A novel genetic tool for metabolic optimization of Corynebacterium glutamicum: efficient and repetitive chromosomal integration of synthetic promoter-driven expression libraries

Fine-tuning the expression level of multiple genes is usually pivotal for metabolic optimization. We have developed a tool for this purpose for the important industrial workhorse Corynebacterium glutamicum that allows for the introduction of synthetic promoter-driven expression libraries of arbitrary genes. We first devised a method for introducing genetic elements into the chromosome repeatedly, relying on site-specific recombinases and the vector pJS31 serving as the carrier. The pJS31 vector contains a synthetic cassette including a phage attachment site attP for integration, a bacterial attachment site attB for subsequent integration, a multiple cloning site, and two modified loxP sites to facilitate easy removal of undesirable vector elements. Meanwhile, we constructed a derivative of the wild-type strain ATCC 13032 carrying an attB site in its chromosome (JS34) and demonstrated that pJS31 readily could integrate into the attB site in this strain providing expression of the corresponding integrase. Subsequent expression of the Cre recombinase promoted recombination between the modified loxP sites, resulting in a strain only retaining the target insertions and an attB site. To simplify the procedure, non-replicating circular expression units for the phage integrase and the Cre recombinase were used. As a showcase, we used the tool to construct a battery of strains simultaneously expressing the two reporter genes, lacZ (encoding β-galactosidase) and gusA (encoding β-glucuronidase), to arbitrary levels. In principle, an unlimited number of genes, whether native, heterologous, or synthetic, can be introduced using the developed approach, and this should greatly facilitate metabolic optimization of this important platform organism.

General information
State: Published
Organisations: National Food Institute, Research Group for Microbial Biotechnology and Biorefining
Authors: Shen, J. (Intern), Chen, J. (Intern), Jensen, P. R. (Intern), Solem, C. (Intern)
Pages: 4737-4746
Publication date: 2017
Main Research Area: Technical/natural sciences

Publication information
Finding the Needle in the Haystack—the Use of Microfluidic Droplet Technology to Identify Vitamin-Secreting Lactic Acid Bacteria

Efficient screening technologies aim to reduce both the time and the cost required for identifying rare mutants possessing a phenotype of interest in a mutagenized population. In this study, we combined a mild mutagenesis strategy with high-throughput screening based on microfluidic droplet technology to identify Lactococcus lactis variants secreting vitamin B2 (riboflavin). Initially, we used a roseoflavin-resistant mutant of L. lactis strain MG1363, JC017, which secreted low levels of riboflavin. By using fluorescence-activated droplet sorting, several mutants that secreted riboflavin more efficiently than JC017 were readily isolated from the mutagenesis library. The screening was highly efficient, and candidates with as few as 1.6 mutations per million base pairs (Mbp) were isolated. The genetic characterization revealed that riboflavin production was triggered by mutations inhibiting purine biosynthesis, which is surprising since the purine nucleotide GTP is a riboflavin precursor. Purine starvation in the mutants induced overexpression of the riboflavin biosynthesis cluster ribABGH. When the purine starvation was relieved by purine supplementation in the growth medium, the outcome was an immediate downregulation of the riboflavin biosynthesis cluster and a reduction in riboflavin production. Finally, by applying the new isolates in milk fermentation, the riboflavin content of milk (0.99 mg/liter) was improved to 2.81 mg/liter, compared with 0.66 mg/liter and 1.51 mg/liter by using the wild-type strain and the original roseoflavin-resistant mutant JC017, respectively. The results obtained demonstrate how powerful classical mutagenesis can be when combined with droplet-based microfluidic screening technology for obtaining microorganisms with useful attributes.

IMPORTANCE The food industry prefers to use classical approaches, e.g., random mutagenesis followed by screening, to improve microorganisms used in food production, as the use of recombinant DNA technologies is still not widely accepted. Although modern automated screening platforms are widely accessible, screening remains as a bottleneck in strain development, especially when a mild mutagenesis approach is applied to reduce the chance of accumulating unintended mutations, which may cause unwanted phenotypic changes. Here, we incorporate a droplet-based high-throughput screening method into the strain development process and readily capture L. lactis variants with more efficient vitamin secretion from low-error-rate mutagenesis libraries. This study shows that useful mutants showing strong phenotypes but with minimal gene mutations can be identified with efficient screening technologies. It is therefore possible to avoid accumulating detrimental mutations while enriching beneficial ones through iterative mutagenesis screening. Due to the low mutation rates, the genetic determinants are also readily identified.

General information
State: Published
Organisations: National Food Institute, Research Group for Microbial Biotechnology and Biorefining, Department of Micro- and Nanotechnology, Fluidic Array Systems and Technology, Center for Intelligent Drug Delivery and Sensing Using Microcontainers and Nanomechanics
Authors: Chen, J. (Intern), Vestergaard, M. (Intern), Jensen, T. G. (Intern), Shen, J. (Intern), Dufva, M. (Intern), Solem, C. (Intern), Jensen, P. R. (Intern)
Number of pages: 12
Publication date: 2017
Main Research Area: Technical/natural sciences

Publication information
Journal: mBio (Print)
Volume: 8
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Article number: e00526-17
ISSN (Print): 2161-2129
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Web of Science (2017): Indexed Yes
Scopus rating (2016): CiteScore 5.79
Scopus rating (2015): CiteScore 4.93
Web of Science (2015): Indexed yes
Scopus rating (2014): CiteScore 4.23
Web of Science (2014): Indexed yes
Scopus rating (2013): CiteScore 4.26
ISI indexed (2013): ISI indexed no
Web of Science (2013): Indexed yes
Metabolic characterization and transformation of the non-dairy Lactococcus lactis strain KF147, for production of ethanol from xylose

The non-dairy lactic acid bacterium Lactococcus lactis KF147 can utilize xylose as the sole energy source. To assess whether KF147 could serve as a platform organism for converting second generation sugars into useful chemicals, we characterized growth and product formation for KF147 when grown on xylose. In a defined medium KF147 was found to co-metabolize xylose and arginine, resulting in bi-phasic growth. Especially at low xylose concentrations, arginine significantly improved growth rate. To facilitate further studies of the xylose metabolism, we eliminated arginine catabolism by deleting the arcA gene encoding the arginine deiminase. The fermentation product profile suggested two routes for xylose degradation, the phosphoketolase pathway and the pentose phosphate pathway. Inactivation of the phosphoketolase pathway redirected the entire flux through the pentose phosphate pathway whereas over-expression of phosphoketolase increased the flux through the phosphoketolase pathway. In general, significant amounts of the mixed-acid products, including lactate, formate, acetate and ethanol, were formed irrespective of xylose concentrations. To demonstrate the potential of KF147 for converting xylose into useful chemicals we chose to redirect metabolism towards ethanol production. A synthetic promoter library was used to drive the expression of codon-optimized versions of the Zymomonas mobilis genes encoding pyruvate decarboxylase and alcohol dehydrogenase, and the outcome was a strain producing ethanol as the sole fermentation product with a high yield corresponding to 83% of the theoretical maximum. The results clearly indicate the great potential of using the more metabolically diverse non-dairy L. lactis strains for bio-production based on xylose containing feedstocks.

General information
State: Accepted/in press
Organisations: Department of Systems Biology, National Food Institute, Research Group for Microbial Biotechnology and Biorefining, Department of Biotechnology and Biomedicine, Metabolic Signaling and Regulation
Authors: Petersen, K. V. (Intern), Liu, J. (Intern), Chen, J. (Intern), Martinussen, J. (Intern), Jensen, P. R. (Intern), Solem, C. (Intern)
Publication date: 2017
Main Research Area: Technical/natural sciences

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Journal: Biotechnology Journal
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Web of Science (2017): Indexed Yes
BFI (2016): BFI-level 1
Scopus rating (2016): SJR 1.29 SNIP 0.969 CiteScore 3.2
Web of Science (2016): Indexed yes
BFI (2015): BFI-level 1
Scopus rating (2015): SJR 1.172 SNIP 0.874 CiteScore 2.91
Web of Science (2015): Indexed yes
BFI (2014): BFI-level 1
Scopus rating (2014): SJR 1.189 SNIP 1.062 CiteScore 2.98
Web of Science (2014): Indexed yes
BFI (2013): BFI-level 1
Scopus rating (2013): SJR 1.136 SNIP 1.093 CiteScore 3.01
ISI indexed (2013): ISI indexed yes
Micro-organism for the production of stereo-specific s, s-2,3-butanediol

The invention relates to a genetically modified lactic acid bacterium capable of producing (S,S)-2,3-butanediol stereo specifically from glucose under aerobic conditions. Additionally the invention relates to a method for producing (S,S)-2,3-butanediol and L-acetoin using the genetically modified lactic acid bacterium, under aerobic conditions in the presence of a source of iron-containing porphyrin or a source of metal ions (Fe3+/Fe2+). The lactic acid bacterium is genetically modified to express heterologous genes encoding enzymes catalysing the stereo-specific synthesis of (S,S)-2,3-butanediol; and additionally a number of genes are deleted in order to maximise the production of (S,S)-2,3-butanediol as compared to other products of oxidative fermentation.

General information
State: Published
Organisations: Systems Biotechnology, National Food Institute, Research Group for Microbial Biotechnology and Biorefining
Authors: Solem, C. (Intern), Jensen, P. R. (Intern), Chen, J. (Intern), Liu, J. (Intern)
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Publication information
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Patent number: WO2016097268
Date: 23/06/2016
Priority date: 27/10/2015
Priority number: EP20150191703
Original language: English
Main Research Area: Technical/natural sciences
Source: espacenet
Source-ID: WO2016097268
Publication: Research › Patent – Annual report year: 2016

Acetoin and 2,3 butanediol isomers synthesis in metabolically engineered Lactococcus lactis

Harnessing the biosynthetic machinery of living cells is a common approach used for producing a broad range of useful chemicals. Here, we divert inherent metabolic routes in L. lactis to produce (3R)-acetoin and the derived 2,3 butanediol isomers. Efficient production of (3R)-acetoin was accomplished using a strain where the competing lactate, acetate and
ethanol forming pathways had been blocked. By introducing different alcohol dehydrogenases into this strain, either EcBdh from Enterobacter cloacae or SadB from Achromobacter xylosooxidans, it was possible to achieve high-yield production of m-BDO or R-BDO respectively. To achieve biosustainable production of these chemicals from dairy waste, we transformed the above strains with the lactose plasmid pLP712. This enabled efficient production of (3R)-acetoin, m-BDO and R-BDO from processed whey waste, with titers of 27, 51, and 32.1 g/L respectively. The corresponding yields obtained were 0.42, 0.47 and 0.40 g/g lactose, which is 82%, 89%, and 76% of maximum theoretical yield respectively. These results clearly demonstrate that L. lactis is an excellent choice as a cell factory for transforming lactose containing dairy waste into value added chemicals.

General information
State: Published
Organisations: National Food Institute, Research Group for Microbial Biotechnology and Biorefining, Systems Biotechnology
Authors: Kandasamy, V. (Intern), Liu, J. (Intern), Dantoft, S. H. (Intern), Solem, C. (Intern), Jensen, P. R. (Intern)
Number of pages: 1
Publication date: 2016
Main Research Area: Technical/natural sciences
Links: http://www.sustain.dtu.dk/

Bibliographical note
Sustain Abstract B-6
Publication: Research - peer-review › Conference abstract for conference – Annual report year: 2016

A novel cell factory for efficient production of ethanol from dairy waste
Sustainable and economically feasible ways to produce ethanol or other liquid fuels are becoming increasingly relevant due to the limited supply of fossil fuels and the environmental consequences associated with their consumption. Microbial production of fuel compounds has gained a lot of attention and focus has mostly been on developing bio-processes involving non-food plant biomass feedstocks. The high cost of the enzymes needed to degrade such feedstocks into its constituent sugars as well as problems due to various inhibitors generated in pretreatment are two challenges that have to be addressed if cost-effective processes are to be established. Various industries, especially within the food sector, often have waste streams rich in carbohydrates and/or other nutrients, and these could serve as alternative feedstocks for such bio-processes. The dairy industry is a good example, where large amounts of cheese whey or various processed forms thereof are generated. Because of their nutrient-rich nature, these substrates are particularly well suited as feedstocks for microbial production. We have generated a Lactococcus lactis strain which produces ethanol as its sole fermentation product from the lactose contained in residual whey permeate (RWP), by introducing lactose catabolism into a L. lactis strain CS4435 (MG1363 Δ(3) ldh, Δpta, ΔadhE, pCS4268), where the carbon flow has been directed toward ethanol instead of lactate. To achieve growth and ethanol production on RWP, we added corn steep liquor hydrolysate (CSLH) as the nitrogen source. The outcome was efficient ethanol production with a titer of 41 g/L and a yield of 70 % of the theoretical maximum using a fed-batch strategy. The combination of a low-cost medium from industrial waste streams and an efficient cell factory should make the developed process industrially interesting. A process for the production of ethanol using L. lactis and a cheap renewable feedstock was developed. The results demonstrate that it is possible to achieve sustainable bioconversion of waste products from the dairy industry (RWP) and corn milling industry (CSLH) to ethanol and the process developed shows great potential for commercial realization.

