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Lactococcus lactis, an attractive cell factory for expression of functional membrane proteins

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Abstract: Membrane proteins play key roles in most crucial cellular processes ranging from cell to cell communication to signaling processes. Despite recent improvements, the expression of functionally folded membrane proteins in sufficient amounts for functional and structural characterization remains a challenge. Indeed, it is still difficult to predict whether a protein can be overproduced in a functional state in some expression system(s), though studies of high throughput screens have issued in recent years. Prokaryotic expression systems present several advantages over eukaryotic ones. Among them, *Lactococcus lactis* (*L. lactis*) has emerged in the last two decades as a good alternative expression system to E. coli. The purpose of this chapter is to describe *L. lactis* and its tightly inducible system, NICE, for the effective expression of membrane proteins from both prokaryotic and eukaryotic origins.

Keywords: *Lactococcus lactis*, membrane proteins, NICE system

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1. Introduction

Membrane proteins (MPs), key proteins in cell physiology and drug targets, are encoded by one third of the human genome [1-2]. MPs have different features: i) they form various topologies from peripheral to intrinsic polytopic proteins with a high number of transmembrane helices, ii) their surface is relatively hydrophobic, iii) detergents are required for their solubilization from the cell membrane, and they often need to be reconstituted into proteoliposomes for functional studies, iv) they are flexible and unstable, v) they must be targeted to membrane for a proper folding, vi) they are expressed at very low levels and/or vii) they are functional in an oligomeric state [3-4]. In order to increase and deepen our knowledge, in particular for pharmaceutical objectives, there is an increasing need for structural and functional studies [5]. During the last 7 years, the number of unique 3D structures of MPs increased from 400 to 1348 (https://blanco.biomol.uci.edu/mpstruc/), which is still far away from the 75000 structures available for soluble proteins. The reason why the number of 3D structures is still so low is linked to the difficulty to obtain sufficient amounts of functionally folded MPs. Functional and structural studies require high amounts of proteins. Therefore, the low concentration of MPs in cells highlights the need for heterologous expression systems. There are different types of expression systems such as cell-free systems [6], prokaryotic systems (*E. coli* and *L. lactis*) and eukaryotic expression systems (yeasts, plants, mammalian or insect cells). All of them have advantages and drawbacks [3-4,7]. Bacteria are the most used systems for the expression of recombinant proteins, including MPs and the first hosts used prior to the other expression systems listed above, because they are easy to handle and inexpensive compared to eukaryotic systems. Furthermore, a wide range of genetic methods and vector systems are well established. Among them, *E. coli* can be considered as the traditional and oldest bacterial gene expression system, which has been developed for many years

and a wide variety of plasmids and host strains are available. In most cases, induction of gene expression is based on IPTG (IsoPropyl β-D-1-ThioGalactopyranoside) [8-9]. However, the yield of functional MPs is often unsatisfactory, which is generally due to the formation of inclusion bodies, the production of endotoxins and proteases by the bacteria, and/or the high translation rate [9-10]. In the last twenty years, another bacterium emerged as a good alternative to *E. coli* for the expression of MPs, i.e. *Lactococcus lactis*.

2. *Lactococcus lactis*

Lactococcus lactis, a Gram positive bacterium, has emerged at the beginning of the twenty first century as a good alternative for the functional expression of prokaryotic and eukaryotic MPs [7,11-12]. This bacterium grows at 30°C with a doubling time of 35 to 60 min and grows with a fermentative or respiration type of metabolism [13]. Although largely used in the food industry for the production of fermented foods, its potential as a host for the overexpression of homologous and heterologous proteins has also been explored [14-16]. *L. lactis* is easy and inexpensive to grow, a large variety of genetic methods and vector systems are available and well developed. Therefore, *L. lactis* is an interesting alternative gene expression host, especially for eukaryotic MPs, because of its moderate proteolytic activity, the absence of inclusion body formation and of endotoxin production, and the efficient targeting of the MPs into a single glycolipid cytoplasmic membrane [11,17-18]. Moreover, this bacterium allows to perform functional studies directly with intact cells and membrane vesicles [11,19].

L. lactis has a genome of half the size of that of *E. coli* and may lack specific chaperone systems and other auxiliary factors which could be necessary for targeting and correct folding of particular MPs [11]. Its codon usage is an approximative 65% biased for AT base pairs. Therefore the gene encoding the protein of interest needs to be optimized for the codon usage in *L. lactis* [19]. One difficulty of working with *L. lactis* is in the cloning efficiency [20]. Therefore, in order to facilitate and obtain a larger number of recombinant clones, different strategies have been developed in the last years in addition to the classical one (see below).

The expression of heterologous proteins in *L. lactis* has been facilitated by the advances in genetic knowledge and new developments in molecular biology techniques. Using these tools, various vectors containing either constitutive or inducible promoters have been developed to obtain increased levels of proteins and to control their production. They currently constitute the basis of all expression systems in *L. lactis* and other lactic acid bacteria [21]. Among the various expression systems, the NICE system represents the most used system for soluble and particularly MPs in *L. lactis* [22]. Moreover, different strains were optimized for MP expression (see below).

2.1. NICE system

The tightly regulated NICE (NIsin Controlled gene Expression) system is the most broadly and commonly used gene expression system in *L. lactis* [16,19]. This promising and effective expression system was developed for lactic acid bacteria and is based on genes involved in the biosynthesis and regulation of the antimicrobial peptide, nisin (product of the nisA gene). This 34-amino acid bacteriocin produced by several strains of *L. lactis* [22] can also be used as a natural food preservative [23]. The genes of the two-component signal transduction system nisK and nisR from the nisin gene cluster were inserted into the chromosome of *L. lactis* subsp. cremoris MG1363 (nisin-negative)[24], creating the strain NZ9000 [25-26]. When a gene of interest is subsequently placed behind the inducible promoter PnisA in a plasmid [27], expression of that gene can be induced by the addition of sub-inhibitory amounts of nisin (0.1-5 ng/ml) to the culture medium [28] (Figure 1), either obtained commercially or by adding the supernatant from the NZ9700 nisin secreting lactococcal strain. In order to obtain higher yields the growth medium, fermentation conditions and nisin induction have been optimized [14].

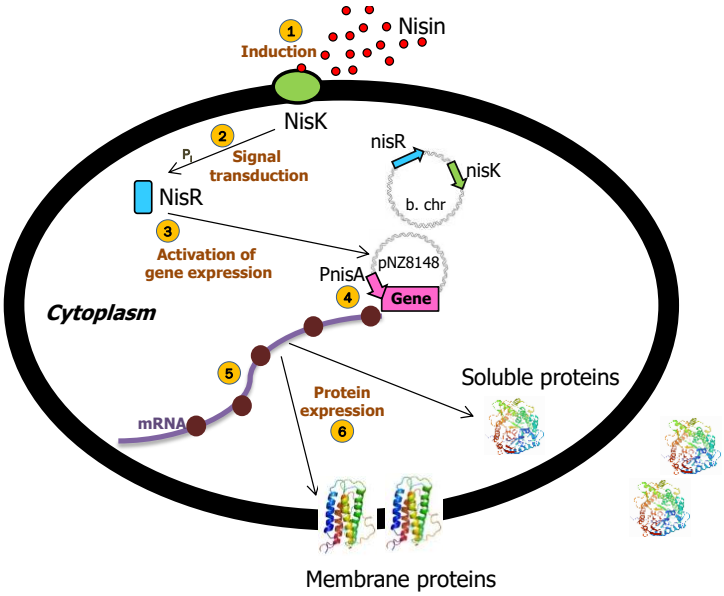

Figure 1: NIsin Controlled gene Expression (NICE) system in *L. lactis*. After detection of nisin by the membrane-located sensor protein (NisK), this histidine protein kinase autophosphorylates and transfers its phosphate group to activate the cytoplasmic response regulator NisR. Activated NisR subsequently induces transcription controlled by the PnisA promoter. Depending on the presence or absence of the corresponding targeting signals, the protein is either expressed into the cytoplasm or the membrane, or secreted into the external medium. B. chr: bacterial chromosome. Adapted from [12,21].

Well-characterized and highly versatile, the NICE system has been widely used for the over-expression and for subsequent functional and structural studies of homologous and heterologous MPs [12]. Moreover, it has been used for other purposes such as pharmaceutical, medical, bio-technology and food-technology applications [15-16,29]. Recently, the NICE system has been combined with the ZIREX system allowing the expression of different proteins at different times during the growth cycle [30].

2.2. *Strains*

Different *L. lactis* host strains derived from *L. lactis* subsp. cremoris MG1363 can be used for expression of cDNAs with the NICE system (Table 1, [24]). The most commonly used host strain for MP expression is the strain NZ9000. The nisin-producing strain NZ9700 [24] has been obtained by conjugation of the nisin-sucrose transposon Tn5276 of the nisin-A-producer NIZO B8 with MG1464, a rifampicin- and streptomycin-resistant derivative of MG1363 [30]. Since expression of MPs in *L. lactis* encounter difficulties due to low expression yields, different strategies have been developed to enhance their production. These strategies are either based on the introduction of a N-terminal fusion protein [31], mutations in the NisK ATPase domain of the sensor kinase (R406C) resulting in the DML1 strain [32], inactivation of the unique protease HtrA [33], selection of a strain enabling a higher plasmid stability (M4; [34]) or the overexpression of the cell envelope stress sensor/regulator CesSR [35].

Table 1: Bacterial strains and plasmids commonly used for the NICE system for overexpression of MPs. nisA, nisRK, genes of the nisin operon; RifR, StrpR and CmR: resistance to rifampicine, streptomycine and chloramphenicol, respectively.

		Characteristics	References
Strains			
L. lactis	NZ9700	Progeny of the conjugation between nisin producer strain NIZO B8 and MG1614 (RifR StrpR derivative of MG1363). Nisin producer strain for nisin induced gene expression	[11,25,36]
	NZ9800	Derivative of NZ9700 with deletion of 4 bp in nisA gene. No nisin production but nisRK transcribed. Host of the NICE system.	[25,36]
	NZ9000	MG1363 strain with nisRK integrated into pepN gene. Most commonly used host for NICE system.	[25]
	NZ9100	MG1363 strain with nisR and nisK integrated in a neutral locus. Standard host strain for nisin regulated gene expression (NICE®).	Mobitec Molecular Biotechnology
	DML1	NZ9000 strain transformed with pNZ-X-GFP-EmrC and selected by increased concentration of erythromycin	[32]
Plasmids			
pNZ8048		*Nco*I site used for translational fusions, CmR	[25]
pNZ8148		pNZ8048 with deletion of 60 bp DNA from B. subtilis, CmR	[19]
pNZ8149		pNZ8048; lacF for food grade selection for growth on lactose; nisA promoter followed by an NcoI site for translational fusions at the ATG.	Mobitec Molecular Biotechnology
pNZ8150		pNZ8148 with *Sca*I site used for translational fusions, CmR	[19]
pNZ8151		pNZ8148 with *Sca*I site used for translational fusions, lacF	Mobitec Molecular Biotechnology
pNZ8152		pNZ8148 with *Sca*I site used for translational fusions, alr gene for food grade selection	Mobitec Molecular Biotechnology

2.3. Cloning

2.3.1. Classical cloning

The cDNA or gene encoding the MP of interest is cloned into the appropriate expression plasmid , i.e. pNZ8048 or its derivatives (Table 1). These plasmids are based on the pSH71 replicon carrying the chloramphenicol resistance gene [27]. Plasmid pNZ8048 is the most commonly used plasmid for translational fusions. Genes of interest are directly fused to the *Nco*I site, which contains the ATG start codon directly downstream of the PnisA promoter. Different variants of pNZ8048 have been constructed. pNZ8148 is a shorter version of pNZ8048 with a deletion of a 60 bp heterologous DNA fragment from *Bacillus subtilis*, the initial cloning host of the pSH plasmid series [37]. pNZ8150 possesses a *Sca*I site directly upstream of the ATG start codon and therefore avoids the obligate use of the *Nco*I site. In this way, it is no longer necessary to change the second amino acid of a protein if that codon does not conform with the sequence of the *Nco*I site. Other plasmids and strains are available and can be used for other purposes [19,29; Mobitec Molecular Biotechnology]. The unidirectional cloning using classical restriction enzymes allows for a higher number of recombinant clones after transformation. Nevertheless, the MCS site is relatively small, containing less than 10 restriction sites and often partial digestions or mutagenesis is required to obtain the desired constructs

2.3.2. Other strategies

In addition to the classical cloning approaches, new strategies were developed to overcome the problem of low efficiency of gene manipulation in *L. lactis* and of the instability of *L. lactis-E. coli* shuttle vectors [38-39], for example: ligation independent cloning (LIC) and Gateway and other technologies developed by Berlec and collaborators. Furthermore, Geertsma and Poolman developed a generic cloning strategy compatible with high-throughput manipulations, which is also suitable for other organisms than *L. lactis* [40]. This method involves ligation-independent cloning (LIC) in an intermediary *E. coli* vector (pRExLIC-geneX), which can rapidly be converted via vector-backbone exchange (VBEx) into an organism-specific plasmid ready for high-efficiency transformation, as for instance pNZxLIC-geneX for *L. lactis*. In both LIC and VBEx procedures, rare restriction sites (*Swa*I and *Sfi*I) were used. This strategy allowed successful expression of MPs from prokaryotic and eukaryotic origins [41-43].

