Non-Pathogenic Clostridia

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Marie Curie Conference on Non-Pathogenic Clostridia
Toulouse, February 24 to 27, 2008

PROGRAMME

Sunday February 24, 2008
***Location**: Comfort Inn Hotel in Ramonville*

17H00-19H00 Registration
18H00-19H00 Plenary lecture
Terry Papoutsakis
Clostridia, biofuels and biorefining: potential and problems
19H00-22H00 Welcome dinner

Monday February 25, 2008
***Location:** INSA Toulouse-Amphitheatre Fourier*

Session I: Metabolism of Non-Pathogenic Clostridia
8H30-9H30 Hubert Bahl
Oxidative stress response and aerotolerance in *Clostridium acetobutylicum*
9H30-10H00 Gaëlle André
The sulphur metabolism and its regulation in *Clostridium acetobutylicum*
10H00-10H30 Katrin Schwartz
System Biology of *Clostridium acetobutylicum*-understanding solvent production
10H30-11H00 Coffee break
11H00-11H30 Stefan Junne
Monitoring of cell polarizability in the acetone-butanol fermentation with electrooptical measurements
11H30-12H30 Wilf Mitchell
Substrate accumulation and its control in solventogenic clostridia
12H30-14H00 Lunch

Session II: Metabolic Engineering of Non-Pathogenic Clostridia
14H00-15H00 Isabelle Meynial-Salles
Metabolic Engineering of *Clostridium acetobutylicum* for the production of 1, 3 propanediol.
15H00-15H30 Agnieska Brandt
Expression of *Clostridium acetobutylicum* butanol synthetic genes in *Escherichia coli*
15H30-16H30 George Bennett
Contributions of Clostridia to the degradation of hazardous materials
16H30-18H00 Poster session I
19H00-22H00 Dinner at the Comfort Inn Ramonville

Tuesday February 26, 2008
Location: *INSA Toulouse-Amphitheatre Fourier*

Session III: Genetics and Genomic of Non-Pathogenic Clostridia
9H00-10h00 Peter Duerre
Regulation of solvent formation in *Clostridium acetobutylicum*
10H00-10H30 John Heap
The Clostron gene knockout system: refinements and application.
10H30-11H00 Coffee break
11H00-11H45 Zhen Shi
Transcriptional analysis of *Clostridium beijerinckii* during the shift from acidogenesis to solventogenesis
11H45-12H30 Philippe Soucaille
Gene deletion and integration without markers in *Clostridium acetobutylicum*
12H30-14H00 Lunch

Session IV: Sporulation of Non-Pathogenic Clostridia
14H00-15H00 Elisabeth Steiner
How do clostridia phosphorylate Spo0A
15H00-15H30 Sara Jabbari
Mathematical modelling of the gene regulation network controlling sporulation initiation
15H30-16H00 Brian Tracy
Development and application of flow-cytometry techniques for *Clostridium* cell cultures
16H00-18H00 Poster session II
19H00-24H00 Congress dinner at the restaurant "Les beaux arts" in Toulouse

Wednesday February 27, 2008
Location: *INSA Toulouse-Amphitheatre Fourier*

Session V: Polysaccharide hydrolysis and sugar transport in Non-Pathogenic Clostridia
9H00-10H00 Ed Bayer
The cellulosome of the Clostridia and related bacteria
10H00-10H30 Julie Watson
Xylose Catabolism in the biofuel producing organism *Clostridium acetobutylicum* ATTC 824
10H30-11H00 Coffee break
11H00-12H00 Mickaël Desvaux
Carbon flux analysis in *Clostridium cellulolyticum*: a continuous culture approach
12H00-13H00 Chantal Tardif
How does *Clostridium cellulolyticum* regulate cellulosome production and composition?
13H00-14H00 Lunch
14H00 Departure

LOCAL ORGANIZING COMMITTEE

Christian Croux, Isabelle Meynial-Salles, Claudia Santos Gai, **Philippe Soucaille**

Oral presentations

Plenary Lecture

Clostridia, biofuels and biorefining: potential and problems

E. Terry Papoutsakis

Department of Chemical Engineering and the Delaware Biotechnology Institute, University of Delaware, Newark, DE 19711

Biofuel production, and biorefining more generally, will have to utilize more complex carbohydrate substrates, such as raw or processed cellulosic material. Based on these needs and for several reasons, butyric-acid solventogenic clostridia (*Clostridium acetobutylicum* and related organisms) have emerged as prototypical organisms for developing new biofuel and biorefining technologies. Clostridia are endospore formers but their differentiation (sporulation) program remains largely ill understood, yet it is of both practical and fundamental importance. E.g., *C. acetobutylicum* produces solvents mostly or only during a distinct stage of its differentiation (sporulation) program. This differentiation stage remains largely uncharacterized at the cellular and molecular levels. Cells that move beyond this differentiation stage to complete their sporulation program do not produce solvents and are thus undesirable from the industrial point of view. Beyond metabolic engineering, “Differentiation Engineering” and “Cellular engineering of complex phenotypes” (such as, solvent and acid tolerance, cellulose or xylan utilization, “use them all”, “produce only one”, etc) for strain development will require advanced genetic, genomic and computational tools (much beyond standard genome-scale models) that remain largely ill developed for clostridia, and most other organisms. Where are we now then with these organisms, and where could we have been or go in the future? What is the dream? Beyond the issues and the dreams, I will show the data from the complete transcriptional analysis of the classical batch fermentation of *C. acetobutylicum* going all the way deeply into the late stationary phase, 25 time points, in all, of extraordinary transcriptional details.

Session I: Metabolism of Non-Pathogenic Clostridia

Invited Speaker

Oxidative stress response and aerotolerance in *Clostridium acetobutylicum*

Hubert Bahl,

University of Rostock, Institute of Biological Sciences, Division of Microbiology, Albert-Einstein-Strasse 3, D-18051, Rostock, Germany.

Although Clostridia are generally considered as obligate anaerobes, they often encounter oxygen or reactive derivatives via NAD(P)H dependent reduction. The deletion of a *perR* homlogous gene fully deregulated the oxygen defence of *C. acetobutylicum* and produced a strain which rapidly consumed oxygen from the environment. In cell free extracts, this oxygen consumption was primarily dependent on NADH and sufficient to allow limited growth in a fully aerobic environment. The mutant strain also revealed higher resistance to H_2O_2, as activities of NADH dependent scavenging of H_2O_2 and organic peroxides in cell free extracts increased by at least one order of magnitude. Surprisingly, only few genes encoding the putative enzymes were upregulated and identified as members of the clostridial PerR regulon. The heat shock protein Hsp21, a reverse rubrerythrin, was massively produced and became the most abundant protein in the absence of PerR. This multifunctional protein is involved in the scavenging of O_2 as well as H_2O_2 and is proposed to play the crucial role in aerotolerance of clostridia.

