ClostNet

The Conference

March 2013

Nottingham, UK

Programme and Abstracts

CLOSTNET PROJECT MANAGER:

Jacque Minton

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The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

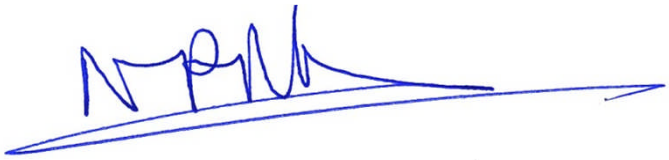
Dear Colleagues,

Clostridia are an important but poorly understood group of bacteria. The EU-funded, Initial Training Network (ITN) CLOSTNET aims to address this deficiency through the creation of a critical mass of clostridial researchers trained to both tackle the threats posed to human health and well being, and stimulate wealth creation from industrial and medical use at the community level.

A four year, 5 million € project , CLOSTNET involves 11 different academic and industrial partners from 7 Member States and funds 22 PhD fellows and a series of 5 workshops and 2 open *Clostridium* conferences . The research undertaken covers both pathogenic and non-pathogenic species, and falls within 5 broad work packages: Global Gene Regulation, Sporulation and Germination, Secretion, Metabolism and Genome Plasticity

Our aim at this final conference is both showcase the accomplishments of the fellows, and provide them with the opportunity to network with acknowledged experts in the field. In the latter respect we would like to thank those experts from outside of the network for agreeing to participate in what we believe will be an excellent forum for discussion and networking.

Nigel P Minton
On behalf of the CLOSTNET partnership
March 2013



Prof. Dr. Hubert Bahl
Universität Rostock Traditio et Innovatio

Dr. John Telford
NOVARTIS VACCINES

Prof. Dr. Wolfgang Liebl
Technische Universität München

Dr. Peter McClure
Unilever

Dr. Paola Mastrantonio
Istituto Superiore di Sanità

Prof. Rick Titball
University of Exeter

Prof. Maja Rupnik

Prof. Philippe Soucaille
metabolic explorer

Prof. Chantal Tardif
cnrs

Prof. Miia Lindstrom
University of Helsinki

GENERAL INFORMATION

VENUE
Nottingham Conference Centre
Burton Street
Nottingham
NG1 4BU

ORAL PRESENTATIONS
Oral presentations will be in the Adams Room, level 2, Nottingham Conference Centre. The length of oral presentations is scheduled from 15 to 30 min (check programme), within that presenters should allow 5 min for discussion. All presentations should be prepared in a form of MS Power Point slide show and stored on USB sticks or CD/DVD. The use of a personal computer or Mac is not possible. All presentations will be uploaded to the computer in the lecture hall. This can be done anytime, but at least 2-3 hours before your session or the evening before for early morning presentations.

POSTER PRESENTATIONS
Poster presentations will be in the Bowden Room, level 2, Nottingham Conference Centre. Please mount your poster any time before your allocated poster session, on your allocated poster board, which will be displayed outside the poster room. Velcro tabs will be provided. The presenting author should stand by his/her poster for the whole length of the session.

SOCIAL EVENTS
Welcome Reception
Sunday, 17th March 2013, 20:00 until 23:00, Wembley Suite, Hilton Hotel.
Conference Dinner
Wednesday, 20th March 2013, 20:00 until 23:00, Wembley Suite, Hilton Hotel.

STAYING IN TOUCH
There is complimentary Wi-Fi access throughout the conference centre.
The Business Centre up the stairs on level 3 provides you with an area to work quietly, collate your presentation, or send emails.

REFRESHMENTS
The refreshment hubs just outside the Adams and Bowden rooms, offer a complimentary self-service selection of refreshments throughout the day.

YOUNG PRESENTER AWARDS
There will be two prizes one for the best oral presentation and one for the best poster.

TAXIS
We always use DG Taxis 0115 9 607607, or ask your hotel to book for you.

CLOSTNET II PROGRAMME (* invited)

Sunday, 17th March 2013

19.00-20.00 **Registration** *Wembley Suite, Hilton Hotel*

20:00-22.00 **Welcome Dinner** *Wembley Suite, Hilton Hotel*

Monday, 18th March 2013

Session I: **Spores** *Adams Room, Nottingham Conference Centre*

Chairperson: Nigel P Minton

09.00 - 09.30 *Adriano O. Henriques - Universidade Nova de Lisboa, Lisbon, Portugal
A Streamlined Sporulation Pathway in *Clostridium difficile*

09.30 - 10.00 *Mike Peck - Institute of Food Research, Norwich, UK
Clostridium botulinum and Foodborne Botulism

10.00 - 10.20 David Kirk - University of Helsinki, Finland
The Sporulation Pathway in *Clostridium botulinum* ATCC 3502

10.20 - 10.40 Carolyn Meaney – Unilever, UK
Strategies for the control of germination and outgrowth of Group I *Clostridium botulinum* in food products.

10.40 - 11.10 **Coffee break**

Session II: **Spores** *Adams Room, Nottingham Conference Centre*

Chairperson: Miia Lindström

11.10 - 11.40 *Simon Cutting - Royal Holloway, University of London, UK
Mucosal vaccination to C. difficile using Bacillus spores for Antigen Delivery

11.40 - 12.00 Henrique Machado - University of Rostock, Germany
The role of granulose in in the obligate anaerobe *Clostridium acetobutylicum*.

12.00 - 12.20 Daniela Heeg - The University of Nottingham, UK
Spore formation and spore germination of *Clostridium difficile*.

12.20 - 12.40 Carlo Rotta – Unilever, UK
Development of a DNA transfer procedure and genetic tools for *Clostridium pasteurianum*.

12.40 - 13.00 Ann-Kathrin Kotte - The University of Nottingham, UK
RNPP-type Quorum sensing in *Clostridium acetobutylicum*.

13.00 - 14.00 **Lunch** *The Old Library, Nottingham Conference Centre*

Session II: **Carbohydrate Binding Proteins** *Adams Room, Nottingham Conference Centre*

Chairperson: Marco Soriani

14.00 - 14:30 *Harry Gilbert – University of Newcastle, UK
Carbohydrate binding proteins: From Cellulosomes to the Microbiota

14:30 - 15.00 *Willem de Vos – University of Helsinki, Finland & Wageningen University, The Netherlands
Microbes Inside

15.00 - 15.20 Hamza Celik - CNRS, Marseille, France
A two-component system (XydS/R) controls the expression of genes encoding CBM6-containing proteins in response to straw in *Clostridium cellulolyticum*.

15.20 - 15.40 Tanja Dapa – Novartis Vaccines, Italy
Multiple Factors Modulate Biofilm Formation by the Anaerobic Pathogen *Clostridium difficile*.

15. 40 - 17.30 **Posters I & Coffee** *Bowden Room, Nottingham Conference Centre*

18.00 – 21.45 **Dinner** - at your convenience *Nicholson's Restaurant, Hilton Hotel*

Tuesday, 19th March 2013

Session III: **Regulation** *Adams Room, Nottingham Conference Centre*

Chairperson: Hubert Bahl

09.00 - 09.30 *Isabelle Martin-Verstraete - Institut Pasteur, Paris, France
Global regulation of cysteine metabolism in Gram positive bacteria

09.30 - 10.00 *Jose Vazquez-Boland - University of Edinburgh, Edinburgh, UK
Listeria pathogenesis: faster, always faster

10.00 - 10.20 Delyana Vasileva - University of Rostock, Germany
Iron and Zinc-dependent Regulation in the Strict Anaerobe *Clostridium acetobutylicum*.

10.20 - 10.40 Zhen Zhang – The University of Helsinki, Finland
Regulation of neurotoxin production in *Clostridium botulinum*.

10.40 - 11.10 **Coffee break**

Session V: **Regulation** *Adams Room, Nottingham Conference Centre*

Chairperson: Philippe Soucaille

11.10 - 11.40 *Wilfrid Mitchell - Heriot Watt University, Edinburgh, UK
Phosphotransferase systems and sugar transport.

11.40 - 12.10 *Bruno Dupuy - Institut Pasteur, Paris, France
Sugar metabolism and virulence in *Clostridium difficile*.

12.10 - 12.30 Niall Bollard – The University of Nottingham, UK
Characterisation of Phosphotransferase Systems (PTSs) in *Clostridium difficile*.

12.30 - 12.50 Justyna Lesiak - Technical University Munich, Germany
Investigation of Xylose and Arabinose Metabolism and Glucose Catabolite Repression in Solventogenic Clostridia

12.50 – 13.10 Thao Nguyen - Metabolic Explorer, (INSA), France
Regulation of *adhE2* gene in *Clostridium acetobutylicum*

13.10 - 14.10 **Lunch** *The Old Library, Nottingham Conference Centre*

14.00 - 17.00 **NETWORKING AFTERNOON**

Session VI: **Metabolism** *Adams Room, Nottingham Conference Centre*

Chairperson: Wolfgang Liebl

17.00 - 17.30 *David Fell - Oxford Brookes University, UK
Construction and Analysis of a *Salmonella typhimurium* Genome-Scale Metabolic Model

17.30 - 18.00 *Peter Dürre - University of Ulm, Germany
Novel approaches to the biotechnological production of butanol and acetone.

17.50 - 18.20 Minyeong Yoo - Metabolic Explorer, (INSA), France
Analysis of metabolic mutants of *Clostridium acetobutylicum.*

18.20 – 18.40 Liu Ziyong - Technical University Munich, Germany
The role of cysteine for acetogenesis and solventogenesis of *Clostridium acetobutylicum*

18.00 – 21.45 **Dinner** - at your convenience *Nicholson's Restaurant, Hilton Hotel*

Wednesday, 20th March 2013

Session VII: **Secretion** *Adams Room, Nottingham Conference Centre*

Chairperson: Chantal Tardif

09.00 - 09.30 *Kim Hardie - The University of Nottingham, UK
How Bacteria Secrete Proteins And How To Investigate The Mechanisms

09.30 - 10.00 *Neil Fairweather - Imperial College, London, UK
Cell surface proteins of *Clostridium difficile*

10.00 - 10.20 Minh-Uyen Dao Thi – CNRS, Marseille, France
Secretion and assembly of cellulosome components in *Clostridium cellulolyticum*.

10.20 - 10.40 Andrea Kovacs Simon - University of Exeter, UK
Role of Lipoproteins in Virulence of *Clostridium difficile*

10.40 - 11.10 **Coffee break**

Session VIII: **Toxins and Disease** *Adams Room, Nottingham Conference Centre*

Chairperson: Rick Titball

11.10 - 11.40 *Filip van Immerseel - University of Gent, Belgium
An update on novel virulence factors and new roles for old virulence factors in animal diseases.

11.40 - 12.00 Sergio Fernandes da Costa – University of Exeter, UK
NetB, the (w)hole story.

12.00 - 12.20 Magdalena Kasendra – Novartis Vaccines, Italy
Destroy the fence or open the gate: how *Clostridium difficile* toxins modulate infection of polarized cells?

12.20 - 12.50 *Matej Butala - University of Ljubljana, Slovenia
Key players of the SOS response as a targets for antiinfectives.

12.50 - 13.10 Beata Walter - Institute of Public Health, Maribor, Slovenia
The Role of DinR and SOS Response in *Clostridium difficile*.

13.10 - 14.10 **Lunch** *The Old Library, Nottingham Conference Centre*

14. 10 – 16:00 **Posters II and Coffee** *Bowden Room, Nottingham Conference Centre*

Session IX: **Antibiotic Resistance** *Adams Room, Nottingham Conference Centre*

Chairperson: Maja Rupnik

16.00 - 16.30 *Peter Mullany - University College, London
Mobile genetic elements from the Clostridia.

16.30 - 17.00 Francois Wasels - Istituto Superiore di Sanità, Rome, Italy
Investigating the Fitness Cost of MLS_B Resistance in *Clostridium difficile.*

17.00 - 17.20 Ines Moura - Istituto Superiore di Sanità, Rome, Italy
Resistance to metronidazole in *Clostridium difficile.*

17.20 – 17.40 Alexander Grosse-Honebrink – The Nottingham of Nottingham, UK
Mariner-Transposon Mediated Random Mutagenesis in *Clostridium species*

20.00 - 22.00 **Conference Dinner** *Wembley Suite, Hilton Hotel*

ABSTRACTS OF ORAL PRESENTATIONS

A Streamlined Sporulation Pathway in *Clostridium difficile*

FÁTIMA C. PEREIRA[1], LAURE SAUJET[2,3], ANA TOMÉ[1,], MÓNICA SERRANO[1], MARC MONOT[3], EVELYNE COUTURE-TOSI[3], ISABELLE MARTIN-VERSTRAETE[2,3], BRUNO DUPUY[3], and ADRIANO O. HENRIQUES[1]

[1]Microbial Development Laboratory, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, PORTUGAL; [2]Univ. Paris Diderot, Sorbonne Paris Cité, Cellule Pasteur, 25 rue du Dr. Roux, Paris, 75015, FRANCE; [3] Laboratoire Pathogenèse des Bactéries Anaérobies, Institut Pasteur, Paris, FRANCE.

Endospores, as those formed by the anaerobic Clostridial species, the aerobic *Bacillus* and related organisms, are highly resilient dormant cell types. Endospore development has been extensively studied in *Bacillus subtilis* where it is governed by four cell type-specific RNA polymerase sigma factors, two active sequentially in the mother cell and two active sequentially in the forespore, the two cells that participate in endospore development. The precise timing of sigma factor activation is coordinated between the mother cell and forespore during development by several mechanisms. By combining studies of gene expression at the single cell level with the phenotypic characterization of mutants for the sigma genes, we describe the regulation of gene expression during endospore development by the intestinal pathogen *C. difficile*. In this organism, the endospore is the vehicle for infection, transmission and persistence of the organism in the host and the immediate environment. The overall picture that emerges is of a developmental program in which the coordination of gene expression between the forespore and mother cell is less tightly controlled. A streamlined version of the cellular differentiation program may be a general feature of endospore formation in the more ancient Clostridia group of bacteria.

Clostridium botulinum and Foodborne Botulism

MICHAEL W. PECK

Gut Health and Food Safety Programme, Institute of Food Research, Norwich Research Park, Colney, Norwich, NR4 7UA, UK

Clostridium botulinum and also some strains of *Clostridium baratii* and *Clostridium butyricum* form botulinum neurotoxin. *C. botulinum* is a heterogeneous species and is defined on the basis of a single physiological property, an ability to form botulinum neurotoxin. *C. botulinum* can be separated into four distinct phylogenetic groups (*C. botulinum* Groups I to IV), with the distinction between each group strong enough to justify separation into four different species.

