The orotate transporter encoded by *oroP* from *Lactococcus lactis* is required for orotate utilization and has utility as a food-grade selectable marker

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A new lactococcal plasmid, pDBORO, was isolated from the *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* strain DB0410. This plasmid is responsible for the sensitivity of DB0410 to the toxic pyrimidine analogue 5-fluoroorotate. The complete nucleotide sequence has been determined and amounts to 16 404 bp. Of 15 ORFs encountered, three were found to be insertion sequence (IS) elements, identified as two IS946 and one IS982. Two ORFs are incomplete due to the insertion of an IS element in their C-terminal region. Homologues for four ORFs were found in the IL1403 sequence: the *copB* gene, coding for a copper-potassium-transporting ATPase B, and the *ysbA*, *ysbB* and *ysbC* genes. The structural organization of the pDBORO replication region is highly similar to other theta-replicating plasmids in both the *cis*- (*repA*) and *trans*-acting (*repB* and *orfX*) sequences. By plasmid deletion analysis and molecular cloning, a single locus on pDBORO was found to confer sensitivity to 5-fluoroorotate. It was identified as *ysbC*, but renamed *oroP* in order to reflect its function. The *oroP* gene was found to be essential for the utilization of orotate as the sole pyrimidine source in a strain deficient in pyrimidine *de novo* synthesis. The amino acid sequence encoded by the ORF showed the characteristic features of a membrane protein. Therefore, *oroP* most probably encodes an orotate transporter. Surprisingly, homologues of *oroP* could be identified in the genomes of both *L. lactis* MG1363 and *L. lactis* IL1403 despite the fact that these strains were unable to significantly utilize orotate. Cloning of *oroP* in *Escherichia coli* and *Bacillus subtilis* showed that the orotate transport phenotype could be transformed to both organisms. The findings presented indicate that *oroP* can be used as a powerful, food-grade selection/counterselection marker in many different organisms.

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INTRODUCTION

Lactococcus lactis strains are widely used in the dairy industry as starter cultures in cheese fermentation. All *L. lactis* species isolated so far from starter cultures contain a number of different plasmids. These plasmids are thought to have been acquired during adaptation, and many plasmid-encoded functions have been related to or are essential for growth in milk. Examples are genes involved in lactose transport and metabolism (Efstathiou & McKay, 1976), genes encoding proteinases necessary for casein degradation (Efstathiou & McKay, 1976; McKay *et al.*, 1976) and di- and oligopeptide transport genes (Yu *et al.*, 1995a, 1996).

Pyrimidine metabolism has been extensively studied in *L. lactis* in our laboratory (Andersen *et al.*, 1996; Jorgensen

et al., 2003, 2004; Kilstrup & Martinussen, 1998; Martinussen *et al.*, 1994, 1995, 2001, 2003; Martinussen & Hammer, 1994, 1995, 1998; Wadskov-Hansen *et al.*, 2000, 2001), and a review of nucleotide metabolism in lactic acid bacteria has recently been published (Kilstrup *et al.*, 2005). In *L. lactis*, pyrimidines can be synthesized *de novo* or can be salvaged from the growth medium (Martinussen *et al.*, 1994). The pyrimidine *de novo* synthesis pathway consists of six steps leading to the formation of UMP and seems to be universal among all organisms. *L. lactis* is also able to metabolize various pyrimidine nucleosides and nucleobases, but not cytosine, due to the lack of cytosine deaminase (Martinussen *et al.*, 1994).

Orotate is an intermediate in the pyrimidine biosynthetic pathway. It is formed in the fourth step, in which dihydroorotate is converted into orotate by dihydroorotate dehydrogenase, encoded by the *pyrD* gene. A surprising discovery in *L. lactis* is the presence of two genes, *pyrDa* and *pyrDb*, both coding for a functional dihydroorotate dehydrogenase (Andersen *et al.*, 1994, 1996) (Fig. 1). Later

The GenBank/EMBL/DDBJ accession number for the *oroP* region is DQ089807; the accession number for the total pDBORO sequence is EF210104.

were obtained by adding 3 ml of an overnight culture (not lysed) grown in GM1 medium to 10 ml fresh GM1 medium and incubation for 4.5 h at 37 °C. The bacterial culture was subsequently diluted 1/10 in GM2 medium and incubated for 1 h at 37 °C. For 168MIU6 (*trpC2 pyrB*) cells, 20 µg uracil ml⁻¹ was added to the GM1 and GM2 media. For the transformation, 1 ml of the competent culture was mixed with 10 or 30 µl of the linearized plasmid solution (100 ng µl⁻¹) and the mixture was incubated for 35 min at 37 °C. Cells were centrifuged for 4 min and the cell pellet was resuspended in 200 µl 0.9% NaCl solution and plated on selective LB agar plates containing 5 µg neomycin ml⁻¹. The agar plates were incubated overnight at 37 °C.

Molecular cloning techniques. Standard molecular cloning techniques were used according to Sambrook *et al.* (1989). Chromosomal DNA from lactococci was prepared as previously described (Johansen & Kibenich, 1992). Chromosomal DNA from *B. subtilis* was prepared according to Saxild & Nygaard (1987). Plasmid DNA was obtained by purification on Qiagen anion-exchange columns using the protocols supplied by the manufacturer. Plasmid DNA from lactococci was purified using the following modifications. Cells were resuspended in P1 buffer containing 20 mg lysozyme ml⁻¹ followed by 20 min incubation at 37 °C. Purification on the Qiagen anion-exchange column was omitted. Isolation of plasmids from DB0410 and 901-1 was carried out by the adapted Qiagen protocol for the isolation of BAC and PAC clones, and of the pDBORO plasmid for sequencing purposes by the Qiagen very-low-copy plasmid purification protocol.

PCR amplification of DNA. Amplification of DNA was performed on 50 ng DNA in a final volume of 50 µl containing 200 µM of each deoxyribonucleoside triphosphate, 200 nM of each oligonucleotide, and *Taq* polymerase. AmpliTaq DNA polymerase (2.5 U) was used in the verification experiments. For cloning purposes either *Pfu* DNA polymerase, *TaqPlus Precision* polymerase mixture or *Elongase* (Invitrogen) was used according to the manufacturer's recommendations. Amplification was performed with 25–30 cycles of 95 °C 50 s, 50 °C 50 s and 72 °C 1–5 min.

Nucleotide sequences. Nucleotide sequences were determined by MWG-Biotech. The sequence of the *oroP* region has been assigned the GenBank accession number DQ089807; the total pDBORO sequence can be accessed by the GenBank number EF210104.

Bacterial strains and plasmids. The bacterial strains, plasmids and primers used in this work are shown in Tables 1 and 2. All plasmids were analysed by restriction enzyme analysis and sequencing; all recombinant strains were verified by PCR.