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State: Published
Organisations: National Food Institute, Research Group for Microbial Biotechnology and Biorefining, Arla Foods Ingredients Group P/S
Authors: Liu, J. (Intern), Dantoft, S. H. (Intern), Würtz, A. (Ekstern), Jensen, P. R. (Intern), Solem, C. (Intern)
Number of pages: 11
Publication date: 2016
Main Research Area: Technical/natural sciences
Publication information
Journal: Biotechnology for Biofuels
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Issue number: 1
Article number: 33
ISSN (Print): 1754-6834
Ratings:
BFI (2017): BFI-level 2
Web of Science (2017): Indexed Yes
BFI (2016): BFI-level 2
Combining metabolic engineering and biocompatible chemistry for efficient production of food ingredients

Biocompatible chemistry, that is non-enzymatic chemical reactions compatible with living organisms, is gaining increasing attention because of its potential within biotechnology for expanding the repertoire of biological transformations carried out by enzymes. Here we demonstrate how biocompatible chemistry can be used for synthesizing valuable food ingredients as well as for linking metabolic pathways to achieve redox balance and rescued growth. By comprehensive rerouting of metabolism, activation of respiration, and finally metal ion catalysis, we successfully managed to convert the homolactic bacterium Lactococcus lactis into a homo-diacetyl producer with high titer (95 mM or 8.2 g/L) and high yield (87% of the theoretical maximum). Subsequently, the pathway was extended to (S,S)-2,3-butanediol (S-BDO) through efficiently linking two metabolic pathways via chemical catalysis. This resulted in efficient homo-S-BDO production with a titer of 74 mM (6.7 g/L) S-BDO and a yield of 82%. The diacetyl and S-BDO production rates and yields obtained are the highest ever reported, demonstrating the promising combination of metabolic engineering and biocompatible chemistry as well as the great potential of L. lactis as a new production platform.

General information

State: Published
Organisations: National Food Institute, Research Group for Microbial Biotechnology and Biorefining, Systems Biotechnology
Authors: Liu, J. (Intern), Solem, C. (Intern), Jensen, P. R. (Intern)
Number of pages: 1
Publication date: 2016
Main Research Area: Technical/natural sciences
Links:
http://www.sustain.dtu.dk/
Combining metabolic engineering and biocompatible chemistry for high-yield production of homo-diacetyl and homo-(S,S)-2,3-butanediol

Biocompatible chemistry is gaining increasing attention because of its potential within biotechnology for expanding the repertoire of biological transformations carried out by enzymes. Here we demonstrate how biocompatible chemistry can be used for synthesizing valuable compounds as well as for linking metabolic pathways to achieve redox balance and rescued growth. By comprehensive rerouting of metabolism, activation of respiration, and finally metal ion catalysis, we successfully managed to convert the homolactic bacterium Lactococcus lactis into a homo-diacetyl producer with high titer (95mM or 8.2g/L) and high yield (87% of the theoretical maximum). Subsequently, the pathway was extended to (S,S)-2,3-butanediol (S-BDO) through efficiently linking two metabolic pathways via chemical catalysis. This resulted in efficient homo-S-BDO production with a titer of 74mM (6.7g/L) S-BDO and a yield of 82%. The diacetyl and S-BDO production rates and yields obtained are the highest ever reported, demonstrating the promising combination of metabolic engineering and biocompatible chemistry as well as the great potential of L. lactis as a new production platform.
Elucidation of the regulatory role of the fructose operon reveals a novel target for enhancing the NADPH supply in Corynebacterium glutamicum

The performance of Corynebacterium glutamicum cell factories producing compounds which rely heavily on NADPH has been reported to depend on the sugar being metabolized. While some aspects of this phenomenon have been elucidated, there are still many unresolved questions as to how sugar metabolism is linked to redox and to the general metabolism. We here provide new insights into the regulation of the metabolism of this important platform organism by systematically characterizing mutants carrying various lesions in the fructose operon. Initially, we found that a strain where the dedicated fructose uptake system had been inactivated (KO-ptsF) was hampered in growth on sucrose minimal medium, and suppressor mutants appeared readily. Comparative genomic analysis in conjunction with enzymatic assays revealed that suppression was linked to inactivation of the pfkB gene, encoding a fructose-1-phosphate kinase. Detailed characterization of KO-ptsF, KO-pfkB and double knock-out (DKO) derivatives revealed a strong role for sugar-phosphates, especially fructose-1-phosphate (F1P), in governing sugar as well as redox metabolism due to effects on transcriptional regulation of key genes. These findings allowed us to propose a simple model explaining the correlation between sugar phosphate concentration, gene expression and ultimately the observed phenotype. To guide us in our analysis and help us identify bottlenecks in metabolism we debugged an existing genome-scale model onto which we overlaid the transcriptome data. Based on the results obtained we managed to enhance the NADPH supply and transform the wild-type strain into delivering the highest yield of lysine ever obtained on sucrose and fructose, thus providing a good example of how regulatory mechanisms can be harnessed for bioproduction.

General information
State: Published
Organisations: National Food Institute, Research Group for Microbial Biotechnology and Biorefining, RWTH Aachen University
Authors: Wang, Z. (Intern), Chan, S. H. J. (Intern), Sudarsan, S. (Ekstern), Blank, L. M. (Ekstern), Jensen, P. R. (Intern), Solem, C. (Intern)
Number of pages: 14
Pages: 344-357
Publication date: 2016
Main Research Area: Technical/natural sciences

Publication information
Journal: Metabolic Engineering
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Ratings:
BFI (2017): BFI-level 2
Integrating biocompatible chemistry and manipulating cofactor partitioning in metabolically engineered Lactococcus lactis for fermentative production of (3S)-acetoin

Biocompatible chemistry (BC), i.e. non-enzymatic chemical reactions compatible with living organisms, is increasingly used in conjunction with metabolically engineered microorganisms for producing compounds that do not usually occur naturally. Here we report production of one such compound, (3S)-acetoin, a valuable precursor for chiral synthesis, using...
a metabolically engineered Lactococcus lactis strain growing under respiratory conditions with ferric iron serving as a BC component. The strain used has all competing product pathways inactivated, and an appropriate cofactor balance is achieved by fine-tuning the respiratory capacity indirectly via the hemin concentration. We achieve high-level (3S)-acetoin production with a final titer of 66 mM (5.8 g/L) and a high yield (71% of the theoretical maximum). To the best of our knowledge, this is the first report describing production of (3S)-acetoin from sugar by microbial fermentation, and the results obtained confirm the potential that lies with BC for producing useful chemicals.

**General information**

State: Published
Organisations: National Food Institute, Research Group for Microbial Biotechnology and Biorefining
Authors: Liu, J. (Intern), Solem, C. (Intern), Jensen, P. R. (Intern)
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Main Research Area: Technical/natural sciences

**Publication information**

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Ratings:
BFI (2017): BFI-level 1
Web of Science (2017): Indexed Yes
BFI (2016): BFI-level 1
Scopus rating (2016): SJR 1.411 SNIP 1.163 CiteScore 4.14
Web of Science (2016): Indexed yes
BFI (2015): BFI-level 1
Scopus rating (2015): SJR 1.613 SNIP 1.37 CiteScore 4.44
Web of Science (2015): Indexed yes
BFI (2014): BFI-level 1
Scopus rating (2014): SJR 1.589 SNIP 1.401 CiteScore 4.16
Web of Science (2014): Indexed yes
BFI (2013): BFI-level 2
Scopus rating (2013): SJR 1.621 SNIP 1.425 CiteScore 4.44
ISI indexed (2013): ISI indexed yes
Web of Science (2013): Indexed yes
BFI (2012): BFI-level 2
Scopus rating (2012): SJR 1.639 SNIP 1.366 CiteScore 4.04
ISI indexed (2012): ISI indexed yes
Web of Science (2012): Indexed yes
BFI (2011): BFI-level 2
Scopus rating (2011): SJR 1.668 SNIP 1.483 CiteScore 4.08
ISI indexed (2011): ISI indexed yes
Web of Science (2011): Indexed yes
BFI (2010): BFI-level 2
Scopus rating (2010): SJR 1.538 SNIP 1.357
Web of Science (2010): Indexed yes
BFI (2009): BFI-level 2
Scopus rating (2009): SJR 1.491 SNIP 1.356
Web of Science (2009): Indexed yes
BFI (2008): BFI-level 1
Scopus rating (2008): SJR 1.238 SNIP 1.288
Web of Science (2008): Indexed yes
Scopus rating (2007): SJR 1.368 SNIP 1.362
Web of Science (2007): Indexed yes
Scopus rating (2006): SJR 1.458 SNIP 1.43
Web of Science (2006): Indexed yes
Stimulation of acetoin production in metabolically engineered Lactococcus lactis by increasing ATP demand

Having a sufficient supply of energy, usually in the form of ATP, is essential for all living organisms. In this study, however, we demonstrate that it can be beneficial to reduce ATP availability when the objective is microbial production. By introducing the ATP hydrolyzing F1-ATPase into a Lactococcus lactis strain engineered into producing acetoin, we show that production titer and yield both can be increased. At high F1-ATPase expression level, the acetoin production yield could be increased by 10%; however, because of the negative effect that the F1-ATPase had on biomass yield and growth, this increase was at the cost of volumetric productivity. By lowering the expression level of the F1-ATPase, both the volumetric productivity and the final yield could be increased by 5% compared to the reference strain not overexpressing the F1-ATPase, and in batch fermentation, it was possible to convert 176 mM (32 g/L) of glucose into 146.5 mM (12.9 g/L) acetoin with a yield of 83% of the theoretical maximum. To further demonstrate the potential of the cell factory developed, we complemented it with the lactose plasmid pLP712, which allowed for growth and acetoin production from a dairy waste stream, deproteinized whey. Using this cheap and renewable feedstock, efficient acetoin production with a titer of 157 mM (14 g/L) acetoin was accomplished.