Foodborne botulism is a severe and often fatal neuroparalytic intoxication caused by consumption of pre-formed botulinum neurotoxin. There are seven botulinum neurotoxins (types A to G), and more than thirty different botulinum neurotoxin sub-types are now recognised. The botulinum neurotoxins are the most potent toxins known, with as little as 30-100ng of neurotoxin sufficient to cause human illness and possibly death.

The majority of outbreaks of foodborne botulism are associated with strains of *C. botulinum* Group I (proteolytic *C. botulinum*) and *C. botulinum* Group II (non-proteolytic *C. botulinum*). *C. botulinum* Group I (proteolytic *C. botulinum*) is a mesophile (minimum growth temperature 10°C-12°C) that forms highly heat resistant spores, while *C. botulinum* Group II (non-proteolytic *C. botulinum*) is a psychrotroph (minimum growth temperature 2.5°C-3.0°C) that forms spores of moderate heat resistance. The extreme severity of foodborne botulism ensures that regulators and industry remain vigilant in order to minimize the foodborne botulism risk.

The Sporulation Pathway in *Clostridium botulinum* ATCC 3502

DAVID G. KIRK, HANNU KORKEALA, AND MIIA LINDSTRÖM

Department of Food Hygiene and Environmental Health, Centre of Excellence in Microbial Food Safety Research, P.O. Box 66, 00014 University of Helsinki, Finland

Clostridium botulinum is notorious food pathogen and produces heat-resistant spores. Spores survive food processing and may germinate into toxic cultures, jeopardizing food safety. Little is known about *C. botulinum* sporulation. The molecular pathway shares components with *Bacillus subtilis*, a model organism for sporulation. The shared genes include those encoding the sporulation "master switch" Spo0A and four sigma factors SigF, SigE, SigG and SigK, in order of activation in *B. subtilis*.

Transcriptional and mutational analyses of the four sigma factors were performed using qRT-PCR and the ClosTron system in *C. botulinum* ATCC 3502. For qRT-PCR analysis, the expression of eight housekeeping genes was analysed over 48 hours of growth to identify the most suitable normalisation reference gene. 16S *rrn* was most stably expressed across all growth phases and was thus selected.

Transcriptional analysis showed bi-phasic expression of *sigK*, corresponding to both early and late stages of sporulation. Sporulation of the *sigK* mutant halted at an early stage, and transcription of *spo0A* was significantly reduced in this mutant, suggesting early-stage involvement of SigK in sporulation of *C. botulinum* (Kirk *et al*. 2012). This is contrary to the role of SigK in *B. subtilis*, in which SigK is restricted to completion of the sporulation process. Transcriptional analysis showed *sigF* to be expressed in late-log growth, and *sigE* expression to peak in the stationary phase. The *sigF* and *sigE* mutants failed to produce spores, halting prior to spore cortex formation. This corresponds to the roles of SigF and SigE in *B. subtilis*. Peak transcription of *sigG* followed that of *sigE* in the stationary phase, suggesting a relatively late involvement of SigG in the sporulation process of *C. botulinum*. This was confirmed by the *sigG* mutant possessing a partial spore cortex. *B. subtilis sigG* mutants do not possess a spore cortex, suggesting a differential role for SigG in *C. botulinum* and in *B. subtilis*.

Reference:

- Kirk *et al* (2012). Involvement of *Clostridium botulinum* ATCC 3502 sigma factor K in early-stage sporulation. Appl. Environ. Microbiol. 78:4590-6.

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement No. 237942, "CLOSTNET".

Strategies for the Control of Germination and Outgrowth of Group I *Clostridium botulinum* in Food Products.

CAROLYN MEANEY, STEPHEN CARTMAN, PETER McCLURE and NIGEL MINTON

Centre for Biomolecular Sciences, Clifton Boulevard, University Park, Nottingham, NG7 2RD

The spore-forming bacterium *Clostridium botulinum* is the causative agent of food-borne botulism, infant botulism and wound botulism. Botulinum neurotoxin is one of the most lethal neurotoxins known to man and is one of the main virulence factors but it is the spore-forming capacity of this bacterium that enables it to persist in the environment and be transmitted from soil to foods and then from person to person.

On one hand, an actively growing vegetative cell can differentiate into a spore in response to nutrient limitation or other adverse conditions, enabling it to remain viable in the environment for long periods of time. Spores can survive without water or nutrients and be highly resistant to extremes of temperature, pH, UV and ionizing radiation. On the other hand, germination is a multi-step process involving the transformation of spores into vegetative cells enabling initiation of growth, toxin production and sometimes infection.

Ultimately it is the aim of the food industry to gain an understanding of these germination and outgrowth processes in order to eradicate these spore forming bacteria as causes of food poisoning and food spoilage.

By exploiting current molecular tools available, such as Allelic Exchange and the Group II intron directed ClosTron technology, it is the aim of this project to focus on the area of spore cortex degradation and cortex lytic enzymes in an attempt to expand our current understanding of germination and outgrowth processes. Similarly, novel mutagenesis strategies such as the *mariner*-based transposon mutagenesis system, which uses a traceable mobile genetic element to randomly disrupt genes in bacterial genomes, has been implemented and utilised to examine a germination phenotype in Group I *C. botulinum* ATCC 3502.

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement No. 237942, "CLOSTNET".

Mucosal Vaccination to *C. difficile* Infection Using *Bacillus* Spores for Antigen Delivery

HONG A. HUYNH, PATIMA PERMPOONPATTANA and SIMON M. CUTTING

School of Biological Sciences, Royal Holloway, University of London, Egham, TW20 0EX, UK.

Clostridium difficile infection is an important nosocomial infection in the developed world and one for which no vaccine currently exists. Two toxins, A and B, produced by most strains of *C. difficile* are implicated yet only recently has the requirement of both been defined. Current vaccine strategies are mostly focused on parenteral delivery of toxoids. In this work we have used bacterial spores (*Bacillus subtilis*) as a delivery vehicle to evaluate the carboxy-terminal domains of toxins A and B as protective antigens.

Our findings are important and show that oral delivery of the cell binding domain of toxin A is sufficient to confer protection in a hamster model of infection designed to closely mimic the human course of infection. Importantly, neutralizing antibodies to the toxin A domain were shown to be cross reactive with the reciprocal domain of toxin B and, being of high avidity, provided protection to challenge with an A^+B^+ *C. difficile* strain. Thus, although both toxins are required for disease only antibodies to toxin A are required for protection. Animals vaccinated with recombinant spores were fully able to survive reinfection, a property that is particularly important for a disease where patients are prone to relapse. We show that in contrast to parenteral delivery, mucosal immunization is required to generate secretory IgA and that production of these neutralizing polymeric antibodies correlates with protection. This work demonstrates that an effective vaccine to *C. difficile* can be designed around two attributes, mucosal delivery and the cell-binding domain of toxin A.

The role of granulose in the obligate anaerobe *Clostridium acetobutylicum*

HENRIQUE MACHADO, HUBERT BAHL and RALF-JÖRG FISCHER

Institute of Biological Sciences, Division of Microbiology, University of Rostock, Rostock, Germany

The transition phase of growth of the Gram-positive, endospore forming anaerobe *Clostridium acetobutylicum* is characterized by several morphological changes. When entering the stationary phase swollen and cigar shaped cells, termed clostridial stages, are formed. In this type of cells a polymeric glycogen-like carbohydrate is accumulated in the form of granules, called granulose. This glucose-polymer is defined as an amylopectin-like structure and only slightly branched (98% α-1.4-linkages and 2% of α-1.6-linkages). Granulose is considered to store energy- and carbon, and hitherto believed to be necessary as a prerequisite for sporulation.

We generated a granulose defect mutant by inactivating (ClosTron® technology) the single glycogen synthase (GlgA) annotated in the genome of *C. acetobutylicum*. The mutant strains showed no differences in growth rate, fermentation pattern and, surprisingly, sporulation, when compared to the wild-type strain, even though it exhibited a granulose defect phenotype. This raised questions regarding the importance and function of the granulose in the lifestyle of *C. acetobutylicum*. Therefore, the importance of this glucose-polymer under periods of nutrient depletion usually affecting soil bacteria was investigated. The strain was able to be complemented for its granulose defective phenotype by plasmid-based *glgA* expression.

The results demonstrate that sporulation and the cell differentiation observed during *C. acetobutylicum* sporulation are independent of granulose accumulation. Furthermore, new insights concerning the enzymatic process of *C. acetobutylicum* granulose synthesis by GlgA will be presented.

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement No. 237942, "CLOSTNET".

Spore formation and spore germination of *Clostridium difficile*

DANIELA HEEG[1], DAVID A. BURNS[2], STEPHEN T. CARTMAN[1] AND NIGEL P. MINTON[1]

[1]*Clostridia Research Group, School of Molecular Medical Sciences, Centre for Biomolecular Sciences, University of Nottingham, Nottingham, UK*
[2]*Pharmacom Media, City Reach, 5 Greenwich View Place, London, UK*

Clostridium difficile is the major underlying cause of antibiotic-associated diarrhoea and recently, *C. difficile*-associated disease has become the most prevalent nosocomial infection. Spores are widely regarded to be the infectious particle, however, in order to cause disease theses spores must abjure dormancy and return to vegetative cell growth through germination. While the mechanisms of sporulation and spore germination are well understood in other spore formers such as *Bacillus*, knowledge of these pathways in *C. difficile* remains incomplete. Previous work has identified *Spo0A*, the master regulator of sporulation in *Bacillus* spp. to be equally involved in the spore formation of *Clostridium* spp., including *C. difficile*. Several other homologues to genes involved in the spore formation pathway of *Bacillus* can be found in the *C. difficile* genome; however, their specific functions have not been defined by experiments yet. Other studies have shown that bile salts and amino acids play an important role in regulating germination in *C. difficile*. Taurocholate has been shown to act as a co-germinant with glycine, while chenodeoxycholate has been shown to inhibit spore germination in a clinical isolate. The absence of homologues to genes involved in sporulation and spore germination of other spore formers, such as the kinases upstream of Spo0A or germinant receptors, has complicated research into *C. difficile* disease. Additionally, increasing incidence of specific types of *C. difficile* has opened up questions about the virulence of certain molecular types and thereby, toxicity, sporulation, and spore germination capacities. In this work, we have used a genotypic approach in order to further characterise the *C. difficile* spore formation and spore germination pathways. Further, we have used phenotypic characterisation in order to gain an understanding of the sporulation and germination abilities of *C. difficile* clinical isolates.

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement No. 237942, "CLOSTNET".

Development of a DNA transfer procedure and genetic tools for *Clostridium pasteurianum*

CARLO ROTTA[1,2], KATRIN SCHWARZ[2], ALAN MCNALLY[3], PETER MCCLURE[1], NIGEL MINTON[2]

[1]*Safety & Environmental Assurance Center, Colworth Science Park, Unilever, Sharnbrook (Bedfordshire), UK;*
[2]*Clostridia Research Group, Centre for Biomolecular Sciences, University Park, University of Nottingham, Nottingham, UK;*
[3]*School of Science and Technology, Nottingham Trent University, Nottingham, UK*

C. pasteurianum is a spore-forming, gram-positive organism associated with the bursting of swollen cans and sealed food packages. Both are caused by carbon dioxide and hydrogen by-products of the organism's metabolism. Butyrate and acetate are the main products. Especially butyrate accounts for an off-odour and off-flavour of the packed food, not acceptable to consumer. Mild processes like e.g. pasteurisation, acidification and increasing salt concentrations are used in food industry to circumvent bacterial outgrowth while preserving the food characteristics. However, due to its spore formation *C. pasteurianum* resists these treatments. To date, comparably little is known about the metabolism, sporulation and germination of the bacterium, mainly caused by the lack of an available genome sequence and its genetic inapproachability.

We, therefore, sequenced the genome of the type strain ATCC 6013 and developed a protocol for the transfer of foreign DNA into the bacterium. A preliminary genome annotation revealed the presence of several restriction systems which can severely inhibit successful DNA transfer.

Protection of foreign DNA against these endogenous restriction systems was, therefore, our first aim leading to the cloning of the respective methyltransferase into modular plasmids exhibiting different Gram$^+$ origins of replication. In a second step, we put our emphasis on the development of a DNA transfer technique. Starting from procedures established for other clostridia (*C. acetobutylicum*, *C. beijerinkii*), we tested parameters such as cell density, composition of the electroporation buffer, electropulse settings and recovery time.

With a reliable electroporation protocol at hand, it will be possible to apply Clostron and ACE to *C. pasteurianum* and study sporulation and germination in more detail.

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement No. 237942, "CLOSTNET".

RNPP-type Quorum Sensing in *Clostridium acetobutylicum*

ANN-KATRHIN KOTTE, KATRIN SCHWARZ, NIGEL MINTON and KLAUS WINZER

Clostridia Research Group, Centre for Biomolecular Sciences, University Park, University of Nottingham, Nottingham, NG7 2RD, UK

This work investigated whether RNPP-type quorum sensing is involved in the regulation of key features such as solventogenesis and sporulation in *Clostridium acetobutylicum*. It identified eight putative RNPP-type Quorum Sensing Systems (*qssA* to *qssH*) in the genome of *C. acetobutylicum,* each consisting of a Quorum Sensing Regulator (*qsr*) and a putative Quorum Sensing Peptide (*qsp*). The regulators were defined by their helix-turn-helix, tetratricopeptide repeat structure which is typical for RNPP-type regulators, peptides by their length and amino acid composition. ClosTron inactivation of *qsrA, qsrC*, *qsrE, qsrF*, *qsrG* and *qsrH* negatively affected solventogenesis, morphological development or sporulation whereas inactivation of *qsrB* had a positive effect. A repression of solvent formation in *qsrB*-overexpressing strains could be relieved by the addition of synthetic QspB-derived peptides to the culture medium, suggesting that QsrB and QspB form a functional quorum sensing system.

This work identified two further *qsr*-type regulators which are not encoded together with a putative signalling peptide. The two orphan *qsr* regulators, *qsrI* and *qsrJ*, are instead encoded in a gene cluster that is conserved across *Clostridium sensu stricto* spp, *qsrI* preceding *qsrJ*. Inactivation of *qsrI* and *qsrJ* reduced total solvent formation to below 15 mM, eliminated sporulation and granulose accumulation. The effect was very similar to what has been reported for *spo0A* inactivation. An RNA-Seq analysis of the *qsrI* mutant combined with co-culture experiments provided strong evidence that QsrI controls *agr*-type quorum sensing.