The MG1363 mutant ED79.1175 harbours deletions of both *pyrDa* and *pyrDb*, resulting in a pyrimidine requirement which can be satisfied by uracil. This mutant was constructed in two steps. In the first step, the MG1363 *ΔpyrDa* mutant ED58.82 was made. To achieve this, plasmids pRL101, pED102 and pED202 were constructed. pRL101 containing *ΔpyrDa* was constructed in the following way. A PCR fragment was amplified with primers pyrDaBamHI and pyrDaNcoI using pIP61 (Andersen *et al.*, 1994) as template. Both pIP61 and the PCR fragment were digested with *Bam*HI and *Nco*I, ligated together and transformed into *E. coli* XL1-Blue at 37 °C with selection for 100 µg ampicillin ml⁻¹, resulting in pRL101. Plasmid pED102 was obtained in the following way. Using pRL101 as template and the primers pyrDaBamHI and pyrDaHindIII the *ΔpyrDa* allele was amplified by PCR. The fragment was subcloned into the vector pCR2.1-TOPO using the TOPO-TA cloning system. Plasmid pED202 was constructed by ligating pED102 and pGhost⁺4 (Maguin *et al.*, 1992) together at the *Xba*I site, which is unique in both plasmids. After transformation into *E. coli* XL1-Blue, the fusion plasmid was

obtained by selection at 37 °C on LB agar plates containing both 100 µg ampicillin ml⁻¹ and 150 µg erythromycin ml⁻¹. Plasmid pED202 was transformed into *L. lactis* MG1363, selecting for erythromycin resistance at 28 °C. Using the method originally developed by Biswas *et al.* (1993) and improved by Martinussen & Hammer (1994), the *ΔpyrDa* allele was exchanged with the wild-type, resulting in the mutant ED58.82.

In the second step, the *pyrD* double mutant ED79.1175 was created by deletion of a 780 bp internal fragment in the *pyrDb* gene. This was carried out in the following way. Two PCR fragments were amplified in separate reactions with MG1363 chromosomal DNA as template using pyrDbIF and pyrDbIR in one reaction and pyrDbIIF and pyrDbIIR in the other. Since the primers pyrDbIR and pyrDbIIF are partially complementary, the two PCR products could be used as templates in a new PCR reaction with primers pyrDbIF and pyrDbIIR. The resulting PCR fragment containing the *pyrDb* deletion was cloned into the vector pCR2.1-TOPO, yielding pED105. Plasmid pED204 was made by ligating pED105 and pGhost⁺4 together at the *Xba*I site and transforming into *E. coli* XL1-Blue as described above for pED202. The wild-type allele *pyrDb* was exchanged with *ΔpyrDb* as described above for ED58.82 (*ΔpyrDa*), thus obtaining the *pyrD* double mutant ED79.1175.

A 3.8 kb DNA fragment was amplified using pDBORO plasmid DNA and the primer set DBORO2/DBORO8. The PCR fragments were cloned into the vector pCR2.1-TOPO and recombinant plasmids were selected at 37 °C on LB agar plates containing 100 µg ampicillin ml⁻¹. The recombinant plasmid pED112 harboured *oroP* and *ysbBA*.

An *Eco*RI fragment from pED112 was ligated into the unique *Eco*RI site of pCI3440 (Hayes *et al.*, 1990) and amplified in *E. coli* XL1-Blue at 37 °C with selection for resistance to 20 µg chloramphenicol ml⁻¹. The resulting plasmid was called pED210.

With pDBORO as template and primers DBORO18 and CS105, a PCR product was obtained and cloned into pCR2.1-TOPO. The recombinant plasmid pMBK701 was obtained after transformation into *L. lactis* ED79.1175 and selection for growth on GSA plates supplied with orotate as sole pyrimidine source.

A DNA fragment containing the *oroP* gene was amplified with primer set DBORO22BamHI/DBORO23EcoRI using pDBORO plasmid DNA as template. The PCR fragment was digested with *Bam*HI and *Eco*RI and ligated into the *B. subtilis* integrative vector pDG268 (Antoniewski *et al.*, 1990) digested with the same restriction endonucleases. The recombinant plasmid pED301 was amplified in *E. coli* XL1-Blue at 37 °C on LB agar plates containing 100 µg ampicillin ml⁻¹. Recombinant plasmid pED307 was constructed in the same way except that primer set DBORO22BamHI/DBORO24EcoRI was used. Plasmid pED301 was integrated into the *B. subtilis* 168 *amyE* gene by a double cross-over recombination through the *amyE* N-terminal and *amyE* C-terminal parts located on the pDG268 vector. The plasmid was linearized by digestion with *Kpn*I prior to transformation in order to ensure chromosomal integration by a double cross-over event. The neomycin-resistant strain ED348 was readily obtained. The control strain ED344 was obtained by transforming strain 168 with *Kpn*I-linearized pGD268. The *B. subtilis* pyrimidine auxotrophic mutant 168MIU5 (*trpC2 pyrB*) (Potvin *et al.*, 1975) was transformed with linearized pED307 DNA to obtain ED358 [*pyrB* (*oroP-ysbB*)⁺]. As a control strain 168MIU5 was also transformed with *Kpn*I-linearized pGD268, resulting in ED364.

Chromosomal integration by a double cross-over event was identified by the lack of α -amylase activity of the *B. subtilis* recombinants. Integration of pED301 or pED307 DNA into the *amyE* gene was investigated at the chromosomal level by PCR with primer sets 268neo/DBORO20 and 268neo/DBORO04.

