The \textit{yicM} (\textit{nepI}) gene of \textit{Escherichia coli} encodes a major facilitator superfamily protein involved in efflux of purine ribonucleosides

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Abstract

The \textit{yicM} gene of \textit{Escherichia coli} was found by selection for resistance to 6-mercaptopurine. Translation and transcription initiation sites of \textit{yicM} were determined. Overexpression of \textit{yicM} increased resistance of sensitive cells to inosine and guanosine, decreased \textit{E. coli} growth rate in medium containing these ribonucleosides as the sole carbon source, led to inosine accumulation by the \textit{E. coli} strain deficient in purine nucleoside phosphorylase and enhanced the rate of inosine excretion by an inosine-producing strain. These results suggest that \textit{yicM} encodes a purine ribonucleoside exporter and we have accordingly renamed it \textit{nepI} (for ‘nucleoside efflux permease – inosine’).

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Keywords: \textit{yicM}; Nucleoside efflux; \textit{Escherichia coli}

1. Introduction

Purine nucleosides and purine nucleotides obtained by microbial biosynthesis are widely used in the pharmaceutical and food industries. For fermentative production of purine derivatives, various auxotrophic mutants and mutants resistant to purine analogues, derived mainly from \textit{Bacillus} spp. and \textit{Corynebacterium ammoniagenes}, are used [1]. Recently, inosine-producing strains of \textit{E. coli} have been obtained using genetic manipulation [2].

Nucleosides are relatively hydrophilic molecules and, therefore, their penetration through the cytoplasmic membrane of cells depends on specific transport proteins. Three transport systems involved in nucleoside uptake have been identified in \textit{E. coli} [3,4]. However, genes and proteins involved in nucleoside transport from inside the cell into the medium remain unknown.

Recently, several transporters involved in excretion of amino acids [5,6], sugars [7,8] and purine bases [9] in bacteria have been found and characterized. Overexpression of genes encoding amino acid efflux transporters considerably improved productivity of the respective amino acid-producing strains [6,10]. It could be predicted that enhancement of nucleoside export may also improve the respective producing strains. Therefore,
we performed a search for the genes involved in excretion of purine derivatives. In this study, the E. coli yicM gene, encoding a protein involved in efflux of purine ribonucleosides, was identified.

2. Materials and methods

2.1. Bacterial strains, phages, plasmids and growth conditions

The E. coli strains, phages and plasmids used in this study are listed in Table 1. The media used were LB or M9 minimal medium [11], supplemented with 40 μM thiamine HCl, 0.4% (w/v) glucose and, when required, purine bases (0.17 mM) and antibiotics at the following concentrations: ampicillin 100 μg ml⁻¹, kanamycin 40 μg ml⁻¹ and chloramphenicol 20 μg ml⁻¹. Minimal inhibitory concentrations (MICs) were determined, as described previously [6].

2.2. In vivo cloning

In vivo cloning of the genes conferring resistance to 6-mercaptopurine was performed using the phagemid Mu s5005, according to the technique described previously [14]. As a bacterial chromosome donor, the MG1655 strain, lysogenic for Mu cts, was used. Cells containing suitable phagemids were selected on M9 medium with kanamycin (40 μg ml⁻¹) and 6-mercaptopurine (2 mM).

2.3. Construction of plasmids

To obtain pYICM1 plasmid, the yicM gene was cut off the pMP1 phagemid, using PstI and PvuII enzymes, and cloned into the PstI and Eco32I sites of the pOK12 vector [15].

To obtain the plasmids pYICM-CTG, pYICM-ATC1 and pYICM-ATC2, containing mutations that destroy potential start codons of the yicM gene, a Quik-Change site-directed mutagenesis (Stratagene) of the pYICM1 plasmid with the putative yicM-tr (Table 1), which is complementary to the region from +36 to +15 bp, relative to the putative yicM initiation codon ATG2, was radiolabeled by T4 polynucleotide kinase (Pharmacia) with γ-[³²P]ATP (6000 Ci mM⁻¹). Aliquots of mRNA (1-μg) were incubated with 10 μM of the labeled oligonucleotide at 70°C for 2 min. Then, the components for the cDNA labeling steps: 1× reverse transcriptase buffer (Qiagen), 500 μM each of deoxyribonucleotide triphosphates (final concentration) and 5 U Omniscript Reverse Transcriptase (Qiagen) were added. Reaction mixtures were incubated at 37°C for 1 h. The synthesized cDNAs were precipitated by 2 volumes of ethanol, dissolved in H₂O, denatured at 65°C for 10 min and analyzed by electrophoresis on 6% acrylamide gel. The same primer was used for the DNA sequence ladder run on the same gel. Gel radioautography visualization was carried out using a Typhoon 9210 imager (Amersham) and Image-Quant version 5.2 software (Molecular Dynamics).

