Improved membrane protein expression in
*Lactococcus lactis* by fusion to Mistic

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Difficulty overexpressing (eukaryotic) membrane proteins is generally considered as the major impediment in their structural and functional research. *Lactococcus lactis* possesses many properties ideal for membrane protein expression. In order to investigate membrane protein expression in *L. lactis*, we created a novel expression system by introducing Mistic, a short peptide previously identified in *Bacillus subtilis*, into *L. lactis*. The potential of this system was demonstrated in the overexpression of a eukaryotic membrane protein (pkjDes4) and a prokaryotic membrane protein (pkjLi), a newly isolated linoleate isomerase from *Lactobacillus acidophilus*. The expression levels reached up to 4.4 % and 45.2 % for pkjDes4 and pkjLi, respectively, which represented an exceptionally robust ability to overproduce membrane proteins. Moreover, the expressed pkjLi was functional, with its catalysing nature characterized for the first time in this species. Up to 0.852 mg ml⁻¹ conjugated linoleic acid was obtained during the linoleic acid conversion catalysed by the recombinant lactococcal strains. In summary, we established a membrane protein expression system in *L. lactis* and examined its functionality. Our results demonstrate that the Mistic chaperoning strategy can be efficiently applied to *L. lactis* hosts and show its extraordinary capacity to facilitate the high-yield production of intractable membrane proteins.

**INTRODUCTION**

Membrane proteins account for 20–25 % of all sequenced open reading frames and play key roles in many essential cellular functions, including homeostasis, ion conductance, nutrient uptake and intercellular signalling (Drew et al., 2003; Midgett & Madden, 2007). For undertaking functional and structural studies, their overexpression is a necessary prerequisite, as the natural levels of most membrane proteins are low (Monné et al., 2005). However, while there are 50 times more known structures of soluble proteins than membrane proteins, the heterologous overproduction of membrane proteins, particularly eukaryotic membrane proteins, remains challenging (Kunji et al., 2005). The problem arises from the fundamental differences in membrane protein biogenesis, as well as growth impairment caused by their overexpression. Even when high-yield expression was achieved, the expressed membrane proteins were often found in inclusion bodies in a misfolded state (Monné et al., 2005).

**Abbreviations:** CLA, conjugated linoleic acid; DHA, docosahexaenoic acid; GFP, green fluorescent protein; LA, linoleic acid; Li, linoleate isomerase; PUFAs, polyunsaturated fatty acid.

The GenBank/EMBL/DDBJ accession number for the gene sequence of *Lb. acidophilus* H42 Li (pkjLi) is J0065335.

One supplementary figure and one supplementary table are available with the online version of this paper.

In contrast to expensive and technically challenging eukaryotic expression systems, bacterial expression systems are convenient and relatively cheap (Kunji et al., 2005). In this respect, *Lactococcus lactis* has been successfully used as an expression host because of the ideal properties it possesses: single-membrane structure, a strong and tightly regulated promoter system, mild proteolytic activity and non-inclusion body targeting (Kunji et al., 2003). Recently, a dozen membrane proteins, including transporting proteins, receptors and a quinone oxidoreductase, have been successfully overexpressed in lactococcal hosts (Kunji et al., 2003; Wieczorek & Martin, 2010; Steen et al., 2011; Murreddy et al., 2011; Bernaudat et al., 2011).