Key outcomes of this work are the affirmation that RNPP-type quorum sensing is not restricted to aerobic *Firmicutes* but also occurs in anaerobes, the first, strong evidence that solvent formation in a *Clostridium* spp. is controlled by quorum sensing and the discovery of two regulators which are equal in importance to the master regulator of sporulation, Spo0A.

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement No. 237942, "CLOSTNET".

Microbes Inside

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Since early life, intestinal microbes dominate our body and outnumber our own cells by one or more orders of magnitude. In the intestinal tract they constitute the largest microbial ecosystem that is close to our heart: our microbes inside. The collective genome of these microbes contributes considerably to the coding capacity of our system. However, unlike our own genome, the intestinal microbiome is not strictly vertically inherited. Moreover, this personalized organ can be modified by a variety of food and pharma treatments that target its composition, stability and activity. These are instrumental in providing cause-effect relations to complement the abundance of associations that are presently being reported.

This contribution will provide an overview of the present state of the art in the human microbiome and focus on Clostridia and other Gram-positive bacteria that are dominant in the human intestinal tract. Specific attention will be given to the successes and future of fecal transplantations that are used to treat *Clostridium difficile* infections.

A Two-Component System (XydS/R) Controls the Expression of Genes Encoding CBM6-Containing Proteins in Response to Straw in *Clostridium cellulolyticum*.

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The composition of the cellulosomes (multi enzymatic complexes involved in the degradation of plant cell wall polysaccharides) produced by *Clostridium cellulolyticum* differs according to the growth substrate. In particular, the expression of a cluster of 14 hemicellulase-encoding genes (called *xyl-doc*) is induced by the presence of straw and not of cellulose.

Genes encoding a putative two-component regulation system (XydS/R) were found upstream of *xyl-doc*. First evidence for the involvement of the response regulator, XydR, in the expression of *xyl-doc* genes was given by the analysis of the cellulosomes produced by a regulator overproducing strain when grown on cellulose. Nano LC MS/MS analysis allowed the detection of the products of all *xyl-doc* genes and of the product of the gene *Ccel_1656*. This last product is a protein of unknown function predicted to bear a family 6 carbohydrate binding module targeting hemicelluloses as the carbohydrate binding modules present in the products encoded by *xyl-doc*. RT-PCR experiments further demonstrated that the regulation occurs at the transcriptional level and that all *xyl-doc* genes are transcriptionally linked.

mRNA quantification in a *xydR* knock-out strain and in its complemented derivative confirmed the involvement of *XydR* in the expression of *xyl-doc* and *Ccel_1656* genes in response to straw. Electrophoretic mobility shift assays further demonstrated that the regulator *XydR* binds to DNA regions located upstream of the first gene of the *xyl-doc* gene cluster and upstream of *Ccel_1656* gene.

As a model, we propose that the sensor *XydS* is involved in sensing the signal linked to the presence and/or the degradation of straw. This signal may be generated by the activity of products encoded by *xyl-doc* and/or *Ccel_1656* genes transcribed at basal level. In response to this signal, *XydR* would be activated and in turn would activate transcription of *xyl-doc* and *Ccel_1656* genes to efficiently degrade hemicelluloses.

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Multiple Factors Modulate Biofilm Formation by the Anaerobic Pathogen *Clostridium difficile*

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Clostridium difficile is an obligate anaerobic, Gram-positive, endospore-forming bacterium. Although an opportunistic pathogen, it is one of most important causes of healthcare-associated infections. *C. difficile* colonizes the gut when the normal intestinal microflora is disrupted by antimicrobial agents and causes *Clostridium difficile* infection (CDI). Recurrent clostridial infections and rapid rise of antibiotic resistant strains have made disease treatment very difficult.

While toxins TcdA and TcdB are the main virulence factors of *Clostridium difficile*, the factors or processes involved in gut colonization during infection remain unclear. Biofilm formation by *Clostridium difficile* could play a role in virulence and persistence of *C. difficile*, as seen for other intestinal pathogens.

We demonstrate that clinical *C. difficile* strain, 630, and the hypervirulent strain R20291, form structured biofilms *in vitro*, with R20291 accumulating substantially more biofilm. Microscopic and biochemical analyses show multiple layers of bacteria encased in a biofilm matrix containing proteins, DNA and polysaccharide. Employing isogenic mutants, we show that virulence-associated proteins, Cwp84, flagella and a putative quorum sensing regulator, LuxS are all required for maximal biofilm formation by *C. difficile*. Interestingly, a mutant in Spo0A, a transcription factor that controls spore formation, was defective for biofilm formation, indicating a possible link between sporulation and biofilm formation. Furthermore, we demonstrate that bacteria in clostridial biofilms are more resistant to high concentrations of vancomycin, a drug commonly used for treatment of CDI.

Our data suggest that biofilm formation by *C. difficile* is a complex multifactorial process and may be a crucial mechanism for clostridial persistence in the host.

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Global regulation of cysteine metabolism in Gram-positive bacteria

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Cysteine has a crucial role in cellular physiology and due to its reactivity its synthesis is tightly controlled. We combined transcriptome and functional genomic approaches to identify cysteine transporters and to characterize the methionine into cysteine conversion pathway. We further deciphered the molecular mechanisms involved in the fine-tuning of cysteine metabolism superimposing premature termination of transcription to transcriptional control by two LysR-type regulators (CysL and YtlI) and a Rrf2-type repressor, CymR. In transcriptome, CymR negatively controls the expression of 24 genes involved in cysteine uptake or biosynthesis. CymR recognizes a 27 bp consensus motif and binds to the promoter region of 7 genes or operons. CymR senses cysteine availability via an original signal transduction pathway, which involves the formation of a complex with the OAS thiol-lyase CysK, a key enzyme of the cysteine biosynthetic pathway. We then demonstrated that CysK and CymR form a complex *in vivo* and *in vitro*. The recent resolution of the structure of CysK and CymR and the identification of the structural determinants required for the complex formation allow us to better understand the molecular bases of the interaction between the different partners of this regulatory system. The effector of the signal transduction pathway is *O*-acetyl-serine (OAS), the direct precursor of cysteine, which prevents the formation of the CysK-CymR complex and its binding to DNA targets. In the presence of cysteine, the size of the intracellular pool of OAS decreased leading to the formation of the CymR-CysK complex and to the CymR-dependent repression of the controlled genes. CymR-like regulators are present in Bacilli, Staphylococci, Listeria and several clostridia. In *Staphylococcus aureus*, CymR also indirectly controls biofilm formation, the oxidative stress response and virulence. This establishes the existence of complex regulatory links between cysteine metabolism, stress and virulence probably via redox changes.

Listeria pathogenesis: faster, always faster

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The gram-positive pathogen *Listeria monocytogenes* is the causative agent of listeriosis, an invasive foodborne infection with severe clinical manifestations including meningoencephalitis, septicemia, stillbirth and neonatal sepsis. *Listeria* virulence depends on the ability of these bacteria to survive and proliferate within macrophages and a variety of non-professional phagocytes. In contrast to other major intracellular pathogens such as *Mycobacterium tuberculosis* or *Salmonella*, *L. monocytogenes* replicates in the cytosol and not in a membrane-bound vacuole. Internalisation into host cells permits *Listeria* to evade extracellular host defenses while vacuolar escape is essential to prevent lysosomal killing. After replication in the cytosol, *Listeria* avoid the cytotoxic immune response directed against infected cells via a “runaway” strategy involving actin-based cell-to-cell spread. *Listeria* possesses dedicated virulence factors to ensure the efficient completion of each of the key steps of its intracellular lifestyle. This presentation will discuss some of the *L. monocytogenes* factors that provide a competitive edge in the race with the host immune response, with a focus on interference with endocytic traffic and the metabolic determinants of intracellular growth.

Iron and Zinc-dependent Regulation in the Strict Anaerobe *Clostridium acetobutylicum*

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In the natural environments, bacteria are challenged with either insufficient or elevated and even toxic amounts of metals. Therefore, they had to establish a tight control on the intracellular metal content in order to meet the metabolic needs of the cell. Classical metal-dependent response in bacteria is carried out by the Fur (ferric uptake regulator) family of regulators. These proteins function as transcriptional repressors that bind to palindromic sequences in the promoters of the target genes. This family includes members like Fur and Zur, which sense two distinct divalent metals certainly of great importance for almost all microorganisms- iron and zinc, respectively.

Clostridium acetobutylicum is a Gram-positive, endospore-forming strict anaerobe, which is characterized by the ability to switch from synthesis of the organic acids acetate and butyrate during exponential growth to production of the solvents acetone, ethanol and butanol upon transition to stationary phase. The regulation of Fe and Zn homeostasis that are required for many important enzymes, including those involved in the central metabolism, is of interest for establishing of fundamental cellular characteristics in *C. acetobutylicum*. The genome of *C. acetobutylicum* revealed two genes encoding a putative ferric uptake regulator (Fur) and a putative zinc uptake regulator (Zur). We inactivated the *fur* and *zur* genes through insertional mutagenesis using the Clostron system and both mutants were physiologically characterized. To gain further insights into the role of the Fur and Zur proteins and the mechanisms for establishment of metal balance in *C. acetobutylicum*, we characterized the gene expression profile of the mutant strains and the iron- respectively zinc-limitation stimulon of the parental strain. Our results demonstrated that *C. acetobutylicum* senses and responds to availability of iron and zinc using a sophisticated system and the Fur family of proteins plays an important role in this process.

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Regulation of Neurotoxin Production in *Clostridium botulinum*

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Botulinum neurotoxin produced by the spore-forming bacterium *Clostridium botulinum* is the most poisonous biological substance known to mankind. By blocking neurotransmission, the neurotoxin causes a flaccid paralysis called botulism which may to lead to death upon respiratory muscle collapse. Two-component signal transduction systems (TCSs) and global regulatory elements have been found to regulate the expression of genes encoding virulence factors in many pathogenic bacteria. However, their roles in regulation of neurotoxin production in *C. botulinum* remain unknown.

We have identified a TCS and global regulators that contribute to neurotoxin regulation in *C. botulinum*. Using the ClosTron mutagenesis system, several target genes were inactivated individually in *C. botulinum* type A strain ATCC3502, and neurotoxin expression was monitored using toxin gene expression profiling and ELISA. Inactivation of *cbo0786*, encoding a TCS response regulator and *cbo0787*, encoding a TCS sensor histidine kinase, resulted in significantly induced neurotoxin gene (*botA*) expression and neurotoxin production. Complementation of the *cbo0786* mutant with a plasmid expressing *cbo0787/cbo0786* restored neurotoxin production to the wild-type level. Further protein-DNA interaction assays suggested recombinant CBO0786 to repress neurotoxin gene expression by blocking transcription from the neurotoxin promoters recognised by the alternative sigma factor BotR (Zhang *et al*. 2013). Inactivation of genes encoding the global regulators and phosphotransferase system (PTS) proteins also affected the neurotoxin production. Further characterization of these mutants is ongoing. These findings suggest that *C. botulinum* modulates neurotoxin production in response to extracellular signals and cellular metabolism.

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Phosphotransferase Systems and Sugar Transport

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Bacteria possess a number of mechanisms for accumulation of sugars and sugar derivatives. Among these mechanisms, the PEP-dependent phosphotransferase system (PTS) is unique in that it catalyses not only the uptake of the substrate but also its phosphorylation. In common with other anaerobic bacteria, the clostridia show a marked dependence on the PTS as a mechanism for uptake of carbohydrates.

The PTS comprises a multi-component system that catalyses the transfer of phosphate from PEP to the sugar substrate, which is phosphorylated as it enters the cell. Phosphoryl-transfer is mediated by the general proteins enzyme I and HPr, and substrate-specific IIA and IIB domains, while substrate translocation across the cell membrane is facilitated by a IIC (and sometimes also a IID) domain. Changes in the phosphorylation state of the PTS components in response to the accumulation of extracellular sugars provide cells with the ability to sense the nutritional status of their environment. The system therefore occupies a central position in carbohydrate metabolism and its control, and it has also been implicated in regulation of virulence in some pathogenic bacteria.

Genome sequencing projects have confirmed that clostridia, both pathogenic and non-pathogenic, carry a wide range of phosphotransferase systems that are representative of all known PTS families. This implies that the bacteria have a considerable metabolic capacity for sugar assimilation, and suggests that the PTS is of major significance in clostridial physiology. Thus, detailed characterization of the PTS can potentially contribute to effective biotechnological exploitation or control of pathogenicity. In this presentation, the PTS complement in different species will be compared, and progress in identifying the function of individual systems in the solventogenic strains *Clostridium acetobutylicum* and *Clostridium beijerinckii* will be reviewed.

Sugar Metabolism and Virulence in *Clostridium difficile*

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Correlation between carbon metabolism and virulence has been shown in several bacterial pathogens. In *Clostridium difficile,* it is now well establish that toxin production is regulated in response to various environmental signals. In the presence of glucose or other rapidly metabolizable sugars, *C. difficile* toxin synthesis is repressed involving the **C**arbon **C**atabolite **R**epression (CCR) mechanism. CcpA, the global transcriptional regulator of the CCR, generally binds a cis-acting element (*cre* site) in the promoter region of the controlled genes. Moreover, CcpA binding activity is enhanced by its interaction with HPr-Ser-P, a component of the phosphotransferase system. We showed by *in vivo* and *in vitro* approaches that CCR is implicated in the glucose-dependent repression of the toxin gene. With the exception of *tcdE*, we demonstrated that CcpA binds the promoter region or the 5' of the PaLoc genes, suggesting a complex regulation of toxin synthesis in response to glucose availability. We further showed in the hamster model that virulence is delayed in the *ccpA* mutant indicating that other factors important for infection and pathogenesis may be under CCR. Thus, to understand the impact of glucose and the regulatory role of CcpA in *C. difficile* physiology, we performed a transcriptome analysis of JIR8094 strain and its isogenic *ccpA* mutant, grown in the presence or absence of glucose. In addition with an *in silico* analysis and a ChIP to chip study, we define the *C. difficile cre* site motif and characterize the CcpA regulon. CcpA plays a central role in several metabolic pathways and cellular processes like stress response, toxin synthesis and sporulation. Finally using an axenic mouse model, we determined the CcpA regulon *in vivo*. Among genes specifically controlled *in vivo* by CcpA, we found genes involved in ethanolamine utilization, an abundant compound of the intestinal tract that can be used as carbon and nitrogen sources during the *C. difficile* gastrointestinal lifestyle

Characterisation of PhosphoTransferase Systems (PTSs) in *Clostridium difficile*

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PhosphoTransferase Systems (PTSs) represent an important method of sugar uptake in bacteria and have been well described in the past. However, research into PTSs within the genus *Clostridium* has been mainly restricted to the non-pathogens. Analysis of the genome of *Clostridium difficile* 630 revealed over 40 intact PTSs, a surprisingly large number; this is over three times as many as in other pathogenic *Clostridia* such as *Clostridium perfringens* and *Clostridium botulinum*. Previously, carbon catabolite repression has been shown to affect toxin production in *C. difficile*. Being capable of utilising different carbohydrates efficiently could be important for *C. difficile* to grow and survive in the human gut. So far, very little work has been done to elucidate the role of individual systems in carbohydrate uptake, sensing of the environment and regulation of toxin expression. A deeper understanding of the PTSs in *C. difficile*, and their importance in virulence, could lead to the development of new drug targets.

