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Two nucleoside transporters in *Lactococcus lactis* with different substrate specificities

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In an alternative to biosynthesis of nucleotides, most organisms are capable of exploiting exogenous nucleotide sources. In order to do so, the nucleotide precursors must pass the membrane, which requires the presence of transporters. Normally, phosphorylated compounds are not subject to transport, and the utilization of nucleotides is dependent on exogenous phosphatases. The composition of transporters with specificity for purine and pyrimidine nucleosides and nucleobases is subject to variation. The ability of *Lactococcus lactis* to transport different nucleosides across the cell membrane was characterized at both genetic and physiological level, using mutagenesis and by measuring the growth and uptake of nucleosides in the different mutants supplemented with different nucleosides. Two high affinity transporters were identified: BmpA–NupABC was shown to be an ABC transporter with the ability to actively transport all common nucleosides, whereas UriP was shown to be responsible for the uptake of only uridine and deoxyuridine. Interestingly, the four genes encoding the ABC transporter were found at different positions on the chromosome. The *bmpA* gene was separated from the *nupABC* operon by 60 kb. Moreover, *bmpA* was subject to regulation by purine availability, whereas the *nupABC* operon was constitutively expressed.

INTRODUCTION

Most organisms exploit two strategies to obtain purine and pyrimidine nucleotides; they either synthesize them *de novo* through the biosynthetic pathways or they utilize pre-formed precursors present in the surroundings. *Lactococcus lactis* is no exception (Kilstrup *et al.*, 2005). Whereas the biosynthesis of both purines and pyrimidines has been investigated in this organism (Andersen *et al.*, 1994, 1996; Kilstrup *et al.*, 1998; Kilstrup & Martinussen, 1998; Martinussen *et al.*, 2001; Martinussen & Hammer, 1998; Nilsson & Kilstrup, 1998), only the pyrimidine salvage has been described in detail (Defoor *et al.*, 2007; Martinussen *et al.*, 1994; Martinussen & Hammer, 1994, 1995). It is known that a variety of nucleosides and nucleobases can be used as a sole purine and pyrimidine source (Kilstrup *et al.*, 2005). In order to utilize exogenous nucleotide precursors, the cells need to transport these compounds across the cytoplasmic membrane. Where the uptake of nucleotide precursors has been studied, each organism has been found to encode a number of transport systems with different and overlapping affinities.

In *L. lactis*, a uracil permease PyrP (Martinussen *et al.*, 2001) and the OroP orotate transporter (Defoor *et al.*, 2007) have been identified experimentally. In addition, two putative purine base transporters PbuO and PbuX have been found by sequence analysis (Kilstrup *et al.*, 2005). Based on the analysis of homologous genes in *Bacillus subtilis*, it is likely that PbuO is responsible for the uptake of all common purine bases except xanthine, which is taken up by PbuX (Kilstrup *et al.*, 2005; Saxild *et al.*, 2001).

With respect to nucleoside uptake, biochemical evidence suggests the presence of at least two different systems: one responsible for purine and cytidine transport and another responsible for uridine (Martinussen *et al.*, 2003). In a preliminary study, the purine and cytidine transport system was identified by transposon mutagenesis followed by sequencing of the mutated locus. The transposon was found to be integrated in the *yngE* gene; the first gene in a putative tri-cistronic operon potentially encoding an ABC transporter (Martinussen & Sørensen, 2005). The genes were consequently renamed *nupABC* (Kilstrup *et al.*, 2005; Martinussen & Sørensen, 2005; Wegmann *et al.*, 2007). In a later study, the *rnsBACD* operon from *Streptococcus mutans* was also shown to encode an ABC transporter facilitating the uptake of all nucleosides, including uridine, in this organism (Webb & Hosie, 2006). Interestingly, the amino acid sequences of NupA, B and C showed extensive homology to RnsA, C and D, respectively, whereas no homologues to RnsB were found in the vicinity of the *L.*

Abbreviations: GM17, M17 broth supplemented with 1 % glucose; GSA, SA-medium supplemented with 1 % glucose.

lactis nupABC operon. It is not common that nucleoside and nucleobase uptake systems belong to the ABC transporter family. Previously characterized transporters from prokaryotes all belong to the electrochemical potential-driven permeases, which is a large and diverse group of secondary transporters that includes uniporters, symporters and antiporters (Saier, 2000).

Transposon IS*S1* mutagenesis has been used successfully to obtain mutants in both the purine and pyrimidine biosynthetic pathways (Kilstrup & Martinussen, 1998; Martinussen *et al.*, 2001). In combination with a positive selection of mutants resistant to 5-fluoro-substituted pyrimidines as described for *L. lactis* (Martinussen & Hammer, 1995) this would be a very powerful means to obtain specifically tagged mutants.

It has been observed that the growth rate of *L. lactis* is significantly increased if purines are present in the growth medium (Kilstrup *et al.*, 2005; Martinussen *et al.*, 2003), making the investigation of nucleoside transport an important issue in the understanding of the physiology of this organism. In this study, we report the isolation and identification of two nucleoside transporters in *L. lactis*. We show that a gene (*bmpA*) located 60 kb upstream of the *nupABC* operon, is subject to regulation by purine availability, and encodes the fourth subunit of the nucleoside ABC transporter. The gene is required for a functional uptake system, facilitating the uptake of all common nucleosides. We also identify and characterize the gene for a second uptake system responsible for the uptake of uridine only. This gene was originally annotated as a gene encoding a putative substrate translocation pore but has been renamed *uriP*. The substrate specificities of both systems are described.