2.4. Construction of yicM disruption mutant

To disrupt the chromosomal yicM gene, the phage λ Red disruption system was used [13]. Plasmid pKD3 was used as a template and also primers with the homology to yicM, YICM1 and YICM2 (Table 1). The resulting strain GS158 carried a substitution of a 1.2-kb fragment of the yicM gene to the cat gene. Then, the ΔyicM::cat mutation was transferred by P1 transduction [16].

2.5. Integration of yicM expressed under the control of nlpD promoter into the E. coli chromosome

The yicM coding region was obtained by PCR, using primers YICM3 and YICM4 (Table 1) and chromosomal DNA of strain MG1655 as a template. The 1.2-kb PCR product was digested with SaI and XbaI, and cloned into the SaI and XbaI sites of the vector pMIV-Pₐₙ₉D (Ziyatdinov, M., unpublished data) to give pMIV-Pₐₙ₉D-yicM. The vector pMIV-Pₐₙ₉D is a derivative of plasmid pM1 [17]. In this vector, a strong promoter of the E. coli nlpD gene [18] and the chloramphenicol resistance gene (cat) are located between the Mu phage attachment sites. Integration of yicM under the control of the nlpD promoter into the chromosome of E. coli MG1655 strain was performed, as described previously [17], using plasmid pMIV-Pₐₙ₉D-yicM. Then, the Pₐₙ₉D-yicM-cat cassette was transferred by P1 transduction [16].

2.6. Primer extension analysis

The E. coli TG1 strain, harboring the pYICM1 or pOK12 plasmid, was grown in M9 medium and total RNAs isolated using RNeasy Minikit (Qiagen), according to the manufacturer’s recommendations. Oligonucleotide YICM-tr (Table 1), which is complementary to the region from +36 to +15 bp, relative to the putative yicM initiation codon ATG2, was radiolabeled by T4 polynucleotide kinase (Pharmacia) with γ-[³²P]ATP (6000 Ci mM⁻¹). Aliquots of mRNA (1-μg) were incubated with 10 μM of the labeled oligonucleotide at 70°C for 2 min. Then, the components for the cDNA labeling steps: 1× reverse transcriptase buffer (Qiagen), 500 μM each of deoxyribonucleotide triphosphates (final concentration) and 5 U Omniscript Reverse Transcriptase (Qiagen) were added. Reaction mixtures were incubated at 37°C for 1 h. The synthesized cDNAs were precipitated by 2 volumes of ethanol, dissolved in H₂O, denatured at 65°C for 10 min and analyzed by electrophoresis on 6% acrylamide gel. The same primer was used for the DNA sequence ladder run on the same gel. Gel radioautography visualization was carried out using a Typhoon 9210 imager (Amersham) and Image-Quant version 5.2 software (Molecular Dynamics).

2.7. Determination of inosine excretion in a short-term fermentation

Inosine-producing strains were incubated at 34°C overnight, cells were harvested and washed three times
with ice-cold 0.9% (w/v) NaCl. Efflux was initiated by suspending the cells up to OD$_{600}$ 2.5–3.0 in pre-warmed E1 medium (10 g l$^{-1}$ D-glucose, 4 g l$^{-1}$ (NH$_4$)$_2$SO$_4$, 0.1 g l$^{-1}$ KH$_2$PO$_4$, 1 g l$^{-1}$ MgSO$_4$·7H$_2$O, 0.01 g l$^{-1}$ FeSO$_4$·7H$_2$O, 0.01 g l$^{-1}$ MnSO$_4$·H$_2$O, 2 g l$^{-1}$ CaCO$_3$). Samples were removed at regular intervals, cells were separated by centrifugation and inosine concentration determined by HPLC.
HPLC was carried out using a Shimadzu analytical system (Shimadzu), including dual absorbance UV detector. The wavelength was set at 250 nm (and 280 nm for a comparison). Separation by HPLC was performed in a column Inertsil ODS-3 (100 × 4 mm, 3 μm) at 20 °C (GL Sciences). Ten microlitre samples of appropriately diluted supernatant were injected into the chromatograph. The mobile phase contained 2% (v/v) CH₃OH, 0.8% (v/v) triethylamine and 0.5% (v/v) CH₃COOH. Flow-rate of the mobile phase was 0.4 ml min⁻¹.

