VI. CHAPTER

PHYSIOLOGICAL RESPONSES OF THE BACTERIUM LACTOCOCCUS LACTIS TO THE PRODUCTION OF HUMAN CFTR

Galileo: How old is this discovery?
Ludovico: I don’t think it was more than a few days old when I left Holland, in any case it hadn’t been longer on the market.
Abstract
Biochemical and biophysical characterization of CFTR (the cystic fibrosis transmembrane conductance regulator) is thwarted by difficulties in obtaining sufficient quantities of correctly folded and functional protein. Here we have produced human CFTR in the prokaryotic expression host Lactococcus lactis. The full-length protein was detected in the membrane of the bacterium, but the yields were too low for in vitro functional and structural characterization and induction of the expression of CFTR resulted in growth arrest. We used quantitative proteomics to find out why production of CFTR in L. lactis was problematic. Protein abundances in membrane and soluble fractions were monitored as a function of induction time, both in CFTR expression cells and in control cells that did not express CFTR. 846 proteins were identified and quantified (35% of the predicted proteome), including 163 integral membrane proteins. Expression of CFTR resulted in a massive increase in abundance of stress-related proteins (e.g. heat-shock and cell envelope stress), indicating the presence of misfolded proteins in the membrane. In contrast to the reported consequences of membrane protein overexpression in E. coli there were no indications that the membrane protein insertion machinery (Sec) became overloaded upon CFTR production in L. lactis. Nutrients and ATP became limiting in the control cells as the culture entered the late exponential and stationary growth phases but this did not happen in the CFTR expressing cells, which had stopped growing upon induction. The different stress responses elicited in E. coli and L. lactis upon membrane protein production indicate that different strategies are needed to overcome low expression yields and toxicity.
**Introduction**

The human cystic fibrosis transmembrane conductance regulator CFTR is an exceptional member of the superfamily of ATP binding cassette (ABC) transporters, because it is a channel (for chloride ions) rather than a transporter. Mutations in CFTR cause cystic fibrosis (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989), the most common genetic disease among Caucasians. Mechanistic studies on CFTR and attempts to rationally design drugs to treat cystic fibrosis are hampered by difficulties to produce the protein in amounts needed for biochemical and biophysical studies, such as X-ray crystallography. A major bottleneck is a lack of suitable overexpression systems to produce recombinant CFTR, a problem which is often encountered for human membrane proteins (Grisshammer & Tate, 1995; Tate & Grisshammer, 1996; Cereghino & Cregg, 2000; Kunji et al., 2003; Tate et al., 2003; Surađe et al., 2006; Bonander & Bill, 2009; Zweers et al., 2009). In an attempt to find suitable hosts for recombinant production of CFTR the cystic fibrosis foundation has funded a project to express CFTR in the bacterium Lactococcus lactis.

*Lactococcus lactis* is a Gram-positive bacterium for which expression plasmids and inducible promoters are available (de Ruyter et al., 1996). Several cases have been reported in which functional overexpression of membrane proteins could be achieved in *L. lactis*, but not in *E. coli* (e.g. the human KDEL receptor and Na+/tyrosine transporter (Tyt1) of Fusobacterium nucleatum) (Kunji et al., 2003; Kunji et al., 2005; Monne et al., 2005; Monne et al., 2007; Quick & Javitch, 2007). The use of *L. lactis* as host for (eukaryotic) membrane protein expression has been reviewed by Kunji et al. (Kunji et al., 2005). Among the potential advantages of *L. lactis* are its growth rate of ~1 doubling per hour, which is much slower than *E. coli* and could be beneficial for expression of proteins that do not fold easily. Also the presence of a different repertoire of chaperones, *e.g.* two copies of the integral membrane chaperone YidC (Luirink et al., 2001; Zweers et al., 2008; Funes et al., 2009), could facilitate insertion and assembly of heterologous membrane proteins. Other factors such as different membrane lipids and cytosolic environment could play a role as well.

Here we have used *L. lactis* for the expression of the human cystic fibrosis transmembrane conductance regulator CFTR. We were able to express full length (1480 amino acids long) CFTR in the bacterial host, but the expression levels were too low for to pursue structural studies, and expression was toxic to the cells. To understand this toxicity and to identify potential remedies to improve expression levels, we investigated the physiological responses that were elicited in *L. lactis* upon CFTR expression by performing a global quantitative proteomics study.

**Results**

**CFTR expression by *L. lactis***

The cfr gene was fused to the coding sequence for an N-terminal (nHis-CFTR) or C-terminal (CFTR-cHis) His-tag, or both and was cloned in plasmid pNZ8048 for expression in *L. lactis*. Expression of genes from this plasmid is under control of the Nisin A inducible promoter. The cfr containing plasmids were transformed to *L. lactis* expression strain NZ9000. No mutations or gene rearrangements were observed in the expression plasmid, even after many generations of growth of the transformed strains. This indicates that the gene is stable and well tolerated by *L. lactis*, in contrast to what has been reported for *E. coli* (Drumm et al., 1990; Gregory et al., 1990). *L. lactis* strains with the plasmids for nHis-CFTR, CFTR-cHis or nHis-CFTR-cHis were cultivated and cfr expression was induced with Nisin A in the exponential growth phase (OD$_{600}$ of 0.5). Induction of the expression with Nisin A severely affected growth, and at the time of harvest (two hours after induction) the cultures of the cfr expression strains had reached much lower cell densities than control cultures (Supplementary Fig. 1). Cells were lysed, membranes were isolated, and the proteins in the membrane fraction were separated by SDS-PAGE. CFTR expression was examined by western blot analysis, using two different monoclonal antibodies, which recognized the engineered His-tags or the native C-terminus of the CFTR protein. The nHis-CFTR protein could be detected by both...
antibodies as a band that migrated at an apparent molecular mass of 130 kDa, showing that both the N-terminus (His-tag) and the C-terminus (epitope of the CFTR specific antibodies) were present, and thus full-length protein had been produced (Fig. 1A and B). For detection of CFTR constructs with C-terminal His-tags (CFTR-cHis and nHis-CFTR-cHis), only the anti-His-tag antibodies could be used, because the His-tag on the C-terminus prevented detection of the protein by the CFTR specific antibodies. Full-length CFTR-cHis was not detected, but nHis-CFTR-cHis was detected and migrated at approximately the same apparent molecular weight as nHis-CFTR on SDS-PAGE, which again confirmed that the full-length CFTR had been produced by \textit{L. lactis}. In addition to the full-length proteins, a number of smaller fragments were detected which are likely to be degradation products (Fig. 1).

Figure 1. EXPRESSION OF HUMAN CFTR IN \textit{L. lactis}

Panel A: Membranes of \textit{L. lactis} expressing His-MBP-CFTR and nHis-CFTR were analyzed by SDS-PAGE/Western blotting. CFTR was detected using anti-CFTR antibodies (clone 24-1, R&D systems). 10 μg protein was loaded per lane.

Panel B: Membranes of \textit{L. lactis} expressing nHis-CFTR, CFTR-cHis and nHis-CFTR-cHis were analyzed as described above, but now using anti-His-tag antibodies.

Panel C: Topology model of His-MBP-CFTR indicating the different domains, and showing positions of the tryptic peptides identified with LC-MS/MS. Peptides derived from all soluble domains of His-MBP-CFTR were found. For a complete list see Supplementary Table 2.

