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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

A comparative study on phosphotransferase activity of acid phosphatases from *Raoultella planticola* and *Enterobacter aerogenes* on nucleosides, sugars, and related compounds

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Abstract Natural and modified nucleoside-5′-monophosphates and their precursors are valuable compounds widely used in biochemical studies. Bacterial nonspecific acid phosphatases (NSAPs) are a group of enzymes involved in the hydrolysis of phosphoester bonds, and some of them exhibit phosphotransferase activity. NSAP containing *Enterobacter aerogenes* and *Raoultella planticola* whole cells were evaluated in the phosphorylation of a wide range of nucleosides and nucleoside precursors using pyrophosphate as phosphate donor. To increase the productivity of the process, we developed two genetically modified strains of *Escherichia coli* which overexpressed NSAPs of *E. aerogenes* and *R. planticola*. These new recombinant microorganisms (*E. coli* BL21 pET22b-phoEa and *E. coli* BL21 pET22b-phoRp) showed higher activity than the corresponding wild-type strains. Reductions in the reaction times from 21 h to 60 min, from 4 h to 15 min, and from 24 h to 40 min in cases of dihydroxyacetone, inosine, and fludarabine, respectively, were obtained.

Keywords Acid phosphatase · Nucleosides-5′-monophosphate · Sugars phosphate · Enzymatic phosphorylation · Fludarabine-5′-monophosphate

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Introduction

Most of the dephosphorylations that occur in prokaryotic cells involve the hydrolysis of phosphoester or phosphoanhydride bonds and are catalyzed by a group of enzymes called phosphohydrolases or phosphatases (Boyer et al. 1961). Some of these enzymes are secreted outside the plasmatic membrane or retained as proteins attached to membrane. It is believed that the function of these enzymes is the hydrolysis of different organic phosphoesters (as nucleotides, sugars phosphate, phytic acid, etc.) that cannot pass through the membrane. Consequently, the inorganic phosphate and dephosphorylated products can be transported into the cell, providing nutrients for cell proliferation (Beacham 1979; Wanner 1996).

The classification of phosphatases was initially based on biochemical and biophysical properties such as optimum pH, affinity for substrate, and molecular size. Since the development of data banks, it was found that phosphatases, as well as other proteins, can be grouped based on their sequence. This approach allowed the classification in families by defining a sequence pattern for each one, which is very useful for the identification of new gene functions (Vincent et al. 1992).

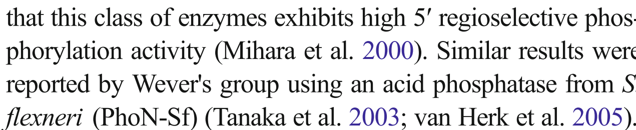
In particular, nonspecific acid phosphatases (NSAPs) (*bacterial nonspecific acid phosphatases*) are a group of enzymes that are able to hydrolyse a broad range of organic phosphoesters and display optimal activity in acidic or neutral

pH. These enzymes are widely distributed among enteric bacteria as soluble proteins in the periplasmic space or membrane bound. At least three molecular classes of NSAPs have been identified according to the amino acid sequences, named as A, B, and C, although members within one family can exhibit distinctive functional differences, like ample or limited substrate specificity. Besides, bacterial species can produce NSAPs from different families, suggesting their participation in different physiological functions (Rossolini et al. 1998).

In addition to their intrinsic phosphatase activity, all class B and some class A acid phosphatases also exhibit phosphotransferase activity. The transference of a phosphate group from different organic compounds to alcohols was first reported by Axelrod (1948) and Appleyard (1948), employing the phosphatases present in orange juice. Some enzymes of the class A have been exploited biotechnologically in the food industry (Asano et al. 1999a) and as tools in environmental bioremediation, e.g., removal of heavy metals from metal-polluted soil and aqueous wastes (Basnakova et al. 1998; Dissing and Uerkvitz 2006). At amino acid level, the class A NSAPs possess a conserved sequence motif, KX_6RP-($X_{12\text{-}54}$)-PSGH-($X_{31\text{-}54}$)-SRX_5HX_3D, shared and conserved by several lipid phosphatases and the mammalian glucose-6-phosphatases (Stukey and Carman 1997). The relevance of sugar monophosphates is due to their role as precursors in the synthesis of nucleosides and their analogues, and also of carbohydrates. Arion et al. (1972) synthesized glucose 6-phosphate from glucose and different organic and inorganic phosphate donors showing the phosphotransferase activity of glucose-6-phosphatase. NSAPs from *Shigella flexneri* and *Salmonella enterica* were also able to catalyze the phosphorylation of a broad range of sugars and alcohols in a regioselective manner using cheap pyrophosphate as the phosphate donor (van Herk et al. 2005).

