCAGGGCCCGGGCGA
TTC1070F	TTC*CATATG*ATGAGCCCCATCCACGTG
TTC1070R	TTC*GAATTC*TCAAACCTCCAGGACGGC
TTC1412F	TTC*CATATG*ATGAACCCCGTGGTCTTCATCCGGGA
TTC1412R	TTC*GAATTC*CTAGATGGCCTCCAGGACCAAGGG
TTC1491F	TTC*CATATG*ATGGACGCGGTGAAGAAGGCCA
TTC1491R	TTC*GAATTC*CTAGGCCCTGAGAAAGGCCACG

[a] Restriction sites for NdeI (*CATATG*) and EcoRI (*GAATTC*) are indicated in italics, and the nucleotide excess sequences for proper anchoring of the restriction enzymes are underlined.

NP assays. Analysis of nucleoside phosphorolysis was performed using 5 mM nucleoside in 50 mM sodium phosphate buffer (pH 7.0) and 5 μg of enzymatic extract ml^{-1}. The reaction was performed at 65°C for 30 min and stopped by the addition of 1 volume of ice-cold methanol. The one-pot synthesis of nucleosides was performed by a coupled enzymatic reaction (Fig. 1B). Standard conditions for the synthetic reactions were as follows: 2.5 mM acceptor nucleobase and 5 mM nucleoside donor in 50 mM sodium phosphate buffer (pH 7.0), 10 μg of NP1 enzymatic extract ml^{-1} (when added), and 5 μg of NP2 enzymatic extract ml^{-1}. The reaction was developed at 65°C for 30 min and stopped by the addition of 1 volume of ice-cold methanol. The abbreviations used for the nucleosides and bases are as follows: 5-FC, 5-fluorocytosine; 5-IU, 5-iodouracil; 6-tFMeU, 5-trifluoromethyluracil; A, adenine; ACV, acyclovir; Ado, adenosine; *ara*-A: adenine-arabinoside; *ara*-U, uracil-arabinoside; AZT, azidothymidine; BI, benzimidazole; C, cytosine; Ctd, cytidine; dAdo, 2′-deoxyadenosine; DAP, 2,6-diaminopurine; dCtd, 2′-deoxycytidine; DFU, 2-deoxy-2-fluorouracil; dGuo, 2′-deoxyguanosine; dIno, 2′-deoxyinosine; dUrd, 2′-deoxyuridine; FMPy, 5-fluoro-2-methoxy-4(1*H*)-pyrimidinone; G, guanine; Guo, guanosine; HX, hypoxanthine; Ino, inosine; T, thymine; Thd, thymidine; U, uracil; and Urd, uridine.

Analysis of the reaction products. After reaction, the samples were analyzed immediately using high-pressure liquid chromatography (Agilent Series 1100) equipped with a Mediterranean Sea C_{18} column (15 by 0.46 cm, 5-μm particle size; Teknokroma) equipped with an UV detector

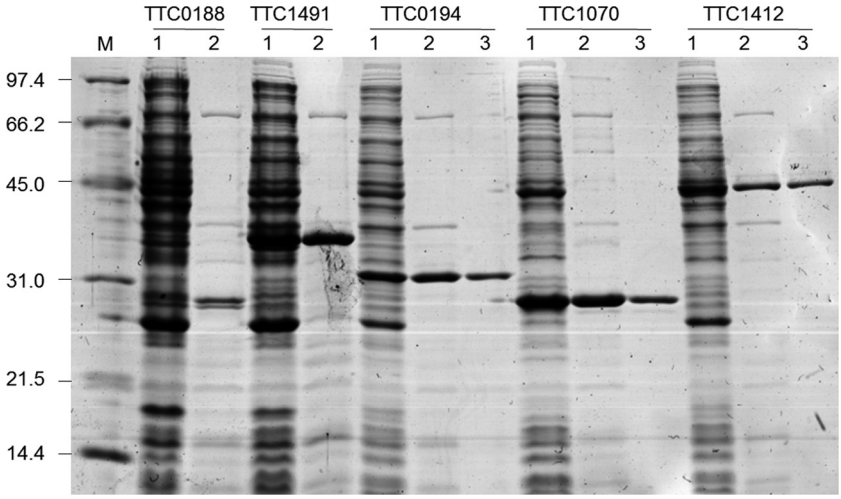

FIG 2 Purification of proteins TTC0188, TTC1491, TTC0194, TTC1070, and TTC1412. The indicated proteins were overproduced from the corresponding pET22b derivatives in *E. coli* BL21 cells. Lanes: 1, whole extract obtained after cell disruption by sonication; 2, soluble fraction after incubation of the whole extract at 80°C for 1 h; 3, purification by ion-exchange chromatography of the heat-treated extract; M, size markers (in kilodaltons).

set at 254 nm and H_2O-CH_3OH (95:5) as the mobile phase. For the phosphorolytic reactions, the disappearance of the nucleoside and appearance of the nucleobase was monitored. For the synthetic reactions, the disappearance of the acceptor nucleobase and the appearance of the new nucleoside were monitored.

