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Cloning and Characterization of the *pyrH* Gene Encoding UMP-Kinase from *Lactobacillus reuteri* ATCC 55739

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Abstract From a genomic library of *Lactobacillus reuteri* ATCC 55739, one clone, NE347, carrying a *pyrH* gene encoding UMP kinase, was identified. pNE347 carried a 1.88 kb *Eco*RI fragment and the *pyrH* was located in the middle of the insert. *pyrH* ORF was 723 bp in size and capable of encoding UMP kinase composed of 240 amino acid residues. *tsf* encoding an elongation factor-Ts and *frr* encoding a ribosomal recycling factor were present upstream and downstream of *pyrH*, respectively. When introduced into *E. coli* KUR1244, a *pyrH*-negative strain, pNE347 restored the ability to grow at 42°C, indicating that *pyrH* from *L. reuteri* synthesized functional UMP kinase in *E. coli*. Northern blot experiment showed that *pyrH* and *frr* were cotranscribed as a 1.4 kb single transcript. *pyrH* was overexpressed in *E. coli* by using a pET26b(+) vector, and a major 25 kDa protein band appeared on SDS-polyacrylamide gel.

Key words: *Lactobacillus reuteri*, UMP-kinase, *pyrH*, ribosomal recycling factor, operon

Nucleotides are the building blocks for DNA and RNA, and they are also essential for the function of several enzymes. Enzymes involved in the *de novo* synthesis of nucleotides are ubiquitous and essential for cell viability [2]. UMP is the precursor for all pyrimidine nucleoside triphosphates and synthesized via six enzymatic reactions directed by six unlinked genes in the enteric bacteria [4]. UMP is subsequently converted into UTP and CTP. UMP kinase further phosphorylates UMP by using ATP or dATP as phosphate donors. A gene encoding UMP-kinase, *pyrH*, was cloned from *Escherichia coli* and characterized [15]. Functional *E. coli* enzyme is known as a homohexamer and subjected to complex regulatory mechanisms in which UTP and GTP act as allosteric effectors [14]. Interestingly, *E. coli* enzyme does not share any sequence similarity with other known NMP-kinases [2]. *pyrH* genes from other microorganisms, including *Lactococcus lactis* subsp. *cremoris* MG1363 [17], *Pseudomonas aeruginosa* [9], and *Thermus thermophilus* [1], were cloned and characterized. In most cases, *pyrH* forms an operon with the downstream *frr* gene encoding a ribosomal recycling factor. *Lactobacillus reuteri* is one of the dominant heterofermentative lactobacilli found in the GI (gastrointestinal) tracts of human and animals. *L. reuteri* has a useful feature of producing reuterin, an antimicrobial compound derived from glycerol with a broad inhibition spectrum [16]. Because of its desirable properties, such as growing well in the GI tracts of human and producing an antimicrobial agent, *L. reuteri* is considered as an important probiotic strain [12]. In contrast to its high potential as a commercial probiotic strain, little is known on this organism in terms of genetic and molecular biological bases for the important metabolic pathways such as nucleotide synthesis. More basic researches are necessary to fill the gap. This communication reports on the cloning and characterization of a *pyrH* gene from *L. reuteri* ATCC 55739, and it is hoped that this will contribute to the better understanding and utilization of *L. reuteri*.

Materials and Methods

Bacterial Strains and Growth Conditions

Lactobacillus reuteri ATCC 55739 was obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). *Escherichia coli* DH5α was used as the host for DNA manipulation and transformation experiments. *E. coli* KUR1244 (*pyrH*88 *thi*-1 *leu*B6 *pro*A2 *lac*Y1 *gal*K2 *mtl*-1 *xyl*-5 *ara*-14 *sup*E44) [15] was used for complementation

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of PyrH$^-$ phenotype by the cloned *Lactobacillus reuteri pyrH* gene. *E. coli* BL21(DE3) (Novagen) was used as the host strain for expression of *pyrH*. pUC19, pBluescriptKS(+), and pGEM®-T Easy vector (Promega) were used for cloning and subcloning. pET26b(+) (Novagen) was used for the overexpression of *pyrH* in *E. coli*. *E. coli* cells were grown in Luria-Bertani (LB) broth with vigorous shaking at 37°C or on an agar plate solidified with 1.5% agar. Antibiotics were used for *E. coli* at the following concentrations: ampicillin (Ap), 100 μg/ml; kanamycin (Km), 60 μg/ml. *L. reuteri* cells were cultivated as previously described [10].