Table 1. Bacterial strains and plasmids used in this work

Strain	Genotype	Parental/host strain	Reference or source
<i>Lactococcus lactis</i>			
1. NCDO712			Gasson (1983)
2. Wg2			Braun <i>et al.</i> (1989)
3. 901-1			Braun <i>et al.</i> (1989)
4. 3107			Braun <i>et al.</i> (1989)
5. MG1363		NCDO712	Gasson (1983)
6. NCDO2118			NCDO*
7. C2			Huggins & Sandine (1977)
8. IL1403			Chopin <i>et al.</i> (1984)
9. FHCY-1			CHCC†
10. CHCC336			CHCC†
11. DB0410	<i>ilv</i>		Curic <i>et al.</i> (1999), CHCC†
ED58.82	Δ <i>pyrDa</i>	MG1363	This work
ED79.1175	MG1363 Δ <i>pyrDa</i> Δ <i>pyrDb</i>	MG1363	This work
<i>Bacillus subtilis</i>			
168	<i>trpC2</i>		Potvin <i>et al.</i> (1975)
168MIU5	<i>trpC2 pyrB</i>	168	Potvin <i>et al.</i> (1975)
ED344	<i>trpC2 amyE neo</i>		This work
ED348	<i>trpC2 amyE neo (oroP-ysbB)⁺</i>	168	This work
ED364	<i>trpC2 pyrB amyE neo</i>	168	This work
ED358	<i>trpC2 pyrB amyE neo (oroP-ysbB-ysbA)⁺</i>		This work
<i>Escherichia coli</i>			
XL1-Blue	<i>endA1 hsdR17 (r_{K12}⁻m_{K12}⁻, r_{K12}⁻m_{K12}⁺) supE44 thi-1 recA1 gyrA96 relA1 lac F'[proAB lacI^qZΔM15 tet]</i>		Stratagene
KUR1351	<i>araD139 ΔlacU169 rpsL thi ΔpyrB usp-4 dctA</i>		Baker <i>et al.</i> (1996)
Plasmids			
pRL101	Δ <i>pyrDa amp</i>	<i>E. coli</i>	This work
pIP61	<i>pyrDa⁺ amp</i>	<i>E. coli</i>	Andersen <i>et al.</i> (1994)
pED201	<i>pyrDa⁺ amp kan</i>	<i>E. coli</i>	This work
pED202	<i>pyrDa⁺ amp kan erm</i>	<i>L. lactis</i> , <i>E. coli</i>	This work
pG ⁺ Host4	<i>erm ori^{ts}</i>	<i>L. lactis</i> , <i>E. coli</i>	Maguin <i>et al.</i> (1992)
pCR2.1-TOPO	<i>amp kan</i>	<i>E. coli</i>	Invitrogen
pDBORO	<i>oroP rep⁺</i>	<i>L. lactis</i>	This work
pDBORO- <i>Xba</i> I	<i>oroP rep⁺</i>	<i>L. lactis</i>	This work
pDBORO- <i>Eco</i> RV	<i>oroP rep⁺</i>	<i>L. lactis</i>	This work
pED105	Δ <i>pyrDb amp kan</i>	<i>E. coli</i>	This work
pED204	Δ <i>pyrDb amp kan erm</i>	<i>L. lactis</i> , <i>E. coli</i>	This work
pED112	<i>(oroP ysbBA)⁺ amp kan</i>	<i>E. coli</i>	This work
pCI3340	<i>cam</i>	<i>L. lactis</i> , <i>E. coli</i>	Hayes <i>et al.</i> (1990)
pED210	<i>(oroP ysbBA)⁺ cam</i>	<i>L. lactis</i> , <i>E. coli</i>	This work
pGD268	<i>neo</i>	<i>E. coli</i>	Antoniewski <i>et al.</i> (1990)
pED301	<i>(oroP ysbB)⁺ neo</i>	<i>E. coli</i>	This work
pMBK701	<i>amp kan oroP⁺</i>	<i>L. lactis</i> , <i>E. coli</i>	This work

*NCDO, National Collection of Dairy Organisms, Reading, UK.

†CHCC, Chr. Hansen's Culture Collection.

Detection of α -amylase activity in *B. subtilis*. The α -amylase phenotype of *B. subtilis* clones on LB agar plates containing 1 % starch was analysed by adding a solution of 0.5 % iodine and 1 % potassium iodine to the plates on which the strains had been growing overnight. The α -amylase-producing colonies formed a clear halo, whereas the α -amylase-negative colonies did not.

Growth experiments and orotate uptake. Starting with fresh colonies from GSA plates with erythromycin, strains were grown for

6–8 h at 30 °C in 10 ml GSA medium with erythromycin. Different dilutions of the growing culture were added to 10 ml medium and grown overnight. From an exponentially growing culture, new medium was inoculated to OD₄₃₆ 0.05. The growth experiments were performed in plastic flasks without stirring. For uptake experiments, 500 μ l cells at OD₄₃₆ 0.8 were transferred to an Eppendorf tube containing [¹⁴C]orotate at a specific activity of 52 mCi mmol⁻¹ (1924 MBq mmol⁻¹). After 1, 3 and 5 min the medium was removed by filtration through a 0.22 μ m filter. The filter

Table 2. Primers used in this work

Name	Sequence
268neo	CTCATTCCCTGATCTCG
pyrDaBamHI	CGGGATCCATGACCGACCAACAGC
pyrDaNcoI	CATGCCATGGCCAAATCCATCTTAGGC
pyrDaHindIII	CGTGAAGCTTGACAAAATAGGCTGACCTC
pyrDbIF	CGGAAGATCTGATGATGACAGTTGTCAG
pyrDbIR	CTGTACTGGTCCATAAGCTCGGATCCACCAAAACAACCTGACGCTG
pyrDbIIF	CAGCGTCAGGTTGTTTTGGTGGATCCGAGCTTATGGACCAGTACAG
pyrDbIIR	TCGGAGATCTATCCAAGGACAAGTGCAG
DBORO2	ACTTATCGTCCGGACTTG
DBORO04	TTCACGCTCACTACCTTC
DBORO8	CATTAGAAAGCGCACGAC
DBORO15	GACCGCCAGTTAGAAGCC
DBORO16	TTGTGATTTTTAGATAGGG
DBORO18	GAATAAGCTGATATTAGCCCC
DBORO20	GGCTCACCATTTTTGGCCTCTGG
DBORO22BamHI	CAGGATCCTACTGACAGACTTGTCAG
DBORO23EcoRI	GAGAATTCTGATTTCGGACAAGGCTTC
DBORO24EcoRI	GAGAATTCAAAGTCGTTTCGCCTCAAG
DBORO26	TAAGTCCTGCCTTTTAACCG
DBORO27	TCATTTCCATAGTTGCCACC
CS105	ACGGTCGACACGCTGTTCCATATTACTAG

was washed twice with 5 ml water. After drying, the radioactivity on the filters was determined in a scintillation counter.

Phylogenetic analysis. The GenBank program protein–protein BLAST at NCBI (<http://www.ncbi.nlm.nih.gov/BLAST>) was used to find similar proteins. Phylogenetic analysis was performed by a CLUSTAL W alignment used in a cluster analysis at the TreeTop - Phylogenetic Tree Prediction server (http://www.genebee.msu.su/services/phree_reduced.html). The corresponding phylogenetic tree was obtained at the Phylogendron Phylogenetic tree printer (<http://www.es.embnnet.org/Doc/phylogendron/treeprint-sample1.html>).

RESULTS

Orotate metabolism by lactococci

As described above, orotate disappears during cheese production using *L. lactis* starter cultures. In order to investigate orotate metabolism, the model strain *L. lactis* subsp. *lactis* MG1363 was analysed for its ability to utilize orotate as measured by its sensitivity to 5-fluoroorotate, as both compounds are metabolized by the same enzymes of nucleotide metabolism (Fig. 1). Surprisingly, MG1363 was found to be completely resistant. Moreover, the pyrimidine-requiring derivative ED79.1175 was only able to grow in the presence of uracil but not orotate. The uptake of uracil has previously been shown to be facilitated by a dedicated uracil transporter encoded by the chromosomally located *pyrP* (Martinussen *et al.*, 2001).

To determine whether the inability to utilize orotate is a general trait among *L. lactis* strains, 11 strains (nos 1–11 in Table 1) were screened for their sensitivity to 5-fluoroorotate.