Analytical ultracentrifugation. The analytical centrifugation experiments were carried with an Optima XL-I (Beckman-Coulter, Inc.) ultracentrifuge equipped with UV absorbance optics, an An-50Ti rotor, and 1.2-cm double-sector center pieces of Epon-charcoal. Protein samples were analyzed at 20°C in 50 mM sodium phosphate buffer (pH 7.0). For sedimentation velocity experiments, the software SEDFIT (25) was used to calculate the sedimentation coefficient (S), and the apparent sedimentation coefficient of distribution [$c(s)$] from the experimental data. For sedimentation equilibrium experiments, the calculated buoyant molar mass (Mb) values were converted into molar mass (M) values according to the formula: $Mb = M(1 - \nu r)$, where ν is the partial specific volume of the protein (calculated from the amino acid sequence), and r is the buffer density. The HeteroAnalysis software developed by James L. Cole (http://www.biotech.uconn.edu/auf/) was used for these analyses.

RESULTS

Cloning, expression, and purification. TTC0188, TTC0194, TTC1070, TTC1412 and TTC1941 were successfully cloned and expressed in the *E. coli* BL21(DE3)/pET22b system. All attempts to express TTC1185 were unsuccessful. Induced cell extracts showed overproduced proteins of the expected size, although the amount produced was different in each case (Fig. 2, lanes 1), with the lowest production levels detected for TTC0188. After heat treatment, ca. 10 to 12% of the total proteins remained in the soluble fraction in the production of TTC0194, TTC1070, and TTC1412, whereas only a 3 to 4% of the total proteins remained soluble in the case of TTC0188 and TTC1491. The purity of the thermostable proteins in the heat-purified fractions was estimated to be ca. 40% by densitometry analysis of Coomassie blue-stained PAGE gels. For enzymatic assays, this value was deemed acceptable, since the amount of NPs recovered was enough to detect activity, and none of the background proteins from the host organism interfered in the assays. Purification by ion-exchange chromatography of TTC0194, TTC1070, and TTC1412 heat-purified extracts yielded purities close to 95%, as analyzed by gel staining.

Detection of NP activity. The heat-purified recombinant enzymes were tested against a set of natural nucleosides in order to detect phosphorolysis. Adenosine (Ado), 2′-deoxyadenosine (dAdo), inosine (Ino), 2′-deoxyinosine (dIno), guanosine (Guo), 2′-deoxyguanosine (dGuo), uridine (Urd), 2′-deoxyuridine (dUrd), thymidine (Thd), cytidine (Cyt), and 2′-deoxycitidine (dCyt) were used in this initial test. The results are summarized in Table 2.

Although sequence analysis and domain prediction identified UDP_PNP_1 domains within TTC0188 and TTC1941, none of them showed phosphorolytic activity against any nucleoside, and so they were consequently rejected from further assays. In contrast, TTC1070 and TTC0194 showed specificities typical of purine NP, where TTC1070 was specific for 6-oxopurine (2′-deoxy)ribosides (hypoxanthine and guanine), and TTC0194 was specific for 6-aminopurine (2′-deoxy)ribosides (adenine). According to the classification of NPs proposed by Pugmire and Ealick (24), these enzymes would be included in the type I category. A third protein, TTC1412, showed specificity for uracil and thymine (2′-deoxy)ribosides, but not for cytosine. According to the same classification, TTC1412 would be included in the type II category. However, the specificity of this enzyme does not correlate with classical uridine phosphorylases (UP) or thymidine phosphorylases (TP) but to a class of pyrimidine NP (PyNP) found in bacteria such as *Haemophilus influenzae* (26) and *Geobacillus stearothermophilus* (19) that do not discriminate between these bases and the presence or absence of a hydroxyl group at the 2′ position of the ribose moiety.

It is interesting that the three enzymes favored the use of 2′-deoxyribosides rather than ribosides and that their specificity did not overlap, covering almost every combination of nucleobase and sugar, in agreement with our previous findings on cell extracts from this strain (2). Due to these results, we renamed TTC1070, TTC0194, and TTC1412 as TtPNPI, TtPNPII, and TtPyNP, respectively.

Analytical ultracentrifugation. Sedimentation velocity and sedimentation equilibrium experiments were performed on puri-

TABLE 2 Phosphorolytic activity of recombinant enzymes on natural nucleosides[a]

Substrate	Mean phosphorolytic activity (%) ± SD				
	TTC0188	TTC0194	TTC1070	TTC1412	TTC1491
Adenosine	ND	24.2 ± 0.2	ND	ND	ND
2′-Deoxyadenosine	ND	27.8 ± 0.4	ND	ND	ND
Guanosine	ND	ND	8.7 ± 0.1	ND	ND
2′-Deoxyguanosine	ND	ND	22.5 ± 0.3	ND	ND
Inosine	ND	ND	19.1 ± 0.2	ND	ND
2′-Deoxyinosine	ND	ND	40.5 ± 0.3	ND	ND
Uridine	ND	ND	ND	15.2 ± 0.5	ND
2′-Deoxyuridine	ND	ND	ND	40.0 ± 1.2	ND
Thymidine	ND	ND	ND	29.7 ± 0.9	ND
Cytidine	ND	ND	ND	ND	ND
2′-Cytidine	ND	ND	ND	ND	ND

