

ClostNet

The Conference

December 2010
Nottingham, UK



Programme and Abstracts

CLOSTNET PROJECT MANAGER:

Jacque Minton

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

Tel: 0115 846 6287

Fax: 0115 8232120

email: jacqueline.minton@nottingham.ac.uk



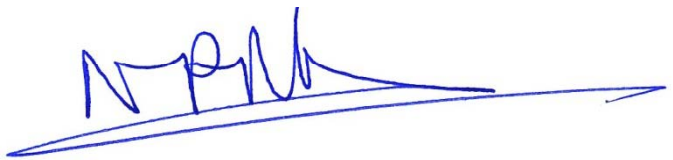
Dear Colleagues,

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 tackle both the threats posed to human health and well being, and to 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 first conference is to highlight some of the activities of the partner laboratories, while at the same time asking experts from outside of the clostridial community to educate and inform our Marie Curie fellows of the ‘state-of-the-art’ in relevant processes in non-clostridial organisms. 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
10th December 2010




Dr. John Telford



Prof. Dr. Wolfgang Liebl



Dr. Peter McClure



Dr. Paola Mastrantonio



Prof. Philippe Soucaille



Prof. Chantal Tardif



Prof. Miia Lindstrom

PROGRAMME

WEDNESDAY 15th - ARRIVAL AT HILTON HOTEL (Check-in from 15:00)		
16:00	Registration	Hilton Hotel
19:30	Reception	Hilton Hotel
20:30	Dinner	Hilton Hotel
THURSDAY 16th December 2010		
Chair: Wolfgang Liebl		
09.00 – 09.10	Jacque Minton	Welcome
09.10 – 10.00	Rolf Thauer <i>Max Planck, Marburg</i>	New Perspectives in Clostridial Fermentations
10.00 – 10.20	Hubert Bahl <i>University of Rostock</i>	Life with Oxygen: The <i>Clostridium acetobutylicum</i> Way
10.20 – 10.40	Philippe Soucaille <i>Metabolic Explorer</i>	From The Discovery Of A New Dehydratase To An Industrial Process Using <i>Clostridium Acetobutylicum</i> For The Production Of 1, 3 Propanediol
10.40 - 11.10	Coffee/Tea Break	
Chair: Philippe Soucaille		
11.10 - 11.45	Isabelle Verstraete <i>Institut Pasteur</i>	Global Regulation of Cysteine Metabolism in <i>Bacillus subtilis</i>
11.45 – 12.05	Chantal Tardif <i>CNRS, Marseille, F</i>	How Does <i>Clostridium Cellulolyticum</i> Regulate Cellulosome Production and Composition?
12.05 – 12.40	Colin Harwood <i>Newcastle University</i>	The Primary and Secondary Sec Protein Secretion Pathways of <i>Bacillus</i>
12.40 – 13.00	Rick Titball <i>University of Exeter</i>	Old and New Toxins from <i>Clostridium perfringens</i>
13.00 – 14.30	Lunch	Hilton Hotel
Chair: Maja Rupnik		
14.30 – 14.50	Davie Serruto <i>Novartis, Sienna</i>	Identification and Characterization of Surface-Associated and Secreted Proteins of <i>Clostridium difficile</i>
14.50 – 15.25	Vesa Kontinen <i>University of Helsinki</i>	PrsA peptidyl-prolyl cis-trans isomerase: role in bacterial cell wall synthesis and post-translocational folding of exported proteins.
15.25 – 16.00	Mickaël Desvaux <i>INRA Clermont</i>	Protein Secretion in Monoderm (Gram-positive) Bacteria: Generic and Rational Secretomic Strategy for Comprehensive Appraisal of the Exoproteome
16.00 – 16.20	Nigel Minton <i>University of Nottingham</i>	The Molecular Pathogenesis of <i>C. difficile</i>
16.20 – 18.00	POSTERS/ Refreshments	
19.30	Dinner	Hilton Hotel

FRIDAY 17 th December 2010		
Chair: Miia Lindstrom		
09.00 – 09.35	Matt Holden <i>Sanger Institute</i>	Getting a Genomic Grip on Strangles and <i>Streptococcus equi</i> Pathogenesis
09.35 – 09.55	Maja Rupnik <i>University of Maribor</i>	Intraspecies Variability in <i>Clostridium difficile</i>
09.55 – 10.25	Aziz Aboobaker <i>University of Nottingham</i>	Understanding Tissue Regeneration with a Worm and Second Generation Sequencing Machine
10.25 – 10.45	Wolfgang Liebl <i>Technical University Munich</i>	Transcriptional Analysis of <i>Clostridium acetobutylicum</i> Growing on Mixtures of D-Glucose and D-Xylose
10.45 - 11.15	Coffee/Tea Break	
Chair: Hubert Bahl		
11.15 – 11.50	Michael Hecker <i>Ernst-Moritz-Arndt University, Greifswald</i>	Aspects of Gene Regulation in <i>B.subtilis</i>
11.50 – 12.10	Miia Lindstrom <i>University of Helsinki</i>	Role of Two-Component Signal Transduction Systems in <i>Clostridium botulinum</i> Stress and Virulence
12.10 - 12.45	Peter Mullany <i>Eastman Dental Inst, UCL</i>	Bacterial Antibiotic Resistance
12.45 – 13.05	Paola Mastrantonio <i>ISS, Rome</i>	Antibiotic Resistance in <i>Clostridium difficile</i>
13.05 – 14.30	Lunch	Hilton Hotel
14.30 – 16.00	PARTNER MEETING	[Free for other delegates to explore 'Shoppingham']
Chair: Paola Mastrantonio		
16.00 – 16.35	Anne Moir <i>University of Sheffield</i>	Spore Germination in <i>Bacillus</i>
16.35 – 17.10	Adriano Henriques <i>University of Lisbon</i>	Developmental Biology of <i>Bacillus subtilis</i> and Related Organisms
17.10 – 17.30	Peter McClure <i>Unilever, Sharnbrook</i>	Risk Assessment for <i>Clostridium botulinum</i>
17.30 – 18.05	Mike Young <i>Aberystwyth University</i>	The Initiation of Sporulation in Endospore-Forming Clostridia
20.00	Dinner/ Disco	Hilton Hotel
SATURDAY 18 th December 2010		
Depart		

ABSTRACTS OF ORAL PRESENTATIONS

New Perspectives In Clostridial Fermentations

R. THAUER

*Max Planck Institute for Terrestrial Microbiology,
D-35043, Marburg, Germany.*

Two years ago we discovered that the cytoplasmic butyryl-CoA dehydrogenase-EtfAB complex from *Clostridium kluyveri* couples the exergonic reduction of crotonyl-CoA to butyryl-CoA with NADH and the endergonic reduction of ferredoxin with NADH via flavin-based electron bifurcation (1). We now found a second cytoplasmic enzyme complex in *C. kluyveri* capable of energetic coupling via this novel mechanism. It is the iron-sulfur flavoprotein complex NfnAB that couples the exergonic reduction of NADP⁺ with reduced ferredoxin and the endergonic reduction of NADP⁺ with NADH in a reversible reaction: $Fd_{red}^{2-} + NADH + 2 NADP^+ + H^+ = Fd_{ox} + NAD^+ + 2 NADPH$ (2). The role of these two and of other novel energy converting enzyme complexes (3) in clostridial fermentations is discussed.