Session I: Metabolism of Non-Pathogenic Clostridia

The Sulfur Metabolism and its Regulation in *Clostridium acetobutylicum*

G. André, O. Soutourina, B. Dupuy and I. Martin-Verstraete

Unité de Génétique des Génomes Bactériens, Institut Pasteur, 28 rue du Docteur Roux,75724 Paris Cedex 15, France

We are studying the sulfur metabolism and its regulation in gram-positive bacteria. In *Bacillus subtilis*, several genes involved in this metabolism have been characterized. The regulation of sulfur metabolism occurs via a premature termination of transcription control and transcriptional regulators: two activators CysL and YtlI, and a repressor CymR. In *C. acetobutylicum*, we have performed sulfur metabolism reconstruction. We have analyzed in details the regulation of an operon (*ubiGmccBA*) involved in methionine to cysteine conversion and shown that this operon was controlled by a complex regulatory system combining RNA-based mechanisms in *B. subtilis*. Two convergent promoters associated with transcriptional antitermination systems, a cysteine-specific T-box and an S-box riboswitch, are located upstream and downstream from the operon. Several antisense RNAs were synthesized from the downstream promoter. The abundance of sense and antisense transcripts was inversely correlated with the sulfur source availability (G. André *et al*, submitted for publication). Our data bring important insights into the mechanism of antisense-mediated regulation suggesting an antisense RNA action *in cis* via transcriptional interference. We would like to show now that this complex regulation system is functional in *C. acetobutylicum* . CymR-like repressors sharing 40 % to 50% identity with CymR from *B. subtilis* are found in clostridia with one to 4 copies per genome. Interestingly, two types of CymR-like proteins seem to be present in clostridia. CymR proteins (CymRcys) contain a cluster of 3 cysteines and the corresponding *cymRcys* gene is usually the first gene of an operon encoding NifS and NifUlike proteins involved in Fe-S cluster biogenesis. It is tempting to think that CymRcys may bind a Fe-S cluster as observed for other Rrf2 regulators to control Fe-S biogenesis and/or sulfur metabolism. The second CymR does not contain the cysteine cluster and the gene is often in operon with *cysK*. It could form a complex with CysK as observed in *B. subtilis* (Hullo et al, 2007) to control the expression of genes involved in cysteine metabolism. We will analyze the role of these two regulators in *C. acetobutylicum*. We plan to disrupt the corresponding genes in *C. acetobutylicum* and to identify the controlled genes with proteomic approach.

Session I: Metabolism of Non-Pathogenic Clostridia

Systems Biology of *Clostridium acetobutylicum* – Understanding Solvent Production

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Clostridium acetobutylicum, the non-pathogenic model organism of clostridia, is able to fundamentally change its metabolism from the production of organic acids (acetate, butyrate) to solvents (acetone, butanol, ethanol) dependent on the culture conditions. This characteristic has already been used in large scale fermentation processes. The involved metabolites and enzymes are well-established, however, little is known about the regulation of the metabolic shift, the characteristics of key-regulatory elements as well as bottlenecks of the metabolism. Goal of the Systems Biology of Microorganisms (´SysMo`) collaborative project ´COSMIC` (*Clostridium acetobutylicum* Systems Microbiology) is to increase the knowledge of this clostridial metabolism and its regulatory patterns and to establish it as a paradigm of the clostridial systems biology. The project is carried out in close collaboration with research groups in Rostock, Ulm, Göttingen, and Nottingham. The focus will be on the key regulatory and metabolic events that occur during the exposure to stress conditions in general and butanol stress, in particular. The dynamic, quantitative data obtained will be used to mathematically model the various interactions at the cellular level. Therefore, *C. acetobutylicum*, grown in a well-known phosphate limited chemostat under defined conditions guaranteeing the direct control of the metabolic shift, will be analysed by different state-of-the-art methods. This includes DNA-microarrays, proteomics and metabolomics using LC-MS-NMR and LC-FTMS. Currently, research focuses on the analysis of the oscillation of the culture`s redox potential under solvent producing conditions. A net change of the redox potential of 10 mV up to 18 mV depending on the dilution rate (0.055 $h^{-1}$ to 0.21$h^{1}$) could be observed. As the dilution rate defines the growth rate and cells` need of metabolites, especially energy and reduction equivalents, this effect will give information about so far unknown regulation patterns.

Session I: Metabolism of Non-Pathogenic Clostridia

Monitoring of Cell Polarizability in the Acetone Butanol Fermentation with Electrooptical Measurements

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By underlying and alternating an electrical field on rod-shaped microorganisms, the polarizability of cells can be monitored with optical sensors. The degree of polarizability is related to the cell compartment as well as to their metabolic state. We investigated the possibility of electrooptical measurements (EOM) for the online-monitoring of main metabolite fluxes in Clo*stridium acetobutylicum* during anaerobic batch fermentations at different pH values.
The obtained spectra of EOM at varying frequencies showed strong changes, when the fermentations switched from acidogenesis to solventogenesis. Polarizability was much lower during this fermentation stage. Alterations in polarizability prior to the change of the culture could be seen very early. The polarizability seems to be a good indicator for the start of solvent synthesis. The early solventogenic phase was characterized by a reascending polarizability. The same was observed for the onset of acidogenesis. Different pH had a strong impact on the time course development of the measured signals. The obtained data allowed the development of a model able to predict appropriately metabolite fluxes.
From EOM, cell length and cell shape could be determined. Since spore formation in *C. acetobutylicum* alters the bacterial shape, on-line monitoring of the sporulation process was possible. Dependencies between cell polarizability and conformational changes could be detected.
The application of EOM seem to be well-suited as a process analytical tool at *C. acetobutylicum* batch fermentations. Since the data reflect the metabolic activity of the cell, changes in the bioprocess' progression can be detected at a very early stage.

Session I: Metabolism of Non-Pathogenic Clostridia

Invited Speaker

Substrate accumulation and its control in solventogenic clostridia

Wilfrid J Mitchell

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The predominant mechanism of carbohydrate accumulation by the butanol-forming solventogenic clostridia is the phosphoenolpyruvate-dependent phosphotransferase system (PTS), a multi-domain complex which catalyses the uptake and concomitant phosphorylation of a range of hexoses, hexitols and disaccharides. The genomes of *C. acetobutylicum* and *C. beijerinckii* respectively encode 13 and 41 phosphotransferases that are structurally similar to those in other bacteria. In addition to its role in carbohydrate accumulation, the bacterial PTS has also been shown to act as an environmental sensor which regulates the accumulation of carbohydrate substrates in response to the nutritional status of the environment. PTS-dependent control mechanisms that are characteristic of low-GC Gram-positive bacteria, involving covalent modification of transcriptional regulators and antiterminators, and phosphorylation of the PTS component HPr at residue ser46, appear to be operative in the clostridia (though perhaps with some differences), and are likely responsible for the observed preference for glucose over other potential substrates during growth. An increased understanding of the control of expression and activity of catabolic uptake and metabolic systems will facilitate development of strains for fermentation processes exploiting lignocellulose and other novel substrates.

