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ORIGINAL PAPER

Enhancement of extracellular purine nucleoside accumulation by *Bacillus* strains through genetic modifications of genes involved in nucleoside export

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Abstract Using a simple method to introduce genetic modifications into the chromosome of naturally nontransformable *Bacillus*, a set of marker-free inosine-producing and 5-aminoimidazole-4-carboxamide (AICA) ribonucleoside-producing *Bacillus amyloliquefaciens* strains has been constructed. These strains differ in expression levels of the genes responsible for nucleoside export. Overexpression of *B. amyloliquefaciens pbuE* and heterologous expression of *Escherichia coli nepI*, which encode nucleoside efflux transporters, each notably enhanced inosine production by a *B. amyloliquefaciens* nucleoside-producing strain. *pbuE* overexpression was found to increase AICA ribonucleoside accumulation, indicating that the substrate specificity of the PbuE pump extends to this nucleoside. These results demonstrate that identifying genes whose products facilitate transport of a desired nucleoside out of cells and enhancing their expression can improve the performance of strains used for industrial production.

Keywords Marker-free *Bacillus* strains · AICA ribonucleoside producer · *nepI* heterologous expression · *pbuE* overexpression

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Introduction

Bacillus subtilis, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, and *Bacillus pumilis* have been widely used as industrial producers of primary metabolites, including vitamins (riboflavin, folic acid, and biotin) and purine nucleosides. The inosine and guanosine nucleosides constitute raw materials for synthesis of the flavor enhancers inosine monophosphate (IMP) and guanosine monophosphate (GMP). In addition, inosine is used therapeutically. Another nucleoside, 5-aminoimidazole-4-carboxamide (AICA) ribonucleoside (AICAr), produced by a chemical method, could also be a potential target for biotechnology. AICAr, also called acadesine, is used for treatment of acute lymphoblastic leukemia and as an anti-ischemic agent, and may have applications in treating other disorders such as diabetes [2, 6, 12]. The remedial effect of AICAr on various metabolic disorders is achieved through its intracellular transformation into the respective nucleotide AICA ribonucleotide (AICAR). AICAR, in turn, exerts its effects through adenosine monophosphate (AMP)-activated protein kinase (AMPK), the regulator of carbohydrate and lipid metabolism in eukaryotes [3]. Moreover, it cannot be ruled out that other cellular pathways are influenced by AICAR; for example, direct suppression of hepatic gluconeogenesis by AICAR has been proposed [19].

Most *Bacillus* strains used for industrial applications have been constructed by the traditional mutagenesis and selection method. A significant disadvantage of this approach is accumulation of mutations, which can cause undesirable changes in physiology and growth retardation. Rational metabolic engineering by specific targeted modifications can overcome this disadvantage. However, a vast majority of industrially important strains are characterized by absence of natural competence for DNA uptake. This

feature complicates genetic manipulation of these microorganisms and makes methods for obtaining recombinant strains relatively time and labor intensive. Recently, a routine method has been developed to introduce marker-free deletions, insertions or point mutations into chromosomes of *Bacillus* strains, including those that are naturally nontransformable [22]. The method is based on a combination of effective plasmid transfer and the replacement recombination procedure, which occurs at a very high frequency due to the use of a thermosensitive rolling-circle replication plasmid as the delivery vector. The resulting colonies are screened using polymerase chain reaction (PCR) analysis. The use of PCR primers with mismatches at the 3′ end enables strains containing a single point mutation in the target gene to be selected. This method permits the generation of any genetic modification without positive selection, as well as the use of a counterselectable marker or a special prerequisite strain, and it can be successfully applied to metabolic engineering of food-grade industrial strains.

As previously shown for amino-acid-producing strains, design of high-efficiency producers requires identification and overexpression of genes encoding amino acid exporters. This overexpression has been shown to markedly improve the productivity of amino-acid-producing strains [4, 5, 11, 20].

Relatively little effort has been devoted to searching for and using nucleoside exporters to create efficient nucleoside-producing strains. It was found that *Escherichia coli* NepI (YicM) and its homologue from *Bacillus*, PbuE (YdhL), which was previously described as a purine base exporter, are involved in export of purine nucleosides [7, 9, 16, 21]. A low-copy plasmid containing the *B. amyloliquefaciens pbuE* gene was shown to increase the production of extracellular purine nucleosides by *E. coli* and *B. amyloliquefaciens* strains [21].