[a] Reaction conditions: 5 mM nucleoside in sodium phosphate buffer (50 mM, pH 7.0), 5 μg of enzyme extract ml^{-1}. Reactions were performed for 30 min at 65°C and stopped in 1 volume of ice-cold methanol. The activities are expressed as a percentage of phosphorolyzed nucleoside with respect to the total amount of nucleoside used as a substrate. ND, not detected.

fied TtPNPI, TtPNPII, and TtPyNP. Sedimentation velocity experiments showed that a single species was present in samples of the purified proteins (Fig. 3). Sedimentation equilibrium experiments were performed to obtain their molar masses. After correction of the buoyant molar masses, the molecular masses calculated for the proteins were 156 ± 1 kDa, 30 ± 0 kDa, and 73 ± 1 kDa for TtPNPI, TtPNPII, and TtPyNP, respectively. Taking into account the theoretical mass deduced from the amino acid sequence of each protein (25, 30, and 45 kDa, in the same order) it was concluded that TtPNPI forms hexamers, TtPNPII monomers, and TtPyNP dimers.

Phosphorolysis of nucleosides. The ability of the three proteins to phosphorolyze natural and synthetic nucleosides was explored. The three enzymes showed high sensitivity to modifications in the sugar moiety. TtPNPI was unable to phosphorolyze acyclovir (ACV) (Table 3), which could be attributed to the presence of an open analogue of ribose rather than to the conjugated guanine, since activity against guanosine and 2′-deoxyguanosine was detected (Table 2). The activities of TtPNPII and TtPyNP against nucleosides carrying an arabinose moiety were dramatically lower than those carrying the natural isomer 2′-deoxyribose, as illustrated by the difference in phosphorolysis between 2′-deoxyadenosine (34%) and adenine-arabinoside (*ara*-A) (1%) or between 2′-deoxyuridine (55%) and uracil-arabinoside (*ara*-U) (3%) (Table 3). In addition, the lack of phosphorolytic activity of TtPyNP against AZT (Table 3), which carries substituents in positions 3′ and 5′, suggests a dependence of the activity for an adequate fitting of the pentose into the active site of the enzyme.

Synthesis of nucleosides. One of the most important features of the NP-catalyzed reaction is its reversible character, which allows it to not only phosphorolyze a nucleoside but also to synthesize a new one by combining an acceptor nucleobase and a pentose-1P. The ability of TtPNPI, TtPNPII, and TtPyNP to perform synthesis of new nucleosides was explored with different base acceptors and 2′-deoxyribose-1P. However, since 2′-deoxyribose-1P is not commercially available, the synthetic reaction was coupled to the phosphorolysis of a 2′-deoxynucleoside, which yields 2′-deoxyribose-1P (Fig. 1B). The phosphorolysis of either Thd or dUrd by TtPyNP was chosen for this end, since these substrates showed the highest phosphorolytic activities (Table 2). The results of the synthesis assays, as well as a schematic view of the

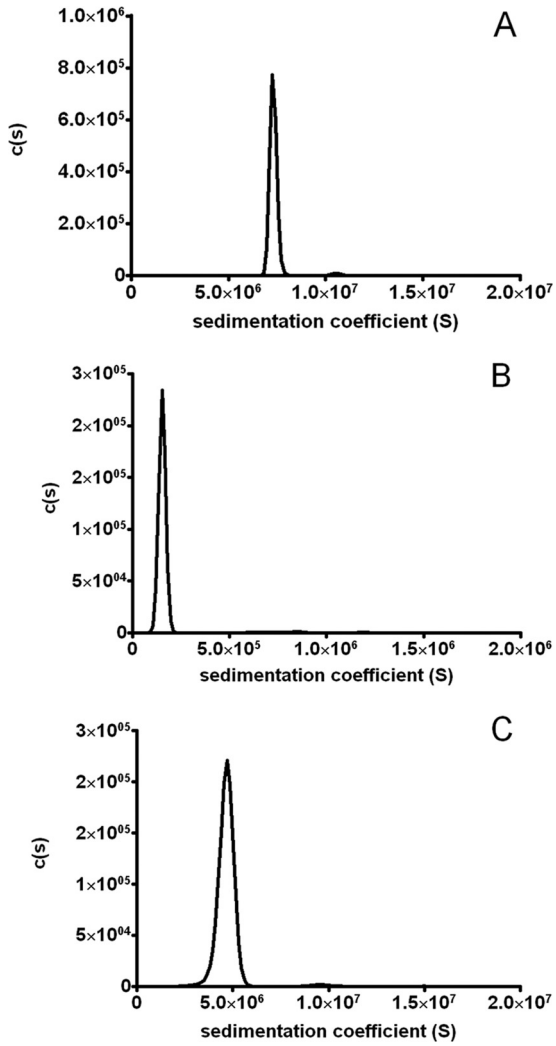

FIG 3 Determination of *T. thermophilus* HB27 NP oligomeric state by analytical ultracentrifugation. (A) TtPNPI, 0.35 mg/ml in 50 mM sodium phosphate (pH 7.0) at 20°C, 50,000 × *g*. (B) TtPNPII, 0.22 mg/ml in 50 mM sodium phosphate (pH 7.0) at 20°C, 50,000 × *g*. (C) TtPyNP, 0.53 mg/ml in 50 mM sodium phosphate (pH 7.0) at 20°C, 50,000 × *g*.

