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Review

Data resources and mining tools for reconstructing gene regulatory networks in *Lactococcus lactis*

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Abstract

DNA is the blueprint and template for tRNA, rRNA, sRNA and mRNA synthesis in all living organisms. Subsequently, the mRNA is translated to proteins a process in which other RNA types, compounds and proteins play important roles. The regulation of transcription and translation is a delicate equilibrium of thousands of factors interacting within an organism. These factors vary from compound- and protein concentrations, to their activity and stability to physical parameters like temperature and pH. Especially the transcription factors and transcription factor binding sites play a central role in the regulation of gene expression. For *lactococci lactis* several data sets on gene expression and regulation are available in databases or literature. Furthermore, the number of (in-) complete sequenced lactococci genomes is increasing, while the majority of the strains will only be studied *in silico*. This review focuses on the visualization and mining of the complex transcription and translation systems via interactive graphics and network reconstruction tools and the amalgamation of DNA microarray data with biological data that together lead to the reconstruction of gene regulatory networks (GRN). These networks are a valuable source of information and knowledge that can be used for studying *L. lactis* physiology and provide clues for improving industrial strains by either non-GMO methods (strain selection, fermentation condition) or by specific engineering.

Key words: DNA microarray, Gene Regulatory Network, Time series, Transcription factor, Transcription factor binding site, *Lactococcus lactis*

Lactococcus lactis

Since 10.000 years humans consume fermented milk products as a rich source for proteins, small peptides, amino acids, fatty acids, calcium and other valuable nutrients. The milk sugar lactose is fermented to lactic acid; the consequent acidification prevents food spoilage and is probably the major driving force of the success of fermented milk products. Although nowadays other food preservation methods are applied, the acidification of the product is still the major growth inhibitor of bacteria other than Lactococci. The global world market of milk-related products is estimated

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at € 240 billion (IMIS Euromonitor 2006) explaining the high interest of the diary industry in research on food process management and improvement. To gain more insight in flavour formation, intensive research is taking place to unraveling genomes, transcriptomes, proteomes and (bio-) chemical pathways. The Holy Grail would be to build a predictive *in silico* model of each bacterial strain involved in milk fermentation. Probably this holistic result will not be obtained soon, not even for one of the organisms, but recent work has already led to models describing various relatively simple processes in the bacterial cell. The basis of this research is the availability of genome sequences of lactic acid bacteria but, although many *L. lactis* genomes have been sequenced, mainly by industrial companies, only a few are available in the public domain (from the strains MG1363, IL1403, SK11 and KF147) at "The Integrated Microbial Genomes (IMG) system" [1]. In this review we will discuss the state of the art bioinformatics tools that facilitate the reconstruction and visualization of gene regulatory networks in *L. lactis*.

Gene prediction

Normally, splicing of mRNA does not occur in prokaryotes, which would suggest that prediction of a protein encoding gene (open reading frame (ORF)) is a simple and straight forward process. Although prediction methods like GeneMarkHMM [2], Glimmer 3 [3], TriTISA [4], TICO [5], Easygene [6], MED 2.0 [7] and PRODIGAL [8] predict up to 90% of the genes correctly in most prokaryotes, still quite a number of genes are incorrect predicted or missed [8]. All methods struggle with predicting correct translation start point (AUG, GUG and UUG) and use Shine-Dalgarno Ribosomal Binding Site (RBS) motifs to improve the prediction of the translation initiation site. An important difference between coding and non-coding DNA regions is the GC bias and this phenomenon has been used to improve the ORF prediction. For each organism this bias can be calculated and is used to plot reading frame information for detection of the spurious upstream gene region. Furthermore, modern prediction methods use an ORF length threshold of 90-120 bp, because of the high rate of false positives for smaller genes. Prediction of a small ORF (smaller than 120-150 bases) is still very complicated. In annotation processes small ORFs are habitually discarded when they do not show homology to known proteins. Of course, in this way, novel small genes will never be discovered. BAGEL2 [9], a bacteriocin mining tool, circumvents this issue by taking the genomic context into account and is able to discover small ORFs encoding novel lantibiotics. The main resources for annotated genomes, NCBI, EBI and JGI, offer multiple ORF predictions tools made by Glimmer3 and PRODIGAL.