Other laboratories developed strategies based on the Gateway technology (Invitrogen), which are now widely used to simplify the cloning of cDNAs into many different expression systems from bacteria to eukaryotic systems [44] and for high-throughput expression screening of integral MPs [45]. Several libraries are currently available in Gateway compatible vectors [46]. However, *L. lactis* plasmids (e.g. pNZ8048 or derivatives) cannot be converted into Gateway destination vectors. Therefore, a strategy for the preservation of the correct reading frame has then been established for rapid transfer of cDNA from Gateway entry vectors into *L. lactis* nisin-inducible vectors [12,47]. This strategy allows the successful expression of MPs from prokaryotic and eukaryotic origins including proteins which could not be expressed using traditional cloning [7,48]. Only one development using an *E. coli-L. lactis* shuttle vector containing the Gateway cassette was proposed. These vectors allowed the expression of two lactococcal phages Tuc2009 and TP901-1 [49] and methyltransferases [50] but not of MPs.

Furthermore, in order to obtain higher number of insert-containing plasmids after transformation, Berlec and Strukelj [51] have developed a TA-cloning expression plasmid. A few years later, Berlec developed pNZ vectors for dual expression of proteins, pNZDual and pNZDualTT and one additional vector for the expression of proteins from polycistronic RNAs, pNZPolycist [52]. For the combinations tested, expression was higher using the latter compared to the pNZDual versions. Only one article showed dual expression of secreted proteins fused to the usp45 secretion signal [53]. This point needs to be further investigated with different combinations of MPs to verify the impact of such constructs on the expression of MPs.

Once gene cloned within the proper vector, recombinant bacteria could be generated and used for MP expression through the NICE system.

3. Expression of membrane proteins using the NICE system

In the last twenty years, the NICE system has proved to be highly versatile for the expression of proteins including MPs using pNZ8048 and its derivatives. Up to now 113 MPs from prokaryotic or eukaryotic origin, with diverse topologies and sizes have been successfully expressed including 79 in 2014 [12 and the present]. This system also allows the expression of MPs in their native oligomeric form (homo or heterodimers) [11-12].

Tables 2, 3 and 4: List of homologous and heterologous prokaryotic and eukaryotic MPs expressed in *L. lactis* using the NICE system. Species, size, expression yields and functions are given for each protein; the classification of MPs has been sorted according to the protein complexity in term of TM helix numbers. UNIPROT (http://www.uniprot.org/) is used as reference for protein information in addition to literature.

a Protein sizes are given in kDa and for full proteins, i.e. including the transit peptide for mitochondrial and chloroplastic MP (truncated for heterologous expression);

b The number of TM helices listed here has either already been demonstrated or predicted with software (such as TNHMM or psipred) with the FASTA sequence published in Uniprot. p for peripheral proteins

c *B. breve* (*Bifidobacterium breve*); *B. longum* (*Bifidobacterium longum*); *B. melitensis* (*Brucella melitensis*); *B. subtilis* (*Bacillus subtilis*); *C. acetobutylicum* (*Clostridium acetobutylicum*); *E. coli* (*Escherichia coli*); *E. faecalis* (*Enterococcus faecalis*); *H. pylori* (*Helicobacter pylori*); *L. brevis* (*Lactobacillus brevis*); *L. innocua* (*Listeria innocua*); *L. monocytogenes* (*Listeria monocytogenes*); *L. plantarum* (*Lactobacillus plantarum*); *Lb; pentosus* (*Lactobacillus pentosus*); *L. salivarius* (*Lactobacillus salivarius*); *M. smegmatis* (*Mycobacterium smegmatis*); *R. palustris* (*Rhodopseudomonas palustris*); *R. prowazekii* (*Rickettsia prowazekii*); *R. sphaeroides* (*Rhodobacter sphaeroides*); *S. agalactiae* (*Streptococcus agalactiae*); *S. aureus* (*Staphylococcus aureus*); *S. mutans* (*Streptococcus mutans*); *S. pneumoniae* (*Streptococcus pneumonia*); *S. thermophilus* (*Streptococcus thermophilus*); *T. maritime* (*Thermotoga maritima*)(Table 3); *A. polyphaga* (*Acanthamoeba polyphaga*); *A. thaliana* (*Arabidopsis thaliana*); *H. sapiens* (*Homo sapiens*); *M. musculus* (*Mus musculus*); *N. patriciarum* (*Neocallimastix patriciarum*); *S. cerevisiae* (*Saccharomyces cerevisiae*); *S. tuberosum* (*Solanum tuberosum*) (Table 4).

d The expression yields are given as a percentage of the recombinant protein compared to the total membrane proteins (TMP)

Table 2: List of homologous prokaryotic MPs

Protein	Function	Size (kDa)[a]	TM helices[b]	Expression level[c]	References
ArcD1	arginine/ornithine antiporter	52.6	13	-	[54]
ArcD2	arginine/ornithine antiporter	54	13	-	[54-55]
BcaP	branched-chain amino acid permease	50	12	20%	[35]
BioY	biotin transporter	20.5	5	5%	[56]
ChoS	glycine betaine ABC transporter permease	55.1	5	2%	[57]
CitP	citrate sodium symporter	48.6	13	1-2%	[58]
CmbT	MFS transporter	50	12	<1%	[59]
DtpT	di-/tripeptide transporter	54.8	12	10%	[11]
GlnP	ABC transporter	78.5	3	<1%	[57,60]
GlnQ	glutamine transport ATP-binding	27	8	2-5%	
LmrA	ABC efflux pump	65	6	30%	[61]
LmrCD	ABC transporter	63+73.7	6+6	5-10%	[62]
LmrP	MFS efflux pump	45	12	5%	[63-65]
MleP	MFS transporter	46.7	11	1-2%	[11]
MscL	large-conductance mechano-sensitive channel	13.8	2	5-10%	[66]
OppB	ABC transporter with OpuC,D,F	35.1	6	<1%	[11]
OppC	ABC transporter with OpuB,D,F	32.3	6	<1%	
OpuABC	ABC transporter with OpuAA	63	8	10%	[11,67]
RibU	riboflavin transporter	23	6	5%	[68]
SerP1	serine permease	51.3	12	-	[69]
SerP2	DL-alanine permease	51.5	12	-	
ThiT	thiamine transporter	20	6	2%	[42]

228
229
230

Table 3: List of heterologous prokaryotic MPs

Protein	Function	Size (kDa)[a]	TM helices[b]	Organism[c]	Expression level[d]	References
abcA	ABC transporter	70	6	*B.breve*	1%	[70]
abcB	ABC transporter	66	6		5-10%	
LanR1	lantibiotic response regulator	24	6	*B. longum*	-	[71]
LanI	ABC transporter	32.76	-		-	
LanT	lantibiotic transporter	80.1	6		-	
BmrA	ABC transporter	65.3	6		5-10%	[72]
tlyC1	hemolysin-like protein	11.2	2		-	[73]
Omp16	peptidoglycan associated lipo-protein	18.2	p	*B. melitensis*	-	[74]
DctA	C4-dicarboxylate transport	45.4	8	*B.subtilis*	0.5-1%	[41]
CA_C2849	proline/glycine betaine ABC-type transport system, permease	57.6	6	*C. acetobutylicum*	2%	[57]
MsbA	lipid A export ATP-binding/per-mease	64.5	6	*E. coli*	20-30%	[75]
EfrA	ABC transporter	56.3	4	*E. faecalis*	-	[76]
EfrB	ABC transporter	60.54	3		-	
Jhp0757	putative osmoprotection binding protein	62.6	6	*H. pylori*	1%	[57]
HpaA	neuraminyllactose-binding he-magglutinin	29.1	p		25-30%	[77]
HorA	Multidrug transporter	64.2	5	*L. brevis*	30%	[78]
ArcD	arginine/ornithine exchangers	51.9	13		-	[79]
Lin0840	ABC transporter	53.2	6	*L. innocua*	<1%	[57]
Lin1461	binding-protein-dependent transport system permease	55.7	6		2%	
Lin2352	ABC transporter	53.4	6		1%	
Lmo1422	binding-protein-dependent transport system permease	55.7	6	*L. monocytogenes*	1%	
Lmo2250	ABC transporter	53.1	6		2%	
cwaA	cell wall anchored adhesion asso-ciated protein	93.7	2	*L. plantarum*	-	[80]
OppA	oligopeptide-binding protein	59.7	p	*L. salivarius*	-	[81]
XylP	xylose-proton symporter	52.7	12	*Lb. pentosus*	20%	[11]
Rv1410	MFS transporter	54.7	14	*M. smegmatis*	-	[82]
CYP201A2	cytochrome-mono-oxygenase	49.7	p	*R. palustris*	1.5%	[7]

TlcA,B,C	ATP/ADP translocator	56.8	12	*R. prowazekii*	5-10%	[11]
NapC	cytochrome-electron transfer	25.6	1	*R. sphaeroides*	0.5%	[7]
BspA	Gram+ anchoring domain containing protein	101	1	*S. agalactiae*	-	[83]
SAR1949	putative extracellular glutamine-binding protein	53.1	4	*S. aureus*	1%	[57]
Sav1866	multidrug export ATP-binding/permease	64.8	6		20-25%	[84]
Cnm	collagen and laminin-binding glycoprotein	58	1	*S. mutans*	-	[85]
PspC	choline binding protein	85.24	1	*S. pneumoniae*	-	[86]
MreC	peptidoglycan synthesis	32	1		1%	[7]
ProWX	ABC transporter permease-choline transporter	55.5	6		2-3%	[57]
SP_0453	AA ABC transporter, AA-binding protein/permease protein	57.4	6		<1%	
SP_1241	AA ABC transporter, AA-binding protein/permease protein	78.4	3		<1%	
LacS	MFS transporter	56.6	12	*S. thermophilus*	1-2%	[11]
SfbA/FbaA	streptococcal fibronectin binding protein A	37.8	1	*Streptococcus*	-	[87]
SfbI	fibronecting binding protein	67.3	1		-	
TM287/288	ABC transporter	60+60	6+6	*T.maritima*	0.5-1%	[88]

Table 4: List of eukaryotic MPs

Protein	Function	Size (kDa)[a]	TM helices[b]	Organism[c]	Expression level[d]	References
ATM1	mitochondrial iron-sulfur cluster transporter	77.5	6	*S. cerevisiae*	-	[89]
GDT1	cation exchanger (homologous to TMEM)	30.3	7		-	[90]
CTP1	tricarboxylate transport protein	32.9	6		5%	[18]
SAM5	mitochondrial S-adenosyl methionine carrier	30.9	4		<1%	
Mdl1	mitochondrial ATP-dependent permease	76	5		<0.1%	[91]
MIR1	mitochondrial phosphate carrier protein	32.8	6		<1%	[18]
DIC1	mitochondrial dicarboxylate transporter	33	6		10%	