METHODS

Growth conditions. Lactococcal cultures were grown, unless otherwise stated, at 30 °C either in M17 broth (Terzaghi & Sandine, 1975) supplemented with 1 % glucose (GM17) or in defined SA-medium (Jensen & Hammer, 1993) with 1 % glucose (GSA). Agar plates were made by adding 15 g agar l^{-1} to the media. Supplements were added to the media at the following concentrations, unless otherwise indicated: uracil at 20 μg uridine ml^{-1}; cytidine and the corresponding deoxyribose derivatives at 40 μg ml^{-1}; 45 μg hypoxanthine ml^{-1}; inosine, guanosine, adenosine and the corresponding deoxyribose derivatives at 100 μg ml^{-1}; 5-fluorocytidine and 5-fluorouridine at 5 μg ml^{-1}; 5-azauracil at 50 μM; 5 μg erythromycin ml^{-1}; 5 μg tetracycline ml^{-1}; 100 μg ampicillin ml^{-1}. *Escherichia coli* strains were grown at 37 °C in Luria–Bertani broth with 150 μg erythromycin ml^{-1} when indicated. For identification of β-galactosidase activity on plates, 180 μg X-Gal ml^{-1} was added.

***In vitro* DNA manipulation and transformation.** Chromosomal lactococcal DNA was prepared as described by Johansen & Kibenich (1992). The methods described by Sambrook *et al.* (1989) were used for general *in vitro* DNA methods and *E. coli* transformation (Sambrook *et al.*, 1989). *L. lactis* was transformed by electroporation (Holo & Nes, 1995). Sequencing reactions were performed by either MWG, Ebersberg, Germany, or Macrogen, Seoul, Korea, on either purified plasmids or PCR products prepared according to the requirements of the company.

ISS*1* transposon mutagenesis and selection for transport mutants. Pools of pG^+host9::IS*S1* and pG^+host8::IS*S1* transposition mutants of the *L. lactis* strain MB420 (*pyrB*), MK185 (*purD*) and CS24 (*purD*, *nupA*) were obtained as described previously (Kilstrup & Martinussen, 1998). After resuspension of the mutant libraries in 0.9 % NaCl, aliquots of 100 μl culture, both undiluted and 10-fold diluted were spread on GSA plates containing either uracil (MB420 background) or hypoxanthine and either 5-fluorouridine or 5-fluorocytidine. Plates were incubated at 37 °C overnight. The integration site of the IS*S1* transposon was determined by sequencing. Chromosomal DNA was isolated, digested with *Hin*dIII, ligated and used as template in a PCR with the primers ISS1-down and M13 forward (Table 1). Curing of the pG^+host9 and pG^+host8 plasmids from the different *L. lactis* strains was performed as described previously (Kilstrup & Martinussen, 1998).

Bacterial strains and plasmids. The bacterial strains, plasmids and primers used in this study are listed in Table 1. MB131 and MB181 were obtained after transforming *L. lactis* MG1363 and MK185, respectively, with plasmid pMBK1031, selecting for resistance to erythromycin. The plasmid was constructed in the following way: *L. lactis* MG1363 chromosomal DNA was amplified by PCR using primers yngE5F and yngE10R and *Taq* polymerase. Using the TOPO cloning strategy from Invitrogen, the fragment was inserted into the TOPO cloning vector pCR2.1-TOPO. Subsequently, the fragment was moved to the vector pRC1 after digestion of both with *Xho*I and *Spe*I, and the resulting plasmid was propagated in *E. coli* DH5α, selecting for resistance to erythromycin.

Strains MBK132 and MBK182 were obtained after transforming *L. lactis* MG1363 and MK185, respectively, with plasmid pMBK1032, which was constructed in exactly the same way as pMBK1031 except that the primers BmpA_F1 and BmpA_R1 were used in the PCR.

Strain MM103 was obtained after transforming *L. lactis* MG1363 with plasmid pMM102, which was constructed as described for pMBK1031, except that primers Urup_F1 and Urup_R1 were used in the PCR, and pCS1966 was used as the integration vector. Strain CBJ443 (MG1363 P_{bmpA}::*lacLM*) was constructed as follows: a PCR product using *Pfu* polymerase was obtained with the primers CBJP135 and CBJP138. The product was purified and digested with *Hin*dIII and *Bgl*II. The digested PCR product was ligated in the presence of plasmid pLB86 digested with the same enzymes. The ligation mixture was transformed into chemically competent *E. coli* DH5α selecting for resistance to ampicillin and erythromycin, and a positive clone was confirmed by sequencing of the resulting plasmid. The plasmid was transformed into electrocompetent MG1363 previously transformed with pLB95. The transformed cells were spread on GM17 plates supplemented with erythromycin and X-Gal, and blue colonies were chosen; these were cured for the presence of pLB95 by growth on GM17 plates with 1 % NaCl and erythromycin at 37 °C (Kilstrup & Hammer, 2000). Strains CBJ431 and CBJ444 were similarly constructed, using the primer pairs CBJP137/CBJ138 and CBJP136/CBJP138, respectively, introducing site-directed single-nucleotide mutations relative to CBJ443.

Strain MM148 (MG1363 P_{nupA}::*lacLM*) was constructed in exactly the same way, except that primers YngE_F1 and YngE_R2 were used, and the PCR fragment was cloned using the TOPO strategy as above. The resulting plasmid was digested with *Xho*I and *Pst*I and used in a ligation with pLB86. All strains were verified by PCR using various primers using isolated chromosomal DNA as template.

