Expression and purification of the 5′-nucleotidase YitU from Bacillus species: its enzymatic properties and possible applications in biotechnology

Yuliya R. Yusupova 1 · Victoria S. Skripnikova 1 · Alexandr D. Kivero 1 · Natalia P. Zakataeva 1

Received: 16 November 2019 / Revised: 17 January 2020 / Accepted: 3 February 2020 / Published online: 10 February 2020
© The Author(s) 2020

Abstract
5′-Nucleotidases (EC 3.1.3.5) are enzymes that catalyze the hydrolytic dephosphorylation of 5′-ribonucleotides and 5′-deoxyribonucleotides to their corresponding nucleosides plus phosphate. In the present study, to search for new genes encoding 5′-nucleotidases specific for purine nucleotides in industrially important Bacillus species, “shotgun” cloning and the direct selection of recombinant clones grown in purine nucleosides at inhibitory concentrations were performed in the Escherichia coli GS72 strain, which is sensitive to these compounds. As a result, orthologous yitU genes from Bacillus subtilis and Bacillus amyloliquefaciens, whose products belong to the ubiquitous haloacid dehalogenase superfamily (HADSF), were selected and found to have a high sequence similarity of 87%. B. subtilis YitU was produced in E. coli as an N-terminal hexahistidine-tagged protein, purified and biochemically characterized as a soluble 5′-nucleotidase with broad substrate specificity with respect to various deoxyribo- and ribonucleoside monophosphates: dAMP, GMP, dGMP, CMP, AMP, IMP and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-monophosphate (AICAR-P). However, the preferred substrate for recombinant YitU was shown to be flavin mononucleotide (FMN). B. subtilis and B. amyloliquefaciens yitU overexpression increased riboflavin (RF) and 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) accumulation and can be applied to breed highly performing RF- and AICAR-producing strains.

Keywords 5′-Nucleotidases (EC 3.1.3.5) · Riboflavin producers · 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR) producers · Bacillus subtilis · Bacillus amyloliquefaciens

Introduction
Bacillus subtilis, Bacillus amyloliquefaciens, and other Bacillus species are gram-positive bacteria widely used for the production of enzymes, recombinant proteins, antimicrobial components (peptide and lipopeptide antibiotics and bacteriocins), insecticides, adsorbents, surfactants, and other industrially important biochemicals such as D-ribose, vitamins, purine nucleosides, and poly(gamma-glutamic acid) (Schallmey et al. 2004; Abriouel et al. 2011; Liu et al. 2013).

The main desirable features for the application of many Bacillus species as microbial cell factories are their generally recognized as safe (GRAS) status, probiotic properties, absence of exotoxins and endotoxin production, fully sequenced genomes, well-studied secretion pathways, and fairly simple cultivation conditions; their available transcriptome, metabolome, and proteome analysis data, and advanced genetic engineering tools are suitable for use with these species. B. subtilis and B. amyloliquefaciens strains have been successfully designed to produce riboflavin (RF), adenosine, inosine, guanosine, and 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), which are widely used in food technology and the pharmaceutical industry (Stepanov et al. 1984; Perkins et al. 1999; Asahara et al. 2010; Lobanov et al. 2011; Sheremet et al. 2011; Zhang et al. 2015). Since the compounds listed can be synthesized from their immediate phosphorylated precursors, flavin mononucleotide (FMN), AMP, IMP, GMP, and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-monophosphate (AICAR-P), respectively,
the construction of industrial producers requires not only enhanced metabolic flux towards the biosynthesis of these phosphorylated compounds but also the oversynthesis of enzymes with respective phosphatase or 5'-nucleotidase activity. 5'-Nucleotidase (EC 3.1.3.5) are enzymes that catalyze the hydrolytic dephosphorylation of 5'-ribonucleotides and 5'-deoxyribonucleotides to nucleosides and phosphate. These enzymes are widely distributed among all domains of life (Zimmermann 1992). Most well-studied soluble 5'-nucleotidases belong to the ubiquitous haloacid dehalogenase superfamily (HADSF) and have been shown to be involved in purine and pyrimidine salvage pathways, nucleic acid repair, cell-to-cell communication, signal transduction, etc. (Bianchi and Spychala 2003; Hunucker et al. 2005; Borowiec et al. 2006). HADSF members, which are multifunctional enzymes with 5'-nucleotidase activity expressed by bacteria, control the intracellular concentrations of key phosphorylated metabolites and thereby participate in regulating cellular metabolism. The identification and investigation of these enzymes are important from both fundamental and applied points of view.

Despite the essential role of soluble 5'-nucleotidases in bacterial metabolism and the design of industrially important strains, little information about the functions of these enzymes from Bacillus species could be found in the literature. Terakawa and coauthors reported the 5'-nucleotidase activities of several B. subtilis proteins (YqeG, YcaA, YutF, YcsE, and YktC) (Terakawa et al. 2016) homologous to earlier described E. coli multifunctional enzymes that exhibit 5'-nucleotidase activity with respect to a remarkably broad and overlapping substrate spectrum (Matsuhisa et al. 1995; Kuznetsova et al. 2006). A HADSF member from B. subtilis, the 5'-nucleotidase YutF, was found to hydrolyze various purine and pyrimidine 5'-nucleotides, showing a preference for 5'-nucleoside monophosphates and, specifically, 5'-XMP (Zakataeva et al. 2010). Recently, enzymes with phosphatase and 5'-nucleotidase activities belonging to the HADSF were shown to catalyze essential steps in the biosynthesis of the key metabolites serine and RF. Thus, YsaA from B. subtilis was found to be a phosphoserine phosphatase, the enzyme that catalyzes the final step of serine biosynthesis (Koo et al. 2017). Another HADSF member, B. subtilis YcsE, was shown to catalyze the dephosphorylation of 5-amino-6-ribitylaminopurine-2,4(1H,3H)-pyrimidinedione 5'-phosphate (ARPP), forming the pyrimidine precursor of RF, 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (Sarge et al. 2015). Moreover, screening of 13 putative HADSF members from B. subtilis revealed that two additional proteins, YwtE and YitU, can catalyze the same reaction at appreciable rates (Sarge et al. 2015). Recently, a homologue of YwtE and YcsE, B. subtilis PhoC, which is probably involved in the phosphosugar stress response, was characterized (Morabbi Heravi et al. 2019).

In the present study, to search for genes encoding 5'-nucleotidases specific to purine nucleotides in B. subtilis and B. amyloliquefaciens, “shotgun” cloning and the direct selection of recombinant clones grown with purine nucleosides at inhibitory concentrations were performed in the E. coli GS72 strain, which is sensitive to these compounds. As a result, the yitU gene was selected, and its product was characterized as a 5'-nucleotidase with broad substrate specificity with respect to various deoxyribo- and ribonucleoside monophosphates. The preferred substrate for YitU was shown to be the redox-active coenzyme FMN. Furthermore, the application of yitU overexpression for the design of industrially important RF- and AICAR-producing strains was demonstrated.

Materials and methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are shown in Table 1. The primers used in this study are shown in Supplementary Table S1. E. coli was used as a host for cloning and protein expression. The B. subtilis and B. amyloliquefaciens strains, except for strain AJ1991purH::spc, were constructed using pNZT1-based delivery plasmids and a two-step replacement recombination procedure (Zakataeva et al. 2010), as described in Table 1. Single crossover was maintained by erythromycin (Em) resistance. Strain AJ1991purH::spc, in which the spc cassette was inserted into purH, was constructed by allele replacement (due to double crossover events) using the delivery plasmid pHY300PLK-purH::Sp.

Growth conditions and preparation of crude cell extracts

E. coli and B. subtilis were grown in Luria-Bertani (LB) or M9 minimal medium (Miller 1972) supplemented with t-glucose (0.4% for E. coli or 2% for Bacillus unless otherwise specified). When required, thiamine HCl (5 μg/ml), RF (25 μg/ml), tryptophan (50 μg/ml), casamino acids (0.1% w/v), ampicillin (Ap, 100 μg/ml), erythromycin (Em, 200 μg/ml for E. coli or 10 μg/ml for Bacillus), kanamycin (Km, 10 μg/ml), tetracycline (Tc, 10 μg/ml), spectinomycin (Spc, 100 μg/ml), or chloramphenicol (Cm, 7 μg/ml) was added to the medium. Solid medium was obtained by adding 20 g/l agar to liquid medium. All reagents were purchased from Sigma-Aldrich (Steinheim, Germany) unless otherwise specified.