GGC1	mitochondrial GTP/GDP carrier protein	33.2	6		4%	
PIC2	mitochondrial phosphate carrier protein 2	33.5	6		1-2%	[92]
AAC3	mitochondrial ADP/ATP carrier protein 3	33.7	6		5%	[11]
ODC2	mitochondrial 2-oxodicarboxylate carrier 2	34	6	*S. cerevisiae*	10%	[18]
AAC1	mitochondrial ADP/ATP carrier protein 1	34.1	6		<1%	
ODC1	mitochondrial 2-oxodicarboxylate carrier 1	34.2	6		8%	
AAC2	mitochondrial ADP/ATP carrier protein 2	34.4	6		<1%	
MPC1/2	mitochondrial pyruvate carrier	15+14.5	2+3		-	[93]
MPC1/2	mitochondrial pyruvate carrier	12.3+14.3	2+2	*M. musculus*	<1%	[93-94]
MPC1/2	mitochondrial pyruvate carrier	12.4+12.2	2+3		-	[93]
ceQORH	quinone oxidoreductase - electron transfer	33.1	p		30%	[47]
LPR1	multi-copper oxidase	60.5	p		<0.1%	[7]
PHF	phosphate transport regulation	42.4	1		1.5%	
AtHMA1	heavy metal transporter	80.1	6	*A. thaliana*	3%	[47]
AtHMA3	heavy metal transporter	81.4	8		1%	
AtHMA6	heavy metal transporter	100	8		3%	
AtHMA4	heavy metal transporter	126.7	8		0.75%	[7]
NTT1	chloroplast ADP/ATP transporter	57.5	12		0.2%	[47]
NRT1 (NPF2.3)	nitrate excretion transporter	61	12		-	[95]
ATM3 (ABCB25)	mitochondrial ABC transporter	80	7	*A. thaliana*	-	[89]
AAC hyd	hydrogenosomal carrier	33.9	6	*N. patriciarum*	<1%	[11]
SUT1	sucrose transporter	54.8	12	*S. tuberosum*	1-2%	[96]
L276	mitochondrial carrier like	27.3	6	*A. polyphaga*	5%	[97]
Bcl-Xl	apoptosis regulation	24.7	1		1%	[7]
CYP3A4	cytochrome-mono-oxygenase	57.4	1	*H. sapiens*	5%	[48]
MGST1	microsomal glutathione S-transferase 1	17.6	4		3%	
ABCG2	breast cancer resistance protein	72	6		0.5-1%	[98]

Erd2	KDEL receptor	24.4	7	*H. sapiens*	<0.1%	[11]
CXCR4	chemokine receptor type 4	37.9	7		<0.1%	[7]
CCR5	chemokine receptor type 5	38.7	7		<0.1%	
PS1Δ9	human alpha secretase component	55	9		0.1-0.2%	[97]
CFTR	cystic fibrosis transmembrane conductance regulator	168	12		<0.1%	[43]
TMEM165	cation transporter	34.9	6		-	[99]
AAC1	mitochondrial ADP/ATP carrier protein 1	34	6		0.5-1%	[100-102]
ANT2 (AAC2)	mitochondrial ADP/ATP carrier protein 2	32.8	6		-	[101]
ANT3 (AAC3)	mitochondrial ADP/ATP carrier protein 3	32.8	6		-	[101]
SLC25A3	mitochondrial pyruvate carrier (homologous to PIC)	40.1	6		-	[103]

Tables 2, 3 and 4 display respectively non-exhaustive lists of prokaryotic MPs (homologous or heterologous expression) and of eukaryotic MPs expressed in *L. lactis* with the NICE system. They include studies of functionally active proteins in which expression yields were not determined. The tables do not display, for some proteins, the percentage of expressed proteins when not available, and of functional proteins out of the proteins expressed; indeed, this information is seldom reported since such a ratio is difficult to measure and necessitates isolating native proteins as controls. *L. lactis* MPs represent 20% of total MPs expressed, prokaryotic MPs 40% and eukaryotic 40% respectively; among the latter, each origin (yeast, plant and human) represents one-third (Figure 2).

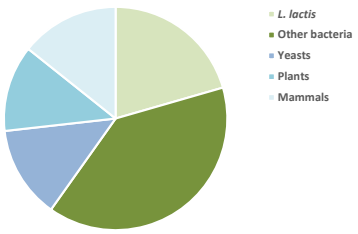

Figure 2: Comparison of MPs expressed in *L. lactis* using the NICE system depending on their origin: *L. lactis*, other bacteria or eukaryotic cells

The membrane proteins listed in Tables 2, 3 and 4 can be plotted as a function of the number of their TM helices and their molecular size. As shown in Figure 3, a large number of MPs have sizes below 100 kDa with many MPs having either 6 or 12 TM helices, whatever they are prokaryotic, from *L. lactis* or other bacteria or are of eukaryotic origin (Figures 3 and 4), highlighting the two large families of proteins expressed (ABC and mitochondrial transporters).

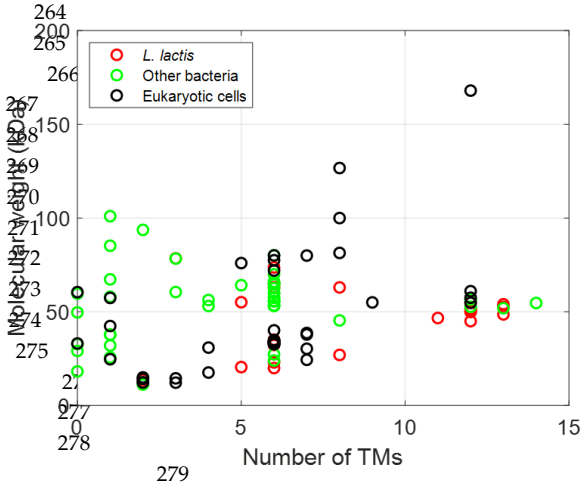

Figure 3: Influence and relationship between origin on MPs expressed in *L. lactis*.

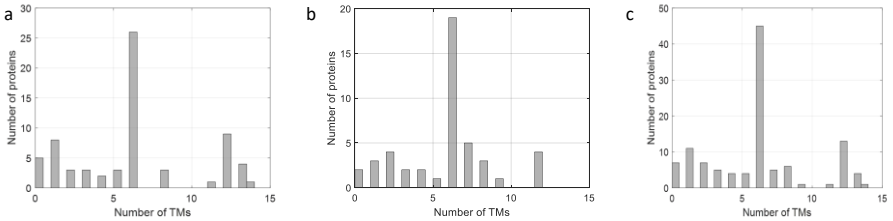

Figure 4: Influence of TM number on expression of MPs expressed in *L. lactis*. a. On expression of MPs from *L. lactis*. b. On expression of MPs from other bacteria. c. On expression of eukaryotic MPs.

3.1. Prokaryotic MPs

Tables 2 and 3 report the successful expressions of 23 homologous and 43 heterologous MPs using the NICE system. Expression yields of prokaryotic MPs were the highest obtained of all reviewed MPs with up to 30% of total MPs (TMP) by heterologous (HorA and MsbA) and homologous (LmrA) expression. The expressed MPs possess up to 13 TM helices and, even with such a high TM helix content, they were produced with expression yields up to 20% TMP (BcaP and XylP). Most homologous MP expression studies have been focused on proteins belonging to the families of amino acid and ABC (ATP-Binding Cassette) transporters, probably related to the specialization of the laboratories working with this system. In addition to the above-mentioned amino acid and ABC transporters, other heterologous MPs have been expressed, belonging to diverse families such as cytochrome, permease and binding proteins (Table 3). The relatively high expression yields obtained with heterologous prokaryotic MPs could be explained by the fact that the codon usage is compatible with AT-rich codon bias of *L. lactis* [104]. *L. lactis* also allowed the expression of a MP with 14 TM domains, like the MFS transporter called Rv1410 (Table 3; [82]).

3.2. Eukaryotic MPs

Expression of eukaryotic MPs in *L. lactis* were initiated and first reported in 2003 by Kunji and collaborators with the expression of mitochondrial carriers from yeast [11]. Since then, several other eukaryotic MPs from yeast, plants and humans have been expressed, with levels from 0.1 to 10% of TMPs (Table 4), mainly from the mitochondrial carrier superfamily but also from other families. Only one MP from protozoa (A. polyphaga; Table 4; [97]) was expressed in *L. lactis*.

3.2.1.Yeast (S. cerevisiae)

16 MPs from yeast have been successfully expressed in *L. lactis*. Two main studies on mitochondrial carriers revealed that all the MPs tested could be expressed with yields from 0.5 to 10% (Table 4). For some of them, expression yields were even improved by rational design of the N-terminus (replacing or truncating these regions or by addition of lactococcal signal peptides) [18].

3.2.2.Plants

13 MPs from three plant species, i.e. *A. thaliana, S.tuberosum* and *N. patriciarum*, have been successfully expressed in *L. lactis*. They belong to different families, as for instance an oxidase and various transport proteins (heavy metal, ATP/ADP or sucrose) and their topologies span from peripheral to intrinsic 12 TM helices (Table 4). The levels of expression obtained were relatively high, up to 30% (Table 4), without modifications of the sequence. These relatively high expression yields allowed performing functional studies to discover and/or go deeper in the function of the MP expressed.

3.2.3.Human MPs

As for yeast mitochondrial carriers, human ADP/ATP translocators (AAC1, AAC2 and AAC3) were also expressed in *L. lactis*. Other human MPs from diverse families and topologies (1 to 12 TM helices), have been expressed with yields from almost undetectable (<0.1%) to 1% (Bcl-Xl) (Table 4) including the ABC transporter, CFTR with a very high TM helix number (12 helices) and size (168 kDa) expressed at very low levels (below 0.1% of TMP; [43]).

3.3. Comparison of expression yields between E. coli and L. lactis

The expression yields obtained for expression of MPs in *L. lactis* are generally lower than those obtained for overexpression of same MPs in *E. coli* [7,12,57]. In some cases, expression in *L. lactis* allowed a higher expression or the expression of proteins produced usually in inclusion bodies in *E. coli*. For proteins produced with both bacterial expression systems, yields were almost 10 times lower after expression in *L. lactis* compared to *E. coli* [12]. The reason for this difference could be a limitation of amino acid import, especially for branched amino acids. This problem could be overcome by supplying the cells with an alternative path, such as a medium containing the appropriate dipeptides or by engineering the transport capacity for branched-chain amino acids [105]. Other strategies have been implemented using optimization of functional expression, i.e. control of transcription rate, nutrient availability in richer medium, gene optimization and/or fusion tags [57].

All MPs listed in Tables 2, 3 and 4 have been expressed in *L. lactis* and were functional in this bacterium, which allowed different assays to be performed and to decipher/discover the function of the MPs in the original organism.

4. Functional expression of MPs

The following section will focus on examples of MPs of either prokaryotic or eukaryotic origin belonging to one functional class such as ABC transporters, secondary transporters etc. *L. lactis* presents three major advantages over *E. coli* for functional MP expression: i) it possesses only one membrane; ii) it does not form inclusion bodies and iii) it expresses proteins in their native oligomeric state. Moreover, the genomes of MG1363 and NZ9000 are completely sequenced and annotated, allowing the generation of mutated strains. These functional characterizations could be performed on: i) whole bacteria using radioactive substrates, ii) membrane vesicles, iii) proteoliposomes after reconstitution with phospholipids and/or iv) solubilized/purified proteins. All MPs expressed (Tables 2,

3 and 4) belong to different families: ABC transporters, secondary transporters, MPs originating from organelle (mitochondria, chloroplast), MCP and other families (Figure 5).