Session II : Metabolic Engineering of Non-Pathogenic Clostridia

Invited Speaker

Metabolic Engineering of *Clostridium acetobutylicum* for the production of 1, 3 propanediol

Isabelle Meynial Salles, Christian Croux, Patricia Sarcabal and Philippe Soucaille

Ingénierie métabolique et évolution moléculaire *in vivo* des procaryotes, Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés, UMR CNRS/INSA 5504 - UMR INRA/INSA 792
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Applications of 1,3-propanediol, in a new polyester called poly(propylene terephtalate) with unique properties for the fiber industry , call for a drastic increase in the production of this chemical. 1,3 propanediol is currently produced by a chemical process by Shell and by a biological process (from glucose) by Dupont. With the development of Biodiesel as a renewable fuel for diesel engines, a lot of glycerol is put on the market and its price has been constantly decreasing starting to make it attractive as a fermentation substrate.

In all the "natural 1, 3 propanediol producers" characterized so far glycerol conversion to 1, 3 propanediol involved a B12-dependent glycerol dehydratase. We have shown that the glycerol dehydratase of *C. butyricum* VPI 1718 is not stimulated by Coenzyme B12 and is extremely oxygen sensitive which suggests that it is a coenzyme-B12 independent enzyme (1).

We have cloned, sequenced and characterized from a molecular point of view the genes encoding the B12 independent glycerol dehydratase of *C. butyricum*VPI 1718 (2). This unique enzyme belongs to a new family of glycerol dehydratase that has never been described so far. By heterologous expression of this new dehydratase and by introducing defined mutations in the central metabolism of *C. acetobutylicum,* we developed a recombinant strain that possesses the best yield and productivity for glycerol conversion to 1, 3 propanediol (3).
This strain was successfully used in an anaerobic continuous process for the conversion of raw glycerol (coming from a biodiesel plant) to 1, 3 propanediol (4). The performances obtained make it highly attractive to the glucose aerobic fed-batch process.

1) SAINT-AMANS S, et al (2001). **J. Bacteriol.** 183, 1748-1754.
2) SARÇABAL P. et al (2003). **Proc. Natl. Acad. Sci**., 100, 5010-5015
3) GONZALEZ-PAJUELO M, et al (2006). **Appl. Env. Microbiol.** 72, 96-101
4) GONZALEZ -PAJUELO M, et al (2006). **Met. Eng.** 7, 329-336

Session II: Metabolic Engineering of Non-Pathogenic Clostridia

Expression of Clostridium acetobutylicum Butanol Synthetic Genes in *Escherichia coli*

A. Brandt[1], O. Berezina[2], W. Schwarz[2] and V. Zverlov[2]

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Clostridium acetobutylicum ferments sugars to a variety of end products: H_2, CO_2 and short chain fatty acids. Furthermore it is able to convert the fatty acids to acetone, ethanol and n-butanol (ABE fermentation), which has been exploited on a commercial scale.
Butanol has been proposed as a next-generation renewable fuel and solvent. Therefore increased effort is being put into improving the performance of the traditional industrial fermentation process. One promising approach is the expression of the butanol synthesis pathway in microorganisms other than *Clostridium* spp.
This work describes the cloning of several genes from a *C. acetobutylicum* strain with the aim of transferring this system into a more suitable host organism.
The genes required for converting acetyl-CoA to butanol were cloned into the intermediate host *Escherichia coli*. A shuttle vector containing the *C. acetobutylicum* genes for the conversion of acetyl-CoA to butyryl-CoA was constructed in *E. coli*. Assays for the respective enzyme activities were established. Specific activities were determined in cell extracts of the *C. acetobutylicum* wild type strain and in extracts of recombinant *E. coli*.
A recent publication (Inui et al, 2007) indicates that similar strategies are followed elsewhere.

Session II: Metabolic Engineering of Non-Pathogenic Clostridia

Invited Speaker

Contributions of Clostridia to the degradation of hazardous compounds

George Bennett, Rice University, Houston, TX

While the activity of various clostridial species in solvent production and in the utilization of many polysaccharides including cellulose as substrates has been studied extensively, there has been less appreciation of the role of these organisms in degrading many man-made pollutants that accumulate in anaerobic environments. The capability of the clostridia to provide reducing equivalents allows a variety of compounds to be transformed by reductive mechanisms. One example of these processes is their action on nitro compounds, such as those in explosives and some pesticides. Clostridial action can convert these compounds to a series of partially or fully reduced nitrogen compounds, some of which may be more toxic than the initial compound, while overall removing most of the original compound and lowering the total toxicity. Clostridial actions on chlorinated solvents and other compounds have also been described. The role of clostridia in communities of organisms that degrade various compounds is beginning to be studied and their contribution to the action of other biodegrading organisms by providing hydrogen or substrates for these ecological co-inhabitants expands their importance in the removal of hazardous compounds from the biosphere.

Session III: Genetics and Genomic of Non-Pathogenic Clostridia

Invited Speaker

Regulation of solvent formation in *Clostridium acetobutylicum*

Peter Duerre

Institute of Microbiology and Biotechnology, University of Ulm, Germany

Session III: Genetics and Genomic of Non-Pathogenic Clostridia

The Clostron Gene Knockout System: Refinements and Application

JT Heap[1], OJ Pennington[1], ST Cartman[1], JC Scott[1], K Winzer[2], and NP Minton[1]

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[2]Department of Biological Sciences, University of Lancaster, UK

To facilitate the genetic modification of *Clostridium* species, we have developed a directed integration tool (the ClosTron) based on the Ll.ltrA mobile group II intron from *Lactococcus lactis*. The original plasmid pMTL007 has proven very effective, and has been used to inactivate numerous genes in several different clostridial species, including *Clostridium acetobutylicum*. Key to the system's ease-of-use is a retrotransposition-activated marker (RAM) based on an *ermB* gene, which allows the direct selection of insertional mutants by acquisition of resistance to erythromycin. However, multiple mutations are not easily constructed using pMTL007, because the initial single mutants are already erythromycin-resistant. To address this and other issues, several refinements have been made to the system, leading to the construction of 'second-generation' ClosTron plasmids. Importantly, insertions may now be made in which the *ermB* gene is flanked by FRT sites, allowing the subsequent removal of *ermB* by expression of FLP recombinase, and therefore opening the way to the construction of multiple mutations. Improvements for ease-of-use have also been made, including facilities for rapid screening of the initial re-targeted plasmids. Second-generation ClosTron plasmids have been used in the mutational analysis of the Agr quorum sensing system of *C. acetobutylicum.*