The aim of our study is to characterise the main PTSs of *C. difficile*, determine their role in carbohydrate uptake and their effect on regulation of virulence. To date, we have chosen what we believe to be the main candidates involved in glucose, mannitol, fructose, and sorbitol uptake, and have inactivated these PTSs using the ClosTron and in-frame deletion methods. We intend to prove their role in uptake of the relevant sugar; and to determine their role in toxin regulation.

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Investigation of Xylose and Arabinose Metabolism and Glucose Catabolite Repression in Solventogenic Clostridia

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Xylose is a common pentose sugar present in majority of biomass feedstock, such as agricultural or municipal by-products and waste, molasses or simply plant material. Research focuses on improvement of xylose utilization by different solventogenic Clostridia for their important role in production of green energy sources and solvents. In some Clostridia xylose metabolism seems to be biochemically and genetically related to arabinose metabolism. In *Clostridium acetobutylicum* genes responsible for xylose and arabinose degradation are placed in the same putative operon.

In a previous study we described a glucose repression of one operon involved in xylose degradation in *C. acetobutylicum* ATCC 824 (1). In media containing glucose and xylose large amounts of xylose remained untapped until nearly all glucose was consumed and we wanted to investigate this catabolite repression. Therefore, we constructed ClosTron mutants and two clean deletion mutants to disrupt both operons presumably involved in xylose degradation in *C. acetobutylicum* to further investigate catabolite repression of glucose on xylose metabolism. Basing on a methylation system developed in our lab, we also created ClosTron mutants in two predicted xylose degradation operons in *Clostridium saccharobutylicum* NCP 262, and made an attempt to describe and classify the restriction-modification systems of the bacterium.

We studied phenotypes of the mutant strains in rich and minimal media, containing different sugars as sole carbon sources. In several mutants catabolite repression was clearly visible, and some mutants showed in batch cultures significant differences in growth with different sugars.

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Regulation of *adhE2* in *Clostridium acetobutylicum*

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In continuous culture, *C. acetobutylicum* can be maintained in three different stable metabolic states: acidogenic, solventogenic and alcohologenic (Girbal and Soucaille, 1998). In alcohologenic culture, the genes involved in the butanol formation (*bdhA, bdhB,* and *adhE*) were not transcribed and the *adhE2* gene of *C. acetobutilycum* ATCC 824 is expressed specifically. The *adhE2* gene was characterized from molecular and bio-chemical points of view by Lisa Fontaine in our laboratory (Fontaine *et al.* 2002). This is the second AADH identified in *C. acetobutylicum* ATCC 824. Both of the genes *adhE* and *adhE2* are carried by the pSOL1 megaplasmid of *C. acetobutylicum* ATCC 824. Regulatory mechanism of *adhE2* expression is unknown. A redox sensing protein encoded by *CA_C2713* was found to bind to the promoter regions of *thl, ldh, crt* and *adhE2* (Wietzke and Bahl 2012). Interestingly, we found two additional palindromes upstream of *adhE2* that could potentially contribute to concomitant control of *adhE2*. Magnetic bead technology was employed to isolate proteins which have affinity to these palindromes from *Clostridium acetobutylicum* cell extracts. Either using double stranded DNA or single stranded DNA preparations, in both of the two palindrome constructs, the major proteins captured by the DNA were surprisingly the AdhE2 and Bcd proteins (analyzed by mass spectrometry). We are examining and characterizing the DNA binding activity of the two dehydrogenase enzymes for better understanding their effects on gene regulation and/or replication, specifically on *adhE2* gene. In parallel, we are elucidating the functions of these two palindromes by employing pGUSA reporter system developed by Girbal (Girbal *et al.* 2003). Controlling of β-glucuronidase expression is studied under different *adhE2* promoter constructs in *Clostridium acetobutylicum* wild type and Δ*adhE2* mutant strains. Understanding the mechanism of *adhE2* regulation would be helpful for further engineering *C. acetobutylicum* to achieve a highly efficient process for the production of butanol.

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Construction and Analysis of a *Salmonella* Typhimurium Genome-Scale Metabolic Model

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The example of *Salmonella* Typhimurium is used to illustrate the theory and practice of building genome-scale models of metabolism in order to infer metabolic phenotypes from genome sequences. Such models are analysed by linear programming (also termed flux balance analysis) to determine optimal states of metabolism during growth. An extension of such an analysis is to scan a series of solutions as the demand for a particular metabolic function is varied; this reveals how global metabolism rearranges to accommodate the changing metabolic demand. As an illustration, we show how the set of reactions responding to increasing demand for ATP, during growth on glucose, identifies a small catabolic core of the larger model. Though this core includes reactions of glycolysis and the TCA cycle, some less-expected reactions are also involved, which shows the advantages of having an objective method of assessment.

This approach is potentially applicable to any sequenced and annotated microbe and any metabolic product it produces or can be engineered to produce. An extension, illustrated in our poster, is to identify the common catabolic core used by *Salmonella* on any of a wide range of metabolisable substrates in order to identify universal weak points in its ability to generate ATP for survival.

Novel Approaches To The Biotechnological Production Of Butanol And Acetone

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Clostridium acetobutylicum is able to produce the industrially important solvents acetone and butanol. The process is tightly regulated by transcription factors such as Spo0A, CodY, CcpA, and AdcR. At least one additional protein, AdcS, and a small, noncoding RNA (SolB) are also involved. Spo0A can be considered the master regulator inducing solventogenesis, while SolB is a negative regulator, completely shutting down acetone and butanol formation when overexpressed.

Although many new fermentation plants have been built in Brazil and China, future biotechnological production will rely on introduction of substrates, not competing with human nutrition. There are two suitable options: lignocellulose and gas mixtures with CO and CO_2/H_2. From lignocellulose, *C. acetobutylicum* can use hexoses and pentoses, rendering that a feasible solution. The other possibility is to use autotrophic anaerobic acetogens and to metabolically engineer them for solvent production. *Clostridium ljungdahlii* is able to ferment synthesis gas (CO/H_2) or CO_2/H_2 mixtures, rendering it interesting for the biotech industry, because of combining industrials needs with sustained reduction of CO and CO_2 in the atmosphere. Experimental data and *in silico* comparisons revealed a novel mode of anaerobic homoacetogenic metabolism. The organism was transformed with plasmids bearing heterologous genes for butanol production. Successful expression of these genes could be demonstrated, leading to formation of the biofuel. Thus, *C. ljungdahlii* can be used as a novel microbial production platform based on synthesis gas. As the organism does not grow very well on CO_2/H_2 mixtures, *Clostridium aceticum* was chosen for this substrate. Again, an electroporation procedure was developed and expression of heterologous acetone-forming enzymes could be demonstrated. Both examples stress the possibility to use CO or CO_2 as novel carbon sources for formation of bulk chemicals, so far derived from crude oil.

Analysis of Metabolic Mutants of *Clostridium acetobutylicum*

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Clostridium acetobutylicum is an anaerobic, non-pathogenic, and spore-forming bacterium which can produce mixtures of organic acids and/or solvents from various sugars and polysaccharides. In phosphate limited continuous cultures of *C. acetobutylicum*, three different metabolic states are able to be observed dependent on the pH of the culture and availability of NAD(P)H: i) an acidogenic state (production of acetic and butyric acids), ii) a solventogenic state (production of acetone, butanol, and ethanol) and iii) an alcohologenic state (formation of butanol and ethanol but not acetone). Although the complex metabolism of *C. acetobutylicum* has been studied for a long while, still the factors regarding metabolic shift from acidogenesis to solventogenesis are not totally comprehended.

To understand the regulation of solvent formation of *C. acetobutylicum* better, several metabolic mutants (deletion of *buk*, *adhE1*, *adhE2*, *alsD*, respectively) have been constructed by the “double crossing over gene deletion” method developed by Metabolic Explorer. The results will provide a physiological characterization of these mutants in batch and continuous cultures under different metabolic states.

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The Role of Cysteine for Acetogenesis and Solventogenesis of *Clostridium acetobutylicum*

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The batch fermentation of *Clostridium acetobutylicum* is characterized by a acetogenic growth phase during exponential growth when mainly acetate and butyrate are fermentation products. Then, at the end of exponential growth and during stationary phase, the organism switches to solventogenic growth and large amounts of acetone, ethanol and butanol are produced. These growth phases can be studied independent from each other in a phosphate-limited continuous culture. In transcription analysis of continuous cultures using DNA microarrays it became evident that, among others, operons involved in sulfur assimilation are strongly up-regulated during solventogenesis. Using the ClosTron technique we constructed two knock-out mutants in the genes CAC0105 and CAC0930 annotated as involved in sulfate reduction and cysteine biosynthesis. We did complementation experiments with sulfite and cysteine to prove the predicted function and show that more reduced sulfur is needed by the organism during solventogenesis. This could be due to synthesis of a high concentration of a sulfur containing redox mediator like e.g. thioredoxin needed during solventogenesis. Construction and analysis of further mutations in sulfur metabolism try to narrow down the sulfur compound involved and its physiologic function.

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How Bacteria Secrete Proteins And How To Investigate The Mechanisms

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Almost 20% of bacterial proteins are localised to the external cell wall or beyond. Some of these proteins are structural, others enable the acquisition of nutrients, and for pathogens many of them are virulence factors that need to be localised outside the bacterium to gain access to the host.

The process of protein secretion presents significant challenges to bacteria as often large proteins with a hydrophilic surface need to be moved across hydrophobic membranes without compromising the integrity of the cell. There is also a need for specificity.

Bacteria have evolved more than seven independent pathways to secrete proteins, some of which are shared between Gram positive and Gram negative bacteria. An overview of the range of complexity of these different molecular machines will be provided. Particular emphasis will be placed on the techniques that can be used to investigate whether a protein is secreted and how the components of the secretion machinery function.

Cell Surface Proteins of *Clostridium difficile*

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Clostridium difficile is now recognised as an important infectious agent associated with health care institutions. Infection control measures within hospitals have been crucial in reducing the rates of infection, but this pathogen remains highly problematic and novel intervention measures are urgently needed. Experimental vaccines against *C. difficile* are being developed based on inducing toxin neutralising activity, but such vaccines do not address carriage of the bacterium within the host.

Cell wall proteins are attractive candidates for antigens to include in a vaccine as they are surface exposed and are often involved in bacterial-host interactions. Immunity to these proteins may reduce carriage. Our lab has characterised a number of cell wall proteins of *C. difficile* and has discovered unexpected functions for several proteins.

The major cell wall proteins in *C. difficile* are the S-layer proteins that form a two-dimensional lattice surrounding the cell. We have shown that the S-layer proteins are part of a large family of proteins that share a conserved architecture that is involved in anchoring the proteins to the underlying cell wall. While some of these proteins are highly conserved between strains, others show considerable sequence diversity in their surface exposed domains. These variable proteins include SlpA, the S-layer precursor, Cwp66 and CwpV. This variation is consistent with evasion of the immune response.

A number of cell wall proteins have been assigned functions by various *C. difficile* research groups. Thus Cwp84 is a cysteine protease that cleaves the SlpA precursor, Cwp22 (CD2963) is a peptidoglycan L-D transpeptidase and CwpV is a bacterial aggregation factor. Recently we have shown that Cwp66, previously described as an adhesin, is necessary for long-term survival of the bacterium in a mouse model of infection. The functions of other cell wall proteins and how they interact with recently discovered polysaccharides that form secondary cell wall polymers is an active avenue of our research.

Secretion and Assembly of Cellulosome Components in *Clostridium cellulolyticum*

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Clostridium cellulolyticum is a cellulolytic, mesophilic and anaerobic bacterium, which secretes multi-enzymatic complexes, termed cellulosomes to hydrolyze plant cell wall polymers into simple sugars. Cellulosomes of *C. cellulolyticum* are composed of a scaffolding protein CipC, which assembles up to eight catalytic enzymes with different mode of action towards plant cell wall degradation. Secretion of cellulases in *C. cellulolyticum* remains little understood. My aim was to identify putative chaperons dedicated to the secretion of one of the most abundant cellulases: Cel48F. A strain producing strep-tagged-Cel48F was used to co-purify proteins interacting with the signal sequence of Cel48F. MS/MS analysis needs to be performed in order to identify putative chaperones.

Second part of the project involves the cellulosome assembly. OrfXp protein is putatively involved in this process. OrfXp- encoding gene is located in the *cip-cel* operon, which encodes three major cellulosome components: CipC, Cel48F and Cel9E. This envelope-attached protein contains a cohesin module and was hypothesized to be a shuttle between the membrane and CipC. Revealing more details of its subcellular localization, OrfXp was found to be an integral membrane protein and not covalently linked to the peptidoglycan layer. Strains overproducing OrfXp or defective in the synthesis of this protein were developed. All these strains produce similar cellulosomes (in terms of protein composition) and display similar growth kinetics on cellulosic substrates. In addition, in terms of cellulose adherence, no differences were observed. Another protein could play a role in the assembly of cellulosomes: CotH protein (Ccel_1101). This protein belongs to the CotH superfamily, a spore coat assembly protein. MS/MS analysis showed that CotH-containing cellulosomes are produced in response to straw, cellulose and xylan. Dockerin module of CotH protein has lower affinity for the cohesin of CipC, when compared to the dockerins of the other cellulases tested, i.e. Cel48F, Cel9G, Cel5A. Thus, CotH could be transiently incorporated in the cellulosomes waiting for the incorporation of new dockerin-containing enzymes produced in response to the substrate modification.