To select 5'-nucleotidase genes by the “shotgun” technique, recombinant plasmids containing DNA fragments from genomic libraries were transferred into E. coli strain GS72, and the resulting transformants were grown in glucose M9 minimal medium supplemented with inhibitory concentrations of guanosine (50 μg/ml) or inosine (1500 μg/ml).
| Strain or plasmid          | Relevant characteristics | Source or description               |
|---------------------------|--------------------------|-----------------------------------|
| **Bacillus subtilis strains** |                          |                                   |
| 168                       | trpC2                    | VKPM B13486                       |
| BsΔDEG                    | Derivative of 168; contains ΔdeoD::kan ΔpbaE::cat ΔpurG::spc | VKPM B13846                       |
| BsC⁺                     | Derivative of 168; contains wild-type trpC                  | pNZT1-trpCwt → 168                |
| BsC⁺ΔU                   | Derivative of BsC⁺; contains a 780-bp in-frame deletion in yitU (ΔU) | pNZT1-ΔyitUBs → BsC⁺               |
| B. subtilis 168 Δrib      | Derivative of 168; contains the kan gene inserted into the ribD region, RF auxotroph, Km⁺ | VKPM B13485                       |
| Y25                      | B. subtilis riboflavin-producing strain; contains ribO335 ribC1 azgR rosR | VKPM B9850                        |
| Y25AU                    | Derivative of Y25; contains a 780-bp in-frame deletion in yitU | pNZT1-ΔyitUBs → Y25               |
| BS168 ΔyutF              | Derivative of BS168; contains a 351-bp in-frame deletion of yutF | Zakataeva et al. 2016             |
| BS168 ΔyutF ΔU           | Derivative of BS168 ΔyutF; contains a 780-bp in-frame deletion in yitU | pNZT1-ΔyitUBs → BS168 ΔyutF       |
| **Bacillus amyloliquefaciens strains** |                      |                                   |
| AJ1991                    | B. amyloliquefaciens inosine- and guanosine-producing strain; contains Ade⁺, Ile⁺, azgR | VKPM B8994                        |
| IAM1523                   | B. amyloliquefaciens K, wild-type                          | Zakataeva et al. 2010             |
| IAMADG                    | Derivative of IAM1523; contains deoD::Km pupG::Cm          | Successful disruption of the deoD and pupG genes in the IAM1523 chromosome using the delivery plasmids pNZT1-DeoD::Km and pNZT1-ΔpupG::Cm, respectively |
| AJ1991purH::spc           | AICAR-producing strain, derivative of AJ1991; contains purH::spc | pHY300PLK-purH::Sp → AJ1991     |
| AJ1ΔU                    | Derivative of AJ1991purH::spc; contains a 753-bp in-frame deletion of yitU | pNZT1-ΔyitUBa → AJ1991purH::spc |
| **Plasmids**              |                          |                                   |
| pMW118                    | Low copy number vector, ori of pSC101, Plac lacZ’ ApR       | Nippon Gene, Tokyo, Japan          |
| pMWAL1                    | Low copy number bireplicon E. coli–B. subtilis shuttle vector; based on the theta-replicating B. subtilis plasmid pBS72 and pMW118 plasmid; ApR (E. coli), CmR (B. subtilis, B. amyloliquefaciens) | Smirnov and Kotliarova 2015       |
| pMWAL1-ΔyitUBa            | pMWAL1 derivative; contains a 1217-bp XbaI-SacI fragment of the B. amyloliquefaciens IAMADG chromosome with the yitU gene and its upstream region (for yitUBa expression under control of its own regulatory elements) | The DNA fragment of IAMADG was PCR amplified (primer pair yitU-Xba/yitU-Sac), digested with XbaI-SacI, and cloned into XbaI-SacI-digested pMWAL1 |
| pMWAL1-ΔyitUBs           | pMWAL1 derivative; contains a 1191-bp XbaI-SacI fragment of the B. subtilis 168 chromosome, with the yitU gene and its upstream region (for yitUBs expression under control of its own regulatory elements) | The DNA fragment of 168 was PCR amplified (primer pair (+)yitU Sac Bs/(-)yitU Xba Bs), digested with XbaI-SacI, and cloned into XbaI-SacI-digested pMWAL1 |
| pMWAL1-PyiitUBa-ΔyitUBa    | pMWAL1 derivative; contains a 344 bp fragment of the yitUBa upstream region fused to promoterless yitUBa | The DNA fragment of 168 was PCR amplified (primer pair (-)yitUB Xba Bs/(+)PBam yitUBs) and fused using OE-PCR with a PCR-amplified fragment of the B. amyloliquefaciens IAMADG chromosome (primer pair (+)yitUB seq1 Bam/(−)yitUBs PBam). Obtained fragment was digested with XbaI-SacI and cloned into XbaI-SacI-digested pMWAL1 |
| pET-15b(+)                | E. coli expression vector, ApR                                 | Novagen (Merck Millipore, Darmstadt, Germany) |
| Strain or plasmid | Relevant characteristics | Source or description |
|------------------|--------------------------|-----------------------|
| pET15-yitUBBs    | pET-15b(+) derivative for the production of YitUBBs | Coding sequence of 168 yitU was PCR amplified (primer pair (+)yitU Neo Bs/(−)yitUBH BHI Bs, digested with Neol-BamHI, and cloned into Neol-BamHI-digested pET-15b(+) |
| pET15-H6-yitUBBs | pET-15b(+) derivative for the production of YitUBBs with an N-terminal hexahistidine tag | Coding sequence of 168 yitU was PCR amplified (primer pair (+)yitU His Bs/(−)yitUBH BHI Bs, digested with Neol-BamHI, and cloned into Neol-BamHI-digested pET-15b(+) |
| pKS1             | Thermosensitive integration vector, EmR, KmR | Shatalin and Neyfakh 2005 |
| pNZT1            | pKS1 derivative, thermosensitive integration vector, EmR | Zakataeva et al. 2010 |
| pNZT1-trpCwt     | pNZT1 derivative to introduce wild-type trpC+ in 168 | The DNA fragment of 168 was amplified using OE-PCR (primers (+)trpC Hind Bs/(−)trpCw splc and (−)trpCw Pst Bs/(+)trpCw splc), digested with HindIII-PstI, and cloned into HindIII-PstI-digested pNZT1 |
| pNZT1-AyatUBBs   | pNZT1 derivative to introduce ΔyitUBBs | The DNA fragment of 168 was amplified using OE-PCR (primers (+)yitU delR Bs/(−)yitU seq1 Bs and (−)yitU del L Bs/(+)yitU seq1 Bs), digested with PvuI-EcoRV, and cloned into EcoRV-Smal-digested pNZT1 |
| pNZT1-xityUBBs   | pNZT1 derivative to introduce ΔyitUBBs | The DNA fragment of B. amyloliquefaciens IAM125 was amplified using OE-PCR (primers: (+)yitU Sal Bam/(−)yitU delL Bam and (−)yitU Pst Bam/(+)yitU delR Bam), digested with PstI-Sall and cloned into PstI-Sall-digested pNZT1 |
| pNZT1-ApupG::Cm   | pNZT1 derivative to introduce ΔpupG::cat | The DNA fragment of B. amyloliquefaciens IAM125 was PCR amplified (primer pair punA-Xho/punA-Pst), digested with XhoI-PstI, and cloned into XhoI-PstI-digested pNZT1, yielding pNZT1-pupG. Then, the cat gene was cut from pUC7-Cm (Blatny et al. 1997) with Sall-Smal and cloned into the Sall-Bsp6I sites of pNZT1-pupG, giving pNZT1-ApupG::Cm |
| pKS1-AdeoD::Km   | pKS1 derivative to introduce ΔdeoD::kan | DNA fragments of B. amyloliquefaciens IAM125 containing the 5′ end and 3′ end of deoD coding region were PCR amplified with primer pairs deoD1-Xho/deoD1-Hind and deoD1-Sma/deoD1-Bcu, respectively. PCR fragments were digested with XhoI-HindIII and SmaI-BcaI, respectively, and successively cloned into their respective sites in pKS1, yielding pKS1-AdeoD::Km |
| pDG1726          | Plasmid containing the spe antibiotic cassette | Guérout-Fleury et al. 1995 |
| pHY300PLK        | E. coli-B. subtilis shuttle vector, ApR (E. coli), TrR (B. subtilis, B. amyloliquefaciens) | Ishiwa and Shibahara-Sone 1986 |
| pHY300PLK-purH   | pHY300PLK derivative with cloned purH | The DNA fragment of B. amyloliquefaciens IAM125 was PCR amplified (primer pair P24/P25), digested with EcoRI, and cloned into EcoRI-digested pHY300PLK |
| pHY300PLK-purH::Sp | pHY300PLK derivative to introduce ΔpurH::spc | DNA fragment containing the spc cassette was cut from pDG1726 using EcoRV-HindIII and cloned into EcoRV-HindIII-digested pHY300-purH |

*pApR, ampicillin resistance; EmR, erythromycin resistance; CmR, chloramphenicol resistance; SpcR, spectinomycin resistance; TrR, tetracycline resistance; KmR, kanamycin resistance; azgR, 8-azaguanine resistance; rosR, roseoflavin resistance

This work unless otherwise specified; VKPM, the Russian National Collection of Industrial Microorganisms; pNZT1-AyatUBBs—BsC+ denotes a strain constructed from BsC+ using the pNZT1-AyatUBBs plasmid; PCR, polymerase chain reaction; OE-PCR, overlap extension PCR.

In agar diffusion assays, drops of cellular suspensions of the B. subtilis 168 strain containing plasmids pMWAL1, pMWAL1-yitUBBs, pMWAL1-yitUBBs, or pMWAL1-PyitUBBs-yitUBBs were placed onto M9 plates supplemented with glucose and tryptophan (without RF) on which a suspension of the RF auxotrophic strain B. subtilis 168 Δrib had previously been spread. After...
16 h of cultivation at 37 °C, the diameters of the growth halos of *B. subtilis* 168 Δrib around the plaques of control strain harboring empty vector and yitU-overexpressing strains were assessed. All experiments were performed in triplicate.

Extracellular AICAR accumulation in AICAR-producing strains was evaluated by tube fermentation as previously described (Sheremet et al. 2011), but the initial glucose concentration in the fermentation media was 60 g/l. AICAR concentration in the culture broth was determined using high-performance liquid chromatography (HPLC) as described (Sheremet et al. 2011). Glucose concentrations were determined by an enzymatic method using an enzyme electrode (BIOSEN C-line; EKF Diagnostic, Germany). Bacterial growth was assayed by measuring the optical density of the culture broth (OD_{600}) using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) at 600 nm.