Session III: Genetics and Genomic of Non-Pathogenic Clostridia

Invited Speaker

Transcriptional analysis of *Clostridium beijerinckii* during the shift from acidogenesis to solventogenesis

Z. SHI

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Clostridium beijerinckii is an anaerobic bacterium which can be used for the fermentative production of acetone and butanol. The recent availability of genomic sequence information for *C. beijerinckii* NCIMB 8052 has allowed for an examination of gene expression by DNA microarray analysis over the time course of a batch fermentation. Expression patterns of the wild-type strain and a mutant strain with increased butanol formation were compared. The results revealed marked differences between the two strains with respect to transcription of a diverse set of genes representing multiple functional classes necessary for solventogenesis, sporulation, cell motility and sugar transport. Taken together, these variations appear to collectively contribute to enhanced butanol formation in the mutant.

Session III: Genetics and Genomic of Non-Pathogenic Clostridia

Invited Speaker

Gene deletion and integration without markers in *Clostidium acetobutylicum*

Philippe Soucaille, Christian Croux, Florence Saint Prix, Maria Gonzalez-Pajuelo and Isabelle Meynial Salles

Ingénierie métabolique et évolution moléculaire *in vivo* des procaryotes, Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés, UMR CNRS/INSA 5504 - UMR INRA/INSA 792 INSA DGBA, 135 Avenue de Rangueil, 31077 Toulouse Cedex 4, France

In the recent past few years, *Clostridium acetobutylicum* ATCC824 has entered in the postgenomic era, due to the completion of the sequencing and annotation of its genome (Nolling *et al.*, 2001), supplying a wealth of information on its protein machinery. This global knowledge thus prompts new approaches for high-throughput genetic analysis, ie to analyse and manipulate its biochemical pathways, as well as to assign functions to unknown genes.
These reverse genetics approaches must be based on simple and highly efficient tools to inactivate and/or introduce genes at their normal chromosomal context, an optimal situation for genetics analysis of the effects of the mutations.
We have developed an original method based on i) deletion and replacement of the target gene by an antibiotic resistance gene by a double crossover integration through homologous recombination of a replicative integrative plasmid, giving segregationally higly stable mutants, ii) removing of the antibiotic resistance gene with the Flp recombinase system from *Saccharomyces cerevisiae* allowing the repeated use of the method for rapid construction of multiple, unmarked mutations in the same strain and iii) a *C. acetobutylicum* strain deleted for the *upp* gene, encoding uracil phosphoribosyl transferase, thus allowing the use of 5-Fluorouracil (5-FU) as a counter selectable marker and a positive selection of the double crossover integrants.
The replicative integrative vector, based on a pIMP13 origin of replication from *Bacillus subtilis* functional in clostridia, contains a replacement cassette consisting of an antibiotic resistance gene (MLS^R) flanked by two FRT sequences and two sequences homologous to selected regions surrounding target DNA sequence to be deleted. The first original method (without *upp*/5-FU system as a counter selection strategy) was successfully used to consecutively delete in the *C. acetobutylicum* ATCC824 chromosome the restriction endonuclease Cac824I and the *upp* genes, yielding a *C. acetobutylicum* strain, free of any selection marker, readily transformable without any previous *in vivo* plasmid methylation and that will serve as the host for the "marker-less" genetic exchange system. Other genes, from metabolic pathways, like *buk* (*cac3075)* and *ctfA,B* (cap 163-164) involved in the butyrate and acetone production respectively, were deleted using the *upp*/5-FU counter selection strategy, thus validating our improved method.
It's likely that this system of maker-less genetic exchange coupled with positive selection, with high efficiency, easy to perform and giving very stable mutants will greatly improve the metabolic engineering of *C. acetobutylicum* for the construction of strains with industrially desirable characteristics.

Session IV: Sporulation of Non-Pathogenic Clostridia

Invited Speaker

The Initiation of Sporulation in *Clostridium acetobutylicum*

E. Steiner[1], D. I. Young[1], J. T. Heap[2], O. J. Pennington[2], J. A. Hoch[3], N. P. Minton[2] and M. Young[1].

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[2]Institute of Infection, Immunity and Inflammation, Centre for Biomolecular Sciences, University of Nottingham, NG7 2RD, UK.
[3]The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037, USA.

The solvent-producing bacterium *Clostridium acetobutylicum* forms heat-resistant endospores but the signal(s) that trigger sporulation in this organism are unknown. In contrast to *Bacillus subtilis*, clostridia do not sporulate under nutrient limitation. Like the bacilli however, the clostridia contain the master transition state regulator, Spo0A. In *B. subtilis*, this phosphorylation-activated transcription factor orchestrates gene expression during the transition from the exponential to the stationary phase of growth. Previous work has shown that Spo0A also controls endospore formation and solventogenesis in clostridia. However, Spo0F and in most cases Spo0B, which are key components of the phosphorelay responsible for activating Spo0A in the bacilli, are seemingly absent from the clostridia. The objective of this investigation is to determine how *C. acetobutylicum* phosphorylates Spo0A.

Some 35 histidine kinases have been annotated in the *C. acetobutylicum* genome. Five of them (CAC0323, CAC0437, CAC0903, CAC2730 & CAC3319) are orphan kinases lacking an adjacent cognate response regulator. By analogy to other organisms, it is these orphan kinases that are most likely to play a role in the phosphorylation of Spo0A.

Recently developed “ClosTron” technology, which uses a mobile re-targeted group II intron to generate knockout mutations, is both reliable and efficient in *C. acetobutylicum*. We have employed it to inactivate the genes encoding these five orphan kinases. The mutant phenotypes have been carefully analysed and the results indicate that some of these kinases are involved in the initiation of sporulation whereas others are not.

Session IV: Sporulation of Non-Pathogenic Clostridia

Mathematical Modelling of the Gene Regulation Network Controlling Sporulation Initiation

S. Jabbari[1], J. Heap[2] , J.R. King[1], N.P. Minton[2]

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[2]Centre for Biomolecular Sciences, School of Molecular Medical Sciences, University Park, University of Nottingham, Nottingham, NG7 2RD, UK.