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Role of Lipoproteins in Virulence of *Clostridium difficile*

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Antibiotic-associated diarrhoea (AAD) and colitis, with the causative agent being the Gram-positive, anaerobe *Clostridium difficile* are one of the most important hospital-acquired infections and significant burden to healthcare services worldwide. Therefore, research to identify novel virulence factors and better understand the pathogenic process of this organism is of great importance. As lipoproteins have been shown to play key roles in virulence of many pathogens, the aim of this project was to investigate whether lipoproteins are involved in the pathogenesis of *C. difficile*.

Lipoproteins are attached to the extracellular side of the cytoplasmic membrane in Gram-positive bacteria with a single enzyme, lipoprotein diacylglycerol transferase (Lgt). Therefore, inactivation of the *lgt* gene allows investigation of the global contribution of lipoproteins in bacterial processes. An *lgt* mutant was generated by the ClosTron system and reduced lipoprotein content on the bacterial cell surface was confirmed. Physiology and virulence-associated functions of the *lgt* mutant was studied and surprisingly, many of the assayed phenotypes were not significantly affected by disruption of the *lgt* gene. Nevertheless, the ability of the *lgt* mutant to adhere to cultured intestinal endothelial cell line (Caco-2) was markedly reduced. In further studies, lipoprotein CD0873 as a potential adhesin was identified and characterised. Involvement of this lipoprotein in adherence of *C. difficile* was investigated by several different assays and the results strongly suggest that CD0873 contributes to the adherence of *C. difficile.*

Taken together, these results suggest that lipoproteins might have a role in virulence of *C. difficile* as colonization is essential for establishment of the infection. Nevertheless, further studies have to be undertaken to determine how lipoproteins are involved in the adherence process.

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An Update on Novel Virulence Factors and New Roles for Old Virulence Factors in Animal Diseases

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Clostridium perfringens is an animal pathogen because of its capacity to produce a wide range of toxins. For most, but not all, animal pathologies induced by this bacterium, the causative toxins are known. In addition, new roles for known toxins and non-toxin virulence factors are being identified the last years. Bovine hemorrhagic enteritis is an example where a new role is discovered for well-known toxins and broiler necrotic enteritis is discussed as a case to present novel non-toxin virulence factors.

In Belgian Blue veal calves, bovine enterotoxemia or hemorrhagic enteritis is a feared disease because of the high mortality. While epsilon and beta2 toxin have been suggested to be important for bovine enterotoxemia, the actual evidence has not yet been given by fulfilling Koch's postulates. We have developed a model for this disease and shown that any *C. perfringens* strain from any origin can induce pathology, suggesting a role for a universal *C. perfringens* toxin. Moreover, by using knockout strains, we showed that alpha toxin and perfringolysin act synergistically in inducing hemorrhagic enteritis. *In vitro* data show that bovine endothelial cells are a key target for these toxins. These data demonstrate that the well-known 'old' toxins alpha toxin and perfringolysin play a role in hemorrhagic enteritis in veal calves.

The pathogenesis of broiler necrotic enteritis has recently been unravelled because of the identification of the causative NetB toxin. What is also noticed is that in the gut of all affected animals within an affected flock, typically usually only one *C. perfringens* clonal population is found, suggesting a role of bacteriocins. It was found that virulent *C. perfringens* strains are more able to inhibit none virulent strains. Subsequently, we have identified perfrin, an intra-species antimicrobial protein belonging to a novel class of bacteriocins, which is associated with *netB* positive strains and not with non-*netB* carrying poultry strains suggesting it is of importance for virulence.

The data described above thus clearly show that in the case of animal diseases, still novel virulence traits can be identified, while novel roles for already well-described toxins can be found.

NetB, the (w)hole Story

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Clostridium perfringens is one of the pathogenic species within the *Clostridium* genus as it is able to produce at least 17 toxins. NetB (Necrotic enteritis toxin B) is a recently identified β-pore-forming toxin produced by *Clostridium perfringens*. This toxin has been reported to play a role in the pathogenesis of avian necrotic enteritis, a severe gastro-intestinal disease causing economic damage to the poultry industry worldwide (around 2 billion US dollars per year). In this study, we present the crystal structure of the NetB multimer and identify amino acids that play an important role in NetB function by site directed mutagenesis. The heptameric structure of NetB shows high similarity to the *Staphylococcus aureus* alpha-hemolysin, the prototype of the related family of small β-pore-forming toxins. However, in particular the region thought to interact with the target cell membrane shows some interesting divergence in amino acid composition. Mutations within this domain significantly affected binding and toxicity of NetB. Subsequently, a NetB genetic toxoid and a formaldehyde NetB toxoid were used to immunise poultry in an *in vivo* necrotic enteritis disease model. Vaccination with any of the two antigens resulted in the induction of antibody responses against NetB and provided significant protection against disease. This work will provide the basis for devising a vaccine to control avian necrotic enteritis.

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Destroy the Fence or Open the Gate: How *Clostridium difficile* Toxins Modulate Infection of Polarized Cells.

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Clostridium difficile is an important opportunistic human pathogen that produces two potent toxins essential for its virulence. Both TcdA and TcdB inactivate small Rho proteins, the central regulators of cell polarity in eukaryotes, leading to damage of human colonic mucosa. Even though the effects of these toxins on epithelial cells have been already described, the question of how they could benefit the bacteria and facilitate host colonization remains open.

The mucosal barrier, a first line of organism defense from pathogens, is composed by polarized epithelial cells with distinct apical and basolateral surfaces separated by tight junctions (TJs). TJs play pivotal roles in tissue integrity and maintenance of cell polarity. In particular, they aim to: a) regulate the paracellular passage of molecules, including pathogens (gate function); b) restrict the movement of plasma membrane components between apical and basolateral region (fence function). However, even such a well-organized barrier can be overcome by specialized microorganisms that have evolved different strategies to infect polarized cells and cause diseases.

In this study, we hypothesized that *C.difficile* toxins interfere with cell polarity in order to enhance bacteria binding and crossing of epithelium. We optimized the use of Caco-2 cell model, which resembles colonic tissue, to mimic natural infection under anaerobic conditions. Then we focused on: 1) the effects of toxins on gate and fence function of TJs, 2) the importance of host cell polarity for their susceptibility to *C. difficile* infection and 3) the influence of toxin-mediated subversion of cell polarity on the bacterial ability to colonize epithelium. As a result we found that *C. difficille* infection of Caco-2 cells is differentiation state dependent and occurs preferentially at the basolateral membrane. Moreover we demonstrated for the first time that *C. difficile* toxins modulate epithelial cell functions to the benefit of invading bacteria.

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Key Players of the SOS Response as a Targets for Antiinfectives

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Factors that confer DNA damage or block the replication forks induce a number of co-ordinately regulated responses in bacteria, including the SOS response. It is an inducible DNA repair system with functions to maintain genetic integrity and to enhance adaptation of bacteria through mutagenesis and genetic exchange. Studies employing therapeutic drugs showed that subinhibitory concentrations of certain antibiotics, that interfere with DNA replication as well as cell wall synthesis, can trigger the SOS response. In some well-characterised pathogens, induction of the SOS response modulates the evolution and dissemination of drug resistance, as well as synthesis, secretion and dissemination of the virulence. Hence, selective SOS-induction inhibitors can enhance antibiotic chemotherapy.

The SOS system requires a transcriptional repressor, the LexA protein, and an inducer, the RecA protein. In response to DNA damage, RecA polymerizes onto single-stranded DNA and interacts with LexA and activates its latent self-cleaving activity. Cleavage inactivates LexA, instigating repressor dissociation from its DNA targets and induction of the LexA regulon. We have conducted most studies with *Escherichia coli* to show how the LexA repressor discerns specific target DNA, operator sequences, and how DNA damage induces the SOS response. In addition, we carried out a mapping of the LexA/RecA interaction area that directed us to generate model of the complex. Insights into the critical step of the DNA damage response offer a platform for the development of novel antibacterials. We are testing small molecules for the ability to modulate the bacterial SOS stress response.

The SOS system is widespread among bacteria and we show that several LexA biochemical processes in *Clostridium difficile* 630 resemble those identified in *E. coli.*

The Role of DinR and SOS Response in *Clostridium difficile*

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The increasing incidence of *C.difficile* infections remains one of the main concerns of a healthcare worldwide. A significant number of research has been devoted to investigation of virulence factors such as toxins, spore formation and antibiotic resistance.

The SOS is a DNA repair system among bacteria, induced by factors that cause DNA damage or block the replication forks. It is controlled by a global transcriptional repressor LexA and an inducer, the recombinase protein RecA. Its functions are to maintain genetic integrity, enhance adaptation of bacteria through increased mutation rate and accelerate the spread of mobile genetic elements. It is best understood in Gram negative model organism *E.coli.* The knowledge regarding the SOS mechanism in *C. difficile* is scarce.

In this project we study the SOS response in *C.difficile* and we focus on the role of DinR (LexA homolog in *C. difficile*). We have generated a ClosTron mutant which does not produce the DinR, thus all the genes up regulated by the protein are continuously expressed. As a consequence, the strain acts like it was under constant stress. We observed noteworthy morphological and physiological changes. The mutant formed long filamentous cells due to inhibited division. The initial data suggested lower sporulation rate but higher toxin production. Finally, different antibiotic resistance patterns were observed between mutant and its wild type.

The, analysis of a major regulator, DinR provides understanding of its contribution to stress response and possibly virulence. The analysis of various pathogenic properties such as toxin production, sporulation and antibiotic resistance of the mutant provides better insight into the processes that occur within the cell exposed to the stress.

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Mobile Genetic Elements from the Clostridia

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The clostridia contain a plethora of mobile genetic elements (MGEs) ranging from simple insertion sequences to very large integrative elements such as conjugative transposons and phage. In some organism such as *Clostridium difficile* up to 25% of the genome is made up of MGEs. These MGEs have a huge impact on the biology of the host organism, often being responsible for the spread of antibiotic resistance, virulence factors and the ability to exploit different ecological niches. In addition they have more subtle effects on the genome as integrative elements can inactive genes into which they insert and can sometimes change how genes near the area of insertion are regulated. Finally the MGEs provide portable areas of homology and can recombine with their host genome and other MGEs to generate novel associations of genes on which natural selection can act; they are one of the major drivers of evolution.

Due to the large numbers of MGEs that can survive in the clostridia this genus has access to a large pan genome. This extends beyond the clostridia as MGEs that have been found in the clostridia are capable of transfer into other genera. Understanding the role of MGEs in the biology and evolution of the clostridia is complex as the factors governing host range and regulation of these different MGEs are not well understood. However work in our lab and others has begun to provide a framework with which to understand how these elements contribute to the biology and pathogenicity of the clostridia.

Investigating the Fitness Cost of MLS_B Resistance in *Clostridium difficile*

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Clostridium difficile is the leading cause of hospital-acquired diarrhoea in Europe and North America. Antimicrobial therapy plays a central role in the development of *C. difficile* infections. Historically, most of the *C. difficile* epidemic isolates were resistant to the macrolide-lincosamide-streptogramin (MLS_B) antibiotics and this resistance is still the most common phenotype in strains isolated in Europe. In *C. difficile*, MLS_B resistance is conferred by *erm*(B) genes located on mobile elements showing heterogeneity in their genetic organization.

Antibiotic resistance mechanisms are often associated with a fitness cost that is typically observed as a reduced growth rate and an altered capability of survival and/or virulence. This cost is the main biological parameter that influences the rate of development of resistance, its stability and the rate at which the resistance might decrease if the use of antibiotics is reduced. A better understanding of the impact of antimicrobial resistance on bacterial fitness may allow us to predict how it could spread and persist in clinical isolates.

Filter-mating assays were performed between *C. difficile* donors, containing different *erm*(B)-elements, and two MLS_B-susceptible recipient strains, CD13 and CD37. Isogenic resistant derivatives from both the recipient strains were obtained. In addition to Tn*5398* from *C. difficile* 630, we were able to transfer to both recipients a Tn*6194*-like transposon as well as a new conjugative element. The genetic structure of the last two elements and the transfer mechanisms were investigated. Interestingly, our results suggest that *C. difficile* CD13 can acquire resistant element by transformation *in vitro*. Furthermore, we observed that Tn*6194*-like elements are widely diffused among clinical strains isolated during a recent European surveillance. Finally, we demonstrated that in most of the cases acquisition of an *erm*(B)-containing element imposes a burden on the fitness of the bacterium *in vitro*.

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Resistance to Metronidazole in *Clostridium difficile*

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Clostridium difficile strains *in vitro* susceptible to metronidazole (MZ), a drug used in the treatment of *C. difficile* infections, have been associated with cases of treatment failure. Susceptibility to MZ of 81 strains, belonging to different PCR-ribotypes, was determined by Etest, agar dilution method (ADM) and agar incorporation method (AIM). Heterogeneity of MIC values was also investigated by ADM and AIM in strains showing MIC increase after exposure to sub-inhibitory concentrations of MZ.

Reduced susceptibility to MZ was observed by both ADM and AIM in isolates belonging to PCR-ribotypes 001 and 010, with higher MIC values by AIM. One strain PCR-ribotype 010 showed a MIC ≥16mg/L by all three methods. After *in vitro* exposure to MZ, four strains showed MIC increase by Etest and ADM, but not by AIM. MICs within these strains were less heterogenic by AIM compared to ADM.

To investigate MZ resistance mechanisms in *C. difficile*, genomes of two strains, one *in vitro* stable resistant (Cd89R) and another reduced-susceptible (Cd85S), both PCR-ribotype 010, were sequenced and quantitative proteomic analysis, in presence and absence of MZ was performed. Comparative transcriptional analysis of Cd89R and Cd85S was also carried out.

DNA sequencing has not shown variation in genes possibly involved in MZ resistance. Proteomic analysis showed stable pyruvate-ferredoxin oxidorreductase concentration in Cd89R before and after MZ exposure, whereas it rose 2-fold in Cd85S following antibiotic exposure. Comparative transcriptomic data showed no variations in the expression of this gene between the two strains, however, other genes codifying enzymes involved in the electron flux essential for MZ activation were down-regulated in Cd89R.

This study shows that *C. difficile* sub-populations with reduced susceptibility to MZ can be highlighted using AIM. An altered regulation of specific metabolic pathways seems to be involved in MZ resistance in *C. difficile* Cd89R, possibly in synergy with other mechanisms.