Operon prediction

An operon is defined as a cluster of genes that are translated from one mRNA, which is thus called a polycistronic messenger, and are under control of the same promoter. To accurately predict operon purely on the basis of genome sequences is still a challenging task. Using gene direction, gene spacing and transcription terminator prediction programs in combination with comparative genomics, up to 95% accuracy of operon prediction can be achieved [10]. Currently, databases provide operons predicted in publically accessible genomes. For the *de novo* annotation or re-annotation of operons, UNIPOP provides an easy, quick and accurate way [11, 12]. DOOR [13] (Database of prOkaryotic OpeRons) using the algorithm developed by Dam [14]) which is believed to be the most accurate prediction method as described in an intensive comparison study [15]. MicrobesOnline is a product of the Virtual Institute for Microbial Stress and Survival; it provides operons for 620 genomes while OperonDB contains operons for 550 genomes, ODB [16] for 203 genomes. RegulonDB [17] basically provides *E. coli* operons and DBTBS [18] those for *B. subtilis*. While operons in OperonDB [19], Microbes Online and ODB [16] are only predicted, operons defined in RegulonDB and DBTBS are mainly based on laboratory experiments.

Transcriptomics

Bacteria often encounter changes in their environment and will adapt the expression of genes

and activity of proteins to optimize growth, which is of utmost importance to survive in a competitive niche. By sensing the changes in the intracellular and/or extracellular environment, the cell can adjust the transcription of genes and subsequently change its protein content, to cope with the new situation. Transcription of genes (gene expression) is tightly regulated and mostly mediated by sigma factors and other *trans*-acting transcription factors (transcription regulator proteins; TFs). Furthermore, micro RNA' s and T-boxes or S-boxes, can be involved in mediating the level of transcription. The TFs can be activated or deactivated when they directly or indirectly "sense" changes in concentration of physical constants (e.g. temperature), ions or other molecules in the cell. Besides these types of TFs, two component signal transduction systems (e.g. by phosphorelay) exist that specifically react on external signals [20]. DNA microarray studies show the expression of all genes at a defined state of the cells transcriptome. Different growth rates, stress or other conditions and also the co-expression of genes can be used to build a gene-to-gene interactive network including their deduced proteins and subsequent metabolic compounds. The co-regulation of a collection of genes by the same TF is called a regulon.

Transcription factors

Transcription of DNA to mRNA can be activated or repressed when a TF binds to a specific cis-regulatory DNA element (TFBS) in the promoter region of an operon. The DNA binding region of TFs ofthen consists of a conserved Helix-Turn-Helix secondary structure, a motif that is prevalent in TFs of prokaryotes [21]. Furthermore, it is common that a co-factor molecule is required to activate a TF *e.g.* branched-chain amino acids (BCAAs) for CodY of *L. lactis* [22]. Such a co-factor influences the secondary or tertiary structure of the TF and thus its DNA binding capability. In general, the type of regulation (activation or repression) depends on the position of the TFBS relative to the binding site of the RNA polymerase. A special class of TFs is formed by the so-called two component systems, where a dedicated sensory kinase activates the TF by phosphorylation.

Transcription regulators in *Lactococcus lactis*

In the genome of *L. lactis* subsp. *cremoris* MG1363 154 genes are annotated as ncodong TFs. Among these, 32 have been studied in either *L. lactis* MG1363 or in *L. lactis* IL1403 and among these, 7 genes encode TFs that are members of a two component system (**Table 1**). Another 55 TFs have high homology to regulators in other bacteria with known function (not shown) while 68 are unknown TFs annotated as putative, based on conserved protein motifs involved in DNA-binding.