Genomic Library Construction and Library Screening

A genomic library of *Lactobacillus reuteri* ATCC 55739 was constructed in *E. coli* DH5α. Chromosomal DNA was isolated according to the method of Kim *et al.* [6]: 30 μg of chromosomal DNA was digested with *Eco*RI, and 1.5–5 kb fragments were recovered from an agarose gel by electroelution. Eluted DNA was ligated with pUC19, and the ligation mixture was introduced into competent *E. coli* cells by electroporation using GenePulser II (BioRad). Competent cells were prepared by the method of Dower *et al.* [3]. White colonies on LB plates containing ampicillin (100 μg/ml), IPTG (isopropyl-β-D-thiogalactopyranoside, 0.5 mM), and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, 40 μg/ml) were pooled and screened by the colony hybridization method. An oligonucleotide, named LI-1 (TDCCWCCWAAYGGYA-ARAARGTHYTDATG), was used as a probe and labeling of the probe was done by using [γ-^{32}P] ATP and T4 polynucleotide kinase [13].

Southern Blot Hybridization

Southern blot was carried out according to the standard methods [13]. Zeta-probe membrane (BioRad) was used under alkaline conditions and the 1.88 kb *Eco*RI insert from pNE347 was labeled with [α-^{32}P]dCTP by using the Rediprime™ II random prime labeling system (Amersham).

DNA Sequencing and Sequence Analysis

DNA sequence was determined by the dideoxy-chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer) [8]. Primers for sequencing were synthesized at Bionics (Seoul, Korea). Homology of the deduced amino acid sequence was analyzed by the Blast program at NCBI. Sequence alignment was performed with the ClustalW program by using the PAM250 matrix. ExPASy Proteomics tools were used to calculate the pIs and molecular weights of the translated protein products.

RNA Isolation and Northern Blot Analysis

Lb. reuteri ATCC 55739 cells were grown in MRS broth (Difco) to an optical density of 0.8 at 550 nm. Total RNA was prepared according to the method of Park *et al.* [11] and the concentration was determined by measuring absorbance at 260 nm. Ten μg of RNA was loaded and separated on a 1.0% agarose-formaldehyde gel, transferred onto a Hybond™-XL membrane (Amersham), and hybridized at 48°C with radio-labeled DNA probes. Then, 302 bp of *tsf*, 432 bp of *pyrH*, and 331 bp of *frr* specific probes were prepared by PCR, respectively. Primer sequences are shown in Table 1.

Overexpression of the *pyrH* Gene in *E. coli*

For overexpression of the *pyrH* gene in *E. coli*, the *pyrH* gene was amplified by PCR and subcloned into pET26b(+) (Novagen). Two oligonucleotide primers containing a unique *Nde*I site (pyrH-expF, see Table 1) and *Xho*I site (pyrH-expR) were used for the amplification. The amplified fragment containing *pyrH* was digested with *Nde*I and *Xho*I, and ligated with pET26b(+). The resulting recombinant plasmid was named pYRHE1. *E. coli* BL21(DE3) harboring pYRHE1 was grown overnight at 37°C, and 1% of culture was inoculated into fresh medium and cultured at 37°C. When the A_{600} of the culture reached about 0.8, IPTG was added to the final concentration of 0.5 mM, and the culture was further incubated for 3 h at 30°C [5]. Cells were recovered by centrifugation at 5,000 ×*g* for 20 min, and cell extract

Table 1. Primers used in this work.

Primer use		Primer name	Primer sequence
Amplification of 5' terminus of *tsf*		pNE347up-tsf1	TGCAACAGTTTGGTCTGGGTTC
		pNE347up-tsf2	TAACAAAGTCTTGGTCGGCCAAGCA
Northern blot	*tsf*	tsf-sF	TGACTCGTGATGATGTATCT
		tsf-sR	GTTCATTTGGTCCTTAACTT
	pyrH	pyrH-sF	CCTTGGAATCACTTGATGTT
		pyrH-sR	TTCCAACTTCTTGTCCCTTA
	frr	frr-sF	TAAATGAAGCAAAGGACAAA
		frr-sR	TCCTTAACAAGCTCTTTTCG
Expression of *pyrH*		pyrH-expF	GGAATTCCATATGTCAGACATTAAATACAATCGTGTC
		pyrH-expR	CCCCTCGAGTTAATCCCCCTCAATCGTGGTT