Mistic is a uniquely hydrophilic membrane protein first identified from *Bacillus subtilis* and found to be exceptionally efficient in chaperoning the expression of other integral membrane proteins at high yields in *Escherichia coli* (Deniaud et al., 2011; Roosild et al., 2005; Petrovskaya et al., 2010). It has been proposed that Mistic is capable of autonomously integrating, in a Sec-independent manner, into the lipid bilayer, providing a novel strategy to be explored in *L. lactis*. Besides, among the several Mistic homologues characterized, M110 (a Mistic homologue first discovered in *Bacillus subtilis*) associated with the inner membrane of *E. coli* most tightly, and thus was predicted to have the best potential to be used as the expression partner (Roosild et al., 2006).
Polyunsaturated fatty acids (PUFAs), particularly docosahexaenoic acid (DHA) and conjugated linoleic acid (CLA), are a group of bioactive compounds beneficial for human health (Ogawa et al., 2005; Tonon et al., 2003). Currently, CLA is produced mainly through chemical isomerization of linoleic acid (LA), which results in the by-production of unexpected isomers, each of which can have distinct health effects. cis-9, trans-11 and trans-10, cis-12 isomers have been paid particular attention because of their remarkable biological activities (Ogawa et al., 2005). Thus, the production of CLA through biological synthesis has significant value.

Large quantities of enzymes that catalyse PUFA biosynthesis are located in cellular membranes, including cytoplasmic and endoplasmic reticulum membranes. Fatty acid Δ4 desaturase is a family of eukaryotic transmembrane proteins responsible for the DHA synthesis in several species of marine microalgae (Tonon et al., 2003; Zhou et al., 2007), with a homologue (pkJDes4) recently identified in Pavlova viridis (Xu et al., 2011). For CLA production, a specific linoleate isomerase (LI) was first isolated and sequenced in Lactobacillus reuteri in 2004 by Rosson et al. (2004). However, due to their intrinsic membrane association, heterologous overproduction of lactobacillus LIs has never, to our knowledge, been accomplished. In this study, both heterologous overproduction of lactobacillus LIs has never, to our knowledge, been accomplished. In this study, both

General DNA techniques and transformation. Molecular cloning techniques were performed as described by Sambrook et al. (1989). Taq DNA polymerase, the relevant restriction enzymes and T4 DNA ligase were purchased from TakaraBio. Lb. acidophilus genomic DNA was isolated according to the manufacturer’s instructions. The competent cells of L. lactis NZ9000 were prepared and transformed through the method described by Wells et al. (1993). PCR amplifications were performed in a 20 μl reaction volume with 100 ng DNA, 4 nM deoxynucleotide mixture, 8 pmol each primer, 2 μl 10× Easytaq buffer and 1 U Easytaq DNA polymerase (TakaraBio). An Eppendorf PCR thermocycler was used to subject samples to 30 cycles of denaturation (94 °C for 35 s), annealing for 35 s, and elongation (72 °C for 1 min for every 1 kb of target DNA). The PCR-amplified LI fragment from Lb. acidophilus H42 was ligated into pMD18T vector (TakaraBio) and sequenced.

Methods

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Lactococcal strains were propagated in M17 (Oxoid) broth or agar [1.5% (w/v)] containing glucose (0.5% [w/v]) at 30 °C without aeration. Lb. acidophilus H42 was grown in MRS medium (Oxoid) at 37 °C without aeration. E. coli was grown in Luria–Bertani medium with vigorous agitation at 37 °C. When needed, the antibiotic chloramphenicol (Sangon) was added to the lactococcal culture at a concentration of 5 μg ml⁻¹ and kanamycin (Sangon) was added to the E. coli culture at a concentration of 30 μg ml⁻¹.