TABLE 3 Phosphorolysis of nucleosides by *T. thermophilus* NPs[a]

Enzyme	Nucleoside	Nucleobase features	Sugar moiety features	Mean (%) phosphorolysis ± SD[b]
TtPNPI	dIno	6-Oxo	2′-Deoxyribose	58.4 ± 0.6
	ACV	6-Oxo,2-amino	Open ring	ND
TtPNPII	dAdo	6-Amino	2′-Deoxyribose	34.0 ± 0.3
	ara-A	6-Amino	Arabinose	1.0 ± 0.1
TtPyNP	dUrd	2,4-Dioxo	2′-Deoxyribose	55.0 ± 1.1
	ara-U	2,4-Dioxo	Arabinose	3.2 ± 0.1
	AZT	2,4-Dioxo,5-methyl	3′-Azido-5′-phosphoribose	ND

[a] Reaction conditions: 2.5 mM nucleoside in sodium phosphate buffer (50 mM, pH 7.0), 5 μg of enzyme extract ml^{-1}, and 30 min at 65°C. Acronyms: dIno, 2′-deoxyinosine; ACV, acyclovir; dAdo, 2′-deoxyadenosine; *ara*-A, adenine-arabinoside; dUrd, 2′-deoxyuridine; *ara*-U, uracil-arabinoside; AZT, azidothymidine.
[b] Percentage of nucleobase liberated with respect to the total amount of nucleoside used as a substrate. ND, not detected.

experimental setup, are depicted in Table 4. TtPNPI was able to synthesize 6-oxopurine nucleosides, in agreement with the specificities previously observed against (2′-deoxy)guanosine and (2′-deoxy)inosine (Table 2). However, it was unable to synthesize nucleosides when the acceptor lacked substituents for its recognition (benzimidazole [BI]) or contained a 6-amino group (2,6-diaminopurine [DAP]). In contrast, TtPNPII synthesized aminopurine nucleosides, showing similar yields with adenine and DAP (49.1 and 41.7%, respectively). Thus, the presence of a substituent in position 2 of the purine ring was not required for ligand recognition by this enzyme, for which the fitting of position 2-substituted adenines in the catalytic pocket may be limited by steric hindrance. However, the presence of a 6-amino group in the purine ring proved to be essential for the ligand recognition by TtPNPII, as shown by the lack of synthetic activity when BI was used as acceptor.

TtPyNP was also able to carry out the synthesis of nucleosides when the pyrimidine acceptor was 2,4-dioxo substituted, and the reaction did not proceed when either one (5-fluorocytosine [5-FC]) or both substituents (2-deoxy-2-fluorouracil [DFU] and 5-fluoro-2-methoxy-4(1*H*)pyrimidinone [FMPy]) were absent. This dependence on the 2,4-dioxo groups could be explained on the basis of the specificities for phosphorolysis observed, where TtPyNP was able to cleave uracil and thymine nucleosides, but not cytosine nucleosides, which are 2-oxo,4-amino substituted (Table 2). Substituents in the pyrimidine ring prevented the synthesis reaction when located in the 6-carbon, as revealed by the lack of activity against FMPy, but substitutions in position 5, such as with 5-IU, were tolerated by the enzyme. This flexibility in position 5 would explain the ability of the enzyme to recognize both uracil and 5-methyluracil (thymine).

TABLE 4 Synthesis of nucleosides by *T. thermophilus* NPs[a]

Enzyme	Acceptor	Feature	% Synthesis (mean ± SD)[b]
TtPNPI	HX	2,6-Dioxo	42.0 ± 0.5
	BI	Backbone only	ND
	DAP	2,6-Diamino	ND
TtPNPII	A	6-Amino	51.0 ± 0.4
	BI	Backbone only	ND
	DAP	2,6-Diamino	41.7 ± 1.5
TtPyNP	U	2,4-Dioxo	49.1 ± 1.3
	5-FC	2-Oxo,4-amino,5-fluoro	ND
	DFU	2,4-Difluoro	ND
	6-tFMeU	2,4-Dioxo,6-trifluoromethyl	ND
	FMPy	5-Fluoro,2-methoxy	ND
	5-IU	2,4-Dioxo,5-iodo	47.8 ± 1.1

[a] The transferred sugar moiety was a 2′-deoxyribose obtained by phosphorolysis of dUrd or Thd by TtPyNP. The reaction conditions for nucleoside synthesis by TtPNPI and TtPNPII were as follows: 5 mM dUrd and 2.5 mM acceptor nucleobase in sodium phosphate buffer (50 mM, pH 7.0), 10 μg of TtPyNP ml^{-1}, 5 μg of TtPNPI or TtPNPII ml^{-1}, and 30 min at 65°C. The reaction conditions for nucleoside synthesis by TtPyNP were as follows: 5 mM Thd and 2.5 mM acceptor in sodium phosphate buffer (50 mM, pH 7.0), 5 μg of TtPyNP ml^{-1}, and 30 min at 65°C. Acronyms: HX, hypoxanthine; BI, benzimidazole; DAP, 2,6-diaminopurine; A, adenine; U, uracil; 5-FU, 5-fluorouracil; DFU, 2-deoxy-2-fluorouracil; 6-tMeFU, 6-trifluoromethyluracil; FMPy, 5-fluoro-2-methoxy-4(1*H*)pyrimidinone; 5-IU, 5-iodouracil.
[b] Percentage of free nucleobase converted into nucleoside with respect to the total amount of nucleobase used as a substrate. ND, not detected.