In the present study, a set of marker-free inosine-producing and AICA ribonucleoside-producing strains has been constructed by introducing genetic modifications into the chromosome of naturally nontransformable *B. amyloliquefaciens* strain using the replacement recombination method mentioned above. The obtained producers differ from each other by expressing different levels of the *pbuE* and *nepI* genes. The role of nucleoside export in purine nucleoside accumulation by these producers was investigated.

Materials and methods

Bacterial strains, bacteriophage, and growth conditions

In this study, we used the following strains: *E. coli* MG1655 (F^- λ^- *ilvG rfb-50 rph-1*) and TG1 [F′ *traΔ36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZΔM15/supE hsdΔ5 thi* Δ(*lac-proAB*)]; *Bacillus subtilis* 168 (*trpC2*) (ATCC 23857) and *B. amyloliquefaciens* wild-type IAM1523 (University of Tokyo, Japan) and its derivative AJ1991 (Ade^-, Ile^-, 8-azaguanine^R) (VKPM, Russian National Collection of Industrial Microorganisms, B 8994). Bacteriophage E40 (VKPM Ph-1629) [10] was used for plasmid transduction of *B. amyloliquefaciens*. *E. coli* and *Bacillus* cells were grown in Luria–Bertani (LB) or M9 minimal medium [15] supplemented with D-glucose (0.4% for *E. coli* or 2% for *Bacillus*). When required, thiamine HCl (5 μg/ml), amino acids (40 μg/ml), purine bases (50 μg/ml) or erythromycin (200 μg/ml for *E. coli* or 5 μg/ml for *Bacillus*) were also added to the medium. Solid medium was obtained by adding 18 g/l agar to the liquid medium.

Genetic methods

All recombinant DNA manipulations were carried out according to standard procedures [17] and the recommendations of the enzyme manufacturer (Fermentas). Plasmid and chromosomal DNA were isolated with the Qiagen miniprep kit and the Qiagen DNA purification kit, respectively, according to the manufacturer's instructions.

Transformation of *B. subtilis* competent cells was performed using a standard method [1]. Introduction of plasmids into *B. amyloliquefaciens* strains was carried out by E40 phage transduction or electrotransformation [22].

PCR amplifications were performed with *Pfu* DNA polymerase (Fermentas) for DNA cloning and site-directed mutagenesis; and with *Taq* DNA polymerase (Fermentas) for colony PCR analysis. To design primers (Table 1) for genetic modifications of *B. amyloliquefaciens* AJ1991, whose nucleotide sequence is unknown, the DNA fragments containing the *pur* operon promoter, as well as the *purH*, *aprE*, and *pbuE* genes of the parental wild-type strain IAM1523, were PCR-amplified and sequenced (GenBank accession numbers GU825964, GU825965, GU825966, and DQ662404, respectively). The primers for these PCR amplifications were designed based on the reported nucleotide sequence of *B. amyloliquefaciens* FZB42 [18]. All primers were purchased from Syntol (Moscow, Russia). The nucleotide sequences of the target chromosome modifications were verified by DNA sequencing using the appropriate primers. Sequence analysis was performed on double-stranded DNA using the dideoxy chain termination method with the BigDye® Terminator v3.1 Cycle Sequencing Kit on a GeneAmp PCR System 2700 (Applied Biosystems). The sequencing reactions were primed with fluorescent oligonucleotides (Applied Biosystems) and analyzed on an automatic sequencer (ABI Prism 3130xl Genetic Analyzer; Applied Biosystems).

Table 1 Primers used in this study

Designation	Sequence (5′–3′)[a]
Z3	CCGTAAAATCCTGA**C**TACAAAAACTGTCC
Z4	GGACAGTTTTTGTA**G**TCAGGATTTTACGG
yicM+Hind	TGAAAGCTTA*AGGTGG*GAACAGAATGAGTGAATTTATTGCCGAA
yicM-Xho	TACTCGAGCGTTGGGTAAGGTAGAATG
(+)AprE_Pst	GTCTGCAGTCTTTCGTTTTTCCGCAATT
(−)AprE	AAATTTTGTTCATTATGGGGCA
(+)PpurPst	CACTGCAGCTTTAATGAAGGAAGAGGGG
(+)purN_BcuI	TTTACTAGTCCCGTCATTCGCCTTTG
(−)purH-del-5′	ATGTTTGAAATGTCTGATGCCTGTGATTGTCATGCCTTTTCACCTCTG
(+)purH-del-5′	CAGAGGTGAAAAGGCATGACAATCACAGGCATCAGACATTTCAAACAT
(−)purD_Pst	ATCCTGCAGGCATTCAATCGCTTCC
(+)purNdw	ATGAAAAAATTTGCAGTATTTG