Table 1. Bacterial strains and plasmids used in this study

<table>
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<tr>
<th>Strain or plasmid</th>
<th>Relative properties</th>
<th>Source or reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>L. lactis NZ9000</td>
<td>MG1363 derivative, nisRK*</td>
<td>Kuipers et al. (1998)</td>
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<tr>
<td>Lb. acidophilus H42</td>
<td>Wild-type strain</td>
<td>Chicken intestine, this study</td>
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<td><strong>Plasmids</strong></td>
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<td>pNZ8148</td>
<td>Cm1, nisin-inducible expression vector</td>
<td>Mierau &amp; Kleerebezem (2005)</td>
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<td>pWaldo-GFPd</td>
<td>Km1, a pET28b derivative fused with GFP moiety</td>
<td>Drew et al. (2006)</td>
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<td>pMGFP</td>
<td>Km1, a pWaldo-GFPd derivative encoding a M110-GFP fusion</td>
<td>Xu et al. (2011)</td>
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<td>pMDes4</td>
<td>Cm1, a pWaldo-GFPd derivative encoding a M110-pkjDes4 fusion</td>
<td>Xu et al. (2011)</td>
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<td>pNDes4</td>
<td>Cm1, a pNZ8148 derivative encoding a pkjDes4 protein</td>
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<td>pNM110</td>
<td>Cm1, a pNZ8148 derivative inserted with M110 and multiple cloning sites</td>
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<td>pNMGFP</td>
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<td>pNMSdes4</td>
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<td>pNMLi</td>
<td>Cm1, a pNZ8148 derivative encoding a M110-pkjLi fusion</td>
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*nisRK, Nisin signal transduction genes.
†Cm, Chloramphenicol.
‡Km, Kanamycin.
the italics are the introduced multiple cloning sites), and the resultant product was cloned into the Ncol–HindIII sites of pNMGFP, resulting in the plasmid pNM110. The LI fragment from Lb. acidophilus H42 was amplified using primers MLAF3 (5’-GCTCTAGTATGTTATTTACATTTGGAAG-3’) and MLAR (5’-CGCGATCTAGTCTGCTAATTTGATTCAAG-3’), and the resultant product was cloned into the Xhol–Sph sites (indicated with italic characters in the sequences) of pNM110, resulting in the plasmid pNMLi. All of the constructed plasmids were transformed into L. lactis NZ9000, generating a series of recombinant strains.

**Overexpression of the membrane proteins pkjDes4 and pkjLi in L. lactis.** All the recombinant NZ9000 strains (except for NZ9000/pMGFP, including NZ9000/pNMDes4, NZ9000/pNDes4, NZ9000/pNMGFP, NZ9000/pNM110 and NZ9000/pNMLi, were induced when OD600 of the cultures reached 0.6 by the addition of 5 ng ml−1 nisin (Sigma) and incubated at 18 °C. After 24 h of induction, the cells from 60 ml cultures were harvested by centrifugation at 6000 g for 5 min and washed once with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4).

For the extraction of membrane proteins, the bacteria harvested were resuspended in 6 ml PBS buffer and subjected to sonication on ice. Cellular debris and unbroken cells were precipitated by centrifugation at 10,000 g for 30 min. The presumptive insoluble material (or enriched membrane proteins) was isolated by collecting the pellets obtained from another round (with the same procedure) of resuspension, sonication and centrifugation. The membranes were isolated from the supernatant by centrifugation at 100,000 g for 30 min, and the pellets were resuspended in PBS buffer containing 0.5% SDS and incubated for 1 h at 25 °C with gentle agitation. After another ultracentrifugation (100,000 g for 30 min) to remove unsolubilized membranes, the extracted membrane proteins (in 600 μl PBS), as well as the presumptive insoluble material (or enriched membrane proteins in 600 μl PBS) was mixed with 5 × SDS loading buffer, and 20 μl of each sample (approx. 30 μg and 20 μg for membrane proteins and presumptive insoluble material or enriched membrane proteins, respectively) were subsequently subjected to standard SDS-PAGE. The expression yields were measured as the percentage of the amount of the objective protein relative to the total proteins on the gel (Glyco Band-Scan, PROZYME).

**GFP detection.** After induction for 3 h at 30 °C, the NZ9000/pNMGFP cells were collected, washed and resuspended in the same amount of PBS buffer. The imaging experiment was carried out using a Nikon Fluorescence Microscope with an excitation wavelength of 485 nm.