Optimization of reaction conditions. The experimental conditions for the phosphorolytic activity of the NPs were optimized. The phosphate buffer concentration was fixed at 50 mM, although higher concentrations did not affect the activity of the enzymes, except in the case of TtPyNP, which showed high sensitivity beyond 100 mM (Fig. 4A). Regarding the pH of the medium, the three enzymes were widely tolerant, showing a pH plateau between 5.0 and 7.0, rather than an optimum (Fig. 4B).

As expected, the three enzymes were active at high temperatures (Fig. 4C), to the extreme that conversion rates kept rising beyond the water boiling point. Therefore, a compromise was reached between high activity values and the convenience of the experimental procedure, and 80°C was set as the standard temperature for the assays. According to this activity at high temperatures, the half-lives of TtPNPI and TtPNPII at 80°C were quite long (132 and 232 h, respectively). However, TtPyNP was much more heat sensitive, with a half-life of 2 h under the same conditions (data not shown).

Synthesis of nucleoside analogues frequently uses organic solvents when either the substrates and/or their products have low solubility in aqueous medium. We assayed the ability of the three enzymes to catalyze NP reactions in the presence of different concentrations (5 to 50% [vol/vol]) of dimethylformamide (DMFA), dimethyl sulfoxide (DMSO), and 2-methyltetrahydrofuran (2-MeTHF). In order to correlate the effect of the solvent to the enzymatic activity, nucleoside phosphorolysis assays were performed as phosphorolytic reactions on 2′-deoxyinosine (TtPNPI), 2′-deoxyadenosine (TtPNPII), or 2′-deoxyuridine (TtPyNP). The heat-purified enzymes were added at a concentration of 5 μg ml^{-1} to a reaction mixture contain-

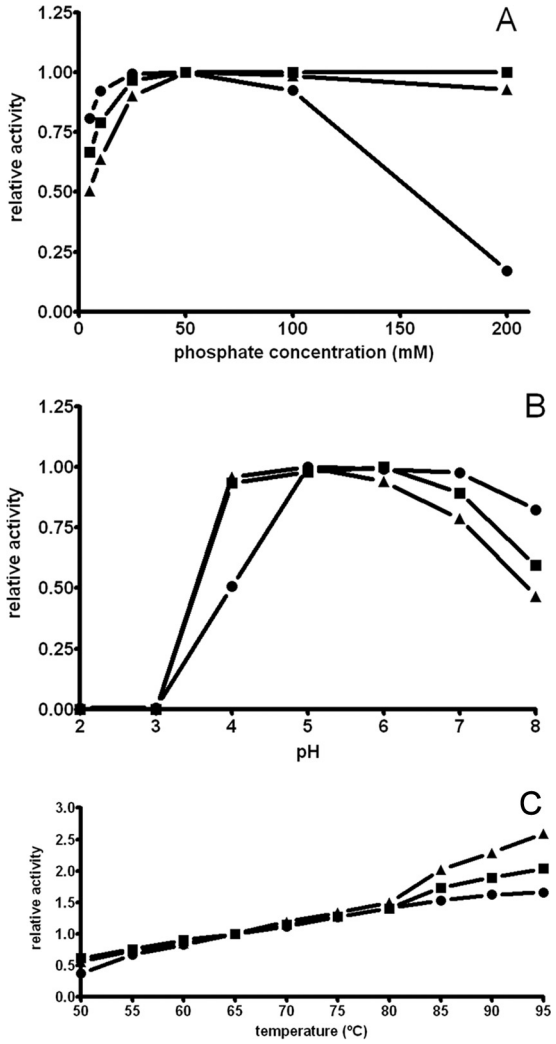

FIG 4 Optimization of reaction conditions, i.e., the phosphate buffer concentration (A), medium pH (B), and temperature (C) in phosphorolytic reactions carried out by TtPNPI (■), TtPNPII (▲), and TtPyNP (●). The values on the *y* axis correspond to the residual activity, where 1 is the activity under standard conditions (50 mM sodium phosphate buffer [pH 7.0], 65°C).

ing 5 mM substrate in 50 mM sodium phosphate buffer (pH 7.0) and the organic solvent in the desired concentration. After 30 min of incubation at 80°C, the percentages of activity detected in the presence of 50% DMFA, DMSO, and 2-MeTHF were, respectively, as follows: TtPNPI, 30, 25, and 25%; TtPNPII, 20, 15, and 62%; and TtPyNP, 34, 20, and 40%. Since these polar nonaqueous solvents are used to solubilize the substrates and products used by these enzymes, such resistance reinforce their potential in industrial applications.