Apparently, the presence of an N-terminal His-tag was necessary for production/detection of full-length CFTR. To investigate further how N-terminal modification affected the production of full-length CFTR, a construct was made with an MBP domain plus a His-tag fused at the N-terminus (His-MBP-CFTR). His-MBP-CFTR was detected in \textit{L. lactis} membranes with the anti-His-Tag antibodies and had an apparent molecular weight of 170 kDa (Fig. 1A), as expected for the full-length protein. To confirm that the full-length protein was produced, and to obtain an estimate of the expression levels, His-MBP-CFTR was partially purified. Membranes containing His-MBP-CFTR were solubilized with the detergent n-dodecyl-β-D-maltoside (DDM) and subjected to Ni-sepharose chromatography. Fractions from different steps of the purification were analyzed by SDS-PAGE. On a Coomassie-stained gel a very faint band was visible in the elution fraction corresponding to a protein with an apparent mass of 170 kDa. The stained 170 kDa protein band was excised from the gel, peptides were generated by trypsin hydrolysis, and the peptides were analyzed by MALDI tandem mass spectrometry. Forty-four peptides were identified covering 28% of the protein sequence and including peptides from both the N-terminal domain (MBP) and the most C-terminally located domain (NBD2), again confirming that \textit{L. lactis} had
produced the full His-MBP-CFTR fusion (Fig. 1C, and supplementary Table 2).

Consequences of CFTR overexpression

The above experiments show that full-length human CFTR was produced in L. lactis membranes. Although this result is extremely encouraging, the expression levels were too low for functional or structural characterization (we estimate <0.01% of the proteins in the membranes); in addition expression of CFTR severely affected the growth of L. lactis (Supplementary Fig. 1). To investigate the effect of CFTR expression on the physiology of L. lactis, and to identify why L. lactis produced only small amounts of CFTR, a proteomic approach was followed using quantitative mass spectrometry. The experimental setup is outlined in Figure 2. Two replicate fermentations (Replicate 1 and 2 in Fig. 2) were carried out both of L. lactis containing the expression plasmid for nHis-CFTR-cHis, and of L. lactis containing the empty plasmid pNZ8048. All cultures were grown in fermenters of 3 liter volume, with temperature (30°C) and pH control (6.5). The inducer Nisin A was added to both the control and the expression strains in the mid-exponential growth phase (OD\textsubscript{600} ~0.5, Supplementary Fig 1). One liter of cells was harvested at each of three time points: just before the addition of Nisin A (time point 0 hr), and at 1 hr and 4 hrs after induction, yielding a total of 12 cell-samples (3 time points per fermentation) (Fig. 2 and supplementary Fig. 1).

Each of the 12 cell-samples was lysed, and membrane and soluble fractions were isolated (abbreviated as M and S, respectively in Fig. 2), resulting in 24 protein samples. The separation of membrane and soluble fractions was done to facilitate the identification of low-abundance membrane proteins, and to follow the possible redistribution of proteins between the membrane soluble fractions upon overexpression (see discussion). The membrane and soluble fractions were kept separate during the subsequent analysis. The 24 protein samples were digested with trypsin, yielding 24 peptide-samples, which were divided into four sets, each containing 6 different peptide-samples (Fig. 2). Three of these peptide-samples were derived from control cells (corresponding to the three timepoints 0 hr, 1 hr and 4 hr of the same fermentation), and the other three from the CFTR expression cells. This was done separately for the membrane and the soluble fractions, and separately for the replicate fermentations.

Each peptide-sample in the set of 6 was labeled with a different isotope label, for which isobaric iTRAQ reagents from the 8-plex iTRAQ kit were used, and the six differentially labeled samples were combined into a ‘Master pool’. The Master-pool was supplemented with two more peptide-samples (labeled with yet two different iTRAQ labels from the 8-plex iTRAQ kit): (1) a ‘technical replicate’, which was a peptide sample identical to one of the six peptide-samples already present, but labeled with a different isotope label; (2) a ‘biological replicate’, which was related to one of the six peptide-samples already present but obtained from the replicate fermentation. The iTRAQ labeling scheme is shown in Supplementary Figure 2. Different label combinations (label swaps) were used in the different Master pools.

The labeled peptide mixtures in the 4 Master pools were fractionated using cation exchange and reversed phase chromatography, and the eluting peptides were analyzed by MALDI-MS/MS, which provides both identification and quantification data. The fragmentation spectrum was used to identify each peptide, and the areas of the 8 different reporter peaks from the iTRAQ labels were measured for later comparative quantification. The identification and quantification data of different peptides originating from the same protein were integrated, and the resulting protein data from the different replicates were combined. This resulted in 2 lists of proteins (744 from the membrane fractions and 688 from the soluble fractions) that were fully quantified using iTRAQ in all replicates (Supplementary Tables 3 and 4).

The experimental design allowed for the comparison of the relative protein abundances (ratios of the quantified iTRAQ reporter peaks) for each pair of samples present in the Master-pool. Because there were 8 peptide samples in the Master pool, a total of 28 different iTRAQ pairs (ratios) could be calculated (8!/2!(8-2)!) for each
VI. CHAPTER: Physiological responses of *L. lactis* to the production of CFTR protein. Only 9 of these ratios are biologically relevant: (a) the changes in protein levels during the time course of the expression (0 hr vs. 1 hr; 1 hr vs. 4 hr; 0 hr vs. 4 hr) for both the control strain and the CFTR expression strain (six iTRAQ ratios in total; Fig. 3, dashed lines); and (b) the differences in protein levels between the control strain and the CFTR expression strain at each of the three time-points (0 hr, 1 hr, 4 hr; three iTRAQ ratios; Fig. 3, solid lines). The technical and biological replicates which had been taken along in each 8-plex iTRAQ experiment resulted in two more relevant ratios for control purposes (Supplement Fig. 2). In Supplementary Tables 3 and 4, the iTRAQ ratios (expressed as logarithms, log_{10}-ratios) of the 9 biologically relevant comparisons are given for each of the identified proteins. Numbers in green and red indicate significant differences (up- and down-regulated, respectively) in the relative protein abundances between the sample pairs that were compared. The iTRAQ log-ratios of the technical and biological replicates are shown in Supplementary Table 5.

![Figure 2. WORKFLOW OF THE PROTEOMICS STUDY](image)

To compare relative protein abundance in the control and CFTR-expression strains, the cells were grown in fermenters under controlled conditions. Each strain was grown twice (biological replicates). To follow the relative changes in expression in the CFTR and the control strain in time, three cell aliquots - 1 L each - were harvested immediately before induction of CFTR-expression, and 1 hour and 4 hours after induction, resulting in 12 cell samples. The cells were lysed and the cell lysate was fractionated into the membrane (M) and soluble (S) fractions by differential centrifugation. Thus 24 protein samples were obtained from 4 cultures. Each protein sample was digested with trypsin to create pep-
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tide samples and the peptides were labeled with the isobaric iTRAQ reagents. Eight different iTRAQ reagents were used (113, 114, 115, 116, 117, 118, 119, 121) for the mass of the reporter fragment. Six peptide samples labeled with different reagents - three from the control strain, and three from the CFTR-expression strain- were combined in one “Master pool” (for details see Supplemental Fig. 2). In total, the 24 labeled peptide samples were combined in four Master pools. Each Master pool of labeled peptides was subjected to 2-dimensional chromatography separation, using off-line Strong Cation Exchanger in combination with the Reversed-phase Chromatography. Separated peptides were collected on a MALDI-target and analyzed by tandem MS/MS. The obtained MS/MS spectra were analyzed by Mascot and ProteinPilot software which provided identification and quantification information for each spectrum. The identified and quantified peptides derived from the same protein were integrated, resulting in identification and quantification information of proteins. The lists of identified and quantified proteins were further subjected to statistical analyses (RankSum, False Discovery Rate) in order to detect proteins with significantly changed relative expression levels. To determine significantly clustered protein groups, the Iterative Group Analysis was applied.