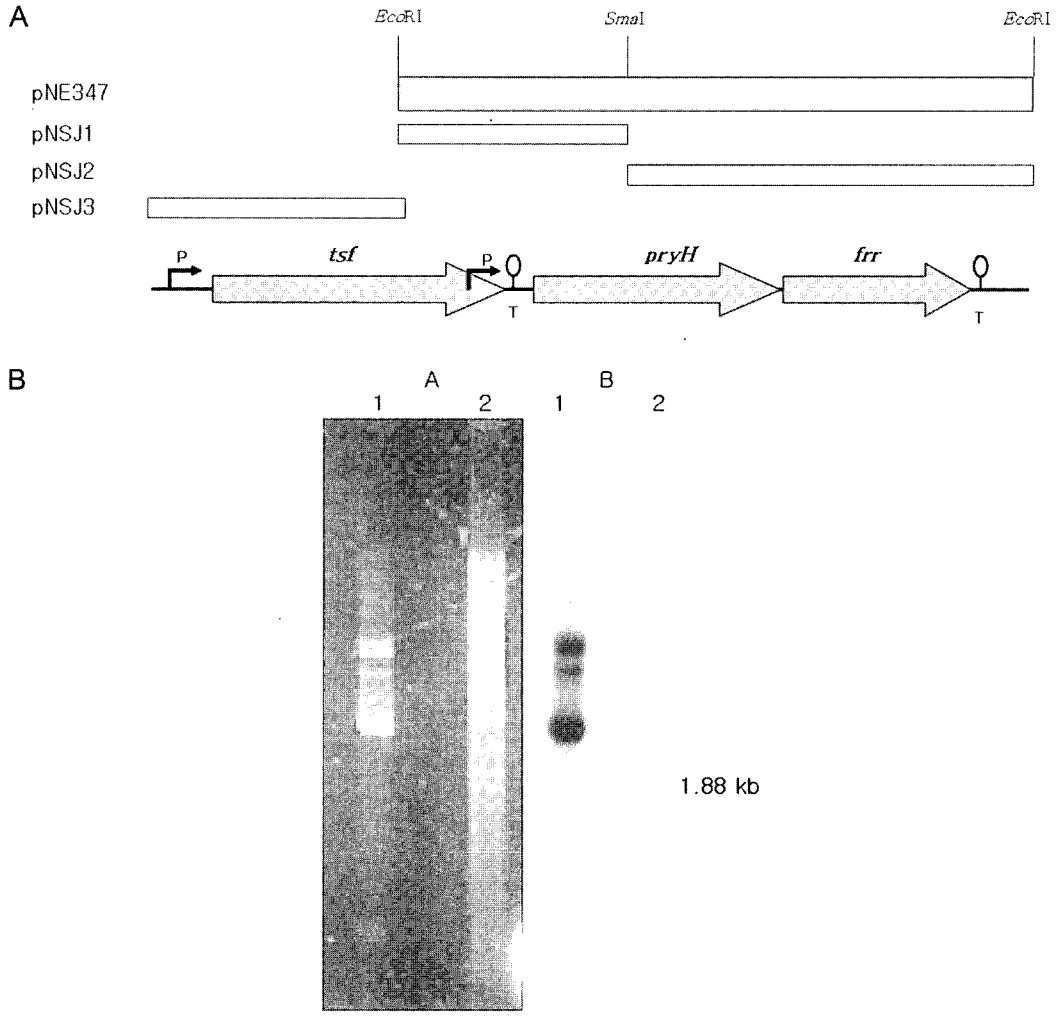

Fig. 1. Physical map of the *pyrH* locus and Southern blot analysis of pNE347.
A. A 1.88 kb *Eco*RI insert in pNE347 and fragments subcloned into pBluescript II KS(+) are shown. A 850 bp PCR product cloned into a pGEM-T easy vector is also shown. The location of *tsf*, *pyrH*, and *frr* are indicated. Putative promoters (P, arrowheads) and transcription terminators (T, loop) are also marked. B. A 1.88 kb fragment was used as the probe for Southern blot, and the result confirmed that the insert was originated from *L. reuteri* ATCC 55739 chromosome. A, Agarose gel; B, Autoradiogram. Lane 1, pNE347 DNA (4.6 kb) undigested; lane 2, *L. reuteri* ATCC 55739 chromosomal DNA digested with *Eco*RI.

was prepared by sonication followed by centrifugation. Then, 12% SDS-PAGE was done according to the method of Laemmli [7].