Regulons

Genes or operons that are under control of the same TF are member of a regulon. Although prediction methods for regulons have been substantially

Table 1. Transcriptional regulators studied in *L. lactis* MG1363 and/or IL1403

Gene	Literature
AhrC	MG1363 [57, 58]
ArgR	MG1363 [57, 58]
BusR	[59, 60]
CcpA	MG1363 [61]
CodY	MG1363 [22, 62]
ComX	[63]
CopR	IL1403 [64]
CtsR	MG1363 [65, 66]
FhuR	IL1403 [67]
FlpA	MG1363 [68]
FlpB	MG1363 [68]
FruR	[69]
GadR	[70, 71]
GntR	MG1363 [72]
HdiR	MG1363 [73]
HisZ	[74, 75]
LlrA	MG1363 [76]
LlrB	MG1363 [76]
LlrC	MG1363 [76]
LlrD	[77]
LlrE	MG1363 [76]
LlrF	MG1363 [76]
LlrG	MG1363 [76]
LmrR	MG1363 [78, 79]
MalR	[80]
PhoU	[81]
PurR	[82]
PyrR	MG1363 [83]
RcfB	[84]
SpxA	[85, 86]
XylR	[87]
ZitR	[88]

improved, they are still far from perfect. A relatively small number of TFs have been studied in detail in *L. lactis* and the same accounts for other well-studied microorganisms such as *E. coli* and *B. subtilis*. By using comparative genomics, regulons can be predicted in bacterial genomes. Although this is true for many regulons, the procedure can also lead to incorrect regulon calling. Comparative genomics fails for the diverged regulon of the *B. subtilis* TF CodY, which regulates quite different genes than does CodY of *L. lactis*. Despite this drawback several regulon databases are available that are based on comparative genomics of regulons (**Table 2**). Probably the most extended and accurate databases of regulons are DBTBS and RegulonDB. RegulonDB is the oldest and is specifically developed for *Escherichia coli* K12. It is updated on a regular basis starting from 1998 [17]. Furthermore, the RegulonDB web server offers the Nebulon regulon mining tool for other but a limited number of prokaryotes. The first release of the database of transcriptional regulation in *B. subtilis* (DBTBS) dates from 1999 and is regularly updated [18]. The latest update brought the total number of *B. subtilis* TFs to 120, promoters to 1475 and regulated operons to 736. In the meantime from which 463 of these operons have been experimentally validated. The most comprehensive manually curated regulon database of *L. lactis* is MolgenRegDB, which is available via the PePPER webserver (http://pepper.molgenrug.nl). Together, RegulonDB, DBTBS and MolgenRegDB are currently the major resources for regulon network mining in dedicated to prokaryotes. PRODORIC [23] is the most extended generic and manually curated database on gene regulation in prokaryotes in general. Besides regulon information it includes TFBSs and bioinformatics tools for prediction, analysis and visualization of gene regulatory networks using ProdoNet [24]. The recently launched web server RegPrecise [25] gives access to a database containing a collection of manually curated regulons grouped together by similar properties such as TFs, biological process and metabolic pathway. The database is limited to six taxonomic groups of closely related genomes (*Shewanella*, *Staphylococcus*, *Streptococcus*, Thermotogales, Bacillales and Desulfovibrionales).

Regulons in *Lactococcus lactis*

Each of the 154 TFs of *L. lactis* MG1363 will probably regulate the transcription of one or more genes. As mentioned before, the functionality of 32 TFs of *L. lactis* strain MG1363 or *L. lactis* IL1403 has been reported in literature, using techniques ranging from DNA microarrays to DNA footprinting. Although the two lactococcal strains are closely related, not all their regulators and regulons are present or similar. Comparative genomic studies showed that large regulons (those of CodY, CcpA, CmbR, CesSR, ArgR, and PurR) as well as small regulons (those of RcfB, ZirR, BusR, LmrR) are well conserved in the two strains. Likewise, the majority of the TFs are highly conserved between MG1363 and IL1403. From the total of 154 TFs in *L. lactis* MG1363, 22 are not present in *L. lactis* IL1403 while 20 out of the 143 TFs identified in *L. lactis* IL1403 are not found in MG1363 (**Table 3**). Furthermore, some regulators, like CmhR, appear to be less conserved (55%) between the two strains. Furthermore, it has been shown (unpublished results) that the CmhR regulons of both strains overlap but are not identical. The conservation of regulons between closely related strains is illustrated by the CmbR regulon of cysteine and methionine biosynthesis which has been studied in detail in *L. lactis* IL1403 [26] and in *L. lactis* MG1363 [27]. Comparative genomics of both CmbR regulons showed that 16 out of 17 proteins in the IL1403 CmbR regulon have high homology to MG1363 proteins, suggesting the good conservation of the regulon between these two species (see **Table 4**).