Assays. Uptake assays on exponentially growing cells in three biological replicates were conducted as previously described

Table 1. Strains, plasmids and primers used in this work

Strain, plasmid or primer	Genotype or construction	Source/reference
E. coli		
DH5α	*φ*80*lacZ*ΔM15 Δ(*lacZYA–argF*) *U169 recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1*	Stratagene
L. lactis		
MG1363	Plasmid-free strain	Gasson (1983)
MK185	*purD*::IS*S1* MK138 cured for pG$^+$host9 (*purD*)	Kilstrup & Martinussen (1998)
CS11	MB420 *pyrB*::IS*S1*, *udk*::pG$^+$host9::IS*S1* (*pyrB udk-1*, Erm^R)	This study
CS12	MB420 *pyrB*::IS*S1*, *udk*::pG$^+$host9::IS*S1* (*pyrB udk-2*, Erm^R)	This study
CS13	MB420 *pyrB*::IS*S1*, *udk*::pG$^+$host9::IS*S1* (*pyrB udk-3*, Erm^R)	This study
CS14	MB420 *pyrB*::IS*S1*, *udk*::pG$^+$host9::IS*S1* (*pyrB udk-4*, Erm^R)	This study
CS16	MK185 *nupA*::pG$^+$host9::IS*S1* (*pyrD nupA-1*, Erm^R)	This study
CS18	MK185 *nupA*::pG$^+$host9::IS*S1* (*pyrD nupA-2*, Erm^R)	This study
CS24	*nupA*::IS*S1* CS16 cured for pG$^+$host9 (*pyrD nupA-1*)	This study
MBK131	MG1363 *nupB*::pMBK1031 (*nupBC*, Erm^R)	This study
MBK132	MG1363 *bmpA*::pMBK1032 (*bmpA*, Erm^R)	This study
MBK181	MK185 *nupB*::pMBK1031 (*purD nupBC*, Erm^R)	This study
MBK182	MK185 *bmpA*::pMBK1032 (*purD bmpA*, Erm^R)	This study
MB420	MG1363 *pyrB*::IS*S1* (*pyrB*)	Martinussen *et al.* (2001)
MM14	CS24 *uriP*::pG$^+$host8::IS*S1* (*purD nupA-1 uriP-1*, Tet^R)	This study
MM16	CS24 *uriP*::pG$^+$host8::ISS1 (*purD nupA-1 uriP-2*,Tet^R)	
MM50	MM14 *uriP*::IS*S1* (*purD nupA-1 uriP*)	This study
MM101	MG1363 *uriP*::pMM102 (*uriP* Erm^R)	This study
MM103	MK185 *uriP*::pMM102 (*purD uriP* Erm^R)	This study
CBJ443	MG1363 P_{bmpA}::*lacLM*, Erm^R	This study
CBJ444	MG1363 P_{bmpA}::*lacLM*, Erm^R (PurBox down mutation)	This study
CBJ431	MG1363 P_{bmpA}::*lacLM*, Erm^R (PurBox up mutation)	This study
MM148	MG1363 P_{nupA}::*lacLM*, Erm^R	This study
Plasmids		
pCR2.1-TOPO	Amp^R, Kan^R	Invitrogen
pG$^+$host9::IS*S1*	Ori^{ts} used for IS*S1* mutagenesis, Erm^R	Maguin *et al.* (1996)
pG$^+$host8::IS*S1*	Ori^{ts} used for IS*S1* mutagenesis, Tet^R	Maguin *et al.* (1996)
pRC1	Erm^R	Le Bourgeois *et al.* (1992)
pLB86	*attP*, *bla*, Erm^R, *lacLM* – promoterless *lacLM* integration vector	Brøndsted & Hammer (1999)
pLB95	*int*, Tet^R – expresses the integrase from TP901-1	Brøndsted & Hammer (1999)
pCS1966	Erm^R, *oroP*	Solem *et al.* (2008)
pMBK1031	pRC1–*nupBC* internal fragment (Erm^R)	This study
pMBK1032	pRC1–*bmpA* internal fragment (Erm^R)	This study
pMM102	pCS1966–*uriP* internal fragment (Erm^R)	This study
Primers*		
ISS1-down	GAAGAAATGGAACGCTC	This study
M13 forward	CTGGCCGGTCGTTTTAC	This study
yngE5F	CATAAGTTGGATGAAATTCGTG	This study
yngE10R	ATGAGGACATCCAATTGGTTC	This study
BmpA_F1	GCAGGATGCCGTTCACATGA	This study
BmpA_R1	AAGTTAGAATCTTTACCGTC	This study
Urup_F1	TCTAGTGATAATGTGGGCTC	This study
Urup_R1	CCACTCGAAGGACCTTCGC	This study
YngE_F1	CGTAGCCTCGAGGGAAAATCAAACCAGAGGG	This study
YngE_R2	TTTAACTGCAGCATTTGCCATAGTGGCTCC	This study
CBJP135	CGAGTTAAGCTTTGTTAGTTATTTCCGAAAGTTAAGCAAG	This study
CBJP136	CGAGTTAAGCTTTGTTAGTTATTTCCCAAAGTTAAGCAAGAAAATGAAATATTCC	This study
CBJP137	CGAGTTAAGCTTTGTTAGTTATTTCCGAACGTTAAGCAAGAAAATGAAATATTCC	This study
CBJP138	CGAGTTAGATCTATAGCACTAACTGCGATTACG	This study

*Primers are given in 5′–3′ orientation.

(Martinussen *et al.*, 2003). For enzyme assays, the different lactococcal strains were grown exponentially for at least eight generations in GSA medium with or without hypoxanthine. Aliquots (2 ml) were harvested at different times during exponential growth between OD_{450} 0.5 and 1.0 and washed in 1 ml ice-cold 0.9 % NaCl; pellets were stored at −20 °C. The amount of β-galactosidase in the cells was assayed by resuspending the pellet in 200 µl ice-cold Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$), and the cell density was measured (OD_{450}) in a 20-fold dilution in Z-buffer. This value was used correcting for loss and inaccuracies during washing and resuspension. For the assay, 100 µl cell suspension was transferred to a mixture of 787.5 µl Z-buffer, 12.5 µl 0.1 % SDS, 25 µl chloroform, thoroughly mixed for 10 s, and incubated at 30 °C. After 2 min, the reaction was started by addition of 100 µl 4 mg *ortho*-nitrophenyl-β-galactoside ml^{-1}. Upon development of a yellow colour, the reaction was stopped by the addition of 500 µl 1 M Na_2CO_3 and vigorous mixing of the tubes, which were then kept on ice. As a blank reference, the cell suspension was substituted with 100 µl Z-buffer. The reaction mixtures were centrifuged for 2 min at 15 000 ***g***, and OD_{420} and OD_{550} of the supernatant was measured at room temperature. The specific enzymic activity was determined as $1000 \times (OD_{420} - 1.75\ OD_{550}) \times [OD_{450}\ min^{-1}\ (ml\ culture)^{-1}]$, similarly to the Miller unit (Sambrook *et al.*, 1989), with standard deviations for three biological replicates.