Natural and modified nucleoside-5′-monophosphates (NMPs) are valuable compounds widely used in biochemical studies as active pharmacological components or as flavor enhancers in the food industry. Remarkably, 9-β-D-arabinofuranosyl-2-fluoroadenine 5′-monophosphate (fludarabine monophosphate, 5′-FAraAMP) has generated considerable attention as chemotherapeutic pro-drugs since it is active in chronic lymphocytic leukemia therapies (Robak et al. 2009). Natural NMPs are usually obtained by microbial fermentation or by isolation from hydrolysates of nucleic acids. Methods for the production of modified NMPs are based on either chemical or enzymatic phosphorylation of the corresponding nucleosides being the enzymatic phosphorylation considered as a green advantageous alternative.

Asano et al. (1999b) reported an enzymatic method to produce inosine 5′-monophosphate (IMP) by the recombinant PhoC from *Morganella morganii*, an A1 class of NSAP, using inorganic pyrophosphate (PPi) as donor. They demonstrated



that this class of enzymes exhibits high 5′ regioselective phosphorylation activity (Mihara et al. 2000). Similar results were reported by Wever's group using an acid phosphatase from *S. flexneri* (PhoN-Sf) (Tanaka et al. 2003; van Herk et al. 2005).

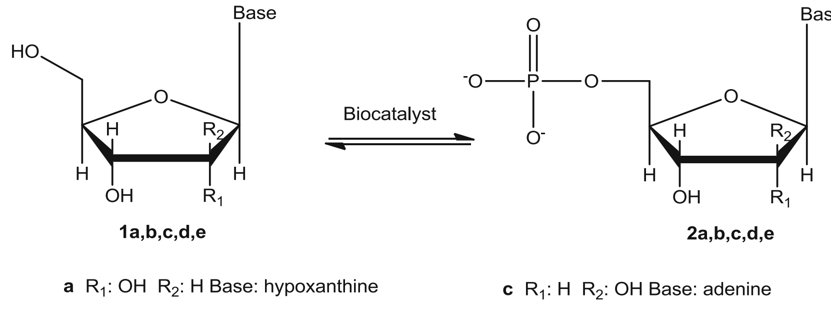
In recent years, enzyme-catalyzed approaches using aldolases have received attention as alternatives to chemical methodologies, allowing the formation of carbon–carbon bonds and the introduction of stereocenters into the product (Greenberg et al. 2004; Samland and Sprenger 2006; Valino et al. 2012). Dihydroxyacetone phosphate (DHAP)-dependent aldolases have a strict requirement for DHAP as the donor substrate. In addition, while DHAP is also a regular metabolite of glycolysis, its use in industrial application is limited due to its low stability and high price. Recent advances to provide DHAP by chemical synthesis or by multienzyme approaches have been reported. For example, Schoevaart et al. (1999) described the synthesis of 5-deoxy-5-ethyl-D-xylulose where DHAP is synthesized in situ by a phytase and glycerol 3-phosphate oxidase. Sanchez-Moreno et al. (2004) used a more straightforward approach, where dihydroxyacetone (DHA) is phosphorylated by a kinase, and ATP is recycled using an acetate kinase. More recently, a bifunctional aldolase/kinase enzyme was developed to use DHAP prepared in situ (Iturrate et al. 2010). Likewise, van Herk et al. (2006) proposed the phosphorylation of DHA using recombinant NSAPs from *S. flexneri* and *S. enterica* ser. *typhimurium* LT2, following the same strategy first applied in the phosphorylation of different polyhydroxy compounds as well as carbohydrates (van Herk et al. 2005).

In this paper, we report the phosphotransferase activity of NSAPs from wild type and recombinant *Raoultella planticola* and *Enterobacter aerogenes* strains selected from our bacteria cell collection. Natural and modified nucleosides (Scheme 1) as well as some hydroxylated molecules (Schemes 2 and 3) were tested as substrates to prepare monophosphate compounds regioselectively using disodium acid pyrophosphate as phosphate donor. Proof of concept on nucleosides and sugar phosphate purification is also described.

Material and methods

Chemicals

Nucleosides (adenosine, guanosine, inosine, cytidine, thymidine, uridine, 9-β-D-arabinofuranosyluracil, 2′-deoxyuridine, 2′-deoxyinosine, 9-β-D-arabinofuranosyladenine, 9-β-D-arabinofuranosyl-2-fluoroadenine), aldehydes and ketones (glyceraldehyde, dihydroxyacetone), and sugars (D-ribose, D-glucose, 2-deoxy-D-ribose, D-arabinose) were from Sigma-Aldrich (St Louis, MO, USA). The culture media components were obtained from Merck (Darmstadt, Germany) and Difco (Sparks, MD, USA). HPLC grade methanol and acetonitrile



Scheme 1 Biocatalysed phosphorylation of natural and modified nucleosides

were from Carlo Erba (Rodano, Italy) or Sintorgan (Buenos Aires, Argentina). Microorganisms were supplied by the Colección Española de Cultivos Tipo (CECT), Universidad de Valencia (Spain). All other chemicals and enzymes were purchased from commercial suppliers and used without purification. Disodium acid pyrophosphate was kindly donated by Saporiti SA (Buenos Aires, Argentina).