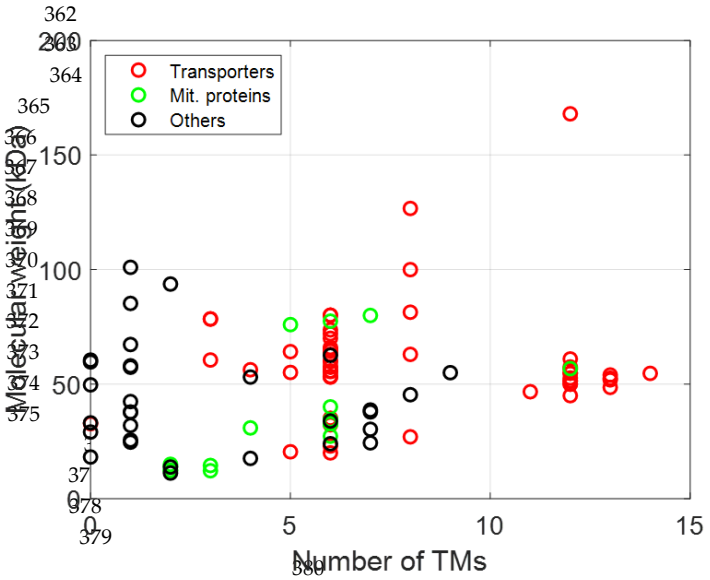

Figure 5: Relationship between function (transporters in red, mitochondrial proteins in green and other functions in black), size and topology for MPs expressed in *L. lactis*.

4.1. ABC transporters

ABC transporters generally consist of four domains, two membrane-embedded domains carrying out substrate recognition and translocation and two hydrophilic nucleotide binding domains (NBDs). They represent one third of MPs expressed in *L. lactis* (Tables 2, 3 and 4; Figure 5). Either transport or ATPase activities can be measured with radioactive or non-radioactive compounds on intact cells or detergent-purified protein within or not proteoliposomes or nanodiscs. In some cases, mutations allowed assigning the role of certain amino acids to the proper function of the proteins. Studies in intact cells were facilitated by the availability of strains deleted in LmrACD, the 3 main ABC transporters present in the *L. lactis* membrane.

The ABC half-transporter LmrA (65 kDa, 6 TM helices), a well-characterized ABC transporter from *L. lactis* was expressed in very high levels (up to 30% of TMP; [61]). The critical role of a carboxylate group in proton conduction to secondary-active transporters could be assigned [106]. Additional studies were performed on mutated versions expressed in *L. lactis* wild type strains or strains with a deletion of LmrA homologs (LmrCD) [62]. Different studies based on nuclear magnetic resonance (NMR) and electron spin resonance (EPR) spectroscopy allowed deciphering the ATP hydrolysis cycle of the protein, nucleotide binding and the induction of the ion-motive force [107-110].

The thiamine high affinity ABC transporter, ThiT (20 kDa, 6 TM helices) belonging to the family of energy coupling factors, has been characterized in *L. lactis*. The expression yield in *L. lactis* was around 1-2% (Table 2; [42]). Mutagenesis studies allowed the determination of some amino acids interacting with the energizing module, necessary for vitamin translocation [111]. EPR performed on purified ThiT and molecular dynamic studies allowed detailed description of the conformational changes of the protein during binding and coupling with the energizing module [80]. The structure of this protein has been solved in 2014 [112].

Moreover, out of the 31 ABC transporters that have been expressed in *L. lactis*, 19 originated from other bacteria. Among them, the half ABC-transporter MsbA from *E. coli* was expressed with a yield slightly lower than that obtained with the homologous expression of LmrA (20-30%). This homodimeric transporter with 6 TM helices and a molecular

size of 64 kDa is involved in lipid A export in *E. coli* [75]. Functional studies have demonstrated that substrate binding to the MsbA dimer caused NBD dimerization [113-115].

A heterodimeric ABC exporter, TM287/288 from *Thermotoga maritima*, has also been expressed in *L. lactis* [88]. TM287 and TM288 with a molecular size of 60 kDa and 6 TM helices each, form a functional heterodimer sharing 36% of sequence identity with LmrCD, a well characterized heterodimeric ABC exporter from *L. lactis* [62]. Functional studies allowed to determine that the NBDs only partially separate, remaining in contact through an interface involving conserved motifs connecting the two ATP hydrolysis sites [88].

Finally, some eukaryotic ABC transporters were expressed in *L. lactis*. Among them, the well-known CFTR [43] and a plant mitochondrial ABC transporter, ATM3/ABCB25. Membrane vesicle assays revealed that glutathione (GSH) polysulfides are likely to be the substrates serving as precursors for iron-sulfur cluster assembly [89].

4.2. Secondary transporters

Secondary active transporters exploit the electrochemical potential of solutes to shuttle specific substrate molecules across biological membranes, usually against their concentration gradient. These proteins are involved in transport of amino acids [116], organic or inorganic anions, through symport or exchange processes [117]. MPs from the MFS superfamily were successfully expressed in *L. lactis* in their functional state [116-117]. Whilst the quantity of protein produced in these studies was not determined, the biological activity of the proteins was however detected using substrates specific to the transporters.

4.3. MPs from organelle

26 MPs out of the 113 possess either a chloroplast or mitochondrial origins (Tables 3 and 4, Figure 5). They are belonging to the families of ADP/ATP carriers (AAC) and of Mitochondrial Pyruvate Carriers (MPC) in mitochondria and chloroplast but also to other families in chloroplasts.

4.3.1.Mitochondrial MPs

AACs represent a large proportion of the MPs with 6 TM domains expressed in *L. lactis* (Figures 3 and 4). Firstly, two mitochondrial carriers from *S. cerevisiae*, CTP1 and AAC3, have been successfully expressed with yields of 5% and shown to be functionally active in *L. lactis* [11]. Subsequently, ten other carriers from *S. cerevisiae* have been successfully expressed with yields ranging from 1 to 10% and activities varying depending on the substrate and the protein studied [18]. The relatively high expression yields obtained for these proteins could most probably be linked to the presence of cardiolipin in the membrane of *L. lactis* (32%; [100]. Indeed, it could be demonstrated that the expression of these proteins is facilitated and the presence of the appropriate lipids could help to drive the protein folding to the right conformation.

The human isoforms of ATP/ADP translocators (AAC1, 2 and 3) displaying TM helix number and size features similar to the mitochondrial carriers of *S. cerevisiae* were also studied. AAC1, expressed at 0.5-1% of TMP, was sensitive to the same inhibitors as its yeast orthologs [100]. Mutants of this MP were shown to be involved in childhood-onset mild skeletal myopathy [101]. Zhang and collaborators [102] tested and compared the efficiency of *L. lactis* versus yeast mitochondria for studying the impact of inhibitors of AACs on the different isoforms. Their studies revealed that *L. lactis* shows a higher specificity in the exchange assay than yeast, it allowed differentiating between direct and indirect inhibitors and it is more reproducible and can be prepared in large quantities.

Among the mitochondrial proteins, the MCPs are remarkable. Indeed, the isoforms of MCP1 and MCP2 from 3 different species, i.e. the yeast *Saccharomyces cerevisiae, Mus musculus* and *Arabidopsis thaliana* have been expressed under a functional state in their

heterodimeric form in *L. lactis* [93-94]. The mouse isoforms were able to transport pyruvate across the membrane in intact recombinant bacteria [94]. This uptake was sensitive to the mitochondrial pyruvate carrier inhibitor UK5099 and to 2-deoxyglucose, which collapses the proton electrochemical gradient. Moreover, artificially increasing the membrane potential by lowering the pH in the buffer from 7.2 to 6.2 significantly increased pyruvate uptake. Co-expression of mMPC1 and mMPC2 in the membrane of *L. lactis* was sufficient to allow the import of pyruvate with properties similar to the mitochondrial pyruvate carrier [118].

4.3.2.Chloroplast MPs

Expression in *L. lactis* using the NICE system proved to be efficient for functional expression of several plant MPs involved in different chloroplast metabolic pathways, i.e. ceQORH, HMA6 and NTT1 proteins from *Arabidopsis thaliana*.

The peripheral ceQORH protein is interacting with the chloroplast envelope through electrostatic interactions [119]. While this protein was produced in *E. coli* in inclusion bodies [119], it was expressed in *L. lactis* at almost 30% of TMP (Table 4;[47]), a surprisingly high expression yield and similar to those obtained for homologous prokaryotic MPs (Tables 2,3 and 4). Functional characterization performed on purified proteins reconstituted in proteoliposomes revealed that ceQORH has a NADPH dependent dehydrogenase activity and requiries a lipid environment. Moreover, when produced in *L. lactis*, ceQORH behaved as the natural chloroplast envelope protein and interacted with the bacterial membrane through electrostatic interactions [47].

Other chloroplast MPs such as the P1B-type ATPase family have also successfully been expressed with yields from 0.7 to 3% of TMP (Table 4; [7,47]). These MPs (6 to 8 TM helices) translocate ions across plasma or organelle membranes at the expense of ATP consumption and are involved in the control of metal homeostasis within the cell [120]. Among the eight P1B-type ATPases encoded by the Arabidopsis genome, four have been successfully expressed in *L. lactis* [47]. Biochemical characterizations using phosphorylation assays were performed using *L. lactis* membranes expressing HMA6 and these assays allowed the identification of this protein as a high affinity Cu+ transporter of the chloroplast envelope [121].

The NTT1 protein is one of the AAC identified in the chloroplast ; it imports ATP in exchange with ADP. This transporter has already been functionally characterized after expression in *S. cerevisiae* and *E. coli* [122-123]. Even expressed at a very low yield (0.2% of TMP), uptake assays of radioactive nucleotides could be performed on intact *L. lactis* cells and showed a time dependent uptake of ATP with a rate similar to the one measured in *E. coli* cells [47].

To conclude, *L. lactis* appears to be an appropriate expression system for functional characterization of mitochondrial and Arabidopsis MPs, especially for chloroplast MPs. This can be explained by the fact that the *L. lactis* membrane contains cardiolipin and glycolipids [124], which are both also present in mitochondria and the inner membrane of chloroplasts [125] in contrast to *E. coli* membranes [126], which have a different composition. The importance of the lipid composition of host cells in the overexpression of functional MPs has also already been underlined by other authors [3,127].

4.4. Other families

The first human MP produced in *L. lactis* was the KDEL receptor, Erd2. This protein of 7 TM helices is involved in the retrieval of proteins of the endoplasmic reticulum (ER) at later stages of the secretory pathway. While expressed at a very low level, the protein could still bind its specific peptide and conserve the pH-dependent activities as in rat Golgi membranes [11].

Two MPs involved in human liver detoxification functions have been successfully expressed in *L. lactis*: the cytochrome-mono-oxygenase (CYP3A4) and the microsomal Glutathione S-Transferase 1 (MGST1). Interestingly, both proteins could successfully be expressed in *L. lactis* with higher yields than those previously obtained with classical expression systems (*E. coli*, *S. cerevisiae*) at 5 and 3% TMPs, respectively. This was also higher than results obtained with other eukaryotic membrane proteins expressed in *L. lactis* [48]. Expression in *L. lactis* of MGST1 isoform from *Rattus norvegicus* was able to exhibit its GSH-transferase activity somewhat lower than values previously reported for rMGST1 from purified microsomes or after heterologous expression in *E. coli*.

As shown in the last two paragraphs about the expression and functional characterization MPs in *L. lactis*, the number of MPs expressed in their functional state is increasing. Additional information has been obtained through structural analysis of some of the proteins listed above.

5. Structures resolved from MPs expressed in *L. lactis*

Because of its numerous advantages for MP expression and functional characterization, *L. lactis* is now also a good alternative bacterial expression system to *E. coli* for structure determination of MPs of interest. The first structure of a homologous MP expressed in *L. lactis* was obtained for OpuAC 10 years ago [128]. Then, the structure of ThiT was obtained with both the wild-type and a selenomethionine labeled protein. This crystal structure has been obtained with an expression yield of 2% of TMPs [111,129]. One year after that, the same group resolved the structure of BioY, another *L. lactis* MP from the ECF family involved in biotin transport [56]. Altogether almost 20 structures of MPs have been resolved in the last ten years after expression in *L. lactis* using the NICE system, including their various conformations and bound to their substrates (Table 5).