[a] Modified bases are shown in bold; restriction sites are underlined. SD sequence is shown in italics

Construction of plasmids containing gene replacement constructs

To construct plasmids that would deliver genetic modifications of interest into the chromosome of the naturally nontransformable *B. amyloliquefaciens* AJ1991, the thermosensitive rolling-circle replication pNZT1 plasmid, based on the pG$^+$host replicon, was used as a vector [13, 22]. To deliver the $pbuE_{T\rightarrow C}$ mutation, plasmid pNZT1-pbuEm was constructed as follows. First, the pYDHL2 plasmid containing the *B. amyloliquefaciens pbuE* gene [21] was cut with *Pvu*II, and the DNA fragment containing 0.4-kb *pbuE* upstream region and 0.1 kb of the gene coding sequence was ligated to *Eco*32I-digested pNZT1, giving pNZT1-pbuE. To generate the $pbuE_{T\rightarrow C}$ mutation, site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis method (Stratagene) and *Pfu* DNA polymerase (Fermentas) with the Z3/Z4 primer pair and pNZT1-pbuE as a template, yielding pNZT1-pbuEm.

To express the *E. coli nepI* gene in *B. amyloliquefaciens*, the pNZT1-aprE-Ppur-nepI plasmid was constructed as follows (Fig. 1). First, the *nepI* gene was cloned under control of the *B. amyloliquefaciens pur* operon promoter (Ppur). With this aim, a DNA fragment containing the *nepI* coding sequence was PCR-amplified using the yicM+Hind/yicM-Xho primer pair and MG1655 genomic DNA as a template. SD sequence suitable for gene translation in *Bacillus* was created using the yicM+Hind primer (Table 1). The obtained fragment was digested with *Hin*dIII—*Xho*I and cloned into *Hin*dIII—*Xho*I-digested pNZT1-Ppur, yielding the pNZT1-Ppur-nepI plasmid. pNZT1-Ppur was constructed by cloning the 270-bp *Pst*I–*Hin*dIII DNA fragment corresponding to the upstream region of the *B. amyloliquefaciens pur* operon between the *Pst*I and *Hin*dIII sites of pNZT1 [22]. To incorporate the Ppur-*nepI* fusion into the chromosome of AJ1991, the *aprE* gene encoding serine alkaline protease was chosen as the target gene. To construct pNZT1-aprE-Ppur-nepI, an *Xho*I–*Sma*I DNA fragment containing Ppur-*nepI* was cut out from pNZT1-Ppur-nepI, blunted with Klenow, and cloned into the *Pvu*II site of *aprE* contained in the pNZT1-aprE plasmid. The pNZT1-aprE plasmid, in turn, was constructed by cloning a 2-kb *Pst*I–*Hin*dIII DNA fragment containing *aprE* [generated by PCR using the (+)AprE_Pst/(−)AprE primer pair and genomic DNA of *B. amyloliquefaciens* AJ1991 as a template] between the *Pst*I and *Hin*dIII sites of pNZT1.

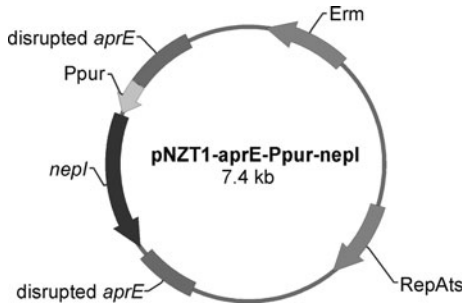

Fig. 1 Schematic representation of the pNZT1-aprE-Ppur-nepI plasmid. RepAts, replication determinant of the pG$^+$host; Erm, erythromycin-resistance gene; Ppur, *pur* operon promoter from *B. amyloliquefaciens*

To obtain a chromosomal 1.5-kb in-frame deletion of the *purH* coding sequence, the pNZT1-ΔpurH delivery plasmid was constructed as follows. First, the DNA fragment containing disrupted *purH* flanked by 400-bp sequences was obtained from AJ1991 genomic DNA by the overlap extension PCR method [8] using the (+)purN_BcuI, (−)purH-del-5′, (+)purH-del-5′, and (−)purD_Pst primers. The resulting fragment was *Bcu*I-*Pst*I-digested and cloned into *Bcu*I-*Pst*I -digested pNZT1, resulting in pNZT1-ΔpurH.