(1) Li, F., Hinderberger, J., Seedorf, H., Zhang, J., Buckel, W. & Thauer, R.K. (2008) Coupled ferredoxin and crotonyl coenzyme A (CoA) reduction with NADH catalyzed by the butyryl-CoA dehydrogenase/Etf complex from *Clostridium kluyveri*. *J. Bacteriol.* 190, 843-50

(2) Wang, S., Huang, H., Moll, J., & Thauer, R. K. (2010) NADP⁺ reduction with reduced ferredoxin and NADP⁺ reduction with NADH are coupled via an electron bifurcating enzyme complex in *Clostridium kluyveri*. *J. Bacteriol.* 192, 5115-5123

(3) Schut GJ & Adams MW (2009) The iron-hydrogenase of *Thermotoga maritima* utilizes ferredoxin and NADH synergistically: a new perspective on anaerobic hydrogen production. *J. Bacteriol.* 191, 4451-4457.

Life with Oxygen: The *Clostridium acetobutylicum* Way

HUBERT BAHL

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

Clostridium acetobutylicum belongs to those bacteria which are considered as obligate anaerobe, e.g. oxygen is harmful or lethal. Nevertheless, it is known that it can survive limited exposure to air, and eliminates oxygen or reactive derivatives via NADH dependent reduction. This system does apparently contribute to survival after oxidative stress, but is insufficient to establish long-term tolerance of aerobic conditions.

Recently, a PerR-homologous protein has been identified in *C. acetobutylicum* as being a key repressor of a reductive machinery for the scavenging of reactive oxygen species and molecular O₂. In the absence of PerR, the full derepression of its regulon resulted in increased resistance to oxidative stress and nearly full tolerance of an aerobic environment. Several genes were upregulated and identified as members of the clostridial PerR regulon, including the heat shock protein Hsp21, a reverse rubrerythrin and unique in clostridia. It was massively produced and became the most abundant protein in the absence of PerR. This multifunctional protein plays a crucial role in the oxidative stress defence.

We used a transcriptomic approach to analyze gene expression in the aerotolerant PerR mutant strain and compared it to the O₂ stimulon of wild-type *C. acetobutylicum*. The genes encoding the components of the alternative detoxification system were PerR regulated. Only few other targets of direct PerR regulation were identified, including two highly expressed genes encoding enzymes that are putatively involved in the central energy metabolism. All of them were highly induced when wild-type cells were exposed to sublethal levels of O₂. Under these conditions, *C. acetobutylicum* also activated the repair and biogenesis of DNA and Fe-S clusters as well as the transcription of a gene encoding an unknown CO dehydrogenase-like enzyme. Surprisingly few genes were down regulated when exposed to O₂, including those involved in butyrate formation. In summary, these results show that the defense of this strict anaerobe against oxidative stress is robust and by far not limited to the removal of O₂ and its reactive derivative

The influence of oxygen on the central carbon flow and the iron homeostasis is in the focus of our ongoing research.

From The Discovery Of A New Dehydratase To An Industrial Process Using *Clostridium Acetobutylicum* For The Production Of 1, 3 Propanediol

PHILIPPE SOUCAILLE

Metabolic Explorer, Biopôle Clermont-Limagne, 63340 Saint-Beauzire, France

Recent applications of 1,3-propanediol, in a new polyester called poly(propylene terephthalate) with unique properties for the fiber industry, call for a drastic increase in the production of this chemical. 1,3 propanediol can be produced by two different chemical processes that both produce toxic intermediates and need a reduction step under high hydrogen pressure. Recently, several patents were filed that describe an environmentally friendly biological process for the conversion of renewable resources like glucose to 1, 3 propanediol. This process uses recombinant microorganisms expressing genes encoding, among other enzymes, a coenzyme-B12 dependent glycerol dehydratase. One of the possible limitations of this biological process is the cost of vitamin B12 that has to be added in high amount to the culture medium.

In all the "natural 1, 3 propanediol producers" characterized so far *ie Klebsiella pneumoniae*, *Citrobacter freundii*, and *Clostridium pasteurianum*, glycerol conversion to 1, 3 propanediol involved a B12-dependent glycerol dehydratase. We have recently shown that the glycerol dehydratase of *C. butyricum* VPI1718 is not stimulated by Coenzyme B12 and is extremely oxygen sensitive which suggests that it is a coenzyme-B12 independent enzyme.

We have cloned, sequenced and characterized from a molecular point of view the genes encoding the glycerol dehydratase and the 1, 3 propanediol dehydrogenase of *C. butyricum* VPI 1718. By both heterologous expression and biochemical characterization, we have demonstrated that this glycerol dehydratase is "coenzyme B₁₂ independent". This unique enzyme belongs to a new family of glycerol dehydratase that has never been described so far. By heterologous expression of this new dehydratase and the 1, 3 propanediol dehydrogenase and by introducing defined mutations in the central metabolism of *C. acetobutylicum*, we obtained a recombinant strain that possesses the best yield and productivity for glycerol conversion to 1, 3 propanediol.

Based on this new enzyme, a "vitamin B12 free" biological process for the production of 50 000 tons/year of 1, 3 propanediol from glycerine has been developed and will be operational in Malaysia in 2012.

Global Regulation of Cysteine Metabolism in *Bacillus subtilis*

ISABELLE MARTIN-VERSTRAETE

Laboratoire de Pathogénèse des Bactéries Anaérobies, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France.

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.

How Does *Clostridium cellulolyticum* Regulate Cellulosome Production and Composition?

CHANTAL TARDIF

*Laboratoire de Chimie Bactérienne - CNRS and Université d'Aix-Marseille
FRANCE*

The mesophilic anaerobic bacterium *Clostridium cellulolyticum* degrades cellulose and plant cell wall associated polymers via multi-enzyme complexes, named cellulosomes, and free enzymes. Each cellulosome potentially combines a big scaffolding protein (CipC) and eight enzymes with different and complementary action modes. Their association with the scaffoldin promotes their synergistic action and substrate proximity. Two-dimensional gel analysis has shown that bacteria grown on cellulose secrete about thirty cellulosomal proteins belonging to various CAZy families. The recent genome release offered the opportunity to analyze the complete degradation system.

Twelve genes encoding several key cellulosomal components are clustered and transcriptionally linked. Among them, the first, second and fifth genes encode the scaffoldin CipC and the two major cellulosomal cellulases Cel48F and Cel9E, respectively. The expression of this operon is regulated at the transcriptional level by a complex mechanism involving carbon catabolite repression. The expression of the genes located in the *cip-cel* operon is also regulated at the post-transcriptional level. The processing of a primary very large transcript which covers the entire cluster leads to the production of several secondary messengers displaying different stabilities; this would contribute to the fine tuning of the expression of individual genes. The majority of the proteins encoded by the *cip-cel* operon are detected in cellulosomes produced by cells grown on microcrystalline cellulose, oat-spelt xylan and wheat straw.

A second large gene cluster encodes enzymes specialized in hemicellulose degradation. These enzymes are only detected in cellulosomes produced by cells grown on wheat straw, the most complex of the substrates studied. The expression of this cluster of genes might be controlled via a two-component system.

Maamar, H.*, Abdou, L.*, Boileau, C., Valette, O., and Tardif, C. (2006) Transcriptional analysis of *cip-cel* gene cluster from *Clostridium cellulolyticum*. *J. Bacteriol* 188, 2614-2624. (*These authors contributed equally to this work)

Abdou, L., Boileau, C., de Philip, P., Pagès, S., Fiérobe, H.-P., and Tardif, C. (2008) Transcriptional regulation of the *Clostridium cellulolyticum* "*cip-cel*" operon: a complex mechanism involving a catabolite-responsive element. *J. Bacteriol* 190, 1499-1506.