General information
State: Published
Organisations: National Food Institute, Research Group for Microbial Biotechnology and Biorefining, Arla Foods
Authors: Liu, J. (Intern), Kandasamy, V. (Intern), Würtz, A. (Ekstern), Jensen, P. R. (Intern), Solem, C. (Intern)
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Publication date: 2016
Main Research Area: Technical/natural sciences

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Volume: 100
Issue number: 2
ISSN (Print): 0175-7598
Ratings:
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Web of Science (2017): Indexed Yes
BFI (2016): BFI-level 1
Scopus rating (2016): CiteScore 3.57 SJR 1.177 SNIP 1.173
Web of Science (2016): Indexed yes
BFI (2015): BFI-level 1
Scopus rating (2015): SJR 1.254 SNIP 1.217 CiteScore 3.43
Web of Science (2015): Indexed yes
BFI (2014): BFI-level 1
Scopus rating (2014): SJR 1.327 SNIP 1.458 CiteScore 3.71
Synthesis of (3R)-acetoin and 2,3-butanediol isomers by metabolically engineered Lactococcus lactis

The potential that lies in harnessing the chemical synthesis capabilities inherent in living organisms is immense. Here we demonstrate how the biosynthetic machinery of Lactococcus lactis, can be diverted to make (3R)-acetoin and the derived 2,3-butanediol isomers meso-(2,3)-butanediol (m-BDO) and (2R,3R)-butanediol (R-BDO). Efficient production of (3R)-acetoin was accomplished using a strain where the competing lactate, acetate and ethanol forming pathways had been blocked. By introducing different alcohol dehydrogenases into this strain, either EcBDH from Enterobacter cloacae or SadB from Achromobacter xylosoxidans, it was possible to achieve high-yield production of m-BDO or R-BDO respectively. To achieve biosustainable production of these chemicals from dairy waste, we transformed the above strains with the lactose plasmid pLP712. This enabled efficient production of (3R)-acetoin, m-BDO and R-BDO from processed whey waste, with titers of 27, 51, and 32 g/L respectively. The corresponding yields obtained were 0.42, 0.47 and 0.40 g/g
lactose, which is 82%, 89%, and 76% of maximum theoretical yield respectively. These results clearly demonstrate that L. lactis is an excellent choice as a cell factory for transforming lactose containing dairy waste into value added chemicals.

**General information**

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Organisations: National Food Institute, Research Group for Microbial Biotechnology and Biorefining
Authors: Kandasamy, V. (Intern), Liu, J. (Intern), Dantoft, S. H. (Intern), Solem, C. (Intern), Jensen, P. R. (Intern)
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Main Research Area: Technical/natural sciences

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BFI (2016): BFI-level 1
Scopus rating (2016): CiteScore 4.63 SJR 1.625 SNIP 1.401
Web of Science (2016): Indexed yes
BFI (2015): BFI-level 1
Scopus rating (2015): SJR 2.057 SNIP 1.684 CiteScore 5.3
Web of Science (2015): Indexed yes
BFI (2014): BFI-level 1
Scopus rating (2014): SJR 2.103 SNIP 1.544 CiteScore 4.75
Web of Science (2014): Indexed yes
BFI (2013): BFI-level 1
Scopus rating (2013): SJR 1.886 SNIP 1.51 CiteScore 4.06
ISI indexed (2013): ISI indexed yes
Web of Science (2013): Indexed yes
BFI (2012): BFI-level 1
Scopus rating (2012): SJR 1.458 SNIP 0.896 CiteScore 2.44
ISI indexed (2012): ISI indexed yes
Web of Science (2012): Indexed yes
ISI indexed (2011): ISI indexed no
Original language: English

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DOIs:
10.1038/srep36769
Source: FindIt
Source-ID: 2349022877
Publication: Research - peer-review › Journal article – Annual report year: 2016

**Adaptation of Lactococcus lactis to high growth temperature leads to a dramatic increase in acidification rate**

Lactococcus lactis is essential for most cheese making, and this mesophilic bacterium has its growth optimum around 30 °C. We have, through adaptive evolution, isolated a mutant TM29 that grows well up to 39 °C, and continuous growth at 40 °C is possible if pre-incubated at a slightly lower temperature. At the maximal permissive temperature for the wild-type, 38 °C, TM29 grows 33% faster and has a 12% higher specific lactate production rate than its parent MG1363, which results in fast lactate accumulation. Genome sequencing was used to reveal the mutations accumulated, most of which were shown to affect thermal tolerance. Of the mutations with more pronounced effects, two affected expression of single proteins (chaperone; riboflavin transporter), two had pleiotropic effects (RNA polymerase) which changed the gene expression profile, and one resulted in a change in the coding sequence of CDP-diglyceride synthase. A large deletion containing 10 genes was also found to affect thermal tolerance significantly. With this study we demonstrate a simple approach to obtain non-GMO derivatives of the important L. lactis that possess properties desirable by the industry, e.g. thermal robustness and increased rate of acidification. The mutations we have identified provide a genetic basis for further investigation of thermal tolerance.
**A New Type of YumC-Like Ferredoxin (Flavodoxin) Reductase Is Involved in Ribonucleotide Reduction**

The trxB2 gene, which is annotated as a thioredoxin reductase, was found to be essential for growth of Lactococcus lactis in the presence of oxygen. The corresponding protein (TrxB2) showed a high similarity with Bacillus subtilis YumC (E value = 4.0E-88), and YumC was able to fully complement the ΔtrxB2 mutant phenotype. YumC represents a novel type of ferredoxin (flavodoxin) reductase (FdR) with hitherto-unknown biological function. We adaptively evolved the ΔtrxB2 mutant under aerobic conditions to find suppressor mutations that could help elucidate the involvement of TrxB2 in aerobic growth. Genome sequencing of two independent isolates, which were able to grow as well as the wild-type strain under aerated conditions, revealed the importance of mutations in nrdI, encoding a flavodoxin involved in aerobic ribonucleotide reduction. We suggest a role for TrxB2 in nucleotide metabolism, where the flavodoxin (NrdI) serves as its redox partner, and we support this hypothesis by showing the beneficial effect of deoxynucleosides on aerobic growth of the ΔtrxB2 mutant. Finally, we demonstrate, by heterologous expression, that the TrxB2 protein functionally can substitute for YumC in B. subtilis but that the addition of deoxynucleosides cannot compensate for the lethal phenotype displayed by the B. subtilis yumC knockout mutant. Ferredoxin (flavodoxin) reductase (FdR) is involved in many important reactions in both eukaryotes and prokaryotes, such as photosynthesis, nitrate reduction, etc. The recently identified bacterial YumC-type FdR belongs to a novel type, the biological function of which still remains elusive. We found that the YumC-like FdR
(TrxB2) is essential for aerobic growth of Lactococcus lactis. We suggest that the YumC-type FdR is involved in the ribonucleotide reduction by the class Ib ribonucleotide reductase, which represents the workhorse for the bioconversion of nucleotides to deoxynucleotides in many prokaryotes and eukaryotic pathogens under aerobic conditions. As the partner of the flavodoxin (NrdI), the key FdR is missing in the current model describing the class Ib system in Escherichia coli. With this study, we have established a role for this novel type of FdR and in addition found the missing link needed to explain how ribonucleotide reduction is carried out under aerobic conditions.

**General information**
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**High-level ethanol production by metabolically engineered Lactococcus lactis using economically renewable feedstocks**

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Increased expression of pyruvate carboxylase and biotin protein ligase increases lysine production in a biotin prototrophic Corynebacterium glutamicum strain

Corynebacterium glutamicum, a Gram-positive bacterium used for the production of various biochemicals, is naturally a biotin auxotroph. We introduced the biotin genes from Bacillus subtilis on a plasmid, pBIO, into a lysine-producing derivative (termed AHP-3) that has been described previously, and achieved biotin prototrophy. We found that AHP-3, containing pBIO, was able to produce lysine in a medium lacking biotin and that the lysine yield on glucose was similar to what is obtained when using a medium containing biotin. However, there was a decrease in specific growth rate of 20% when the strain was cultivated without biotin, indicating a suboptimal intracellular concentration of biotin. In an attempt to locate the potential bottleneck, we added pimelic acid, an early biotin precursor, and found that growth rate could be restored fully, which demonstrates that the bottleneck is in pimeloyl-CoA (or pimeloyl-acyl Carrier Protein [ACP]) formation. Pyruvate carboxylase (pycA), a biotin-dependent enzyme needed for lysine biosynthesis and biotin ligase (birA), which is responsible for attaching biotin to pyruvate carboxylase, were overexpressed by replacing the native promoters with the strong superoxide dismutase (sod) promoter, to see whether growth could be restored. Neither pycA nor birA overexpression, whether alone or in combination, had an effect on specific growth rate, but they did have a positive effect on lysine yield, which increased by 55% in the strain overexpressing both enzymes.
Acetate Kinase Isozymes Confer Robustness in Acetate Metabolism

Acetate kinase (ACK) (EC no: 2.7.2.1) interconverts acetyl-phosphate and acetate to either catabolize or synthesize acetyl-CoA dependent on the metabolic requirement. Among all ACK entries available in UniProt, we found that around 45% are multiple ACKs in some organisms including more than 300 species but surprisingly, little work has been done to clarify whether this has any significance. In an attempt to gain further insight we have studied the two ACKs (AckA1, AckA2) encoded by two neighboring genes conserved in Lactococcus lactis (L. lactis) by analyzing protein sequences, characterizing transcription structure, determining enzyme characteristics and effect on growth physiology. The results show that the two ACKs are most likely individually transcribed. AckA1 has a much higher turnover number and AckA2 has a much higher affinity for acetate in vitro. Consistently, growth experiments of mutant strains reveal that AckA1 has a higher capacity for acetate production which allows faster growth in an environment with high acetate concentration. Meanwhile, AckA2 is important for fast acetate-dependent growth at low concentration of acetate. The results demonstrate that the two ACKs have complementary physiological roles in L. lactis to maintain a robust acetate metabolism for fast growth at different extracellular acetate concentrations. The existence of ACK isozymes may reflect a common evolutionary strategy in bacteria in an environment with varying concentrations of acetate.

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Development of droplets-based microfluidic systems for single-cell high-throughput screening

High-throughput screening (HTS) plays an important role in the development of microbial cell factories. One of the most popular approaches is to use microplates combined with the application of robotics, liquid handling and sophisticated detection methods. However, these workstations require large investment, and a logarithmic increase to screen large combinatorial libraries over the decades also makes it gradually out of depth. Here, we are trying to develop a feasible high-throughput system that uses microfluidics to compartmentalize a single cell for propagation and analysis in monodisperse picoliter aqueous droplets surround by an immiscible fluorinated oil phase. Our aim is to use this system to facilitate the screening process for both the biotechnology and food industry.

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Elucidating Flux Regulation of the Fermentation Modes of Lactococcus lactis: A Multilevel Study
The long history of application to the dairy industry has established Lactococcus lactis (L. lactis), the lactic acid bacterium, as one of the most extensively characterized low GC organisms. The relatively simple metabolism of L. lactis has also made it an attractive target for metabolic engineering for the production of non-food related chemicals. Moreover, the status of being the first genetically modified organism to deliver immunoproteins alive to human has brought L. lactis considerable fame in biomedical research. Beside the exceptional industrial relevance of L. lactis, it is also an important subject for basic research in cellular metabolism because L. lactis exhibits an interesting metabolic shift. Under anaerobic conditions, on fast fermentable sugars, L. lactis produces lactate as the primary product, known as homolactic fermentation but on slowly fermentable sugars, significant amounts of formate, acetate and ethanol are formed, known as mixed-acid fermentation. This shift is termed the mixedacid shift. This type of shift between a low-yield and a high-yield metabolism has drawn a lot of research focus and has similarly been observed in other bacteria, yeast and even tumor cells. Efforts have been put to find out the mechanism regulating the mixed-acid shift as well as to answer questions such as why L. lactis prefers such a switch. Until now, some pieces of evidence have been reported and several factors and models have been proposed as the keys to regulating the shift, including the expression level of certain genes in glycolysis
and fermentation pathways, the levels of the cofactors NADH, NAD+, ATP and ADP, the balance between catabolism and anabolism, etc. In this project, we studied the mixed-acid fermentation of L. lactis by (i) examining the roles of the enzymes in the mixed-acid fermentation pathway under different growth conditions; (ii) testing the predicted effect of the cofactors NADH, NAD+ on the mixed-acid shift proposed in previous studies; (iii) looking into the connection between amino acid metabolism and the mixed-acid shift; and (iv) contrasting the difference regarding the mixed-acid shift between two widely studied laboratory strains of L. lactis, MG1363 that shifts significantly and IL1403 that does not shift. We have measured the promoter activities of several mixed-acid genes which suggested that the regulatory elements governing the transcriptional regulation of the mixed-acid genes in MG1363 and IL1403 were different. This led us to performing experimental control analysis of the role of pyruvate formate-lyase (PFL) in MG1363 and IL1403. The expression of PFL in MG1363 appeared to be optimized for growth rate when growing on maltose whereas overexpressing PFL in IL1403 was probably detrimental. The two homologous acetate kinases in MG1363 were also characterized with respect to the transcription and enzyme activities. The isozymes were found to have complementary physiological roles that became important in acetate-producing or acetate-assimilating conditions respectively. The proposed roles of NADH and NAD+ on the mixed-acid shift were tested by perturbation via introducing activities of 2,3-butanediol dehydrogenase and supplying extracellular acetoin as an oxidizing agent. The additional NAD+ regenerating activities allowed a faster growth of MG1363 on maltose by shifting ethanol production into acetate production and also stimulated formate and acetate production in IL1403. Dependence of the mixed-acid fermentation of MG1363 on amino acid availability was observed and the impact of individual amino acids could differ significantly. Meanwhile, a computational method for combining metabolic flux analysis and elementary mode analysis was developed and applied to analyse a case of amino acid metabolism of L. lactis.
Microbial production of lysine from sustainable feedstock