2.8. Evaluation of inosine production

Inosine-producing strains were cultivated at 34 °C for 18 h in l-broth, 0.3 ml of the obtained culture was inoculated into 3 ml of a fermentation medium in a 20 × 200 mm test tube, and cultivated at 34 °C for 72 h in a rotary shaker. The fermentation medium has been described previously [2]. After the fermentation, inosine concentration in the culture broth was determined, as described above. Optical densities were measured with a Shimadzu spectrophotometer at 600 nm.

3. Results

3.1. Cloning of E. coli genes involved in resistance to purine base analogues into mini-Mu phagemid and identification of a gene involved in resistance to guanosine

It is known that the overexpression of genes encoding components of efflux pumps increases cell resistance to the respective inhibitors [19]. We proposed that amplification of genes involved in excretion of purine nucleosides might increase the resistance of E. coli cells to purine base analogues. Therefore, E. coli genes involved in resistance to the hypoxanthine analogue, 6-mercaptopurine, were cloned in vivo using the mini-Mud5005 phagemid (see Section 2). Recombinant phagemids, carrying 6-mercaptopurine resistance to cells, were isolated and transformed into the GS72Mu strain. This strain contains the gsk-3 mutation in the structural gene for guanosine kinase, which makes it extremely sensitive to guanosine [20]. The obtained transformants were checked for their ability to grow with the inhibiting concentration of guanosine (10 mg l⁻¹), and several phagemids conferring cells resistance to the nucleoside were selected. Then, the flanks of the inserted DNA fragments were sequenced using the Mu-L and Mu-R primers (see Section 2) to the left and right Mu phage attachment sites, respectively. The obtained sequence data were compared to GenBank information and the cloned fragments identified. It appeared that several types of inserts, located in the different chromosome regions, had been cloned. One of them, an 11,970-bp chromosomal insert of the pMP1 phagemid, conferring resistance to 6-mercaptopurine and guanosine, carried several genes from the 83-min region of the E. coli chromosome, encoding membrane proteins. After subcloning into a moderate copy number pOK12 vector [15], a minimal PstI–PstII fragment, containing the yicM gene 342 bp upstream of the predicted start codon (GeneBank accession number U00096), was found to be responsible for the mentioned phenotype.

3.2. Effect of the yicM gene inactivation or overexpression on E. coli cell resistance to 6-mercaptopurine and purine ribonucleosides

To study the role of yicM in cell physiology, a disruption mutant, containing the ΔyicM::cat mutation, was constructed (see Section 2). Inactivation of yicM did not affect the viability of the bacteria grown in rich or minimal media at different temperatures (results not shown). However, this inactivation made the GS72 cells more susceptible to purine ribonucleosides (Table 2). On the other hand, amplification of yicM cloned into a multi-copy plasmid increased cell resistance to inosine, adenosine, guanosine and 6-mercaptopurine – the resistance to inosine being increased more than 100-fold (Table 2).

The yicM gene was also expressed under a strong promoter of the nlpD gene [18]. After this construction was inserted into the chromosome, cell resistance to

<table>
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<tr>
<th>Strain</th>
<th>Minimal inhibitory concentration (MIC, μg ml⁻¹)a</th>
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<tbody>
<tr>
<td></td>
<td>6-Mercaptopurine</td>
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<tr>
<td>GS72</td>
<td>150</td>
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<tr>
<td>GS72 (pOK12)</td>
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<tr>
<td>GS72ΔyicM</td>
<td>150</td>
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<tr>
<td>GS72 (pYICM)</td>
<td>300</td>
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<tr>
<td>GS72P&lt;sub&gt;ΔyicM&lt;/sub&gt;yicM</td>
<td>n.d.&lt;sup&gt;b&lt;/sup&gt;</td>
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a The MIC was determined as lowest concentration of an inhibitor preventing growth in minimal agar plates after 40 h incubation. The experiments were repeated three to five times and revealed consistent MICs.

b n.d., not determined.
guanosine was further increased (Table 2). Thus, yicM overexpression increased cell resistance to ribonucleosides, but its inactivation decreased resistance.