Strains, plasmids, and culture conditions

All standard recombinant DNA procedures were performed as described by Sambrook et al. (2001). The host strains *Escherichia coli* DH5α (Invitrogen) and *E. coli* BL21 (DE3) ATCC 47092 (acid phosphatase deficient) were used in subcloning and expression experiments. *R. planticola* CECT 843 (ATCC 33531) and *E. aerogenes* CECT 684 (ATCC 13048) were used as DNA source for *pho-Rp* and *pho-Ea* gene cloning. Bacteria were routinely grown at 37 °C in liquid Luria-Bertani broth medium (NaCl, 10 g L^{-1}; tryptone, 10 g L^{-1}; yeast extract, 5 g L^{-1}) containing 100 μg/ml ampicillin (LBAmp) when required. pGEMT-easy (Promega) was used as cloning vector and pET-22b (Novagen) as expression vector.

Recombinant DNA techniques

R. planticola pho gene was cloned in the pET-22b as follows. The sequence containing the secretion signal was PCR amplified from *R. planticola* chromosomal DNA using the forward primer 5′-CATATG AAAAAGCGTGTACTCGCC-3′ and the reverse primer 5′-GGATCC CGCCGACTATTTCTGCTGTT-3′ (the *Nde*I and *BamH*I sites, respectively, are underlined). Likewise, *E. aerogenes pho* gene was PCR amplified using forward primer 5′-CATATG AAAAAGCGCGTTCTCG-3′ and the reverse primer 5′-GGATCC TTACTTCTGCGTTTTGGCG-3′ (the *Nde*I and *BamH*I sites, respectively, are underlined).

The PCR were performed using Taq polymerase (Fermentas) with the following conditions: 0.1 μg of *R. planticola* or *E. aerogenes* chromosomal DNA, 0.2 μM of each primer, 500 μM of each dNTPs, 1.5 mM $MgCl_2$, and two units of Taq polymerase mix in a final volume of 50 μl. A "hot start" of 2 min at 95 °C was followed by 35 cycles of denaturation (30 s at 95 °C), annealing (1 min at 58 °C), and extension (1 min at 72 °C) using a programmable heating block (Eppendorf Mastercycler 5415B).

The PCR products were ligated on pGEMT-easy, and the plasmids were first established in *E. coli* DH5α. The resulting clones were confirmed by DNA sequencing using an Applied

Scheme 2 Biocatalysed phosphorylation of sugars

3a,b,c
Biocatalyst
4a,b,c

a R_1: OH R_2: H D-ribose
b R_1: H R_2: H 2-D-deoxyribose
c R_1: H R_2: OH D-arabinose

Scheme 3 Biocatalysed phosphorylation of hydroxylated molecules

1,3-dihydroxyacetone dimer ⇌ (Biocatalyst) dihydroxyacetone-3-phosphate

DL-glyceraldehyde ⇌ (Biocatalyst) DL-glyceraldehyde-3-phosphate

Biosystems 3730XL DNA sequencer, restricted with *Nde*I and *BamH*I, and cloned into the corresponding sites of pET-22b.

Expression of the recombinant enzymes

E. coli BL21 (DE3) ATCC 47092 carrying the recombinant plasmids were grown at 37 °C in LBAmp medium until the absorbance of the culture suspension reached A_{600}=0.4–0.6. The expression of the recombinant Pho-Rp and Pho-Ea was induced by adding 0.5 mM isopropyl isothio-β-D-galactoside (IPTG), and the growth was continued at 20 °C for 4 h. The bacterial cells were harvested by centrifugation, and the pellets were used for further protein expression analysis or washed with 0.1 M sodium acetate buffer pH 4.0 and directly employed as biocatalysts.

Standard screening conditions

The standard reaction mixture comprising per milliliter: 74.5 mmoles nucleoside, 0.2 mg (1 mmol) $MgSO_4 \cdot 7H_2O$, 58 mg (260 mmoles) disodium acid pyrophosphate, and 50 mg biocatalyst expressed as wet cell weight, dissolved in 0.1 M sodium acetate buffer pH 4 (final volume, 0.50 mL), was stirred at 200 rpm and 35 °C for 48 h. Aliquots were drawn at different times, quenching the reaction by the addition of HCl 2 N, centrifuged at 10,000×*g* for 30 s, and the supernatants were analyzed by thin-layer chromatography (TLC).