Kinetic parameters of the enzymes. The K_m and k_{cat} values were determined for the phosphorolysis of different substrates at 80°C for the three enzymes using heat-purified extracts. The results are summarized in Table 5. In agreement with the NP test assays (Table 2), the k_{cat}/K_m ratios were higher for 2′-deoxyribosides than for their respective ribosides. TtPNPI showed a higher specificity against 6-oxo,2-aminopurine (guanine) than versus 6-oxopurine (hypoxanthine), while in the case of TtPyNP, higher k_{cat}/K_m values were obtained with uracil nucleosides than with thymidine. Moreover, the activation energy with one substrate per enzyme (dGuo for TtPNPI, dAdo for TtPNPII, and dUrd for TtPyNP) was calculated between 60 and 90°C using the Arrhenius plot. The calculated activation energies (45.8, 46.0, and 45.1 kJ mol^{-1}, respectively) were very similar for the three enzymes, suggesting shared catalytic mechanisms.

DISCUSSION

A screening for putative thermostable NPs identified six putative genes encoding homologues to these enzymes. We attempted to overproduce the corresponding proteins in *E. coli*, where the differences in thermostability would allow us to get a high enrichment degree by differential denaturation of the host proteins through heating.

One of the proteins selected, TTC1185, could not be produced. We cannot assess the reason for this failure, but we hypothesize that folding interferences during translation or toxicity could be responsible for it. In this sense, it is relevant to note that the development of structural genomic programs reveals that ca. 20 to 40% of the open reading frames encoded by thermophiles cannot be overproduced in *E. coli* (12). Two other genes (TTC0188 and TTC1491) produced soluble proteins that could be purified but did not show any detectable NP activity with the substrates assayed. Further *in silico* analysis showed a high similarity of TTC0188 to a futalosine hydrolase (EC 3.2.2.26). A homologue of

TABLE 5 Kinetic parameters of NPs[a]

	TtPNPI			TtPNPII			TtPyNP		
Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m
Guo	0.53 ± 0.01	296 ± 2	558.78						
dGuo	1.01 ± 0.03	830 ± 6	822.53						
Ino	2.08 ± 0.02	173 ± 2	83.20						
dIno	2.06 ± 0.02	627 ± 6	304.33						
Ado				0.32 ± 0.02	440 ± 3	1,376.47			
dAdo				0.36 ± 0.03	563 ± 4	1,563.53			
Urd							0.12 ± 0.01	202 ± 6	1,683.33
dUrd							0.36 ± 0.3	562 ± 17	1,561.11
Thd							0.42 ± 0.02	400 ± 12	952.38

[a] Calculated kinetic parameters of *T. thermophilus* HB27 NPs with different substrates at 80°C. Values are presented as means ± the standard deviations where applicable. Acronyms: Guo, guanosine; dGuo, 2′-deoxyguanosine; Ino, inosine; dIno, 2′-deoxyinosine; Ado, adenosine; dAdo, 2′-deoxyadenosine; Urd, uridine; dUrd, 2′-deoxyuridine; Thd, thymidine.

this protein from *T. thermophilus* HB8 was confirmed to have this activity that has been proposed to be involved in a novel pathway for the synthesis of menaquinones. In this pathway, futalosine is synthesized from chorismate and inosine (10). It is likely that the use of inosine as a substrate requires the presence of a purine-binding domain in TTC0188, leading to mistaking this enzyme for an NP on the basis of sequence comparisons and domains presence. On the other hand, TTC1491 showed high similarity with anthranilate phosphoribosyl transferases (EC 2.4.2.18) from several thermophilic genera, including *Thermus*. This enzyme is involved in the synthesis of aromatic amino acids by performing the reversible phosphorolysis of *N*-(5-phospho-D-ribosyl) anthranilate and does not have NP activity, although structural analyses include them within the same family (13).

Three other proteins—TTC1070, TTC0194, and TTC1412—are *bona fide* NPs, with specificities for 6-oxopurines, 6-aminopurines, and 2,4-dioxopyrimidines, respectively; we renamed these proteins as TtPNPI, TtPNPII, and TtPyNP. Our analysis shows that TtPNPI could be classified as a high-molecular-mass PNP, despite the fact that the strict 6-oxo specificity constitutes an exception with respect to other high-molecular-mass PNPs. The hexameric structure of this enzyme is in agreement with the three-dimensional structure of a PNP from *T. thermophilus* HB8, with which it shares a sequence identity of 91% (28). In contrast, the monomeric nature of TtPNPII was surprising. No other PNP has yet been reported to be monomeric, and homologues, such as the human PNP, with which it shares 46% of identity (1), are trimers. We cannot rule out the possibility that this enzyme could form trimers *in vivo*, but our data clearly show that it is functional as a monomer *in vitro*.

Hexameric PNPs from most organisms accept both adenine and guanine. However, there are species, such as *G. stearothermophilus*, in which the activity is split between two different PNPs with nonoverlapping specificities. This also seems to be the case for *T. thermophilus* HB27, with a high-molecular-mass PNP (TtPNPI) specific for 6-oxopurines (guanine and hypoxanthine) and a low-molecular mass PNP (TtPNPII) specific for 6-aminopurines (adenine). The relevance of these split activities is not clear, but it could be related to the higher requirements for more efficient repair systems at high temperatures.