RESULTS

Identification of the purine nucleoside/cytidine transporter

The pyrimidine analogue 5-fluorocytidine is toxic to *L. lactis* after intracellular conversion through the pyrimidine salvage pathways (Fig. 1), primarily due to the formation of 5-fluoro-dUMP (Martinussen & Hammer, 1995). This compound is a transition state inhibitor of the thymidylate synthase (*thyA*), preventing the formation of dTTP. Mutations in pyrimidine salvage and uptake machinery, confer resistance towards 5-fluorocytidine. Four independent libraries with more than 8000 pG^+host9::IS*S1* transposon mutants were established in *L. lactis* MK185 (*purD*::IS*S1*) as described previously (Kilstrup & Martinussen, 1998). From each library, 10^4 cells were spread on plates of chemically defined GSA medium supplemented with 5-fluorocytidine and hypoxanthine to satisfy the purine requirement. Mutants were readily obtained, and four 5-fluorocytidine-resistant clones from each of the libraries were screened for their ability to grow on inosine as a sole purine source. We have previously shown that cytidine and purine nucleosides, including inosine, could share a common transport system (Martinussen *et al.*, 2003). Consequently, it was expected that the 5-fluorocytidine-resistant clones had lost the ability to efficiently utilize the purine nucleoside inosine if the resistance was due to an impaired cytidine uptake. Growth of all 16 5-fluorocytidine-resistant mutants was impaired on inosine. This strongly suggested that mutants in the cytidine/purine nucleoside transporter were obtained. The integration sites were identified by sequencing in two selected mutants (CS16 and CS18). They were both found at different sites in the *nupA* gene (previously annotated as *yngE*), as shown in Fig. 2. This strongly indicates that *nupA* does indeed encode a cytidine/purine nucleoside transporter. The *nupA* gene showed homology to the ATP binding subunit of ABC transporters, and is the first gene of a putative tri-cistronic operon *nupABC*. The *nupB* and *nupC* genes showed homology to the permease subunits of ABC transporters. After insertion of a non-replicating plasmid by homologous recombination in the *nupA* gene of MK185 (*purD*), an *L. lactis* (*nupBC*, *purD*) mutant was isolated (MBK181). Similarly to the selected clones from the mutant library, this strain was unable to grow on inosine as a sole purine source and was resistant towards 5-fluorocytidine, verifying that *nupABC* does encode the cytidine/nucleoside transporter. For further analysis, we created an erythromycin-sensitive, temperature-resistant

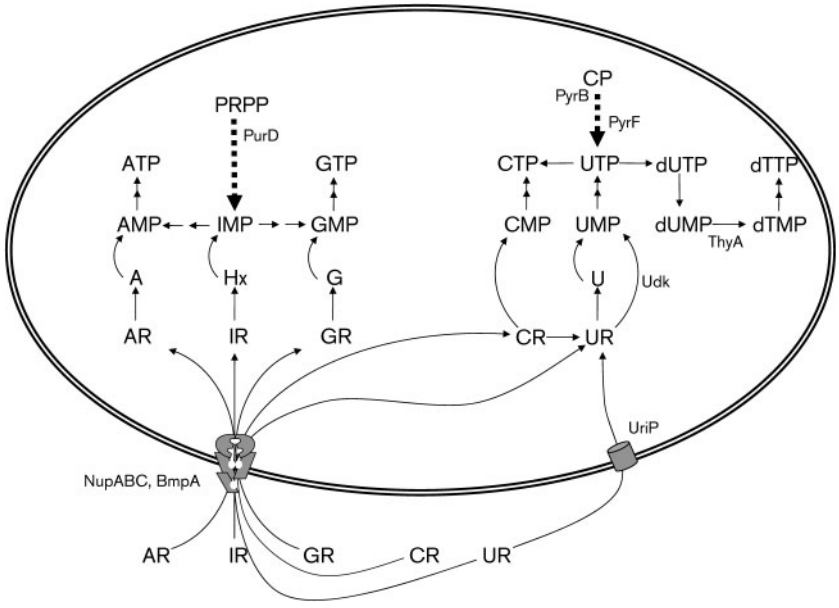

Fig. 1. The uptake and salvage of ribonucleosides in *L. lactis*. The purine and pyrimidine biosynthetic pathways, starting with 5-phosphoribosyl-1-pyrophosphate (PRPP) and carbamoylphosphate (CP), respectively, are shown by dashed arrows symbolizing the 11 and six enzymic steps. Arrows with double arrowheads indicate two enzymic reactions. BmpA, NupABC represents the general nucleoside ABC uptake system; UriP represents the uridine transporter. AR, adenosine; GR, guanosine; IR, inosine; CR, cytidine; UR, uridine; A, adenine; Hx, hypoxanthine; G, guanine; U, uracil. Where appropriate, the relevant enzymes are indicated. For a full overview refer to the paper by Kilstrup *et al.* (2005).