Results and Discussion

Cloning of the *pyrH* Gene from *L. reuteri* ATCC 55739

An *Eco*RI library of *Lactobacillus reuteri* ATCC 55739 was constructed in *E. coli* as described in the Methods section. The library was screened by colony hybridization using an oligonucleotide probe, which was designed based on an experimentally determined amino acid sequence of a protein. One clone, NE347, was inadvertently selected as a false positive (result not shown), and the following restriction mapping and DNA sequencing showed that pNE347 harbored a 1.88 kb insert encompassing a gene homologous to known *pyrH* genes encoding UMP-kinases. Southern blotting confirmed that the insert was originated from *L. reuteri* ATCC 55739 chromosome (Fig. 1B).

Functional Complementation of an *E. coli* PyrH⁻ Temperature-Sensitive Phenotype by pNE347

To confirm the function of cloned *pyrH*, pNE347 was introduced into *E. coli* strain KUR1244, which carried a temperature-sensitive *pyrH* mutation. *E. coli* KUR1244 cannot grow at 42°C because of the mutation [15], but transformants no longer showed the temperature sensitive phenotype of KUR1244 and were able to growth at 42°C (Table 2) [17]. The result confirmed that *pyrH* from *Lactobacillus reuteri* was expressed in *E. coli* and successfully complemented the mutation. Together with the sequence

Table 2. Complementation of *E. coli* PyrH⁻ temperature-sensitive phenotype by pNE347.

Strain	Growth	
	37°C	42°C
KUR1244	+	-
KUR1244 [pUC19]	+	-
KUR1244 [pNE347]	+	+

homology data with the known *pyrH* genes, the functional complementation result proved conclusively that the cloned 1.88 kb fragment contained the functional *pyrH* gene from *Lactobacillus reuteri* ATCC 55739.

Nucleotide Sequence of *pyrH* and Neighboring Genes

*Sma*I digestion of the 1.88 kb *Eco*RI insert generated two *Eco*RI-*Sma*I fragments of 0.68 and 1.2 kb in size. Each fragment was subcloned into pBluescriptII KS(+), generating pNSJ1 and pNSJ2, respectively (Fig. 1A), and the complete nucleotide sequence was determined by primer walking. The nucleotide sequence of *pyrH* and neighboring genes was deposited into the GenBank under the accession number AF401482. Blast analysis located *pyrH* in the middle of the insert and two other genes: 3' part of *tsf* upstream and a complete *frr* downstream of *pyrH*, respectively. Nucleotides 1 to 306 correspond to the 3'-end of *tsf*, encoding an elongation factor Ts. To obtain a complete *tsf* gene, a 850 bp fragment was amplified by using DNA Walking *SpeedUp*™ Kit (Seegene, Korea) and two primers (see Table 1). The amplified fragment was subcloned into a pGEM®-T Easy Vector, resulting in pNSJ3 (Fig. 1A). Thus, genomic DNAs totaling 2,699 bp in size and containing *pyrH* and neighboring genes were cloned and sequenced. The physical map of the 2,699 bp fragment is shown in Fig. 1A. The nucleotide sequence and the deduced amino acid sequence of open reading frames (ORFs) are shown in Fig. 2. Nucleotides from 250 to 1.125, corresponding to *tsf*. *tsf* ORF, can encode a protein of 291 amino acids, which has a molecular weight of 31,813 Da and pI of 4.65. A ribosome binding site (AAGGAG, nucleotides 234–239) was located ten nucleotides upstream of the ATG start codon. Putative −10 and −35 promoter sequences were located at 89 and 105. A putative rho-independent transcription terminator (AAAAAGGGCGT-ACTCTTTGAAGTGCGCCTTTTTT, nucleotides 1,139–1,179) was found 13 nucleotides downstream from the stop codon of *tsf* with ΔG of −10.2 kcal/mol at 37°C. Nucleotides 1,202 to 1,924 correspond to *pyrH*. *pyrH* ORF can encode a protein of 240 amino acids, which has a molecular weight of 25,957 Da and pI of 5.41. A ribosome binding site (AGGAGG, nucleotides 1,189–1,195) was located seven nucleotides upstream of the ATG start codon. Putative −10 and −35 promoter sequences were located at 983 and 1,007. Nucleotides 1,928 to 2,468 correspond to *frr* encoding a ribosomal recycling factor and which starts three nucleotides downstream from the stop codon (TAA) of *pyrH*. A ribosome binding site (AGGGGG, nucleotides 1,914–1,920) was located eight nucleotides upstream of the