Construction of marker-free genetically modified strains

Genetic modifications were introduced into the chromosome of *B. amyloliquefaciens* using the previously described two-step replacement recombination procedure [22]. In the first step, a *Bacillus* strain bearing the thermosensitive rolling-circle replication delivery plasmid containing the gene replacement construct was cultivated with aeration in LB at 37°C (a nonpermissive temperature for plasmid replication) to initiate integration of the entire plasmid into the chromosome. This integration was accomplished via a single crossover between the target gene and a homologous sequence in the plasmid. In the second step, a separate clone of the integrant was cultivated with aeration in LB at 30°C for 48 h to initiate a second single-crossover event and excision of the plasmid, which yielded erythromycin-sensitive (Erm^S) clones with either a parental or mutant allele on the chromosome.

Colony PCR analysis was used to examine several Erm^S clones for presence of the desired mutation [22]. The PCR products (4 µl each) were analyzed by electrophoresis using 1% (w/v) agarose gel.

Tube-fermentation conditions and determination of exogenous nucleoside accumulation

After cultivating nucleoside producer cells at 34°C for 18 h in LB with maltose (20 g/l), 0.3 ml of the obtained culture was inoculated into 3 ml fermentation medium in a 20 × 200 mm test tube and incubated at 30°C for 72 h in a rotary shaker (until all glucose was exhausted). The fermentation medium contained (per liter): 80.0 g glucose, 15.0 g NH_4Cl, 1.0 g KH_2PO_4, 0.4 g $MgSO_4{\cdot}7H_2O$, 0.01 g $FeSO_4{\cdot}7H_2O$, 0.01 g $MnSO_4{\cdot}4H_2O$, 0.8 g total nitrogen of Mameno (soybean hydrolysate), 0.3 g adenine, and 25 g $CaCO_3$. Glucose and $CaCO_3$ were sterilized separately. pH was adjusted to 6.5 before sterilization.

Inosine, guanosine, and AICAr concentrations in the culture broth were determined using high-performance liquid chromatography (HPLC). HPLC was carried out using a Shimadzu analytical system (Shimadzu) including a dual absorbance ultraviolet (UV) detector. The wavelength was set at 250 nm (and 280 nm for comparison). Separation by HPLC was performed in an Inertsil ODS-3 (100 × 4 mm, 3 µm) (GL Sciences) column at 37°C. Samples (10 µl) of the appropriately diluted supernatant were injected into the chromatograph. The mobile phase contained 2% (v/v) C_2H_5OH, 0.8% (v/v) triethylamine, and 0.5% (v/v) CH_3COOH. The flow rate of the mobile phase was 0.45 ml/min. Optical density of the culture broth was measured using a Shimadzu spectrophotometer at 562 nm.

Results and discussion

Overexpression of genes responsible for nucleoside efflux enhances inosine production

To study the effects of *pbuE* overexpression or heterologous *E. coli nepI* expression on nucleoside production, plasmid-free derivatives of the naturally nontransformable *B. amyloliquefaciens* AJ1991 were constructed. AJ1991 is able to produce simultaneously about 3 g/l inosine and 2 g/l guanosine due to mutations useful for de novo inosine and guanosine oversynthesis, namely adenine auxotrophy and a mutation conferring resistance to the 8-azaguanine purine analog [21].

pbuE gene expression is positively regulated by an adenine-controlled riboswitch, and a $T \rightarrow C$ mutation ($pbuE_{T\rightarrow C}$) in the sequence motif forming the riboswitch structure markedly enhances the gene's expression, regardless of adenine [14, 21]. This mutation was used to enhance *pbuE* expression levels. To introduce the $pbuE_{T\rightarrow C}$ mutation into the chromosome of AJ1991, the pNZT1-pbuEm delivery plasmid was constructed in the *E. coli* TG1 host. The plasmid was constructed to include the *pbuE* gene with the mutation encompassed by the 0.25-kb flanks for successful homologous recombination. AJ1991 lost its natural competence for DNA uptake. Therefore, pNZT1-pbuEm was transferred into this strain by electrotransformation. Further incorporation of the $pbuE_{T\rightarrow C}$ mutation into the chromosome was carried out by the two-step replacement recombination procedure as described in "Materials and methods" section. The resulting plasmid-free Erm^S clones were examined by colony PCR analysis using two primer pairs matching either the wild-type or the modified template, as described previously in detail [22]. The selected $pbuE_{T\rightarrow C}$ mutants were then sequenced to confirm the accuracy of the genetic modification. Thus, the AJ1991pbuE_{TC} strain was obtained with an overexpressed PbuE efflux pump.