For the detection of in-gel fluorescence, both the extracted membrane proteins and the soluble proteins (obtained according to ‘Overexpression of the membrane protein pkjDes4 and pkjLi in L. lactis’ above) were mixed with 2× SB buffer [200 mM Tris/HCl (pH 8.8), 20% glycerol, 5 mM EDTA (pH 8.0), 0.02% bromophenol blue, 4% SDS and 0.05 M DTT] and incubated for 10 min at 37 °C before SDS-PAGE. After the electrophoresis, the gel was rinsed with distilled water and exposed to a STORM840 scanner for the detection of fluorescent bands.

**Bioconversion assays.** A stock emulsion (50 mg ml−1) of linoleic acid (LA) (Sigma) was prepared by stirring in a solution of 2% (v/v) Tween 80. After 4 h of induction, the free LA was added to the cultures of NZ9000/pNMLi and NZ9000/pNM110 at a concentration of 3 mg ml−1, respectively. The cultures supplemented with LA were continuously cultivated under nitrogen gas at 25 °C for 24 h. After cultivation, the cultures were centrifuged at 6000 g for 5 min and the pellets were subsequently lyophilized for CLA analysis.

**Extraction and methylation.** The lyophilized biomass was resuspended in 1 ml 2 M HCl–CH3OH. After filling the tube with nitrogen gas, the esterification reaction proceeded at 95 °C for 1 h. Then, 1 ml hexane was added to the solution and mixed vigorously before centrifuging at 4000 g for 5 min. The organic phase was aspirated into a new tube, dried by nitrogen gas and dissolved in 600 μl hexane for GC analysis.

**GC analysis.** FAMEs (fatty acid methyl esters) were analysed in a GC-2010 Shimazu GC System, mainly according to Macouzet et al. (2010): injection (1 μl) was carried out automatically with an inlet temperature of 240 °C and a split ratio of 10:1. Nitrogen gas was used as a carrier through an Aoc-20i capillary column (30 m × 320 μm). The initial temperature of the GC oven was 140 °C, with four ramps to attain 176 °C, 200 °C, 205 °C and 250 °C at rates of 9 °C min−1, 4.5 °C min−1, 0.25 °C min−1 and 10 °C min−1, respectively. Detection was done by flame ionization at 280 °C.

**RESULTS**

**Construction of the M110-fused vector pNM110**

The vector pNZ8148, carrying the nisin-inducible controlled promoter PnisA, was used as the backbone of the constructed vector. In addition to the M110-encoding sequence, the vector was constructed to harbour a TEV protease recognition site, as well as a multiple cloning site, including XbaI, SacI, EcoRI, SphI and HindIII sites downstream of M110 and TEV (Fig. 1). The resultant plasmid, designated pNM110, was used to overexpress eukaryotic and prokaryotic membrane proteins in L. lactis.

**Mistic behaviour in L. lactis by fluorescence detection of M110-GFP fusion**

As GFP is a common biological reporter for quantitative analysis of gene expression in bacteria (Fernández de Palencia et al., 2000; Niu et al., 2008), it was chosen to monitor M110 expression and localization in L. lactis. According to Fig. 2, most of the recombinant lactococcal

![Fig. 1. Schematic representation of the expression vector pNM110. ‘Cm’ represents the chloramphenicol acetyltransferase gene; ‘M110 peptide’ refers to the M110 peptide-encoding sequence.](image-url)
cells fluoresced following nisin induction, indicating the presence of M110-GFP fusion in these cells. Given that GFP fluorescence can only be detected when GFP is properly folded, this result demonstrated that Mistic (M110) could be expressed and aid in fusion expression in *L. lactis*.