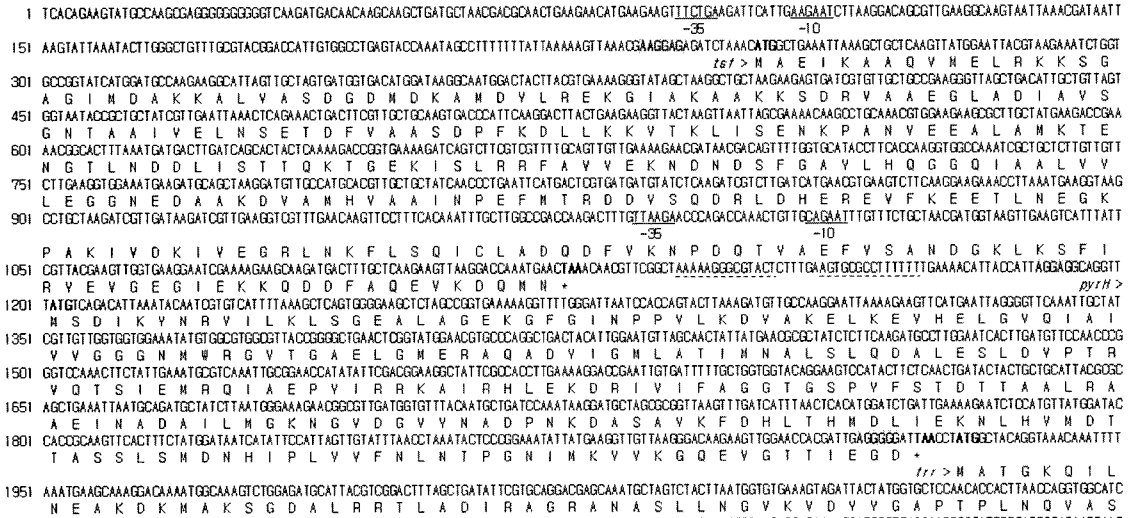

Fig. 2. Nucleotide and translated amino acid sequence of *pyrH* and surrounding genes.
Ribosome-binding sites are indicated by shadow. The possible transcription terminators are underlined by a dotted line (.....). Putative promoter sequences (−35 and −10 boxes) are underlined. The sequence has been deposited to the GenBank under the accession no. AF401482.