Extracellular RF accumulation in RF-producing strains and BsC⁺-based strains was evaluated by flask fermentation. Cells were incubated on glucose medium plates for 18 h at 34 °C and then resuspended in 40 ml of fresh M9 medium supplemented with glucose (1% for RF-producing strains and 0.4% for BsC⁺-based strains) to an OD_{600} of 0.3 (for RF-producing strains) or 0.1 (for BsC⁺-based strains). Cm was added to plasmid-containing strains. Strains were incubated in 750-ml flasks at 34 °C in a rotary shaker for 72 h (for RF-producing strains) or 192 h (for BsC⁺-based strains). Every 24 h, samples were taken from each strain and analyzed for biomass accumulation (OD_{600}) and RF and glucose concentrations.

RF concentrations in culture broth were determined using a UPLC Acquity system (Waters, USA) with a fluorescence detector. Samples (5 μl) of appropriately diluted cell-free supernatants were applied to a Nucleosil 100-5 C18 MPN column (4 × 125 mm, 5 μm; Macherey & Nagel). The following solvent system was used at a flow rate of 0.7 ml/min: 25% (vol/vol) acetonitrile–50 mM formic acid–50 mM ammonium formate (pH 4.3). Detection was carried out with a fluorescence detector (excitation, 325 nm; emission, 513 nm; Waters Associates, Inc., USA).

To analyze culture broth by liquid chromatography-tandem mass spectrometry (LC-MS/MS), cells were grown for 70 h in 20 ml of M9 medium supplemented with 0.2% glucose and Cm in a rotary shaker in 750-ml flasks. For LC-MS/MS analysis, cell-free supernatants of the culture broth were used.

To prepare crude cell extracts, cells grown with aeration to mid-log phase in LB or M9 medium (*E. coli*) and M9 medium (*Bacillus*) supplemented with thiamine HCl and Ap for *E. coli* or tryptophan, casamino acids and Cm (when required) for *Bacillus* were harvested by centrifugation, washed with 0.9% NaCl, resuspended in 0.7 ml of buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 10% glycerol, 1 mM AEBSF), and then lysed by sonication (3× 60 s), following which debris was removed by centrifugation at 13,200×g for 20 min at 4 °C. The protein concentration in the crude extract was 3 mg/ml.

### DNA manipulation and genetic methods

All recombinant DNA manipulation was conducted according to standard procedures (Sambrook and Russell 2001) and the recommendations of the enzyme manufacturer (Thermo Scientific, Lithuania, Vilnius). Plasmid and chromosomal DNA was isolated using the Qiagen Miniprep kit (Germany, Hilden) and Qiagen DNA purification kit (Germany, Hilden), respectively, according to the manufacturer’s instructions.

Transformation of *B. subtilis* competent cells, E40 bacteriophage transduction to transfer plasmids into *B. amyloliquefaciens* cells, PCR amplification, and DNA sequence analyses were performed as previously described (Zakataeva et al. 2010). Primers were purchased from Evrogen (Moscow, Russia). All constructs were verified by DNA sequencing.

### Heterologous expression of YitU and purification

The pET15-H6-yitUBₜₜ expression construct was transferred into *E. coli* BL21(DE3). The recombinant hexahistidine-tagged YitUBₜₜ (Ht-YitUBₜₜ) protein was overexpressed in the obtained transformants as previously described (Zakataeva et al. 2016) and purified by immobilized metal affinity chromatography on a HisTrap HP column (GE Healthcare) according to the manufacturer’s instructions. Imidazole-eluted recombinant protein was transferred to buffer A (50 mM Tris-HCl buffer, pH 7.1, 5 mM MgCl₂, 20% glycerol) by gel filtration on a Sephadex G-25 column (Pharmacia) and stored at −70 °C until required. The protein concentration was assayed using a Bio-Rad protein assay kit (Bio-Rad) with bovine serum albumin as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 15% polyacrylamide gels and subsequent staining with Coomassie brilliant blue R250.

Gel filtration analysis was performed on a Superose 6 Increase 10/300 GL column (GE Healthcare Life Sciences) in PBS (10 mM phosphate buffer, 140 mM NaCl, pH 7.4) according to the manufacturer’s recommendation. The column was calibrated using a sample from a molecular mass standard kit (Gel Filtration Markers Kit for Protein Molecular Weights 29,000–700,000 Da, Sigma-Aldrich, St. Louis, USA).

#### Enzymatic assay

General phosphodiesterase activity was measured spectrophotometrically at 25 °C in a reaction mixture (0.5 ml) containing 50 mM Tricine buffer (pH 8.5), 0.5–5 mM Mg²⁺ (MgCl₂ or MnCl₂), 5 mM bis(p-nitrophenyl) phosphate (bis-pNPP) or 5 mM p-nitrophenyl phosphorylcholine (pNPC) as a substrate and purified Ht-YitUBₜₜ (3 μg) diluted in a stabilization buffer (50 mM Tris-HCl buffer, pH 7.0, 5 mM MgCl₂, 20%...
glycerol, 1 mg/ml BSA). The reaction was initiated by substrate addition, and p-nitrophenol (pNP) production was monitored at 410 nm ($\varepsilon_{410 \text{ nm}} = 15,460 \text{ M}^{-1} \text{ cm}^{-1}$). The specific phosphodiesterase activity towards 1 mM flavin adenine dinucleotide (FAD) was assessed using shrimp alkaline phosphatase as an auxiliary enzyme as previously described (Podzelinska et al. 2009).

General phosphatase activity towards the artificial substrate pNPP (pNPPase) was assayed spectrophotometrically at 25 °C. The standard reaction mixture (0.5 ml) contained 50 mM imidazole buffer, pH 7.0, 5 mM MgCl₂, 10 mM pNPP, and purified Ht-YitUBs (3 μg) or crude cell extract (0.1 mg of total protein). The reaction was initiated by the addition of pNPP and monitored by continuously following the production of pNP at 410 nm. No activity was detected in the control reaction, which excluded the enzyme.

Specific phosphatase (5′-nucleotidase) activity towards physiological substrates was assayed by the rate of inorganic phosphate (Pi) release. A standard reaction mixture (0.5 ml) contained 50 mM imidazole buffer, pH 7.0, 5 mM MgCl₂, 3 mM or 15 mM substrate for purified Ht-YitUBs (from 0.08 to 3 μg), and crude cell extract (0.1 mg of total protein), respectively. The assay was initiated by substrate addition and carried out at 30 °C for 10 min. The reaction rate was linear under these conditions. The amount of released inorganic phosphate (Pi) was assessed by a previously described colorimetric method (Chen et al. 1956). For acid-labile substrates (all di- and triphosphates, sugar phosphates, NADP, pyridoxal 5-phosphate, phosphonoacetic acid, phosphoenolpyruvate, PRPP), Pi was assessed by the method of Cariani (Cariani et al. 2004). Pi concentrations were estimated from a standard curve obtained with KH₂PO₄. To exclude the influence of nonenzymatic factors, the background phosphate level was monitored in parallel using a control reaction without enzyme.

The activity was calculated by subtracting nonspecific substrate addition, and p-nitrophenol (pNP) production was monitored at 410 nm ($\varepsilon_{410 \text{ nm}} = 15,460 \text{ M}^{-1} \text{ cm}^{-1}$). The specific phosphodiesterase activity towards 1 mM flavin adenine dinucleotide (FAD) was assessed using shrimp alkaline phosphatase as an auxiliary enzyme as previously described (Podzelinska et al. 2009).

The pH dependence of the phosphatase activity towards pNPP (pNPPase) was assayed spectrophotometrically at 25 °C. The standard reaction mixture (0.5 ml) contained 50 mM imidazole buffer, pH 7.0, 5 mM MgCl₂, 10 mM pNPP, and purified Ht-YitUBs (3 μg) or crude cell extract (0.1 mg of total protein). The reaction was initiated by the addition of pNPP and monitored by continuously following the production of pNP at 410 nm. No activity was detected in the control reaction, which excluded the enzyme.

Specific phosphatase (5′-nucleotidase) activity towards physiological substrates was assayed by the rate of inorganic phosphate (Pi) release. A standard reaction mixture (0.5 ml) contained 50 mM imidazole buffer, pH 7.0, 5 mM MgCl₂, 3 mM or 15 mM substrate for purified Ht-YitUBs (from 0.08 to 3 μg), and crude cell extract (0.1 mg of total protein), respectively. The assay was initiated by substrate addition and carried out at 30 °C for 10 min. The reaction rate was linear under these conditions. The amount of released inorganic phosphate (Pi) was assessed by a previously described colorimetric method (Chen et al. 1956). For acid-labile substrates (all di- and triphosphates, sugar phosphates, NADP, pyridoxal 5-phosphate, phosphonoacetic acid, phosphoenolpyruvate, PRPP), Pi was assessed by the method of Cariani (Cariani et al. 2004). Pi concentrations were estimated from a standard curve obtained with KH₂PO₄. To exclude the influence of nonenzymatic factors, the background phosphate level was monitored in parallel using a control reaction without enzyme.

The activity was calculated by subtracting nonspecific substrate addition, and p-nitrophenol (pNP) production was monitored at 410 nm ($\varepsilon_{410 \text{ nm}} = 15,460 \text{ M}^{-1} \text{ cm}^{-1}$). The specific phosphodiesterase activity towards 1 mM flavin adenine dinucleotide (FAD) was assessed using shrimp alkaline phosphatase as an auxiliary enzyme as previously described (Podzelinska et al. 2009).

General phosphatase activity towards the artificial substrate pNPP (pNPPase) was assayed spectrophotometrically at 25 °C. The standard reaction mixture (0.5 ml) contained 50 mM imidazole buffer, pH 7.0, 5 mM MgCl₂, 10 mM pNPP, and purified Ht-YitUBs (3 μg) or crude cell extract (0.1 mg of total protein). The reaction was initiated by the addition of pNPP and monitored by continuously following the production of pNP at 410 nm. No activity was detected in the control reaction, which excluded the enzyme.