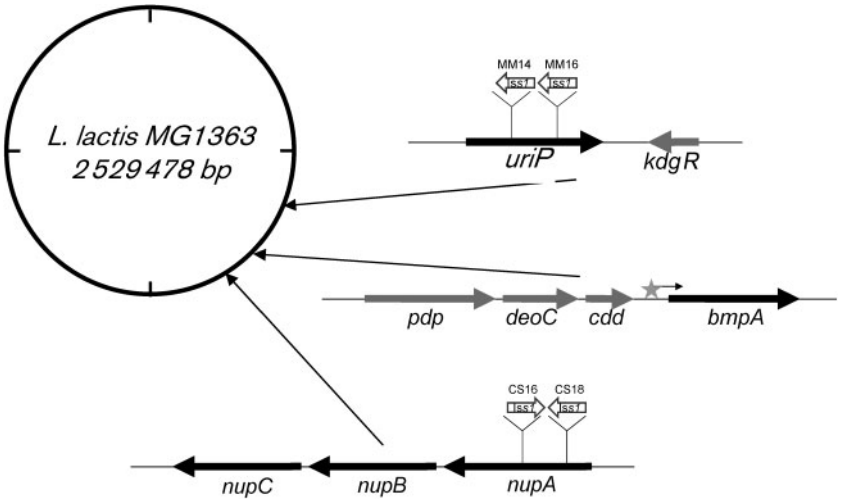

Fig. 2. Genetic organization of the genes encoding the nucleoside transporters in *L. lactis* MG1363. Their positions on the chromosome are indicated. The genes *nupA*, *nupB*, *nupC* and *bmpA* encode the general nucleoside ABC transporter; *uriP* [originally annotated as *llmg_0856* (Wegmann *et al.*, 2007)] encodes the uridine permease. The PurR-activated promoter of the *bmpA* gene is indicated by an asterisk and an arrow. The *kdgR* gene encodes a putative GntR family transcription regulator; *pdp*, *deoC* and *cdd* encode the pyrimidine phosphorylase, deoxyribose aldolase and the cytidine deaminase, respectively. The positions of the IS*S1* elements in the different mutant strains are shown by arrows.

nupA::IS*S1* derivative (CS24) of CS16, leaving a single IS*S1* element in the *nupA* gene.

In a previous study, a putative protein BmpA with unknown function was found among genes regulated by purine availability (Beyer *et al.*, 2003). BmpA belongs to the type 1 superfamily of periplasmic binding protein, consisting primarily of substrate-binding subunits in ABC transporters.

This could indicate that this protein was involved in transport of purine nucleotide precursors across the membrane. To clarify this, a mutant strain MBK182 (*bmpA*, *purD*) was constructed and its phenotype was determined. This strain was unable to grow on inosine as a sole purine source, and had simultaneously acquired resistance towards 5-fluorocytidine, i.e. the same phenotype as CS16 (*purD*, *nupA*) and MBK181 (*purD*, *nupBC*). Despite the fact that *bmpA* and *nupABC* are separated by approximately 60 kb, the experimental data strongly suggest that BmpA and NupABC constitute a nucleoside ABC transporter.

To verify that BmpA together with NupABC is the general purine nucleoside transporter identified in our previous study (Martinussen *et al.*, 2003), the ability of common purine nucleosides and deoxynucleosides to act as the sole purine source was investigated in CS24 (*purD*, *nupA*) and MK185 (*purD*) by plating on mimimal media supplemented with the different purines (Table 2). All derivatives fulfilled the purine requirement of strain MK185 (*purD*), but none was able to facilitate growth in the transport mutant CS24 (*purD*, *nupA*), showing that BmpA–NupABC is indeed responsible for the uptake of all common purine nucleosides.

Previous published data suggest that cytidine and purine nucleosides are taken up by the same transporter, while uridine uptake is transported by an alternative system (Martinussen *et al.*, 2003). To test whether the mutants in *bmpA* and *nupABC* were still able to transport uridine, CS24 (*purD*, *nupA*) and MBK182 (*purD*, *bmpA*) were spread on agar plates containing defined medium supplemented with 5-fluorouridine and hypoxanthine. As seen in the reference strain MK185 (*purD*), both CS24 and MBK182 were sensitive to 5-fluorouridine, indicating that uridine was still taken up by the mutants. This was further substantiated in an uptake experiment using radioactive adenosine and uridine. These physiological experiments were confirmed (Fig. 3); the mutations in either MBK131 (*nupBC*) or MBK132 (*bmpA*) result in complete loss of adenosine transport, whereas uridine uptake was still observed. Interestingly, uridine uptake was slower in both mutants compared with the reference strain. Together with the fact that CS24 (*purD*, *nupA*), MBK181 (*purD*, *nupBC*) and MBK182 (*purD*, *bmpA*) are resistant to 5-fluorocytidine and hence unable to convert cytidine and to utilize purine nucleosides, these observations demonstrate the existence of an additional nucleoside uptake system that enables the uptake of uridine but neither cytidine nor purine nucleosides. On the other hand, it cannot be ruled out that the BmpA–NupABC transporter can also

Table 2. Substrate specificity of BmpA–NupABC and UriP

Nucleoside	Substrate for the uptake system	
	BmpA–NupABC	UriP
Inosine	+	−
Deoxyinosine	+	−
Adenosine	+	−
Deoxyadenosine	+	−
Guanosine	+	−
Deoxyguanosine	+	−
Xanthosine	+	−
Uridine	+	+
Deoxyuridine	+	+
Cytidine	+	−
Deoxycytidine	+	−