**Figure 3. SCHEMATIC REPRESENTATION OF A MASTER POOL AND AN OUTLINE OF THE MEANINGFUL COMPARISONS OF THE RELATIVE PROTEIN ABUNDANCES**

Each Master pool contained peptide samples representing three time points of the control strain and three time points of the CFTR-expression strain (0 hr, 1 hr, 4 hrs after induction of expression). The expression levels of proteins could be followed as function of the time within the control and CFTR-expression strains (dashed lines). Furthermore, the relative changes in protein abundance could also be calculated between the control and the CFTR-expression strains at three the time points (solid lines).

**Control experiments: technical and biological replicates**

To assess the experimental noise of the relative quantification by iTRAQ, the data from the technical replicates were used (Supplementary Fig. 2, indicated with double asterisk). The iTRAQ log-ratios were close to zero for all identified proteins. A full statistical analysis, using RankSum and False Discovery Rate analyses detected only 3 proteins with significantly changed iTRAQ log-ratio in the technical replicates, confirming that the applied method yields a low number of false positives (Supplementary Table 5).

To assess the extent of biological variation (variation between replicate fermentations), each master-pool of peptide-samples contained two peptide-samples derived from the same condition (1 hr of induction; control cells) but from two different fermentations (Supplementary Fig. 2, indicated with asterisk). Statistical analysis using Rank-Sum and False Discovery Rate analyses did not find any proteins that had significantly changed in abundance in the replicates (Supplementary Table 5). As a final control for the significance of any observed differences in protein levels, we compared the protein abundances of the control and CFTR-expression strains at time point zero (immediately before induction of expression), when no differences are expected (Supplementary Table 5). The iTRAQ log-ratios very close to zero were detected for the large majority of proteins, and again, the statistical analysis did not find any significantly up- or downregulated proteins, confirming that the biological noise was low.

**Time series: patterns**
In contrast to the control experiments described above, we did observe large changes in the protein abundances when the samples taken after 1 hr and 4 hrs of induction were compared with the time point 0 hr, both in the control and the CFTR-expressing cells, and both in the membrane and soluble fractions (Supplementary Tables 3 and 4). We identified several patterns of change as a function of induction time. The expression level of a protein either remained unchanged, or increased or decreased in the time. The increment or decline could follow several patterns: (1) ‘gradual’, i.e. it increased or decreased after 1 hr of expression when compared to the zero time point, and increased or decreased even more after 4 hrs of expression (e.g. FruC and FruD, Fig. 4A); (2) ‘leveling off’, i.e. the change within 1 hr was followed by constant levels upon further expression (e.g. the Pur proteins in the control cells, Fig. 4A); (3) ‘delayed’, i.e. no changes after 1 hr followed by a change after 4 hrs (e.g. the Pur proteins in the CFTR expressing cells or the subunits of the pyruvate dehydrogenase complex in both the control and expression strains, Fig. 4A); (4) ‘opposite’, i.e. a change in expression after 1 hr is followed by an opposite change after 4 hrs (e.g. RibA in Fig. 4A).

Importantly, the patterns of change in time for different subunits of known complexes, or for different proteins coded by the same operon, were very similar, indicating a high level of consistency in the results. For instance, the four subunits of the pyruvate dehydrogenase complex mentioned above (Pdh proteins), all showed the ‘delayed’ pattern of change (Fig. 4A). Similarly, the subunits of the Opp system, an ABC transporter for peptides, showed the same pattern of expression (‘gradual’ or ‘delayed’ in the control and expression strains, respectively, Fig. 4A). In addition, PepO, which is coded by the same operon as the opp genes, also displayed the same pattern of changes. Another example of proteins coded by an operon are the Pur proteins, all of which showed the same pattern of change (‘leveling off’ and ‘delayed’ in the control cells and expression cells, respectively, Fig. 4A).

Control versus CFTR-expressing cells

The relative protein abundances between control cells and CFTR expressing cells at the time points 1 hr and 4 hr after induction were also compared. Again, levels of proteins were either the same or different (elevated or lowered in the CFTR expression cells compared to the control cells). Differences resulted from unequal patterns of time-dependent changes in protein levels between the control and the CFTR-expression cells. At each time point thirteen different combinations of changes in proteins levels in the control and expression cells are possible (Fig. 5). If no differences are observed for the abundance of a protein at a time point, this could be the result of the absence of time-dependent changes in both the control and CFTR-expressing cultures, (Fig. 5, middle row, pattern #1), but it also could result from similar extents of up- or down-regulation of in both strains (Fig. 5, middle row, patterns #2 and 3, respectively). An example of pattern #2 is the pyruvate dehydrogenase complex where all subunits were up-regulated to a similar extent in both the control- and the expression-strain, resulting in an apparently unchanged expression when comparing the two strains with each other. An apparent elevation of a protein level as a consequence of CFTR-overexpression could be the result of up-regulation in the CFTR-strain and concomitantly either no changes (Fig. 5, bottom row, pattern #1), down-regulation (Fig 5, #2), or up-regulation to a lesser extent (Fig 5, #3) in the control strain. An apparent up-regulation also could be a result of down-regulation in the control strain and either no changes in the CFTR expression strain (Fig. 5, #5) or a weaker down-regulation (Fig 5, #4). An example of pattern #1 is, of course, the CFTR protein itself: its abundance ‘gradually’ increased in time in the CFTR-expressing strain, whereas the protein was absent in the control strain (Fig. 4A). In theory, the iTRAQ ratio, when comparing CFTR abundance in the expression strain and in the control strain, should be infinite (division by zero). However, this was not the case, because iTRAQ quantification tends to dampen to ratio of proteins that are of very low abundance in one of the two strains (Ow et al., 2009). Nonetheless, the CFTR protein had one of the highest iTRAQ ratios found.
Figure 4: EXAMPLES OF PROTEINS CHANGING ABUNDANCE UPON INDUCTION
Each bar in the panels and insets represents one protein. The height of the bar indicates relative difference in abundance of protein (logarithm of the iTRAQ ratio). On the left-hand side of each panel, the relative changes in protein expression within the control strain upon induction are presented (comparing 0 hr and 1 hr, and 0 hr and 4 hrs). On the right-hand side of each panel, the relative changes in protein expression in the CFTR-expression strain are presented. The apparent changes in protein
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abundance between the CFTR- and the control strain (at time-point 0 hr, 1 hr and 4 hrs) are shown in the insets. Color: red, green and yellow colors indicate significantly decreased, increased and unchanged protein abundance, respectively.


Apparent down-regulation at the 1 hr and 4 hr time-points also could be the results of several scenarios (Fig. 5, upper row). For instance, the proteins of the oligopeptide transport system \textit{Opp} followed pattern #4: in the control strain the abundances of all subunits were increased after 1 hr of expression and further increased after 4 hours (‘gradual’, Fig. 4A). In contrast, the same proteins had not changed in abundance in the CFTR strain after 1 hour, but did increase in abundance after 4 hours (‘delayed’). So, even though the levels of the \textit{Opp} proteins increased in time in the CFTR expression strain, they increased at a lower rate than in the control strain, resulting in an apparent decrease of protein abundances when comparing the two strains at any given time point. Another example for the apparent lowering of protein levels as the consequence of CFTR-overexpression was seen for the proteins of the purine metabolism. \textit{PurB}, \textit{PurC}, \textit{PurD}, \textit{PurH}, \textit{PurK} and \textit{Purl} were upregulated in the control cells when comparing timepoint 1 hr with time point 0 hrs, but remained constant when comparing time points 4 hrs with time point 1 hr (‘leveling off’, Fig. 4A). In contrast, in the CFTR-expressing cells the protein levels remained constant or increased slightly only during the first hour of induction, but increased during the next 3 hrs to levels comparable to the control cells (‘gradual’ or ‘delayed’, Fig. 4A). Therefore, when comparing the CFTR-expression strain to the control, the relative abundances of these proteins were apparently decreased at 1 hr (Fig. 5, upper row, pattern #4 or #5), but were unchanged after 4 hrs (Fig. 5, middle row, pattern #2).