The biocatalysts employed in the screenings, which belong to the *Enterobacteriaceae* family present in our culture collection, are listed: *Citrobacter amalonaticus* CECT 863, *Citrobacter freundii* CECT 401, *Citrobacter koseri* CECT 856, *Klebsiella* sp. CECT 367, *E. aerogenes* CECT 684, *Enterobacter cloacae* CECT 194, *E. cloacae* CECT 960, *E. cloacae* CECT 4502, *Enterobacter gergoviae* CECT 857, *Erwinia carotovora* CECT 225, *Erwinia crysanthemi* CECT 509, *E. coli* CECT 45, *E. coli* CECT 100, *E. coli* CECT 105, *E. coli* CECT 731, *E. coli* CECT 877, *E. coli* BL21 (DE3) ATCC 47092, *Proteus mirabilis* CECT 4101, *Proteus vulgaris* CECT 165, *P. vulgaris* CECT 174, *Proteus rettgeri* CECT 4557, *P. vulgaris* CECT 4077, *Providencia rettgeri* CECT 171, *Providencia rettgeri* CECT 865, *R. planticola* CECT 843, and *Serratia rubideae* CECT 868.

Standard phosphorylations

For the production of phosphorylated nucleosides, the reaction mixture consisted of 100 mM sodium acetate buffer pH 4.0, 260 mM disodium acid pyrophosphate (PPi), 1 mM $MgSO_4 \cdot 7H_2O$, 50 mg/mL wet weight of biocatalyst, and nucleoside (74.5 mM inosine, adenosine, cytidine, uridine, 2′-deoxyuridine, thymidine, 2′-deoxyinosine; 30 mM uracil arabinoside and 15 mM guanosine, adenine arabinoside, and fludarabine), in a volume of 1 mL and was stirred at 35 or 40 °C and 200 rpm. In case of sugars, aldehydes, and ketones, the substrate final concentration was increased (100 mM) as well as the phosphate donor concentration (300 mM). At different reaction times, aliquots were taken, stopped after the addition of 1.5 μL 2 N HCl and after centrifugation, the supernatants were stored at −20 °C until analysis.

Analytical methods

Chromatography

The phosphorylated nucleoside formation was followed by HPLC using a modular Gilson instrument (321Pump, 156 UV/VIS detector and 234 Autoinjector Series) (Middleton, WI, USA) and an Alltech Apollo RP18 column (150× 4.6 mm, 5 μm particle size) (Deerfield, IL, USA) at a flow rate of 1 mL.min^{-1}. The UV detector was set at 254 nm, and the column was operated at room temperature, employing 100 mM triethylammonium acetate (TEAA), pH 7.0, and

acetonitrile mixtures as mobile phase. The other operating conditions are shown in Supplementary Material Table S1.

TLC analyses were performed using Silicagel 60 F254 plates (Merck, Rahway, NJ) and a solution of 1-propanol: NH_4OH (28 % *w*/*w*): H_2O in a ratio 20:15:3 as mobile phase for nucleosides and nucleosides phosphate or a mixture of *n*-butanol: 2-propanol: H_2O in a ratio 3:12:4 for sugars. The formation of sugar phosphates, glyceraldehyde-3-phosphate (G3P), and dihydroxyacetone phosphate were followed using this technique by the appearance of low RF's spots, detected by 1 % (*v*/*v*) anisaldehyde and 2 % (*v*/*v*) H_2SO_4 in acetic acid, e.g., glucose-5-phosphate: 0.14, 2-deoxy-D-ribose-5′-phosphate: 0.16, ribose-5′-phosphate: 0.13, arabinose-5-phosphate: 0.18, glyceraldehyde-3-phosphate: 0.15, dihydroxyacetone-3-phosphate: 0.16.

^{31}P NMR

Phosphorylated products of aldehydes, ketones and sugars, and PPi and Pi were quantified by phosphorous magnetic resonance (^{31}P NMR) using a 500-MHz Bruker instrument. Chemical shifts (δ) are expressed in parts per million relative to 85 % of phosphoric acid (Supplementary Data Table S2). Concentrations of products and reagents were determined using 10 mM of dimethylmethylphosphonate in deuterated water as internal standard.

Purification of 5′-IMP

A 5-mL phosphorylation reaction was carried out following the standard conditions using inosine as substrate and *E. coli* BL21 pET22b-phoRp as biocatalyst. After 20 min, the conversion reached 10 % based on initial inosine. IMP isolation was performed following a modified strategy to the one reported by van Herk et al. (2005) for glucose-6-phosphate purification. First, the pH was adjusted to 5.0 with NaOH 10 N, and the inorganic phosphate was precipitated in two sequential steps by the addition of 1.75 and 0.85 ml of 1.5 M barium acetate. The solids obtained were separated from the reaction mixture by centrifugation and discarded. The supernatant was cooled down in an ice-bath; the pH was adjusted at 7.5, and 1.5 volumes of cold ethanol were slowly added. The produced precipitate (IMP barium salt) was separated by centrifugation, washed with cold ethanol, and dried under vacuum. The solid was dissolved in 6 mL of deionised water, placed onto a cation exchange resin (Dowex 50WX2-200, H^+ form), and eluted with deionised water. The collected fractions were neutralized with NaOH and freeze-dried. The presence of IMP and inosine in the different fractions was detected by TLC and quantified by HPLC. A portion of the solid was dissolved in deuterated water and analyzed by ^{31}P NMR and ^{13}C NMR. This isolation resulted in 62 % recovery (99 % purity) of the formed IMP.