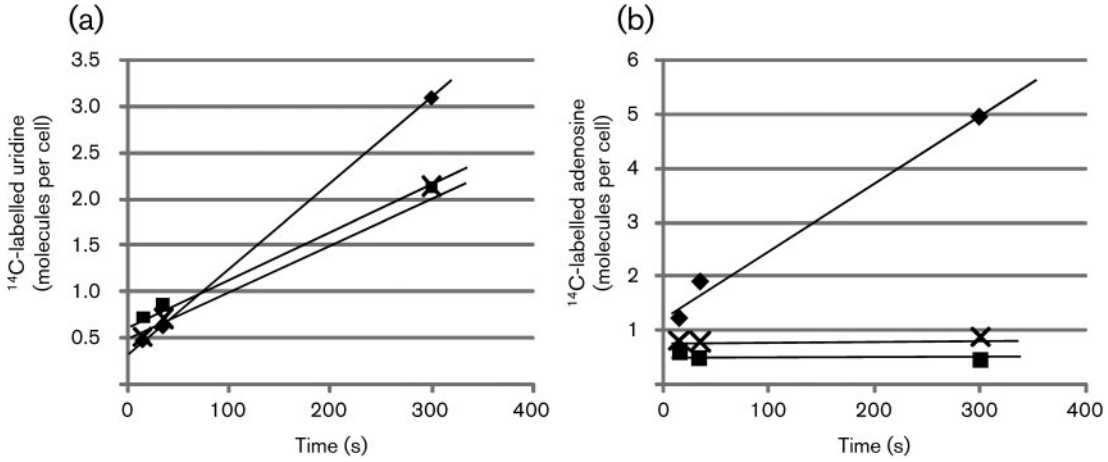

Fig. 3. Uptake of ^{14}C-labelled uridine (a) and adenosine (b) in *L. lactis*. ◆, MG1363 (wild-type); ■, MBK131 (*nupBC*); ×, MBK132 (*bmpA*).

transport uridine, as uridine uptake is slower in the mutants. Despite several attempts, it was not possible to establish the *bmpA* and *nupABC* mutations in a pyrimidine-requiring background.

Expression of *bmpA* but not *nupA* is regulated by purine availability

As mentioned above, the level of BmpA was reduced in the presence of exogenous purines. A consensus PurR binding site motif (WWWWCCGAACWWW) – the PurBox – with an optimal spacing of 57 with respect to the −10 sequence has previously been identified as the putative activator binding in the *bmpA* promoter (Beyer *et al.*, 2003; Kilstrup & Martinussen, 1998). To investigate whether expression of *bmpA* is subject to PurR-mediated regulation in *L. lactis* MG1363, transcriptional fusions between different derivatives of the *bmpA* promoter and the β-galactosidase-encoding *lacLM* were constructed and integrated on the chromosome. The PurBox in the derivatives of the *bmpA* promoter sequence was either destroyed or optimized to fit the consensus (Table 3). The strains were cultured in the chemically defined GSA medium in the absence or presence of hypoxanthine, and the β-galactosidase activity was measured during exponential growth (Table 3). The results clearly show that the expression of *bmpA* is regulated by exogenous purine addition (4.9-fold) and that this regulation is mediated by PurR, as in the absence of hypoxanthine, a mutation in the PurBox leads to reduced activity, whereas an optimization of the PurBox results in a significant increase in expression with conservation of the regulation. It should be noted that some expression is still seen, even though the PurBox is destroyed, most likely due to the presence of a functional −35 sequence.

With respect to the regulation of the expression of the *nupABC* operon, analysis of the 200 bp untranslated sequence upstream of *nupA* and after *llmg_1124*, a conserved gene with unknown function, revealed that no putative PurBox is present. This suggests that either the expression of *nupABC* is unregulated by PurR or the *nupABC* genes are expressed from a promoter further upstream. To investigate

Table 3. β-Galactosidase activity in strains carrying different *bmpA–lacLM* or *nupA–lacLM* fusions

Strain	Chromosomal *lacLM* fusion	β-Galactosidase activity*		Fold induction
		No supplement	Hypoxanthine	
CBJ443	P_{bmpA}::*lacLM*†	22.8±4.1	4.7±0.4	4.9
CBJ444	P_{bmpA}::*lacLM* disrupted PurBox‡	11.6±0.6	4.6±0.4	2.5
CBJ431	P_{bmpA}::*lacLM* optimized PurBox‡	73.8±6.3	17.4±0.6	4.2
MM148	P_{nupA}::*lacLM*	1.1±1	0.6±0.1	1.9

*The β-galactosidase activity is given as $1000\times(OD_{420}-1.75\times OD_{550})\times[OD_{450}\ min^{-1}\ (ml\ culture)^{-1}]\pm SD$.

†The sequence of the *bmpA* promoter is GTTATTTCCGAAAGTTAAGCAAGAAAATGAAATATTCCGCTAAATTATGCTTGCAAAAAGATACA-ATATAGCTTAAAAT (the PurBox, −35 and −10 sequences are underlined).

‡The sequences of the disrupted PurBox and the optimized PurBox are ATTTCCCAAAGTT and ATTTCCGAACGTT, respectively.

whether the expression of the *nupABC* operon is subject to regulation as *bmpA*, a *nupABC* transcriptional fusion to *lacLM* was constructed in *L. lactis* MG1363, and the β-galactosidase activity was measured during exponential growth in the presence and absence of hypoxanthine (Table 3). This reveals that the expression of the *nupABC* operon is 1.9-fold lower in the presence of hypoxanthine. Approximately the same degree of change was observed in the *bmpA* mutant with a disrupted PurBox. It should be emphasized that we generally observe a twofold lower expression from promoters unrelated to purine metabolism, including a number of synthetic derivatives (J. Martinussen, unpublished data). We therefore conclude that only the expression of *bmpA* is subject to PurR-mediated regulation.