To improve the confidence of our statistical analyses, we repeated the comparison CFTR versus control at the time point 4 hrs two more times, so as to get 4 replicates for these values. The time point 4 hrs was chosen for the extra replicates, because we had noticed that the relative abundances of almost all proteins (CFTR expression strain versus the control strain) were qualitatively similar at time points 1 hr or 4 hrs after induction (down- or up-regulation or no change), but the extent of change (absolute values of the iTRAQ ratios) were generally larger, and thus more reliable, at the 4 hrs time point. When all 4 replicates were combined, 709 and 644 proteins were identified and quantified in the membrane and soluble fractions, respectively (Supplementary Table 3 and 4).

**Figure 5. PATTERNS OF RELATIVE CHANGES**

At any given time point, the abundance of a protein in the CFTR expression strain can be the same (yellow), increased (green) or decreased (red) compared to the control strain. These apparent effects depend on the changes in abundance of the proteins as function of the time within the control and CFTR-expression strains. Arrows pointing upward and downward indicate
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that protein abundance respectively increased or decreased in time. A horizontal bar indicates unchanged levels in time. An apparently unchanged expression level in the CFTR/control comparison is the result of equal changes in the control and CFTR-expression strains, respectively (middle panel). An apparent down-regulation in the CFTR/control comparison at a time point (upper panel) can be caused by decreased protein levels in the CFTR-expression strain and either unchanged levels in the control strain (upper panel, pattern #1), increased levels in the control strain (upper panel, pattern #2), or decreased levels in the control strain but of a lower magnitude than in the CFTR-strain (upper panel, pattern #3). Furthermore, an apparent down-regulation can be the consequence of increased protein expression levels in the control strain, combined with either unchanged levels in the CFTR-strain (upper panel, pattern #5), or increased levels in the CFTR strain of a lower magnitude than in the control strain (upper panel, pattern #4). An apparent increase in protein abundance between the CFTR- and control strains (bottom panel) is caused by the opposite effects as an apparent decrease.

To find proteins with significantly different abundances in the two strains the two lists were analyzed by the RankSum and FDR algorithms. At the 4 hrs time point, 147 proteins had significantly changed in abundance in the membrane fraction (70 up and 77 down) and 202 proteins in the soluble fraction (104 up and 98 down) (FDR-corrected p-values <0.1; Supplementary Tables 3 and 4) These lists were used as the basis for the biological discussion below.

Discussion

Production of sufficient amounts of well-folded membrane protein is a major bottleneck in membrane protein research. CFTR is no exception, and biochemical/biophysical studies on the protein are hampered by low yields of correctly folded and stable protein. Here we have used the prokaryotic expression host L. lactis to express full-length human CFTR. To the best of our knowledge this is the first report of bacterial expression of full-length human CFTR. Although the results are encouraging, the yields of CFTR were too low (<0.01% of membrane protein) for functional characterization. In addition, growth of the cells was severely compromised when expression of CFTR was induced, resulting in low biomass yield and indicating toxicity to the cell. Low yields and growth arrest have been observed upon expression of numerous human membrane proteins in L. lactis, also for proteins that could be assayed for function. For instance the human KDEL receptor was shown to be functional in the membrane of L. lactis by a ligand binding assay, despite low levels of expression and growth arrest (Kunji et al., 2003). The ligand binding assay for the KDEL receptor was possible because a high-affinity radiolabeled ligand was available. Such ligands are not available for CFTR.

In an attempt to understand why the CFTR yields were low, and possibly to remedy the expression bottlenecks, we used quantitative proteomics to characterize the response of L. lactis to expressing CFTR in its plasma membrane. In the combined membrane and soluble fractions we identified and quantified a total of 846 proteins, representing 35% of the predicted L. lactis proteome. Among the identified proteins were 163 integral membrane proteins, which were strongly enriched in the isolated membrane fractions. The large number of identified proteins allows reliable analysis of the physiological responses of L. lactis to the expression of CFTR. The major responses are summarized in Figure 6, and will be discussed below. To our knowledge this is the first study in which the stress response of L. lactis upon membrane protein production is systematically analyzed.

Stress from protein misfolding

For the majority of the proteins that had higher abundance in the CFTR-expression strain than in the control strain at the 4 hr time point (Supplementary Table 3 and 4), the abundance had increased as a function of time in the expression strain, but remained unchanged in the control strain (Fig. 5, bottom row, pattern #1). Almost all of the proteins following this pattern were found to be stress related. A striking example is PacL, a putative cation transporting P-type ATPase (Berkelman et al., 1994). When comparing the expression and control strains at the 4 hrs time point, PacL displayed the highest level of upregulation (highest iTRAQ ratio) of all identified proteins (Supplementary Table 3 and 4). The
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The biological role of PacL is not known, but expression of the protein is under control of the CesSR two-component system, which orchestrates the response of *L. lactis* to cell envelope stress caused by, for instance, anti-bacterial peptides (Martinez et al., 2007; Roces et al., 2009). CesSR regulates the expression of numerous proteins, many of which were also affected in the CFTR expression strain: the membrane protein chaperone/insertase OxaA2 (YidC homologue), the peptidyl-prolyl isomerase PpiB, CesR itself, and a number of proteins of unknown function were all upregulated, indicating cell envelope stress.

A second group of stress related proteins that was collectively upregulated comprised proteins of the heat shock response (van de Guchte et al., 2002). The chaperones DnaK, GrpE, GroEL and GroES were all upregulated in the CFTR expression strain, indicating a response to misfolded proteins. Similarly, ClpB and ClpE, involved in the degradation of misfolded proteins (Ingmer et al., 1999), were upregulated but, surprisingly, DnaJ was not, and its expression pattern was very different from the other heat-shock related proteins. In contrast to the heat shock proteins, the cold shock protein CspE decreased in abundance in the CFTR expression cells.

Other proteins that have been associated with stress responses also increased in abundance upon CFTR expression, including the major stress regulator SpxA, NAD synthase NadE, and endo-1,4-beta-xylanase D XynD (involved in cell wall stress). In addition, a number of universal stress proteins, hydrolases and peptidases (PepQ, PepXP, PepC, PepM) were upregulated, indicating that these proteins may also be stress-related.

Two proteins that have been reported to be involved in the oxidative stress response (thioredoxin TrxH and thioredoxin reductase TrxB1 (Jobin et al., 1999; Prieto-Alamo et al., 2000)) were also upregulated in the CFTR-expressing strain, whereas their expression levels were nearly unchanged in the control sample. In contrast, many other proteins involved in the oxidative stress response were very differently regulated. In control cells numerous proteins involved in the response to oxidative stress were upregulated at the 1 hr and 4hr time-points, including SodA (superoxide dismutase), NoxB (NADH dehydrogenase), NoxE (NADH oxidase), Rex (redox sensing transcriptional regulator), and the manganese transporters MntT and MtsAB (van de Guchte et al., 2002; Miyoshi et al., 2003). In the CFTR expression cells these proteins were not changed, upregulated to a lesser extent, or downregulated, resulting in an apparent strong downregulation when comparing the CFTR expression cells with the control cells (Fig. 5, upper row, patterns #2, 4 or 5). This finding suggests that in the late-exponential and stationary growth phases *L. lactis* normally upregulates the proteins involved in oxidative stress, even in the absence of excess oxygen (the cultures were grown semi-anaerobically), but that this response is largely absent in the CFTR expression cells.