Table 5: Structures obtained after expression in *L. lactis*

Protein	Organism	Code	Structure	References
OpuA	*L. lactis*	7AHH	OpuA inhibited inward-facing, SBD docked	[130]
		7AHC	OpuA apo inward-facing	
		7AHE	OpuA inhibited inward facing	
		7AHD	OpuA (E190Q) occluded	
PrgL	*E. faecalis*	7AED	VirB8 domain of PrgL from *Enterococcus faecalis* Pcf10	[131]
MhsT	*A. halodurans*	6YU2	Crystal structure of MhsT in complex with L-isoleucine	[132]
		6YU3	Crystal structure of MhsT in complex with L-phenylalanine	
		6YU4	Crystal structure of MhsT in complex with L-4F-phenylalanine	
		6YU5	Crystal structure of MhsT in complex with L-valine	
		6YU6	Crystal structure of MhsT in complex with L-leucine	
		6YU7	Crystal structure of MhsT in complex with L-tyrosine	
GLNPQ	*L. lactis*	6FXG	Crystal structure of substrate binding domain 1 (SBD1) OF ABC transporter GLNPQ in complex with Asparagine	[133]

ECF	*L. delbrueckii subsp. Bulgaricus*	5D0Y	Substrate bound S-component of folate ECF transporter	[112]
ATP-Mg/Pi carrier (APC)	*H. sapiens*	4ZCU	Structure of calcium-bound regulatory domain of the human ATP-Mg/Pi carrier in the P2 form	[134]
		4ZCV	Structure of calcium-bound regulatory domain of the human ATP-Mg/Pi carrier in the P212121 form	
ThiT	*L. lactis subsp. cremoris MG1363*	4POP	ThiT with LMG139 bound	[129]
		4POV	ThiT with LMG135 bound	
ECF	*L. lactis subsp. cremoris MG1363*	4DVE	Crystal structure at 2.1 A of the S-component for biotin from an ECF-type ABC transporter	[56]
OpuAC	*L. lactis*	3L6G	Crystal structure of lactococcal OpuAC in its open conformation	[128]
		3L6H	Crystal structure of lactococcal OpuAC in its closed-liganded conformation complexed with glycine betaine	

This opens up the road to the elucidation of other MP structures in the future since the expression yields obtained for almost all the proteins is close to 1-2% and higher (Tables 2, 3 and 4). Furthermore, the ability to label the MPs with SelenoMet allows resolving the diffraction data [135] and the availability of specific protocols developed for this purpose [136].

6. Conclusion

Over the last two decades, *Lactococcus lactis* emerged and proved to be an alternative and promising expression system to other bacterial systems. Numerous prokaryotic and eukaryotic MPs with diverse topologies, origins and functions were successfully expressed in *L. lactis* using the tightly regulated NICE system and a yield although lower than *E.coli*, still allowing functional and structural characterizations. Finally, twenty crystal structures of MPs after expression in *L. lactis* have been resolved and opened up the road to others in the future. This promising cell factory will enrich the knowledge of MPs in their functional and structural states, and allow further biotechnological and biotherapeutical applications in a near future.

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References

1. Wallin, E.; von Heijne, G. Genome-wide analysis of integral membrane proteins from eubacterial, archaean, and eukaryotic organisms. *Protein Sci* **1998**, *7*, 1029-1038. doi: 10.1002/pro.5560070420.
2. Lundstrom, K. Structural genomics and drug discovery. *J Cell Mol Med* **2007**, *11*, 224-238. doi: 10.1111/j.1582-4934.2007.00028.x.
3. Junge, F.; Schneider, B.; Reckel, S.; Schwarz, D.; Dötsch, V.; Bernhard, F. Large-scale production of functional membrane proteins. *Cell Mol Life Sci* **2008**, *65*, 1729-1755. doi: 10.1007/s00018-008-8067-5.
4. Kesidis, A.; Depping, P.; Lodé, A.; Vaitsopoulou, A.; Bill, R.M.; Goddard, A.D.; Rothnie, A.J. Expression of eukaryotic membrane proteins in eukaryotic and prokaryotic hosts. *Methods* **2020**, *180*, 3-18. doi: 10.1016/j.ymeth.2020.06.006.

5. Lacapere, J.J.; Pebay-Peyroula, E.; Neumann, J.M.; Etchebest, C. Determining membrane protein structures: still a challenge. *Trends Biochem Sci* **2007**, *32*, 259-270. doi: 10.1016/j.tibs.2007.04.001.
6. Fogeron, M.L.; Lecoq, L.; Cole, L.; Harbers, M.; Böckmann, A. Easy Synthesis of Complex Biomolecular Assemblies: Wheat Germ Cell-Free Protein Expression in Structural Biology. *Front Mol Biosci* **2021**, *8*, 639587. doi: 10.3389/fmolb.2021.639587.
7. Bernaudat, F.; Frelet-Barrand, A.; Pochon, N.; Dementin, S.; Hivin, P.; Boutigny, S.; Rioux, J.B.; Salvi, D.; Seigneurin-Berny, D.; Richaud, P.; Joyard, J.; Pignol, D.; Sabaty, M.; Desnos, T.; Pebay-Peyroula, E.; Darrouzet, E.; Vernet, T.; Rolland, N. Heterologous expression of membrane proteins: choosing the appropriate host. *PLoS One* **2011**, *6*, e29191. doi: 10.1371/journal.pone.0029191.
8. Gordon, E.; Horsefield, R.; Swarts, H.G.; de Pont, J.J.; Neutze, R.; Snijder, A. Effective high-throughput overproduction of membrane proteins in Escherichia coli. *Protein Expr Purif* **2008**, *62*, 1-8. doi: 10.1016/j.pep.2008.07.005.
9. Kaur, J.; Kumar, A.; Kaur, J. Strategies for optimization of heterologous protein expression in E. coli: Roadblocks and reinforcements. *Int J Biol Macromol.* **2018**, *106*, 803-822. doi: 10.1016/j.ijbiomac.2017.08.080.
10. Schlegel, S.; Klepsch, M.; Gialama, D.; Wickström, D.; Slotboom, D.J.; de Gier, J.W. Revolutionizing membrane protein overexpression in bacteria. *Microb Biotechnol* **2010**, *3*, 403-411. doi: 10.1111/j.1751-7915.2009.00148.x.
11. Kunji, E.R.S.; Slotboom, D.J.; Poolman, B. Lactococcus lactis as host for overproduction of functional membrane proteins. *Biochim Biophys Acta* **2003**, *1610*, 97-108. doi: 10.1016/s0005-2736(02)00712-5.
12. Bakari, S.; André, F.; Seigneurin-Berny, D.; Delaforge, M.; Rolland, N.; Frelet-Barrand, A. Lactococcus lactis, recent developments in functional expression of membrane proteins. In: *Membrane Proteins Production for Structural Analysis*; Mus−Vuteau, I. Eds; Springer eBook, 2014, pp 107−132. doi.org/10.1007/978-1-4939-0662-8_5
13. Gasson, M.J.; de Vos, W.M. *Genetics and biotechnology of lactic acid bacteria*. Gasson, M.J. and de Vos, W.M. Eds; Blackie, London. 1994
14. Mierau, I.; Olieman, K.; Mond, J.; Smid, E.J. Optimization of the Lactococcus lactis nisin-controlled gene expression system NICE for industrial applications. *Microb Cell Fact* **2005**, *4*, 16. doi: 10.1186/1475-2859-4-16.
15. Morello, E.; Bermúdez-Humarán, L.G.; Llull, D.; Solé, V.; Miraglio, N.; Langella, P.; Poquet, I. Lactococcus lactis, an efficient cell factory for recombinant protein production and secretion. *J Mol Microbiol Biotechnol* **2008**, *14*, 48–58. doi: 10.1159/000106082.
16. Song, A.A.; In, L.L.A.; Lim, S.H.E.; Rahim, R.A. A review on Lactococcus lactis: from food to factory. *Microb Cell Fact* **2017**, *16*, 55. doi: 10.1186/s12934-017-0669-x.
17. Kunji, E.R.S.; Chan, K.W.; Slotboom, D.J.; Floyd, S.; O'Connor, R.; Monné, M. Eukaryotic membrane protein overproduction in Lactococcus lactis. *Curr Opin Biotechnol* **2005**, *16*, 546-551. doi: 10.1016/j.copbio.2005.08.006.
18. Monné, M.; Chan, K.W.; Slotboom, D.J.; Kunji, E.R.S. Functional expression of eukaryotic membrane proteins in Lactococcus lactis. *Protein Sci* **2005**, *14*, 3048-3056. doi: 10.1110/ps.051689905.
19. Mierau, I.; Kleerebezem, M. 10 years of the nisin-controlled gene expression system (NICE) in Lactococcus lactis. *Appl Microbiol Biotechnol* **2005**, *68*, 705-717. doi: 10.1007/s00253-005-0107-6.
20. Surade, S.; Klein, M.; Stolt-Bergner, P.C.; Muenke, C.; Roy, A.; Michel, H. Comparative analysis and "expression space" coverage of the production of prokaryotic membrane proteins for structural genomics. *Protein Sci* **2006**, *15*, 2178-2189. doi: 10.1110/ps.062312706.
21. Pontes, D.S.; de Azevedo, M.S.; Chatel, J.M.; Langella, P.; Azevedo, V.; Miyoshi, A. Lactococcus lactis as a live vector: heterologous protein production and DNA delivery systems. *Protein Expr Purif* **2011**, *79*, 165-175. doi: 10.1016/j.pep.2011.06.005.
22. Lubelski, J.; Rink, R.; Khusainov, R.; Moll, G.N.; Kuipers, O.P. Biosynthesis, immunity, regulation, mode of action and engineering of the model lantibiotic nisin. *Cell Mol Life Sci* **2008**, *65*, 455-476. doi: 10.1007/s00018-007-7171-2.
23. Delves-Broughton, J.; Blackburn, P.; Evans, R.J.; Hugenholtz, J. Applications of the bacteriocin, nisin. *Antonie Van Leeuwenhoek* **1996**, *69*, 193-202. doi: 10.1007/BF00399424.
24. Gasson, M.J. Genetic transfer systems in lactic acid bacteria. *Antonie Van Leeuwenhoek* **1983**, *49*, 275-282. doi: 10.1007/BF00399503.
25. Kuipers, O.P.; de Ruyter, P.G.G.A.; Kleerebezem, M.; de Vos, W.M. Quorum sensing-controlled gene expression in lactic acid bacteria. *J Biotechnol* **1998**, *64*, 15–21. 10.1016/s0168-1656(98)00100-x.
26. Hasper, H.E.; de Kruijff, B.; Breukink, E. Assembly and stability of nisin-lipid II pores. Biochemistry 2004, 43, 11567-11575. doi: 10.1021/bi049476b.
27. de Ruyter, P.G.; Kuipers, O.P.; Beerthuyzen, M.M.; Alen-Boerrigter, I.; de Vos, W.M. Functional analysis of promoters in the nisin gene cluster of Lactococcus lactis. *J Bacteriol* **1996**, *178*, 3434-3439. doi: 10.1128/jb.178.12.3434-3439.1996.
28. de Ruyter, P.G.; Kuipers, O.P.; de Vos, W.M. Controlled gene expression systems for Lactococcus lactis with the food-grade inducer nisin. *Appl Environ Microbiol* **1996**, *62*, 3662-3667. doi: 10.1128/aem.62.10.3662-3667.1996.
29. Zhou, X.X.; Li, W.F.; Ma, G.X.; Pan, Y.J. The nisin-controlled gene expression system: construction, application and improvements. *Biotechnol Adv* **2006**, *24*, 285-295. doi: 10.1016/j.biotechadv.2005.11.001.
30. Mu, D.; Montalbán-López, M.; Masuda, Y.; Kuipers, O.P. Zirex: a Novel Zinc-Regulated Expression System for Lactococcus lactis. *Appl Environ Microbiol* **2013**, *79*, 4503-4508. doi: 10.1128/AEM.00866-13.