Specific phosphatase (5′-nucleotidase) activity towards physiological substrates was assayed by the rate of inorganic phosphate (Pi) release. A standard reaction mixture (0.5 ml) contained 50 mM imidazole buffer, pH 7.0, 5 mM MgCl₂, 3 mM or 15 mM substrate for purified Ht-YitUBs (from 0.08 to 3 μg), and crude cell extract (0.1 mg of total protein), respectively. The assay was initiated by substrate addition and carried out at 30 °C for 10 min. The reaction rate was linear under these conditions. The amount of released inorganic phosphate (Pi) was assessed by a previously described colorimetric method (Chen et al. 1956). For acid-labile substrates (all di- and triphosphates, sugar phosphates, NADP, pyridoxal 5-phosphate, phosphonoacetic acid, phosphoenolpyruvate, PRPP), Pi was assessed by the method of Cariani (Cariani et al. 2004). Pi concentrations were estimated from a standard curve obtained with KH₂PO₄. To exclude the influence of nonenzymatic factors, the background phosphate level was monitored in parallel using a control reaction without enzyme.

The activity was calculated by subtracting nonspecific substrate hydrolysis measured in the absence of protein, which was no more than 5% of the total activity. One unit of activity was defined as 1 μmol of Pi released per minute at 30 °C.

The pH dependence of the phosphatase activity towards pNPP (10 mM) or 5′-GMP (3 mM) was determined in the presence of 5 mM MgCl₂ and purified Ht-YitUBs. The assays were performed in the following buffer systems (50 mM): MES buffer between pH 5.5 and 6.5, imidazole buffer between pH 6.0 and 7.5, Tris-HCl buffer between pH 7.1 and 8.9, and CHES buffer between pH 9 and 9.5.

The metal dependence of the phosphatase activity of purified Ht-YitUBs, towards pNPP (10 mM) or 5′-IMP (3 mM) was determined in 50 mM imidazole buffer, pH 7.0, using various divalent metal ions (Mg²⁺, Mn²⁺, Co²⁺, Ni²⁺).

To determine the Michaelis constant ($K_m$) and maximal initial velocity ($V_{max}$), kinetic analyses were performed using the appropriate activity assay with at least ten different concentrations of substrate in the range of 0 to 20 mM for nucleotides and 0 to 3 mM for FMN. The measured activities were analyzed using the Lineweaver–Burk plot or Hill plot (for AICAR-P) with the nonlinear curve-fitting program GraphPad Prism 8 software (GraphPad Software, Inc., San Diego, CA, USA). All $k_{cat}$ values correspond to the turnover number per monomer. All kinetic parameters were obtained from at least three measurements.

**LC-MS/MS analysis**

Detection of RF in samples was performed by LC-MS/MS using an Acquity system with a Xevo TQD mass detector (Waters) and a previously described method (Guo et al. 2006) with the following modifications. Chromatographic separation was achieved with an Acquity UPLC BEH C18 (1.7 μm, 2.1 × 100 mm) column. UPLC conditions were set as follows: column temperature 30 °C, λ = 222 nm, injection volume 5 μl, flow rate 0.3 ml/min, buffers: [A], 5%, and [B], 95% methanol in water. The gradient was as follows: [B] was increased from 5 to 70% over 10 min, then held for 2 min at 70%, decreased to 5% over 0.5 min and held for 2.5 min at 5%. The MS/MS conditions were as follows: electrospray ionization (ESI), positive ion mode, multiple reaction monitoring mode, capillary voltage 3.5 kV, desolvation temperature 600 °C, source gas flow 800 L/H, cone gas flow 3 L/H, source temperature 150 °C, cone voltage 32 V, and collision energy 25 V. The precursor-to-product ion transitions $m/z$ 377 → $m/z$ 243, $m/z$ 377 → $m/z$ 198, $m/z$ 377 → $m/z$ 172, $m/z$ 377 → $m/z$ 117, and $m/z$ 377 → $m/z$ 99 were used for quantification. Standards were prepared by dissolving RF in Milli-Q water. The calibration range for the mass spectrometer was from 45 to 4500 μg/l. The limit of detection was 10 μg/l.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA). One-way ANOVA and Tukey’s multiple-comparison test were used to determine significant differences among sample means. Tests were considered to be statistically significant if $P$ values lower than 0.05 were obtained.

**Results**

**Search for B. subtilis 5′-nucleotidases using the selection of clones resistant to purine nucleosides**

To identify genes encoding 5′-nucleotidases in Bacillus species, a method to exploit the hydrolytic dephosphorylation activity of the gene products was applied. This method was based on “shotgun” cloning followed by the direct selection of DNA fragments containing 5′-nucleotidase genes identified...
by the resistance of recombinant E. coli cells to the purine nucleosides guanosine and inosine at inhibitory concentrations.

The uptake of extracellular nucleosides at even high concentrations is not toxic for wild-type E. coli cells (Petersen 1999). However, the phosphorylation of intracellular guanosine (inosine) catalyzed by guanosine-inosine kinase (EC 2.7.1.73) encoded by the gsk gene leads to the formation of GMP (IMP) (Fig. S1). GMP is further converted to IMP, AMP, and ADP, which, at high concentrations, inhibit the activity of 5-phosphoribosyl-1-pyrophosphate (PRPP) synthetase (Willemoës et al. 2002), resulting in PRPP deficiency and growth arrest (Petersen 1999). Proper functioning of PRPP synthetase is essential for life because PRPP is the biosynthetic precursor of the amino acids histidine and tryptophan, as well as purine, pyrimidine, and pyridine (NAD+, NADP+) nucleotides. Growth arrest is prevented in wild-type E. coli cells by the degradation of guanosine (inosine) to guanine (hypoxanthine) and ribose-1-phosphate (catalyzed by purine nucleoside phosphorylase encoded by deoD) and feedback inhibition of guanosine-inosine kinase activity by GMP (Fig. S1). However, the addition of guanosine (inosine) to the growth medium of E. coli strain GS72 (TG1 ΔdeoD gsk-3) caused an uncontrolled increase in intracellular GMP (IMP) and then AMP/ADP pools, followed by PRPP synthetase inhibition and growth arrest (Petersen 1999). Based on these data, we hypothesized that the dephosphorylation of excess nucleotides via 5′-nucleotidase gene overexpression in the gsk-3 ΔdeoD strain would remove PRPP synthetase inhibition and rescue sensitivity to the purine nucleosides guanosine and inosine.

Therefore, to find genes encoding enzymes with 5′-nucleotidase activity in B. subtilis and B. amyloliquefaciens, genomic libraries for the B. subtilis BsΔDEG (168 ΔdeoD ΔpupG ΔpupF) and B. amyloliquefaciens IAMADG (IAM1523 ΔdeoD::Km, ΔpupG::Cm) strains were first obtained. Both strains contain deletions of purine nucleoside phosphorylase genes (unlike E. coli, these bacteria have two purine nucleoside phosphorylase genes, deoD and pupG) to exclude the selection of these genes in this search. Then, genomic DNA was digested with EcoRI and ligated to the EcoRI-digested low copy number vector pMW118 to obtain recombinant plasmids for the expression of cloned genes controlled by their own regulatory elements. The resulting recombinant plasmids containing DNA fragments from the genomic libraries were transferred into E. coli strain GS72 (TG1 ΔdeoD gsk-3), which is sensitive to purine nucleosides due to deoD and gsk-3 mutations, to select clones resistant to guanosine (50 μg/ml) and inosine (1500 μg/ml) at inhibitory concentrations. More than 50 plasmids in which DNA fragments ranging in size from 1600 to 6000 bp had been inserted were selected. These insertions were identified by sequence analysis, followed by an NCBI database sequence similarity search (Altschul et al. 1990). Plasmids conferring the highest level of resistance to purine nucleosides that simultaneously contained open reading frames (ORFs) encoding putative phosphatases were selected for further investigation. Identification of genes responsible for the resistance phenotype revealed the B. subtilis and B. amyloliquefaciens yutU genes (yitUBa and yitUBs, respectively), which encode putative phosphatases. These genes were recloned into the low copy number E. coli/B. subtilis shuttle vector, pMWAL1, under the control of their own regulatory elements, yielding the plasmids pMWAL1-yitUBa and pMWAL1-yitUBs, respectively. Resistance to inosine and guanosine conferred upon GS72 cells by these plasmids was confirmed (Supplementary Table S2). Moreover, pMWAL1-yitUBa and pMWAL1-yitUBs were also found to increase resistance to the purine analog 2,6-diaminopurine (DAP) (Supplementary Table S2).

In silico analysis of the 5′-untranslated regions (UTRs) of yitUBa and yitUBs did not reveal sequences that exactly matched consensus sequences from known SigA promoters. However, according to published data (Nicolas et al. 2012), B. subtilis yitU is transcribed from the SigA promoter as part of a tricistronic transcript that also includes the downstream ORFs BSU_11136 and yitC, both of which have unknown functions (Supplementary Fig. S2). Indeed, no putative Rho-independent transcription terminators immediately downstream of the yitU ORF were predicted using the ARNold: finding terminators web server (http://rna.igmors.u-psud.fr/toolbox/arnold/index.php). Based on low-level matching with the optimum consensus sequence of the identified yitU promoter, moderate expression of this gene, at least during exponential growth, was suggested. The UTRs of yitUBa and yitUBs demonstrated differences in their promoter and Shine–Dalgarno (SD) sequences, suggesting that these genes are expressed at different levels (Supplementary Fig. S3). Indeed, the pMWAL1-yitUBa and pMWAL1-yitUBs plasmids, in which yitUBa and yitUBs, respectively, are expressed under the control of their own regulatory elements, conferred different levels of resistance to purine nucleosides and DAP to GS72 cells (Supplementary Table S2). Moreover, pMWAL1-PyitUBa-yitUBs, which contained a DNA fragment in which the coding region of yitUBs was placed under control of the yitUBa UTR, conferred a higher level of resistance than pMWAL1-yitUBs.