To sum up, multiple stress responses were observed in *L. lactis* upon CFTR expression, the most obvious of which are the responses to heat shock (misfolded protein) and cell envelope stress. Similar responses to the expression of (nonnative) proteins have also been observed in other organisms (e.g. *E. coli* or *B. subtilis* (Mogk et al., 1998; Wagner et al., 2007)). The stress responses observed in *L. lactis* could be related directly to the presence of CFTR, i.e. CFTR misfolds, and the misfolded protein affects the integrity of the membrane leading to cell envelope stress. Alternatively, the stress responses may be an indirect consequence of CFTR production, similar to the cellular responses that have been observed in *E. coli* as a consequence of membrane protein overexpression. Overexpression of membrane proteins in *E. coli* caused overloading of the membrane proteins insertion machinery (the Sec machinery), resulting in misfolding/aggregation of endogenous proteins targeted for secretion (Wagner et al., 2007). To distinguish between the two possibilities, we examined the effects of CFTR expression on the translation-targeting-membrane insertion machinery.

**Translation and targeting**

The majority of integral membrane proteins are targeted to the Sec translocase as ribosome-bound nascent chains, which are cotranslationally inserted into the membrane upon docking on the Sec translocon (Valent et al., 1998; de Gier & Lutirink, 2001; Urba-
Higher rates of membrane protein translation may result in a higher fraction of ribosomes associated with the membrane (Bisle et al., 2006), e.g. induced synthesis of bacterio-rhodopsin increases the amounts of ribosomes isolated with the membrane fraction (Gropp et al., 1992). In *E. coli* an increased fraction of membrane bound ribosomes caused by membrane protein overexpression resulted in overloading of the Sec insertion machinery, and consequently toxicity to the cell (Wagner et al., 2007). In *L. lactis* the situation is very different: The abundance of ribosomal proteins in the membrane fraction decreased as a function of time in both the control cells and in the CFTR-expressing cells, albeit at different pace (Fig. 4B). Also in the soluble fraction the amounts of ribosomal proteins went down in time, both in the control and CFTR-expressing cells (Fig. 4B).

**Figure 6. THE PHYSIOLOGICAL RESPONSES OF THE BACTERIUM LACTOCOCCUS LACTIS TO THE PRODUCTION OF THE HUMAN CFTR**

Red and green colors indicate proteins that are lower and higher in abundance respectively in the CFTR expressing strain compared to the control strain at time point 4 hrs after induction. Yellow proteins have the same abundance. (a) Nascent chains of membrane proteins emerging from the ribosome are recognized by trigger factor (tig) and the signal recognition particle (containing the ffh pro-
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tein) and the complex is targeted to the membrane via the receptor FtsY. The nascent chain is cotranslationally inserted into the membrane upon docking on the SecYEG translocon. (b) The proper folding of the protein is assisted by membrane-integral (OxaA) and/or cytosolic (DnaJK) chaperones. Misfolded proteins trigger heat-shock and cell envelope stress responses. (c) Upon production of CFTR, the stalled ribosomes response is triggered. Also proteins involved in general and oxidative stress responses are upregulated in response to CFTR production (not shown). (d) CFTR production leads to growth arrest which correlates with the reduced ribosomal biogenesis. (e) Proteins that become de-repressed in response to limited nitrogen were collectively lower in abundance in the CFTR expressing strain than in the control strain (Opp, DtpT, CtrA, Pep proteases, IlvC, IlvE, GlnA, GlnB, GltBD). The reduced abundance was caused by a strong up-regulation of these proteins in the control strain.

Thus, the absolute amounts of ribosomes decreased, both in the control and in the CFTR expression cells, and both in the membrane and in the soluble fractions. In contrast, the abundance of translocon channel SecY, as well as SecA, the motor protein for protein secretion, remained unchanged in the membrane fraction. As the majority of integral membrane proteins are targeted to the Sec translocase as ribosome-bound nascent chain/Ffh/FtsY complex (Luirink et al., 2005), this result indicates that it is very unlikely that the Sec translocon became overloaded in *L. lactis* upon CFTR expression. The unchanged level of SecY in itself also points at the absence of jammed translocons, because jammed translocons are degraded rapidly (van Stelten et al., 2009). Therefore, we tentatively conclude that the misfolded protein response is not triggered by secondary effects (Sec overloading), but rather directly by CFTR expression.

When the abundances of ribosomal protein were compared between the control and expression cells at the 1 and 4 hrs time point, we found that many ribosomal proteins had apparently increased in abundance in the membrane fraction of the CFTR expressing cells compared to the control cells whereas their levels in the soluble fraction remained unchanged (Fig. 4B). The apparent up-regulation of ribosomal proteins in the membrane fraction was caused by a stronger down-regulation in the control cells in comparison to the CFTR-expressing cells (Fig. 5, top row, pattern #4), and the unchanged levels of ribosomal proteins in the soluble fraction resulted from their down-regulation in the control and CFTR-expressing cells to the same extent (Fig. 5, middle row, pattern #2). Statistical analysis using iterative Group Analysis (iGA, Wiederhold et al., 2009) confirmed that almost all ribosomal subunits cluster among the proteins with the highest iTRAQ ratios in the membrane fraction but not in the soluble fraction. These results show that the distribution of ribosomes over the membrane and soluble fractions becomes different in the control and CFTR expressing cells as a function of induction time. The redistribution takes place predominantly in the control cells, where the fraction of membrane bound ribosomes decreases to a much larger extent than in soluble fraction. In the CFTR-expressing cells the distribution remains approximately the same. This finding is surprising, and shows that normal (control) *L. lactis* cells entering the late exponential/stationary growth phase specifically decrease the amounts of membrane bound ribosomes, perhaps indicating a lower need for integral membrane and secreted proteins.

The signal recognition particle protein Ffh increased in abundance at the membrane upon CFTR expression. Because the receptor FtsY remained unchanged, and membrane associated ribosomal proteins decreased upon expression, a possible explanation for the increased Ffh abundance is that the ribosome-nascent chain complexes stayed attached for longer with the signal recognition particle after targeting to the membrane. Increased association times at the membrane could be indicative of hindered translation, in which case the ribosomes, mRNA and other components of the translation machinery have to be recycled from the stalled ribosomes. In particular peptidyl-tRNAs must be degraded because they are toxic to the cell (Atherly & Menninger, 1972; Menninger, 1979). Several proteins involved in ribosome recycling, including Pth (peptidyl tRNA hydrolase), Frr (ribosomal recycling factor),
InF (initiation factor) and RelA (GTP pyrophosphokinase), increased in abundance upon CFTR expression (Kossel & RajBhandary, 1968; Menninger et al., 1983; Heurgue-Hamard et al., 1998; Karimi et al., 1998; Rao & Varshney, 2001; Singh & Varshney, 2004). We therefore tentatively conclude that CFTR expression leads to a higher extent of stalled ribosomes, which necessitates their rescue.

Ribosomal biogenesis was reduced when comparing the CFTR expressing cells with the control cells (Fig. 5, top row, pattern #5), as indicated by apparent down-regulation of almost all polypeptides of the ribosomal RNA methyltransferase, the ribosomal biogenesis GTPases Era and IImg_1175, the ribosome maturation factor RimM and the ribonuclease Rnc, which is involved into rRNA processing together with the ribosome maturation factor RimM (Dunn & Studier, 1973; Drider et al., 2002; Comartin & Brown, 2006). The apparent reduction of ribosome biogenesis correlates with the observed growth stasis (Fig. 6).