Identification of the uridine transporter

In order to identify a gene responsible for uridine uptake, a pG$^+$host8::IS*S1* transposon library was established in both MB420 (*pyrB*) and CS26 (*purD*, *nupA*). From four independent libraries of more than 10 000 clones each, 5-fluorouridine-resistant strains were obtained on GSA supplemented with (i) 5-fluorouridine, uracil and tetracycline in the MB420 background and (ii) 5-fluorouridine, hypoxanthine and tetracycline in the CS26 background. In the pyrimidine-requiring (*pyrB*) background, 16 analysed mutants were still able to grow on uridine as a sole pyrimidine source, indicating that they have kept their uridine transport ability. DNA from four independent mutants (CS11–14) was isolated, and the position of the transposon was determined. All four mutants had an IS*S1* integrated in the *udk* gene encoding the uridine kinase (Fig. 1). The same trend was observed in an alternative experiment, where spontaneous 5-fluorouridine-resistant mutants were isolated in the *pyrB* background. All of the 48 independent resistant mutants were able to grow on uridine as a sole pyrimidine source, indicating that mutants impaired in uridine transport are not easily obtained. That is consistent with the presence of more than one route of uridine uptake in *L. lactis*.

In CS26 (*purD*, *nupA*) several 5-fluorouridine-resistant mutants were obtained. The chromosomal DNA from two 5-fluorouridine-resistant strains, MM14 and MM16, isolated from independent libraries were analysed by sequencing of the DNA flanking the IS*S1* element, as previously described. In both cases, the IS*S1* element was integrated in *llmg_0856*, a conserved hypothetical protein of 480 aa which belongs to the major facilitator superfamily. Consequently, we renamed *llmg_0856* as *uriP*. A BLAST search revealed that the gene is found abundantly and annotated in general as a major facilitator superfamily permease. One of the clones, MM14, was chosen for further study, and the temperature-sensitive plasmid and the tetracycline resistance gene were cured by homologous recombination between the duplicated IS*S1* elements, leaving a single IS*S1* copy in the *uriP* gene, thus obtaining MM50 (*purD*, *nupA*, *uriP*).

To verify that *uriP* is not required for the survival of a purine-requiring mutant on purine nucleosides, MM103 (*purD*, *uriP*) was constructed. It grew on all tested purine nucleoside sources, confirming that *uriP* is not involved in purine nucleoside uptake.

In order to test whether UriP is the sole uridine transporter, MB420 (*pyrB*) was transformed with pMM102 in order to establish the *uriP* mutation in a pyrimidine-requiring background. Despite several attempts, no transformants were obtained, as found with the *nup* deletion plasmids pMBK1031 and pMBK1032 mentioned above. Alternatively, an inhibitor of pyrimidine biosynthesis could be used to simulate pyrimidine auxotrophy in a *uriP* strain. Therefore, we tested whether 5-azauracil, an inhibitor of the OMP decarboxylase encoded by *pyrF* (Handschumacher, 1963; O'Sullivan & Ketley, 1980), was able to inhibit growth of *L. lactis*. Growth curves of *L. lactis* MG1363 propagated in GSA medium supplemented with 0–10 mM 5-azauracil were obtained, which showed that reduced growth rates correlated with the increasing concentrations of 5-azauracil. Concentrations above 500 μM resulted in a total inhibition of growth whereas 50 μM gave a reduction in growth rate of approximately 50 % (data not shown). The latter concentration was used to test whether uridine could counteract the 5-azauracil toxicity in the *nupA* and *uriP* single and double mutants (Fig. 4). In both the *nupA* and *uriP* strains, the toxic effect of 5-azauracil was abolished in the presence of uridine, whereas uridine had no effect on the mutant lacking both nucleoside transporters. This suggests that both *uriP* and *bmpA–nupABC* encode uridine transporters.

To further prove that both *bmpA–nupABC* and *uriP* encode proteins involved in uridine transport activity, the uptake of uridine was measured in the *nupA* and *uriP* single and double mutants (Fig. 5). Both UriP and BmpA–NupABC are uridine transporters, as only the double mutant was impaired in uridine transport. The uptake rates were slower in both single mutants compared with the wild-type strain. This is consistent with the previous experiments, indicating that both uptake systems contribute significantly to the *in vivo* uptake of uridine.

DISCUSSION

The uptake of nucleotide precursors stimulates growth of *L. lactis* (Kilstrup *et al.*, 2005; Martinussen *et al.*, 2003). Therefore, uptake of these compounds plays an important role in the overall cell physiology. The aim of this work was to get a full overview of nucleoside transport in *L. lactis*, and indeed we succeeded in identifying two different transport systems, BmpA–NupABC and UriP, that were fully responsible for all nucleoside transport in *L. lactis*. The BmpA–NupABC system is an ABC transporter capable of transporting all common nucleosides, whereas UriP has specificity for uridine and deoxyuridine only.

Previous studies have shown that *B. subtilis* nucleoside uptake is accomplished by the purine nucleoside transporter NupG (encoded by *yxjA*) (Johansen *et al.*, 2003) and the pyrimidine transporter NupC encoded by *nupC* (Saxild