Results

Biocatalyst selection

Phosphorylation of nucleosides

Microorganisms are an invaluable source of biocatalysts with broad substrate specificities. In order to make use of this enzymatic diversity for the synthesis of nucleosides-5′-monophosphates, different screenings were carried out to identify the best strains with the proper phosphotransferase activity, modifying the methodology reported by Asano et al. (1999a). A total of 26 bacteria belonging to the family *Enterobacteriaceae* from the genera *Citrobacter*, *Escherichia*, *Erwinia*, *Proteus*, *Klebsiella*, and *Serratia* were tested for the regioselective phosphorylation of C5′ position of inosine, adenosine, guanosine, cytidine, uridine, thymidine, and 2′-deoxyuridine using PPi as phosphate donor. As expected, the enzymatic activity was widely distributed among the bacteria assessed. The time course of the reactions were followed by TLC and quantified by HPLC. The biocatalysts selected for each substrate are listed in Table 1.

Phosphorylation of sugars, aldehydes, and ketones

The same methodology was later on applied to the preparation of phosphorylated nucleoside precursors, such as D-deoxyribose-5-phosphate (dR5P), D-ribose-5-phosphate (R5P), dihydroxyacetone phosphate (DHAP), and G3P. Glucose was also included in the screening as control. Higher concentration of substrates and phosphate donor (100 mM and 300 mM, respectively) were employed for these biotransformations due to the higher solubilities of the substrates in the reaction mixture. The reaction conversions, semiquantitatively estimated by TLC, are shown in Table 2.

Recombinant expression of the acid phosphatases of *E. aerogenes* and *R. planticola*

E. aerogenes ATCC 13048 and *R. planticola* ATCC 33531 were selected as the most versatile biocatalysts. *E. aerogenes*

Table 1 Nucleoside phosphorylation: best biocatalysts obtained after screening from our *Enterobacteriaceae* collection

Product	Microorganism	Conversion (mM)	Time (h)
5′-IMP	*E aerogenes* CECT 684	7.4	4
5′-AMP	*E. aerogenes* CECT 684	7.8	5
5′-GMP	*E. gergoviae* CECT 857	1.5	24
5′-CMP	*E. gergoviae* CECT 857	7.6	24
5′-UMP	*Klebsiella sp*. CECT 367	8.0	48
5′-TMP	*R. planticola* CECT 843	2.8	12
5′-dUMP	*R. planticola* CECT 843	1.7	12

Table 2 Biocatalyst selection for the synthesis of phosphorylated sugars, aldehydes, and ketones

Product	Microorganism	Conversion (%)[a]	Time (h)
dR5P	*E. aerogenes* CECT 684	++	24
	C. amalonacticus CECT 863	++	20
	P. rettgeri CECT 4557	++	20
R5P	*E. aerogenes* CECT 684	++	12
	R. planticola CECT 843	++	12
G6P	*E. aerogenes* CECT 684	++	4
	R. planticola CECT 843	++	4
	E. gergoviae CECT 857	++	2
DHAP	*E. aerogenes* CECT 684	+	20
	R. planticola CECT 843	+	20
G3P	*R. planticola* CECT 843	++	12

[a] Conversion estimated by semiquantitative TLC: ++ stands for approximate 10–20 %, + stands for <10 %

Table 3 Phosphorylated products obtained employing *E. aerogenes* (ATCC 13048) and *R. planticola* (ATCC 33531) whole cells

Entry	Products	*E. aerogenes*		*R. planticola*	
		Conversion (mM)	Time (h)	Conversion (mM)	Time (h)
Nucleosides phosphate					
1	5′-IMP	7.4	4	4.5	4
2	5′-AMP	7.8	5	4.6	5
3	5′-GMP	0.9	24	nr	76
4	5′-CMP	nr	24	nr	76
5	5′-UMP	2.0	24	5.6	24
6	5′-TMP	1.8	50	2.8	12
7	5′-dUMP	1.4	76	1.7	12
8	5′-dIMP	1.1	50	1.7	12
9	5′-AraUMP	2.9	50	4.0	12
10	5′-AraAMP	0.3	30	0.4	9
11	5′-FaraAMP	1.7	24	2.8	8
Sugars, aldehyde, and ketone phosphate					
12	G3P	2.3	12	13.1	12
13	DHAP	1.9	21	2.3	21
14	R5P	14.3	12	9.9	12
15	dR5P	2.3	27	11.5	27
16	Ara5P	5.4	21	8.4	21
17	G6P	31.4	4	63.8	4

nr stands for no formation of phosphorylated product in the conditions assayed

and *R. planticola pho* gene sequences were obtained from the corresponding genomic DNA using primers designed on the basis of deposited sequences of *E. aerogenes* IFO 12010 and *R. planticola* ATCC 33531 (Genbank accession numbers AB044338 and AB04435, respectively) under standard PCR conditions.