Table 2. Main webservers for regulon databases and mining

Web-server	Organisms	Main Tools	url
DBTBS	*Bacillus subtilis* 168	-	http://dbtbs.hgc.jp/
RegulonDB	*Escherichia coli* K12	Nebulon	http://regulondb.ccg.unam.mx/
Regprecise	+/- 50	-	http://regprecise.lbl.gov/
PRODORIC	>200	Virtual Footprint	http://prodoric.tu-bs.de/
FITBAR	>500	PSSM/LLM	http://archaea.u-psud.fr/fitbar/
ProdoNet	>500	Network	http://www.prodonet.tu-bs.de
RegAnalyst	Myco's	MoPP	http://www.nii.ac.in/~deepak/RegAnalyst/
PePPER	All NCBI	Genomics	http://pepper.molgenrug.nl/

Transcription Factor Binding Sites (TFBSs)

The relation of conserved TFBSs in promoter regions with the expression of genes was already discovered in 1983 [28]. Experimental methods like Electrophoretic Mobility Shift Assays (EMSA), Surface Plasmon Resonance (SPR), nuclease protection assays (footprinting) and Chromatine Immuno Precipitation (ChIP) can all be used to prove that an interaction exists between TF and DNA. Experimentally validated TFBSs have been described in literature and are available via publicly accessible databases (DBTBS, RegulonDB, PRODORIC, RegPrecise, PePPER). Besides experiments on protein-DNA interaction, TFBS discovery algorithms have been developed to predict conserved DNA regions. Motif mining is based on a collection of genes having a correlation; subsequently a search starts for the uncovering of conserved DNA patterns in the upstream intergenic region of the gene or the operon to which the gene belongs. The gene-to-gene correlation can be derived from transcriptome data, regulon data or from functional relations like metabolic pathways, COGs or GO classes. An overview of the major prediction algorithms is shown in **Table 5**.

TFBS mining is a complex and challenging task for biologists and bioinformaticians, but many different approaches in developing TFBS discovery tools are being undertaken and progress is very encouraging.

Table 3. Comparative genomics of TF in IL1403 and MG1363

MG1363 TFs not in IL1403	IL1403 TFs not in MG1363
ArsR	CitR
Llmg_0069	LlrH
Llmg_0089	Pi103
Llmg_0147	Pi104
Llmg_0161	Pi356
Llmg_0424	Pi357
Llmg_0432	Ps111
Llmg_0709	Ps314
Llmg_0865	RliB
Llmg_0962	RliDA
Llmg_1051	RliDB
Llmg_1324	RlrA
Llmg_1527	RlrC
Llmg_1868	RmaF
Llmg_1903	RmaJ
Llmg_2334	YcfA
Llmg_2441	YdcG
Ps116	YneE
Ps205	YrfA
Ps404	YwjD
Ps408	
RgrB	

Regulon prediction in prokaryotes

The number of fully sequenced bacterial genomes is rapidly increasing thanks to Next Generation Sequencing (NGS) technology, but experimental

Table 4. Comparative genomics of the CmbR regulon in *L. lactis* strains IL1403 and MG1363

IL1403 gene	MG1363 gene	IL1403 locus	MG1363 locus	Description
cysD	*cysD*	LL0073	llmg_0091	O-acetylhomoserine sulfhydrylase
cysK	*cysK*	LL0782	llmg_1775	Cysteine synthase
cysM	*llmg_0508*	LL0540	llmg_0508	Cysteine synthase
metA	*metA*	LL1918	llmg_2182	Homoserine O-succinyltransferase
metB1	*metB1*	LL1917	llmg_2181	Cystathionine gamma-synthase
metB2	*metC*	LL0181	llmg_1776	Prenyl transferase
plpA	*plpA*	LL0318	llmg_0335	Outer membrane lipoprotein
plpB	*plpB*	LL0319	llmg_0336	Outer membrane lipoprotein
plpC	*plpC*	LL0320	llmg_0338	Outer membrane lipoprotein
plpD	*plpD*	LL0321	llmg_0340	Outer membrane lipoprotein
ydcB	*metN*	L121289	llmg_0341	amino acid ABC transporter ATP binding protein
ydcD	*llmg_0343*	LL0324	llmg_0343	UPF0397 protein ydcD
yhcE	*metE2*	LL0718	llmg_1849	Putative uncharacterized protein yhcE
yjgC	*llmg_1593*	LL0937	llmg_1593	Amino acid ABC transporter substrate binding
yjgD	*llmg_1591*	LL0938	llmg_1591	Amino acid ABC transporter permease protein
yjgE	*llmg_1590*	LL0939	llmg_1590	Amino acid ABC transporter ATP binding protein
ytjE	-	LL1916		Aminotransferase