Blouzard J.-C., Coutinho P.M., Fiérobe H.-P., Henrissat B., Lignon S., Tardif C., Pages S., and de Philip, P. (2010) Modulation of cellulosome composition in *Clostridium cellulolyticum*: adaptation to the polysaccharide environment revealed by proteomic and carbohydrate-active enzyme analyses. *Proteomics*, 10: 541–554.

The Primary and Secondary Sec Protein Secretion Pathways of *Bacillus*

COLIN R HARWOOD

*Centre for Bacterial Cell Biology, Baddiley-Clark Building, Newcastle University,
Newcastle upon Tyne, NE2 4AX, UK*

The transport of secretory proteins from their site of synthesis in the cytoplasm to their functional location is of importance for both pathogenesis and biotechnology. In *Bacillus* the majority of secreted proteins are transported across the cytoplasmic membrane in an essentially unfolded form by the Sec translocase. Consequently, they must fold into their functional configuration in an environment that is dominated by a high density of immobilised negative charge. Native *Bacillus* proteins therefore have intrinsic folding characteristics that facilitate their rapid folding, assisted by a variety of extrinsic folding factors, including enzymes, chaperone-like peptides and metal ions. Despite these intrinsic and extrinsic folding factors, secretory proteins do misfold, and consequently, *Bacillus* species encode membrane and cell wall proteases that act to clear misfolded from the translocase and the cell wall.

In recent years a number of Gram-positive pathogens, including *Bacillus anthracis*, have been shown to encode paralogues of Sec translocase components, namely SecA (SecA1/A2), SecY (SecY1/Y2) and PrsA (PrsAA/AB/AC), implying that they have distinct primary and secondary secretion systems. Our evidence suggests that *Bacillus anthracis* SecA2 and SecY2 are unlikely to form a discrete accessory translocase, but that SecY2 functions to maintain secretion at low growth rates. The major surface-associated proteins of *B. anthracis*, namely Sap and EA1, are specific substrates of SecA2 and their secretion efficiency is improved in the presence of a newly identified secretion pathway component, SecH. Following their translocation through the Sec translocase, the essentially unfolded SecA2 substrates must fold rapidly to avoid intermolecular aggregation and/or illegitimate interaction with cell wall growth sites. We show that this folding is aided specifically by one of the three extracytoplasmic peptidyl-prolyl *cis/trans* isomerases encoded by this bacterium, namely PrsAB.

Old and New Toxins from *Clostridium perfringens*

RICHARD W. TITBALL

Bacterial Pathogenicity Research Group, Biosciences, University of Exeter, Exeter, EX4 4QD, UK

Clostridium perfringens is a prolific producer of toxins, many of which have been shown to play key roles in the pathogenesis of disease. The alpha-toxin is produced by all strains and has been shown to play a major role in gas gangrene. This toxin is a membrane active phospholipase C and the molecular architecture of the toxin has revealed much about the mode of action of the toxin. Calcium-dependent binding of the toxin to cell membranes is associated with opening of the active site and hydrolysis of membrane phospholipids.

More recently we have been investigating the roles of the alpha-toxin in diseases of animals. For many years it has been suggested that the alpha-toxin plays a role in bovine enterotoxaemia. Compared to alpha-toxin produced by gas gangrene strains of *C. perfringens* we have found that toxin produced bovine enterotoxaemia isolates is resistant to proteases. This is consistent with the different sites of production of the toxin – in soft tissues or in the gut. We have now identified the molecular basis of protease resistance.

Necrotic enteritis in fowl has also been associated with alpha-toxin produced by *C. perfringens*. However, the precise role of the toxin in disease is not clear. Whilst alpha-toxoid vaccines can provide protection against experimental necrotic enteritis, the evidence now points towards a newly discovered toxin, NetB, as playing a key role in disease.

There may yet be other toxins to be discovered. We have recently genome sequenced an isolate of *C. perfringens* from a case of bovine enterotoxaemia, and this has revealed a number of potential toxins which are not encoded by genome sequenced gas gangrene isolates. The roles of these putative toxins in disease is currently being investigated

Identification and Characterization of Surface-Associated and Secreted Proteins of *Clostridium difficile*

D. SERRUTO, M. SORIANI, R. LEUZZI, M. UNNIKRISHNAN N. NORAI, V. CAFARDI, L. FIASHI, M. BIAGINI, T. DAPA, L. TULLI, J. TELFORD, M. PIZZA, M. SCARSELLI

Novartis Vaccines and Diagnostics, Via Fiorentina 1, 53100 Siena, Italy.

Clostridium difficile causes *Clostridium difficile* associated disease (CDAD), which includes a range of gastrointestinal diseases ranging from diarrhea to pseudomembranous colitis. Usually, *C. difficile* infections are associated with antibiotic therapy in noscomial settings. Antibiotics are believed to disrupt normal gut flora, allowing *C. difficile* to colonize. While the major virulence factors of this bacterium, cytotoxins TcdA and TcdB, have been well studied, the precise roles of *C. difficile* of surface-associated and secreted proteins in the pathogenesis are unknown. The most widely studied surface proteins are the proteins that constitute the S-layer, a paracrystalline array surrounding the cell. Few other putative cell wall proteins (CWPs) have been reported. The availability of different genomic sequences of *C. difficile* has recently provided an excellent opportunity to identify novel potentially CWPs, likely involved in the pathogenesis of the bacterium.

Through the application of genomic and proteomic approaches we aimed to identify new surface-associated and secreted *C. difficile* proteins. A reverse vaccinology approach using multiple *C. difficile* genomes, based on presence of canonical sequence signatures of extra-cellular localization and homology to known surface-exposed or secreted proteins of other Gram-positive bacteria, has led to the identification of new potential candidates. The genes have been cloned and express in recombinant forms and used to immunize sera. Mice immune sera are currently used to prove the expression of the selected proteins on the surface of *C. difficile* and to define their ability to impair bacterial adhesion to human cells. In addition, a proteomic approach has been applied in order to characterize the bacterial surface and the culture supernatant of *C. difficile* strains.

The biological functions of selected surface-exposed and secreted proteins in *C. difficile* physiology and pathogenesis will be studied by characterizing them in various *in vitro* and *ex vivo* assays.

PrsA Peptidyl-Prolyl *Cis-Trans* Isomerase: Role in Bacterial Cell Wall Synthesis and Post-Translocational Folding of Exported Proteins

VESA P. KONTINEN

*Haartman Institute, Haartmaninkatu 3 (P.O. Box 21)
FIN-00014 University of Helsinki, Finland*

The PrsA peptidyl-prolyl *cis-trans* isomerase and its homologues are abundant lipoproteins found in most Gram-positive bacteria of the Firmicutes group. The established function of PrsA is to assist post-translocational folding of secretory proteins and possibly large extracytoplasmic domains of some integral membrane proteins. The property of PrsA to enhance secretion of bacillar α -amylases is utilized in the industrial production of the hyperthermoresistance α -amylase of *Bacillus licheniformis*.