The fragments amplified were first ligated into a cloning vector. The presence of both 747 bp open reading frames was confirmed by sequencing and later subcloned into pET-22b(+). The peptide signal pelB was first removed from the expression vector since the exporting signals from the *E. aerogenes* and *R. planticola* proteins may be recognized and excised through *E. coli* peptidases (Choi and Lee 2004).

Recombinant enzymes were expressed in *E. coli* BL21 (DE3) ATCC 47092 from which its periplasmic acid phosphatase/phytase (appA) gene was inactivated. SDS-PAGE gels revealed the overexpression of approximately 27 kDa proteins in the soluble form, in agreement with the calculated enzyme molecular weights (26.96 kDa in case of *E. aerogenes* and 26.74 kDa for *R. planticola*) without post-translational modification.

E. coli transformants were cultured in LB media containing ampicillin and IPTG, and the enzymatic activities of the whole cells were measured under standard reaction conditions.

Phosphotransferase activity of wild-type *E. aerogenes* and *R. planticola* whole cells

Phosphotransferase activities of the *E. aerogenes* and *R. planticola* whole cells were assessed on a wide variety of natural and modified nucleosides, sugars, aldehydes, and ketones. The best results are shown in Table 3.

The phosphorylation of purine nucleosides such as inosine and adenosine by *E. aerogenes* afforded higher yields than those obtained with *R. planticola* at the same reaction time (Table 3, entries 1 and 2) except when 2′-deoxyinosine was the substrate (entry 8). Moreover, when phosphotransferase activity was assayed for guanosine, only *E. aerogenes* produced 5′-GMP (Table 3, entry 3).

Regarding pyrimidine nucleosides, the yields obtained using *R. planticola* were higher than those afforded by *E. aeorgenes* cells (Table 3, entries 5, 6, and 7), especially, doubling the 5′-UMP concentration (5.6 mM vs. 2.0 mM). In particular, for 5′-CMP synthesis none of the microorganisms showed phosphotransferase activity (Table 3, entry 4).

Moreover, *R. planticola* cells exhibited higher yields and shorter reaction times in the synthesis of arabinonucleosides, glyceraldehyde, and dihydroxyacetone phosphates (Table 3, entries 9 to 13). Mainly due to the low solubility of 9-β-D-arabinofuranosyladenine (AraA); low concentration of 5′-AraAMP was accumulated (Table 3, entry 10).

With regard to sugars, D-ribose was more efficiently phosphorylated by *E. aerogenes* cells (Table 3, entry 14), whereas the phosphorylation activity on 2-deoxy-D-ribose and arabinose was significantly lower compared to *R. planticola* cells

(Table 3, entries 15 and 16). At the same reaction time (4 h), G6P was accumulated in a 63.8 % conversion in the reaction media containing *R. planticola* cells and in 31.4 % conversion in those containing *E. aerogenes* cells (Table 3, entry 17).

Phosphotransferase activity of recombinant *E. coli* whole cells

When the same reactions were carried out by *E. coli* BL21 cells heterologously expressing *E. aerogenes* and *R. planticola* NSAPs, employing nucleosides as substrates, increased yields and substantial reductions in the reaction times (from days to no more than 2.5 h) were observed in most of the cases (Table 4). Phosphorylation products were hardly detected on *E. coli* BL21 (DE3) ATCC 47092 cells. Furthermore, a faster dephosphorylation rate of the formed products was also detected in the recombinant strains compared to the wild type ones (data not shown).

Unlike the *E. aerogenes* wild-type cells, 5′-AMP was barely formed using the recombinant strain (7.8 and 1.1 mM, respectively, Table 4, entry 2). Adenine formation was observed at short incubation times.

Table 4 Phosphorylated products obtained employing *E. coli* BL21 pET22b-phoEa and *E. coli* BL21 pET22b-phoRp whole cells

Entry	Products	*E. coli* BL21 pET22b-phoEa		*E. coli* BL21 pET22b-phoRp	
		Conversion (mM)	Time (min)	Conversion (mM)	Time (min)
Nucleosides phosphate					
1	5′-IMP	7.6	20	8.1	30
2	5′-AMP	1.1	60	6.4	25
3	5′-GMP	0.7	50	1.8	30
4	5′-CMP	3.0	20	6.6	20
5	5′-UMP	4.7	40	6.7	25
6	5′-TMP	2.5	150	4.6	25
7	5′-dUMP	1.8	120	2.0	40
8	5′-dIMP	1.7	120	2.7	40
9	5′-AraUMP	6.5	80	6.1	30
10	5′-AraAMP	1.0	80	1.3	25
11	5′-FaraAMP	2.1	50	1.5	35
Sugars, aldehyde and ketone phosphate					
12	G3P	7.3	60	17.3	60
13	DHAP	2.3	60	6.9	60
14	R5P	14.4	60	28.4	60
15	dR5P	2.7	60	15.7	60
16	Ara5P	6.6	60	4.5	60
17	G6P	29.2	40	66.9	40