Lysine is produced in a fermentation process using Corynebacterium glutamicum. And even though production strains have been improved for decades, there is still room for further optimization.
Synthetic promoter libraries for \textit{Corynebacterium glutamicum}

The ability to modulate gene expression is an important genetic tool in systems biology and biotechnology. Here, we demonstrate that a previously published easy and fast PCR-based method for modulating gene expression in lactic acid bacteria is also applicable to \textit{Corynebacterium glutamicum}. We constructed constitutive promoter libraries based on various combinations of a previously reported \textit{C. glutamicum} -10 consensus sequence (ggnT\textgro/TA(c/t)aaTgg) and the \textit{Escherichia coli} -35 consensus, either with or without an AT-rich region upstream. A promoter library based on consensus sequences frequently found in low-GC Gram-positive microorganisms was also included. The strongest promoters were found in the library with a -35 region and a \textit{C. glutamicum} -10 consensus, and this library also represents the largest activity span. Using the alternative -10 consensus TATAAT, which can be found in many other prokaryotes, resulted in a weaker but still useful promoter library. The upstream AT-rich region did not appear to affect promoter strength in \textit{C. glutamicum}. In addition to the constitutive promoters, a synthetic inducible promoter library, based on the \textit{E. coli} lac-promoter, was constructed by randomizing the 17-bp spacer between -35 and -10 consensus sequences and the sequences surrounding these. The inducible promoter library was shown to result in β-galactosidase activities ranging from 284 to 1,665 Miller units when induced by IPTG, and the induction fold ranged from 7–59. We find that the synthetic promoter library (SPL) technology is convenient for modulating gene expression in \textit{C. glutamicum} and should have many future applications, within basic research as well as for optimizing industrial production organisms.
Transforming Lactococcus lactis into a microbial cell factory

Biological conversion of lignocellulosic biomass to biofuels and -chemicals is a promising technology to reduce dependency on fossil fuels. This is important considering the environmental problems associated with consumption of the fossil fuels together with the fact that the reserves are limited and will be depleted if the increasing demand continues. However, one of the main challenges in the biological conversion is the identification of suitable platform organisms that can convert all the sugars present in the lignocellulosic biomass, including xylose. The aim of this PhD project was to investigate the potential of Lactococcus lactis as a platform organism for production of biofuels and-chemicals with a focus on characterization and optimization of the xylose metabolism. The plant isolate L. lactis KF147 was selected as the potential platform organism due to its natural ability to utilize both the pentose sugars xylose and arabinose. One of the desirable traits of a good platform organism is that it is easy to manipulate genetically. Since genetic manipulation usually involves introducing exogenous DNA, it is important that suitable methods are available. For this reason a standard protocol for preparing competent L. lactis KF147 cells was optimized resulting in a 100-fold increase in the transformation efficiency. Tools for introducing genes are likewise important. To expand the repertoire of genetic engineering tools available for L. lactis a novel tool named Repetitive Marker-Free Site-Specific Integration was developed. This tool facilitates repetitive rounds of site-specific integration of genes into the chromosome of L. lactis without leaving behind undesired vector elements. The site-specific integration is based on elements from the temperate lactococcal phage TP901-1, whereas excision of undesirable elements relies on a modified Cre-loxP system and 5-fluoroorotate mediated counter-selection. The plasmid used for the site-specific integration was termed pKV6 and when it is used for integrating
genes, a new attachment site, attBmin, is likewise introduced, which can subsequently be used for the next round of integration. The xylose metabolism in L. lactis KF147 was characterized in a defined medium supplemented with 0.2%, 1% or 3% (w/v) xylose. The defined medium contains free arginine, and it was found that L. lactis KF147 co-metabolizes the arginine through the arginine deiminase pathway. To simplify further analysis arcA encoding the arginine deiminase was deleted, thus eliminating the arginine catabolism. We found that in L. lactis KF147 xylose is metabolized through two pathways namely the phosphoketolase pathway and the non-oxidative part of the pentose phosphate pathway. The only products formed were lactate, formate, acetate and ethanol, and the composition of the products depended on the xylose concentration. As xylose concentration increased, the proportion of xylose metabolized through the pentose phosphate pathway also increased. The effect from deleting and over-expressing the phosphoketolase pathway was also studied. The latter was achieved by introducing an additional copy of the ptk gene encoding the phosphoketolase. When the phosphoketolase pathway was deleted the product composition was similar to a typical mixed-acid pattern where formate, acetate and ethanol are formed in the ratio 2:1:1 in addition to lactate. For the strain with an inactive phosphoketolase pathway as well as for its parent, lactate production increased with xylose concentration, but in the phosphoketolase deficient strain up to almost three times more lactate was formed. In contrast when ptk was overexpressed the flux through the phosphoketolase pathway was 1.5 times higher compared to the parent strain when grown with 3% xylose. In addition, a clear shift towards a more mixed-acid fermentation profile was observed. The effect of adding additional arginine to the medium was likewise investigated in regards to growth of L. lactis KF147 and product composition. At low xylose concentration (0.2%) additional arginine greatly stimulated the growth of L. lactis KF147. In contrast, no effect of growth was observed at the higher xylose concentrations (1%, 3%). Irrespective of xylose concentration, arginine did affect the xylose and product fluxes, which all decreased as the arginine concentration increased. The final product yields were, however, not affected. L. lactis KF147 was also grown in rich M17 medium with xylose which showed that product composition strongly depends on the growth medium as the yield of lactate per xylose increase significantly. The pentose phosphate pathway present in L. lactis KF147 and other genome sequenced L. lactis strains is, however, a modified version of the known pentose phosphate pathway as no transaldolase gene is present in any of the these strains. A codon optimized version of the transaldolase gene ywhf from B. subtilis 168 was introduced in the phosphoketolase deficient strain. The effect of the introduced transaldolase gene was investigated in defined medium with 0.2% and 3% (w/v) xylose; however, no significant effects on either the growth rates or product formation were observed; even though expression of the introduced transaldolase was confirmed. The xyIT gene, predicted to encode a D-xylose/H+ linked symporter, was deleted and the effect on growth evaluated in defined medium with 0.2% and 1% (w/v) xylose. Only at the low xylose concentration was a reduced growth rate observed for the ΔxyIT strain compared to the parent strain. Based on this it was deduced that more than one transport system for xylose is present in L. lactis KF147. An adaptive evolution experiment was carried out with the goal of isolating mutants with improved growth on xylose. For this purpose the phosphoketolase deficient strain was applied. Of the 44 evolved strains screened for improved growth on xylose, three were selected for more detailed studies where specific growth rate and product formation were determined. These three strains underwent whole genome sequencing as well. The most interesting evolved strain was AD29 that, as the only of the investigated strains, exhibited both a pronounced accelerated growth on xylose (62.5% faster), and a changed fermentation profile with a clear increase in lactate production and corresponding drop in the production of formate, acetate, and ethanol. Three adaptive mutations were identified in AD29. Two is by all accounts involved in regulatory mechanisms either to stress (yhbB) or more globally (ytgF), and the last facilitate improved uptake of xylose (ptnC). Based on the above findings we conclude that L. lactis KF147 possesses many of the features a platform organism need, however whether the industry will find it attractive remains to be seen.

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Characterization of Lactococcus lactis mutants with improved performance at high temperatures and potential dairy applications
Lactococcus lactis (L. lactis) is a Gram-positive mesophile, which has considerable importance in the dairy industry for production of cheese and butter milk, and which carries the “GRAS” (generally recognized as safe) designation. Temperature has a great impact on dairy fermentation processes through its effect on the starter culture. The optimum and maximum temperature for most L. lactis strains are approximately 30°C and 38°C, respectively. Increasing the fermentation temperature could have several beneficial effects, e.g. reduce bacteriophage attacks and increase acidification rate, the latter because the increased energy consumption at high temperatures potentially could stimulate glycolysis. However in many cases the fitness is affected and mostly negative effects on productivity are observed. In this study, the non-GMO approach, experimental adaptation, was employed for isolating thermo-tolerant L. lactis. The adaptation was carried out using a serial-transfer regime at steadily increasing temperatures, and the strain used was L.
lactis subsp. cremoris MG1363, which is a well-characterized dairy isolate. After exposure to increasing temperatures over 900 generations, one mutant (TM29) capable of growing at 40ºC was successfully isolated. By determining the temperature dependent growth rate profile, a shift of the optimum temperature from 30ºC to 36ºC was observed for TM29. Meanwhile, metabolic flux analysis revealed that TM29 was able to hold higher glucose consumption and lactate production rates when compared to MG1363 at high temperatures.

Whole genome re-sequencing identified 13 SNPs, one DIP and one large deletion in TM29, and additional sequencing of the isolated intermediates indicated dynamic accumulation of mutations with rising fitness in a temporal order. DNA microarray analysis revealed apparent differences in the transcriptional response to heat between the mutant and parent. It was found that SNPs preceding gene groESL and ribU resulted in over-expression of chaperone proteins GroES-GroEL, and membrane associated riboflavin transporter protein RibU in TM29, respectively. Moreover, a large deletion in TM29 caused the inactivation of 10 genes (llmg_1349-llmg_1358).

Through allelic replacement and gene knockout followed by fitness assessment, four main positive mutations were eventually discovered. The SNP preceding groESL and deletion of llmg_1349-llmg_1358 contributed to 30% and 10% increase in the growth rate of MG1363 at 38ºC, respectively. The over-expression that was caused by the SNP preceding ribU relived FAD starvation, which results in insufficient pyruvate dehydrogenase and NADH oxidase at high temperatures. Through replacing the mutated rpoC allele, that encodes the β subunit of the RNA polymerase, MG1363 exhibited extended maximum growth temperature with concomitant phenotypic changes, e.g. with respect to morphology and cellular fatty acid composition.

At last, acidification capability in milk was compared at different temperatures, for the endpoint mutant TM29 and the wild-type MG1363. It was found that TM29 was able acidify milk at 40ºC, whereas MG1363 could not. Tradeoffs, as a consequence of using a synthetic medium for the evolution, were also observed. TM29 acidified milk slower than MG1363 at 36ºC, which was not in accordance with growth in synthetic medium.

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### Engineering strategies aimed at control of acidification rate of lactic acid bacteria
The ability of lactic acid bacteria to produce lactic acid from various sugars plays an important role in food fermentations. Lactic acid is derived from pyruvate, the end product of glycolysis and thus a fast lactic acid production rate requires a high glycolytic flux. In addition to lactic acid, alternative end products - ethanol, acetic acid and formic acid - are formed by many species. The central role of glycolysis in lactic acid bacteria has provoked numerous studies aiming at identifying potential bottleneck(s) since knowledge about flux control could be important not only for optimizing food fermentation processes, but also for novel applications of lactic acid bacteria, such as cell factories for the production of green fuels and chemicals. With respect to the control and regulation of the fermentation mode, some progress has been made, but the question of which component(s) control the main glycolytic flux remains unanswered. © 2012 Elsevier Ltd.