Streaking the strains on defined SA medium containing 50 µg 5-fluoroorotate ml⁻¹ showed that only two strains, DB0410 and 901-1, were affected by 5-fluoroorotate: their growth was inhibited, suggesting that they were able to metabolize orotate in significant amounts. Both strains were further analysed on defined GSA medium with different 5-fluoroorotate concentrations (5, 10, 20 and 50 µg ml⁻¹). No growth of either strain was observed at a concentration of 20 µg ml⁻¹. Growth was severely reduced at 5 µg 5-fluoroorotate ml⁻¹ for both strains.

The genes encoding the enzymes for lactose and casein degradation, required for growth in milk, have been found to be plasmid-borne in lactococci. Since orotate is present in high amounts in cow's milk (Motyl *et al.*, 1991), we examined whether the ability of DB0410 and 901-1 to utilize orotate was plasmid dependent. Plasmids were indeed present in DB0410 and 901-1 as judged by agarose gel electrophoresis of crude extracts (not shown). The plasmid preparations were used to transform the pyrimidine-auxotrophic strain *L. lactis* ED79.1175 (*pyrDa pyrDb*), a derivative of *L. lactis* MG1363, and streaked on defined GSA medium supplemented with 100 µg orotate ml⁻¹ as sole pyrimidine source. MG1363 is unable to metabolize orotate since the strain is resistant to 5-fluoroorotate, and ED79.1175 is unable to utilize orotate as sole pyrimidine source. After 2 days, more than 1000 transformants were obtained with the total plasmid preparation of DB0410, while after 4 days, only 5 transformants were obtained with the plasmid preparation isolated from 901-1. No colonies appeared on GSA plates without orotate, indicating that the transformants obtained had acquired orotate-utilization

genes. Thirty-six DB0410 transformants and the five 901-1 transformants were restreaked on GSA agar plates supplemented with 100, 50 and 20 μg orotate ml^{-1} , or with 20 μg uracil ml^{-1} . Strain ED79.1175 transformed with plasmid DNA from DB0410 grew equally well on orotate and uracil. In contrast, ED79.1175 transformed with plasmid DNA from strain 901-1 did not grow efficiently on orotate when supplied at 50 μg ml^{-1} . It was decided to further characterize the strains originating from the transformation with DB0410 DNA.

The plasmid profile of five transformants was analysed and all of them harboured only a single plasmid. It was named pDBORO, and the size was roughly estimated by restriction fragment analysis to be about 16 kbp. The restriction enzyme map is shown in Fig. 2. The complete DNA sequence of the plasmid was determined on both strands with an error rate less than 0.01 % and a redundancy of at least 2.

Genetic organization of pDBORO

The sequence assembly of pDBORO was confirmed by PCR and by restriction endonuclease analysis (not shown). pDBORO amounts to 16404 bp and has a G+C content of 35.16 mol% (GENEMARK program) (Lukashin & Borodovsky, 1998), which is in agreement with the overall G+C content (35.4 mol%) of the *L. lactis* subsp. *lactis* strain IL1403 genome sequence (Bolotin *et al.*, 2001). Sequence analysis revealed the presence of 19 ORFs, but ORF prediction carried out with the software programs GENEMARK (Lukashin & Borodovsky, 1998) and EasyGene (Larsen & Krogh, 2003) suggested only 15 ORFs (Table 3). Coding density is 77.8 %, which is lower than the coding density (86 %) of the IL1403 genome (Bolotin *et al.*, 2001). The general organization is shown in Fig. 2(a).

Detailed sequence analysis and comparison with DNA databases showed that the pDBORO plasmid contains

Fig. 2. Map of the orotate plasmid pDBORO isolated from *L. lactis* subsp. *lactis* biovar *diacetylactis*. (a) Genetic map of pDBORO. Refer to Table 3 for a description of the individual ORFs. (b) Restriction map and deletion analysis of pDBORO. The black lines represent the DNA sequences present in the plasmids obtained by restriction enzyme digestion followed by ligation using pDBORO DNA. The ability of the deletions to confer growth on orotate as sole pyrimidine source in a pyrimidine-requiring mutant is shown as +. (c) Physical map of the *oroP* region. The plasmids shown could all confer growth on orotate as sole pyrimidine source in a pyrimidine-requiring mutant.

Table 3. Predicted ORFs in pDBORO

ORF	Strand	No. of aa	Start	Stop	Mol% G + C	RBS	% aa identity	Homology with <u>IL1403</u> or <u>SWISS-PROT</u> database
ORF10	+	530	1474	3063	35.66		42 (520 aa)	Contains part of SunT, an ABC-type bacteriocin/lantibiotic exporter
IS946	+	226	3116	3793	37.30		100 (226 aa)	IS946 transposase (226 aa) present on <i>L. lactis</i> DPC3147 plasmid pMRC01
ORF12	+	151	4300	4752	33.11	AAGGAG	98 (151 aa)	ORFB (151 aa) of <i>L. lactis</i> pCI2000 plasmid with unknown function
ORF13	+	199	4759	5355	39.00	AGGAG	99 (199 aa)	Contains resolvase domain of ORFC (199 aa) putative resolvase of <i>L. lactis</i> pCI2000 plasmid
<i>repB</i>	+	383	5934	7082	36.11	AAGGAG	86 (383 aa)	RepB protein (383 aa) of <i>L. lactis</i> pS7A plasmid
<i>orfX</i>	+	258	7082	7855	36.04	AAGG	47 (270 aa)	OrfX (270 aa) of <i>L. lactis</i> pAH82 plasmid
ORF2	+	313	7869	8807	38.54	GAGG	99 (97 aa)	N-terminal 97 aa: HsdR restriction subunit of a type I restriction/modification system of <i>L. lactis</i> subsp. <i>lactis</i> pAH82 plasmid, restriction subunit domain
							96 (216 aa)	C-terminal 216 aa: <u>AroB</u> , 3-dehydroquinate synthase, aromatic amino acid family
<i>oroP</i>	+	307	8921	9841	38.20	AGAGG	98 (307 aa)	<u>YsbC</u> (307 aa), <u>unknown protein</u> contains two copies of COG3238, uncharacterized protein conserved in bacteria
<i>ysbB</i>	+	114	9956	10297	34.49	GAGG	97 (114 aa)	<u>YsbB</u> (199 aa), <u>hypothetical protein</u> contains truncated COG4493, uncharacterized protein conserved in bacteria
<i>ysbA</i>	+	193	10305	10883	30.93	GGAGG	95 (190 aa)	<u>YsbA</u> (198 aa), <u>conserved hypothetical protein</u> contains COG0546, Gph domain, predicted phosphatase
IS946	+	226	14176	14853	37.30		100 (226 aa)	IS946 transposase (226 aa) present on <i>L. lactis</i> DPC3147 plasmid pMRC01
ORF8	+	165	15022	15516	34.14			Contains part of COG5527, protein involved in initiation of plasmid replication
IS982	–	229	11780	11094	40.29		98 (296 aa)*	IS982, transposase (296 aa)
<i>copB</i>	–	692	14070	11995	37.57	AGGAG	98 (695 aa)	<u>CopB</u> (695 aa), <u>copper-potassium transporting ATPase B</u> contain COG2217, ZntA, cation transport ATPase (inorganic ion transport)
ORF19	–	103	16390	16079	36	AGGAG	42 (87 aa)	<u>Hup</u> (91 aa), <u>DNA-binding protein HU</u> contains a HimA domain characteristic of a bacterial nucleoid DNA-binding protein (DNA replication, recombination, and repair)