31. van Gijtenbeek, L.A.; Robinson, A.; van Oijen, A.M.; Poolman, B.; Kok, J. On the Spatial Organization of mRNA, Plasmids, and Ribosomes in a Bacterial Host Overexpressing Membrane Proteins. *PLoS Genet.* **2016**, *12*, e1006523. doi: 10.1371/journal.pgen.1006523.
32. Linares, D.M.; Geertsma, E.R.; Poolman, B. Evolved Lactococcus lactis strains for enhanced expression of recombinant membrane proteins. *J Mol Biol* **2010**, *401*, 45-55. doi: 10.1016/j.jmb.2010.06.002.
33. Poquet, I.; Saint, V.; Seznec, E.; Simoes, N.; Bolotin, A.; Gruss A. HtrA is the unique surface housekeeping protease in Lactococcus lactis and is required for natural protein processing. *Mol Microbiol.* **2000**, *35*, 1042-1051. doi: 10.1046/j.1365-2958.2000.01757.x.
34. Noreen, N.; Hooi, W.Y.; Baradaran, A.; Rosfarizan, M.; Sieo, C.C.; Rosli, M.I.; Yusoff, K.; Raha, A.R. Lactococcus lactis M4, a potential host for the expression of heterologous proteins. *Microb Cell Fact* **2011**, *10*, 28. doi: 10.1186/1475-2859-10-28.
35. Pinto, J.P.; Kuipers, O.P.; Marreddy, R.K.; Poolman, B.; Kok, J. Efficient overproduction of membrane proteins in Lactococcus lactis requires the cell envelope stress sensor/regulator couple CesSR. *PLoS One* **2011**, *6*, e21873. doi: 10.1371/journal.pone.0021873.
36. Kuipers, O.P.; Beerthuyzen, M.M.; Siezen, R.J.; de Vos, W.M. Characterization of the nisin gene cluster nisABTCIPR of Lactococcus lactis. Requirement of expression of the nisA and nisI genes for development of immunity. *Eur J Biochem* **1993**, *216*, 281-291. doi: 10.1111/j.1432-1033.1993.tb18143.x.
37. de Vos, W.D. Gene cloning and expression in lactic streptococci. *FEMS Microbiol Lett* **1987**, *46*, 281–295. https://doi.org/10.1016/0378-1097(87)90113-3.
38. Kok, J.; van der Vossen, J.M.; Venema, G. Construction of plasmid cloning vectors for lactic streptococci which also replicate in Bacillus subtilis and Escherichia coli. *Appl Environ Microbiol* **1984**, *48*, 726-731. doi: 10.1128/aem.48.4.726-731.1984.
39. de Vos, W.M.; Simons, G.F.M. Gene cloning and expression systems in Lactococci. In: *Genetics and Biotechnology of Lactic Acid Bacteria*; Gasson, M.J. and de Vos, W.M. Eds; Blackie Academic and Professional, London. 1984.
40. Geertsma, E.R.; Poolman, B. High-throughput cloning and expression in recalcitrant bacteria. *Nat Methods* **2007**, *4*, 705-707. doi: 10.1038/nmeth1073.
41. Groeneveld, M.; Weme, R.G.; Duurkens, R.H.; Slotboom, D.J. Biochemical characterization of the C4-dicarboxylate transporter DctA from Bacillus subtilis. *J Bacteriol* **2010**, *192*, 2900-2907. doi: 10.1128/JB.00136-10.
42. Erkens, G.B.; Slotboom, D.J. Biochemical characterization of ThiT from Lactococcus lactis: a thiamin transporter with picomolar substrate binding affinity. *Biochemistry* **2010**, *49*, 3203-3212. doi: 10.1021/bi100154r.
43. Steen, A.; Wiederhold, E.; Gandhi, T.; Breitling, R.; Slotboom, D.J. Physiological adaptation of the bacterium Lactococcus lactis in response to the production of human CFTR. *Mol Cell Proteomics* **2011**, *10*, M000052MCP200. doi: 10.1074/mcp.M000052-MCP200.
44. Hartley, J.L.; Temple, G.F.; Brasch, M.A. DNA cloning using in vitro site-specific recombination. *Genome Res Nov* **2000**, *10*, 1788-1795. doi: 10.1101/gr.143000.
45. Eshaghi, S.; Hedrén, M.; Nasser, M.I.; Hammarberg, T.; Thornell, A.; Nordlund, P. An efficient strategy for high-throughput expression screening of recombinant integral membrane proteins. *Protein Sci* **2005**, *14*, 676-683. doi: 10.1110/ps.041127005.
46. Yashiroda, Y.; Matsuyama, A.; Yoshida, M. New insights into chemical biology from ORFeome libraries. *Curr Opin Chem Biol* **2008**, *12*, 55-59. doi: 10.1016/j.cbpa.2008.01.024.
47. Frelet-Barrand, A.; Boutigny, S.; Moyet, L.; Deniaud, A.; Seigneurin-Berny, D.; Salvi, D.; Bernaudat, F.; Richaud, P.; Pebay-Peyroula, E.; Joyard, J.; Rolland, N. Lactococcus lactis, an alternative system for functional expression of peripheral and intrinsic Arabidopsis membrane proteins. *PLoS One* **2010**, *5*, e8746. doi: 10.1371/journal.pone.0008746.
48. Bakari, S.; Lembrouk, M.; André, F.; Orlowski, S.; Delaforge, M.; Frelet-Barrand, A. Expression in Lactococcus lactis of two human membrane proteins involved in liver detoxification, cytochrome P450 3A4 and microsomal glutathione S-transferase MGST1. *Mol Biotechnol.* **2016**, *58*, 299-310. doi: 10.1007/s12033-016-9928-z.
49. Douillard, F.P.; Mahony, J.; Campanacci, V.; Cambillau, C.; van Sinderen, D. Construction of two Lactococcus lactis expression vectors combining the Gateway and the NIsin Controlled Expression systems. *Plasmid* **2011**, *66*, 129-135. doi: 10.1016/j.plasmid.2011.07.001
50. Murphy, J.; Klumpp, J.; Mahony, J.; O'Connell-Motherway, M.; Nauta, A.; van Sinderen, D. Methyltransferases acquired by lactococcal 936-type phage provide protection against restriction endonuclease activity. *BMC Genomics.* **2014**, *15*, 831. doi: 10.1186/1471-2164-15-831.
51. Berlec, A.; Štrukelj, B. Generating a custom TA-cloning expression plasmid for Lactococcus lactis. *Biotechniques* **2012**, *52*, 51-53. doi: 10.2144/000113800.
52. Berlec, A.; Škrlec, K.; Kocjan, J.; Olenic, M.; Štrukelj, B. Single plasmid systems for inducible dual protein expression and for CRISPR-Cas9/CRISPRi gene regulation in lactic acid bacterium Lactococcus lactis. *Sci Rep.* **2018**, *8*, 1009. doi: 10.1038/s41598-018-19402-1.
53. Plavec, T.V.; Mitrović, A.; Perišić Nanut, M.; Štrukelj, B.; Kos, J.; Berlec, A. Targeting of fluorescent Lactococcus lactis to colorectal cancer cells through surface display of tumour-antigen binding proteins. *Microb Biotechnol.* **2021**, *14*, 2227-2240. doi: 10.1111/1751-7915.13907.
54. Noens, E.E.; Lolkema, J.S. Physiology and substrate specificity of two closely related amino acid transporters, SerP1 and SerP2, of Lactococcus lactis. *J Bacteriol.* **2015**, *197*, 951-958. doi: 10.1128/JB.02471-14.

55. Pols, T.; Singh, S.; Deelman-Driessen, C.; Gaastra, B.F.; Poolman, B. Enzymology of the pathway for ATP production by arginine breakdown. *FEBS J.* **2021**, *288*, 293-309. doi: 10.1111/febs.15337.
56. Berntsson, R.P.; ter Beek, J.; Majsnerowska, M.; Duurkens, R.H.; Puri, P.; Poolman, B.; Slotboom, D.J. Structural divergence of paralogous S components from ECF-type ABC transporters. *Proc Natl Acad Sci USA* **2012**, *109*, 13990-13995. doi: 10.1073/pnas.1203219109.
57. Marreddy, R.K.R.; Geertsma, E.R.; Poolman, B. Recombinant Membrane Protein Production: Past, Present and Future. In: *Supramolecular Structure and Function*; Brnjas-Kraljević, J. and Pifat-Mrzljak, G. Eds; Springer, Heidelberg. 2011.
58. Pudlik, A.M.; Lolkema, J.S. Rerouting citrate metabolism in Lactococcus lactis to citrate-driven transamination. *Appl Environ Microbiol* **2012**, *78*, 6665-6673. doi: 10.1128/AEM.01811-12.
59. Filipic, B.; Golic, N.; Jovcic, B.; Tolinacki, M.; Bay, D.C.; Turner, R.J.; Antic-Stankovic, J.; Kojic, M.; Topisirovic, L. The cmbT gene encodes a novel major facilitator multidrug resistance transporter in Lactococcus lactis. *Res Microbiol* **2013**, *164*, 46-54. doi: 10.1016/j.resmic.2012.09.003.
60. Fulyani, F.; Schuurman-Wolters, G.K.; Slotboom, D.J.; Poolman, B. Relative Rates of Amino Acid Import via the ABC Transporter GlnPQ Determine the Growth Performance of Lactococcus lactis. *J Bacteriol.* **2016**, *198*, 477-485. doi: 10.1128/JB.00685-15.
61. Venter, H.; Shilling, R.A.; Velamakanni, S.; Balakrishnan, L.; Van Veen, H.W. An ABC transporter with a secondary-active multidrug translocator domain. *Nature* **2003**, *426*, 866-870. doi: 10.1038/nature02173.
62. Lubelski, J.; de Jong, A.; van Merkerk, R.; Agustiandari, H.; Kuipers, O.P.; Kok, J.; Driessen, A.J. LmrCD is a major multidrug resistance transporter in Lactococcus lactis. *Mol Microbiol* **2006**, *61*, 771-781. doi: 10.1111/j.1365-2958.2006.05267.x.
63. Schaedler, T.A.; Tong, Z.; van Veen, H.W. The multidrug transporter LmrP protein mediates selective calcium efflux. *J Biol Chem.* **2012**, *287*, 27682-27690. doi: 10.1074/jbc.M112.372334.
64. Debruycker, V.; Hutchin, A.; Masureel, M.; Ficici, E.; Martens, C.; Legrand, P.; Stein, R.A.; Mchaourab, H.S.; Faraldo-Gómez, J.D.; Remaut, H.; Govaerts, C. An embedded lipid in the multidrug transporter LmrP suggests a mechanism for polyspecificity. *Nat Struct Mol Biol.* **2020**, *27*, 829-835. doi: 10.1038/s41594-020-0464-y.
65. Swain, B.M.; Guo, D.; Singh, H.; Rawlins, P.B.; McAlister, M.; van Veen, H.W. Complexities of a protonatable substrate in measurements of Hoechst 33342 transport by multidrug transporter LmrP. *Sci Rep.* **2020**, *10*, 20026. doi: 10.1038/s41598-020-76943-0.
66. Folgering, J.H.; Moe, P.C.; Schuurman-Wolters, G.K.; Blount, P.; Poolman, B. Lactococcus lactis uses MscL as its principal mechanosensitive channel. *J Biol Chem* **2005**, *280*, 8784-8792. doi: 10.1074/jbc.M411732200.
67. Tassis, K.; Vietrov, R.; de Koning, M.; de Boer, M.; Gouridis, G.; Cordes, T. Single-molecule studies of conformational states and dynamics in the ABC importer OpuA. *FEBS Lett.* **2021**, *595*, 717-734. doi: 10.1002/1873-3468.14026.
68. Duurkens, R.H.; Tol, M.B.; Geertsma, E.R.; Permentier, H.P.; Slotboom, D.J. Flavin binding to the high affinity riboflavin transporter RibU. *J Biol Chem* **2007**, *282*, 10380-10386. doi: 10.1074/jbc.M608583200.
69. Noens, E.E.; Kaczmarek, M.B.; Żygo, M.; Lolkema, J.S. ArcD1 and ArcD2 Arginine/Ornithine Exchangers Encoded in the Arginine Deiminase Pathway Gene Cluster of Lactococcus lactis. *J Bacteriol.* **2015**, *197*, 3545-3553. doi: 10.1128/JB.00526-15.
70. Margolles, A.; Flórez, A.B.; Moreno, J.A.; van Sinderen, D.; de los Reyes-Gavilán, C.G. Two membrane proteins from Bifidobacterium breve UCC2003 constitute an ABC-type multidrug transporter. *Microbiology* **2006**, *152*, 3497-3505. doi: 10.1099/mic.0.29097-0.
71. Yu, L.; Liu, X.; O'Sullivan, D.J. Use of Lactococcus lactis as a production system for peptides and enzymes encoded by a Lantibiotic gene cluster from Bifidobacterium longum. *Microbiology (Reading)* **2018**, *164*, 1481-1490. doi: 10.1099/mic.0.000721.
72. Xu, Q.; Zhai, Z.; An, H.; Yang, Y.; Yin, J.; Wang, G.; Ren, F.; Hao, Y. The MarR Family Regulator BmrR Is Involved in Bile Tolerance of Bifidobacterium longum BBMN68 via Controlling the Expression of an ABC Transporter. *Appl Environ Microbiol.* **2019**, *85*, e02453-18. doi: 10.1128/AEM.02453-18.
73. Liu, Y.; An, H.; Zhang, J.; Zhou, H.; Ren, F.; Hao, Y. Functional role of tlyC1 encoding a hemolysin-like protein from Bifidobacterium longum BBMN68 in bile tolerance. *FEMS Microbiol Lett.* **2014**, *360*, 167-173. doi: 10.1111/1574-6968.12601.
74. Rezaei, M.; Rabbani Khorasgani, M.; Zarkesh Esfahani, S.H.; Emamzadeh, R.; Abtahi, H. Production of Brucella melitensis Omp16 protein fused to the human interleukin 2 in Lactococcus lactis MG1363 toward developing a Lactococcus-based vaccine against brucellosis. *Can J Microbiol.* **2020**, *66*, 39-45. doi: 10.1139/cjm-2019-0261.
75. Woebking, B.; Reuter, G.; Shilling, R.A.; Velamakanni, S.; Shahi, S; Venter, H; Balakrishnan, L; van Veen, HW. Drug-lipid A interactions on the Escherichia coli ABC transporter MsbA. *J Bacteriol* **2005**, *187*, 6363-6369. doi: 10.1128/JB.187.18.6363-6369.2005.
76. Hürlimann, L.M.; Corradi, V.; Hohl, M.; Bloemberg, G.V.; Tieleman, D.P.; Seeger, M.A. The Heterodimeric ABC Transporter EfrCD Mediates Multidrug Efflux in Enterococcus faecalis. *Antimicrob Agents Chemother.* **2016**, *60*, 5400-5411. doi: 10.1128/AAC.00661-16.
77. Zhang, R.; Wang, C.; Cheng, W.; Duan, G.; Shi, Q.; Chen, S.; Fan, Q. Delivery of Helicobacter pylori HpaA to gastrointestinal mucosal immune sites using Lactococcus lactis and its immune efficacy in mice. *Biotechnol Lett.* **2018**, *40*, 585-590. doi: 10.1007/s10529-017-2502-3.