When the yitU gene was identified in our previous study (Yusupova et al. 2014), the yitU product was annotated in the NCBI protein database (http://www.ncbi.nlm.nih.gov/protein) as a putative phosphatase and assigned to the CoT-type HAD-IIB subfamily of the HADSF and Cluster of Orthologous Groups of proteins (COG) no. 0561 (hydroxymethylpyrimidine pyrophosphatase and other HAD family phosphatases, ftp://ftp.ncbi.nih.gov/pub/COG/COG2014/static/byCOG/COG0561.html) due to the
presence of specific domains and its similarity with E. coli Cof hydrolase. The yitU Bs and yitU Ba genes possess a high nucleotide sequence similarity of 75.2%. The translated YitU Bs and YitU Ba proteins have 78.9% identical and 87.0% similar amino acid residues (Supplementary Fig. S4), suggesting an identical function for the YitU Bs and YitU Ba proteins.

**Heterologous expression and purification of YitU Bs**

To characterize the biochemical properties of YitU, two variants of yitU Bs (to translate YitUBs in its native form and as an N-terminally hexahistidine-tagged protein) were cloned into the expression vector pET15b(+), yielding the expression plasmids pET15-yitUBs and pET15-H6-yitUBs, respectively. After the introduction of these plasmids into E. coli strain BL21(DE3), both proteins were produced in a soluble form. The electrophoretic patterns of total extracted proteins by SDS-PAGE revealed protein bands with molecular masses of approximately 30 kDa, which was consistent with the predicted molecular masses of YitUBs and Ht-YitUBs (30.6 and 31.9 kDa, respectively). Moreover, these bands were not detected in the control strain, which contained empty pET15b(+) vector (Supplementary Fig. S5).

pNPPase activity towards the artificial substrate pNPP was assayed in BL21(DE3) (pET15b(+)), BL21(DE3) (pET15-yitUBs) and BL21(DE3) (pET15-H6-yitUBs) crude cell extracts (Fig. 1). YitUBs was shown to possess pNPPase activity. Moreover, the histidine tag at its N-terminus did not alter this activity. Therefore, further study was performed with the purified recombinant Ht-YitUBs protein.

The recombinant enzyme was purified to near homogeneity from the supernatant of disrupted BL21(DE3) (pET15-H6-yitUBs) cells using immobilized metal affinity chromatography (Supplementary Fig. S5).

The Ht-YitUBs subunit structure was analyzed by gel filtration. The protein eluted as a single symmetric peak with a retention time that corresponded to a molecular mass of approximately 32 ± 5 kDa, suggesting that the active form of the enzyme is monomeric (Supplementary Fig. S6).

**Biochemical characterization of recombinant Ht-YitUBs**

General phosphatase screening with respect to artificial chromogenic substrates demonstrated that Ht-YitUBs has no activity towards bis-pNPP and pNPPC (contrary to pNPP), suggesting the absence of phosphodiesterase activity. The optimum pH for Ht-YitUBs was estimated to be 7.0 in 50 mM imidazole buffer with pNPP and GMP as artificial and physiological substrates, respectively (Supplementary Fig. S7). Similar to other members of the HADSF, Ht-YitUBs absolutely requires Mg\(^{2+}\) for its activity. The optimal concentration of Mg\(^{2+}\) was shown to be 5 mM (Supplementary Fig. S8). A maximum pNPPase activity of 160 nmol/mg min was observed in imidazole buffer, pH 7.0, in the presence of 5 mM MgCl\(_2\).

Under optimal conditions, the phosphatase activity of purified Ht-YitUBs with respect to a wide spectrum of physiological substrates (deoxyribo- and ribonucleoside tri-, di-, and monophosphates; sugar phosphates; and other phosphorylated metabolites) was evaluated as described in the “Materials and methods” section. Ht-YitUBs demonstrated the highest activity towards deoxyribo- and ribonucleoside monophosphates (Table 2). FMN, dAMP, GMP, dGMP, CMP, AMP, XMP, IMP, and AICAR-P proved to be its preferred substrates.

The kinetic parameters with FMN, dAMP, GMP, dGMP, CMP, AMP, XMP, IMP, and AICAR-P were studied (Table 3). Ht-YitUBs was shown to have low substrate specificity (K\(_\text{m}\) values in the mM concentration range) and modest catalytic efficiencies with respect to all tested substrates except for FMN, for which the Michaelis constant was almost three orders of magnitude lower, and the catalytic efficiency was two orders of magnitude higher than those of the other tested substrates. The kinetic behavior of the enzyme in the hydrolysis of the tested substrates, except AICAR-P, followed Michaelis-Menten kinetics. For AICAR-P, the kinetic curve indicated allosterism with a Hill coefficient of 1.83 ± 0.15.

**Overexpression of yitU increased the extracellular accumulation of RF by wild-type B. subtilis**

Inactivation of yitU in the chromosome of the wild-type B. subtilis strain, BsC\(^+\) (B. subtilis 168 trpC\(^+\)), had essentially
no effect on cell growth and the glucose consumption rate during its cultivation in minimal medium (Supplementary Fig. S9). When the expression plasmids pMWAL1-yitUBs, pMWAL1-yitUBa, and pMWAL1-PyitUBa-yitUBs or the empty vector pMWAL1 were transferred into BsC+, the resulting transformants were cultivated in minimal medium, and the culture broth of cells overexpressing yitU developed a yellow-green color (Fig. 2a). Moreover, the intensity of the color depended on the type of the yitU expression plasmid and was most intense in the case of the pMWAL1-PyitUBa-yitUBs plasmid. Comparison of the 5′-nucleotidase activities in BS168 Δyuf harboring pMWAL1, pMWAL1-yitUBs, pMWAL1-yitUBa, and pMWAL1-PyitUBa-yitUBs showed that pMWAL1-PyitUBa-yitUBs conferred the highest level of activity, suggesting the highest level of yitU expression due to the 5′ UTR of the B. amyloliquefaciens gene (Fig. 3). The BS168 ΔyutF strain, in which another 5′-nucleotidase gene, yutF, was disrupted, was used in this assay to exclude the impact of yutF on 5′-nucleotidase activity. Interestingly, we observed a more intense yellow-green color with BS168 ΔyutF (pMWAL1-PyitUBa-yitUBs) strain (Fig. 2c vs Fig. 2a).

Since FMN is the preferred substrate of YitU, we supposed that the colored compound that accumulated in the culture broth is the product of FMN dephosphorylation, RF. Consistent with this suggestion, fluorescence of the colored culture broth was observed under UV light (Fig. 2b, d). Moreover, agar diffusion assays demonstrated that an RF auxotrophic strain, B. subtilis 168 Δrib, formed halos of growth around cells containing the pMWAL1-yitUBs, pMWAL1-yitUBa, and pMWAL1-PyitUBa-yitUBs plasmids expressing yitU, most likely due to RF feeding (Fig. 4).

Indeed, LC-MS/MS analysis of cell-free culture broth supernatants of BsC+ bearing the empty vector, pMWAL1, or the pMWAL1-PyitUBa-yitUBs plasmid confirmed the presence of RF (Supplementary Fig. S10). Moreover, the RF concentration in the strain overexpressing yitU was 20 times higher than that in the strain harboring empty vector (2 mg/l vs 0.1 mg/l, respectively).

The kinetics of RF accumulation in the culture broths of BsC+ cells in which yitU was disrupted or overexpressed were studied. In this experiment, the BsC+ strain and its ΔyitU derivative did not accumulate RF at detectable levels (Fig. 5, Supplementary Table S3). Derivatives of BsC+ harboring the pMWAL1-yitUBs, pMWAL1-yitUBa, and pMWAL1-PyitUBa-yitUBs plasmids accumulated in culture broths from 1 to 5 mg/l RF. The plasmid expression of yitUBs under control of the yitUBs promoter region (pMWAL1-PyitUBa-yitUBs) led to a nearly fivefold increase in RF accumulation compared with the plasmid expression of yitUBs under native regulation (pMWAL1-yitUBs).