In contrast to the separation of the activities on purines, TtPyNP uses thymine as well as uracil as substrates. This broad specificity is shared among PyNPs from organisms such as *G. stearothermophilus* (19) and *Haemophilus influenzae* (26), as is the fact that neither accepts cytosine nucleosides as a substrate. PyNPs are usually divided into three categories: uridine phosphorylases (UP), thymidine phosphorylases (TPs), and general-purpose PyNPs, which accept both uracil and thymine nucleosides. Whereas UPs are generally hexamers (and can also be classified as PNPs), TPs and PyNPs are homodimers. In this sense, TtPyNP follows this rule, showing the same quaternary structure with the PyNP from *G. stearothermophilus* (23).

The chemical structure of the sugar moiety is fundamental for substrate recognition, being ribose and 2′-deoxyribose recognized, but not arabinose (*ara*-A or *ara*-U), 3-azido-5-phosphoribose (AZT), or open chains (acyclovir). Interestingly, the three NPs are more prone to cleave 2′-deoxyribosyl nucleosides than the corresponding ribonucleosides. This behavior is in contrast to that described for mesophilic NPs (24) and could have evolved to get nutrients from free DNA produced by accidental access of mesophiles to the thermophilic environments in which *T. thermophilus* lives. Actually, *T. thermophilus* HB27 expresses constitutively an exceptionally efficient natural competence apparatus that allows the internalization of external DNA at huge rates (40 kb s^{-1} $cell^{-1}$) (3). Internalized DNA is degraded into nucleosides and phosphorolyzed by NPs, yielding 2′-deoxyribose-1P and the corresponding base. These can either be used for synthesis or degraded to conserve energy. This way, *T. thermophilus* could survive and grow in an otherwise nutrient-poor environment.

These three enzymes were active in a broad temperature range, even at temperatures bordering the water boiling point, although TtPyNP lost half of the activity after 2 h at 80°C. This relatively low thermostability could be related with the lability of the interaction between monomers of this dimeric enzyme, which would also explain the sensitivity to high ionic strength (Fig. 4A). Alternatively, it could be the consequence of imperfect folding during its synthesis in *E. coli*. This relatively low thermostability does not seem to be shared by TtPNPI and TtPNPII, which remain active after 5.5 and 9.6 days at 80°C, respectively. In this case, it seems that the hexameric TtPNPI does not disassemble under these conditions.

The characterization of these thermostable NPs from the thermophile *T. thermophilus* is of great interest in the field of biocatalysis, since their broad specificity against natural substrates, as well as their ability to use analogue substrates, permits the synthesis of a new range of analogues by combining one or more enzymes with different substrates. The ability of TtPNPII to accept position 2-modified adenines and of TtPyNP to accept position 5-substituted pyrimidines is especially interesting, since these are positions commonly substituted in drugs used for the treatment of human diseases. In this respect, the synthesis of 5-IU nucleosides (Table 4) could prove to be an excellent property, since this compound is an inhibitor of cancer metastasis (18). Moreover, the thermophilic origin of these enzymes has some notable associated advantages, such as an easy purification method with high protein recovery, tolerance to a wide range of pH that lessens the need to finely regulate the pH of the reactor, and thermostability at high temperatures. Also, the three enzymes showed a remarkable resistance to aggressive organic solvents such as DMFA, DMSO, or 2-MeTHF, which are required in the synthesis of compounds with low water solubility. All of these features indicate that the NPs characterized here would be reliable biocatalysts for the synthesis of nucleoside analogues.

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REFERENCES

1. **Afshar S, Sawaya MR, Morrison SL.** 2009. Structure of a mutant human purine nucleoside phosphorylase with the prodrug, 2-fluoro-2′-deoxyadenosine and the cytotoxic drug, 2-fluoroadenine. Protein Sci. **18**: 1107–1114.
2. **Almendros M, Sinisterra JV, Berenguer J.** 2009. *Thermus thermophilus* strains active in purine nucleoside synthesis. Molecules **14**:1279–1287.
3. **Averhoff B, Müller V.** 2010. Exploring research frontiers in microbiology: recent advances in halophilic and thermophilic extremophiles. Res. Microbiol. **161**:506–514.