In *Bacillus subtilis*, inactivation of PrsA is lethal in normal growth conditions. However, in the presence of high concentration of magnesium, *B. subtilis* can grow in the absence of PrsA and the *prsA* gene can be deleted, but the mutant grows poorly and exhibits various changes in cell morphology. Electron and fluorescence microscope images of PrsA-depleted cells indicate that PrsA is an important component in cell elongation. PrsA is required for the stability and folding of several penicillin-binding proteins (PBPs), but not those of the MreC cell shape-determining protein. The PrsA-dependent PBPs are PBP2a, PBP2b, PBP3, PBP4 and PBP5. In contrast, PBP1a/b is not affected by PrsA depletion. Van-FL staining and mucopeptide analyses indicated that PrsA-depletion causes a defect in peptidoglycan cross-linking consistent with the effects on PBPs. PrsA is a dimeric or multimeric protein which is localized in spots organized in a spiral-like manner along the cell membrane. This pattern is similar to the focal localization of several PBPs and also to the synthesis of cell wall peptidoglycan in distinct foci.

Our data demonstrate that PrsA has a house-keeping role in the folding and stability of penicillin-binding proteins at least in some rod-shaped bacteria. If PrsA activity could be inhibited, it would be an alternative way to β -lactams to inhibit PBP activity.

Protein Secretion in Monoderm (Gram-Positive) Bacteria: Generic and Rational Secretomic Strategy for Comprehensive Appraisal of the Exoproteome

MICKAËL DESVAUX

INRA, UR454 Microbiology, Clermont-Ferrand Research Centre, Saint-Genès
Champanelle, F-63122, France

As the main tools used by bacteria to interact with their environment, secreted proteins are relevant to the bacterial lifestyle. By definition, secreted proteins are proteins actively transported via secretion systems but they can have radically different final destinations. In monoderm (single cellular membrane) bacteria, such secreted proteins can be located in the (i) cytoplasmic membrane, (ii) cell wall, or (iii) extracellular milieu. In any case, the gating systems involved in the secretion of such effectors remain a key event in the maturation process. In monoderm bacteria, seven protein secretion systems are currently recognized, namely the (i) Sec (Secretion), (ii) Tat (Twin-arginine translocation), (iii) FPE (Fimbrilin-Protein Exporter), (iv) ABC (ATP-binding cassette) transporters, (v) FEA (Flagellum Export Apparatus), (vi) holins (hole-forming), and (vii) Wss (WXG100 secretion system) pathways.

Taking *Clostridium acetobutylicum* as a case study, encoded protein secretion systems were identified following genomic analysis. In *Listeria monocytogenes*, a step further was taken in considering also protein substrates of these pathways. An original bioinformatic analysis was developed, which rationale stands in mimicking as close as possible the different molecular steps encountered by a protein in the course of secretion based on current knowledge in the field. From there, 90 proteins could be predicted as secreted and located in the extracellular milieu (GO:0005576), i.e. the theoretical exoproteome. Out of the 50 exoproteins experimentally detected as part of the exoproteome investigated by 2-DE and subsequent MALDI-TOF MS for protein spot identification, 31 (62%) were predicted as located extracellularly and 30 (60%) were predicted as secreted in a Sec-dependent manner. This secretomic strategy has been purposely designed to be generic and can be applied for investigating subcellular localization of proteins in a large variety of monoderm bacteria.

-Desvaux *et al.* 2005. Genomic analysis of the protein secretion systems in *Clostridium acetobutylicum* ATCC 824. *Biochim Biophys Acta-Mol Cell Res.* 1745:223-253.

-Desvaux *et al.* 2006. The protein secretion systems in *Listeria*: inside out bacterial virulence. *FEMS Microbiol Rev.* 30:774-805.

-Desvaux *et al.* 2009. Secretion and subcellular localizations of bacterial proteins: a semantic awareness issue. *Trends Microbiol.* 17:139-145.

-Desvaux *et al.* 2010. Comprehensive appraisal of the extracellular proteins from a monoderm bacterium: theoretical and empirical exoproteomes of *Listeria monocytogenes* EGD-e by secretomics. *J Proteome Res.* 9:5076-5092.

Molecular Pathogenesis of *Clostridium difficile*

NIGEL P. MINTON

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

Clostridium difficile has emerged as one of the most important causes of healthcare-associated infections in the western world. Despite its infamy, the molecular basis of pathogenesis remains little understood. Two large cytotoxins (A & B) are the only definitive virulence factors, although the relative role of Toxin A has been questioned. The situation has been exacerbated by the emergence and spread of hyper-virulent strains, responsible for more severe disease. Presently, explanations for hyper-virulence remain conjecture. They include, increased toxin production as a consequence of defects in TcdC repressor, more prolific sporogenesis and enhanced gut epithelial cell adherence.

The elucidation of the molecular basis of pathogenesis has been impeded by ineffective gene systems for forward and reverse genetic studies. We have developed a battery of tools with which both directed (ClosTron) and random (transposon *mariner*) insertional mutants can be made, as well as allelic exchange technologies which allow gene replacement, gene addition and in-frame deletions. Mutants carrying a variety of altered alleles have been created and the physiological consequences assessed.

Through the generation of appropriate multiple mutants, and the use of an *in vivo* infection model, our studies have re-established the role of Toxin A in disease. Furthermore, we have determined a correlation between Quorum Sensing and increased toxin production which may prove to be more telling than variations in the sequence of TcdC. In parallel, we have identified, for the first time, several factors essential to the process of spore germination, and amassed data that suggests the link between hyper-virulence and spore prolificacy is debatable. Whilst our data support the view that hyper-virulent strains adhere more strongly to epithelial cells, the assumption that flagella have a role to play in adhesion has been called into question.

Getting a Genomic Grip on Strangles and *Streptococcus equi* Pathogenesis

MATTHEW T. G. HOLDEN

The Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK.

Streptococcus equi subsp. *equi* (*S. equi*) and *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*) are closely related pathogens that have different hosts ranges and disease associations; *S. equi* is the causative agent of equine strangles, and *S. zooepidemicus* is responsible for diverse diseases in a wide range of hosts, including humans. Recent studies into the population genetics of these organisms using multilocus sequence typing (MLST) suggest that *S. equi* evolved from an ancestral *S. zooepidemicus* strain.

Comparative analysis of the complete genomes of *S. equi* strain 4047 and *S. zooepidemicus* strain H70 reveals evidence of functional loss due to mutation and deletion that has led to a reduction in ancestral functions in *S. equi*. Concurrent with this, pathogenic specialization appears to be accompanied by gene gain, through the acquisition of bacteriophages encoding a phospholipase A2 toxin, and four superantigens, and an integrative conjugative element (ICE) carrying a novel iron acquisition system with similarity to the high pathogenicity island of *Yersinia pestis*. Analysis of the *S. equi* strain 4047 prophages suggests that *S. equi* and the human pathogen *S. pyogenes* share a common phage pool that enhances cross-species pathogen evolution. The complex interplay of functional loss, pathogenic specialization and genetic exchange between *S. equi*, *S. zooepidemicus* and *S. pyogenes*, continues to influence the evolution of these important streptococci.

Using the second generation sequencing technology platforms we have further explored the genomic landscapes of *S. equi*, by sequencing an additional 52 *S. equi* strains. This collection includes both globally diverse strains collected over a 53-year period, and strains from single outbreak, thereby encompassing a spectrum of variation. Using the sequence data we have been able to generate a high-resolution genotypes for the strains, and investigate the macro- and micro-evolution of the strangles pathogen.