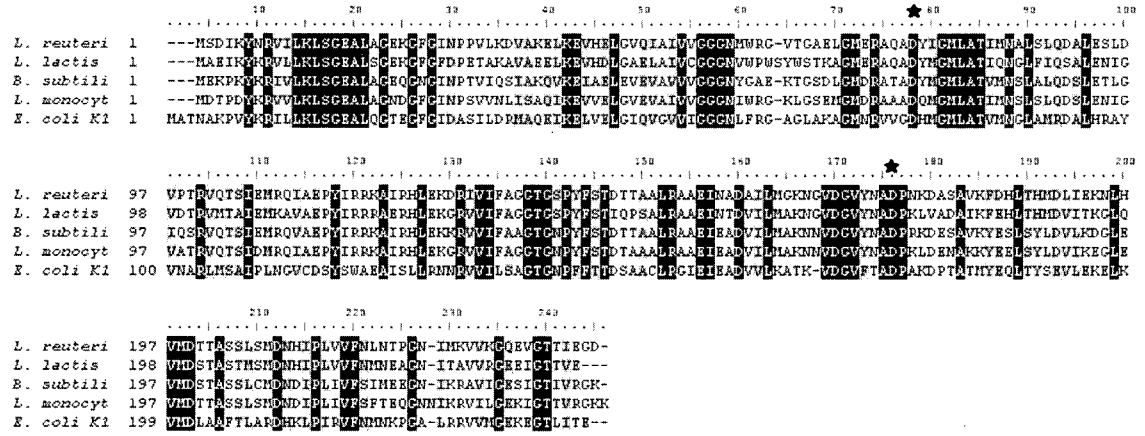

Fig. 3. ClustralW multiple alignment of the five UMP kinases.
Amino acid sequences of UMP kinases from *L. reuteri* (AAL60143), *Lactococcus lactis* subsp. *cremoris* MG1363 (CAB38122), *Bacillus subtilis* (O31749), *Listeria monocytogenes* (Q92C41), and *E. coli* K-12 (CAA55388) were aligned. Identical amino acid residues are indicated by an asterisk, and similar amino acid residues are indicated by a dot. The aspartate residue (D172) is believed to be essential for binding UMP, and another aspartate residue (D74) is believed to be essential for interaction with UTP and GTP [11] and they are marked by symbols of star.

ATG start codon. A putative rho-independent transcription terminator (AAGAGGGGGAAAATGAATTAGGAAG-CTAGTTCATTCTCGCTCTT, nucleotides 2,488–2,532) was found 20 nucleotides downstream from the stop codon of *frr* with a ΔG of −8.9 kcal/mol at 37°C. There is only three nucleotides between the stop codon (TAA) of *pyrH* and the start codon (ATG) of *frr*. The lack of space between *pyrH* and *frr* ORFs strongly indicates that both genes are transcribed together. In other words, *pyrH* and *frr* belong to the same operon.

Comparison of Deduced Amino Acid Sequence of UMP Kinase with Homologues

Blast analysis showed that *pyrH* from *L. reuteri* ATCC 55739 was similar to other *pyrH* genes at the nucleotide sequence level as well as the amino acid sequence level. UMP kinases, most closely resembling the *L. reuteri* enzyme, are those from *Lactococcus lactis* IL1403 (AE006430, 72% similarity), *Lactococcus lactis* MG1363 (AJ011960, 68%), and *Staphylococcus aureus* (AP003133, 66%). In Fig. 3, the amino acid sequence of *L. reuteri* enzyme was aligned with four other homologues from three Gram-positive and one Gram-negative bacterium. Bacterial UMP kinases have similar sizes (between 238 and 247 amino acids long), whereas eukaryotic homologues have additional peptides in the N-termini. UMP kinases appear to have conserved aspartate (D) residues. For UMP kinase of *Lactococcus lactis*, D172 (D174 in *E. coli* enzyme) is believed to be essential for UMP binding and D74 (D77 in *E. coli* enzyme) for interaction with the allosteric effectors, UTP and GTP [2]. Corresponding aspartic acid residues were also found in the same positions (D172 and D74, indicated by a star in Fig. 3) in *L. reuteri* enzyme.

The Organization of *pyrH* and *frr*

The organization of *pyrH-frr* in *L. reuteri* ATCC 55739 was the same as those of *Lactococcus lactis* subsp. *lactis* IL1403 (AE006430), *Lactococcus lactis* subsp. *cremoris*, MG1363 (AJ011960), *Pseudomonas aeruginosa* (AB010087), *Bacillus subtilis* (Z99112), *Aquifex aeolicus* (AE000703), *Escherichia coli* (X78809), and *Synechocystis* sp. (D90915). The fact that the organization of *pyrH-frr* on the chromosome is highly conserved among such diverse microorganisms indicates that *pyrH-frr* is essential for cell survival [14].

Northern Blot Analysis of *pyrH* and Neighboring Genes

The size of the mRNA transcripts from *pyrH* and neighboring genes were analyzed by Northern blot with radiolabeled *tsf*-, *pyrH*- and *frr*-specific 302, 432, and 331 bp PCR fragments as a probe, respectively. The autoradiogram (Fig. 4A) showed that only a single 1.0 kb transcript hybridized with the *tsf*-specific probe, in good agreement with the size of the *tsf* transcript predicted from the DNA sequence data. If the putative promoter and terminator marked in Fig. 2 are real ones, the size of the *tsf* transcript should be around 1,050 nucleotides. The result also confirmed that *tsf* is a monocistronic gene, as expected from its sequence data and the transcription terminator downstream of the stop codon of *tsf* functions *in vivo*. When *pyrH*- and *frr*-specific probes were used for hybridization, a single 1.4 kb transcript was detected (Figs. 4B, 4C). The results confirm that *pyrH* is transcribed with *frr* as part of an operon. In *Lactococcus lactis* subsp. *cremoris* MG1363, three genes were shown to constitute an operon [17]. The order of gene was *orfA-pyrH-frr*, and transcription started from a promoter immediately upstream of *orfA*. It is not clear why *pyrH* forms an operon with *frr*, since the former is involved in pyrimidine biosynthesis and the latter gene