Sporulation is the process by which symmetrical division of a bacterial cell is replaced by asymmetric division resulting in a mother cell and a forespore. The former goes on to engulf the forespore, providing it with a coating which is resistant to extreme conditions. The decision to enter sporulation is carefully monitored by the bacteria as the process requires a great deal of energy and is not easily reversible. Thus the decision is made as a result of an intricate combination of regulatory genes and proteins which detect the sporulation inducing signals. Such networks lend their selves readily to mathematical modelling where the interactions occurring within the system can be represented by differential equations. *Bacillus subtilis* a gram positive spore forming bacterium which is relatively well understood due to the ease with which it can be genetically manipulated. Consequently, *B.subtilisserves* as the model organism for many other gram - positive bacteria, particularly those with the ability to sporulate, such as the *Clostridia* species. Given the ease of testing hypotheses mathematically as opposed to experimentally, we use a theoretical approach to present a mathematical model of the regulatory network governing sporulation initiation in *B.subtilis*, with a view to extending the work to represent that of *Clostridium acetobutylicum*.

Session IV: Sporulation of Non-Pathogenic Clostridia

Development and Application of Flow Cytometry Techniques for Clostridium Cell Cultures

Bryan P. Tracy[1], Stefan M. Gaida[2] and Eleftherios T. Papoutsakis[1,3].

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[2]Institute of Biotechnology, Technical University of Berlin, Berlin, Germany
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The improvement of microorganisms for specialty chemical production, bioremediation, and biofuel generation, remains an immediate scientific and industrial goal. Particularly for *Clostridium* species, tremendous efforts are concentrated on developing strains capable of efficiently converting low value biomass into transportation biofuels (butanol). Subsequently, reverse genetics and genome scale approaches have been and continue to be adapted to *Clostridium* for accelerating the discovery phase of strain development. These approaches have been successful for developing more solvent tolerant strains in *C. acetobutylicum*. However, genome scale techniques are often limited to single parameter detection (fitness) and are not high-throughput.

Flow-cytometry epitomizes high-throughput and is ideal for multi-parametric detection of individual cells. Thus, we developed and adapted multiple flow cytometry assays for the analysis of *C. acetobutylicum* cell cultures. Specifically, we developed and adapted a light scattering assay, a *Bac*Light™ (Invitrogen) DNA staining assay, a pencillin-binding-protein (PBP) assay, a *Bac*Light™ membrane potential assay, and an intracellular-immunofluorescence (ICIF) assay. We demonstrate the ability of these assays to identify and quantify cellular morphologies, phenotypes, and physiology. Additionally, we demonstrate the application of multi-parametric fluorescence assisted cell sorting (FACS) for enriching phenotypes within a morphologically heterogeneous cell culture. Our future goal is to employ multi-parametric FACS to rapidly screen genome scale libraries for desirable phenotypes that likely would not be enriched for via fitness selection alone.

Session V:
Polysaccharide hydrolysis and sugar transport in Non-Pathogenic Clostridia

Invited Speaker

Cellulosomes of the Clostridia and Related Bacteria

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Cellulosomes are intricate multi-enzyme machines produced by many cellulolytic bacteria. The first example of a cellulosome was discovered in the anaerobic thermophilic bacterium, *Clostridium thermocellum*. Subsequently, cellulosomes were discovered in other clostridia, including *C. cellulolyticum, C. cellulovorans, C. josui, C. acetobutylicum* and *C. papyrosolvens*. Additional cellulosomes were also discovered in members of the clostridial cluster and other related bacteria, including *Acetivibrio cellulolyticus, Bacteroides cellulosolvens, Ruminococcus flavefaciens* and *R. albus*.

Cellulosomes are designed for efficient degradation of plant cell wall polysaccharides, notably cellulose -- the most abundant organic polymer on Earth. The cellulosomes are composed of a conglomerate of subunits, each of which comprises a set of discrete interacting functional modules. A multi-functional integrating subunit (called scaffoldin) is responsible for organizing the cellulolytic subunits into the multi-enzyme complex. This is accomplished by the interaction of two complementary classes of domain, located on the two separate types of interacting subunits, i.e., a cohesin domain on scaffoldin and a dockerin domain on each enzymatic subunit. The high-affinity cohesin-dockerin interaction defines the cellulosome structure. Attachment of the cellulosome to its substrate is mediated by a cellulose-binding module (CBM) part of the scaffoldin.

The lecture will cover the experimental approach that led to the original discovery of the cellulosome concept, the surprising results and the excitement of the times. We will also discuss cellulosome ultrastructure, cellulosome architecture, the crystal structures of cellulosome components and cellulosome diversity among the different species.

Session V:
Polysaccharide hydrolysis and sugar transport in Non-Pathogenic Clostridia

Xylose Catabolism in the Biofuel Producing Organism *Clostridium acetobutylicum* ATCC 824

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Lignocellulosic waste provides a potentially important resource for biofuel production in the light of issues such as space and competition with food crops potentially causing great hindrance to the development of the biofuel industry. Traditionally yeasts, namely *Saccharomyces cerevisiae*, have been employed in bioethanol production. However yeasts are often unable to utilise pentose sugars found in hemicelluloses, a component of lignocelluloses. Hemicellulose can constitute up to 35% of the contents of lignocellulose and this is composed of a backbone of xylan, a polymer of the pentose xylose. *Clostridium acetobutylicum* is a solvent producing bacterium that can utilise a wide variety of substrates to produce end-products of Acetone, Butanol and Ethanol, the so called ABE fermentation process. Butanol is now recognised as a superior second generation biofuel due to its higher energy, lower volatility and the fact that it can be blended with diesel. The potential therefore exists to employ *C. acetobutylicum* to produce bio-butanol from lignocellulosic waste – however very little is known about this metabolism in clostridia. In this study we report the identification of a potential pathway for uptake and metabolism of xylose by *C. acetobutylicum*. A gene from this pathway is cloned in *E. coli* and the *in vivo* activity of the product is demonstrated by complementation analysis.

Session V:
Polysaccharide hydrolysis and sugar transport in Non-Pathogenic Clostridia

Invited Speaker

Cellulose metabolism in mesophilic cellulolytic clostridia: *Clostridium cellulolyticum* as a case study

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Compared to other saccharolytic clostridia and as most truly cellulolytic clostridia, *Clostridium cellulolyticum* is characterised by limited carbon consumption, and subsequent limited bacterial growth. Early metabolic studies performed in batch cultures suggested that it resulted from inefficient cellulolysis, nutrient(s) limitation, and/or by-product(s) inhibition. Instead, metabolic flux analysis (MFA) in chemostat cultures, using either cellobiose (a soluble cellodextrin resulting from cellulose hydrolysis) or cellulose (an insoluble biopolymer) as sole carbon and energy source, suggests self-intoxication of bacterial metabolism resulting from inefficiently regulated carbon fluxes. In fact, MFA revealed that (i) in comparison to cellobiose, cellulose hydrolysis by cellulosomes introduces an extra regulation of entering carbon flow resulting in lower metabolic fluxes on cellulose than on cellobiose, (ii) glucose 1-phosphate/glucose 6-phosphate branch point controls the carbon flow directed towards glycolysis and dissipates carbon excess towards the formation of cellodextrins, glycogen and exopolysaccharides, (iii) the pyruvate/acetyl-CoA metabolic node is essential for regulating electronic and energetic fluxes. This in-depth carbon flux analysis on actively growing bacteria strengthens the idea of cellulolytic clostridia particularly well adapted, and even restricted, to a cellulolytic lifestyle, and further served as basis for the improvement of cellulose degradation by *C. cellulolyticum*.