Intraspecies Variability in *Clostridium difficile*

MAJA RUPNIK

Institute of Public Health Maribor and Faculty of Medicine, University of Maribor

Previous studies on variability of specific genomic regions in *C. difficile* as well as recent comparative genomic studies have shown marked intraspecies variability. Best studied examples are variant virulence factors such as two large toxins (toxin A and B) and surface layer protein SlpA.

C. difficile toxinotypes are groups of strains defined by changes in PaLoc region encoding two main virulence factors, toxins TcdA and TcdB. Currently 31 toxinotypes (I to XXXI) are known, whereas strains with PaLoc identical to the reference strain VPI 10463 represent the toxinotype 0. Changes in PaLoc are insertions, deletions and point mutations. Interestingly, the three types of mutations seem to prevail at specific parts of PaLoc. Deletions are found mostly in *tcdA* gene and to date no form of significant deletion in *tcdB* gene is known. Point mutations are more common in *tcdB* gene. Sequencing of some representative *tcdB* genes showed 87% identity in DNA sequence. However, if the toxinotypes with large deletions are excluded from comparison, minimal identity between two forms of variant toxin genes is 90% and mutations are distributed in the genes in specific patterns. Other parts of PaLoc, in particular *tcdC*, can also show great variability. Interestingly, such variant strains were in the past more likely associated with animal hosts but in recent years number of reports of such strains in human population is increasing. Some of toxinotypes have hypervirulent potential and are emerging in many countries as a cause of severe cases and outbreaks.

Variant strains – toxinotypes – could be useful model to study the variability in other genomic loci and molecular mechanisms underlying this variability which are currently not known.

Understanding Tissue Regeneration with a Worm and Second Generation Sequencing Machine

A. AZIZ ABOOBAKER

Deep Seq, Centre for Genetics and Genomics, Queens' Medical Centre, University of Nottingham, Nottingham, NG7 2UH, UK

Understanding how we might repair and regenerate lost or damaged organs is a major goal of biomedical research. However, an integrated understanding of how stem cells can be used to replace and repair damaged or ageing tissue remains, for the most part, a distant goal. The planarian model system provides a simple model in which to understand how this process is regulated in a natural context. These amazing animals are able to regenerate entire animals from small starting fragments after amputations and constantly replace their somatic tissues during homeostasis as part of their normal life history. This ability can be traced to a population of collectively totipotent planarian adult stem cells (pASCs), classically called neoblasts.

We are particularly interested in understanding the control of regenerative axial polarity and stem cell differentiation during regeneration and have started to understand the molecular mechanisms that control this process.

We have generated and describe a transcriptome from a mixed time course of regenerative stages of the asexual strain of the planarian *S. mediterranea* to use as a basis for genome wide expression analyses, such as RNAseq. We have integrated data from two next generation sequencing platforms in an attempt to increase gene discovery and transcript representation as well as avoid single platform bias. We have then used massively parallel sequencing from different time points in regeneration to look at how transcriptional activity changes across different regenerative scenarios. This represents the first regenerative RNAseq transcriptome data set from any animal. A broad view of the data identifies key time points during regeneration that agree with both cell biological observations and classical manipulative experiments originally performed by T.H. Morgan. More fine grained analysis identifies key candidate pathways for our ongoing functional genomic studies of regenerative events.

Transcriptional Analysis of *Clostridium Acetobutylicum* Growing on Mixtures of D-Glucose and D-Xylose

A. EHRENREICH, C. DÖRING, C. HELD, W. LIEBL

Technische Universität München, Department of Microbiology, D-85354 Freising, and Georg-August-Universität, Department of Genomic and Applied Microbiology, D-37077 Göttingen, Germany

Clostridium acetobutylicum, a strict anaerobic organism that is used for biotechnological butanol production, ferments various hexoses and pentoses to solvents but prefers glucose presumably using a catabolite repression mechanism. Accordingly, during growth of *C. acetobutylicum* ATCC 824 on a mixture of D-glucose and D-xylose a typical diauxic growth pattern was observed. We have used DNA microarrays and real-time RT PCR to study gene expression during growth on D-glucose, D-xylose and the diauxic growth lag on a defined minimal medium, while monitoring substrate consumption and product formation. Two putative operons involved in D-xylose degradation were identified. The first operon includes genes for a transporter, a xylulose kinase, a transaldolase, a transketolase, an aldose-1-epimerase and a putative xylose isomerase that has been annotated as an arabinose isomerase. Expression of the genes of this operon was induced by D-xylose but was catabolite repressed by D-glucose. The second putative xylose utilization operon consists of genes for a xylulose kinase and a hypothetical protein, and a gene that has been annotated as a L-fucose isomerase gene but might in fact code for a xylose isomerase. This operon was induced by D-xylose but was not subject to catabolite repression. In accordance with these results, during a genome wide search for CRE sites we identified a CRE site in the catabolite repressed operon but not in the operon that was not subject to catabolite repression.

Aspects of Gene Regulation in *B.subtilis*

MICHAEL HECKER

Ernst-Moritz-Arndt University, Greifswald, Germany

Unfortunately abstract not received in time for publication.

Role of Two-Component Signal Transduction Systems in *Clostridium botulinum* Stress and Virulence

MIIA LINDSTRÖM

Department of Food Hygiene and Environmental Health, Centre of Excellence in Microbial Food Safety, Faculty of Veterinary Medicine, University of Helsinki, Finland

Clostridium botulinum produces extremely potent botulinum neurotoxins (Bot; A to G) that cause a potentially lethal paralysis, botulism, to humans (A, B, E, F) and animals (C, D). The classical food poisoning botulism is due to ingestion of pre-formed Bot with food or drink, while infant and wound botulism are toxicoinfections as a result of *in vivo* Bot formation from spores. Apart from being a serious threat to food producers and a potential bioweapon, Bot is being widely used as a therapeutic agent in a range of spastic muscular disorders. Nevertheless, the environmental factors affecting the growth of *C. botulinum* and Bot production under various environmental conditions and the regulation of Bot synthesis are still poorly characterized.

Central mechanisms that bacteria utilize in sensing and adaptation to environmental changes include the two-component signal transduction systems (TCS). The membrane-located histidine kinases sense environmental stimuli with an extracytoplasmic sensor domain and send autophosphorylation signals to the cognate response regulators. The response regulators possess DNA-binding activity, ultimately resulting in a specific response in gene expression. TCS's have been associated in the regulation of virulence or stress response in pathogenic and non-pathogenic clostridia. The role of TCS's in *C. botulinum* growth and toxin production is unknown. Using the Clostron mutagenesis, we have shown that TCS's play a central role in stress response of and Bot production by *C. botulinum* strain ATCC 3502.

Bacterial Antibiotic Resistance

PETER MULLANY

*Eastman Dental Institute, University College London, 256 Gray's Inn Road London
WC1X 8LD*

Antibiotic resistance is one of the great medical problems and it is frequently spread between bacteria on specialised mobile genetic elements, which include conjugative and mobilizable plasmids and transposons. In this talk I will review our current understanding of the mechanisms underlying the spread of these genetic elements and how the application of novel metagenomic approaches is allowing the discovery of new mobile genetic elements.