In Fig. 3, a fluorescent band approximately 40 kDa (the upper band), corresponding to M110-GFP fusion, was detected in the membrane fraction of NZ9000/pNMGFP. The other fluorescent bands observed in the gel should represent the degradation products of M110-GFP, for instance M1-GFP (M1 is a Mistic homologue lacking the first 26 amino acid residues from M110) (Roosild *et al.*, 2006). So, Mistic (M110) was successfully inserted into cellular membrane of *L. lactis*, which means that it has the potential of facilitating membrane protein expression in this host bacterium. Moreover, the soluble form of the M110-GFP fusion was also observed in *L. lactis* (Fig. 3), which was in agreement with Mistic behaviour in *E. coli* (Dvir *et al.*, 2009).

**Overexpression of pkjDes4 in *L. lactis***

The eukaryotic membrane protein pkjDes4 was recently identified in *Pavlova viridis* (Xu *et al.*, 2011). This protein and its family, namely microalgal front-end fatty acid desaturases, have not, to our knowledge, been overexpressed in *L. lactis* so far. Depending on the strategy of Mistic chaperoning and the use of pNMDes4, pkjDes4 overexpression was achieved in *L. lactis* (Fig. 4a). Compared to the uninduced control, the induction of NZ9000/pNDes4 did not show any additional protein bands in SDS-PAGE gels (Fig. 4b). The results proved the feasibility of Mistic as a promising tool for the overproduction of intractable eukaryotic membrane proteins.

The expression level of pkjDes4 was up to 4.4% of total membrane proteins, which was high compared to that of other eukaryotic membrane proteins expressed in *L. lactis* (Kunji *et al.*, 2003; Bernaudat *et al.*, 2011). However, the percentage might be underestimated, as the membrane is readily contaminated with soluble and peripheral membrane proteins (up to 50%) (Kunji *et al.*, 2003). Besides, the enzymatic activity of pkjDes4 was not tested here because the electron carriers of the bacterial system often cannot complement the system of eukaryotic microalga, and the approach of adding substrates and necessary compounds into bacterial culture did not work. The activity of pkjDes4 will be further tested *in vitro*.

**Overexpression and functional determination of pkjLi in *L. lactis***

The 1776bp gene encoding the putative LI of 591 aa was isolated from *Lb. acidophilus* H42. The molecular mass of the isomerase (named pkjLi) (GenBank: JQ065335) was 65.1 kDa. The isomerase showed 92% identity with a putative LI from *Lb. acidophilus* AS1.1854 (GenBank: ABB43157.1) and 72% identity with the *Lb. reuteri* putative LI gene (Rosson *et al.*, 2004). pkjLi was predicted to be a membrane protein with a cytoplasmic N terminus (Krogh, 2009).
Using M110 as the chaperone molecule, pkjLi was the first lactobacillus Li overexpressed in \textit{L. lactis} cytoplasmic membrane (Fig. 5), to our knowledge. The expression level reached up to 45.2 \% in the membrane fractions, which could be viewed as a very significant amount for membrane protein expression in \textit{L. lactis}. No apparent corresponding bands were observed in control strains, indicating that the system established here, based on the NICE system (see Mierau \& Kleerebezem, 2005), was strong and tightly regulated. The proteins observed in lanes 3 and 4 of Fig. 5 may be insoluble material, but this has not yet been investigated in detail.

To investigate the activity of the M110-pkjLi fusion, the substrate LA (3 mg ml\(^{-1}\)) was directly added to the induced cultures of NZ9000/pNMLi and NZ9000/pNM110 for bioconversion assays. Chromatograms of FAMEs from these recombinant lactococcal strains are presented in Fig. 6. Fig. 6(a) shows the chromatograms from the mixture of the standard methyl esters of LA and CLA. In comparison with NZ9000/pNM110 (Fig. 6b), a unique peak representing the presumptive methyl ester of \textit{cis}-9, \textit{trans}-11-CLA was detected in the NZ9000/pNMLi strain, along with the methyl ester of substrate LA (Fig. 6c), demonstrating that LA was successfully converted to CLA through the overexpression of M110-pkjLi fusion in \textit{L. lactis}.