**Metabolism**

### Nitrogen metabolism

CFTR expression strongly affected nitrogen metabolism. CodY is a global repressor of genes that become expressed only when nitrogen sources are limiting (den Hengst et al., 2005; Guedon et al., 2005). Proteins of which the expression is regulated by CodY were collectively lower in abundance in the CFTR expression strain than in the control strain, indicative of higher intracellular levels of branched-chain amino acids (co-repressors of CodY) and presumably higher levels of amino acids in general. Among the proteins with the strongest downregulation were the subunits of the oligopeptide transport system Opp (Fig. 4A), the peptidase PepO, enzymes of the branched-chain amino acid synthetic pathway (IlvC, IlvE), glutamate synthase GltBD, the branched chain amino acid transporter CtrA, and asparagine synthase AsnB. Without exception, the apparent downregulation of these proteins was caused by strong upregulation in the control cells, which did not occur in the CFTR expressing cells (Fig. 5, upper row, patterns #4 and #5, see also Fig. 4A for the Opp proteins). Also other proteins regulated by nitrogen limitation were apparently downregulated, such as the nitrogen regulatory protein GlnB (P-II), the glutamine synthetase regulator GlnR, glutamine synthetase GlnA, the di- and tripeptide transporter DptT, and the numerous peptidases involved in the breakdown of imported peptides (PepA, PepT, PepO2, PepN, PepP, PepDA and PepDB). The data indicates that the control cells become starved for nitrogen at time points 1 hr and 4 hrs, but that the CFTR expressing cells experience no shortage of nitrogen supply. This is consistent with the fast growth of the control cells (Supplementary Fig. 1), which deplete the available nitrogen compounds for biomass production, and the growth arrest of the CFTR expressing cells, which lowers the need for nitrogen compounds. It is remarkable that the strong upregulation in the control cells is noticeable already after 1 hr of induction when the cells still appear to be growing exponentially (albeit in the late exponential phase). Clearly *L. lactis* begins to experience nitrogen shortage in this phase already.

### Sugar metabolism

The effects of CFTR expression on the sugar metabolism are not as clear-cut as in the case of the nitrogen metabolism, possibly because many of the enzymes involved in glycolysis, and pathways downstream of pyruvate are not primarily regulated at the level of expression, but rather by allosteric and feedback regulation using molecules that sample the energetic status (such as the NADH/NAD^+ and ADT/ADP ratio). Nonetheless there are indications that the control cells become starved for sugar, as opposed to the CFTR expression cells. For example, the PTS transport systems for alternative sugars (cellobiose and mannose) become more abundant as function of time in the control cells, but not in the expression cells. Similarly, AdhE (alcohol acetaldehyde dehydrogenase) increases in abundance in the control cells only, indicating that these cells are switching to mixed acid fermentation to produce more ATP. Shortage of ATP is also indicated by upregulation of the F-type ATPase in the control cells only.

Taken together the data indicates that the direct effects of CFTR expression on metabolism are minor. The main difference between the control cells and the expression cells is that the control cells continue to
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grow rapidly after induction, thereby depleting their sugar and nitrogen supplies, whereas the expressing cells stop growing, and -initially- do not experience energy and nitrogen shortage. In *E. coli* the situation is very different: Upon overexpression of membrane proteins the Sec translocon becomes overloaded, which negatively affects the levels of respiratory enzymes in the membrane, and leads to activation of the Arc two-component system, which mediates adaptive responses to changing respiratory states. The acetate-phospho-transacetylase pathway for ATP production was induced and the tricarboxylic acid cycle was down-regulated. *E. coli* thus switches to a less efficient energy metabolism, which strongly affects biomass production (Wagner et al., 2007).

**Outlook**

The different responses of *E. coli* and *L. lactis* to stress caused by membrane protein expression imply that different strategies must be used to remedy expression bottlenecks. In *E. coli*, careful tuning of the expression levels (to prevent overloading of the Sec machinery) has been used successfully to optimize expression levels (Wagner et al., 2008). What can be done to improve the expression of CFTR in *L. lactis*? The answer hinges on two possibilities.

1. It is possible that CFTR was folded properly upon expression in *L. lactis*, but that the protein was recognized as a misfolded protein (because it is non-endogenous). In that case the answer could be to trick *L. lactis* and force it not to use the stress responses and thus to prevent growth arrest, *e.g.* by deleting heat shock proteins (Skretas & Georgiou, 2009).

2. If CFTR was not properly folded, then the protein could be helped to fold properly, *e.g.* by including (human) chaperones, or by mutagenesis, such as changing all the phosphorylation sites in the R-domain into negatively charged residues. Chaperone co-expression has been used with mixed success to improve heterologous expression in *E. coli* (Chen et al., 2003; Link et al., 2008; Kolaj et al., 2009). Also, the production of recombinant proteins under thermal stress could be improved by co-expression of GroESL in *E. coli* (Kim et al., 2009). The fact that the expression of CFTR improved at higher temperatures (Supplementary Fig. 3) indeed suggests that the upregulation of heat shock proteins helps *L. lactis* to deal better with the expression stress. Again, this is very different from what is normally observed in *E. coli*, where lower temperatures, and thus lower expression rates, usually improve production, possibly because overloading of the Sec machinery is prevented.

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**Experimental procedures**

**DNA manipulations and cloning of CFTR**

Human *cftr* cDNA (gift from Christine Bear, Toronto) (accession nr M28668) was cloned into the *E. coli* vectors pREnLIC, and pREcLIC (Supplementary Table 1), which introduce the sequences coding for N- and C-terminal His<sub>10</sub>-tags, respectively, by Ligation Independent Cloning (LIC) as described by Geertsma *et al.* (Geertsma & Poolman, 2007), yielding plasmids pREnCFTR and pREcCFTR. Plasmid pREncCFTR, which contains the *cftr* coding region fused to sequences coding for both an N- and C-terminal His<sub>10</sub>-tag, was constructed by exchanging the NcoI-XhoI fragment of pREnCFTR with the NcoI-XhoI fragment of pREcCFTR. pRE_MBP-CFTR was constructed by amplifying *cftr* by PCR and subsequent cloning in the NcoI and SpeI sites of pRE_MBP (Supplementary Table 1). The pRE vectors were converted into pNZ8048-related vectors for *L. lactis* by Vector Backbone Exchange as described by Geertsma *et al.* (Geertsma & Poolman, 2007).

**Expression of CFTR in *L. lactis* and sample preparation**

*L. lactis* NZ9000 transformed with pNZ8048-derived plasmids was cultivated in M17 medium (Oxoid, Basingstoke, UK) containing 1% glucose, and 5 μg/mL chloramphenicol. To test for expression of CFTR *L. lactis* was grown in 10 mL cultures (inoculated with O/N cultures that were diluted 1:50) to an OD<sub>600</sub> of 0.5 at 30°C, after which Nisin A (1:5000 dilution of the culture supernatant of the nisin producing strain *L. lactis*).
NZ9700 (Kunji et al., 2003) was added and the cells were incubated for another 2 hrs. A volume of culture containing the equivalent amount of cells as 1 mL of OD<sub>600</sub> of 5 was spun down (20,000 x g, 2 min, RT) and the pellet was resuspended in 400 μL 50 mM potassium phosphate buffer (KP) pH 7.5, 10% glycerol. PMSF (1 mM) was added and the cells were disrupted in a Fastprep machine (Bio101, Vista, CA) by vigorous shaking in the presence of glass beads (two times at force 6.0 for 30 sec, with 10 min incubation on ice in between the two runs). The crude cell extracts were supplemented with EDTA (15 mM final concentration) and centrifuged for 15 min at 20,000 x g at 4°C. The supernatant was subsequently centrifuged at 300,000 x g (30 min, RT) and the proteins were solubilized on ice for 1 hr (4°C). The supernatant was centrifuged for 15 min at 4°C. The supernatant was centrifuged at 100,000 x g for 30 min at 4°C. The supernatant was carefully recovered and the proteins were solubilized on ice for 1 hr. Solubilized membranes were centrifuged at 100,000 x g for 30 min at 4°C. The supernatant was incubated with Ni-sepharose resin (GE Healthcare) for 1 hr (400 μL slurry, which had been pre-equilibrated with solubilization buffer), with gentle rotation. The resin was washed with 10 mL of the same buffer containing 0.05% DDM and 50 mM imidazole and finally proteins were eluted with buffer containing 500 mM imidazole and 0.05% DDM (100, 200, 200 μL fractions).