*Th pDBORO IS982 transposase gene contain a C688T mutation resulting in an early stop. The pDBORO IS982 gene product is truncated and consists of only 229 aa residues.

several regions with very high identity either to the IL1403 chromosomal sequence or to other lactococcal plasmids. Regions 8159–10873 and 11975–14104 have 96 % and 97 % identity to the IL1403 sequence and regions 3887–5373 and 7589–8158 have 95 % and 99.1 % identity to pCI2000 (accession no. AF178424, unpublished) and pAH82 (O'Sullivan *et al.*, 2001), respectively. Similarities between plasmid sequences have been described for other lactococcal

plasmids such as pAH90, which indicates a high frequency of horizontal transfer among the lactococci and an overall plasticity in plasmid structures (O'Sullivan *et al.*, 2001).

Table 3 lists the properties of the ORFs and their closest relatives based on BLAST searches. Two of the three IS elements are identical and nearly identical (96 %) to IS946, including the 18 bp inverted repeats (Romero &

Klaenhammer, 1990). One IS946 is inserted before ORF8 and the second IS946 is inserted in the end of ORF10. The third IS element is nearly identical (98 %) to IS982 (Yu *et al.*, 1995b) but the 999 bp IS982 on pDBORO is flanked by 16-perfect instead of 18-perfect inverted repeats and has a C to T mutation at position 778. This mutation creates a stop codon in the ORF encoding the transposase, which results in a truncated transposase of 229 instead of 296 aa.

An ORF encoding a protein of 383 aa with high similarity to RepB proteins, an ORF encoding a 258 aa OrfX-like protein and ORF2 are part of the replication module and are described below. The *copB* gene most probably codes for a copper-potassium-transporting ATPase B first identified in *Enterococcus hirae* (Odermatt *et al.*, 1993) as they are 98 % identical. The deduced amino acid sequence of *copB* located in the IL1403 genome contains an HNM repetition in the N-terminal part which is lacking in the deduced amino acid sequence of *copB* in pDBORO. Three ORFs, ORF10, ORF8 and ORF19, encode proteins with low amino acid sequence identity (42 % or less) to known proteins but in two ORFs a (partial) functional domain could be identified (Table 3). ORF10 encodes a protein of 530 aa showing homology to SunT of the ABC-type bacteriocin/lantibiotic exporter from *B. subtilis* (Paik *et al.*, 1998). The 50 C-terminal amino acids are however missing, and the last 22 bp of ORF10 overlap with the downstream IS946 element and a new stop codon is created by a read-through into the IS946 sequence. ORF19 encodes a 103 aa protein with a domain found in bacterial nucleoid DNA-binding proteins. The deduced amino acid sequence of ORF8 shows no significant similarity to any protein in the databases searched. The DNA fragment between position 3887 and 5373 contains two ORFs, ORF12 and ORF13, which are nearly identical to ORF8 (98 %), with unknown function, and ORF14 (98.5 %), a putative resolvase. Both homologues are found on plasmid pCI200.

The replication module of pDBORO

Between positions 5934–7082 and 7082–7855, a non-coding sequence and ORFs were found that are normally involved in the replication of theta-type plasmids (Frere *et al.*, 1993; Gravesen *et al.*, 1995; Hayes *et al.*, 1991). The *repB* gene codes for a protein of 383 aa with a high amino acid sequence identity to initiator replication proteins of other theta-replicating lactococcal plasmids. The highest identity was found with RepB of the pS7A plasmid (86.4 %; accession no. NC_004652), the food-grade cloning vector pVS40 (85.6 %) (von Wright & Raty, 1993), pCI305 (82 %) (Hayes *et al.*, 1990), pCI605 (82 %; accession no. NC_002138), pAH33 (82 %) (O'Sullivan *et al.*, 2001) and pDR1-1 (82 %; accession no. NC_004163).

Upstream of the *repB* gene, between positions 5625 and 5933, a region is found that shows high identity at the nucleotide level (80 %) with the *cis*-acting *ori* region and the upstream sequence of the *repB* gene in the lactococcal plasmid pCD4 (Emond *et al.*, 2001). Moreover, this region

shows the same organization of secondary structures, including a 41 bp AT-rich box flanked by a GCC and GCGTGG cluster and followed by a tandem of three and a half perfect direct repeats of 22 bp.

The *oroP* gene found on pDBORO encodes an orotate transporter

To identify the genes responsible for orotate utilization, pDBORO deletion derivatives were constructed (Fig. 2b). Three deletions were made. The pDBORO DNA was digested with *Xba*I, *Eco*RV or *Cl*aI and diluted prior to ligation to enhance self-ligation. Ligation mixtures were transformed into *L. lactis* ED79.1175 (*pyrDa pyrDb*) and plated on GSA agar plates containing 20 µg orotate ml⁻¹. Only pDBORO derivatives containing the replication module and the orotate transporter coding genes are able to grow. Very few transformants were obtained from the *Cl*aI deletion, but they all contained both *Cl*aI fragments. As shown above, all the functions required for replication are present on one *Cl*aI fragment (Fig. 2b). Several hundred clones were isolated from the two other deletions. This leads to the conclusion that the DNA between the *Cl*aI site at 8.5 kb and the *Xba*I site at 10.5 kb (Fig. 2b) is required for growth on orotate as sole pyrimidine source. The sequence of the *Cl*aI–*Xba*I fragment revealed the presence of two complete ORFs, *oroP* and *ysbB*, and two truncated ORFs, *orf2* and *ysbA* (Fig. 2c). As *ysbA* is truncated in the *Xba*I deletion, and orotate utilization is unaffected, it can be concluded that *ysbA* is not required for orotate metabolism.

To verify that this region of pDBORO is indeed responsible for orotate uptake, a PCR fragment containing the *oroP* and *ysbB* genes was amplified with primers DBORO2 and DBORO8 and cloned into the pCI3340 vector, resulting in construct pED210. By electroporation pED210 was introduced into ED79.1175 and transformants were selected on rich medium containing chloramphenicol. As control the vector pCI3340 was also established in ED79.1175. Transformants were screened on GSA agar plates containing chloramphenicol in the absence and presence of 20 µg orotate ml⁻¹ as the sole pyrimidine source. Only transformants that harboured pED210 could grow on orotate. This confirmed the results of the plasmid deletion analysis. The plasmid pED210 carries only the *oroP* and *ysbB* genes, showing that the ability to metabolize orotate is linked to *oroP* and/or *ysbB*.