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Investigation of glycerol assimilation and cofactor metabolism in Lactococcus lactis
The production of biodiesel has been steadily increasing during the last decade, and with it crude glycerol as a byproduct. Despite being rich in glycerol, the increased supply has saturated the demand for glycerol, making purification a non-viable option. The background for this project was to investigate the suitability of lactic acid bacteria as production organisms for the production of biofuels and biochemicals. Specifically, the goal was to adapt the model organism Lactococcus lactis to convert crude glycerol, to value-added fuels or chemicals. Work was divided between four main
areas: life cycle assessment of the GLYFINERY project, screening of L. lactis spp. for glycerol utilization, engineering of glycerol metabolism in L. lactis and finally an investigation into perturbation of energy metabolism in L. lactis. The work from the life cycle assessment resulted in two reports, detailing the technological requirements for the GLYFINERY processes. These have been included in the appendix (section A).

The screening did not reveal any L. lactis strains capable of assimilating glycerol nor did it reveal any conditions favorable to glycerol dissimilation in L. lactis. The conditions evaluated were: anaerobic, aerobic and respiration permissive growth in combination with either glycerol as a sole substrate or with co-metabolization of glycerol with common sugar substrates. Although no growth on glycerol was seen, both positive and detrimental effects were observed from cultures with glycerol supplementation.

The positive effects were observed from cultivation of L. lactis IL1403 with trehalose as a substrate under aerated conditions. Under these conditions, the supplementation of glycerol would cause an increase in biomass production of over night cultures. The growth rate of the cultures with glycerol supplementation were determined to be 84% of the reference cultures without glycerol. The detrimental effects of glycerol were observed as reduced growth rate and decreased biomass formation. The effects were observed when cultivating plant isolates of L. lactis on xylose. The effect manifested itself under both anaerobic and respiration permissive conditions, but was not found to have the same profound effect on other sugar substrates such as galactose or ribose.

Supplementation of nucleosides to the growth medium or increased substrate concentration were found to counteract the inhibitory effects and improve the growth rate, though not completely to the level of the reference strain. The fact that this effect was predominantly observed while utilizing xylose implicates the involvement of the pentose phosphate pathway. A possible mechanism underlying the observed growth characteristics under anaerobic conditions could be a rise in triosephosphate levels (the entry point of glycerol in glycolysis) regulating pyruvate formate-lyase. Under aerobic and respiration permissive conditions, the rise in the redox level from channeling glycerol into metabolism could possibly regulate both glyceraldehyde-3-phosphate dehydrogenase and the pyruvate dehydrogenase complex, disrupting flow through the central metabolism and ATP production.

If this is the case, the question remains, as to why the excess redox is not simply removed by respiration. The results from this investigation have provided an initial characterization of the inhibitory effects and some possible directions for future investigations, but more work is needed to fully elucidate the mechanism and target of inhibition.

The engineering of glycerol metabolism in L. lactis was initiated from three different perspectives: overexpression of glycerol kinase from L. lactis, introduction of a heterologous glycerol assimilation pathway and construction of a library of NADH oxidase activity. Based on a preliminary analysis of transcript level data, an attempt was made to stimulate glycerol assimilation by overexpressing the glycerol kinase already present in L. lactis. The construction and verification of a strain with increased glycerol kinase activity was not fully completed and is still ongoing.

Similarly the construction of mutants expressing a heterologous pathway for glycerol dissimilation is also an ongoing task. An artificial glycerol assimilation operon was designed based on components from known glycerol metabolizers. Three genetic elements were placed in the operon: the glycerol facilitator glpF from E. coli, the glycerol dehydrogenase dhaD from Citrobacter freundii and the dihydroxyacetone kinase dhaK also from Citrobacter freundii. These were arranged in an operon structure where glpF was placed in front of dhaD and dhaK. Ribosomal binding sites from glycolytic promoters in L. lactis were placed in front of each gene. The operon was introduced into L. lactis with expression modulated by a synthetic promoter library.

Lastly, to prevent possible issues with redox accumulation during growth on glycerol, a library of mutants with NADH oxidase activity was constructed and verified by enzymatic assays. Despite the NADH oxidase activity, no growth could be detected in defined medium supplemented with glycerol as sole carbon and energy source. This could possibly be connected to the expression levels of the library, which were in the lower range.

Investigations were also made into the response of L. lactis mutants to perturbations in energy metabolism. The motivation was to apply, transcriptomic and metabolomic techniques that were not available at the time of the previous characterization by Koebmann et al., in 2002. To minimize noise and pleiotropic effects, strains with mild perturbations were selected for transcriptomic analysis. For the purpose of investigating the changes in internal metabolite concentrations, a mutant with very high ATPase activity was included with the mildly perturbed strains. The data obtained from the metabolomic study of internal metabolites, did not provide any novel observations and did not substantiate the results from the transcriptomic investigation.

Although significance of the transcriptomic analysis was affected by technical issues, the overall impression gathered from the response to perturbation of ATP levels, was that the genes were generally downregulated. Glycolysis along with most of the anabolic pathways were downregulated in what resembled a starvation response. During hydrolysis of ATP two signals were generated, lowered energy state and increased inorganic phosphate levels (Pi). The exact contribution of each signal along with many other interesting observations will need to be confirmed by additional experiments and further investigation in future studies.

The task of making Lactococcus lactis grow on glycerol as a sole carbon and energy source still remains to be accomplished. It will require continued efforts in the three areas investigated in this work and others, to fulfill this task. Hopefully, future investigations can successfully bridge the integration of the complex challenges encountered, when engineering central carbon metabolism, to complete the goal.

**General information**

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Optimization of lysine metabolism in Corynebacterium glutamicum

Commercial pig and poultry production use the essential amino acid lysine as a feed additive with the purpose of optimizing the feed utilization. Lysine is produced by a fermentation process involving either Corynebacterium glutamicum or Escherichia coli. The global annual production is around 1,000,000 tons. The aim of this project is to optimize the yield of lysine in C. glutamicum using metabolic engineering strategies.

According to a genome scale model of C. glutamicum, theoretically there is much room for increasing the lysine yield (Kjeldsen and Nielsen 2009). Lysine synthesis requires NADPH, and increased NADPH availability is therefore a potential way to enhance lysine production. The generation of NADPH is mainly located in the pentose phosphate pathway (PPP). Using the genome scale model the phosphoglucoisomerase enzyme (PGI) has been identified as a possible bottleneck in the metabolism, which the project intends to eliminate. PGI catalyzes the conversion of alpha-D-glucose-6-phosphate to fructose-6-phosphate just downstream of the branch in the glycolysis, but it also catalyzes the reverse reaction. It is unknown whether up- or down-regulation of the pgi is required to increase the flux through the PPP, increasing the NADPH synthesis and enabling increased lysine production.

Synthetic promoter libraries (SPL) enable fine tuning of the expression of genes. To test the feasibility of SPL in C. glutamicum four constitutive SPLs and one inducible SPL were constructed. The libraries were placed in front of a β-galactosidase gene and the activity of the enzyme was measured to quantify the different promoter strengths in the libraries. The results demonstrate that the SPL technology is an applicable technique for metabolic engineering in C. glutamicum.

SPL was used to both up- and down-regulate the expression of pgi, and it was measured how the regulations affected the lysine production. In this thesis it is shown that under the applied growth conditions it is possible to increase the lysine yield by a factor of 1.6 from 0.0086 mole lysine • mole glucose⁻¹ to 0.0138 mole lysine • mole glucose⁻¹.

Oxidative Stress at High Temperatures in Lactococcus lactis Due to an Insufficient Supply of Riboflavin.

Lactococcus lactis MG1363 was found to be unable to grow at temperatures above 37°C in a defined medium without riboflavin, and the cause was identified to be dissolved oxygen introduced during preparation of the medium. At 30°C, growth was unaffected by dissolved oxygen and oxygen was consumed quickly. Raising the temperature to 37°C resulted in severe growth inhibition and only slow removal of dissolved oxygen. Under these conditions, an abnormally low intracellular ratio of [ATP] to [ADP] (1.4) was found (normally around 5), which indicates that the cells are energy limited. By adding riboflavin to the medium, it was possible to improve growth and oxygen consumption at 37°C, and this also normalized the [ATP]-to-[ADP] ratio. A codon-optimized redox-sensitive green fluorescent protein (GFP) was introduced into L. lactis and revealed a more oxidized cytoplasm at 37°C than at 30°C. These results indicate that L. lactis suffers from heat-induced oxidative stress at increased temperatures. A decrease in intracellular flavin adenine dinucleotide (FAD), which is derived from riboflavin, was observed with increasing growth temperature, but the presence of riboflavin made the decrease smaller. The drop was accompanied by a decrease in NADH oxidase and pyruvate dehydrogenase activities, both of which depend on FAD as a cofactor. By overexpressing the riboflavin transporter, it was possible to improve FAD biosynthesis, which resulted in increased NADH oxidase and pyruvate dehydrogenase activities and improved fitness at high temperatures in the presence of oxygen.
We present a tool for repetitive, marker-free, site-specific integration in Lactococcus lactis, in which a nonreplicating plasmid vector (pKV6) carrying a phage attachment site (attP) can be integrated into a bacterial attachment site (attB). The novelty of the tool described here is the inclusion of a minimal bacterial attachment site (attBmin), two mutated loxP sequences (lox66 and lox71) allowing for removal of undesirable vector elements (antibiotic resistance marker), and a counterselection marker (oroP) for selection of loxP recombination on the pKV6 vector. When transformed into L. lactis expressing the phage TP901-1 integrase, pKV6 integrates with high frequency into the chromosome, where it is flanked by attL and attR hybrid attachment sites. After expression of Cre recombinase from a plasmid that is not able to replicate in L. lactis, loxP recombinants can be selected for by using 5-fluoroorotic acid. The introduced attBmin site can subsequently be used for a second round of integration. To examine if attP recombination was specific to the attB site, integration was performed in strains containing the attB, attL, and attR sites or the attL and attR sites only. Only attP-attB recombination was observed when all three sites were present. In the absence of the attB site, a low frequency of attP-attL recombination was observed. To demonstrate the functionality of the system, the xylose utilization genes (xylABR and xylT) from L. lactis strain KF147 were integrated into the chromosome of L. lactis strain MG1363 in two steps.

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Rewiring Lactococcus lactis for Ethanol Production

Lactic acid bacteria (LAB) are known for their high tolerance toward organic acids and alcohols (R. S. Gold, M. M. Meagher, R. Hutkins, and T. Conway, J. Ind. Microbiol. 10:45–54, 1992) and could potentially serve as platform organisms for production of these compounds. In this study, we attempted to redirect the metabolism of LAB model organism Lactococcus lactis toward ethanol production. Codon-optimized Zymomonas mobilis pyruvate decarboxylase (PDC) was introduced and expressed from synthetic promoters in different strain backgrounds. In the wild-type L. lactis strain MG1363 growing on glucose, only small amounts of ethanol were obtained after introducing PDC, probably due to a low native alcohol dehydrogenase activity. When the same strains were grown on maltose, ethanol was the major product and lesser amounts of lactate, formate, and acetate were formed. Inactivating the lactate dehydrogenase genes ldhX, ldhB, and ldh and introducing codon-optimized Z. mobilis alcohol dehydrogenase (ADHB) in addition to PDC resulted in high-yield ethanol formation when strains were grown on glucose, with only minor amounts of by-products formed. Finally, a strain with ethanol as the sole observed fermentation product was obtained by further inactivating the phosphotransacetylase (PTA) and the native alcohol dehydrogenase (ADHE).
Tunable promoters in synthetic and systems biology.