The conversion rate of LA was approximately 28.4 \%, resulting in CLA production of 0.852 mg ml\(^{-1}\), which was higher than that obtained for lactobacillus strains capable of hydrogenizing LA (0.58 mg ml\(^{-1}\)) (Xu \textit{et al.}, 2008). Moreover, when the concentration of the substrate LA was increased to 1 mg ml\(^{-1}\), the conversion rate reached 30.8 \%. This phenomenon was consistent with what Xu \textit{et al.} (2008) have shown, in that higher CLA production rate was usually reached when the lower concentration of LA was supplemented.

When a membrane protein is expressed with a fusion partner like GFP, the partner moiety does not necessarily interfere with the functioning of the target protein (Drew \\textit{et al.}, 2003). This was the case for pkjLi: upon overexpression in \textit{L. lactis}, the enzymatic activity was readily released without cleavage from the M110-pkjLi fusion (Fig. 5), which was supposed to be another favourable characteristic of using Mistic as the expression partner.

**DISCUSSION**

To date, dozens of membrane proteins have been successfully overexpressed in \textit{L. lactis}, including several...
eukaryotic membrane proteins (Kunji et al., 2003; Wieczorek & Martin, 2010; Steen et al., 2011; Marreddy et al., 2011; Bernaudat et al., 2011). However, the majority of prokaryotic membrane proteins were from *L. lactis* sources, and the eukaryotic membrane proteins were, to a large degree, limited to membrane transporters or carriers. Therefore, it is suggested that membrane protein expression in *L. lactis* is still a matter of ‘trial and error’, and it is impossible to predict whether or not a membrane protein can be successfully overexpressed (Kunji et al., 2005).

Fig. 6. GC analysis of FAMEs from *L. lactis* expressing pkjLi with exogenous substrate linoleic acid (LA). (a) Methyl ester standards of LA and conjugated LA (CLA); (b) extract from induced NZ9000/pNM110 cell pellet after 24 h of LA supplementation; (c) extract from induced NZ9000/pNMLi cell pellet after 24 h of LA supplementation. LA, methyl ester of LA; CLA, methyl ester of CLA; t, trans, c, cis. Asterisk indicates presumptive isomer. Each feeding experiment was repeated twice, and results of a representative experiment are shown.
There are a series of bottlenecks affecting the heterologous expression of membrane proteins in *L. lactis*, such as protein-folding capacity, AT-rich codon usage, protein degradation and particularly membrane space and accommodation of foreign structures (Kunji et al., 2005; Wagner et al., 2006). To alleviate the bottlenecks, the membrane proteins were often expressed with an added N-terminal signal peptide (Monné et al., 2005). However, as the targeting and translocation capacity of lactococcal cells (mainly referring to signal recognition [SRP] and secretory [Sec] pathways, respectively) became very restrained upon membrane protein expression, this strategy did not necessarily lead to improved production yields. One of the most intriguing properties of Mistic is its ability to insert into cytoplasmic membranes independent of the Sec pathway. Another approach frequently adopted to break the bottlenecks is the use of fusion proteins because they could assist in stabilizing the target protein, as well as protect the fusions from protease degradation. GFP was often used as the fusion partner. While GFP could speed up the identification of membrane proteins that could be successfully overexpressed, it did not improve the process for proteins that could not be overexpressed or were poorly expressed (Drew et al., 2003). In comparison, Mistic was able to improve the overexpression of these proteins, as demonstrated for pkjDes4 (Fig. 4) and pkjLi (Fig. 5).

Whether a eukaryotic membrane protein could be overexpressed was tightly associated with its toxicity towards host cells. In the case of several multidrug transporters and KDEL receptors, their expression in *L. lactis* led to growth ceasing almost instantly, resulting in little or no protein expression (Kunji et al., 2003). Although pkjDes4 was also shown to be seriously detrimental to host bacteria growth (Xu et al., 2011), the optical density (OD) of induced NZ9000/pNMDes4 culture was only slightly lower than that of NZ9000/pNM110 (Table S1). Therefore, it was deduced that successful pkjDes4 overexpression in *L. lactis* was at least partly due to the capacity of Mistic to circumvent growth impairment in lactococcal cells. In summary, the Mistic-fusion strategy has unique advantages and is amenable to the overexpression of a wider range of prokaryotic and eukaryotic membrane proteins in *L. lactis*.