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Mariner-Transposon Mediated Random Mutagenesis in *Clostridium species*

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Clostridium species are obligate anaerobic bacteria in which research interest lies in the fields of medicine (e.g. *C. sporogenes*) and bioenergy (e.g. *C. acetobutylicum*). In both fields major efforts are done to develop molecular tools and we therefore present a novel conditional replicon and show the usability of the mariner-transposon in both of the mentioned species.

We applied the transposon system to different *Clostridium* species to obtain mutant libraries. By combining the conditional vector and the *Mariner*-Transposon system, we managed to induce plasmid loss and select for transposon insertion concurrently. These libraries were tested on randomness of insertion and screened for obvious phenotypes such as sporulation/germination deficiency and auxotrophy.

The discussed genetic tools open up new ways in research in these *Clostridium sp.* and will benefit widely in *Clostridium* research community.

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement No. 237942, "CLOSTNET".

ABSTRACTS OF POSTER PRESENTATIONS

(please note, abstracts for posters by MC fellows,
are in the oral section)

Characterisation and Comparison of the Mechanisms Involved in *C. botulinum* and *C. sporogenes* Germination

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Foodborne botulism is a neuroparalytic disease caused by the ingestion of botulinum neurotoxin formed by strains of proteolytic *Clostridium botulinum* and non-proteolytic *C. botulinum* during their growth in food. *Clostridium sporogenes* is usually involved in food spoilage and is often regarded as the non-toxigenic equivalent of proteolytic *C. botulinum*. Both species form highly resistant spores during harsh conditions and germinate when conditions become more favourable.

To improve the control of botulinum neurotoxin-forming clostridia, it is imperative to comprehend the mechanisms involved in spore germination. For clostridia spores, initiation of germination involves the recognition of germinants by specific receptors located in the spore inner membrane. These germinant receptors are encoded by *ger* genes. The number of germinant receptor operons varies from species to species. Genome mining of proteolytic *C. botulinum* (ATCC3502) shows it contains two tricistronic (CBO0123–0125 (*gerXA1-XB1-XC1*), CBO2797–2795 (*gerXA3-XB3-XC3*) and one pentacistronic *ger* receptor operon CBO1974–1978 (*gerXB-XA2-XB2-XC2-XB*), *C. sporogenes* (ATCC15579) has three tricistronic and one tetracistronic *ger* receptor operons. Each strain has also an additional orphan *gerXB*-subunit homolog.

We are currently dissecting spore germination in proteolytic *C. botulinum* and *C. sporogenes*. Preliminary results show that spore germination can be initiated with single amino acids, and also by a combination of germinants. Furthermore, we are beginning to dissect the mechanisms involved and align receptors with their cognate germinants. This should lead to an increased understanding of the germination process in these important clostridial species.

Suppression of *C. difficile* Infection using *Bacillus subtilis* Spores

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Clostridium difficile is one of the major causes of nosocomial infections in the developed world. Clinical outcomes of infection with this Gram-positive bacterium range from diarrhoea to severe intestinal inflammatory conditions including pseudomembranous colitis. Antibiotic treatment and the corresponding disruption of the resident gut microflora can facilitate colonisation of *C. difficile* in the gastro-intestinal (GI) tract.

CDAD (*Clostridium difficile* associated diarrhoea) is caused by the production of two toxins, A and B, by vegetative cells in the GI tract. Production of resistant spores facilitates the spread of the infection, particularly in the hospital environment. Currently, there is no vaccine for this disease, and existing treatments using antibiotics are not effective, with up to 30% of patients experiencing relapse of the infection.

In this work, we will explore the use of *Bacillus subtilis* spores as probiotic treatment of CDAD. *B. subtilis* forms heatstable, resistant spores which are already used worldwide in probiotic applications. Using a murine model of *C. difficile* infection, we show that these spores can be used to suppress symptoms of this infection, and investigate the potential mechanisms behind this effect.

Role of Two-Component System CBO0365/CBO0366 in Cold Stress of *Clostridium botulinum* ATCC 3502

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The mechanisms by which the foodborne pathogen *Clostridium botulinum* senses and responds to environmental stress conditions are poorly characterized. We have previously shown that the genes encoding a two-component system (TCS) CBO0365/CBO0366 are induced after a temperature downshift in *C. botulinum* ATCC 3502 (Lindström *et al*. 2012). Furthermore, inactivation of either of the system components via ClosTron mutagenesis resulted in a cold sensitive phenotype, suggesting an important role for this TCS in response and adaptation to low temperature.

Here, we identified the putative regulon of CBO0365/CBO0366 by DNA microarray analysis. In total, 150 genes were differently expressed between the *cbo0365* regulator mutant and wild type at 37 °C. The affected operons included those encoding components of butyryl-CoA synthesis, motility, arsenic resistance, and phosphate uptake and transport pathways. Quantitative real-time PCR analysis confirmed differential expression of these operons at 15 °C.

Insertional inactivation of *crt, ctfA* and *bdh* encoding 3-hydroxybutyryl-CoA dehydratase, butyrate-acetoacetate CoA-transferase subunit A and NADH-dependent butanol dehydrogenase, respectively, involved in butanol biosynthesis, resulted in deteriorated cold tolerance, whereas inactivation of *bcd* encoding butyryl-CoA dehydrogenase did not. No differences in growth at 37 °C were observed between the mutants and wild type. Moreover, inactivation of *arsR* encoding an arsenical resistance operon repressor completely abolished growth at low temperature, and inactivation of *pstS* encoding a phosphate ABC transporter substrate-binding protein also resulted in poor growth at low temperature. Preliminary electrophoretic mobility shift assays suggest recombinant CBO0365 to bind to at least *crt*, *arsR* and *pstS* promoters.

Reference: Lindström M, Dahlsten E, Söderholm H, Selby K, Somervuo P, Heap JT, Minton NP, Korkeala H. Appl Environ Microbiol. 2012, 78:5466-70.

Photodynamic Antimicrobial Chemotherapy (PACT): Fighting *Clostridium difficile* with Light.

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Clostridium difficile is the leading cause of hospital and community-acquired antibiotic-associated diarrhoea and pseudo membranous colitis in the developed world. The emergence of a new 'hypervirulent' lineage of *C. difficile* in 2003 led to an increased number of outbreaks, raising the impellent need for an effective therapy. This study aims at developing Photodynamic Antimicrobial Chemotherapy (PACT) for the treatment of *C. difficile* infections.

PACT utilises the ability of light-activated photosensitisers (PS) to produce free radical species that are lethal to the target pathogen. In this research, 15 PS were screened in order target *C. difficile* vegetative cells and spores via red laser irradiation. These included both clinically approved PS drugs and experimental PS.

In the presence of oxygen, nine PS were successful in killing 99.99% of the 'hypervirulent' *C. difficile* strain R20291 after exposure to laser light (665nm, 0.2 J/cm^2). Remarkably, although PACT is believe to require molecular oxygen for toxicity, four PS also showed the ability to kill 99.9% of bacteria in anaerobic conditions that are likely to be similar to those present in the colonic environment.

The efficacy of the treatment was confirmed in five different *C. difficile* strains belonging to different ribotypes (173, 011, 249, 027 and 020). Applicability of PACT to eradicate *C. difficile* spores was also shown, using a two-phase approach first inducing germination by the bile salt taurocholic acid, followed by PACT. Toxicity of effective compounds was tested on the colorectal adenocarcinoma cell-line HT-29, showing that taurocholic acid and the PS chlorin e6, S4 and talaporfin were not toxic to human cells at antimicrobial concentrations.

Therefore, PACT shows promise as a new treatment for *C. difficile* colitis and its recurrence, targeting both vegetative cells and spores with light delivered at the site of infection.

Global Regulation in Response to Cysteine Availability in *Clostridium difficile*

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Clostridium difficile pathogenicity is linked to its ability to produce two toxins: TcdA and TcdB. The level of toxin synthesis is influenced by several environmental signals such as PTS sugars, biotin and amino acids like cysteine. We are interested to understand the molecular mechanisms of cysteine-dependent repression of toxin production. We first performed a reconstruction of sulfur metabolism in *C. difficile* and tested the growth of the bacterium in the presence of various sulfur sources to confirm the metabolic pathways identified. We also noticed that providing large amount of cysteine to *C. difficile* is correlated with hydrogen sulfide production indicating that cysteine is actively metabolized by cysteine desulfhydrases of the bacterium. Then we looked at regulatory network involved in cysteine-dependent response by a global approach. The transcriptomic analysis of the 630Δerm strain indicated that 242 genes were differentially expressed after addition of high concentration of cysteine in a rich medium: 105 genes were repressed whereas 137 were induced. Genes the most highly regulated by cysteine are those involved in cysteine metabolism, fermentation and energy metabolism, amino acids biosynthesis and stress response. Interestingly, most of genes belonging to the Fur regulon are also induced as if hydrogen sulfide production chelates iron mimicking an iron depletion of the media. However, the cysteine-dependent repression of toxin synthesis seems to be independent of the Ferric uptake regulator (Fur) since a *fur* mutant still exhibits an efficient cysteine toxin repression. In order to identify regulators involved in the cysteine-dependent regulation of toxin synthesis, strains inactivated for sigma factor (σ^{54}, σ^{H}) and global regulators (CcpA, CodY) were tested for the production of toxin in presence of cystein. We showed that cysteine-dependent repression of toxin production was abolished in the σ^{54} mutant strain indicating that cysteine effect is mediated by σ^{54}.

The Physiologic Role of Pyruvate-Formate-Lyase in *Clostridium acetobutylicum*

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The Gram-positive *Clostridium acetobutylicum* has become a model organism for the acetone–butanol–ethanol (ABE) fermentation among solventogenic Clostridia.
To gain more insight in the biphasic fermentative metabolism, we used the ClosTron technology for targeted gene inactivation and generated knock-out mutants of the pyruvate-formate-lyase (*pflB*) and its activating enzyme (*pflA*). Pfl is catalyzing the reversible, coenzyme-A dependent and nonoxidative cleavage of pyruvate to acetyl-coenzyme-A. It has been demonstrated in a number of anaerobic bacteria, including many species of the genus *Clostridium*. The *pflB*-encoding gene CAC0980 is expressed in *C. acetobutylicum* during growth, as shown in a previous DNA time series microarray study. It was also detected in the proteome reference map of *C. acetobutylicum* ATCC 824. Since *C. acetobutylicum* contains neither a formate-hydrogen-lyase as Enterobacteria, nor a formate-dehydrogenase, the physiological role of Pfl in *C. acetobutylicum* remained obscure, due to the fact that so far no accumulation of formic acid in the growth medium has been observed. Therefore, it has been proposed that the clostridial enzyme may have a biosynthetic role.
This is supported by our observation that both mutants, in contrast to the WT, are not able to grow in minimal medium. However, when adding formate to these cultures the knock-out in the pyruvate-formate-lyase (*pflB*) and its activating enzyme (*pflA*) could be complemented. Since, the same was achieved by adding the purine nucleosides Adenosine and Guanosine we found out that formate synthesized by the pyruvate-formate lyase is used as a one-carbon-unit donor for the syntheses of the purine nucleotides.

Characterization of Glucose Family Phosphotransferase Systems in *Clostridium beijerinckii*

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In many bacteria, including the solventogenic clostridia, glucose is a dominant sugar that represses metabolic systems for alternative carbon sources. Elimination of this carbon catabolite repression (CCR) would be beneficial for the ABE fermentation since it would allow for the simultaneous utilization of carbon sources present in the substrate. According to the mechanism proposed for firmicutes, CCR is controlled by the PEP-dependent phosphotransferase system (PTS), a multi-component system that catalyses uptake and phosphorylation of its sugar substrates (including glucose) and acts as a sensor of the nutrient status of the environment. Understanding of the mechanism of repression will be aided by a detailed characterization of glucose uptake system(s).

The *Clostridium beijerinckii* genome encodes 42 complete phosphotransferase systems. Fifteen of these systems belong to the glucose-glucoside family, of which three are members of the phylogenetic branch that incorporates the glucose phosphotransferases of other bacteria. We have been studying the functions of these three phosphotransferases by an experimental approach involving gene cloning, complementation of *Escherichia coli* mutants and characterization of the recombinants. One of the systems, encoded by the gene *Cbei0751*, has been shown to be a functional glucose PTS that also recognises mannose as a substrate. A second system, encoded by the divergent genes *Cbei4532* and *Cbei4533*, is a N-acetylglucoamine PTS that can also transport and phosphorylate glucose. The third system is encoded by *Cbei4983* and *Cbei4982*, and the proximity of the gene *Cbei4984* encoding a putative glycoside hydrolase suggests that the primary function of this system may be to transport and phosphorylate a disaccharide. While it is already clear that there is not a single route of glucose uptake in *C. beijerinckii*, the contribution of each glucose uptake system to CCR can be assessed by mutational analysis.

Phenotypic characterisation of *lspA* mutants of *Clostridium difficile*

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The onset of infection by pathogenic bacteria is usually achieved by invading and colonising a particular host niche. A fundamental role in this process is adherence, a phenomenon which may be mediated by cell surface molecules such as lipoproteins. In addition to adherence, lipoproteins of many pathogens play an important role in different stages of disease. Therefore, the correct processing and localisation of these molecules play an important role in pathogenesis. These processes are predominantly mediated by two enzymes; Lgt (prolipoprotein diacyl-glyceryl transferase, encoded for by *lgt*, CD2659), which transfers a diacyl-glyceryl moiety on to the side chain of a conserved amino terminal cysteine residing within a 'lipobox', and LspA (Lipoprotein signal peptidase II) which cleaves the amino terminal signal peptide. We have previously demonstrated that *C. difficile* encodes two functional lipoprotein signal peptidases, CD2597 and CD1903. Data will be presented relating to the phenotypic characteristics of *C. difficile* mutated in these genes.

Iron Uptake Systems and Its Regulation in *Clostridium difficile*

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Clostridium difficile, a Gram-positive, anaerobic, spore-former is the major cause of antibiotic associated diarrhoea and is also associated with more severe, sometimes life threatening disease. To date little research has been carried out on iron uptake mechanisms and their regulation in *C.difficile*. In common with other pathogens, iron is likely to be an essential growth factor necessary for the survival of the organism and analysis of available genome sequences reveals the presence of several potential iron uptake systems and regulators.