To investigate whether *oroP* by itself has the ability to facilitate orotate transport, or the *ysbB* gene is required, plasmid pMBK701 carrying only the *oroP* gene and the replication module from pDBORO was constructed by PCR amplification and subsequent cloning in the *E. coli* pTOPO vector. Transformation into *E. coli* was unsuccessful. Therefore, the ligation mixture was used to transform *L. lactis* ED79.1175 with selection for growth on orotate as sole pyrimidine source. Transformants were readily obtained, and a restriction enzyme and PCR analysis

revealed that they all harboured a plasmid corresponding to pMBK701. It was verified that the plasmid contained the *oroP* gene in addition to the pTOPO vector. This finding indicates that the only gene on pDBORO conferring the ability to utilize orotate is *oroP*. The purified plasmid was transformed into *E. coli* by selecting for ampicillin resistance, and an analysis of transformants showed that the plasmids suffered from deletions. As pTOPO is a high-copy-number plasmid, it is reasonable to conclude that overexpression of *oroP* alone is detrimental to *E. coli*.

To verify that *oroP* facilitates orotate transport, an uptake assay using [14 C]orotate was performed. Fig. 3(a) shows a representative experiment in which the *L. lactis* strains MG1363, DB0410 and MG1363/pED210 (*oroP*) were assayed for their ability to facilitate orotate uptake. The presence of plasmid pED210 conferred orotate uptake ability to MG1363. Moreover, the uptake was linear right from the start, indicating that orotate is not converted extracellularly.

In order to verify that orotate is directly taken up and not converted prior to transport, growth of a

pyrimidine-requiring strain in the presence of orotate and 6-azauracil was assayed. 6-Azauracil is converted to 6-azaUMP, known as a very potent inhibitor of the orotate monophosphate (OMP) decarboxylase (Miller *et al.*, 2000). During such a condition, it would be expected that orotate would be phosphoribosylated to OMP, but not further decarboxylated to UMP (Fig. 1), and hence unable to support growth. In the experiment shown in Fig. 3(b) strain ED79.1175 (*pyrDa pyrDb*) harbouring pDBORO was grown with uridine or orotate as sole pyrimidine source in the presence and absence of 6-azauracil. Only the utilization of orotate, and not that of uridine, was impaired by 6-azauracil. Since OMP decarboxylase is inhibited by 6-azaUMP, this result strongly suggests that orotate is indeed taken up by the cell and not converted prior to transport. It could be argued that OMP is formed outside the cell and subsequently transported, but this is extremely unlikely, since 5-phospho-D-ribosyl 1-pyrophosphate (PRPP) is required for the reaction, and phosphorylated compounds with a few exotic exceptions are unable to cross the cytoplasmic membrane.

Based on the DNA sequence, OroP was determined to consist of 207 aa. The deduced sequence was analysed using the TMHMM Server (<http://www.cbs.dtu.dk/services/TMHMM>), and the overall structure suggests that OroP is a membrane protein with nine transmembrane regions (Fig. 4a). Using the SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP>) the signal peptide cleavage site in OroP was predicted to be between Ala23 and Asp24 (Fig. 4b). A BLASTP search showed that OroP belongs to a group of conserved hypothetical membrane proteins. Thus, these observations indicate that *oroP* encodes an orotate transporter, as found in the uptake experiments.

The *oroP*, *ysbA*, and *ysbB* genes are found on the chromosome of *L. lactis*

The *L. lactis* strains IL1403 and MG1363 are resistant to 5-fluoroorotate, indicating that they are unable to metabolize significant amounts of exogenous orotate. Moreover, introduction of the *oroP* gene conferred 5-fluoroorotate sensitivity to MG1363. Hence, lack of orotate transport is the reason why MG1363 is unable to metabolize this compound.

As mentioned previously, homology searches using the *ysbA*, *ysbB* and *oroP* reading frames as queries surprisingly revealed that homologues of these genes are present on the chromosome of *L. lactis* IL1403. The genes are annotated as *ysbA*, *ysbB* and *ysbC*, coding for proteins with unknown functions (Bolotin *et al.*, 2001). The *oroP* gene was originally annotated as *ysbC*, but is here renamed according to its function. The gene order *oroP-ysbB-ysbA* is conserved in both the plasmid pDBORO and the IL1403 chromosome. Compared to IL1403, the deduced *oroP* amino acid sequence has five amino acid substitutions. Four substitutions conserve a hydrophobic side-chain, whereas the fifth changes an arginine to a glutamine residue. An

Fig. 3. Orotate uptake. (a) Uptake of [14 C]orotate by *L. lactis* MG1363 (Δ), *L. lactis* MG1363/pED210 (○) and *L. lactis* DB0410 (+). The radioactivity in 500 μ l filtered culture was determined in a scintillation counter after 1, 3 and 5 min incubation with 5 μ M [14 C]orotate. (b) Growth of *L. lactis* ED79.1175/pDBORO in GSA medium at 30 °C with uridine or orotate as sole pyrimidine source in the absence and presence of 10 μ M azauracil. ◇, Uridine; ×, uridine and azauracil; Δ, orotate; ○, orotate and azauracil.

Fig. 4. Analysis of the orotate transporter encoded by *oroP* present on the orotate plasmid pDBORO isolated from *L. lactis* subsp. *lactis* biovar *diacetylactis*. (a) Prediction of the nine transmembrane helices (I–IX) in the deduced amino acid sequence using the TMHMM server (<http://www.cbs.dtu.dk/services/TMHMM>). The α -helices are shown by rectangles and the numbers refer to the amino acid sequence. The first α -helix (I) is predicted to be part of the signal sequence. (b) The signal sequence predicted by the SignalP server (<http://www.cbs.dtu.dk/services/SignalP>). The signal peptide cleavage site in OroP was predicted to be between Ala23 and Asp24.

oroP homologue exhibiting 89 % identity with the *oroP* ORF was also found in the chromosome of *L. lactis* MG1363 by searching the genome sequence at GenBank (accession no. AM406671). The gene order *oroP* (lmg_1937 in the MG1363 sequence)–*ysbB* (lmg_1936) was conserved in MG1363 whereas *ysbA* seems to be absent. An *oroP* allele was also found in the chromosome of *L. lactis* SK11 by a protein–protein BLAST search at NCBI (<http://www.ncbi.nlm.nih.gov/BLAST>).

oroP homologues are primarily found in Gram-positives

In order to determine whether putative orotate transporters are widespread, a bioinformatics approach was used to analyse the presence of *oroP* homologues in the databases. A protein–protein BLAST search using the server at NCBI revealed a number of putative proteins with homology to OroP from pDBORO. Two kinds of protein sequences showed homology to OroP: a long form with similar length to OroP and a short version. Interestingly, the long form seems to have emerged from a duplication of a conserved domain, DUF606, found in ORFs with unknown function. The long version was only found in Gram-positives.