78. Sakamoto, K.; Margolles, A.; van Veen, H.W.; Konings, W.N. Hop resistance in the beer spoilage bacterium Lactobacillus brevis is mediated by the ATP-binding cassette multidrug transporter HorA. *J Bacteriol* **2001**, *183*, 5371-5375. doi: 10.1128/JB.183.18.5371-5375.2001.
79. Majsnerowska, M.; Hänelt, I.; Wunnicke, D.; Schäfer, L.V.; Steinhoff, H.J.; Slotboom, D.J. Substrate-induced conformational changes in the S-component ThiT from an energy coupling factor transporter. *Structure* **2013**, *21*, 861-867. doi: 10.1016/j.str.2013.03.007.
80. Zhang, B.; Zuo, F.; Yu, R.; Zeng, Z.; Ma, H.; Chen, S. Comparative genome-based identification of a cell wall-anchored protein from Lactobacillus plantarum increases adhesion of Lactococcus lactis to human epithelial cells. *Sci Rep.* **2015**, *5*, 14109. doi: 10.1038/srep14109.
81. Martín, C.; Escobedo, S.; Pérez-Martínez, G.; Coll-Marqués, J.M.; Martín, R.; Suárez, J.E.; Quirós, L.M. Two alkaline motifs in the Lactobacillus salivarius Lv72 OppA surface are important to its adhesin function. *Benef Microbes.* **2019**, *10*, 101-109. doi: 10.3920/BM2018.0052.
82. Hohl, M.; Remm, S.; Eskandarian, H.A.; Dal Molin, M.; Arnold, F.M.; Hürlimann, L.M.; Krügel, A.; Fantner, G.E.; Sander, P.; Seeger, M.A. Increased drug permeability of a stiffened mycobacterial outer membrane in cells lacking MFS transporter Rv1410 and lipoprotein LprG. *Mol Microbiol.* **2019**, *111*, 1263-1282. doi: 10.1111/mmi.14220.
83. Rego, S.; Heal, T.J.; Pidwill, G.R.; Till, M.; Robson, A.; Lamont, R.J.; Sessions, R.B.; Jenkinson, H.F.; Race, P.R.; Nobbs, A.H. Structural and Functional Analysis of Cell Wall-anchored Polypeptide Adhesin BspA in Streptococcus agalactiae. *J Biol Chem.* **2016**, *291*, 15985-16000. doi: 10.1074/jbc.M116.726562.
84. Velamakanni, S.; Yao, Y.; Gutmann, D.A.; van Veen, H.W. Multidrug transport by the ABC transporter Sav1866 from Staphylococcus aureus. *Biochemistry* **2008**, *47*, 9300-9308. doi: 10.1021/bi8006737.
85. Freires, I.A.; Avilés-Reyes, A.; Kitten, T.; Simpson-Haidaris, P.J.; Swartz, M.; Knight, P.A.; Rosalen, P.L.; Lemos, J.A.; Abranches, J. Heterologous expression of Streptococcus mutans Cnm in Lactococcus lactis promotes intracellular invasion, adhesion to human cardiac tissues and virulence. *Virulence.* **2017**, *8*, 18-29. doi: 10.1080/21505594.2016.1195538.
86. Asmat, T.M.; Klingbeil, K.; Jensch, I.; Burchhardt, G.; Hammerschmidt, S. Heterologous expression of pneumococcal virulence factor PspC on the surface of Lactococcus lactis confers adhesive properties. *Microbiology (Reading).* **2012**, *158*, 771-780. doi: 10.1099/mic.0.053603-0.
87. Mu, R.; Kim, B.J.; Paco, C.; Del Rosario, Y.; Courtney, H.S.; Doran, K.S. Identification of a group B streptococcal fibronectin binding protein, SfbA, that contributes to invasion of brain endothelium and development of meningitis. *Infect Immun.* **2014**, *82*, 2276-2286. doi: 10.1128/IAI.01559-13.
88. Hohl, M.; Briand, C.; Grütter, M.G.; Seeger, M.A. Crystal structure of a heterodimeric ABC transporter in its inward-facing conformation. *Nat Struct Mol Biol* **2012**, *19*, 395-402. doi: 10.1038/nsmb.2267.
89. Schaedler, T.A.; Thornton, J.D.; Kruse, I.; Schwarzländer, M.; Meyer, A.J.; van Veen, H.W.; Balk, J. A conserved mitochondrial ATP-binding cassette transporter exports glutathione polysulfide for cytosolic metal cofactor assembly. *J Biol Chem.* **2014**, *289*, 23264-23274. doi: 10.1074/jbc.M114.553438.
90. Colinet, A.S.; Sengottaiyan, P.; Deschamps, A.; Colsoul, M.L.; Thines, L.; Demaegd, D.; Duchêne, M.C.; Foulquier, F.; Hols, P.; Morsomme, P. Yeast Gdt1 is a Golgi-localized calcium transporter required for stress-induced calcium signaling and protein glycosylation. *Sci Rep.* **2016**, *6*, 24282. doi: 10.1038/srep24282.
91. Hofacker, M.; Gompf, S.; Zutz, A.; Presenti, C.; Haase, W.; van der Does, C.; Model, K.; Tampé, R. Structural and functional fingerprint of the mitochondrial ATP-binding cassette transporter Mdl1 from Saccharomyces cerevisiae. *J Biol Chem* **2007**, *282*, 3951-3961. doi: 10.1074/jbc.M609899200.
92. Vest, K.E.; Leary, S.C.; Winge, D.R.; Cobine, P.A. Copper Import into the Mitochondrial Matrix in Saccharomyces cerevisiae is Mediated by Pic2, a Mitochondrial Carrier Family Protein. *J Biol Chem* **2013**, *288*, 23884-23892. doi: 10.1074/jbc.M113.470674.
93. Furumoto, T. Pyruvate transport systems in organelles: future directions in C4 biology research. *Curr Opin Plant Biol* **2016**, *31*, 143-148. doi: 10.1016/j.pbi.2016.04.007.
94. Herzig, S.; Raemy, E.; Montessuit, S.; Veuthey, J.L.; Zamboni, N.; Westermann, B.; Kunji, E.R.; Martinou, J.C. Identification and functional expression of the mitochondrial pyruvate carrier. *Science* **2012**, *337*, 93-96. doi: 10.1126/science.1218530.
95. Taochy, C.; Gaillard, I.; Ipotesi, E.; Oomen, R.; Leonhardt, N.; Zimmermann, S.; Peltier, J.B.; Szponarski, W.; Simonneau, T.; Sentenac, H.; Gibrat, R.; Boyer, J.C. The Arabidopsis root stele transporter NPF2.3 contributes to nitrate translocation to shoots under salt stress. *Plant J.* **2015**, *83*, 466-479. doi: 10.1111/tpj.12901.
96. Marreddy, R.K.; Pinto, J.P.; Wolters, J.C.; Geertsma, E.R.; Fusetti, F.; Permentier, H.P.; Kuipers, O.P.; Kok, J.; Poolman, B. The response of Lactococcus lactis to membrane protein production. *PLoS One* **2011**, *6*, e24060. doi: 10.1371/journal.pone.0024060.
97. Monné, M.; Robinson, A.J.; Boes, C.; Harbour, M.E.; Fearnley, I.M.; Kunji, E.R. The mimivirus genome encodes a mitochondrial carrier that transports dATP and dTTP. *J Virol* **2007**, *81*, 3181-3186. doi: 10.1128/JVI.02386-06.
98. Janvilisri, T.; Venter, H.; Shahi, S.; Reuter, G.; Balakrishnan, L.; van Veen, H.W. Sterol transport by the human breast cancer resistance protein (ABCG2) expressed in Lactococcus lactis. *J Biol Chem* **2003**, *278*, 20645-20651. doi: 10.1074/jbc.M301358200.
99. Stribny, J.; Thines, L.; Deschamps, A.; Goffin, P.; Morsomme, P. The human Golgi protein TMEM165 transports calcium and manganese in yeast and bacterial cells. *J Biol Chem.* **2020**, *295*, 3865-3874. doi: 10.1074/jbc.RA119.012249.