### Table 2

| Substrate                      | $A$ (μmol/mg min$^{-1}$) | Source or reference |
|-------------------------------|---------------------------|---------------------|
| FMN (0.1 mM)                  | 24.8 ± 3.6                | Sarge et al. 2015   |
| ARPP (0.3 mM)                 | 17 ± 2                    |                     |
| dAMP                          | 1.7 ± 0.4                 |                     |
| GMP                           | 13.1 ± 1.9                |                     |
| CMP                           | 12.1 ± 1.7                |                     |
| dAMP                          | 10.9 ± 1.5                |                     |
| AMP                           | 9.7 ± 1.4                 |                     |
| GMP                           | 9.5 ± 1.6                 |                     |
| XMP                           | 8.4 ± 1.4                 |                     |
| IMP                           | 6.4 ± 0.9                 |                     |
| AICAR-P                       | 2.8 ± 0.6                 |                     |
| 2′AMP                         | 2.2 ± 0.4                 |                     |
| CDP                           | 1.4 ± 0.4                 |                     |
| UMP                           | 1.3 ± 0.3                 |                     |
| GDP                           | 1.3 ± 0.4                 |                     |
| 6-Phospho-gluconate           | 1.2 ± 0.3                 |                     |
| IDP                           | 0.90 ± 0.19               |                     |
| Pyridoxal 5-phosphate         | 0.78 ± 0.12               |                     |
| NADP                          | 0.75 ± 0.11               |                     |
| Ribose-5-phosphate            | 0.54 ± 0.08               |                     |
| TDP                           | 0.52 ± 0.07               |                     |
| Mannose 6-phosphate           | 0.52 ± 0.07               |                     |
| 3′AMP                         | 0.37 ± 0.06               |                     |
| Glucose 6-phosphate           | 0.33 ± 0.05               |                     |
| ADP                           | 0.32 ± 0.05               |                     |
| Fructose 6-phosphate          | 0.29 ± 0.04               |                     |
| ITP                           | 0.21 ± 0.04               |                     |
| Erythrose 4-phosphate         | 0.19 ± 0.03               |                     |
| CTP                           | 0.16 ± 0.03               |                     |
| GTP                           | 0.14 ± 0.02               |                     |
| Phosphoribosyl pyrophosphate  | 0.13 ± 0.02               |                     |
| UDP                           | 0.13 ± 0.02               |                     |
| ATP                           | 0.12 ± 0.02               |                     |
| FAD (1 mM)                    | < 0.03                    |                     |
| UTP                           | < 0.03                    |                     |
| Phosphoenolpyruvate           | < 0.03                    |                     |
| Glucose 1-phosphate           | < 0.03                    |                     |
| Phosphonoacetic acid          | < 0.03                    |                     |

1 The rates of substrate (3 mM unless otherwise specified) hydrolysis by purified Ht-YitU (0.12 μg) were measured as described in the “Materials and methods” section. The specific activity is presented as μmoles of Pi released per min per milligram of protein. The results are expressed as the means of three independent experiments ± standard error of the mean.

Disruption of yitU decreased, while enhancement of yitU expression increased, RF accumulation in an RF-producing strain

To further investigate the influence of yitU on RF production, the gene was disrupted and overexpressed in an RF-producing
strain. *B. subtilis* Y25 can produce RF due to the increased expression of purine biosynthetic genes, overexpression of *rib* operon genes and deficiency of RF kinase activity (*ribC1*). This strain was obtained by the traditional selection of clones resistant to the purine analog 8-azaguanine and the RF analog roseoflavin (Mironov et al. 2002). Inactivation of *yitU* in strain Y25 reduced both RF accumulation and the glucose consumption rate at the productive phase but slightly increased the accumulated biomass (Fig. 6a, b). In contrast, *yitU* expression from plasmids pMWAL1-*yitUBs* and pMWAL1-*yitUBa* increased RF accumulation and slightly enhanced glucose consumption in strain Y25 at the productive phase (Fig. 6c, d).

| Substrate | $K_m$ (mM) | $V_{max}$ (U mg$^{-1}$) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ M$^{-1}$) | Source or reference |
|-----------|-----------|-------------------------|-------------------|------------------------|-------------------|
| FMN       | 0.096 ± 0.015 | 43.18 ± 1.52              | 22.97 | 2.39 × 10$^5$ | |
| ARPP      | 0.081 ± 0.006 | –                      | 0.88   | 1.09 × 10$^4$ | Sarge et al. 2015 |
| dAMP      | 8.24 ± 0.42   | 49.35 ± 1.10              | 26.25   | 3.19 × 10$^3$ | |
| GMP       | 7.00 ± 0.29   | 39.97 ± 0.69              | 21.26   | 3.04 × 10$^3$ | |
| dGMP      | 5.61 ± 0.79   | 25.95 ± 1.33              | 13.80   | 2.46 × 10$^3$ | |
| CMP       | 17.45 ± 1.82  | 73.65 ± 3.94              | 39.18   | 2.25 × 10$^3$ | |
| AMP       | 14.32 ± 1.01  | 52.94 ± 2.13              | 28.16   | 1.97 × 10$^3$ | |
| XMP       | 21.74 ± 2.93  | 70.47 ± 6.56              | 37.49   | 1.72 × 10$^3$ | |
| IMP       | 17.81 ± 3.15  | 49.51 ± 4.53              | 26.34   | 1.48 × 10$^3$ | |
| AICAR-P   | 10.19 ± 1.50  | 21.20 ± 4.12              | 11.28   | 1.11 × 10$^3$ | |

*The kinetic parameters were determined using the activity assay described in the “Materials and methods” section with at least ten different substrate concentrations. The results are expressed as the means of three independent experiments ± standard error of the mean.*

Disruption of *yitU* decreased, while enhancement of *yitU* expression increased, AICAR accumulation in an AICAR-producing strain

Since we observed the specific behavior of Ht-YitUBs in AICAR-P hydrolysis, the effect of *yitU* disruption and overexpression on the performance of an AICAR-producing strain was studied. Strain AJ1991purH::spc can produce AICAR due to enhanced de novo purine biosynthesis and the blockade of the conversion of AICAR-P to IMP. Several derivatives of AJ1991purH::spc have been constructed. First, *yitU* was disrupted in the chromosome of AJ1991purH::spc, yielding strain AJΔU. The *yitU* overexpression plasmids pMWAL1-*yitUBa* and pMWAL1-*yitUBs* and the
empty vector pMWAL1 (used as a control) were transferred into AJ1991purH::spc and AJΔU. The resulting strains were tested by test tube fermentation to evaluate the kinetics of cell growth, glucose consumption, and AICAR accumulation (Fig. 7). yitU deletion in AJ1991purH::spc had essentially no effect on cell growth but drastically decreased the glucose consumption rate and AICAR production (Fig. 7a, b).

**Fig. 3** 5’-Nucleotidase activity towards 15 mM IMP in *B. subtilis* strains overexpressing yitU<sub>Bs</sub> and yitU<sub>Ba</sub>. The values are the means ± standard deviations of three independent experiments.

**Fig. 4** Agar diffusion assay. Halos of growth of the RF auxotrophic strain *B. subtilis* 168 Δrib around plaques of the following strains: (1) BS168 ΔyutF, (2) BS168 ΔyutF ΔU, (3) BS168 ΔyutF (pMWAL1), (4) BS168 ΔyutF (pMWAL1-yitUBs), (5) BS168 ΔyutF (pMWAL1-yitUBa). Some error bars are smaller than the data point icons.

**Fig. 5** Extracellular RF accumulation in *B. subtilis* BsC<sup>+</sup> harboring pMWAL1-yitUBs, pMWAL1-yitUBa, and pMWAL1-PyitUBa-yitUBs. The values are the means ± standard deviations of three independent experiments.

Appl Microbiol Biotechnol (2020) 104:2957–2972
yitU_{Ba} overexpression in AJΔU restored AICAR accumulation, which was lost in this strain due to yitU disruption (Fig. 7d). Moreover, compared with the control strain AJ1991purH::spc (pMWAL1), strain overexpressing yitU_{Ba} (AJ1991purH::spc (pMWAL1-yitU_{Ba})) demonstrated 1.6-fold increase in AICAR accumulation (Fig. 7d), less accumulated biomass, which nevertheless did not lead to a reduction in the glucose consumption rate (Fig. 7c) most likely due to more active product biosynthesis. The same effects on growth, glucose consumption, and AICAR accumulation were observed in AJ1991purH::spc and AJΔU due to yitU_{Ba} expression from pMWAL1-yitU_{Ba} (Supplementary Table S4, Supplementary Fig. S11).

Discussion

Despite the important role of 5′-nucleotidases in cellular metabolism, only a few of these enzymes have been characterized in the gram-positive bacteria B. subtilis and B. amyloliquefaciens, the workhorses among industrial microorganisms. To identify genes encoding 5′-nucleotidases in Bacillus species, a search for genes homologous to earlier characterized 5′-nucleotidase genes in other bacteria, for example, E. coli, is often used as a suitable tool (Terakawa et al. 2016; Zakataeva et al. 2016). In this study, another method exploiting 5′-nucleotidase activity in gene products was applied. This method was based on “shotgun” cloning followed by the direct selection of DNA fragments containing 5′-nucleotidase genes due to the resistance of recombinant E. coli GS72 (TG1 deoD gsk-3) cells to the purine nucleosides guanosine and inosine at inhibitory concentrations.

Using this strategy, orthologous yitU genes were selected from genomic libraries of B. subtilis and B. amyloliquefaciens strains. Their products belong to the HADSF and have a high sequence similarity of 87%, suggesting the identical functions of these proteins. The B. subtilis yitU gene was produced in E. coli as an N-terminal hexahistidine-tagged protein, purified, and biochemically characterized as a soluble 5′-nucleotidase with a broad substrate specificity. Like many 5′-nucleotidases of the HADSF, YitU can dephosphorylate a wide range of substrates, including deoxyribo- and ribonucleotides. Among these compounds, the enzyme has the highest catalytic efficiency with respect to the monophosphates dAMP, GMP, dGMP, CMP,
AMP, XMP, IMP, and AICAR-P. However, the preferred substrate with a Michaelis constant almost three orders of magnitude lower than the $K_m$ values for the listed monophosphates was shown to be FMN ($K_m = 0.096$ mM). While this work was in progress, Sarge and coauthors reported that the products of the $B.\ subtilis$ genes $ycsE$, $ywtE$, and $yitU$ catalyze the dephosphorylation of ARPP (designated as 6 in the original publication) with high catalytic efficiency, forming the pyrimidine precursor of RF, 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (Sarge et al. 2015). The relatively high specific activities of YcsE, YwtE, and YitU towards FMN were also demonstrated (Sarge et al. 2015). In our experiments, we did not study the activity and kinetic characteristics of Ht-YitUBs towards ARPP due to the commercial inaccessibility of this compound and instead used data obtained by Sarge and coauthors for comparison. We found that although the Michaelis constants for ARPP and FMN were approximately the same, the catalytic constant ($k_{cat}$) and catalytic efficiency ($k_{cat}/K_m$) of the enzyme for FMN were considerably higher than those for ARPP (Table 3). While Sarge and coauthors did not report the kinetic parameters for YitU with respect to FMN as a substrate, their data on the specific activity of purified YitU towards this compound are consistent with the value we report here (Table 2).