In order to establish their relationship, a cluster analysis based on a CLUSTAL W alignment was conducted. Only the long versions were included. The resulting phylogenetic tree is shown in Fig. 5. OroP homologues from *L. lactis* are tightly clustered, and in general, the relationships among the different OroP homologues are clustered according to the relationship of the different strains. The OroP homologue from *Lactobacillus plantarum* is of special interest. The original sequence is most likely wrongly annotated, since it is elongated by 47 aa compared to the OroP homologues from other organisms, and a much better Shine–Dalgarno sequence is present in front of the second methionine codon (data not shown). The *oroP* homologue from *Lb. plantarum* (annotated as lp_2696 in

the genome sequence) is located immediately downstream of the *pyrE* gene of the pyrimidine biosynthetic operon, and since no terminators are found between *pyrE* and lp_2696 they could be co-transcribed, making the *oroP* homologue a member of the *pyr* regulon of *L. plantarum*. This finding, and the membrane protein characteristics of lp_2696, suggests that it is involved in pyrimidine transport.

The orotate transporter system confers 5-fluoroorotate sensitivity to *L. lactis* wild-type strains

Plasmids pCI3340 and pED210 were transformed into *L. lactis* NCDO712 and its plasmid-cured counterpart MG1363, both resistant to 5-fluoroorotate and prototrophic for pyrimidines. Transformants were selected by the acquired chloramphenicol resistance. Subsequently, the transformants were screened for 5-fluoroorotate sensitivity. No growth of *L. lactis* NCDO712 or MG1363 harbouring pED210 was seen on 5-fluoroorotate-containing agar plates, indicating that *oroP* confers sensitivity to 5-fluoroorotate in both strains.

The orotate transporter is functional in both *E. coli* and *B. subtilis*

In order to investigate whether the orotate transport system is functional in other organisms, *E. coli* and *B. subtilis* strains harbouring the *oroP* gene from pDBORO were analysed either by the ability of orotate to fulfil a pyrimidine requirement of an auxotrophic mutant or by sensitivity to 5-fluoroorotate of a wild-type strain. Both methods probe for the presence of a functional orotate-uptake system.

Plasmid pED301 was transformed into *E. coli* KUR1351 (*pyrD* *dctA*) and *E. coli* XL1-Blue. The KUR1351 transformants were plated on selective ABTG medium supplemented

Fig. 5. Phylogenetic tree of OroP homologues obtained from alignment of related protein sequences found by a protein–protein BLAST search. The cluster analysis was performed at http://www.genebee.msu.su/services/phree_reduced.html, and the phylogenetic tree was constructed using PhyloDendron Phylogenetic tree printer server at <http://www.es.emblnet.org/Doc/phyloDendron/treeprint-sample1.html>. GenBank accession numbers and strain names are as follows: Arthrobac, ZP_00411831 *Arthrobacter* sp. FB24; Nocardia, BAD54985 *Nocardia farcinica* IFM 10152; Brev.lin, ZP_00381369 *Brevibacterium linens* BL2; Bifido.ad, ZP_01028822 *Bifidobacterium adolescentis*; Lla.pDBOR, *Lactococcus lactis* plasmid pDBORO (this work); Lla.IL140, AAK05853 *Lactococcus lactis* subsp. *lactis* IL1403; Lla.SK11, ZP_00382583 *Lactococcus lactis* subsp. *cremoris* SK11; Lla.MG136, BH770678 *Lactococcus lactis* subsp. *cremoris* MG1363; Leuco.mes, ZP_00063719 *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293; Lba.plan, CAD64939 *Lactobacillus plantarum* WCFS1; Oeno.oeni, ZP_00319123 *Oenococcus oeni* PSU-1; Exiguo.sp, ZP_00537982 *Exiguobacterium* sp.; Pedio.pen, ZP_00322824 *Pediococcus pentosaceus* ATCC 25745; Coryne.gl, BAB98464 *Corynebacterium glutamicum* ATCC 13032; Staph.aur, CAG42360 *Staphylococcus aureus* subsp. *aureus* MSSA476; Staph.epi, AAO03989 *Staphylococcus epidermidis* ATCC 12228; Staph.sap, BAE19246 *Staphylococcus saprophyticus* subsp. *saprophyticus* ATCC 15305; Staph.hae, BAE05588 *Staphylococcus haemolyticus* JCSC1435.

with orotate as sole pyrimidine source, whereas XLI-Blue transformants were screened on 5-fluoroorotate (Table 4). *B. subtilis* strain ED358 (*pyrB* *oroP*⁺) was streaked on selective MM agar plates containing 5 µg neomycin ml⁻¹ without or with 20 µg orotate ml⁻¹ or 20 µg uracil ml⁻¹ as sole pyrimidine source. *B. subtilis* strain ED348 (*oroP*⁺) was streaked on selective MM agar plates containing 5 µg neomycin ml⁻¹ with and without 20 µg 5-fluoroorotate ml⁻¹. Results are shown in Table 4.

The *L. lactis* orotate transporter is able to mediate 5-fluoroorotate sensitivity to both *B. subtilis* 168 and *E. coli* XLI-Blue. Surprisingly, XLI-Blue was resistant to 20 µg 5-fluoroorotate ml⁻¹. *E. coli* wild-type is normally sensitive to this drug due to the presence of the *dctA* gene product (Baker *et al.*, 1996). Since XLI-Blue is sensitive to 5-fluoroorotate only in the presence of *oroP*, it is reasonable to believe that this highly manipulated strain has an additional mutation in *dctA*. Moreover, *oroP* is required for the ability of the pyrimidine-auxotrophic derivative of *B. subtilis*, ED358, and of *E. coli* KUR1351, to grow on orotate as sole pyrimidine source. These results show that OroP is also capable of facilitating the uptake of orotate and 5-fluoroorotate in both *E. coli* and *B. subtilis* and, consequently, must be correctly folded and inserted in the plasma membrane.

DISCUSSION

Orotate uptake is mediated by specific transporters

Kneifel *et al.* (1992) and Saidi & Warthesen (1989) observed that the orotate concentration in milk decreased during fermentation with lactic acid bacteria, including *L. lactis* cultures, suggesting that at least some *L. lactis* strains are able to metabolize orotate. As orotate and 5-fluoroorotate are both metabolized by the same enzymes in the pyrimidine biosynthesis pathway (Fig. 1), sensitivity to 5-fluoroorotate is a powerful method to demonstrate orotate uptake. Among 11 tested strains only two, DB0410 and 901-1, were 5-fluoroorotate sensitive, meaning that these two strains can utilize orotate; this implies that orotate utilization is not a general property of *L. lactis*. Both strains were sensitive at a low concentration, suggesting the presence of a high-affinity transporter for orotate. The two sensitive strains belong to different subspecies: DB0410 is a strain of *L. lactis* subsp. *lactis* biovar *diacetylactis*, used as a starter culture for production of cheese and cultured buttermilk (Curic *et al.*, 1999), while 901-1 belongs to the *cremoris* subspecies (Braun *et al.*, 1989). The property to utilize orotate seems therefore to be strain-dependent rather than species-dependent.