Table 5. TFBS prediction and mining methods

Method	url
MEME	http://meme.sdsc.edu/
GLAM2	http://meme.sdsc.edu/
MDScan	http://robotics.stanford.edu/~xsliu/MDscan/
Genome2D	http://genome2D.molgenrug.nl
DISCLOSE	http://bioinformatics.biol.rug.nl/standalone/disclose/
SCOPE	http://genie.dartmouth.edu/scope/
MotifMiner	http://mnm.engr.uconn.edu/
YMF	http://wingless.cs.washington.edu/YMF/
PhyloGibbs	http://www.phylogibbs.unibas.ch
Clover	http://zlab.bu.edu/clover/
TAMO	http://fraenkel.mit.edu/TAMO/
SwissRegulon	http://www.swissregulon.unibas.ch
MARA	http://www.swissregulon.unibas.ch
TCS	http://www.swissregulon.unibas.ch
RSAT	http://rsat.ulb.ac.be/rsat/index.html

verification of the loads of annotations and in particular the regulons is still a huge task. The *in silico* prediction of regulons is usually based on operons that share the same TFBS, information is supplemented with comparative genomics to known regulons. Powerful tools have been developed for prokaryotes; these combine regulon knowledge databases with modern TFBS search algorithms. As mentioned before PRODORIC contains an extended database of regulon data of many organisms and, in addition, offers the tool "virtual footprint" to mine for novel regulons. Whereas FITBAR [29] is more dedicated to TFBS mining and discovery, RegAnalyst [30] and ProdoNet: [24] are webservers enabling integration of data on proteomics and metabolic pathways and provide subsequent graphical representation of networks. An overview of webservers dedicated to regulons and TFBS is shown in **Table 2**.

Techniques for measuring and discovering *cis*-regulatory elements

The combined DNA microarray and ChIP-on-Chip data is the major global source for reconstruction of gene regulation networks (GRNs). Detailed information can be retrieved from EMSA and SPR studies on TF-DNA interactions. In addition, footprinting is used to locate the exact binding site of the TF on its target DNA. Non-protein regulatory elements like microRNA (miRNA) and small interfering RNA (siRNA) have recently been discovered as being very important in gene regulation in prokaryotes and they can be discovered by deep RNA sequencing. Novel promising techniques are being developed such as, *in vivo* digital genomic footprinting: by mapping DNaseI cleavage sites using massive parallel DNA sequencing, the locations of *cis*-regulatory elements on the genome of *Saccharomyces cerevisae* were uncovered[31]. Novel bioinformatics tools and genetic techniques have led to a valuable resource for GRN reconstruction and subsequently to get insight in the complex regulation machinery.

Visualization

In contrast to computers, humans are not able handling large quantities of numbers but are evolved more towards recognition of patterns, colors and movements. Therefore, extensive datasets have to be transformed into a format that is more comprehensible for human. Furthermore, integration of data originating from different sources should be integrated in such a way that it enables understanding the interactions and the relations within these sources. The TIGR4 Multi Experiment Viewer (TM4) is one of the best freeware tools to analyze and visualize DNA microarray data. For integration of such data in metabolic pathways, the KEGG webserver offers several tools; one can either use the website directly or link information of KEGG to stand-alone tools (DISCLOSE, Genome2D, TM4, Cytoscape). The software package Genome2D [32] combines data derived from DNA microarray studies, motif mining, prediction of operons, terminator and DNA motifs to genome annotation data and creates a graphical representation of the genome in concert with the various resources. For studying complex relations between multiple data sources, the Cytoscape platform [33] enables construction of GRNs and sophisticated data mining.