Purification of His-MBP-CFTR

*L. lactis* NZ9000 pNZ_MBP-CFTR was grown in a fermenter (Applikon) in 2 L M17 supplemented with glucose (1%) and chloramphenicol (5 μg/mL) as described below. The cells were harvested 2 hrs after induction by centrifugation (6,800 x g, 15 min, 4°C) and membranes were prepared as described below. The membranes were stored at -80°C in 50 mM KP, pH7.5, 10% glycerol at a concentration of 10 mg/mL protein. Membranes containing 10 mg protein were resuspended in 10 mL of 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 20% glycerol, 10 mM Imidazole. n-Dodecyl-β-D-maltoside (DDM) was added (1% final concentration) and the proteins were solubilized on ice for 1 hr. Solubilized membranes were centrifuged at 100,000 x g for 30 min at 4°C. The supernatant was incubated with Ni-sepharose resin (GE Healthcare) for 1 hr (400 μL slurry, which had been pre-equilibrated with solubilization buffer), with gentle rotation. The resin was washed with 10 mL of the same buffer containing 0.05% DDM and 50 mM imidazole and finally proteins were eluted with buffer containing 500 mM imidazole and 0.05% DDM (100, 200, 200 μL fractions).

In-gel trypsin digest

Bands were excised from a Coomassie Blue-stained SDS PAGE and cut into ~1 mm<sup>3</sup> pieces. Gel slices were incubated 3-4 times for 15 min in 150 μL of destaining solution (50% ACN, 50 mM ammonium bicarbonate). The gel slices were dehydrated in 150 μL of 100% acetonitrile for 10 min, the supernatant was discarded and the gel slices were dried by evaporation. The reduction of cysteine residues was performed by incubating the gel slices in 30 μL of 10 mM DTT in 50 mM ammonium bicarbonate for 45 min at 55°C. The supernatant was removed, 30 μL of 55 mM iodoacetamide in 50 mM ammonium bicarbonate was added to each gel slice and incubated for 30 min at RT. The gel slices were dehydrated as above. To each dried gel slice, 7-10 μL of 10 ng/μL trypsin gold (Cat.: V5280, Promega) in 40 mM ammonium bicarbonate/10% acetonitrile were added and allowed to re-swell for approximately 20 min at 37°C. The gel slices were overlaid with 20 μL of 40 mM ammonium bicarbonate, 10% acetonitrile and incubated overnight at 37°C. The peptides were extracted by adding 50 μL of 2% TFA to each gel slice without removing the overlay. The extraction was repeated twice with 33% acetonitrile/1.3% TFA and 63% acetonitrile/0.7% TFA. The extracted peptides were combined and the peptide mixture was dried. The peptide mixture was resuspended in 10 μL of 0.1% TFA and subjected to tandem MS/MS analysis directly by the mixing 1:2 with 20 mg/mL α-cyano-4-hydroxyxycinnamic acid matrix solution (LaserBio Labs, Sophia-Antipolis, France) onto a MALDI target.

Growth in fermenters

*L. lactis* NZ9000 pNZ8048 and *L. lactis* NZ9000 pNZncCFTR were grown in 3 L fermenters (Applikon) in M17 medium supplemented with glucose (1%) and chloramphenicol (5 μg/mL). The temperature was set at 30°C and the pH was maintained at 6.5 during growth by addition of KOH. At an OD<sub>600</sub> of 0.5 900 mL of the culture was removed and to the remaining culture Nisin A was added (1:5000 dilution of the supernatant of a culture of *L. lactis* NZ9700). After 1 hr and 4 hrs of induction 900 mL of the culture was collected. Cell were spun down (6,800 x g for 15 min, 4°C), and pellets were washed once with 10 mM KP, pH 7.5. The washed cell pellets were frozen in liquid nitrogen and stored at -80°C.

Isolation of membrane and soluble protein fractions

The cell pellets were resuspended in 10 mM KP, pH 7.5 at an OD<sub>600</sub> of 50. To 6 mL of the suspension MgCl₂ was added (1 mM final concentration) and the cells were disrupted at 39 kPsi with a Constant Systems cell disrupter. The cells were passed through the disrupter cell twice. EDTA was added (15 mM) to the suspensions and they were incubated on ice for 15 min. To remove non-broken cells the crude cell lysates were centrifuged for 15 min at 12,000 x g at 4°C. The supernatant was carefully recovered and subsequently centrifuged at 267,000 x g for 15 min at 4°C. The supernatant, containing the soluble protein fraction was carefully pipetted off and stored at -80°C. Residual supernatant was completely removed from the membranes pellet. The membranes were washed once with 1 mL 10 mM KP, containing 10% glycerol. The pellets were finally resuspended in 500 μL 10 mM KP, 10% glycerol and stored at -80°C. Protein con-
centrations were determined with the BCA kit (Pierce).

Sample preparation for SCX/RP-LC and iTRAQ labeling

For trypsinization, 100 µg of protein (when used for 4-plex iTRAQ labeling, experiment A) or 50 µg (when used for 8-plex iTRAQ labeling, experiment B) was resuspended in 20 µL of 500 mM TEAB, 2% acetonitrile plus 0.08% SDS. Reduction of disulfide bonds with Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), cysteine-modification with methylmethanethiosulfonate (MMTS) were performed according to the manufacturer’s protocol for iTRAQ (Applied Biosystems). For enzymatic digestion, trypsin gold (Cat.: V5280, Promega) was reconstituted in 500 mM TEAB and 5 mM calcium chloride, and used in 1:6 (µg/µg) trypsin-to-protein ratio. Digestion was performed overnight at 37°C. Undigested material was spun down for 10 min at 14,000 x g. The pellets were suspended in TEAB/acetonitrile/SDS solution as before and digested for 5 h at 37°C with 0.8 µg trypsin per sample. The corresponding samples from two digests were combined, freeze-dried and suspended in 15 µL 500 mM TEAB. The 8-plex iTRAQ labeling was performed according to the manufacturer’s protocol with a few modifications. Each label was reconstituted in 210 µL 100% isopropanol and to each sample of 15 µL, 100 µL reconstituted label was added, so that each label was used for 2 samples. The 4-plex iTRAQ labeling was performed according to the manufacturer’s protocol except that each label was resuspended in 200 µL ethanol and combined with 20 µL trypsic digest. The samples were incubated for at least 2.5 h at RT and stored at -20 until required. Organic solvent (isopropanol or ethanol) was removed by evaporation. Each sample was suspended in 100 µL water. From each sample, 50 µL were combined (200 µg) and concentrated to a volume of 250 µL. The same volume of 2-fold concentrated SCX buffer A (s. below) was added, the pH was adjusted to 2.7 with phosphoric acid. The peptide mixture was subjected to chromatography and mass spectrometry analysis.