Synthetic and systems biologists need standardized, modular and orthogonal tools yielding predictable functions in vivo. In systems biology such tools are needed to quantitatively analyze the behavior of biological systems while the efficient engineering of artificial gene networks is central in synthetic biology. A number of tools exist to manipulate the steps in between gene sequence and functional protein in living cells, but out of these the most straight-forward approach is to alter the gene expression level by manipulating the promoter sequence. Some of the promoter tuning tools available for accomplishing such altered gene expression levels are discussed here along with examples of their use, and ideas for new tools are described. The road ahead looks very promising for synthetic and systems biologists as tools to achieve just about anything in terms of tuning and timing multiple gene expression levels using libraries of synthetic promoters now exist.
Towards a quantitative prediction of the fluxome from the proteome

The promise of proteomics and fluxomics is limited by our current inability to integrate these two levels of cellular organization. Here we present the derivation, experimental parameterization, and appraisal of flux functions that enable the quantitative prediction of changes in metabolic fluxes from changes in enzyme levels. We based our derivation on the hypothesis that, in the determination of steady-state flux changes, the direct proportionality between enzyme concentrations and reaction rates is principal, whereas the complexity of enzyme–metabolite interactions is secondary and can be described using an approximate kinetic format. The quality of the agreement between predicted and experimental fluxes in Lactococcus lactis, supports our hypothesis and demonstrates the need and usefulness of approximative descriptions in the study of complex biological systems. Importantly, these flux functions are scalable to genome-wide networks, and thus drastically expand the capabilities of flux prediction for metabolic engineering efforts beyond those conferred by the currently used constraints-based models.
Metabolic and Transcriptional Response to Cofactor Perturbations in Escherichia coli

Metabolic cofactors such as NADH and ATP play important roles in a large number of cellular reactions, and it is of great interest to dissect the role of these cofactors in different aspects of metabolism. Toward this goal, we overexpressed NADH oxidase and the soluble F1-ATPase in Escherichia coli to lower the level of NADH and ATP, respectively. We used a global interaction network, comprising of protein interactions, transcriptional regulation, and metabolic networks, to integrate data from transcription profiles, metabolic fluxes, and the metabolite levels. We identified high-scoring networks for the two strains. The results revealed a smaller, but denser network for perturbations of ATP level, compared with that of NADH level. The action of many global transcription factors such as ArcA, Fnr, CRP, and IHF commonly involved both NADH and ATP, whereas others responded to either ATP or NADH. Overexpressing NADH oxidase invokes response in widespread aspects of metabolism involving the redox cofactors (NADH and NADPH), whereas ATPase has a more focused response to restore ATP level by enhancing proton translocation mechanisms and repressing biosynthesis. Interestingly, NADPH played a key role in restoring redox homeostasis through the concerted activity of isocitrate dehydrogenase and UdhA transhydrogenase. We present a reconciled network of regulation that illustrates the overlapping and distinct aspects of metabolism controlled by NADH and ATP. Our study contributes to the general understanding of redox and energy metabolism and should help in developing metabolic engineering strategies in E. coli.
Phosphoglycerate Mutase Is a Highly Efficient Enzyme without Flux Control in Lactococcus lactis

The glycolytic enzyme phosphoglycerate mutase (PGM), which catalyzes the conversion of 3-phosphoglycerate to 2-phosphoglycerate, was examined in Lactococcus lactis with respect to its function, kinetics and glycolytic flux control. A
library of strains with PGM activities ranging between 15-465% of the wild-type level was constructed by replacing the
native promoter of pgm with synthetic promoters of varying strengths. The specific growth rate and glucose flux were
found to be maximal at the wild-type level at which PGM had no flux control. Low flux control of PGM was found on mixed
acid fluxes at highly reduced PGM activities. At the wild-type level PGM operated very far from V-max. Consequently, in a
strain with only 15% PGM activity, the catalytic rate of PGM was almost six times higher than in the wildtype. K-m of PGM
for 3-phosphoglycerate was 1.0 m M and k(cat) was 3,200 s(-1). The L. lactis PGM was dependent on 2,3-
bisphosphoglyceric acid for activity, which showed that the enzyme is of the dPGM type in accordance with its predicted
homology to dPGM enzymes from other organisms. In conclusion, PGM from L. lactis is a highly efficient catalyst, which
partially explains why this enzyme has limited control in wild-type L. lactis.

General information
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Authors: Solem, C. (Intern), Petranovic, D. (Ekstern), Købmann, B. (Intern), Mijakovic, I. (Intern), Jensen, P. R. (Intern)
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Scopus rating (2012): SJR 0.893 SNIP 0.767 CiteScore 1.87
ISI indexed (2012): ISI indexed yes
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ISI indexed (2011): ISI indexed yes
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BFI (2010): BFI-level 1
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Web of Science (2009): Indexed yes
BFI (2008): BFI-level 1
Scopus rating (2008): SJR 1.424 SNIP 0.613
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Scopus rating (2005): SJR 1.578 SNIP 0.769
Web of Science (2005): Indexed yes
Scopus rating (2004): SJR 1.314 SNIP 0.682
Scopus rating (2003): SJR 1.399 SNIP 0.808
Scopus rating (2002): SJR 1.367 SNIP 0.693
Scopus rating (2001): SJR 1.214 SNIP 0.867
Co-factor engineering in lactobacilli: Effects of uncoupled ATPase activity on metabolic fluxes in Lactobacillus (L.) plantarum and L. sakei

The hydrolytic F-1-part of the F1F0-ATPase was over-expressed in Lactobacillus (L.) plantarum NC8 and L. sakei Lb790x during fermentation of glucose or ribose, in order to study how changes in the intracellular levels of ATP and ADP affect the metabolic fluxes. The uncoupled ATPase activity resulted in a decrease in intracellular energy level (ATP/ADP ratio), biomass yield and growth rate. Interestingly, the glycolytic and ribolytic flux increased in L. plantarum with uncoupled ATPase activity compared to the reference strain by up to 20% and 50%, respectively. The ATP demand was estimated to have approximately 80% control on both the glycolytic and ribolytic flux in L. plantarum under these conditions. In contrast, the glycolytic and ribolytic flux decreased in L. sakei with uncoupled ATPase activity. (C) 2008 Elsevier Inc. All rights reserved.
Triosephosphate isomerase (TPI), which catalyses the conversion of dihydroxyacetone phosphate (DHAP) to glyceraldehyde-3-phosphate (G3P), was studied for its control on glycolysis and mixed acid production in L. lactis subspecies lactis IL1403 and L. lactis subspecies cremoris MG1363. Strains in which the TPI activity was modulated from 3%-225% (IL1403) or 13%-103% (MG1363) of the wild-type level were constructed by changing the expression of the tpiA gene. The enzyme was found to be present in high excess in the wild-type cells and 10% TPI activity still supported more than 70% of the wild-type glycolytic flux in both strains. Homolactic product formation was preserved throughout the range of TPI activities studied, although a slight increase in the amount of acetate and formate production was observed in the strains with strongly reduced TPI activity for both IL1403 and MG1363. The upstream metabolites glucose-6-phosphate, fructose-1,6-bisphosphate and DHAP in the IL1403 derivatives were essentially unchanged for TPI activities from 26% to 225%. At a TPI activity of 3%, the level of DHAP increased four times. The finding that an increased level of DHAP coincides with an increase in formate production is surprising and indicates that pyruvate formate lyase is not inhibited by DHAP under these conditions.
Increased biomass yield of *Lactococcus lactis* during energetically limited growth and respiratory conditions

*Lactococcus lactis* is known to be capable of respiration under aerobic conditions in the presence of haemin. In the present study the effect of respiration on ATP production during growth on different sugars was examined. With glucose as the sole carbon source, respiratory conditions in *L. lactis* MG1363 resulted in only a minor increase, 21%, in biomass yield. Since ATP production through substrate-level phosphorylation was essentially identical with and without respiration, the increased biomass yield was a result of energy-saving under respiratory conditions estimated to be 0.4 mol of ATP/mol of glucose. With maltose as the energy source, the increase in biomass yield amounted to 51% compared with an aerobic culture that lacked haemin. This higher ATP yield was obtained by redirecting pyruvate metabolism from lactate to acetate production, and from savings through respiration. However, even after subtracting these contributions, approx. 0.3 mol of ATP/mol of glucose remained unaccounted for. A similar response to respiratory conditions (0.2 mol of ATP/mol of glucose) was observed in a mutant that had a decreased glucose uptake rate during growth on glucose caused by disruption of the PTSmannose (glucose/mannose-specific phosphotransferase system). Amino acid catabolism could be excluded as the source of the additional ATP. Since mutants without a functional H+-ATPase produced less ATP under sugar starvation and respiratory conditions, the additional ATP yield appears to come partly from energy saved on proton pumping through the H+-ATPase due to respiration and partly from a reversed function of the H+-ATPase towards oxidative phosphorylation. These results may contribute to the design and implementation of carbon-efficient high-cell-density cultures of this industrially important species of bacterium.
Plasmid pCS1966, a new selection/counterselection tool for strain construction in Lactic Acid Bacteria based on the oroP gene encoding an orotate transporter from Lactococcus lactis

General information
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Organisations: Center for Systems Microbiology, Department of Systems Biology
Authors: Solem, C. (Intern), Defoor, E. M. C. (Intern), Jensen, P. R. (Intern), Martinussen, J. (Intern)
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Main Research Area: Technical/natural sciences
The extent of co-metabolism of glucose and galactose by L. lactis changes with the expression of the lacSZ operon from Streptococcus thermophilus

The lactose transporter and β-galactosidase from Streptococcus thermophilus, encoded by the lacSZ operon, were introduced into the lactose-negative strain Lactococcus lactis MG1363 and the expression of the lacSZ operon was modulated by substitution of the native promoter with randomized synthetic promoters. A series of strains with various expression levels of lacSZ were examined for their fermentation of lactose. Strains with a high expression level were found to metabolize lactose in a similar manner to S. thermophilus, i.e. the galactose moiety of lactose was excreted to the growth medium and only glucose was metabolized in glycolysis. Interestingly, strains with low expression of the operon showed a mixed acid metabolism and co-metabolism of galactose and glucose. The lactose flux increased gradually with increasing expression of the lacSZ operon until an optimum was observed at intermediate β-galactosidase activities of 2000-3000 Miller units. At higher expression levels, the flux decreased. These strains had a glycolytic flux comparable with those of reference strains with the standard lactococcal PTSlac (lactose phosphotransferase transport system) lactose transporter, which indicates that lactose transport is not rate-limiting for glycolysis in Lactococcus. Finally, an additional ATP drain was introduced into the fastest growing strain, CS2004, to test whether the ATP demand controlled glycolysis under these conditions, but in fact no increase in glycolytic flux was observed. © 2008 Portland Press Ltd.
The las enzymes control pyruvate metabolism in Lactococcus lactis during growth on maltose

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Organisations: Department of Systems Biology
Authors: Solem, C. (Intern), Købmann, B. J. (Intern), Yang, F. (Ekstern), Jensen, P. R. (Intern)
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Web of Science (2015): Indexed yes
BFI (2014): BFI-level 1
Scopus rating (2014): SJR 2.069 SNIP 0.937 CiteScore 2.72
Web of Science (2014): Indexed yes
BFI (2013): BFI-level 1
Scopus rating (2013): SJR 2.136 SNIP 1.018 CiteScore 3
ISI indexed (2013): ISI indexed yes
Web of Science (2013): Indexed yes
BFI (2012): BFI-level 1
Scopus rating (2012): SJR 2.103 SNIP 1.092 CiteScore 3.42
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BFI (2011): BFI-level 1
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Control analysis of the importance of phosphoglycerate enolase for metabolic fluxes in Lactococcus lactis subsp. lactis IL1403.

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Organisations: Center for Microbial Biotechnology, Department of Systems Biology
Authors: Købmann, B. J. (Intern), Solem, C. (Intern), Jensen, P. R. (Intern)
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Journal: IEE Proceedings - Systems Biology
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Control analysis as a tool to understand the formation of the las operon in Lactococcus lactis

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Web of Science (2014): Indexed yes
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BFI (2011): BFI-level 1
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Control analysis as a tool to understand the formation of the las operon in Lactococcus lactis

In Lactococcus lactis the enzymes phosphofructokinase (PFK), pyruvate kinase (PK) and lactate dehydrogenase (LDH) are uniquely encoded in the las operon and we here apply Metabolic Control Analysis to study the role of this organisation. Earlier work showed that LDH at wildtype level has zero control on glycolysis and growth rate but high negative control on formate production. We find that PFK and PK have zero control on glycolysis and growth rate at the wildtype enzyme level but both enzymes exert strong positive control on the glycolytic flux at reduced activities. PK has high positive control on formate and acetate production, whereas PFK has no control on these fluxes. Decreased expression of the entire las operon resulted in a strong decrease in growth rate and the glycolytic flux; at 53% expression of the las operon the glycolytic flux was reduced to 44% and the flux control coefficient increased towards 3. Increased las expression resulted in a slight decrease in the glycolytic flux. At the wildtype level the control was close to zero on both glycolysis and the pyruvate branches. The sum of control coefficients for the three enzymes individually was comparable to the control coefficient found for the entire operon; the strong positive control by PK almost cancels out the negative control by LDH on formate production. The analysis suggests that co-regulation of PFK and PK provides a very efficient way to regulate glycolysis, and co-regulating PK and LDH allows the cells to maintain homolactic fermentation during regulation of glycolysis.