The expression of several enzymes (YcsE, YwtE, and YitU) with substrate specificity towards ARPP and FMN but different affinities for each of these substrates in $B.\ subtilis$ might be necessary for fine-tuning cellular pools of the important flavins RF, FMN, and FAD. The main derivatives of RF, FMN and FAD, are redox-active coenzymes that associate with proteins to form flavoproteins. Flavoproteins function in a large variety of metabolic pathways, including electron transport, DNA repair, nucleotide biosynthesis, the synthesis of cofactors and heme groups, the $\beta$-oxidation of fatty acids, and amino acid catabolism (Abbas and Sibirny 2011). The role of flavoproteins in cellular redox metabolism is ensured by the ability of flavins to transfer electrons. Importantly, unlike other electron transfer cofactors, flavins can mediate both one-electron and two-electron transfer processes (Edwards 2014), making them one of the most important types of cofactors in cells. The intracellular concentrations, composition, and ratios of free flavins should be strongly regulated. FMN controls the biosynthesis and transport of RF by regulating related genes at the level of transcription or translation through a
riboswitch mechanism (Gelfand et al. 1999; Winkler et al. 2002). YcsE, YwtE, and YitU, phosphatases with different flavin specificities, most likely exert their regulatory effects in conjunction with another enzyme involved in the conversion of RF to FMN and FMN to FAD, bifunctional flavokinase/flavin adenine dinucleotide synthetase (encoded in B. subtilis and B. amyloliquefaciens by ribC) (Mack et al. 1998).

ARP deficiency is commonly assumed not to be a bottleneck in RF production even in industrial producers that strongly overexpress other RF biosynthetic genes (Hübelin et al. 1999; Perkins et al. 1999). In this study, we have shown that enhanced activity of the 5′-nucleotidase YitU in B. subtilis not only further elevated RF production in the RF-producing strain Y25 but also significantly increased RF accumulation in the wild-type strain BscC+, making it an RF producer. The positive effect of yitU overexpression on RF production can be attributed to at least two factors: enhanced de novo RF synthesis due to activation of one of its steps (ARP deficiency) and the enhanced conversion of FMN to RF, leading to a reduction in the FMN pool and thus upregulating RF biosynthesis.

Interestingly, the pMWAL1-P-yitUBs plasmid, which was shown to support the highest level of YitU activity, led to the severe retardation of Y25 (pMWAL1-P-yitUBs-yitUBs) growth (data not shown), most likely due to a drastic deficiency in the redox-active cofactors FMN and FAD caused by the simultaneously impaired activity of bifunctional RF kinase/FMN adenyltransferase (ribCl) and enhanced activity of FMN hydrolase. In contrast, the disruption of yitU in the chromosome of the Y25 strain reduced RF accumulation but increased cell growth, most likely due to a decrease in the conversion of FMN to RF, making FMN and FAD more available for various flavoproteins that catalyze important redox reactions in metabolism. In this study, we did not investigate the reason for increased RF accumulation due to yitU overexpression under the genetic background in which another 5′-nucleotidase gene, yutF, was deleted. The elimination of YutF function may have reduced the hydrolysis of some phosphorylated metabolites involved in RF biosynthesis.

AICAR-P, an intermediate in the purine nucleotide biosynthetic pathway and a byproduct of histidine biosynthesis, is a natural analog of AMP and a very important regulatory compound in bacteria, yeast, and humans. By both direct and indirect mechanisms, AICAR-P affects the biosynthesis of purines, thiamine, and histidine as well as one-carbon, carbohydrate, and lipid metabolism (Hürllmann et al. 2011; Daigean-Fornier and Pinson 2012; Bazurto et al. 2015; Ducker and Rabinowitz 2015; Malykh et al. 2018). In our study of the kinetic parameters of recombinant YitU, contrary to hydrolysis of the other tested substrates, which followed Michaelis-Menten kinetics, the kinetics of AICAR-P hydrolysis exhibited a sigmoidal behavior with a Hill coefficient of 1.83 ± 0.15, indicating positive cooperation. Gel filtration experiments showed that the active form of the enzyme is a monomer. Although cooperativity is traditionally observed in enzymes with multiple ligand-binding sites and/or multimeric assemblies, a few monomeric enzymes with single ligand-binding sites that display cooperativity have been described (Porter and Miller 2012). For example, among such enzymes is the best-studied mammalian glucokinase, which demonstrated a special type of allosteric regulation in which cooperativity was observed due to the rates of substrate transformation associated exclusively with conformational reorganization that occurs during substrate association (Storer and Cornish-Bowden 1976; Larion and Miller 2012).

The $K_m$ value of YitU for AICAR-P as a substrate is in the millimolar concentration range and significantly higher than the physiological concentrations of AICAR-P (from 1.6 to 21.8 μM in exponentially grown yeast cells (Daigean-Fornier and Pinson 2012)). Therefore, YitU might hydrolyze AICAR-P under conditions in which this metabolite is oversynthesized. Moreover, positive cooperativity of the enzyme during AICAR-P hydrolysis could allow the cell to adapt to conditions in which the AICAR-P pool sharply increases. Indeed, in strain AJ1991purH::spc, in which the de novo purine biosynthetic pathway is enhanced and the conversion of AICAR-P to IMP is blocked, the plasmid expression of both B. subtilis and B. amyloliquefaciens yitU resulted in the increased accumulation of the product of AICAR-P hydrolysis, AICAR. The disruption of yitU in the chromosome of the AICAR producer AJ1991purH::spc had essentially no effect on cell growth but led to a decrease in AICAR production. This effect can be explained by the inhibition of purine biosynthesis by the drastically increased AICAR-P pool and supports the suggestion that YitU in B. amyloliquefaciens possesses major AICAR-P dephosphorylation activity.

To summarize, in this study, a new approach was used to search for 5′-nucleotidase genes, following which the yitU gene was selected. The product of this gene belongs to the HADSF and not only exhibits specificity for a wide spectrum of deoxyribo- and ribonucleoside monophosphates but also is involved in de novo (Sarge et al. 2015) and salvage RF biosynthesis (from FMN) pathways. Due to its ability to dephosphorylate the important redox-active cofactor FMN and an AMP analog with multiple regulatory functions, AICAR-P, YitU is involved in regulating cellular metabolism. It was also demonstrated for the first time that the overexpression of yitU can be successfully applied to breed highly performing RF- and AICAR-producing strains.

Acknowledgments We thank O. Soshnikova for participating in “shotgun” cloning experiments.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.
Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

Abbas CA, Sibimy AA (2011) Genetic control of biosynthesis and transport of riboflavin and flavin nucleotides and construction of robust biotechnological producers. Microb Mol Biol Rev 75(2):321–360. https://doi.org/10.1128/MMBR.00030-10

Abriouel H, Franz CM, Ben Omar N, Galvez A (2011) Diversity and applications of Bacillus bacteriocins. FEMS Microbiol 35(1):201–232. https://doi.org/10.1111/j.1574-6976.2010.02244.x

Altschul SF, Gish W, Miller W, Myers E.W, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215(3):403–410

Asahara T, Mori Y, Zakataeva NP, Livshits VA, Yoshida K, Matsuno K (2005) The haloacid dehalogenase-like protein halO from Escherichia coli encodes a major facilitator superfamily protein involved in efflux of purine ribonucleosides. FEMS Microbiol Lett 250(1):39–47. https://doi.org/10.1016/j.femsle.2005.06.051

Guérout-Fleury AM, Shazand K, Frandsen N, Stragier P (1995) Antibiotic-resistance cassettes for Bacillus subtilis. Gene 167(1–2):335–336. https://doi.org/10.1016/0378-1119(95)00652-4

Guo J, Lu Y, Dong H (2006) HPLC–MS analysis of the riboflavin crude product of semisynthesis. J Chromatogr Sci 44(9):552–556

Hümbelin M, Grieser V, Keller T, Schurter W, Haiker M, Hohmann HP, Ritz H, Richter G, Bacher A, van Loon APGM (1999) GTP cyclohydrolase II and 3,4-dihydroxy-2-butanoate 4-phosphate synthase are rate-limiting enzymes in riboflavin synthesis of an industrial Bacillus subtilis strain used for riboflavin production. J Ind Microbiol Biotechnol 22:1–7

Hunsucker SA, Mitchell BS, Spychala J (2005) The 5′-nucleotidases as regulators of nucleotide and drug metabolism. Pharmacol Ther 107(1):1–30. https://doi.org/10.1016/j.pharmthera.2005.01.003

Hürlimann HC, Laloo B, Simon-Kayser B, Saint-Marc C, Couplier F, Lemoine S, Guérout-Fleury AM, Shazand K, Frandsen N, Stragier P (2011) Physiological and toxic effects of purine intermediate 5-amino-4-imidazolecarboxamide ribonucleotide (AICAR) in yeast. J Biol Chem 286(35):30994–31002. https://doi.org/10.1074/jbc.M111.262659

Ishiwa H, Shiba-hara-Sone H (1986) New shuttle vectors for Escherichia coli and Bacillus subtilis. IV. The nucleotide sequence of pHY300PLK and some properties in relation to transformation. Jpn J Genet 61:515–528