nr stands for no formation of phosphorylated product in the conditions assayed

5′-GMP synthesis by wild-type *R. planticola* was not detected; however, when the recombinant *E. coli* strain was used for this biotransfomation, 1.8 mM of 5′-GMP was obtained. In the same way, 3.0 and 6.6 mM of 5′-CMP were produced by *E. coli* BL21 pET22b-phoEa and *E. coli* BL21 pET22b-phoRp respectively, while both wild-type strains did not synthesized this compound.

5′-Arabinonucleosides were in major extent more efficiently phosphorylated by the modified *E. coli* strains. Using the recombinant strains, 5′-AraUMP concentration raised to natural 5′-NMPs levels. In particular, shorter reaction times were observed, employing *E. coli* BL21 pET22b-phoRp cells. The only exception was 5′-FAraAMP that was produced in a lower yield by this biocatalyst than *R. planticola* whole cells (Table 4, entry 11).

Moreover, when other hydroxylated molecules, which included carbohydrates from 3 to 6 carbon atoms were subjected to phosphorylation by the recombinant strains, again increased yields were obtained in shorter time. Using *E. coli* strain containing NSAP from *R. planticola*, higher conversions were achieved for G3P, dR5P, and almost triple amount for DHAP and R5P (Table 4, entry 12 to 15). Unexpectedly, Ara5P synthesis was less efficient for this strain. On the contrary, with the exception of G3P, no considerable differences were observed with *E. coli* pET22b-PhoEa cells compared to the *E. aerogenes* wild-type strain.

D-glucose phosphorylation to D-glucose-6-phosphate did not afford better results using the recombinant cells as biocatalysts, only a large reduction from 4 h to 40 min in the reaction time.

Purification of 5′-IMP

To isolate the phosphorylated product from the reaction mixture, several chromatographic strategies were evaluated using as a reaction model the synthesis of 5′-IMP.

Different types of resins were assessed in this study based on literature reports including hydrophobic resins as Sepabeads SP2017 (Ishii et al. 1989); anion exchange resins as Amberlite IRA 400 Cl^- (Asano et al. 1999a) and Dowex 1x8 (Hokse 1983). However, in all cases, the separation of 5′-IMP was not satisfactory due to its co-elution with another reaction component.

Barium salts were applied by van Herk et al. (2005) for the selective precipitation of glucose-6-phosphate from mixture of PPi, inorganic phosphate, and glucose. In our case, two moles of barium salts were added to the reaction mixture per mole of PPi. Sequential precipitation steps were performed in order to maximize the removal of phosphate. After each precipitation step, the remaining supernatants were analyzed by HPLC. Unspecific precipitation of 5′-IMP (ca. 20 %) was observed together with orthophosphate after the addition of barium acetate solution.

The remaining 5′-IMP present in the supernatant was precipitated using cold ethanol in the form of barium salt (BaIMP). Traces of inosine and acetate were also observed. BaIMP fraction was applied onto a column containing a cation exchange resin (Dowex 50WX2-200, H^+ form) to obtain the 5′-IMP in the form of free acid. Later, inosinic acid was transformed into the sodium salt by neutralization with NaOH. The purity of NaIMP was higher than 99 % with respect of inosine (determined by HPLC).

To quantify the concentration of inorganic phosphate present in the collected fractions, they were first freeze-dried; the solids were dissolved in deuterated water and analyzed by ^{31}P RMN. The phosphate area was equivalent to the 7 % of the IMP area, yielding an impurity of the 0.6 % regarding the initial phosphate concentration (Fig. 1). The yields of each 5′-IMP purification step are shown in Table 5.

Table 5 Inosine-5′-monophosphate recovery after each step of purification

Purification step	5′-IMP recovery (%)
Barium acetate precipitation	75.2
Ethanol precipitation	89.9
Dowex 50WX2-200 column	92.1
Total	**62.3**

The number in bold corresponds to the overall recuperation value, the reason to be in bold was to stress this meaning, but regular numbers could also be used

Discussion

In this study, natural and modified nucleosides as well as some hydroxylated molecules were subjected to phosphorylation using intact cells of wild type and recombinant *E. coli* cells carrying *E. aerogenes* and *R. planticola* acid phosphatases using disodium acid pyrophosphate (PPi) as phosphate donor. Even though acid phosphatases from the same species and highly homologue class A1 NSAPs (e.g., the ones from *Morganella morganii*, *Escherichia blattae*, and *Providencia stuartii* cells) were previously cloned and characterized by Mihara et al. (2001); their research was focused on the use of these biocatalysts in the synthesis of 5′-IMP. This prompted us to study their application in the phosphorylation of other compounds using whole cells instead of purified enzymes as employed by van Herk et al. (2005).