We present studies on the ferric uptake regulator Fur. The ClosTron mutagenesis system developed in Nottingham has been used to generate knockout mutants in the single *fur* homologue in *C.difficile*630Δ*erm*. Fur mutant exhibited clear growth phenotype and was hypersensitive to hydrogen peroxide. Cytotoxicity assays revealed decreased levels of toxins TcdA and B in the *fur* mutant. The *fur* mutant showed delayed sporulation and produced less spores than wild type. In addition, the *fur* mutant was more motile than wild type and showed increased cellular iron content. RNA Seq analysis was carried out in order to investigate Fur regulon in *C. difficile* and contribution to our experimental data. RNA-Seq analysis demonstrated over 1600 genes differentially expressed in the *fur* mutant.
These findings further highlight the role of Fur in regulating key and diverse aspects of physiology and virulence of *C.difficile*.

FeFe-Hydrogenases: Functions, Structures and O_2 Tolerance

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FeFe-Hydrogenases are large metallo-enzymes capable of both hydrogen evolution and uptake [1]. These enzymes, widely distributed in several micro-organisms and involved in energy metabolism, could after studies and engineering, lead to a useful catalyst for hydrogen production from water in a clean industrial process [2]. FeFe-Hydrogenases possess a conserved inorganic catalytic site, the H-cluster, catalysing the $H_2 \leftrightarrow 2H^+ + 2e^-$ reaction, but HydA, the *Clostridium acetobutylicum* hydrogenase has also accessory domains functioning in electron transfer. Despite this enzyme is known to be one of the most active FeFe-Hydrogenase, it is deeply sensitive to O_2, which is unfortunate regarding the usefulness of an O_2 tolerant enzyme [1][2].

To study structure-function relationships of Fe-hydrogenase, we developed in *Clostridium acetobutylicum* a system for homologous expression, production and purification of Fe-Fe Hydrogenase in active forms for biochemical characterization concerning both hydrogen production and consumption [3].

Currently we focused our work on the selection of oxygen tolerant enzymes that remain active in H_2 production, using a genetic screening based on recombinant *C. acetobutylicum* and *E. coli* strains, in which survival of the bacteria is directly linked to the expression of an active and/or O_2 tolerant modified enzyme [4]. Random or site-directed variants of HydA and also chimeric enzymes assembled from parts of different origins will be studied for the selection of new enzymes less O_2 sensitive. This biochemical approach will be associated with electrochemistry experiments which could lead to new structural insights of the mechanism and O_2 tolerance of the selected enzymes [5][6].

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The determinants of *R. equi* host tropism: sequencing of the bovine type plasmid pVAPN and comparative roles of v*apA*, *vapB* and *vapN* in virulence

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Rhodococcus equi is a soil-dwelling actinomycete that also has the ability to colonise animal host tissues, causing pyogranulomatous infections in different animal species including humans. *R. equi* is a facultative intracellular parasite of macrophages that replicates within a modified endosomal compartment. Intracellular survival depends on the *vap* pathogenicity island (PAI) carried in a virulence plasmid. A recent molecular epidemiological study from our laboratory demonstrated unique associations between specific virulence plasmid types and host animal species. Besides the known link between *vapA* plasmids and horse isolates, the study showed that *vapB* plasmids are characteristic of porcine isolates and identified a novel bovine-specific plasmid type, characterised by the absence of *vapA* and *vapB* markers. Using a unified nomenclature these plasmids were designated, respectively, pVAPA, pVAPB and pVAPN (for virulence associated plasmid noA noB). Each plasmid was almost exclusively found in the cognate animal species, suggesting host-driven selection of virulence plasmid types (Ocampo-Sosa et al. 2007, J Infect Dis 196:763-769). In contrast, human isolates carried any of the three plasmid types, consistent with the opportunistic nature of the infection. This finding also clearly suggested that human rhodococcosis is zoonotic in origin. Sequencing of a pVAPB plasmid showed that the porcine type shares the same housekeeping backbone with pVAPA; it differs however in *vap* multigene complement in the plasmid pathogenicity island (PAI), suggesting that the Vap proteins are involved in the determination of *R. equi* host tropism (Letek *et al*. 2008, J Bacteriol 190:5797–5805). Using the draft genome of bovine isolate PAM1571, we have now identified and determined the complete nucleotide sequence of a pVAPN plasmid. The *vap* PAI is present but the linear replicon in which it is inserted is different from that of the pVAPA/B plasmids. The *vap* gene complement also differs, including a novel allelic variant of the *vapA* and *vapB* genes, designated *vapN*. In collaboration with the Roslin Institute, we are currently analyzing the role of the *vapA*, *vapB* and *vapN* allelic variants and corresponding *vap* PAIs in *R. equi* virulence and host tropism using *in vitro* infection models as well as in the natural host species.

Homologous recombination promotes the evolution of *Clostridium difficile* 16S-23S rRNA intergenic spacer region

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PCR-ribotyping, method based on size variation in 16S-23S rRNA intergenic spacer region (ISR), is one of the most widely used methods for typing of *Clostridium difficile.* Whereas in *C. difficile* genome, the rRNA operon is present in several copies that differ in the length of ISRs, amplification of ISRs with only a single primer pair results in a pattern of bands ranging from 200 - 700 bp.

The aim of this study was to investigate the sequence diversity of *C. difficile* ISRs and to explore presence of type specific markers within the ISRs that could be used for rapid identification of ribotypes. Sequences of 370 ISRs from 23 strains, representing 22 different PCR-ribotypes were determined (ISRs of published *C. difficile* genomes - strains 630, R20291 and CD196 - were also included in the analysis).

Based on previously described structure, presence or absence of $tRNA^{Ala}$ genes and different combinations of spacers of different lengths (33 or 53 bp) separated with 9 bp direct repeats (Indra *et al*., JMM, 2010), all sequences could be manually grouped into 20 different groups and in majority of cases the ISR structural group coincided with the sequence length. ISRs that were of the same or comparable sizes had very similar sequence (> 88%). The only two exceptions were found in ISRs of 287 bp and 329 bp where the similarities were lower than expected (73% and 88%, respectively) due to inverse arrangements of the sequence blocks. NeighbourNet and statistical test PHI confirmed recombination in *C. difficile* ISRs.

Modular structure of ISRs and high sequence similarities of ISRs of the same sizes suggests concerted evolution of ISRs, and that homologous recombination, as already suggested by Indra *et al*. (2010), is responsible for rearrangements of the sequence blocks rather than accumulation of mutations which would generate type specific ISRs.

Genome-wide identification of small RNAs in *Clostridium difficile*

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Clostridium difficile is a major hospital acquired pathogen that can cause symptoms ranging from mild diarrhoea to more serious, sometimes life-threatening inflammation of the colon. Despite its medical importance, very little is known about the regulatory networks that are operating in this organism, and almost nothing about the roles played by small regulatory RNAs (sRNAs). sRNAs have been found in many other bacteria where they accomplish important functions in the regulation of cellular processes such as sporulation, quorum sensing, metabolism, and virulence (e.g. toxin production). Unravelling the sRNA regulatory networks in *C. difficile* would be an important step toward understanding how this pathogen survives in the gut environment and causes disease.

To identify and map *C. difficile* sRNAs on a genome-wide scale, total RNA was extracted from triplicate cultures at different time points during the growth cycle (mid exponential, transition to stationary phase, and stationary phase) and subjected to RNA-Seq analysis. After filtering out abundant tRNAs and rRNAs, data were normalised and analysed for regions of intergenic and antisense transcription. Numerous putative sRNAs were identified (~260) and the existence of several of these were independently confirmed by Northern blot analysis. The biological function and regulatory targets of selected sRNA candidates is currently being investigated. As the regulatory function of trans-encoded sRNAs is generally mediated by the chaperone protein Hfq, *C. difficile hfq* mutants were also generated by ClosTron mutagenesis and are currently being characterised.

Bacterial Resistance to Biocides: Development of a Predictive Protocol

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In the last 10 years questions have been raised about the contribution of microbicides to the reported increase in antibiotic resistance in pathogenic bacteria. This is due to their increased use in a range of environments (e.g. medical, domestic, food industry, veterinary, farming). Bacteria have been reported to show reduced susceptibility to other antimicrobials after frequent exposure to low microbicide concentrations, due to expression of common resistance mechanisms. The EU Biocidal Product Regulation now requires information on the occurrence of resistance development in organisms targeted by the biocidal product. There is currently no protocol available to predict the likelihood of bacteria becoming resistant to a biocidal product or microbicides contained therein. Identification of useful markers of microbicide resistance will aid development of a protocol predictive of bacterial resistance and cross resistance development.

Salmonella enterica serovar Typhimurium strain SL1344 was exposed to a range of sub-lethal concentrations of benzalkonium chloride (BZC) and chlorhexidine gluconate (CHG). Minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs) and antibiotic susceptibility profiles of surviving bacteria were measured and compared to baseline data. Efflux was also measured by accumulation of fluorescent dye. DNA microarrays were used to identify potential resistance marker genes.

Exposure of *S. enterica* to low concentrations of BZC and CHG resulted in a significant increase in MIC and MBC of both microbicides, up to 100-fold. However, these alterations in MIC and MBC were unstable. SL1344 remained clinically sensitive to all antibiotics tested. Efflux assays demonstrated up-regulation of efflux pump activity after microbicide exposure. Microarray experiments showed that the greatest up-regulation occurred in genes involved in metabolism and efflux.

Changes in phenotype and gene expression of survivors were identified following exposure to sub-lethal concentrations of CHG and BZC. Changes in MIC and MBC, up-regulated efflux activity and altered gene expression can be used as microbicide resistance markers but need to be considered together with resistance stability.

An Integrative Workflow for the Multi-Level Annotation of the *Clostridium ljungdahlii* Genome

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The characterization of genomic features is foundational to our understanding of living systems. To better comprehend physiological states, the genome annotation must expand beyond coding genes to include elements spanning transcription, translation and regulation (e.g., promoters, ribosome binding sites (RBSs), transcription factors (TF), untranslated regions (UTRs)). However, bioinformatics tools alone cannot accurately predict the annotation of these distinct genomic features. Here, a workflow is presented that utilizes multi-omic datasets in conjunction with computational approaches to yield a highly refined genome annotation. Experimentally, this approach is solely dependant on next-generation sequencing to characterize transcription, translation and regulation via RNA-Seq, transcription start site determination, ribosome profiling and ChIP-Exo. Doing so enables us to rapidly and efficiently increase the knowledge base for under-characterized organisms—in particular the acetogen *Clostridium ljungdahlii*. The generation of these omics datasets refines gene coordinates, defines transcription units and reveals novel coding and non-coding features. These expansions to the annotation are then used to guide bioinformatics tools for the genome-scale elucidation of promoters and RBSs. The expanded annotation also serves as a scaffold for interrogation of regulatory features. Furthermore, the study of the *C. ljungdahlii* genome complements the recently developed metabolic model, which consists of 618 genes, 675 reactions, and 693 metabolites. Together with omics datasets, this model revealed critical components of energy metabolism in *C. ljungdahlii*. Thus, the multi-level genome annotation and the metabolic model of *C. ljungdahlii* represent valuable resources to the scientific community studying *Clostridia* and will reveal novel biological insights into the lifestyle of acetogenic microorganisms.

Polysaccharide capsule and *Rhodococcus equi* virulence

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Rhodococcus equi is a multihost pathogenic actinomycete that causes pyogranulomatous infections in a variety of animal species, including humans. A soil-dwelling organism, *R. equi* uses volatile fatty acids from herbivore manure as growth substrate, colonizes the intestine of animals and is ubiquitous in the farm environment. Soil is the main source of infection and inhalation of dust aerosols carrying the organism causes tuberculosis-like pulmonary abscesses in horses and cavitary bronchopneumonia in immunosuppressed individuals. *R. equi* has long been known to possess capsular material, on the basis of which a serotyping scheme was established. Analysis of the *R. equi* genome sequence, recently determined in our laboratory, identified a horizontally acquired (HGT) genomic island encoding products involved in polysaccharide biosynthesis. We hypothesized that this HGT island could correspond to the *R. equi* capsule (*rcp*) locus. Mutants were constructed in putative glycosyl transferase and polysaccharide polymerase genes. The two mutants have altered colony morphology with loss of the mucoid phenotype and no detectable bacteria-associated polysaccharide as determined by gel electrophoresis and Alcian blue staining. The chemical structure of the 103S (serotype 6) capsule has been elucidated and forms a highly negatively-charged shield. It consists of a trisaccharide backbone containing α-l,3-1inked α-D-mannopyranosyl (α-D-Man*p*), α-D-galactopyranosyl (α-D-Gal*p*) and α-D-glucopyranosyl (α-D-Glc*p*) residues, in which the α-D-Man is substituted at the 2-position by a β-D-glucuronic acid (β-D-Glc*p*A) residue and the α-D-Gal*p* carries a 4,6-bridging pyruvic acid acetal moiety. We are currently determining the chemical structure of the altered polysaccharide produced by specific mutants in the *rcp* locus. Analysis of the *rcp* mutants in murine macrophages indicates that the loss of *R. equi* exopolysaccharide production does not affect uptake. However, intracellular survival is significantly impaired, suggesting that the capsule plays a protective role in the harsh environment of the macrophage vacuole. Preliminary data also suggest that the exopolysaccharide material protects *R. equi* from environmental aggressions, important for survival in soil and transmission of the pathogen.

Identification and Analysis of the Catabolic Reaction-Superset in *Salmonella typhimurium*

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Previous work in the CSMG has shown that extraction of catabolic core networks from genome-scale models (GSMs) can be a useful method for model-analysis. A catabolic core network is a set of reactions in the GSM that change flux as a response to an imposed change in energy demand, modelled as ATP hydrolysis. Here, this approach is applied to *Salmonella typhimurium*, a major cause of food-borne infections. During infection *S. typhimurium* is able to use a broad range of nutrients for growth and energy. Previously, identification of catabolic core networks has assumed a single carbon source. In this contribution the analysis was applied to a model capable of utilizing several carbon sources that had been shown to support growth experimentally.

The results of the analysis suggest that approximately 500 reactions are needed to generate energy on all carbon sources. However, distribution of reactions over the solutions is not even – the majority of reactions are unique to a given carbon source and a limited number of reactions (approximately 170) occur in a majority of carbon sources. Analysis of the simulated flux-data shows that the reactions that occurred most frequently over all carbon sources were primarily involved in glycolysis and amino acid biosynthesis.