Time-resolved (chrono) transcriptomics

Bacteria are equipped with an elaborate sensory system that can detect most stressful conditions. They use the obtained information to redirect their metabolism to meet the challenges. Adaptation to

changes in their environment can be detected at the level of the transcriptome through changes in gene expression. To get an insight in the responses to (environmental) changes taking place during batch fermentation and to reveal classes of genes (genes as part of pathways, operons, regulons, COGs) that are important during bacterial growth, transcriptome studies were performed on *L. lactis* during growth in rich media (GM17 and milk) (unpublished results). This transcriptome time-series analysis provided insights in gene expressions over time and showed that *L. lactis* adapted quickly to environmental changes. As an example: the expression profiles during growth in milk of the genes encoding for the oligopeptide permease system (*opp*) and its regulator gene *codY* encoding for the TF CodY is shown in **Figure 1**. The transcription profiles show that expression of *opp* is stimulated during late exponential growth in milk. The increase in expression of *codY* precedes that of *opp*, which points to a specific response time of the regulon or to the availability of co-factors (branched-chain amino acids) needed to establish an active CodY regulator. A low correlation between the TF and its regulon in chrono transcriptomics is not unique for *codY*, it is more common and does not allow to automatically link TFs to the network when network reconstruction is done purely on the basis of DNA microarray data [18].

Data resources for reconstructing Gene Regulatory Networks

Building a GRN relies on data describing interactions or associations between elements. The two resources for a GRN are derived from; i) gene-to-gene interactions based on DNA microarray data, and ii) databases containing biological data as listed in **Table 6**.

Gene Regulatory Network Reconstruction

Inferring GRN from DNA microarray data is a challenging task and many papers handling this problem have been published in recent years [34]. The basic idea of these networks is to gain insight into interactions between biological processes. The number of computational methods that reconstruct such GRNs (synonym; Transcription Regulatory Networks (TRNs)) from genome-wide expression data is rapidly increasing but the methods are not always an improvement [35]. In principle two main approaches for reconstruction networks are being used, namely Bayesian networks or Graphical Gaussian networks. This last model, first used in GeneNet [36], is currently considered as the most reliable approach and is used

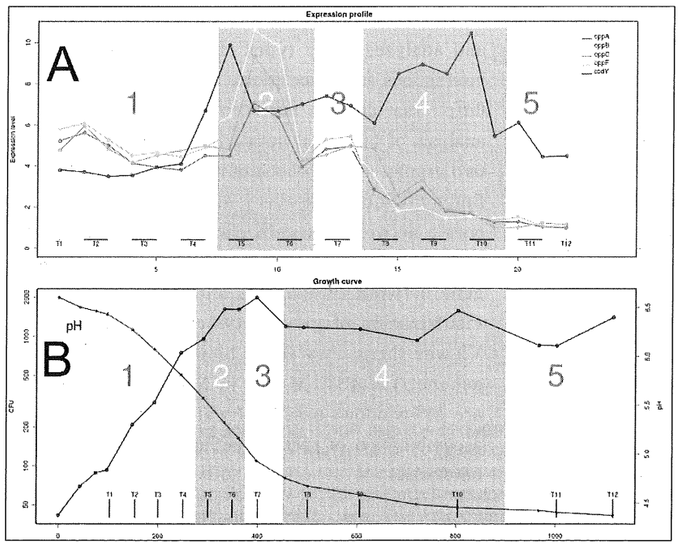

Figure 1. Expression profile of the *opp* operon of *L. lactis* MG1363
Results derived from a time-series experiment of *L. lactis* MG1363 grown for 1100 min at 30℃ in milk. Samples were taken for DNA microarray analysis, as indicated by horizontal or vertical lines on the x-axis numbered from T1 to T12. Grey blocks in the graphs indicated the growth phases: 1. early exponential; 2. late exponential; 3. transition; 4. early stationary; 5. late stationary. A) Expression profiles of *oppABCF* and *codY*. B) Growth and acidification of MG1363.