Heterologous expression of *E. coli nepI* was achieved by cloning of the gene coding region under control of the *B. amyloliquefaciens pur* operon promoter and subsequent insertion of the resulting fusion into the chromosomal *aprE* gene of AJ1991. It was found that the *aprE* disruption had no influence on nucleoside accumulation by AJ1991 (data not shown). Delivery plasmid pNZT1-aprE-Ppur-nepI was constructed and transferred into AJ1991 using plasmid transduction with E40 bacteriophage. E40 lysate was prepared from the *B. subtilis* 168 plasmid-containing intermediate host obtained by transformation. Thus, AJ1991 bearing pNZT1-aprE-Ppur-nepI was selected. After the two-step replacement recombination procedure, the AJ1991Ppur-nepI strain was identified using colony PCR analysis of Erm^S clones with the primers (+)PpurPst and

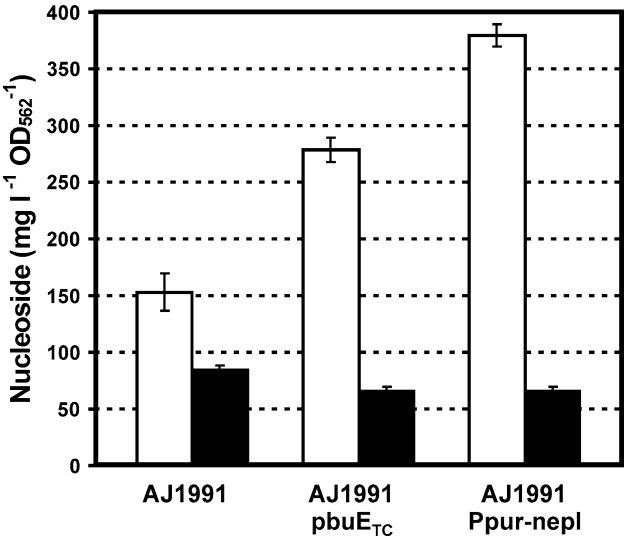

Fig. 2 Effects of PbuE overexpression and NepI heterologous expression on exogenous accumulation of inosine and guanosine by the nucleoside-producing strain AJ1991. Purine nucleoside accumulation in the culture medium was calculated per unit of optical density at 562 nm. The values are means of three experiments; *error bars* indicate standard deviations. *White columns*, inosine accumulation; *black columns*, guanosine accumulation

(−)AprE, which are complementary to the 5′ end of the *pur* operon promoter and the sequence downstream of the *aprE* gene, respectively. Sequence analysis confirmed successful insertion of the Ppur-*nepI* fusion into the *aprE* gene in the AJ1991Ppur-nepI strain.

The performance of the strains overproducing the PbuE and NepI exporters was examined in tube fermentation (Fig. 2). *pbuE* overexpression and heterologous expression of *E. coli nepI* enhanced inosine productivity by 100% and 190%, respectively. At the same time, production of guanosine by AJ1991pbuE$_{TC}$ and AJ1991Ppur-nepI was about the same or even slightly lower than that by the AJ1991 parental strain. This result can be explained by a relatively lower affinity of these transporters for guanosine, and/or reduced GMP formation from IMP (and consequently reduced intracellular guanosine accumulation) due to the enhanced conversion of IMP to the effectively exported inosine (Fig. 3). Remarkably, the NepI membrane protein from a Gram-negative bacterium was able to provide nucleoside export in a microorganism as unrelated as the Gram-positive *B. amyloliquefaciens*. Previously, we found that the *B. amyloliquefaciens* PbuE exporter can function in *E. coli* strains [21]. Recently, Diesveld and co-authors showed that heterologous expression of the *rhtC*, *rhtA*, and *leuE* (*yeaS*) *E. coli* amino acid exporter genes enhanced L-threonine production by a *Corynebacterium glutamicum* strain [4]. Thus, in many cases, heterologously expressed metabolite exporters retain their function in a new host.