The outcome of this analysis is a core model of *S. typhimurium* that accounts for the energy generation during growth on the major growth-supporting carbon sources. This core model can be used for identification of enzymes that are suitable as targets for antimicrobial agents.

Intracellular Metabolite Analysis during the Acid/Solvent Shift in *Clostridium acetobutylicum*

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Despite the long history in using *C. acetobutylicum* for the production of butanol, still little is known about the regulation of the metabolic shift from acids to solvents. It is the goal of the collaborative systems microbiology project ´COSMIC2` to increase the knowledge on the key regulatory and metabolic events that occur during the metabolic shift. Especially key metabolites like butyryl-phosphate and acetyl-phosphate, but also other glycolytic intermediates, may play a crucial role. A set of knockout strains are being developed and their fermentative metabolism is analysed by transcriptomics, proteomics and metabolomics. While the analysis of transcript levels and proteins is rather straightforward, analysis of intracellular metabolites is still problematic. Especially the massive leakage of metabolites during the cold quenching of cells is a common problem in many metabolomics protocols.

We compared various sampling techniques, quenching solutions and extraction methods. Cell leakage and metabolite recovery was determined by ATP measurements. The developed metabolomics protocol was used to analyse the metabolites from steady state acidogenic and solventogenic cells, as well as cells from different points of the intermediate shift. A targeted set of metabolites was analysed and quantified by a validated ion-pair LC-MC method (Orbitrap). The results of the development of the metabolomics protocol and the dynamics of various metabolites during the acid/solvent shift, will be presented. The COSMIC2 project is carried out in close collaboration with research groups in Berlin, Munich, Rostock, Ulm and Nottingham.

The Regulatory Cascade of Sporulation in *Clostridium difficile*

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Clostridium difficile, a Gram positive, anaerobic, spore-forming bacterium is a major cause of nosocomial infections leading to antibiotic-associated diarrhea. *C. difficile* produces highly resistant spores that facilitate the persistence of this bacterium in the environment in particular in aerobic conditions and contaminate hospital environments contributing to the establishment of a reservoir. Transmission of *C. difficile* is mediated by contamination of the gut by spores. The regulatory cascade controlling spore formation that involved four sigma factors, σ^F and σ^G in the forespore and σ^E and σ^K in the mother cell, is still poorly characterized in *C. difficile* compared to *Bacillus subtilis*.

In this work, we combined transcriptional analyses and transcriptional start sites (TSS) mapping to define the σ^F, σ^E, σ^G and σ^K regulons in *Clostridium difficile.* A total of about 225 genes were under the control of these sigma factors: 25 in the σ^F regulon, 97 σ^E-dependent genes, 50 σ^G-governed genes and 56 genes specifically controlled by σ^K. The genome wide determination of TSS of the 630 strain using RNA-seq allows us to map about 1000 TSS, to identify σ^F-, σ^E-, σ^G- and σ^K-dependent promoters among genes regulated in transcriptome and to determine a consensus sequence for each sigma factor.

A significant fraction of genes in each regulon are of unknown function and we can propose new candidates for spore coat proteins synthesized under the control of σ^E or σ^K among proteins previously detected in the spore proteome (Lawley et *al.*, 2009). Global analysis of developmental gene expression under the control of these sigma factors also indicated deviations from the *B. subtilis* model regarding the communication between mother cell and forespore in *C. difficile.*

Lawley et *al.*, 2009. Journal of Bacteriology
Proteomic and genomic characterization of highly infectious Clostridium difficile 630 spores

Sporulation Morphodynamics in *Clostridium difficile*

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Endosporulation is an ancient prokaryotic cell differentiation program that culminates with the formation of a highly resistant dormant cell, the endospore (spores, for simplicity). In the enteric pathogen *C. difficile*, a strict anaerobe, spore formation is central to the infectious/transmission cycle. Two large toxins are the main virulence factors required for the development of symptoms of *C. difficile* infection. However *C. difficile* virulence potential is also linked to the ability of these species to form highly resistant spores, which have a central role in persistence of the organism in the environment, infection, recurrence and transmission of the disease. Following ingestion, spores of this organism germinate in the colon, to establish a population of vegetative cells that will produce the toxins and more spores. However, the molecular mechanisms involved in sporulation and germination are still poorly studied in *C. difficile.*

The regulatory network of sporulation is hierarchical, with the top level defined by cell type-specific RNA polymerase sigma factors that govern gene expression in the forespore and mother cell, the two cells that participate in spore formation. In this study we have combined studies of gene expression at single cell level using a fluorescence reporter gene, with the phenotypic characterization of mutants for the sigma genes. This way we were able to dissect the regulation of gene expression during endospore development by *C. difficile*. The overall picture that emerges is that while the main periods of activity of the sigma factors appear conserved, the coordination of gene expression between the forespore and mother cell is less tightly controlled when compared with the model organism *B. subtilis* were sporulation has been extensively studied. A streamlined version of the cellular differentiation program may be a general feature of endospore formation in the more ancient Clostridia group of bacteria.

PCR-rbotype 126/078 and PCR-ribotype 018: Comparison Between Two Emerging *Clostridium difficile* Types

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The European surveillance performed in 2008 indicated *Clostridium difficile* PCR-ribotypes 078/126 and 018 as two emerging virulent types. The first type includes hypervirulent strains that cause infections in both hospital settings and community and that are currently spread in different European countries; the second type is predominantly isolated in Italy where it is the major cause of severe infections and outbreaks occurred in the last years. While PCR-ribotype 078/126 has variations in the locus of pathogenicity (PaLoc), in particular in the toxin genes, and it is considered a toxin variant type, PCR-ribotype 018 is a non-toxin variant type, as the majority of strains causing infections in Europe. In this study, Italian clinical isolates belonging to these PCR-ribotypes were characterized for sporulation and adhesion to Caco-2 cells. Strains were also compared by competition assays "*in vitro*".

Interestingly, results indicate that sporulation was strain-dependent and more abundant for strains 018 after 48h. Also, a significantly higher adhesion to Caco-2 cells was observed for strains 018. Furthermore, growth competition assays indicated that strains 018 rapidly overcome strains 078/126 with a decrease in fitness for the last.

These data suggest that the strains PCR-ribotype 018 examined in this study show peculiar characteristics that allow them to overcome strains PCR-ribotype 078/126 in "*in vitro*" assays. This behaviour could partially explain the rapid spread and persistence of PCR-ribotype 018 in Italy, whereas PCR-ribotype 078/126 remains the second most frequently type detected in our Country. The results also emphasize the importance of a careful surveillance and characterization of non-toxin variant strains.

Identification of a Novel Zinc Metalloprotease through a Global Analysis of *Clostridium difficile* Extracellular Proteins

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Clostridium difficile is a major cause of infectious diarrhea worldwide. Recurrent infections and an increase in antibiotic-resistant strains have made treatment of *C. difficile* infections extremely difficult. Although the cell surface proteins are recognized to be important in clostridial pathogenesis, biological functions of few are known. Also, apart from the toxins, proteins exported by *C. difficile* into the extracellular milieu have been poorly studied.

We analyzed bacterial culture supernatants prepared from clinical isolates, 630 and R20291, using liquid chromatography-tandem mass spectrometry. The majority of the proteins identified were non-canonical extracellular proteins. These could be largely classified into proteins associated to the cell wall (CWPs, LPXTG-like, extracellular hydrolases), transporters and flagellar proteins. Seven unknown hypothetical proteins were also identified. One of these proteins, CD630_28300, shared sequence similarity with the anthrax lethal factor, a known zinc metallopeptidase. We demonstrated that CD630_28300 (named Zmp1) binds zinc and is able to cleave the extracellular matrix protein fibronectin *in vitro* in a zinc-dependent manner. Using site-directed mutagenesis, we identified residues important in zinc binding and enzymatic activity. Furthermore, we demonstrated that Zmp1 destabilizes the fibronectin network produced by human fibroblasts.

Thus, by analyzing the exoproteome of *C. difficile*, we identified a novel extracellular metalloprotease that may be important in key steps of clostridial pathogenesis such as gut colonization and dissemination.

Alpha Toxin and Perfringolysin Act Synergistically to Induce Hemorrhagic Enteritis in Veal Calves

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Bovine enterotoxemia or hemorrhagic enteritis is a major cause of mortality in veal calves, especially of the Belgian Blue breed, causing important economic losses. It is characterized by a high case fatality rate, sudden death and necrohemorrhagic lesions in the small intestine. *Clostridium perfringens* is considered as the causative agent, but there has been controversy on the toxins responsible for bovine enterotoxemia. Epsilon toxin and Beta2-toxin have been proposed as possible causative toxins. A recent study from our lab however, demonstrated that type A strains from any origin could induce necrohemorrhagic lesions in a calf intestinal loop assay. These results put forward alpha toxin and perfringolysin, since both are produced by nearly all *C. perfringens* strains.

In the current study, the roles of alpha toxin and perfringolysin in the development of lesions were evaluated by the use of a wild-type strain and isogenic mutants, genetically deficient in the production of alpha toxin, perfringolysin or both, and either or not complemented with the wild-type genes. After experimental inoculation in intestinal loops, the alpha toxin mutant and the perfringolysin mutant induced necrosis to a lower level as compared to the wild-type and only the presence of both toxins could restore the activity to that of the wild-type.

The first microscopic effects induced in the intestinal mucosa were hemorrhages, pointing to endothelial damage as a critical trigger of the lesions. Therefore, we studied the effect of the mutants on bovine endothelial cells. Bovine endothelial cells appeared to be sensitive to alpha toxin and perfringolysin and both toxins had a synergistic cytotoxic effect. These results underscore the importance of endothelial cell damage and may explain why capillary hemorrhages are an initial step in the development of bovine enterotoxemia. Taken together, our results show that perfringolysin and alpha toxin contribute synergistically to the pathogenesis of bovine hemorrhagic enteritis and the toxins may act by targeting bovine endothelial cells.

Fructose Utilization in *Clostridium acetobutylicum*

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As a member of the saccharolytic Clostridia, a variety of different carbohydrates as carbon and energy source like glucose, fructose or xylose can be utilized by *Clostridium acetobutylicum*. Generally, carbohydrates were taken up via three types of transporters: symporter, ATP-binding cassette (ABC) transporter and phosphotransferase systems (PTS). For the uptake of hexoses, hexitols and disaccharides thirteen PTS have been identified in *C. acetobutylicum*.[1]

Although fructose is an important sugar in food industry and part of technical substrates such as molasses, up to now fructose metabolism gained only little attention. The uptake and catabolism of fructose in *C. acetobutylicum* was investigated, to get new insights in its physiology.

For the transport of fructose into the cell of *C. acetobutylicum* there are mainly three PTS responsible.[2] The apparent primary fructose transport system is encoded by a polycistronic operon (*cac0231-cac0234*) including a putative DeoR-type transcriptional regulator (FruR, CAC0231), a 1-phosphofructokinase (FruB, CAC0232), a PTS IIA (FruC, CAC0233) and a PTS IIBC (FruD, CAC0234). Thus the other two PTS (CAP0066-CAP0068 and CAC1457-CAC1460) seem to play a secondary role in fructose uptake.

Here, we elucidate the importance of the fructose (*fru*) operon CAC0231-CAC0234 for the growth of *C. acetobutylicum* on fructose as sole carbon source.

[1]Nölling et al., 2001. *Appl Environ Microbiol* 57, 2534–2539.
[2]Servinsky et al., 2010. *Microbiology* 156:: 3478-3491.

Genetic characterization of enterotoxic *Clostridium perfringens* type A foodborne isolates and niche specialization indicated by their MLST profiles

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Clostridium perfringens endospores or vegetative cells in foods can lead to food poisoning cases due to release of *cpe*-encoded enterotoxin upon spore formation in the human gastrointestinal tract. To investigate the prevalence of enterotoxic strains in food borne isolates, we screened 98 suspected *C. perfringens* isolates obtained from a nationwide survey on different foods, and 59 could be identified as *C. perfringens*. Using PCR-based techniques, the *cpe* gene was detected in eight isolates, showing a chromosomal location for 7 isolates and a plasmid location for one. This study shows that classical culturing and confirmation methods for *C. perfringens* give false positive results in approximately 30% of cases. Moreover, in approximately only 10% of cases, the *cpe* toxin gene that can cause food poisoning was detected.

$(GTG)_5$-rep-PCR fingerprinting patterns of all identified *C. perfringens* strains did not show significant correlation between the presence of the *cpe* gene and chromosomal structure. Further typing was performed using Multi-Locus Sequencing Typing (MLST) on the eight enterotoxic strains and eight *cpe*-negative foodborne isolates. Results were interpreted using data from a worldwide survey, and revealed three *C. perfringens* groups that correlate with different environmental niches. The seven chromosomal *cpe* food isolates belonged to a cluster that encompassed all food poisoning strains from the worldwide survey (Group I), while plasmid-*cpe* strains, gas gangrene and isolates from healthy humans clustered in Group II. These results suggest that different groups of *C. perfringens* have undergone niche specialization and that a distinct group of food borne isolates can be distinguished based on core genome sequences. These findings have epidemiological and evolutionary significance.

Clostridium difficile Cell Wall Protein Anchoring

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Clostridium difficile is a pathogen of the human intestine and the leading cause of hospital acquired diarrhea. However, how *C. difficile* colonizes the human intestinal tract is still poorly understood.

The surface of *C. difficile* is likely to play an important role in host colonization. The major surface structure is the S-layer, comprised of a complex between the High Molecular Weight S-layer protein (HMW SLP) and Low Molecular Weight S-layer protein (LMW SLP). There are 28 paralogues of the HMW SLP in the *C. difficile* 630 genome, which together form the cell wall protein family. All *C. difficile* cell wall proteins contain three PFAM-04122 (PF04122) repeats at their N or C termini, which are believed to be responsible for anchoring the proteins to the underlying peptidoglycan or other cell wall polymer. PF04122 repeats are widespread throughout surface proteins of Gram positive bacteria and the Archaea but their ligand or anchoring mechanism are unknown.

To gain insight into the role of the PF04122 repeats, we used mutagenesis to systematically delete the repeats from two members of the cell wall protein family; Cwp2 and Cwp66. We found that deletion of any single repeat abrogated anchoring of these proteins to the cell wall and caused them to be shed into the culture supernatant. This suggest that these proteins have a common mechanism of attachment to the underlying cell wall perhaps involving formation of a complex containing three PF04122 domains. This data and the latest results from our on-going research into the PF04122 repeats will be presented.

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