Session V:
Polysaccharide hydrolysis and sugar transport in Non-Pathogenic Clostridia

Invited Speaker

How does *Clostridium cellulolyticum* regulate cellulosome production and composition?

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The mesophilic anaerobic bacterium *Clostridium cellulolyticum* degrades cellulose and plant cell wall associated polymers via multi-enzyme complexes, named cellulosomes. Each cellulosome potentially combines a big scaffolding protein (CipC) and eight enzymes with different and complementary action modes. Their association with the scaffoldin promotes their synergistic action and substrate proximity. 2-D gel analyses have shown that the cellulosomes produced in cellulose culture contain approx. thirty proteins [5], and at least 59 ORF that potentially encode cellulosomal proteins were recently found from the partial genome sequence of the strain. To gain further insight about the role of each subunit, we studied *in vivo* modified systems obtained by using genetic engineering methods, spontaneous mutant selection and complementation studies [1-3].

Twelve genes encoding several key cellulosomal components were found to be clustered. Among them, the first, second and fifth genes encode the scaffoldin CipC and the two major cellulosomal cellulases Cel48F and Cel9E, respectively. The study of the *cipC*Mut1 mutant strain showed that the presence of an insertion sequence interrupting *cipC* induced a strong polar effect, suggesting a transcriptional linkage of all these genes [1]. The production of a primary very large transcript covering the entire cluster was hypothesized. The processing of this transcript would lead to the production of several secondary messengers displaying different stabilities, which would contribute to the fine tuning of the expression of individual genes [4]. The search for promoter activity using transcriptional fusion with the reporter gene *catP* revealed the presence of a strong regulated promoter upstream from *cipC* [6]. A catabolite-responsive element (CRE) has been shown to be involved in regulating this operon by a carbon catabolite repression mechanism. Sequences surrounding promoter sequence may also be involved in direct (sequence-dependent DNA curvature), or indirect (unknown regulator binding) regulation. Physiological and biochemical studies of the *cipC* insertional mutant showed that this strain was affected in cellulosome synthesis and severely impaired in its ability to degrade crystalline cellulose. In a *cipC*-complemented strain, large complexes were found to be produced. They contain at least a dozen of different dockerin-containing proteins, encoded by genes located outside of the "*cip-cel*" cluster. None of the major wild-type cellulosomal processive cellulases Cel48F and Cel9E are present. Interestingly, the *cipC* and *cipC*-complemented strains share the same cellulolysis-minus phenotype [1]. Conversely, a *cipC-cel48F*-complemented strain exhibits a cellulolytic phenotype suggesting that the major Cel48F processive cellulase would be essential for the building of cellulosomes efficient in crystalline cellulose degradation. This hypothesis is consistent with the results obtained using antisense-RNA (asRNA) directed to *cel48F* mRNAs. The cellulolytic system secreted by the asRNA-producing strain showed markedly lower amount of Cel48F compared to the control strain. This was correlated with a 20 to 30% decrease in the activity of the cellulolytic system on crystalline cellulose [5].

The composition of the cellulosomes produced by *Clostridium cellulolyticum* can be engineered by structural gene expression. This was demonstrated by overexpressing the *man5K* gene coding for a mannanase. The *trans*-produced protein was found to be incorporated into the cellulosomes and became one of the major enzymatic components. Modified cellulosomes displayed 20-fold higher specific activities than control fractions on galactomannan substrates, whereas the specific activity on crystalline cellulose was reduced [3]. These results suggest that the enzymatic composition of the cellulosomes is directly regulated by the availability of the different dockerin-containing enzymes.

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Posters

CHARACTERISATION OF A PUTATIVE *AGR* SYSTEM IN *CLOSTRIDIUM BOTULINUM* AND *CLOSTRIDIUM SPOROGENES*

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One of the most notorious members of the genus *Clostridium* is *C.botulinum*, the causative agent of botulism. Aside from wound botulism in intravenous drug users, the major clinical concerns relate to intestinal toxaemia (common in the USA) and foodborne botulism the major UK concern. To help prevent botulism, it would be useful to understand the regulatory mechanisms that control changes in growth rate, virulence factor production and spore formation/ germination. Such adaptive responses are reliant on the coordinate control of gene expression mediated by celltocell communication systems, i.e., Quorum Sensing (QS).

The genome sequence of the *C.botulinum* Group I strain ATCC 3502 has recently been determined. *In silico* analysis has revealed the presence of two distinct loci capable of encoding proteins with homology to AgrB and AgrD of the *Staphylococcus aureus agr* quorum sensing system. We have begun the functional characterisation of these genes in order to determine whether they play a role in quorum sensing. The equivalent regions were shown to be present in *C.sporogenes*, and to be highly conserved. Regions of conservation are also apparent with similar loci in other clostridia and, to a lesser extent, in staphylococci.

Modulation of the expression of the identified agr genes is a prerequisite to determining their function. We have used antisense RNA expression to down regulate the *agrB* genes in *C.sporogenes* and have demonstrated that this inhibits sporulation. Two *agrD* mutants have also been generated in *C.botulinum* using our newly developed ClosTron system, and these mutants also show reduced sporulation efficiency.

SCREENING AND CHARACTERISATION OF A REDUCTASE FROM A MICRO-AEROPHILIC THERMOPHILIC CULTURE COLLECTION

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Amines serve as building blocks for pharmaceutical, cosmetic and agricultural agents and are of high commercial interest.

At Manchester, a number of mesophilic anaerobes that can hydrogenate C-C and C-N double bonds, aliphatic nitro groups, and amides have been found. Reduction of these compounds can be done using synthetic catalysts but the selectivity and environmental acceptability is poor. Moreover, biocatalytic routes are often preferred as they offer 'green' options to products of high enantioselectivities.

Therefore, there is a need to enlarge the pool of wild type organisms (and further understand how they are) able to perform these useful reductions. In seraching for these biocatalysts, we screened through a culture collection (consisting of over 700 thermophiles) made available by Green Biologics Limited Oxford. For these processes to be employed in industrial scale, it would also be beneficial if these 'new' biocatalysts are thermophilic and therefore thermostable since this is often associated with much better resistance to toxic/inhibitory chemicals and solvents. A 2-step screening method was devised. The initial step used a fluorescent dye to identify potential 'hits' and verified by GC-and LC-MS analysis. Simultaneously, 12 representative strains were assayed against 13 nitro-containing compounds as well as an amide and nitrile. This unique screening method was validated performing already established biotransformations with *Clostridium sporogenes* using conventional solvents such as ethanol and heptane, and more sophisticated solvents such as ionic liquids.