100. Mifsud, J.; Ravaud, S.; Krammer, E.M.; Chipot, C.; Kunji, E.R.; Pebay-Peyroula, E.; Dehez, F. The substrate specificity of the human ADP/ATP carrier AAC1. *Mol Membr Biol* **2013**, *30*, 160-168. doi: 10.3109/09687688.2012.745175.
101. King, M.S.; Thompson, K.; Hopton, S.; He, L.; Kunji, E.R.S.; Taylor, R.W.; Ortiz-Gonzalez, X.R. Expanding the phenotype of de novo SLC25A4-linked mitochondrial disease to include mild myopathy. *Neurol Genet.* **2018**, *4*, e256. doi: 10.1212/NXG.0000000000000256.
102. Zhang, Y.; Tian, D.; Matsuyama, H.; Hamazaki, T.; Shiratsuchi, T.; Terada, N.; Hook, D.J.; Walters, M.A.; Georg, G.I.; Hawkinson, J.E. Human Adenine Nucleotide Translocase (ANT) Modulators Identified by High-Throughput Screening of Transgenic Yeast. *J Biomol Screen.* **2016**, *21*, 381-390. doi: 10.1177/1087057115624637.
103. Boulet, A.; Vest, K.E.; Maynard, M.K.; Gammon, M.G.; Russell, A.C.; Mathews, A.T.; Cole, S.E.; Zhu, X.; Phillips, C.B.; Kwong, J.Q.; Dodani, S.C.; Leary, S.C.; Cobine, P.A. The mammalian phosphate carrier SLC25A3 is a mitochondrial copper transporter required for cytochrome c oxidase biogenesis. *J Biol Chem.* **2018**, *293*, 1887-1896. doi: 10.1074/jbc.RA117.000265.
104. Schleifer, K.H.; Kraus, J.; Dvorak, C.; Kilpper-Bälz, R.; Collins, M.D.; Fischer, W. Transfer of Streptococcus lactis and related streptococci to the genus Lactococcus gen. nov. *Syst Appl Microbiol* **1985**, *6*,183-195. doi.org/10.1016/S0723-2020(85)80052-7.
105. Marreddy, R.K.; Geertsma, E.R.; Permentier, H.P.; Pinto, J.P.; Kok, J.; Poolman, B. Amino acid accumulation limits the overexpression of proteins in Lactococcus lactis. *PLoS One* **2010**, *5*, e10317. doi: 10.1371/journal.pone.0010317.
106. Shilling, R.; Federici, L.; Walas, F.; Venter, H.; Velamakanni, S.; Woebking, B.; Balakrishnan, L.; Luisi, B.; van Veen, H.W. A critical role of a carboxylate in proton conduction by the ATP-binding cassette multidrug transporter LmrA. *FASEB J* **2005**, *19*, 1698-1700. doi: 10.1096/fj.04-3558fje.
107. Agboh, K.; Lau, C.H.F.; Khoo, Y.S.K.; Singh, H.; Raturi, S.; Nair, A.V.; Howard, J.; Chiapello, M.; Feret, R.; Deery, M.J.; Murakami, S.; van Veen, H.W. Powering the ABC multidrug exporter LmrA: How nucleotides embrace the ion-motive force. *Sci Adv.* **2018**, *4*, eaas9365. doi: 10.1126/sciadv.aas9365.
108. Hellmich, U.A.; Glaubitz, C. NMR and EPR studies of membrane transporters. *Biol Chem.* **2009**, *390*, 815-834. doi: 10.1515/BC.2009.084.
109. Hellmich, U.A.; Lyubenova, S.; Kaltenborn, E.; Doshi, R.; van Veen, H.W.; Prisner, T.F.; Glaubitz, C. Probing the ATP hydrolysis cycle of the ABC multidrug transporter LmrA by pulsed EPR spectroscopy. *J Am Chem Soc* **2012**, *134*, 5857-5862. doi: 10.1021/ja211007t.
110. Hellmich, U.A.; Mönkemeyer, L.; Velamakanni, S.; van Veen, H.W.; Glaubitz, C. Effects of nucleotide binding to LmrA: A combined MAS-NMR and solution NMR study. *Biochim Biophys Acta.* **2015**, *1848*, 3158-3165. doi: 10.1016/j.bbamem.2015.10.003.
111. Erkens, G.B.; Berntsson, R.P.; Fulyani, F.; Majsnerowska, M.; Vujičić-Žagar, A.; Ter Beek, J.; Poolman, B.; Slotboom, D.J. The structural basis of modularity in ECF-type ABC transporters. *Nat Struct Mol Biol* **2011**, *18*, 755-760. doi: 10.1038/nsmb.2073.
112. Swier, L.J.; Guskov, A.; Slotboom, D.J. Structural insight in the toppling mechanism of an energy-coupling factor transporter. *Nat Commun.* **2016**, *7*, 11072. doi: 10.1038/ncomms11072.
113. Woebking, B.; Velamakanni, S.; Federici, L.; Seeger, M.A.; Murakami, S.; van Veen, H.W. Functional role of transmembrane helix 6 in drug binding and transport by the ABC transporter MsbA. *Biochemistry* **2008**, *47*, 10904-10914. doi: 10.1021/bi800778d.
114. Doshi, R.; Woebking, B.; van Veen, H.W. Dissection of the conformational cycle of the multidrug/lipidA ABC exporter MsbA. *Proteins* **2010**, *78*, 2867-2872. doi: 10.1002/prot.22813.
115. Doshi, R.; van Veen, H.W. Substrate Binding Stabilizes a Pre-translocation Intermediate in the ATP-binding Cassette Transport Protein MsbA. *J Biol Chem* **2013**, *288*, 21638-21647. doi: 10.1074/jbc.M113.485714.
116. Trip, H.; Mulder, N.L.; Lolkema, J.S. Cloning, expression, and functional characterization of secondary amino acid transporters of Lactococcus lactis. *J Bacteriol* **2013**, *195*, 340-350. doi: 10.1128/JB.01948-12.
117. Ter Horst, R.; Lolkema, J.S. Rapid screening of membrane topology of secondary transport proteins. *Biochim Biophys Acta* **2010**, *1798*, 672-680. doi: 10.1016/j.bbamem.2009.11.010.
118. Halestrap, A.P. Stimulation of pyruvate transport in metabolizing mitochondria through changes in the transmembrane pH gradient induced by glucagon treatment of rats. *Biochem J.* **1978**, *172*, 389-398. doi: 10.1042/bj1720389.
119. Miras, S.; Salvi, D.; Ferro, M.; Grunwald, D.; Garin, J.; Joyard, J.; Rolland, N. Non-canonical transit peptide for import into the chloroplast. *J Biol Chem* **2002**, *277*, 47770-47778. doi: 10.1074/jbc.M207477200.
120. Kühlbrandt, W. Biology, structure and mechanism of P-type ATPases. *Nat Rev Mol Cell Biol* **2004**, *5*, 282-295. doi: 10.1038/nrm1354.
121. Catty, P.; Boutigny, S.; Miras, R.; Joyard, J.; Rolland, N.; Seigneurin-Berny, D. Biochemical characterization of AtHMA6/PAA1, a chloroplast envelope Cu(I)-ATPase. *J Biol Chem* **2011**, *286*, 36188-36197. doi: 10.1074/jbc.M111.241034.
122. Neuhaus, H.E.; Thom, E.; Möhlmann, T.; Steup, M.; Kampfenkel, K. Characterization of a novel eukaryotic ATP/ADP translocator located in the plastid envelope of Arabidopsis thaliana L. *Plant J* **1997**, *11*, 73-82. doi: 10.1046/j.1365-313x.1997.11010073.x.
123. Tjaden, J.; Schwöppe, C.; Möhlmann, T.; Quick, P.W.; Neuhaus, H.E. Expression of a plastidic ATP/ADP transporter gene in Escherichia coli leads to a functional adenine nucleotide transport system in the bacterial cytoplasmic membrane. *J Biol Chem* **1998**, *273*, 9630-9636. doi: 10.1074/jbc.273.16.9630.
124. Oliveira, A.P.; Nielsen, J.; Förster, J. Modelling Lactococcus lactis using a genome-scale flux model. *BMC Microbiol* **2005**, *5*, 39. doi: 10.1186/1471-2180-5-39.

125. Block, M.A.; Douce, R.; Joyard, J.; Rolland, N. Chloroplast envelope membranes: a dynamic interface between plastids and the cytosol. *Photosynthesis Res* **2007**, *92*, 225-244. doi: 10.1007/s11120-007-9195-8.
126. Ingram, L.O. Changes in lipid composition of Escherichia coli resulting from growth with organic solvents and with food additives. *Appl Environ Microbiol* **1977**, *33*, 1233-1236. doi: 10.1128/aem.33.5.1233-1236.1977.
127. Opekarova, M.; Tanner, W. Specific lipid requirements of membrane proteins – a putative bottleneck in heterologous expression. *Biochim Biophys Acta* **2003**, *1610*, 11-22. doi: 10.1016/s0005-2736(02)00708-3.
128. Wolters, J.C.; Berntsson, R.P.; Gul, N.; Karasawa, A.; Thunnissen, A.M.; Slotboom, D.J.; Poolman, B. Ligand binding and crystal structures of the substrate-binding domain of the ABC transporter OpuA. *PLoS One.* **2010**, *5*, e10361. doi: 10.1371/journal.pone.0010361.
129. Swier, L.J.; Monjas, L.; Guskov, A.; de Voogd, A.R.; Erkens, G.B.; Slotboom, D.J.; Hirsch, A.K. Structure-based design of potent small-molecule binders to the S-component of the ECF transporter for thiamine. *Chembiochem.* **2015**, *16*, 819-826. doi: 10.1002/cbic.201402673.
130. Sikkema, H.R.; van den Noort, M.; Rheinberger, J.; de Boer, M.; Krepel, S.T.; Schuurman-Wolters, G.K.; Paulino, C.; Poolman, B. Gating by ionic strength and safety check by cyclic-di-AMP in the ABC transporter OpuA. *Sci Adv.* **2020**, *6*, eabd7697. doi: 10.1126/sciadv.abd7697.
131. Jäger, F.; Lamy, A.; Guerini, N.; Sun, W.S.; Berntsson, R.P.A. Structure of the enterococcal T4SS protein PrgL reveals unique dimerization interface in the VirB8 protein family. *bioRxiv* **2020**, 10.30.342212. doi.org/10.1101/2020.10.30.342212
132. Focht, D.; Neumann, C.; Lyons, J.; Eguskiza Bilbao, A.; Blunck, R.; Malinauskaite, L.; Schwarz, I.O.; Javitch, J.A.; Quick, M.; Nissen, P. A non-helical region in transmembrane helix 6 of hydrophobic amino acid transporter MhsT mediates substrate recognition. *EMBO J.* **2021**, *40*, e105164. doi: 10.15252/embj.2020105164.
133. Ploetz, E.; Schuurman-Wolters, G.K.; Zijlstra, N.; Jager, A.W.; Griffith, D.A.; Guskov, A.; Gouridis, G.; Poolman, B.; Cordes, T. Structural and biophysical characterization of the tandem substrate-binding domains of the ABC importer GlnPQ. *Open Biol.* **2021**, *11*, 200406. doi: 10.1098/rsob.200406.
134. Harborne, S.P.; Ruprecht, J.J.; Kunji, E.R. Calcium-induced conformational changes in the regulatory domain of the human mitochondrial ATP-Mg/Pi carrier. *Biochim Biophys Acta.* **2015**, *1847*, 1245-1253. doi: 10.1016/j.bbabio.2015.07.002.
135. Berntsson, R.P.; Alia Oktaviani, N.; Fusetti, F.; Thunnissen, A.M.; Poolman, B.; Slotboom, D.J. Selenomethionine incorporation in proteins expressed in Lactococcus lactis. *Protein Sci* **2009**, *18*, 1121-1127. doi: 10.1002/pro.97.
136. Martens, C. Membrane Protein Production in Lactococcus lactis for Structural Studies. *Methods Mol Biol.* **2020**, *2127*, 29-45. doi: 10.1007/978-1-0716-0373-4_3.