4. **Bzowska A, Kulikowska E, Shugar D.** 2000. Purine nucleoside phosphorylases: properties, functions, and clinical aspects. Pharmacol. Ther. **88**: 349–425.
5. **Condezo LA, Fernandez-Lucas J, Garcia-Burgos CA, Alcantara AR, Sinisterra JV.** 2006. Enzymatic synthesis of modified nucleosides, p 401–423. *In* Patel RN (ed), Biocatalysis in the pharmaceutical and biotechnology industries. CRC Press, Inc, Boca Raton, FL.
6. **Engelbrecht HL, Sadoff HL.** 1969. Properties of purine nucleoside phosphorylases from spores and vegetative cells of *Bacillus cereus* and their modification by orthophosphate. J. Biol. Chem. **244**:6228–6232.
7. **Gardner R, Kornberg A.** 1967. Biochemical studies of bacterial sporulation and germination. V. Purine nucleoside phosphorylase of vegetative cells and spores of *Bacillus cereus*. J. Biol. Chem. **242**:2383–2388.
8. **Hammer-Jespersen K, Buxton RS, Hansen TD.** 1980. A second purine nucleoside phosphorylase in *Escherichia coli* K-12. II. Properties of xanthosine phosphorylase and its induction by xanthosine. Mol. Gen. Genet. **179**:341–348.
9. **Henne A, et al.** 2004. The genome sequence of the extreme thermophile *Thermus thermophilus*. Nat. Biotechnol. **22**:547–553.
10. **Hiratsuka T, et al.** 2008. An alternative menaquinone biosynthetic pathway operating in microorganisms. Science **321**:1670–1673.
11. **Hori N, Watanabe M, Sunagawa K, Uehara K, Mikami Y.** 1991. Production of 5-methyluridine by immobilized thermostable purine nucleoside phosphorylase and pyrimidine nucleoside phosphorylase from *Bacillus stearothermophilus* JTS 859J. Biotechnol. **17**:121–131.
12. **Jenney FE, Jr, Adams MW.** 2008. The impact of extremophiles on structural genomics (and vice versa). Extremophiles **12**:39–50.
13. **Kim C, et al.** 2002. The crystal structure of anthranilate phosphoribosyltransferase from the enterobacterium *Pectobacterium carotovorum*. FEBS Lett. **523**:239–246.
14. **Koszalka GW, Vanhooke J, Short SA, Hall WW.** 1988. Purification and properties of inosine-guanosine phosphorylase from *Escherichia coli* K-12. J. Bacteriol. **170**:3493–3498.
15. **Krenitsky TA, Koszalka GW, Tuttle JV.** 1981. Purine nucleoside synthesis, an efficient method employing nucleoside phosphorylases. Biochemistry **20**:3615–3621.
16. **Krenitsky TA, et al.** 1982. Pyrazolo[3,4-*d*]pyrimidine ribonucleosides as anticoccidials. 1. Synthesis and activity of some nucleosides of purines and 4-(alkylthio)pyrazolo[3,4-*d*]pyrimidines. J. Med. Chem. **25**:32–35.
17. **Mikhailopulo IA.** 2007. Biotechnology of nucleic acid constituents: state of the art and perspectives. Curr. Org. Chem. **11**:317–335.
18. **Miyadera K, Emura T, Wierzba K, Yamada Y. July** 2001. Cancerous metastasis inhibitors containing uracil derivatives. U.S. patent 6,255,314 B1.
19. **Okuyama K, Hamamoto T, Noguchi T, Midorikawa Y.** 1996. Molecular cloning and expression of the pyrimidine nucleoside phosphorylase gene from *Bacillus stearothermophilus* TH 6-2. Biosci. Biotechnol. Biochem. **60**:1655–1659.
20. **Parker WB, Allan PW, Waud WR, Hong JS, Sorscher EJ.** 2011. Effect of expression of adenine phosphoribosyltransferase on the in vivo antitumor activity of prodrugs activated by *Escherichia coli* purine nucleoside phosphorylase. Cancer Gene Ther. **18**:390–398.
21. **Parker WB, et al.** 1997. In vivo gene therapy of cancer with *Escherichia coli* purine nucleoside phosphorylase. Hum. Gene Ther. **8**:1637–1644.
22. **Prasad AK, Trikha S, Parmar VS.** 1999. Nucleoside synthesis mediated by glycosyl transferring enzymes. Bioorg. Chem. **27**:135–154.
23. **Pugmire MJ, Ealick SE.** 1998. The crystal structure of pyrimidine nucleoside phosphorylase in a closed conformation. Structure **6**:1467–1479.
24. **Pugmire MJ, Ealick SE.** 2002. Structural analyses reveal two distinct families of nucleoside phosphorylases. Biochem. J. **361**:1–25.
25. **Schuck P.** 2000. Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and Lamm equation modeling. Biophys. J. **78**:1606–1619.
26. **Scocca JJ.** 1971. Purification and substrate specificity of pyrimidine nucleoside phosphorylase from *Haemophilus influenzae*. J. Biol. Chem. **246**: 6606–6610.
27. **Swords R, Giles F.** 2007. Troxacitabine in acute leukemia. Hematology **12**:219–227.
28. **Tahirov TH, et al.** 2004. Crystal structure of purine nucleoside phosphorylase from *Thermus thermophilus*. J. Mol. Biol. **337**:1149–1160.
29. **Tai CK, et al.** 2010. Enhanced efficiency of prodrug activation therapy by tumor-selective replicating retrovirus vectors armed with the *Escherichia coli* purine nucleoside phosphorylase gene. Cancer Gene Ther. **17**:614–623.
30. **Taran SA, Verevkina KN, Feofanov SA, Miroshnikov AI.** 2009. Enzymatic transglycosylation of natural and modified nucleosides by immobilized thermostable nucleoside phosphorylases from *Geobacillus stearothermophilus*. Bioorg. Khim. **35**:822–829.
31. **Trelles JA, et al.** 2004. Immobilized *Escherichia coli* BL21 as a catalyst for the synthesis of adenine and hypoxanthine nucleosides. Chem. Biodivers. **1**:280–288.
32. **Vieille C, Zeikus GJ.** 2001. Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. Microbiol. Mol. Biol. Rev. **65**:1–43.
33. **Vorbrüggen H, Ruh-Pohlenz C.** 2001. Handbook of nucleoside synthesis. John Wiley & Sons, Inc, New York, NY.
34. **Wiltshire H, et al.** 2005. Pharmacokinetic profile of ganciclovir after its oral administration and from its prodrug, valganciclovir, in solid organ transplant recipients. Clin. Pharmacokinet. **44**:495–507.