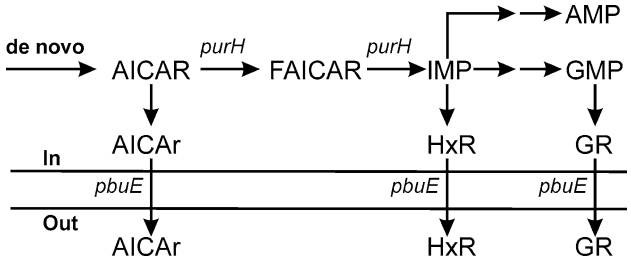

Fig. 3 Simplified metabolic map of the *Bacillus* nucleotide biosynthetic pathway. AICAR, 5-aminoimidazole-4-carboxamide (AICA) ribonucleotide; AICAr, AICA ribonucleoside; FAICAR, 5-formamidoimidazole-4-carboxamide ribotide; HxR, inosine; GR, guanosine

Construction of an AICA ribonucleoside-producing strain

The *purH* gene encodes a bifunctional enzyme, AICAR transformylase [EC 2.1.2.3]/IMP cyclohydrolase [EC 3.5.4.10], which catalyzes a two-step AICAR-to-IMP conversion in the de novo purine biosynthesis pathway (Fig. 3). To construct an AICAr-producing strain, disruption of *purH* was performed on the chromosome of AJ1991. This gene belongs to the *pur* operon and is located between *purN* and *purD*. To avoid a polar effect on the expression of the distal gene, a marker-free in-frame deletion of *purH* was generated through several successive steps: (1) PCR splicing to generate a DNA fragment containing the in-frame deletion encompassed by 400-bp flanks for successful homologous recombination with the respective chromosome regions during plasmid integration and excision, (2) cloning of the resulting DNA fragment into the pNZT1 plasmid to construct the pNZT1-ΔpurH delivery plasmid, and (3) replacement of the wild-type *purH* with Δ*purH* on the chromosome by the two-step replacement recombination method.

After the replacement recombination procedure, several Erm^S clones were examined by colony PCR analysis with (+)purNdw and (−)purD_Pst primers, which are complementary, respectively, to the sequences upstream and downstream of the *purH* gene. Clones with a 1.1-kb instead of 2.6-kb PCR fragment were chosen as Δ*purH* mutants. Sequence analysis confirmed the accuracy of the gene disruption. The obtained strains deficient in the AICAR transformylase/IMP cyclohydrolase enzyme were expected to produce AICAr instead of inosine and guanosine. Indeed, tube fermentation showed that the AJ1991ΔpurH strain produced up to 5.5 g/l AICAr in the culture broth (Table 2).

Effect of PbuE and NepI overexpression on production of AICA ribonucleoside

To study the role of the PbuE and NepI exporters in AICAr production, the *pbuE*$_{T\rightarrow C}$ and *aprE*::Ppur-*nepI*

Table 2 Effects of *pbuE* and *nepI* overexpression on AICA ribonucleoside accumulation

Strain	OD_{562}	AICAr (g/l)
AJ1991ΔpurH	26.0	5.5 ± 0.4
AJ1991ΔpurHpbuE$_{TC}$	25.5	6.6 ± 0.4
AJ1991ΔpurHPpur-nepI	24.1	4.8 ± 0.3

genetic modifications were introduced into the chromosome of AJ1991ΔpurH as described above, yielding AJ1991ΔpurHpbuE$_{TC}$ and AJ1991ΔpurHPpur-nepI strains, respectively. Overexpression of *pbuE* led to increase in accumulation of this nucleoside (Table 2). This result indicates that the substrate specificity of PbuE also extends to the AICA ribonucleoside, and thus, *pbuE* overexpression can be used to improve the respective producing strains. However, the heterologous expression of *E. coli nepI* did not increase AICAr production. Thus, despite the homology between the NepI and PbuE proteins (23% amino acid identity) and their broad substrate specificity, the compounds that can be excreted from cells by these exporters are different.

Conclusions

A simple method to introduce marker-free genetic modifications into naturally low- and nontransformable *B. amyloliquefaciens* was used to genetically engineer marker-free nucleoside-producing strains.

We found that overexpression of the nucleoside efflux transporters *B. amyloliquefaciens* PbuE and *E. coli* NepI notably increased inosine production by a *B. amyloliquefaciens* strain. Using a genetic engineering method, a marker-free strain that overproduces AICAr was constructed. The productivity of this strain was improved by enhancing *pbuE* expression. These data demonstrate that identifying and overexpressing genes encoding metabolite exporters can improve the efficiency of producing strains used for industrial applications.

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