Through a fluorescence-based screen, we have therefore identified organisms with novel (nitro) reductase activity and the characterisation of the new biocatalysts will be reported.

BUTANOL AND HYDROGEN PRODUCTION IMPROVEMENT IN CA BY MODIFYING TWO ENZYMES OF THE ELECTRONIC FLUX PATHWAY: NADH-FERREDOXIN REDUCTASE AND FERREDOXIN-NAD+ REDUCTASE

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The hydrogen production by a biological pathway became one of the most promising alternatives to the use of fossil energy sources. The microorganism ideal for the industrial production of bio-H2 should be able to grow on cheap substrates, and produce large quantities of H2. However, these properties have never been identified simultaneously with the same microorganism.

However, *Clostridium acetobutylicum*, anaerobic soil bacterium, has important assets:
- it is one of the most effective bacteria for the production of H2 with a productivity of 2.4 litres of H2 / l fermenter x h.
- it has a broad spectrum of carbon substrates, and it is able to grow on xylan (polysaccharide main hemicellulose, which account for 30% of lignocellulosic biomass).

But a restriction on its use in a bioprocess is its low yield 2 moles of hydrogen H2 produced per mole of glucose consumed. In order to remove this limitation and achieve maximum theoretical yield of 4 moles H2 produced per mole of glucose consumed, we have embarked on a strategy of metabolic engineering of *Clostridium acetobutylicum*.

ABE FERMENTATION IN *C. ACETOBUTYLICUM* - A SYSTEMS BIOLOGY APPROACH

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The metabolism of *C. acetobutylicum* is characterized by the Acetone-Butanol-Ethanol (ABE) fermentation. Exponentially growing cells mainly produce the organic acids acetate and butyrate. During the transition phase *C. acetobutylicum* switches towards the generation of the solvents butanol and acetone as dominant fermentation products, a process called solventogenic shift. The results of the experiments show that the crucial prerequisite for the induction of this metabolic change is the external pH. The definite inducement of the switch is not figured out completely yet. Thus, we aim to discover the biological background of the switch by creating mathematical models and focussing our attention to the pH-dependency and the ratio of the fermentation products.

PH-DEPENDENT METABOLIC CHANGES IN *CLOSTRIDIUM ACETOBUTYLICUM* – DETECTION AND ANALYSIS OF KEY PROTEINS

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C. acetobutylicum is characterized by the Acetone-Butanol-Ethanol (ABE) fermentation. Exponentially growing cells mainly produce the organic acids acetate and butyrate. Situation changes in the transition phase when *C. acetobutylicum* switches towards the generation of butanol and acetone as dominant fermentation products, a process called solventogenic shift. A crucial prerequisite for the induction of this dramatic metabolic change in a growth limited chemostate culture is the external pH (1, 2). Knowledge about the regulation is still limited. Thus, we established a continuous chemostate culture under phosphate limitation at pH 4.5 (solvent production), switched to the synthesis of acids by applying an external pH of 5.3, and investigated differences in the steady state proteomes, respectively. Main objective of this project is to present in detail key proteins which are not only important in response to the change of the external pH, but furthermore are suitable candidates for a basic mathematic model of the solventogenic shift. Such a model is going to be established in collaboration with the Systems Biology & Bioinformatics Group at the University of Rostock. Both approaches are parts of the COSMIC-project within SysMO.

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MAXIMAL BUTANOL YIELD BY DIRECTED ENGINEERING

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The need to reduce CO_2 emissions has promoted the use of renewable resources as starting material for the production of fuels and chemicals. The acetone-butanol-ethanol fermentation (ABE fermentation) has a great potential for the production of butanol from lignocellulosic biomass. Butanol represents an important source of additives to be blended with existing fuels, and therefore contributes to meeting the increasing demand for renewable fuels. However, to again make the ABE process economically viable, higher butanol yields and higher volumetric productivity are needed to reduce recovery costs. We want, by means of genetic engineering, to increase the butanol yield and reduce by-product formation like acids and acetone. To achieve these aims a genetic tool is needed to knockout genes and to engineer a *Clostridium acetobutylicum* strain with a single-route fermentation pathway. Recently the pMTL007 system was introduced and this tool, along with others, will help us to achieve aforementioned goals. This single-route mutant should convert glucose into 1-butanol, with a theoretical maximum yield of 1 mol BuOH/mol glucose. The wild type metabolic network includes enzymes that catalyse competing reactions whose production is at the expense of butanol production. These products include acids such as lactate, acetate, and butyrate, but also solvents like ethanol and acetone. Enzymes involved in these reactions are our first knock out targets.

This project is financially supported by the Netherlands Ministry of Economic Affairs and the B-Basic partner organizations (www.b-basic.nl) through B-Basic, a public-private NWO-ACTS programme (ACTS = Advanced Chemical Technologies for Sustainability).

THE INITIATION OF SPORULATION IN *CLOSTRIDIUM BOTULINUM*

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The ability of pathogenic clostridia to form spores is considered to be one of the most important virulence factors, so it is important that the processes that control and regulate sporulation are elucidated. A better understanding of the physiology of *Clostridium botulinum* (the principal target of food processing in the UK) would make it possible to meet the increasing requirement of achieving tighter food safety margins without compromising product safety.

The proteins involved in the initiation of sporulation (Stage 0) are best studied in the model Gram-positive bacterium *Bacillus subtilis*. Sporulation is controlled by the phosphorylation status of the master regulator Spo0A. The intracellular and extracellular signals which induce sporulation are conveyed to this transcription factor by a variety of proteins (sensor histidine kinases, response regulators, and a phosphotransferase) which together constitute a phosphorelay.

The recent completion of several clostridial genome sequencing projects has highlighted that clostridia do not possess a recognisable phosphorelay. They all contain a Spo0A homologue, and *C. tetani* does posses a protein with weak homology to the Spo0B response regulator phosphotransferase. However, none of the clostridia contain a homologue to response regulator Spo0F. Therefore, how is Spo0A phosphorylated in clostridia?

One hypothesis being tested is that Spo0A is directly phosphorylated by sensor histidine kinases which do not possess any cognate response regulator, so called 'orphan' sensor histidine kinases. Analysis of the genome sequence of *C. botulinum* highlighted the presence of five orphan sensor histidine kinases. Work is presented here on the inactivation and characterisation of these orphan sensor histidine kinases utilising the clostridial universal knockout technology recently developed at the University of Nottingham (ClosTron technology).