Table 6. Main resources for genome annotation

Name	Class	url
NCBI	Genome resource	http://www.ncbi.nlm.nih.gov/
EBI	Genome Review	http://www.ebi.ac.uk/
EMBL	Genome resource	http://www.ebi.ac.uk/embl/
JGI	Integrated Microbial Genomes	http://img.jgi.doe.gov/
COG	Classification	http://www.ncbi.nlm.nih.gov/COG/
GO	Classification	http://www.geneontology.org/
KEGG	Metabolic pathways	http://www.genome.jp/kegg/
PSI	Structural Biology of proteins	http://www.sbkb.org/
PFAM	Protein	http://pfam.sanger.ac.uk/
STRING	Protein protein interaction	http://string-db.org/
Uniprot	Protein functional information	http://www.uniprot.org/
Microbes online	Genome functional annotation	http://www.microbesonline.org/

as the fundament in current network reconstruction methods. The following section describes the state of GRN building systems to date.

Gene Regulation Network inference methods

ARACNE is an algorithm for the reconstruction of GRNs in a mammalian cellular context [37] subsequently this package was further improved (TimęDelay-ARACNE) to use time-series data as a source information to reconstruct gene networks[38]. Whereas ARACNE is dedicated to mammalians, SEREND (SEmi-supervised REgulatory Network Discoverer) [39], SIRENE [40] and DISTILLER [41] are gene network reconstruction systems developed and tested specific for data derived from experiments with E. *coli*. Subsequent experimental validation of the performance of these methods at the genome scale had to be done. Therefore, an extensive validation study comparing the transcriptional regulation database RegulonDB with 445 Affymetrix DNA microarrays of the transcriptional regulation of E. coli was done using CLR [42]. The predicted interactions for three TFs was tested with chromatin immune precipitation (ChIP); 21 novel interactions derived from their analysis using CLR were confirmed. CLR and ARACNE were further improved in MRNET: [43]. Afterwards C3NET claims that it outperforms MRNET by inferring the conservative causal core of gene regulatory networks [44]. More detailed information of the performance and pro' s and cons of these tools have recently be published in a review [45]. Inferring the GRN using a combination of methods as described above is possible using SEBINI, a software environment allowing to evaluate, compare, refine or combine inference techniques [46, 47]. Also MINET, like the methods mentioned above, uses transcriptomics data to quantify the statistical evidence of specific gene-to-gene interactions and provides a set of functions to infer mutual information networks from other datasets. The strength of the MINET package [48] is that it is developed in the statistical environment R and is freely available at the Bioconductor project [49], which makes it accessible and adaptable for wide-ranging research. Furthermore, MINET claims not to outperform other packages but uses mutual information from four different entropy estimators (empirical, Miller-Madow, Schurmann-Grassberger and shrink) and four inference methods (ARACNE, CLR and MRNET).

Visualization of networks

Mainly three methods are used to visualize networks: i) via web-servers; ii) via stand alone package; iii) using algorithms in a visualization platform (*e.g.*, Cell Designer Cytoscape). The webbased system VisANT [50, 51] is one of the few visualization tools for biological networks. VisANT visualizes and analyzes many types of networks of biological interactions and associations. The NeAT webserver [52] (http://rsat.ulb.ac.be/neat/) provides a toolbox for the analysis of biological networks (comparison between two graphs, neighborhood of a set of input nodes, path finding, cliques discovery and mapping of clusters onto a network), clusters, classes and pathways. The well known GeneNet system uses a database on gene network components and provides automated visualization of gene networks via the GeneNet Viewer (http://wwwmgs.bionet.nsc.ru/mgs/gnw/genenet/). CAST, MAST in MTOM and SEED [53] are MS-windows-based software packages and offer a combination of reconstruction and visualization of networks.

Network reconstruction and visualization

A GRN describes the interactions between TFs and the genes they regulate [54]. Time-series and perturbation transcriptome data derived from DNA microarray studies provide knowledge of timing of gene expression, as well as gene-to-gene and gene-to-class correlation. Inference of GRNs based on DNA microarray studies is referred to as reverse engineering, as the obtained gene expression levels are the outcome of gene regulation. Unfortunately, the ultimate network inference method still does not exist [35] meaning that a good choice of statistics should be made, one that reflects the co-expression of genes and gene classes for the given experiments. Furthermore, due to the fact that expression of TF genes does not always coincide with that of the TFs regulate, regulon network reconstruction will often be limited to TF targets. Besides this, reliable TF studies other than DNA microarray studies are necessary to complement the GRN.