Many functions related to growth in milk are found on plasmids in *L. lactis*, presumably acquired during their adaptation process, as the original niche of lactococci is believed to be plants (Efstathiou & McKay, 1976; McKay *et al.*, 1976; Yu *et al.*, 1995a, 1996). It was therefore interesting to investigate if the orotate transporter genes were also plasmid-borne. For both *L. lactis* DB0410 and 901-1, single plasmids were isolated after transferring total plasmid preparations into the pyrimidine-auxotrophic strain ED79.1175 and analysing the transformants for their orotate utilization as sole pyrimidine source, but only pDBORO from *L. lactis* DB0410 was further characterized.

The *oroP* gene responsible for orotate uptake in *L. lactis* was shown to code for a protein of 307 aa with the overall

Table 4. Growth of *E. coli* and *B. subtilis* harbouring the orotate transporter from *L. lactis*

+, Growth; –, no growth. Agar plates were incubated for 1 day at 37 °C. ND, Not determined.

Species	Strain	Plasmid	Relevant genotype	Supplement*			
				None	Uracil ₂₀	Orotate ₂₀	5-FO ₂₀ †
<i>E. coli</i>	KUR1351	pGD268		–	+	–	ND
<i>E. coli</i>	KUR1351	pED301	(<i>oroP-ysbBA</i>) ⁺	–	+	+	ND
<i>E. coli</i>	KUR1351	pED112	(<i>oroP-ysbBA</i>) ⁺	–	+	+	ND
<i>E. coli</i>	XLI-Blue	pGD268		+	ND	ND	+
<i>E. coli</i>	XLI-Blue	pED301	<i>neo (oroP-ysbB)</i> ⁺	+	ND	ND	–
<i>B. subtilis</i>	ED364			+	ND	ND	+
<i>B. subtilis</i>	ED368		(<i>oroP-ysbB-ysbA</i>) ⁺	+	ND	ND	–
<i>B. subtilis</i>	ED344		<i>pyrB</i>	–	+	–	ND
<i>B. subtilis</i>	ED358		<i>pyrB (oroP-ysbB)</i> ⁺	–	+	+	ND

*The supplements to ABT glucose agar plates for *E. coli* and MM glucose plates for *B. subtilis* are in µg ml^{–1}.

†5-FO, 5-fluoroorotate.

structure of a membrane protein consisting of nine transmembrane regions. To our knowledge, this is the first time an orotate transporter has been identified and cloned in a Gram-positive bacterium. The C₄-dicarboxylate transport protein DctA was identified to mediate orotate uptake in the Gram-negative bacteria *E. coli*, *Salmonella typhimurium* and *Sinorhizobium meliloti* (Baker *et al.*, 1996; Yurgel & Kahn, 2005). OroP shows no homology to DctA.

An *oroP* homologue is present in the chromosome of *L. lactis* strains

Surprisingly, an *oroP* homologue was found in the sequenced genome of IL1403 but IL1403 is resistant to 5-fluoroorotate, indicating that this homologue does not confer the ability to utilize orotate in significant amounts.

The question to be answered is why one homologue codes for a transporter protein that is able to mediate orotate uptake and the other does not. Both deduced amino acid sequences have the overall structure of a membrane protein consisting of nine transmembrane regions, and they differ by only five conservative substitutions. This suggests that the changes in the amino acid sequence do not account for the inability of the IL1403 *oroP* homologue to facilitate significant orotate uptake. More likely, it could be a matter of gene expression; both a higher promoter strength and the fact that *oroP* is present in multiple copies when encoded on pDBORO may account for its capability to facilitate transport of sufficient 5-fluoroorotate for the cell to become sensitive to the drug. We are currently investigating whether a low expression of the chromosomally located gene is responsible for the inability to mediate orotate transport, and whether an increased expression of the chromosomal allele could confer sufficient orotate uptake capability.

The potential of *oroP* as a selection/counterselection marker

The functionality of *oroP* in other organisms has been assessed and we showed that the *L. lactis* orotate transporter is able to mediate 5-fluoroorotate sensitivity in *B. subtilis* 168, *E. coli* XLI-Blue, and 5-fluoroorotate sensitive lactococci. Moreover, *oroP* is required for the ability of the pyrimidine-auxotrophic derivative of *B. subtilis*, ED358, *E. coli* KUR1351 and *L. lactis* ED1175 to grow on orotate as sole pyrimidine source.

Based on the results obtained, the *oroP* orotate transporter system has potential as a selection/counterselection marker. For application in food, vectors should contain selection markers that are acceptable in the food industry. In general, selection is mostly carried out with the use of antibiotic markers, and food-grade alternatives are rare (Pedersen *et al.*, 2005). The *oroP* gene originates from the *L. lactis* plasmid pDBORO and can be applied as a food-grade selective marker. In order for *oroP* to function as a selection marker, the host must have a pyrimidine requirement caused by a mutation in a gene encoding an enzyme in the first part of the biosynthetic pathway, since the pathway from orotate to UMP encoded by *pyrE* and *pyrF* must be intact for orotate taken up by OroP to be metabolized (Fig. 1). Mutants in the first part of the pyrimidine biosynthetic pathway can be obtained either by genetic engineering or by mutagenesis followed by a penicillin counterselection against growth in the absence of uracil. Both methods have successfully been utilized to obtain uracil auxotrophic mutants in *L. lactis* (Martinussen *et al.*, 2001).

The presence of *oroP* in the cell results in sensitivity to 5-fluoroorotate. The *oroP* gene can be exploited as a counterselection marker to select for the loss of a function/plasmid simply by streaking the strains on defined medium containing 5-fluoroorotate. Whereas the use of

oroP as a selection marker requires a specific genetic background, its utilization as a counterselection marker only requires that the background strain is unable to transport 5-fluoroorotate. Many organisms are unable to utilize orotate, and those strains that do encode an orotate transporter can easily be modified simply by selecting a 5-fluoroorotate-resistant derivative. Whereas different selection markers are abundantly available, counterselection markers are rare. Therefore, the counterselection property of the *oroP* system is particularly useful, especially when taking into account that the system may be functional in a large number of organisms (Martinussen & Defoor, 2005).

Previously, an alternative counterselection system has been developed in our lab. It exploits the *upp* gene, whose presence results in sensitivity to 5-fluorouracil (Breuner *et al.*, 1999; Martinussen & Hammer, 1994, 1995). This system requires that the background strain is mutated in the *upp* gene. Since the *upp* gene is very abundant, and not always very easy to obtain, the exploitation of *oroP* as a counterselection marker is more straightforward.

Interestingly, *oroP* could only be maintained in *E. coli* in the presence of the downstream gene *ysbB*, whereas *oroP* could be established in *L. lactis* in the absence of *ysbB*. The reason for the toxic effect of *oroP* in the *E. coli* host in the absence of *ysbB* could be that *ysbB* encodes either a regulatory protein, or a chaperone involved in assembly and/or translocation of OroP to the membrane. We are currently trying to solve this problem, making the utilization of the gene easier in different organisms.

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