Pre-fractionation of peptides on SCX

For off-line peptide pre-fractionation, a silica-based Polysulfoethyl Aspartamide strong cation exchange (SCX) column was used (Cat.: 2025E0502 PolyLC Inc., Columbia USA). The column was run at a flow rate of 200 µL/min on an AKTA purifier (GE Healthcare). Gradient solutions A: 10 mM triethylammonium phosphate, pH 2.7, 25% acetonitrile; B: 10 mM triethylammonium phosphate, pH 2.7, 25% ACN, 500 mM KCl. Gradient conditions: column equilibration with 5 column volumes (CV) (1 CV = 0.7 ml) of 100% A. Peptides were loaded in 100% A and the column was washed with 10 CV at 100% A. Peptides were eluted: 1) 0 to 5% B in 5 CV; 2) followed by 12 to 30% B in 10 CV; and 3) 24-60% B in 5 CV. Fractions of elution steps 1 and 2 were collected every 45 sec, and fractions of the elution step 3 were collected every 1 min in a 96-well plate. Eluted peptides were dried in a vacuum centrifuge and resuspended in 50 µL of 0.1% TFA. Depending on the complexity, either separate fractions or pools of two fractions were analyzed by RP-LC MALDI-TOF/TOF.

RP-LC and MALDI-TOF/TOF analysis

Peptides were trapped on a pre-column (300 µm x 5 mm, C18 PepMap300, LC Packing) and then separated on a C18 capillary column (C18 PepMap 300, 75 µm x 150 mm, 3 µm particle size, LC-Packing) mounted on the Dionex Ultraplex 3000 LC system (LC Packings, Amsterdam, The Netherlands). Mobile phase solutions contained A: 0.05% TFA; B: 0.05% TFA, 80% acetonitrile. Gradient conditions: equilibration of column, binding and washing of peptides was performed with 3% B, elution with 3 to 50% B in 60 min at a flow rate of 300 nL/min. The eluting peptides were mixed 1:4 with 2.2 mg/mL α-cyano-4-hydroxycinnamic acid matrix (LaserBio Labs, Sophia-Antipolis, France) and spotted directly onto a MALDI target (12 sec x 260 spots), using a Probot system (LC Packings, Amsterdam, The Netherlands). Peptides were analyzed with a 4800 Proteomics analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA).

The MALDI-TOF/TOF was operated in reflectron positive ionization mode in the m/z range 900-4000. The 15 most intense peaks above the signal-to-noise (S/N) threshold of 120 from each MS spectrum of odd-numbered RP-LC runs were selected for MS/MS fragmentation in the m/z range from 900 to 2000. The 10 most intense peaks above the S/N of 50 were selected from each MS spectrum of even-numbered RP-LC runs in the m/z range from 2000 to 4000. The MS/MS spectra were acquired using 2 kV acceleration voltage and air as collision gas at 5 x 10⁻⁷ Torr. The precursor mass transmission window was set to 300 (full width at half maximum, FWHM) for peptides in the m/z range of 900-2000, and to 200 (FWHM) in the range of 2000-4000 m/z. The peak-lists of the acquired MS/MS spectra were generated, using default settings and the S/N threshold of 10. The MS spectra were calibrated in the plate model mode, using 4700 calibration mixture (Applied Biosystems). MS/MS calibration of the instrument was performed when required, using ACTH 18-39 (m/z = 2465.199) fragment ions.

Database search and criteria for protein identification
MS/MS peak-lists were extracted by the ProteinPilot software, version 2.0, using default parameters and were automatically submitted to a database search. All MS/MS spectra were analyzed using Mascot (Matrix Science, London, UK; version 2.0) and X!Tandem (www.thegpm.org; version 2007.01.01.1). Mascot and X!Tandem were set up to search a combined L. lactis sp. cremoris MG1363 database, allowing one missed cleavage of the digestion by trypsin. The database was created by combining forward and reversed entries of the L. lactis proteome (release version 31.08.07) and included sequences of porcine trypsin (NCBI accession: P00761), human keratin (P35908, P35527, P13645, NP_006112), chloramphenicol acetyltransferase (P00485), replication protein A (Q04138) and the human CFTR (NCBI accession: NP_004843) containing in total 4,902 protein entries. Mascot and X!Tandem searches were performed with a fragment ion mass tolerance of 0.30 Da and a parent ion tolerance of 200 ppm. MMTS modification of cysteine and Applied Biosystems 4-plex or 8-plexed iTRAQ quantitation chemistry of lysine and the N-terminus were specified in Mascot and X!Tandem as fixed modifications. Deamidation of asparagine and glutamine, oxidation of methionine and Applied Biosystems 4-plex or 8-plexed iTRAQ quantitation chemistry of tyrosine were specified in Mascot and X!Tandem as variable modifications.

Scaffold (version Scaffold-2_02_03, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 uniquely identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principle of parsimony. Those peptides were removed from the dataset when quantification was performed. The false positive rate was calculated by dividing 2 times the number of proteins identified in the reversed database by 4902, the sum of all proteins identified in forward and reversed versions of the database. In all measured samples, no hits from the reversed database were detected, using the criteria described above.

Relative quantification of protein expression

The relative quantification was based on peptides that were chemically labeled with isobaric reagents, using the 4-plex or 8-plex iTRAQ technique. The quantification information was obtained from the peak areas of the reporter ions (m/z 112.2, 113.2, 114.2, 115.2, 116.2, 117.2, 118.2, 119.2 and 121.2). The peak areas were extracted from the MS/MS spectra by the ProteinPilot software using default settings as specified by the ProteinPilot for the 4800 MALDI instruments (Applied Biosystems). The peak areas were corrected for isotopic impurities by the ProteinPilot using the information provided by the manufacturer in the Certificate of Analysis for each iTRAQ batch. To select quantification data, those ratios were removed where the peak area of one reporter ion was below the signal-to-noise threshold of 10.

The global bias correction was performed for all identified peptides. The bias correction factor for a given iTRAQ ratio (e.g. 113/114) was calculated as the sum of all reporter peak areas in all measured spectra from one iTRAQ reagent (e.g. 114) divided by the sum of reporter peak areas of another reagent (e.g. 113). To obtain the bias-corrected peptide iTRAQ ratios, all measured ratios (in this example all 113/114 ratios) were multiplied by the correction factor. The bias-corrected peptide ratios of the same protein were weight-averaged and protein iTRAQ ratios were obtained according to the method utilized by the ProteinPilot software (Applied Biosystems). Peptides that matched to multiple proteins were excluded from quantification. The data from two independent biological samples were integrated. The relevant protein and peptide data and given in Supplementary Tables 6 and 7.

Statistical analysis

Rank Sum analysis, a non-parametric statistical method based on the Rank Product analysis (Breitling et al., 2004b; Breitling & Herzyk, 2005), was used to identify significant changes in protein expression iTRAQ ratios. The weighted protein ratios were calculated as described above and sorted in descending order. Ranks were assigned to each protein, so that the protein with the highest ratio had rank 1, and the protein with the lowest ratio had a rank corresponding to the total number of identified proteins. To combine the protein ranks of all measured replicates (two or four), the sum of ranks across replicates was calculated, sorted in descending order and ranked again. The p-value for each protein was calculated by comparing its rank sum with the result of 1000 permutations of the list using the RankProd package for R (Hong et al., 2006). The resulting p-values were then corrected for multiple testing using the adaptive FDR control method (Benjamini & Hochberg, 2000), giving the so-called q-values. This was done using the fdrtool R package (Strimmer, 2008). An FDR rate of 10% was used as the threshold for selecting proteins with significantly changed expression. The lists of proteins sorted by the RankSum were used as input for iterative Group Analysis.
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*(Breitling et al., 2004a)* as described before
*(Wiederhold et al., 2009).*