Antibiotic Resistance in *Clostridium difficile*

PAOLA MASTRANTONIO and PATRIZIA SPIGAGLIA

Department of Infectious Diseases, Istituto Superiore di Sanità, Rome, Italy

Clostridium difficile has been identified as one of the major causative agents of antibiotic-associated diarrhoea. The antibiotic susceptibility of *C. difficile* strains, including epidemic clones, is changing. Most of the prevalent types responsible for disease in humans were resistant to clindamycin and/or erythromycin due to an *ermB* gene encoding for an rRNA methyltransferase, whereas, currently, we are witnessing an increase in the number of resistant *ermB*-negative *C. difficile* isolates, including the epidemic strain *C. difficile* BI/NAP1/027. Resistance to tetracycline has also been observed in both historic and recent isolates. Tetracycline resistance is commonly conferred by a *tet(M)* gene that encodes for a ribosomal protection protein. This gene is usually carried by a Tn5397 transposon, but recent strains can harbour elements belonging to the Tn916 family, which are widely dispersed in Gram-positive and negative bacteria. Moreover, *C. difficile* clinical isolates resistant to fluoroquinolones have recently appeared and their number is on the rise too. In particular, resistance rate to moxifloxacin has increased dramatically over the last years. The current standard treatment for *C. difficile* Infection (CDI) is oral metronidazole or vancomycin, the latter as the antibiotic of choice for severe cases. Few *C. difficile* strains resistant to metronidazole have been described in the world, but recently an increase in Minimum Inhibitory Concentrations (MIC) and heteroresistance have been observed. The exact mechanism of reduced susceptibility to metronidazole remains to be determined since this pathogen does not show nitroimidazole genes, commonly associated with this resistance in other bacteria. A comprehensive picture of different phenotypic and molecular characteristics of the antibiotic resistance in a very large sample of clinical isolates collected during an European survey of CDI will be presented and will serve as useful source of data for future studies.

Spore Germination in Bacillus

ANNE MOIR

*Krebs Institute, Dept of Molecular Biology & Biotechnology, University of Sheffield,
Sheffield S10 2TN, UK*

Spore germination is an extreme example of a signal transduction system, in which interaction between small molecule germinant and receptor protein results in dramatic changes in spore structure and properties, without the need for any new macromolecular synthesis.

Nutrient germinants – amino acids, nucleosides or sugars - traverse the outer layers of the spore, and interact with germinant receptors, in the spore inner membrane. Such receptors represent a diverged family of proteins, members of which are encoded in endospore forming Bacilli and Clostridia, often but not always in three-gene operons. The proteins of the receptor probably interact, and one component is a homologue of the APC family of single component membrane transporters. *C. difficile* is the only species so far described that does not have such receptor genes, although it does respond to specific triggers. Alternative pathways may include germination in response to high levels of DPA, or activation of a Ser/Thr kinase by peptidoglycan fragments, both of which bypass the need for receptor proteins.

Receptor-mediated release of ions and small molecules across the inner membrane is followed by lysis of cortex peptidoglycan, mediated in Bacilli by SleB and CwlJ. How SleB is activated is not clear, but activation of CwlJ requires dipicolinic acid, which is released in quantity from the spore core early in germination.

Lysis of cortex peptidoglycan will lead to complete rehydration of the spore core; without the activity of cortex lytic enzymes, the spore core, although slightly less dehydrated, is still trapped inside the cortex and cannot outgrow. Some Clostridia encode homologues of SleB, but others encode an alternative cortex lytic enzyme SleC, which is proteolytically activated.

Spore coat or exosporium may contain enzymes that moderate the effects of germinants – for example, alanine racemase and inosine hydrolase are present in the exosporium of *B. cereus*.

Developmental Biology of *Bacillus subtilis* and Related Organisms

HENRIQUES, A.O.

*Microbial Development Laboratory, Instituto de Tecnologia Química e Biológica,
Universidade Nova de Lisboa, Oeiras, Portugal*

Endospores are formed by *Bacillus* and related aerobic endospore forming bacteria as well as by *Clostridium* as a strategy to remain viable in the environment during unfavourable conditions. Spore development has been extensively studied in the model organism *B. subtilis*, in which sporulation at least under laboratory conditions, is triggered by severe nutrient deprivation. The process however, follows the same morphological sequence in all endospore-forming organisms that have been examined. Most spore formers are rod-shaped and in those organisms sporulation involves an asymmetric cell division, closer to one of the cell poles rather than mid-cell, accompanied by partitioning of one copy of the genome into each of the sister cells. The smaller cell, or forespore (a germinal cell line), will develop into the mature endospore while the larger, or mother cell (a terminal cell line) contributes to the differentiation process but will eventually undergo autolysis to release the mature endospore into the medium. Following asymmetric division, the mother cell engulfs the forespore. At later stages in development several protective structures are assembled around the engulfed forespore. The structure and composition of the spore surface layers differs greatly among species and even within the same species, presumably reflecting niche-specific requirements. In *B. subtilis*, morphogenesis is controlled by a cascade of 4 cell type-specific RNA polymerase sigma subunits. σ^F and σ^E control early stages of development in the forespore and in the mother cell, respectively. Following engulfment completion, σ^F is replaced by σ^G , whereas σ^K substitutes σ^E . The forespore and mother cell lines of gene expression are deployed co-ordinately and in close register with the course of morphogenesis. The sporulation sigma factors are conserved among spore formers and studies in some organism suggest that their function is conserved. We will review the main stages in spore morphogenesis as well as the underlying genetic events using *B. subtilis* as our model. We will also extend our description to other important emerging model organisms, including some of the pathogenic spore formers.

Risk Assessment for *Clostridium botulinum*

PETER J. MCCLURE

*Safety and Environmental Assurance Centre, Unilever R&D, Colworth Science Park,
Sharnbrook, Beds., MK44 1LQ, UK*

Clostridium botulinum continues to be a major concern and target for the food industry. Although the safety record for many foods in relation to *C. botulinum* is excellent, and is based on good data and understanding, outbreaks of botulism still occur. In addition, the increasing consumer demands for fresher, less heavily preserved and processed foods are driving the food industry to look for novel approaches and technologies to meet these needs. About 30 years ago, advanced systems for managing pathogens in foods were based on hazard assessment that considered severity of the hazard and specified adequate safety margins to minimise the hazard. These approaches offered significant advantages over purely hazard-based approaches that consider possible presence of a hazard, often resulting in the conclusion that the product is unsafe or unfit for human consumption. Recent development of risk-based approaches has allowed an evolution of qualitative and quantitative tools that characterise the hazard in terms of likely presence and severity.

We have developed a number of tools and approaches that enable the product developer to design safety into foods, some of which utilise combined thermal inactivation and thermal injury effects data, targeting non-proteolytic and proteolytic *Clostridium botulinum*. Some examples of these tools and approaches are provided, together with the steps used in establishing safe product and process designs. Such applications offer significant advantages over hazard-based approaches by identifying new product design 'windows' and provide an equivalent level of safety compared to conventional processes.

The Initiation of Sporulation in Endospore-Forming Clostridia

MICHAEL YOUNG

*Institute of Biological Environmental and Rural Sciences, Aberystwyth University,
Aberystwyth, SY23 3DD, UK*

Spo0A controls the initiation of sporulation and solvent production in *Clostridium acetobutylicum*. Clostridia lack a recognizable phosphorelay (homologues of Spo0F and, in most cases, Spo0B are absent), and the mechanism of Spo0A activation is unresolved. Three alternatives have been proposed: (i) direct phosphorylation by sensory histidine kinases or (ii) by a metabolite such as acetyl phosphate or butyryl phosphate; (iii) indirect phosphorylation by a novel phosphorelay whose components are cannot be deduced by bioinformatic analyses.