Here illustrate the GRN reconstruction using our knowledge of *L. lactis* in combination with chrono transcriptomics data. The regulons of *L. lactis* are shown in a Cytoscape network visualization **(Fig. 2)** using the most comprehensive database on *L. lactis*: MolgenRegDB (ref PePPER; de Jong, in preparation). This relatively simple network shows how the regulons of the TFs CodY, CcpA, ArgR and AhrC are interconnected. To be able to visualize co-expression of genes, a Pearson correlation was calculated between the genes using time-series transcriptional data. Genes having a Pearson correlation *p*-value higher than or equal to 0.80 are considered as co-expressed. The members of the CmbR regulon are shown in Figure 6B, where the width of the lines represents the degree of co-expression (*p*-value). The study of CmbR, which regulates methionine and cysteine metabolism, was done in *L. lactis* IL1403 [55], while the genes of *L. lactis* MG1363 where derived from comparative genomics using PePPER. On the basis of highly interconnected Pearson correlated nodes, four sub-graphs (cliques) of the CmbR regulon emerged: i) *metC, cysK*; ii) *plpABCD, dar*; iii) *llmg_1589-1591, llmg_2294-2295, llmg_0352, metB1* and iv) a group that does not show expression correlation at all. This last group of genes was found to be related to CmbR on the basis of DNA microarray studies in *L. lactis* IL1403 and this relation could not be re-established using the MG1363 chrono transcriptomics data. The example mentioned above shows the power of GRN reconstruction, while even more data can still be linked to the network. A logical subsequent step is to link metabolic processes to the network. The data source can either be complete metabolic processes derived from KEGG pathways or metabolic reactions derived from the reaction

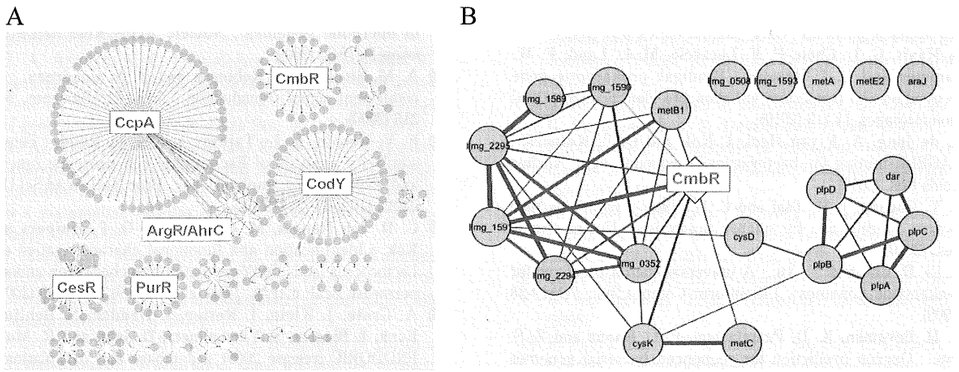

Figure 2. *L .lactis* regulons and CmbR regulon cliques
Cytoscape Network of known regulons in *L. lactis* MG1363. A) The known regulons in *L. lactis*; diamond shapes are TFs and circle shapes are genes. An arrow indicates activation and a blunt-end line shows repression of the gene by the TF. B) All CmbR regulon members connected via Pearson correlations derived from time-series transcriptomics data. The thickness of the lines (edges) corresponds to the strength of co-expression.

database. For *L. lactis* MG1363 a complete model was built by coupling the production and consumption of compounds by enzymes to the reaction database [56]. This production and/or consumption of compounds can directly be integrated into the Cytoscape model by linking enzyme functions to genes.

Conclusion

The current state of bio-informatics algorithms and tools allows gene network reconstruction in prokaryotes. Especially the progress made during the last two years amplifies the accessibility of these tools. As a consequence, there is a move from the more conceptual model-based approach to real applications implemented by biologists. It is exciting that models can now be built for *L. lactis* by using the currently available valuable biological resources like (time-series) transcriptomics data, annotation databases combined with bio-informatics tools.

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