3.3. Determination of the translation and transcription start sites of the yicM gene

The predicted amino acid (aa) sequence length of the YicM protein varies in several databases. According to Genbank, the protein (NP 418118) is composed of 451 aa, while according to SWISS-PROT, the protein (P31438) is composed of 412 aa. The protein (EG11689) is composed of 396 aa according to EcoGene. The respective translation start codons are shown in Fig. 1(a). To identify the real translation start point of yicM, each of the potential start codons of the gene contained in the pYICM1 plasmid was destroyed (see Section 2). Thus, the first potential start codon (GTG) was changed to CTG, the second and the third potential start codons (ATG1 and ATG2) were changed to ATC. The plasmids pYICM-CTG, pYICM-ATC1 and pYICM-ATC2 containing these mutations were introduced into the GS72 cells, and growth of the resulting strains in the presence of 500 µg ml⁻¹ inosine was checked. It proved that mutations which affect the GTG and ATG1 codons had no effect on resistance to the nucleoside. However, if the ATG2 codon was destroyed, the cells became sensitive to inosine. These results indicate that the real start codon of the yicM gene is ATG2. In fact, only this codon is preceded by a sequence resembling the ribosome-binding site (Fig. 1(a)).

The transcription start site of yicM was determined by primer extension. As seen in Fig. 1(b), initiation of yicM transcription occurred with C, 32 bp upstream of yicM start codon (Fig. 1(a)). A weaker signal (initiation with G) was observed 1 bp upstream of the strong signal. Six bp upstream of the main site, sequence (TAaAgT) resembling the consensus −10 element (TAAT) of a putative promoter was found (Fig. 1(a)). Furthermore, 19 bp upstream of this −10 regions, sequence (TTaAat) resembling the consensus −35 element (TTGACA) exists.

Our data indicate that yicM encodes a protein composed of 396 amino acid residues having a calculated molecular mass of 41.85 kDa. The YicM protein belongs to the major facilitator superfamily of transporters [21,22] and to arabinose efflux permease COG 2814 [23]. Another protein belonging to this COG is YdeA of E. coli, involved in arabinose efflux [7], and YdhL of Bacillus subtilis, involved in purine base efflux [9]. Therefore, it could be predicted that yicM gene encodes a protein involved in the efflux of some metabolites. The fact that inactivation of the gene increased cell sensitivity to nucleosides, while its overexpression conferred cell resistance to inosine, adenosine and guanosine, indicates purine nucleosides as possible substrates.

3.4. Overexpression of the yicM gene decreases the growth rate of E. coli in a minimal medium containing purine ribonucleosides as the sole carbon source

E. coli can utilize nucleosides as the sole carbon and energy source [24]. It is known that in the case of an efflux pump gene overexpression, accumulation of the respective substance in cells could be decreased [19]. Therefore, the effect of yicM overexpression or inactivation on growth properties of the MG1655 strain was studied. The parent strain and the ΔyicM::cat mutant grew identically in minimal medium, containing 2 mM glucose, ribose, cytidine, inosine, guanosine or adenosine as the sole carbon and energy source (Fig. 2). However, the strain with yicM expressed under the strong P_{nlpD} promoter grew slowly compared to the parent strain with inosine, guanosine and adenosine. At the same time, yicM overexpression had no effect on growth with purine deoxyribonucleosides. These results indicate that yicM overexpression decreases ribonucleoside accumulation in cells.

3.5. Overexpression of yicM leads to inosine accumulation by E. coli strain deficient in purine nucleoside phosphorylase

To obtain direct evidence of the involvement of YicM in inosine excretion, accumulation of purine derivatives by different E. coli strains was studied. It is known that entry of E. coli cells into the stationary
phase is accompanied by degradation of rRNA and excretion of nucleobases [25]. Obviously, these nucleobases are products of ribonucleoside degradation, predominantly catalyzed by nucleoside phosphorylases [24]. It is proposed that strains deficient in purine nucleoside phosphorylase will not accumulate purine nucleobases but purine ribonucleosides. Therefore, purine ribonucleoside accumulation by *E. coli* strains TG1deoD and TG1deoD*\textsubscript{nlpD}\textsubscript{yicM} grown to stationary phase was studied (Fig. 3). As shown in Fig. 3, TG1deoD accumulated only traces of inosine. Under the same conditions TG1deoD*\textsubscript{nlpD}\textsubscript{yicM} accumulated up to 10 mg l\textsuperscript{-1} inosine. Thus, yicM overexpression significantly increases inosine accumulation by an *E. coli* strain defective in purine nucleoside phosphorylase. This accumulation might be the result of rRNA degradation and/or enhanced de novo purine biosynthesis released from feedback control due to a decrease in the intracellular purine derivative pool caused by inosine overexcretion.