In the present work, the two pho genes from *E. aerogenes* (747 bp) and *R. planticola* (747 bp) were cloned, sequenced, and successfully expressed in *E. coli* BL21 (DE3). On the nucleotide level, the two pho sequences presented an 84 % identity. Furthermore, the overall sequence identity between the two enzymes was found to be 97 %. The protein alignment showed that the enzymes differ only in eight amino acids, one present in the exporting signal (position 18) and the other seven (residues number 75, 77, 99, 106, 112, 131, and 246) in residues not directly involved in the catalysis. However, the residue 131 (serine in case of *E. aerogenes* and glycine in case of *R. planticola*) is close to the residue Lys133 (motif 1 KX6RP), which in *E. blattae*, acid phosphatase crystal

	mmoles	%
5'-IMP initial	0.0360	100.00
5'-IMP final	0.0223	62.29
Pi initial	2.5500	100.00
Pi final	0.0016	0.06

Fig. 1 Quantification of residual orthophosphate impurified 5′-IMP by ^{31}P-NMR. 5′-IMP concentrations were determined by HPLC

structure was indicated to play a pivotal role maintaining the phosphate group close to His207, thus favoring the nucleophilic attack (Ishikawa et al. 2000). Besides the high similarity, several differences concerning substrate acceptance were observed among this two enzymes.

Regarding the synthesis of 5′-NMPs by the wild-type enzymes, higher affinities were observed in case of ribonucleosides than 2′-deoxyribonucleosides. The yields of arabinosides-5′-monophosphates is variable but in agreement with the solubility of the substrates in the reaction media (AraU>5′-FAraA>AraA).

In general, *E. aerogenes* cells were more efficient in the phosphorylation of purines, while *R. planticola* cells were for 2′-deoxynucleosides and arabinosides. Regarding the synthesis of 5′-CMP, the presence of uridine in the reaction media after 24 h evidenced the cytidine deaminase (EC 3.5.4.5) action on the substrate.

Compared to nucleosides, the phosphorylation of sugars was markedly more effective, especially that catalyzed by *R. planticola* whole cells. The only exception was R5P, confirming the highest affinity of *E. aerogenes* NSAP for ribosides.

To enhance the phosphorylation of the different substrates, the two NSAPs were heterologously expressed in *E. coli* BL21 acid phosphatase deficient cells. The obtained reaction times for recombinant enzymes were significantly reduced compared to those produced by native strains, principally due to the higher enzyme accumulation inside the cells. In most of the cases, the shorter reaction times also led to higher substrate conversion as competitive side-reactions, e.g., nucleoside degradation by nucleoside phosphorylases and deaminases, were avoided. This may explain why 5′-CMP and 5′-GMP were only synthesized by the recombinant strains. However, 5′-AMP yields were unexpectedly lower than those obtained by *E. aerogenes* and *R. planticola*. Even though small amounts of adenine were present at short reaction times, adenosine remained largely unconverted after 1 h, suggesting that the phosphorylation reaction in the wild-type strains may not be catalyzed by the class A NSAPs. Recently, the existence of putative acid phosphatases (e.g., class B acid phosphatases) in *E. aerogenes* and *R. planticola* strains has been predicted or inferred by homology (The UniProt Consortium 2012), supporting the previous hypothesis.

The use of the recombinant strains for the phosphorylation of sugars, aldehydes, and ketones resulted in a noteworthy decrease in the reaction times together with similar conversions in most of the cases compared to the wild-type *E. coli* BL21 pET22b-phoEa but higher conversions using *E. coli* BL21 pET22b-phoRp cells.

van Herk et al. (2005) phosphorylated several carbohydrates and alcohols in a regioselective manner by using purified acid phosphatases from *Shigella flexneri* and *Salmonella enterica*. In that work, 60, 2.5, 1.7, and 5 mM of G6P, R5P, Ara5P, and DHAP were obtained, respectively, in 200 min, starting from 100 mM of the corresponding substrates. Our experiments overcame these amounts and decreased reaction times while avoiding the enzyme purification path.

Even though the purification of phosphorylated organic compound from inorganic ones is usually a problematic step, the strategy here employed produced IMP in 67 % (99 % purity).

As conclusion, two recombinant NSAPs containing whole cells were prepared in order to be evaluated as biocatalysts in the phosphorylation of nucleosides, sugars, and analogues. While the increase in yields was moderate, there was a large improvement in reaction times. Among the achieved compounds, it is remarkably the preparation of FAraAMP (2.1 mM, 50 min), of high interest in pharmaceutical industry, and DHAP (6.9 mM, 60 min), a specific donor substrate for some aldolases, used in biotransformation.

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