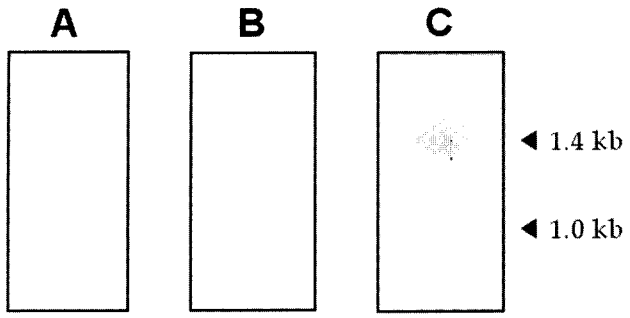

Fig. 4. Northern blot anaysis of the transcripts from *tsf*, *pyrH*, and *frr* genes in *Lb. reuteri* ATCC 55739.
The sizes of the different transcripts are indicated by arrows. A, *tsf*-specific 302 bp PCR product was used as a probe; B, *pyrH*-specific 432 bp PCR product was used as a probe; C, *frr*-specific 331 bp PCR product was used as a probe.

is involved in protein synthesis. However, this operon structure is well conserved among various organisms, including *Escherichia coli*, *Bacillus subtilis*, *Aquifex aeolicus*, *Synechocystis*, *Pseudomonas aeroginosa*, *Mycobacterium tuberculosis*, *Mycoplasma pneumoniae*, and *Rickettsia prowazekii* [17]. Thus, it is likely that this operon structure might be important for the survival of organisms in some ways.

Expression of the *pyrH* Gene in *E. coli*

After PCR amplification and ligation of the product into pET26b(+), pYRHE1 carrying the entire *pyrH* gene was obtained and transformed into *E. coli* BL21(DE3). In this plasmid, *pyrH* transcription is under the control of the T7*lac* promoter in the vector and would be induced by IPTG. At exponential phase of growth, IPTG was added to the culture at the final concentration of 0.5 mM, and the culture was further incubated for 3 h at 30°C [5]. Cells were recovered by centrifugation, and total cellular proteins were obtained and analyzed by SDS-PAGE (Fig. 5). An ~25 kDa protein band was induced, which was in good agreement with the molecular weight (25,957 Da) of UMP kinase calculated from the amino acid sequence. Thus, it can be concluded that *pyrH* from *L. reuteri* was successfully overexpressed in *E. coli*, judging from the intensity of the band. Overexpression of *pyrH* in *E. coli* can be a convenient alternative when purified UMP kinase is needed in high quantity.

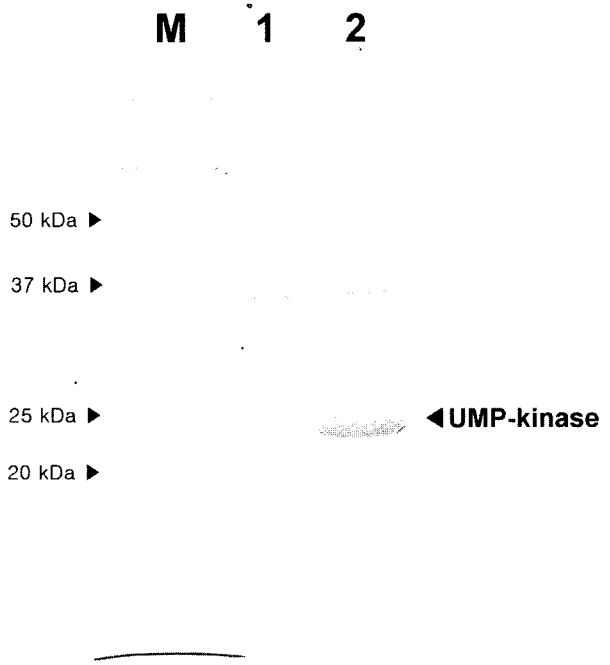

Fig. 5. Expression of UMP kinase in *E. coli* BL21(DE3).
M, Precision Plus Protein™ standard (Bio-Rad); 1, Uninduced BL21(DE3) harboring pYRHE1; 2, IPTG-induced BL21(DE3) harboring pYRHE1.

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