3.6. Overexpression of yicM enhances the rate of inosine excretion and improves inosine productivity of the inosine producing strain

FADRadd (pMWKQ) is an inosine-producing strain having several mutations necessary for inosine overproduction and harbouring the pMWKQ plasmid containing the *purF\textsubscript{KQ}* gene coding for the PRPP amidotransferase insensitive to GMP [2].

As shown in Fig. 4(a), at 6 h of a short-term fermentation (see Section 2), the amount of inosine in the medium was approximately 3-fold higher in the case of FADRadd*\textsubscript{nlpD}\textsubscript{yicM} (pMWKQ) compared to the parent FADRadd (pMWKQ). Reducing the electrochemical proton gradient across the cytoplasmic membrane by the addition of the protonophore, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), led to a drastic reduction in the rate of inosine excretion (Fig. 4(a)).
These results suggest the involvement of YicM in excretion of inosine driven by proton motive force. Inosine production by the FADRadd (pMWKQ) strain and its derivatives was determined after cultivation for 72 h in the fermentation medium [2]. As seen in Fig. 4(b), specific inosine productivity of FADRaddP\_nlpD\_yicM\_pMWKQ was ~2-fold higher than that of the parental strain. Thus, yicM overexpression improved inosine productivity of the respective producing strain. Furthermore, the strain FADRaddP\_nlpD\_yicM\_pMWKQ accumulated less hypoxanthine than the parental strain (Fig. 4(b)). Inactivation of the yicM gene has no effect on inosine and hypoxanthine accumulation (Fig. 4(b)).

4. Discussion

Purine analogues, which are non-toxic until converted to nucleotides, have been used extensively in the selection of mutants defective in uptake and the study of purine metabolism [3]. In this study, the yicM gene, encoding a MFS member [21,22] involved in ribonucleoside transport out of cells, was found by selection for resistance to the hypoxanthine analogue, 6-mercaptopurine. The results presented indicate inosine as the primary substrate of YicM and YicM as the main transporter involved in inosine excretion. Besides inosine, YicM is involved in guanosine efflux. Adenosine also seems to be a substrate for YicM. However, increased susceptibility or resistance to adenosine (Table 2), and a decreased growth rate of the wild-type strain in the medium containing adenosine as the sole carbon source (Fig. 2), may be related to adenosine deamination to inosine by adenosine deaminase present in E. coli [24].

Although proteins capable of transporting both nucleosides and nucleobases are known [26], we believe that YicM is not involved in hypoxanthine or guanine excretion. This proposal is based on the following observations. The level of resistance to 6-mercaptopurine is rather low (Table 2), and may be a result of excretion of the analogue ribonucleoside, the product of the respective ribonucleotide degradation. Indeed, after cultivation of strains TG1deoD and TG1deoDP\_nlpD\_yicM for 24 h with a subinhibitory 6-mercaptopurine concentration (1.3 mM), about 50% and 85% of the drug, respectively, was found in the culture broth as 6-mercaptopurine riboside (Novikova, A.E., unpublished results). Furthermore, the inosine-producing strain, deficient in purine nucleoside phosphorylase, accumulated some amount of hypoxanthine (Fig. 4(b)), obviously due to the activity of the second purine nucleoside phosphorylase (XapA) and/or riboside hydrolase (RihC) present in E. coli [27,28]. While improving inosine productivity, yicM overexpression...
at the same time decreased hypoxanthine accumulation (Fig. 4(b)). Besides, yicM overexpression had no effect on hypoxanthine accumulation by the \( \text{deoD}^+ \) derivative of the strain FADRadd (pMWKQ), which accumulated this purine base instead of inosine (Gronskiy, S.V., unpublished results).

\( \text{yicM} \) gene expression is induced in cells entering the stationary phase (Gronskiy, S.V., unpublished results), when rRNA is degraded [25]. We propose that, under some conditions, purine ribonucleosides are excreted as the degradation products, and YicM mediates this excretion.

Based on the above findings in support of a primary inosine exporter function for YicM, we propose that the gene be redesignated nepI (for 'nucleoside efflux permease – inosine').

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References