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Triosephosphate isomerase has no control on the glycolytic flux and metabolic shift in Lactococcus lactis IL1403

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Scopus rating (2011): SJR 1.157 SNIP 1.064 CiteScore 2.87
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Scopus rating (2009): SJR 1.216 SNIP 1.235
Web of Science (2009): Indexed yes
BFI (2008): BFI-level 1
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Scopus rating (2007): SJR 1.132 SNIP 1.273
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Hvad kontrollerer syringseffektiviteten af den primære starter?

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Authors: Købmann, B. J. (Intern), Solem, C. (Intern), Jensen, P. R. (Intern)
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Kontrolanalyse af glykosen i mikrobielle systemer

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Experimental control analysis of glycolysis in Lactococcus lactis

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Main Research Area: Technical/natural sciences
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Publication: Communication › Report – Annual report year: 2003

Glyceraldehyde-3-phosphate dehydrogenase has no control over glycolytic flux in Lactococcus lactis MG1363
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has previously been suggested to have almost absolute control over the glycolytic flux in Lactococcus lactis (B. Poolman, B. Bosman, J. Kiers, and W. N. Konings, J. Bacteriol. 169:5887-5890, 1987). Those studies were based on inhibitor titrations with iodoacetate, which specifically inhibits GAPDH, and the data suggested that it should be possible to increase the glycolytic flux by overproducing GAPDH activity. To test this hypothesis, we constructed a series of mutants with GAPDH activities from 14 to 210% of that of the reference strain MG1363. We found that the glycolytic flux was unchanged in the mutants overproducing GAPDH. Also, a decrease in the GAPDH activity had very little effect on the growth rate and the glycolytic flux until 25% activity was reached. Below this activity level, the glycolytic flux decreased proportionally with decreasing GAPDH activity. These data show that GAPDH activity has no control over the glycolytic flux (flux control coefficient = 0.0) at the wild-type enzyme level and that the enzyme is present in excess capacity by a factor of 3 to 4. The early experiments by Poolman and coworkers were performed with cells resuspended in buffer, i.e., nongrowing cells, and we therefore analyzed the control by GAPDH under similar conditions. We found that the glycolytic flux in resting cells was even more insensitive to changes in the GAPDH activity; in this case GAPDH was also present in a large excess and had no control over the glycolytic flux.
Experimental determination of control of glycolysis in Lactococcus lactis

The understanding of control of metabolic processes requires quantitative studies of the importance of the different enzymatic steps for the magnitude of metabolic fluxes and metabolite concentrations. An important element in such studies is the modulation of enzyme activities in small steps above and below the wild-type level. We review a genetic approach that is well suited for both Metabolic Optimization and Metabolic Control Analysis and studies on the importance of a number of glycolytic enzymes for metabolic fluxes in Lactococcus lactis. The glycolytic enzymes phosphofructokinase (PFK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate kinase (PYK) and lactate dehydrogenase (LDH) are shown to have no significant control on the glycolytic flux in exponentially growing cells of L. lactis MG1363. Introduction of an uncoupled ATPase activity results in uncoupling of glycolysis from biomass production. With MG1363 growing in defined medium supplemented with glucose, the ATP demanding processes do not have a significant control on the glycolytic flux; it appears that glycolysis is running at maximal rate. It is likely that the flux control is distributed over many enzymes in L. lactis, but it cannot yet be excluded that one of the remaining glycolytic steps is a rate-limiting step for the glycolytic flux.

General information

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Organisations: Center for Microbial Biotechnology, Department of Systems Biology, Bacterial Physiology and Genetics Group
Authors: Købmann, B. J. (Intern), Andersen, H. W. (Intern), Solem, C. (Intern), Jensen, P. R. (Intern)
Pages: 237-248
Publication date: 2002
Expression of genes encoding F-1-ATPase results in uncoupling of glycolysis from biomass production in Lactococcus lactis

We studied how the introduction of an additional ATP-consuming reaction affects the metabolic fluxes in Lactococcus lactis. Genes encoding the hydrolytic part of the F-1 domain of the membrane-bound (F1F0) H+-ATPase were expressed
from a range of synthetic constitutive promoters. Expression of the genes encoding F-1-ATPase was found to decrease the intracellular energy level and resulted in a decrease in the growth rate. The yield of biomass also decreased, which showed that the incorporated F-1-ATPase activity caused glycolysis to be uncoupled from biomass production. The increase in ATPase activity did not shift metabolism from homolactic to mixed-acid fermentation, which indicated that a low energy state is not the signal for such a change. The effect of uncoupled ATPase activity on the glycolytic flux depended on the growth conditions. The uncoupling stimulated the glycolytic flux threefold in nongrowing cells resuspended in buffer, but in steadily growing cells no increase in flux was observed. The latter result shows that glycolysis occurs close to its maximal capacity and indicates that control of the glycolytic flux under these conditions resides in the glycolytic reactions or in sugar transport.

**General information**

State: Published  
Organisations: Center for Microbial Biotechnology, Department of Systems Biology  
Authors: Købmann, B. J. (Intern), Solem, C. (Intern), Pedersen, M. (Ekstern), Nilsson, D. (Ekstern), Jensen, P. R. (Intern)  
Pages: 4274-4282  
Publication date: 2002  
Main Research Area: Technical/natural sciences

**Publication information**

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Volume: 68  
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BFI (2014): BFI-level 2  
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Scopus rating (2013): SJR 1.899 SNIP 1.414 CiteScore 4.25  
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BFI (2012): BFI-level 2  
Scopus rating (2012): SJR 1.975 SNIP 1.429 CiteScore 4.29  
ISI indexed (2012): ISI indexed yes  
Web of Science (2012): Indexed yes  
BFI (2011): BFI-level 2  
Scopus rating (2011): SJR 1.914 SNIP 1.455 CiteScore 4.12  
ISI indexed (2011): ISI indexed yes  
Web of Science (2011): Indexed yes  
BFI (2010): BFI-level 2  
Scopus rating (2010): SJR 1.887 SNIP 1.436  
Web of Science (2010): Indexed yes  
BFI (2009): BFI-level 2  
Scopus rating (2009): SJR 1.972 SNIP 1.528  
Web of Science (2009): Indexed yes  
BFI (2008): BFI-level 2  
Scopus rating (2008): SJR 2.156 SNIP 1.572  
Web of Science (2008): Indexed yes  
Scopus rating (2007): SJR 2.043 SNIP 1.647  
Web of Science (2007): Indexed yes
Modulation of gene expression made easy

A new approach for modulating gene expression, based on randomization of promoter (spacer) sequences, was developed. The method was applied to chromosomal genes in Lactococcus lactis and shown to generate libraries of clones with broad ranges of expression levels of target genes. In one example, overexpression was achieved by introducing an additional gene copy into a phage attachment site on the chromosome. This resulted in a series of strains with phosphofructokinase activities from 1.4 to 11 times the wild-type activity level. In this example, the pfk gene was cloned upstream of a gusA gene encoding beta-glucuronidase, resulting in an operon structure in which both genes are transcribed from a common promoter. We show that there is a linear correlation between the expressions of the two genes, which facilitates screening for mutants with suitable enzyme activities. In a second example, we show that the method can be applied to modulating the expression of native genes on the chromosome. We constructed a series of strains in which the expression of the las operon, containing the genes pfk, pyk, and ldh, was modulated by integrating a truncated copy of the pfk gene. Importantly, the modulation affected the activities of all three enzymes to the same extent, and enzyme activities ranging from 0.5 to 3.5 times the wild-type level were obtained.

General information
State: Published
Organisations: Department of Systems Biology
Authors: Solem, C. (Intern), Jensen, P. R. (Intern)
Pages: 2397-2403
Publication date: 2002
Main Research Area: Technical/natural sciences

Publication information
Journal: Applied and environmental microbiology
Volume: 68
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Ratings:
BFI (2017): BFI-level 2
Web of Science (2017): Indexed Yes
BFI (2016): BFI-level 2
Scopus rating (2016): CiteScore 4.08
Web of Science (2016): Indexed yes
BFI (2015): BFI-level 2
Scopus rating (2015): SJR 1.891 SNIP 1.308 CiteScore 4.14
Web of Science (2015): Indexed yes
BFI (2014): BFI-level 2
Scopus rating (2014): SJR 1.857 SNIP 1.384 CiteScore 4.02
Web of Science (2014): Indexed yes
The extent to which ATP demand controls the glycolytic flux depends strongly on the organism and conditions for growth. Using molecular genetics we have introduced uncoupled ATPase activity in two different bacterial species, Escherichia coli and Lactococcus lactis, and determined the elasticities of the growth rate and glycolytic flux towards the intracellular [ATP]/[ADP] ratio. During balanced growth in batch cultures of E. coli the ATP demand was found to have almost full control on the glycolytic flux (FCC=0.96) and the flux could be stimulated by 70%. In contrast to this, in L. lactis the control by ATP demand on the glycolytic flux was close to zero. However, when we used non-growing cells of L. lactis (which have a low glycolytic flux) the ATP demand had a high flux control and the flux could be stimulated more than two fold. We suggest that the extent to which ATP demand controls the glycolytic flux depends on how much excess capacity of glycolysis is present in the cells.
Totalt regulerbare promotorer i skræddersyede starterkulturer

General information
State: Published
Organisations: Department of Systems Biology
Authors: Johansen, A. H. (Intern), Andersen, H. W. (Intern), Solem, C. (Intern), Jensen, P. R. (Intern)
Pages: 274-277
Twofold reduction of phosphofructokinase activity in Lactococcus lactis results in strong decreases in growth rate and in glycolytic flux

Two mutant strains of Lactococcus lactis in which the promoter of the las operon, harboring pfk, pyk, and ldh, were replaced by synthetic promoters were constructed. These las mutants had an approximately twofold decrease in the activity of phosphofructokinase, whereas the activities of pyruvate kinase and lactate dehydrogenase remained closer to the wild-type level. In defined medium supplemented with glucose, the growth rate of the mutants was reduced to 57 to 70% of wild-type levels and the glycolytic flux was reduced to 62 to 76% of wild-type levels. In complex medium growth was even further reduced. Surprisingly, the mutants still showed homolactic fermentation, which indicated that the limitation was different from standard glucose-limited conditions. One explanation could be that the reduced activity of phosphofructokinase resulted in the accumulation of sugar-phosphates. Indeed, when one of the mutants was starved for glucose in glucose-limited chemostat, the growth rate could gradually be increased to 195% of the growth rate observed in glucose-saturated batch culture, suggesting that phosphofructokinase does affect the concentration of upstream metabolites. The pools of glucose-6-phosphate and fructose-6-phosphate were subsequently found to be increased two- to fourfold in the las mutants, which indicates that phosphofructokinase exerts strong control over the concentration of these metabolites.
Scopus rating (2012): SJR 2.103 SNIP 1.092 CiteScore 3.42
ISI indexed (2012): ISI indexed yes
Web of Science (2012): Indexed yes
BFI (2011): BFI-level 1
Scopus rating (2011): SJR 2.444 SNIP 1.158 CiteScore 3.83
ISI indexed (2011): ISI indexed yes
Web of Science (2011): Indexed yes
BFI (2010): BFI-level 1
Scopus rating (2010): SJR 2.6 SNIP 1.147
Web of Science (2010): Indexed yes
BFI (2009): BFI-level 1
Scopus rating (2009): SJR 2.675 SNIP 1.18
BFI (2008): BFI-level 1
Scopus rating (2008): SJR 2.591 SNIP 1.092
Web of Science (2008): Indexed yes
Scopus rating (2007): SJR 2.626 SNIP 1.151
Web of Science (2007): Indexed yes
Scopus rating (2006): SJR 2.632 SNIP 1.133
Web of Science (2006): Indexed yes
Scopus rating (2005): SJR 2.611 SNIP 1.167
Web of Science (2005): Indexed yes
Scopus rating (2004): SJR 2.449 SNIP 1.185
Web of Science (2004): Indexed yes
Scopus rating (2003): SJR 2.669 SNIP 1.145
Web of Science (2003): Indexed yes
Scopus rating (2002): SJR 2.479 SNIP 1.111
Web of Science (2002): Indexed yes
Scopus rating (2001): SJR 2.675 SNIP 1.189
Web of Science (2001): Indexed yes
Scopus rating (2000): SJR 2.418 SNIP 1.159
Web of Science (2000): Indexed yes
Scopus rating (1999): SJR 2.641 SNIP 1.212
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DOIs: 10.1128/JB.183.11.3458-3467.2001
Source: orbit
Source-ID: 45805
Publication: Research - peer-review › Journal article – Annual report year: 2001