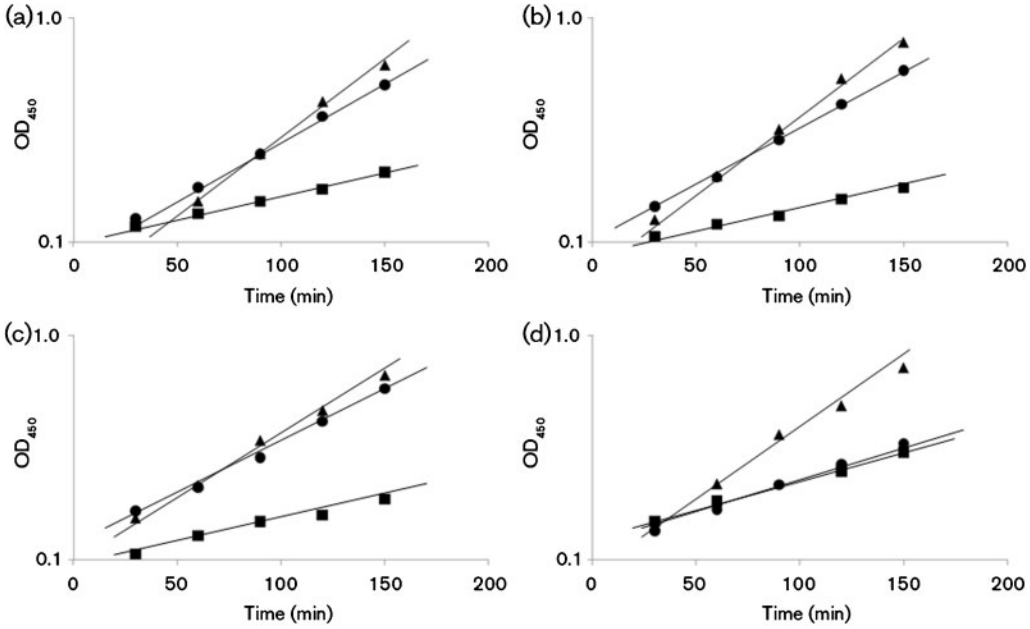

Fig. 4. Growth of *L. lactis* strains MK185 (*purD*; a), CS24 (*purD*, *nupA*; b), MM103 (*purD*, *uriP*; c) and MM50 (*purD*, *nupA*, *uriP*; d) in GSA at 30 °C with 45 μg hypoxanthine ml^{-1} as the purine source (▲). Growth in the presence of 5-azauracil (50 μM) (an inhibitor of pyrimidine biosynthesis; ■) and 50 μM 5-azauracil and 100 μg uridine ml^{-1} (●) of the four strains is shown. Representative results are shown.

et al., 1996). At the sequence level, these transporters are completely unrelated to BmpA–NupABC and UriP in *L. lactis*. Single mutants were constructed in *B. subtilis*, and an 80 % reduction in purine transport was observed in a *nupG* (*yxjA*) mutant, whereas pyrimidine transport was unaffected (Johansen *et al.*, 2003). The opposite was observed in the *nupC* mutant (Saxild *et al.*, 1996). Since the analysis of a *nupC–yxjA* double mutant has not been reported, the question of whether the residual transport is due to overlapping specificities of the two transport systems remains open. Whereas no UriP homologue can be identified in *B. subtilis*, a putative operon consisting of four genes (*yufN*, *yufO*, *yufP* and *yufQ*) show a high degree of similarity to the *bmpA*, *nupA*, *nupB* and *nupC* genes, respectively. The identity at the protein level is between 45 and 62 %. No function has been annotated to the *B. subtilis* genes, but it could be speculated that this operon is responsible for the residual nucleoside transport observed in the *nupG* and *nupC* mutants in *B. subtilis*.

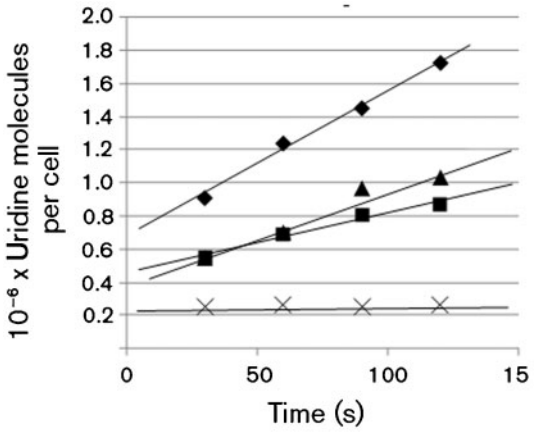

Fig. 5. Uptake of ^{14}C-labelled uridine in GSA at 30 °C in *L. lactis* MG1363 (wild-type; ◆) and the derivatives CS24 (*nupA*; ■), MM103 (*uriP*; ▲) and MM50 (*nupA*, *uriP*; ×). Representative results are shown.

The four subunits of BmpA–NupABC constitute a classical ABC transporter (Saier, 2000); an intracellular ATPase domain NupA, two integral membrane domains formed by NupB and NupC, and one extracellular solute-binding domain BmpA. Surprisingly, it was found that *bmpA* encoding the solute-binding subunit is located 60 kb downstream of the *nupABC* gene cluster that most likely constitutes an operon. A BLAST search revealed that all four genes are commonly found among Gram-positive organisms, including members of the genera *Streptococcaceae*, *Enterococcaceae*, *Listeriaceae*, *Lactobacillaceae*, *Leuconostocaceae* and *Bacillaceae*. The homologous transporter from *Streptococcus mutans* has been subjected to a detailed investigation, and it has specificity for all common nucleosides and to some of the toxic derivatives (Webb & Hosie, 2006).

The *uriP* gene encoding a uridine transporter facilitates the uptake of uridine and deoxyuridine, and its gene product belongs to the major facilitator superfamily of permeases.

Many of the homologues were found to be drug efflux pumps. In one case, analysis of the uridine utilization operon from *Corynebacterium glutamicum* ATCC 130032 demonstrated the presence of a uridine transporter *uriT* by the inability of a *uriT* mutant to grow on uridine as a sole carbon source (Brinkrolf *et al.*, 2008). Analysis of the specificity of the *C. glutamicum* UriT transporter was not performed.

Surprisingly, only *bmpA* and not the other genes encoding the ABC transporter is subject to regulation by the availability of nucleosides or nucleobases. The biological rationale behind this finding is not clear. It could be speculated that *L. lactis* harbours an additional gene encoding an alternative solute-binding domain that changes the substrate specificity of the transporter. However, no evidence favours this idea. If the PurBox upstream of *bmpA* is optimized to consensus, an increase in expression is observed under induced as well as uninduced conditions. This is because PurR activates expression from the PurBox when forming a complex with 5-phosphoribosyl-1-pyrophosphate (PRPP) and the level of PRPP is believed to increase in the absence of exogenous purines (Kilstrup *et al.*, 1998; Kilstrup & Martinussen, 1998). Since PRPP is also present when purine levels are high, a fraction of the PurR proteins will be in their active conformation; by increasing the PurR binding affinity, an increase in transcription of PurR-activated genes is observed.

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