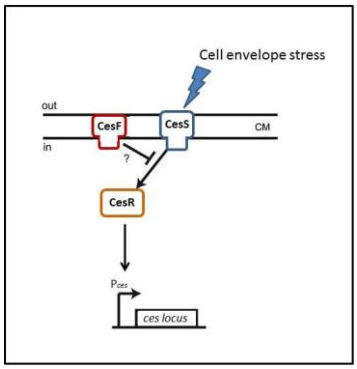
LactococCES

Membrane proteins (MPs) comprise up to 30% of the proteome of any organism and they are the direct targets of 60% of all pharmaceuticals used in human medicine. Although the biological and medical relevance of membrane proteins is obvious, they have been largely neglected due to the poor yield in the production and purification of these proteins. The bacterium *Escherichia coli* is the standard prokaryotic host for protein production but, since MPs encompass molecules that greatly differ with respect to structure, sugar decoration, lipid requirements and folding-factors needed, a broad set of hosts may have to be screened to find one best suited for production of a certain protein.

The lactic acid bacterium ***Lactococcus lactis*** is a microorganism of great economic significance, as it is used in dairy fermentations. It contributes to healthy and tasteful nutrition of millions of humans world-wide. *L. lactis* is rapidly emerging as an alternative protein expression host, due to many properties that make it ideally suited for enhanced expression of MPs, such as [1] Its relatively mild proteolytic activity; [2] Methods for genetic manipulation and for tight control of gene expression are available; [3] It provides a good membrane environment for proteins from both pro- and eukaryotic sources; [4] The small genome size of *L. lactis* (~2.5 Mbp) facilitates complementation studies: in case a given activity is present, a single gene deletion is generally sufficient to obtain the appropriate phenotype; [5] Growth of *L. lactis* to high cell densities at relatively low temperatures is rapid and does not require aeration, features that significantly save on energy costs in industrial fermentations; [6] *L. lactis* is Generally Regarded As Safe (GRAS) and widely used in food industry, which should make it easier to market products of e.g., therapeutic interest made with *L. lactis*.

Cell-envelope stress induced by MP overproduction

Overproducing heterologous as well as homologous MPs provokes a cell-envelope stress in bacteria. Induction of MP overproduction in *L. lactis* triggers the activity of the two-component system (2-CS) CesSR, in which CesS is the membrane embedded histidine kinase and CesR the corresponding response regulator controlling gene expression. This 2-CS monitors the integrity of the cell envelope and activates a response facilitating the production of a broad range of MPs, as was demonstrated by extensive transcriptome and proteome analyses. The regulon of CesSR is quite extended, comprising over 30 genes, confirming its importance in *L. lactis*. The obtained information was successfully used to rationally design *L. lactis* strains for improved functional production of MPs of different types and origins, including eukaryotic ones, by overexpressing certain genes of the CesSR regulon. Apparently, CesSR is able to sense cell envelope stresses of different origins (MP overproduction, treatment of cells with lysozyme or several antibiotics) and drive the expression of quite a large number of genes required to avert the stressful conditions. The nature of the signal driving CesSR activity is as yet unknown. By analyzing the upstream regions of the CesSR target genes a DNA motif was identified and shown to bind the response regulator CesR. The *cesSR* genes are in one operon with the gene *cesF* (*cesFSR*). CesF is anticipated to be a negative regulator of CesR-regulated gene expression. In fact, CesFSR signal transduction system has been postulated to represent a **cell envelope stress-sensing three-component system**.



Small RNAs and control of 2- and 3 component systems

Very recent advances show that a tight interplay exists between bacterial 2-CSs and small regulatory RNAs (sRNAs), a phenomenon that seems to be a global regulatory feature in many bacteria. It emerges that sRNAs might in many cases be the missing links between a 2-CS and its target genes. sRNAs can link 2-CSs and their regulons to other regulatory systems responding to different environmental stimuli. Thus, dense networks are formed to fine-tune gene expression upon integration and transduction of environmental cues. sRNAs can have multiple targets while, on the other hand, multiple sRNAs can regulate a single target under different conditions. The advantage of having an additional layer of control via sRNAs presumably lies in the fact that they do not require translation, take up only limited space in the genome and, through a wide range in half-lives, could determine the duration of control required by the cell. It is evident now that sRNAs are very important regulatory factors in quite a number of different stress responses. Recent RNA sequencing data reveal that an sRNA is produced from the promoter region of *cesFSR*, which indicates that sRNA might also be involved in the CesFSR-executed response to cell envelope stress.

The objective of this project is to unravel the functioning of the *L. lactis* CesFSR regulatory system, its regulon, and the roles that sRNAs may play in its functioning during membrane protein overproduction.

Experimental approach used in LactococCES

L. lactis strains were submitted to membrane protein overproduction stress.A detailed map of the stress response provoked in this way was generated employing DNA microarray data and RNA sequencing using Illumina technology.

A set of mutants were engineered to study the functioning of the CesFSR proteins. Each of the CesFSR components were tagged, overproduced and their genes were deleted. Promotors of the *cesFSR* operon were fused to the Luciferin gene (lux…) to measure their activity against several cell envelope stressors.

Fluorescence microscopy was used to shed more light on the functioning of CesFSR regulatory system. Different fluorescent proteins were fused to the CesF and CesS proteins. This gave valuable information on the mutual interactions and the intracellular dynamics of the components under different stress conditions.

One sRNA was identified as directly involved in the CesFSR system regulation. Knock out and overexpression mutants of this sRNA gene were made in different *L. lactis* genetic backgrounds. Thus, several other sRNAs were found to be upregulated when *L. lactis* faced cell envelope stress, although their actual relation to the CesFSR system has still to be confirmed and determined.

Impact of LactococCES, present and future

The research on the CesFSR system is still ongoing. The progress of LactococCES can be followed on http://www.molgenrug.nl/index.php/lactococcus/lactococces. This project already has given valuable information as to how CesFSR works, and has provided the tools to generate efficient MP overproducing *L. lactis* strains. LactococCES is part of a bigger research effort in our laboratory, in co-operation with international partners, to develop *L. lactis* into a host for the production of industrially or medically important proteins and to improve it for (oral) vaccination.