Projects:

Improving the thermotolerance of the mesophilic starter
National Food Institute
Period: 01/06/2017 → 31/05/2020
Number of participants: 3
Phd Student: Dorau, Robin (Ekstern)
Supervisor: Jensen, Peter Ruhdal (Intern)
Main Supervisor: Solem, Christian (Intern)

Financing sources
Improving the thermotolerance of the mesophilic starter

National Food Institute
Period: 01/06/2017 → 31/05/2020
Number of participants: 3
Phd Student: Dorau, Robin (Intern)
Supervisor: Jensen, Peter Ruhdal (Intern)
Main Supervisor: Solem, Christian (Intern)

NOPROBLEM - Novel tasty dairy products obtained through intelligent resource management

Diacetyl, an important contributor to the butty aroma of many fermented dairy products, is formed by lactic acid bacteria present in the starter culture. Mesophilic starters are efficient producers of diacetyl, but are unsuited for production of certain harder cheeses, because of the high temperatures needed to attain cheese firmness. Such cheeses are made using thermophilic starters, that unfortunately are poor diacetyl formers, and taste is thus compromised (pers. comm. Søren Lillevang, Arla Foods). Besides the butter flavour content, another important factor is butter flavour formation rate. There are several cheese products where butter flavour is formed very slowly, in the course of several weeks of storage, and for some dairy products, technical issues limit butter flavor formation. In the current project we wish to address these issues while at the same time create value from processed whey streams that currently are discarded as pig-feed. 1) We want to make the mesophilic starter more thermotolerant, so that it can be used for making harder cheese variants. 2) Produce diacetyl from whey side-streams which can be added to various dairy products/sold. One way to make the mesophilic starter more thermotolerant is through adaptive evolution, an approach we previously have used with great success (Chen et al., 2015), and which will be applied in this project as well. We have optimized one of the starter culture bacteria into being extremely efficient at producing diacetyl from sugar (Liu et al., 2016). To attain a rich buttery flavor in dairy products, less than <10 mg/kg is needed. Our strain can generate 5-10 g/l under non-optimized conditions. This strain as well as its non-GMO version (to be constructed) will be used in the current project.

National Food Institute
Research Group for Microbial Biotechnology and Biorefining
Arla Foods

Financing sources
Source: Internal funding (public)
Name of research programme: Samfinansieret - Andet
Project: PhD

Optimization of flavour formation in hard cheeses

Hard cheeses are normally made using thermophilic starters because of the high cooking temperatures (>39°C) involved. Mesophilic starters cannot presently be used because the high temperature would affect the subsequent acidification and flavor formation. Thermophilic starters tolerate the high temperature, but are unable to produce some of the desirable
flavor compounds produced by their mesophilic counterparts. In this project we wish to study whether this problem can be solved by changing process parameters and/or starter so that harder cheeses can be made using mesophilic starters.

National Food Institute
Research Group for Microbial Biotechnology and Biorefining

Arla Foods
Arla Foods Ingredients Group P/S
Period: 01/01/2017 → 21/12/2019
Number of participants: 1
Project Coordinator:
Solem, Christian (Intern)

Financing sources
Source: Public research council
Name of research programme: The Danish Dairy Research Foundation
Amount: 2,824,000.00 Danish Kroner

Biofuels of the future - Development of a Lactic Acid Bacteria platform for sustainable production of higher alcohols

National Food Institute
Period: 01/11/2015 → 31/10/2018
Number of participants: 4
Phd Student:
Mar, Mette Dysseholm (Intern)
Supervisor:
Kandasamy, Vijayalakshmi (Intern)
Solem, Christian (Intern)
Main Supervisor:
Jensen, Peter Ruhdal (Intern)

Financing sources
Source: Internal funding (public)
Name of research programme: Samfinansieret - Andet
Project: PhD

Lactic Acid Bacteria as cell factories

National Food Institute
Period: 01/06/2014 → 31/05/2017
Number of participants: 6
Phd Student:
Liu, Jianming (Intern)
Supervisor:
Jensen, Peter Ruhdal (Intern)
Solem, Christian (Intern)
Main Supervisor:
Examiner:
Hansen, Egon Bech (Intern)
Kleerebezem, Michiel (Ekstern)
Zeng, An-Ping (Ekstern)

Financing sources
Source: Internal funding (public)
Name of research programme: Institut stipendie (DTU)
Project: PhD

Lysine production in Gram-positive bacteria

National Food Institute
Period: 01/06/2014 → 17/02/2015
Number of participants: 3
Phd Student:
Grishkova, Maria (Intern)
Supervisor:
Solem, Christian (Intern)
Main Supervisor:
Jensen, Peter Ruhdal (Intern)

Financing sources
Source: Internal funding (public)
Name of research programme: Forskningsrådsfinansiering
Project: PhD

Protein production in Gram-positive bacteria under adverse conditions
National Food Institute
Period: 01/04/2014 → 31/03/2017
Number of participants: 6
Phd Student:
Vestergaard, Mike (Intern)
Supervisor:
Solem, Christian (Intern)
Main Supervisor:
Jensen, Peter Ruhdal (Intern)
Examiner:
Bang, Dang Duong (Intern)
Jönsson, Håkan N. (Ekstern)
Mijakovic, Ivan (Intern)

Financing sources
Source: Internal funding (public)
Name of research programme: Institut stipendie (DTU)
Project: PhD

Metabolic optimization of Corynebacterium glutamicum for enhanced lysine production
National Food Institute
Period: 15/12/2012 → 21/04/2016
Number of participants: 6
Phd Student:
Wang, Zhihao (Intern)
Supervisor:
Jensen, Peter Ruhdal (Intern)
Main Supervisor:
Solem, Christian (Intern)
Examiner:
Hobley, Timothy John (Intern)
Kalinowski, Jörn (Ekstern)
Mijakovic, Ivan (Intern)

Financing sources
Source: Internal funding (public)
Name of research programme: Institut stipendie (DTU)
Project: PhD

Production of organic acids in Gram-positive bacteria
National Food Institute
Period: 01/12/2012 → 03/05/2018
Number of participants: 3
Phd Student:
Shen, Jing (Intern)
Supervisor:
Jensen, Peter Ruhdal (Intern)
Main Supervisor:
Solem, Christian (Intern)

Financing sources
Source: Internal funding (public)
Name of research programme: Institut stipendie (DTU)
Project: PhD

Elucidating and comparing flux regulation across bacterial species
Department of Systems Biology
Period: 15/11/2011 → 27/05/2015
Number of participants: 6
Phd Student:
Chan, Siu Hung Joshua (Intern)
Supervisor:
Solem, Christian (Intern)
Main Supervisor:
Jensen, Peter Ruhdal (Intern)
Examiner:
Hobley, Timothy John (Intern)
Molenaar, Douwe (Ekstern)
Snoep, Jacob Leendert (Ekstern)

Financing sources
Source: Internal funding (public)
Name of research programme: Forskningsrådsfinansiering
Project: PhD

Characterization of a high-temperature adaptive Lactococcus lactis mutant and it’s application in milk fermentation
Department of Systems Biology
Period: 01/11/2011 → 28/03/2014
Number of participants: 6
Phd Student:
Chen, Jun (Intern)
Supervisor:
Solem, Christian (Intern)
Main Supervisor:
Jensen, Peter Ruhdal (Intern)
Examiner:
Kilstrup, Mogens (Intern)
Poolman, Bert (Ekstern)
Sørensen, Kim (Intern)

Financing sources
Source: Internal funding (public)
Name of research programme: Institut stipendie (DTU)
Project: PhD

Transforming Lactococcus lactis into a microbial cell factory
Department of Systems Biology
Period: 01/01/2011 → 30/09/2014
Number of participants: 7
Phd Student:
Petersen, Kia Vest (Intern)
Supervisor:
Jensen, Peter Ruhdal (Intern)
Martinussen, Jan (Intern)
Main Supervisor:
Solem, Christian (Intern)
Examiner:
Mijakovic, Ivan (Intern)
Jørgensen, Sten Troels (Ekstern)
Kok, Jan (Ekstern)

**Financing sources**
Source: Internal funding (public)
Name of research programme: Institut stipendie (DTU)
Project: PhD

**Biofuels production in yeast**
National Food Institute
Period: 01/12/2010 → 29/05/2017
Number of participants: 5
Phd Student:
Phadnavis, Ambareesh Govind (Intern)
Main Supervisor:
Jensen, Peter Ruhdal (Intern)
Examiner:
Solem, Christian (Intern)
Blank, Lars M. (Ekstern)
Pedersen, Per Amstrup (Ekstern)

**Financing sources**
Source: Internal funding (public)
Name of research programme: Institut stipendie (DTU) Samf.
Project: PhD

**Lactococcus lactis as microbial platform for production of biochemicals**
National Food Institute
Period: 01/03/2010 → 01/06/2016
Number of participants: 6
Phd Student:
Belmont, Martin (Intern)
Supervisor:
Jensen, Peter Ruhdal (Intern)
Main Supervisor:
Solem, Christian (Intern)
Examiner:
Martinussen, Jan (Intern)
Neves, Ana Rute (Intern)
van Niel, Eduard Willibrordus Johannes (Ekstern)

**Financing sources**
Source: Internal funding (public)
Name of research programme: Stipendie fra udlandet
Project: PhD

**Comparative Systems Biology**
Department of Systems Biology
Period: 01/01/2010 → 18/12/2013
Number of participants: 6
Phd Student:
Dehli, Tore Ibsen (Intern)
Supervisor:
Solem, Christian (Intern)
Main Supervisor:
Jensen, Peter Ruhdal (Intern)
Examiner:
Mijakovic, Ivan (Intern)
Axelsson, Lars (Ekstern)
Westermann, Peter (Intern)

**Financing sources**
Source: Internal funding (public)
Name of research programme: Institut stipendie (DTU)
Project: PhD

**Udvikling af mikroorganismer til biobrændselsproduktion**
Department of Systems Biology
Period: 01/12/2008 → 01/03/2013
Number of participants: 7
Phd Student:
Hansen, Anders Cai Holm (Intern)
Supervisor:
Solem, Christian (Intern)
Workman, Mhairi (Intern)
Main Supervisor:
Jensen, Peter Ruhdal (Intern)
Examiner:
Kilstrup, Mogens (Intern)
Købmann, Brian (Intern)
Mijakovic, Ivan (Intern)

**Financing sources**
Source: Internal funding (public)
Name of research programme: Anden EU-finansiering
Project: PhD

**Energimetabolismen i mælkesyrebakterier**
Department of Systems Biology
Period: 01/08/2000 → 30/11/2001
Number of participants: 2
Phd Student:
Solem, Christian (Intern)
Main Supervisor:
Jensen, Peter Ruhdal (Intern)

**Financing sources**
Source: Internal funding (public)
Name of research programme: DTU-lønnet stipendie
Project: PhD