Several orphan kinases feed phosphate into the *B. subtilis* phosphorelay. We inactivated the genes encoding all five orphan kinases of *C. acetobutylicum*. The results suggested that *cac0323*, *cac0903* and *cac3319* are involved in Spo0A activation, since sporulation was either reduced or abolished in mutants lacking one or more of these kinases. Mutants that lack *cac0437* sporulate precociously and at a higher frequency than the wild type. Moreover, inactivation of *cac0437* restores sporulation to at least wild type levels in *cac0323*, *cac0903* and *cac3319* mutants. Furthermore, sporulation of the wild type harbouring multiple copies of *cac0437* is greatly reduced. *Cac0437* therefore functions to prevent premature sporulation initiation by interfering with Spo0A activation.

Phosphotransfer experiments indicate that the products of these genes interact directly with Spo0A, either adding (*Cac0903*, *Cac3319*) or removing (*Cac0437*) phosphoryl groups.

During exponential growth, the phosphorylation status of Spo0A is controlled by the competing activities of *Cac0903* and *Cac0437*. Sporulation is initiated in early stationary phase when *spo0A* expression is rising rapidly. In response to an unknown signal(s), *cac0903* expression suddenly increases and *cac0437* expression simultaneously declines. This shifts the Spo0A \leftrightarrow Spo0A~P equilibrium strongly in favour of Spo0A~P, which induces the expression of *spoIIAA* and *sigG*, initiating endospore development.

ABSTRACTS OF POSTER PRESENTATIONS

Regulation of the Composition of the Hemicellulolytic System of *C. cellulolyticum*

H. CELIK¹, S. PAGES^{1,2}, H.P. FIEROBE¹, C. TARDIF^{1,2} and P. de PHILIP^{1,2}

¹*Laboratoire de Chimie Bactérienne, CNRS UPR9043, IMM Marseille, France*

²*Université Aix Marseille, Marseille, France*

Degradation of plant cell wall polysaccharides has remarkable practical applications, especially in the renewable bioenergy sector. In plant cell wall, crystalline cellulose is embedded in a network of hemicellulose polymers and a gel of pectins. Because of this complex composition and organization, plant cell wall degradation involves a set of enzymes of different mode of action and specificity (cellulase, hemicellulase...). *C. cellulolyticum* is a model anaerobic mesophilic bacterium, which secretes a degradation system, composed mainly of multienzymatic complexes (cellulosomes), to hydrolyse plant cell walls into simple sugars for its growth. Cellulosomes are composed of a scaffolding protein bearing 8 cohesin modules able to interact with dockerin modules of 62 putative dockerin-containing enzymes. Proteomic analyses of the composition of the cellulosomes produced by *C. cellulolyticum* upon growth on different substrates (cellulose and straw) indicate that modulation of cellulosomes composition occurs according to the growth substrate. In particular, the expression of a cluster of 14 hemicellulase-encoding genes (so called *xyl-doc*) seems to be induced by the presence of straw. Interestingly, genes encoding a two-component regulation system are present upstream of *xyl-doc*.

Our aim is to demonstrate the involvement of the two-component regulation system in the regulation of *xyl-doc*. First, inactivation and over-expression of the regulator encoding gene and then analysis of the composition of the cellulosomes produced on cellulose will be performed. This work will be completed by the purification of this protein and its use in electrophoretic mobility shift assays with the predicted target promoter(s) of *xyl-doc*. Another part of my project is to determine if *xyl-doc* has an operonic structure; this investigation will involve transcriptional fusions experiments, RT PCRs ...

Secretion and Assembly of Cellulosome Components in *Clostridium cellulolyticum*

MINH UYEN DAO THI, SANDRINE PAGÈS, STÉPHANIE PERRET,
CHANTAL TARDIF

Laboratory of Bacterial Chemistry, CNRS Marseille, France

Clostridium cellulolyticum is a cellulolytic, mesophilic and anaerobic bacterium whose genome has recently been released. This organism secretes multienzymatic complexes termed cellulosomes to hydrolyze plant cell wall polymers into simple sugars for its growth. Cellulosomes are composed of a scaffolding protein which assembles numerous enzymatic subunits displaying various and complementary specificities and mode of action. However, the secretion and assembly of the cellulosome components remains little understood. Our project aims to identify key chaperones involved in the secretion of the cellulosome components and the factors which mediate or influence the cellulosome assembly.

Several approaches will be used. The genome data will be explored to find out chaperone gene candidates from whom specific mutants using ClosTron will be constructed and studied. Random mutants will be also constructed using Tn1545 derivative delivery tool and screened for secretion and assembly defect. Chaperones for abundant and/or large cellulases such as Cel48F, Cel9E, Cel9G will be particularly considered. Pull down experiment will be performed to identify Cel48F polypeptide interacting chaperones. In addition, fluorescent reporters will be used to analyze the intracellular traffic and secretion of the cellulosome components.

Actually, the putative involvement of OrfXp – a cohesion containing protein associated with the membrane - in the cellulosome assembly is being studied. We have obtained *C.cellulolyticum* strains overproducing either OrfXp (with and without His-tag) or antisense RNAs binding *orfX* messengers. The phenotypes of these strains will be studied and compared.

Identification and Characterization of Surface-Associated and Secreted Proteins of *Clostridium difficile*

T. DAPA, V. CAFARDI, M. BIAGINI, M. PIZZA, M. SCARSELLI, N. NORAIS,
D. SERRUTO AND M. UNNIKRIISHNAN

Novartis Vaccines and Diagnostics, Via Fiorentina 1, 53100 Siena, Italy.

Clostridium difficile is an obligately anaerobic, Gram-positive and endospore-forming bacterium. Although it is an opportunistic pathogen, it is one of most important causes of health care-associated infections. *C. difficile* colonizes the gut when the normal intestinal microflora is disrupted by antibiotics. *Clostridium difficile* infections (CDI) are a group of toxin-mediated intestinal diseases. Clinical outcomes can range from asymptomatic colonization, mild diarrhoea to severe disorders such as pseudomembranous colitis.

While it is known that toxins TcdA and TcdB are the main virulence factors of *C. difficile*, the roles of surface-associated and secreted proteins during infection remain unclear. The most widely studied surface proteins are the proteins that constitute the S-layer, a paracrystalline array surrounding the cell. Few other putative cell wall proteins (CWPs) have been reported.

We aim to identify and characterize surface-associated and secreted *C. difficile* proteins and determine their role in disease. Comparative expression analyses will be performed with various clinically important *C. difficile* strains under different growth conditions using genomic and proteomic methods. Surface-exposed and secreted proteins that are common or unique to pathogenic strains will be identified. The biological functions of selected proteins in *C. difficile* physiology and pathogenesis will be studied by generating isogenic mutants and characterizing them in various *in vitro* and *ex vivo* assays.

Inactivation of *glgC* and *glgD* in the Granulose Operon of *Clostridium acetobutylicum*

ANA BELÉN CORREDERA ESPEJO AND HUBERT BAHL

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

Clostridium acetobutylicum is a strictly anaerobic spore forming Gram-positive bacterium and its cell cycle is characterized by metabolic and morphological differentiations. During exponential growth, sugars are fermented to acetate and butyrate and in the transition phase the metabolism switches to the production of the solvents acetone and butanol. The process is accompanied by the initiation of endospore formation. Sporulation of *C. acetobutylicum* is preceded by the accumulation of granulose. This polymer consists of a high molecular weight polyglucan containing α -1.4-linked D-glucopyranose units and which is slightly branched (2 % α -1.6-linkages).