Koo BM, Kritikos G, Farello JD, Todor H, Tong K, Kimsey H, Wapinski I, Galardini M, Cabal A, Peters JM, Hachmann AB, Rudner DZ, Allen KN, Typas A, Gross CA (2017) Construction and analysis of two genome-scale deletion libraries for Bacillus subtilis. Cell Syst 4(3):291–305. https://doi.org/10.1016/j.cels.2016.12.013

Kunst F, Ogawasa N, Moszer I, Albertini AM, Alloni G, Azevedo V, Barerio MG, Bessières P, Bolotin A, Borchert S, Borsírs R, Bourrier L, Brans A, Braun M, Brignell SC, Bron S, Brouillet S, Bruschi CV, Caldwell B, Capuano V, Carter NM, Choi SK, Cordani J, Connerton IF, Cummings NJ, Daniel RA, Denzio F, Devine KM, Dürstöerhöft A, Ehrlich SD, Emmerson PT, Entian KD, Errington J, Fabret C, Ferrari E, Foulger D, Fritz C, Fujita M, Fujita Y, Fuma S, Galizia A, Galleron N, Ghim SY, Glaser P, Goffeau A, Golightly EJ, Grandi G, Guiseppe G, Guy BJ, Haga K, Hauich J, Harwood CR, Henraut A, Hilibert H, Holsapple S, Hosono S, Hullo MF, Itayama Jones L, Ioris B, Karamata D, Kasahara Y, Klarer-Blanchard M, Klein C, Kobayashi Y, Koetter P, Koningstein G, Krogh S, Kumano M, Kurita K, Lapidos A, Lardinosins D, Lauber J, Lazarevic V, Lee SM, Levine A, Liu H, Masuda S, Mauel C, Médigue C, Medina N, Mellado RP, Minozono M, Moestl D, Nakai S, Nobeak M, Noone D, Orelli M, Ogawa K, Ogawa I, Oudega B, Park SH, Parro V, Pohl TM, Portelle D, Porwollik S, Prescott AM, Presecan E, Pujić P, Purnelle B, Rapoport G, Rey M, Reynolds S, Rieger M, Rivolta C, Rocha E, Roche B, Rose M, Sadaie Y, Sato T, Scanlan E, Schleif C, Schreoter R, Scoffone F, Sekiguchi J, Sekowska A, Seror SJ, Seror P, Shin BS, Soldo B, Sorokin A, Taconi C, Takagi T, Takahashi H, Takemaru K, Takeuchi M, Tamakoshi A, Tanaka T, Terpsstra P, Togoni A, Tosato V, Uchiyama S, Vandelob M, Vannier E, Vassarotti A, Viana A, Wambutt R, Wedler H, Weizenegger T, Winter P, Wipat A, Yamamoto H, Yamane K, Yasumoto K, Yata K, Yoshida K, Yoshikawa HF, Zumstein E, Yoshikawa H, Danchin A (1997) The complete genome sequence of the gram-positive bacterium Bacillus subtilis. Nature 390(6657):249–256. https://doi.org/10.1038/36786

Kuznetsova E, Proudfoot M, Gonzalez CF, Brown G, Omelchenko MV, Borozan I, Carmel L, Wolf YI, Mori H, Savchenko AV, Arrowsmith CH, Koonin EV, Edwards AM, Yakunin AF (2006) Genome-wide analysis of substrate specificities of the Escherichia coli halolacid dehalogenase-like phosphatase family. J Biol Chem 281(47):36149–36161. https://doi.org/10.1074/jbc.M605449200
Matsuhisa A, Suzuki N, Noda T, Shiba K (1995) Inositol 2972 Appl Microbiol Biotechnol (2020) 104:2957

Lobanov KV, Ernais Lopes L, Korol’kova NV, Tyaglov BV, Glazunov AV, Shakulov RS, Mironov AS (2011) Reconstruction of purine metabolism in Bacillus subtilis to obtain the strain producer of AICAR: a new drug with a wide range of therapeutic applications. Acta Nat 3(2):79–89

Mack M, van Loon AP, Hofmann HP (1998) Regulation of riboflavin biosynthesis in Bacillus subtilis is affected by the activity of the flavokinase/flavin adenine dinucleotide synthetase encoded by rlyB. J Bacteriol 180(4):950–955

Malych EA, Butov IA, Ravcheeva AB, Krylov AA, Mashko SV, Kozlov YI, Dehabov VG (2002) Method for preparing riboflavin, strains Bacillus subtilis as producers of riboflavin. French patent application №9801196. 1998

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor, New York

Mironov AS, Korolkova NV, Tyaglov BV, Glazunov AV, Stoyanova NV (2018) Specific features of L-histidine production by Escherichia coli concerned with feedback control of AICAR formation and inorganic phosphate/metal transport. Microb Cell Factories 17(1):42–15. https://doi.org/10.1186/s12934-018-0890-2

Matsushita A, Suzuki N, Noda T, Shiba K (1995) Inositol monophosphatase activity from the Escherichia coli subB gene product. J Bacteriol 177(1):200–205. https://doi.org/10.1128/jb.177.1.200-205.1995

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor, New York

Mironov AS, Korolkova NV, Ernais LL, Semenova LE, Perumov DA, Kroneva RA, Glazunov AV, Akishina RI, Iomantas JV, Galuchkina ZM (1984) Method for preparing riboflavin. French patent application №8401176. 1984

Noirot P (2012) Condition-dependent transcriptome reveals high-level regulatory architecture in Bacillus subtilis. Science 335(6072):1103–1106. https://doi.org/10.1126/science.1205167

Porter CM, Miller BG (2012) Cooperativity in monomeric enzymes with single ligand-binding sites. Bioorg Chem 43:44–50. https://doi.org/10.1016/j.bioorg.2011.11.001

Sambrook J, Russell DW (2001) Molecular cloning: laboratory manual, 3rd edn. Cold Spring Harbor, New York

Sarge S, Haase I, Illarionov B, Lausert D, Hohmann HP, Bacher A, Fischer M (2015) Catalysis of an essential step in vitamin B2 biosynthesis by a consortium of broad spectrum hydrolases. ChemBioChem 16(17):2466–2469. https://doi.org/10.1002/cbic.201500352

Shalluey M, Singh A, Ward OP (2004) Developments in the use of Bacillus species for industrial production. Can J Microbiol 50:1–17. https://doi.org/10.1139/w03-076

Shatalin KY, Neyfakh AA (2005) Efficient gene inactivation in Bacillus anthracis. FEMS Microbiol Lett 245(2):315–319

Sheremet AS, Gronsky SV, Akhmadyshin RA, Novikova AE, Livshits VA, Shakulov RS, Zakataeva NP (2011) Enhancement of extracellular purine nucleoside accumulation by Bacillus strains through genetic modifications of genes involved in nucleoside export. J Ind Microbiol Biotechnol 38(1):65–70. https://doi.org/10.1007/s10295-010-0829-z

Smirnov SV, Kotliarova VA (2015) Method for producing isoprene using bacterium. Patent WO 2015056813. 2015;A1

Stepanov AI, Zhdanov VG, Kukanov AI, Khakinson MY, Rabinovich PM, Iomantas JV, Galuchkina ZM (1984) Method for preparing riboflavin. French patent application №3599355

Storer AC, Cornish-Bowden A (1976) Kinetics of rat liver glucokinase: co-operative interactions with glucose at physiologically significant concentrations. Biochem J 159(1):7–14

Terakawa A, Natsume A, Okada A, Nishihata S, Kuse I, Tanaka K, Takanaka S, Ishikawa S, Yoshida KI (2016) Bacillus subtilis 5′-nucleotidases with various functions and substrate specificities. BMC Microbiol 16(1):249–213. https://doi.org/10.1186/s12866-016-0866-5

Willemsø M, Hove-Jensen B, Larsen S (2002) Steady state kinetic model for the binding of substrates and allosteric effectors to Escherichia coli phosphoribosyl-diphosphate synthase. J Biol Chem 275(45):35408–35412. https://doi.org/10.1074/jbc.M006346200

Winkler WC, Cohen-Chalamish S, Breaker RR (2002) An mRNA structure that controls gene expression by binding FMN. Proc Natl Acad Sci U S A 99(25):15908–15913

Yusupova YR, Skripnikova VS, Zakataeva NP (2014) Gen e cloning and characterization of a new 5′-nucleotidase from Bacillus subtilis. Proceedings of the 16th International Biotechnology Symposium and Exhibition, Fortaleza, Brazil, Sept, 14–19th, 2014, A257:586–587. http://target.com.br/newclients/ibs2014.org/downloads/IBS_2014_final_e-Book.pdf

Zakataeva NP, Nikitina OV, Gronsky SV, Romanenkov DV, Livshits VA (2010) A simple method to introduce marker-free genetic modifications into the chromosome of naturally nontransformable Bacillus amyloliquefaciens strains. Appl Microbiol Biotechnol 85(4):1201–1209. https://doi.org/10.1007/s00253-009-2276-1

Zakataeva NP, Romanenkov DV, Yusupova YR, Skripnikova VS, Asahara T, Gronsky SV (2016) Identification, heterologous expression, and functional characterization of Bacillus subtilis YutF, A HAD superfamily 5′-nucleotidase with broad substrate specificity. PLoS One 11(12):e0167580. https://doi.org/10.1371/journal.pone.0167580

Zhang C, Du S, Liu Y, Xie X, Xu Q, Chen N (2015) Strategy for enhancing adenosine production under the guidance of transcriptional and metabolic pool analysis. Biotechnol Lett 37(7):1361–1369. https://doi.org/10.1007/s10529-015-1801-9

Zimmermann H (1992) 5′-Nucleotidase: molecular structure and functional aspects. Biochem J 285(2):345–365. https://doi.org/10.1042/bj2850345

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.