REGULATION OF ACETONE FORMATION IN *CLOSTRIDIUM ACETOBUTYLICUM* BY TRANSCRIPTIONAL REGULATORS

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Clostridium acetobutylicum is a Gram-positive, spore-forming, anaerobic soil bacterium and well known for its solvent formation. The master regulator Spo0A as well as the potential transcription factors AdcR and AdcS play a role in the regulation of the *adc* gene and therefore in the production of acetone. As mutations of the Spo0A binding sites (0A boxes) in the promoter region of the *adc* gene did lower but not completely abolish the activity of the *adc* promoter, AdcR as additional transcriptional regulator was identified by DNA affinity chromatography. RT-PCR experiments indicate an operon structure of *adcR* with the downstream gene *adcS*. Spo0A, AdcR, and AdcS were purified as his tag fusion proteins in *Escherichia coli* and used for gel retardation and protein-protein interaction assays. The binding-site of AdcR was determined by footprint analyses. The effect of AdcR and AdcS on solvent production was investigated using antisense constructs of the *adcR* and *adcS* gene which were transformed in *C. acetobutylicum*. Knockout mutants of all three genes are under construction to validate their physiological function. So far the data indicate a role of AdcR as a repressor and AdcS as an positive regulator of acetone formation.

PRODUCTION OF LESS TOXIC 1-BUTANOL DERIVATIVES BY SOLVENTOGENIC *CLOSTRIDIA*

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Current butanol production by microbial fermentation is not economically viable. The final product concentration in the fermentation process is relatively low mainly due to toxicity of butanol. Although this inhibitory effect of butanol has been subject of research for many decades, butanol tolerance is still insufficient (about 1-2%). The molecular basis of this toxicity most likely relates to the hydrophobic nature of butanol, allowing it to dissolve into the lipid bilayer, resulting in significantly disturbed membrane structure and function.

A novel approach is based on metabolic engineering aiming at the production of butanol derivatives with lower toxicity. Candidate derivatives are considered in the beginning of the project. The downstream processing of these products is expected to be more efficient, and they have a significantly reduced toxicity. Since the feasibility of this novel approach is unpredictable, a parallel random mutagenesis approach (UV, chemicals) will be initiated that aims at obtaining mutants with enhanced tolerance towards 1-butanol or derivatives thereof.

GENOMIC ANALYSIS AND TRANSCRIPTOMICS OF THE HYDROGEN PRODUCING THERMOPHILIC BACTERIUM *CALDICELLULOSIRUPTOR SACCHAROLYTICUS*

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Hydrogen is becoming a realistic option for the sustainable energy economy, provided that production occurs from renewable primary energy sources and not from fossil fuels. In addition to thermochemical technologies, fermentative hydrogen production is gaining a lot of interest. For thermodynamic reasons fermentative hydrogen production is easier at elevated temperatures and comparison of various hydrogen-producing anaerobes shows that thermophilic bacteria and archaea indeed have better hydrogen yields and produce less other reduced end products. Among the different thermophilic saccharolytic hydrogen producers that are available to date, *Caldicellulosiruptor saccharolyticus* has excellent characteristics for efficient hydrogen production. It grows optimally at 70ºC, it can grow on many different carbohydrates (including cellulose, xylan and pectin) and it produces high levels of hydrogen. Moreover, it can simultaneously convert C6-and C5-sugars, the main constituents of commonly used biomass feedstocks. For these reasons *C. saccharolyticus* is one of the key microorganisms of the dark fermentation within the EU-KP6 program HYVOLUTION. For the same reason this organism was selected by the DOE for a genome sequencing project, which was completed in May 2007. In the current presentation we describe the results of the genome annotation and of a genome-wide transcriptional analysis of glucose/xylose utilization.

XYLOSE CATABOLISM IN THE BIOFUEL PRODUCING ORGANISM *CLOSTRIDIUM ACETOBUTYLICUM* ATCC 824

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Lignocellulosic waste provides a potentially important resource for biofuel production in the light of issues such as space and competition with food crops potentially causing great hindrance to the development of the biofuel industry. Traditionally yeasts, namely *Saccharomyces cerevisiae*, have been employed in bioethanol production. However yeasts are often unable to utilise pentose sugars found in hemicelluloses, a component of lignocelluloses. Hemicellulose can constitute up to 35% of the contents of lignocellulose and this is composed of a backbone of xylan, a polymer of the pentose xylose. *Clostridium acetobutylicum* is a solvent producing bacterium that can utilise a wide variety of substrates to produce end-products of Acetone, Butanol and Ethanol, the so called ABE fermentation process. Butanol is now recognised as a superior second generation biofuel due to its higher energy, lower volatility and the fact that it can be blended with diesel. The potential therefore exists to employ *C. acetobutylicum* to produce bio-butanol from lignocellulosic waste – however very little is known about this metabolism in clostridia. In this study we report the identification of a potential pathway for uptake and metabolism of xylose by *C. acetobutylicum*. A gene from this pathway is cloned in *E. coli* and the *in vivo* activity of the product is demonstrated by complementation analysis.

SLH MODULES IN *CLOSTRIDIUM THERMOCELLUM* SURFACE

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Clostridium thermocellum is an anaerobic, thermophilic, cellulolytic, and ethanogenic bacterium capable of directly converting cellulosic substrate into ethanol. In this bacterium, degradation of the cellulosic materials is carried out by a large extracellular cellulase system called the cellulosome.
The cellulosome ability to attach to the cell surface is a result of the existence of S-layer homology (SLH) modules as part of its scaffold protein. The interaction between the cell wall (peptidoglycan) and the S-layer proteins is very strong, although not covalent. Thus, the high level of expression of these proteins, together with their efficient binding to the cell wall, makes this system very attractive for studying cell surface anchoring. Despite the vital role of the SLH domain in the cellulosome system, it is still one of the few components of this system for which no three dimentional structure is available.
Deep search in the *C. thermocellum* genome revealed several putative SLH modules, one of them is a part of a huge multi-domain surface protein, termed Anak. This 0.8 Mda protein is comprised of a signal peptide, multiple domains possibly involved in protein/protein interactions and a C-terminal S-layer homology (SLH) domain.
Our hypothesis about Anak and its SLH modules is that its binding to the cell surface is mediated by the SLH modules, in a similar manner to the cellulosome attachment to the cell surface. We therefore suggest that the SLH modules in *C. thermocellum* play as a common anchoring mechanism to the cell surface.
In our research we cloned and over-expressed the putative SLH modules in order to check their ability to bind to the cell surface, The Anak SLH modules showed positive binding to native peptidoglycan-containing sacculi.
We also attempt to crystallize the SLH modules in order to solve the SLH 3D structure. Once structural data become available, it will aid in the understanding of the SLH binding properties and provide an insight to the anchoring of the cellulosome.

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