According to the NMR structure of Mistic (M110) (Roosild et al., 2005), its C terminus is extracellular, so the GFP moiety of Mistic-GFP fusion was anticipated to cross the cellular membrane to be folded. Depending on the observation that GFP can only fold and become fluorescent in the cytoplasm of *E. coli* (Drew et al., 2006), the Mistic-GFP fusion had been hypothesized not to be fluorescent. It was, however, not the case in *L. lactis*, as confirmed in Fig. 2 and Fig. 3. It is possible that GFP is able to fold properly and become fluorescent outside of *L. lactis* cells, which could represent a different post-translational-folding route for GFP in *L. lactis* compared to *E. coli*. The mechanisms underlying the translocation of GFP across the cellular membrane remain to be investigated because its fluorescence profile in *E. coli* was also an empirical conclusion (Feilmeier et al., 2000). A similar phenomenon was observed in a GFP display system on the surface of *L. lactis* (Li et al., 2009).

To date, efforts to functionally express the lactobacillus-presumptive LIs in a wide range of microbial systems have failed (Macouzet et al., 2010). We previously failed to express pkjLi using the common NICE system (unpublished data). Among the several lactobacillus species capable of converting LA to CLA, *Lb. acidophilus* showed major tolerance to LA and the greatest CLA-producing ability (Xu et al., 2008). Thus, we selected the LI from *Lb. acidophilus* H42 for overexpression in *L. lactis* in this study. Depending on the Mistic-fusion strategy, expression was accomplished, and a recombinant CLA-producing *L. lactis* strain, NZ9000/pNMLi, was established. This strain should be expected to have extensive applications.

In the bioconversion assays, there was no CLA, and substantially less LA was extracted from the supernatant of the pkjLi-producing strain, suggesting that almost all of the CLA produced was accumulated as intracellular or cell membrane-associated lipids, which was basically in agreement with what was reported by Ogawa et al. (2005). It was hypothesized that membrane incorporation was a strategy used by lactic acid bacteria to avoid the inhibitory effect of PUFAs on cell growth. In addition, when washed cells (resting cells) were used for the LA conversion experiment, as suggested by Ogawa et al. (2005), no detectable CLA products were obtained.

With different CLA isomers having different biological effects on human health, the biogenesis of CLA is favoured, compared to chemical synthesis, because it allows for the production of high-purity, single-isomer CLA (Rosson et al., 2004). However, there were always at least three CLA isomers detected in CLA-producing lactobacilli (Macouzet et al., 2010). Using NZ9000/pNMLi and LA as the LI source and substrate, respectively, it was shown that only one isomer (presumptive cis-9, trans-11-CLA) was produced in the conversion (Fig. 6). In combination with the above phenomena, we hypothesize that there are other enzymes with no significant homology with the identified LIs that are responsible for CLA production in *Lb. acidophilus*.

**CONCLUSIONS**

In this study, we established a high-yield membrane protein expression system in *L. lactis*, the exceptional efficiency of which was demonstrated by expressing two intractable membrane proteins: pkjDes4 and pkjLi. The system was based on the use of Mistic, and it was proven to efficiently improve membrane protein expression in *L. lactis*. Additionally, the LI from *Lb. acidophilus* was for the first time, to our knowledge, functionally characterized using this expression system. In conclusion, the work presented here was an innovative attempt to investigate the high-yield expression of membrane proteins in *L. lactis*. 
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REFERENCES

[http://www.cbs.dtu.dk/services/TMHMM].


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