The accumulation of granulose occurs via the ADP glucose pathway. In the genome of *C. acetobutylicum* ATCC 824 the open reading frames *cac2237* (*glgC*), *cac2238* (*glgD*) and *cac2239* (*glgA*) putatively encode the key enzymes of granulose synthesis. Glucose-1-phosphate-adenyltransferase is encoded by *glgC*, ADP-glucose pyrophosphorylase (EC 2.7.7.27) by *glgD*, and granulose synthase (E.C. 2.4.1.21) by *glgA*.

We are interested in the role of granulose formation in the cell cycle of *C. acetobutylicum* and have started with the functional analysis of the *glgCDA* operon. A knock out of *glgA* abolished granulose formation. Furthermore, we were unable to detect any sporulating cells or spores in cultures of this mutant. The functional analysis of *glgC* and *glgD* is in progress.

Identification of Proteins Involved in Spore Formation and Germination in *Clostridium difficile*

DANIELA HEEG, DAVID A. BURNS, STEPHEN T. CARTMAN, ALAN COCKAYNE
and NIGEL P. MINTON

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

Clostridium difficile is the major cause of nosocomial diarrhoea particularly in the elderly and those that have undergone antibiotic therapy. *C. difficile* infection can lead to mild or severe diarrhoea that may progress to potentially fatal pseudo-membranous colitis. The ability to form spores allows *C. difficile* to persist in the environment for long periods and residing spores are a serious problem in health care units as they are thought to be the infectious agent. However, in order to cause disease the spore needs to return to vegetative growth via germination. Sporulation and spore germination pathways have been extensively studied in *Bacillus sp.*, but the precise mechanisms within *C. difficile* remain unclear. In this work, reverse and forward genetic approaches are being used to generate and study mutants defective for spore formation and germination processes. Initially, sporulation/germination defective mutants are identified by measuring the development of heat resistant CFU after 5 days growth in BHIS medium. Interesting mutants are then subjected to more detailed analyses. To date, 80 random *mariner*-transposon mutants have been screened, resulting in 8 potentially interesting sporulation/germination defective genotypes. Further to this, 5 proteins that are potentially involved either in spore formation or spore germination have been identified by literature study. The corresponding ClosTron mutants are being constructed at present and preliminary tests showed that at least 2 of these mutants seem to be defective for either spore formation or spore germination. This work has the potential to improve our understanding of the mechanisms of *C. difficile* spore formation and germination.

Does Exposure of Sub-cidal Levels of Biocide Increase *Clostridium difficile* Spore Adherence?

LOVLEEN T. JOSHI, JENNY HAWKINS & LES BAILLIE

Welsh School of Pharmacy, Cardiff University, King Edward VI Avenue, Cardiff, CF10 3NB

Clostridium difficile is an anaerobic Gram positive bacterium implicated as the primary cause of antibiotic associated diarrhoea (CDAD) in the UK. Indeed, between 2004 - 2008 *C. difficile* was involved in 10.5 /1000 deaths in England and Wales. *C. difficile* produces highly resistant endospores which facilitate environmental survival and disease transmission. The ability of the spores to resist the attention of general-purpose disinfectants and biocides is thought to contribute to the spread of the organism around the hospital environment. Hence we sought to ascertain the effects of biocide exposure on spore adherence ability.

21 strains of *C. difficile* were exposed to sub-cidal concentrations (500 ppm) of the biocide Sodium dichloroisocyanurate (NaDCC) for 30 s, 1 min, 5 min and 10 min. Sensitivity and resistance to NaDCC exposure differed between strains, and log reductions in cfu/ml increased with biocide exposure time. Five strains were then tested with 1000 ppm NaDCC for an exposure time of 30 s.

The most resistant strain (PCR Ribotype 027) and most sensitive strain (PCR Ribotype 027) were selected to be exposed to 1000 ppm NaDCC for times of 1 min, 5 min and 10 min. A replica plating method was used to determine the ability of these biocide-exposed spores to adhere to stainless steel. As NaDCC exposure time increased, the ability of spores to stick to stainless steel decreased. This suggests that unless appropriate contact times are employed for biocide use, not only will the biocide fail to inactivate *C. difficile* spores, but also may potentiate spore spread.

The varying response of *C. difficile* spores to biocide treatment and stainless steel suggests that the surface properties of spores, including those with the same PCR ribotype, may differ. It is also intriguing to speculate that exposure to sub-optimal biocide concentrations could increase the adherence of hypervirulent spores, thus aiding environmental contamination and onward patient transmission.

Cell-type Dependent Binding of *Clostridium difficile* TcdA and B to the Host Cell Surface

MAGDALENA KASENDRA, MARIA SCARSELLI, MARIAGRAZIA PIZZA, MARCO SORIANI and ROSANNA LEUZZI

Novartis Vaccines and Diagnostics Srl, Siena, Italy

Clostridium difficile is the leading cause of antibiotic-associated diarrhea and pseudomembranous colitis. Toxins A and B are principally responsible for the disease manifestations by causing damage to human colonic mucosa and inflammation of the colon.

The toxins bind to host receptors by the C-terminal located binding domain, enter host cells via receptor-mediated endocytosis and release the N-terminal catalytic moiety into the cytosol, where they modify and thereby inactivate Rho GTPases.

Toxin A and B are structurally similar and display an identical modality of action *in vitro*. However, the potency of their cytotoxicity is extremely cell-type dependent and the determinants of this biological diversity are not elucidated. In the attempt to investigate the molecular mechanism at the basis of this cell-type diversity, we planned to study each step of the intoxication process, including the binding to the host surface, the time of internalization and the intracellular processing.

In the present study we evaluated the contribution of the binding step to the cell-type diversity. To address this point, we are studying the distribution of the purified toxins and the recombinant C-terminal binding domains on the surface of different cell lines by confocal microscopy. The degree of association is then correlated to the cytotoxicity potency of the two toxins on each cell line.

Investigating the Role of sigma-Factors in *Clostridium botulinum* Neurotoxin Production

DAVID KIRK, HANNU KORKEALA, ZHEN ZHANG, ELIAS DAHLSTEN,
MIIA LINDSTRÖM

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

Groups I and II *Clostridium botulinum* produce potent neurotoxins, as little as 30 ng can be fatal to humans. Food-borne botulism has caused concern in chilled foods such as ready-meals and sous-vide foods. The neurotoxin also has a range of applications in both medicine and cosmetics. Botulism can also occur in wounds and in infants whose gastrointestinal tract has yet to be colonised by commensal bacteria. There are few fatal cases of botulism in developed countries due to improved care. Still, very little is understood about the regulation of this deadly neurotoxin.

In *C. botulinum* type A, the production of neurotoxin is primarily regulated by the Group 5 sigma factor BotR. Sigma factors bind to RNA polymerase and act as the DNA-binding subunit for transcription. The *botR* gene is located in the toxin gene cluster between the neurotoxin complex genes and three hemagglutinin genes. Recently, it has been shown that loss of the putative *botR* promoter did not stop toxin production in type A5 *C. botulinum* (Carter *et al*, 2010). This could indicate that other sigma factors are influencing transcription of the toxin gene cluster.

The aim of this work is to identify if and how other sigma factors play a role in neurotoxin production. Preliminary work has focused on one gene (further referred to as *sig*). We have disrupted this gene using the CloStron tool (Heap *et al*, 2007, 2010), inserting a mutation to disable the gene. This mutant has been evaluated with a toxin ELISA and has shown a significant difference in toxin production suggesting Sig may be inducing, or acting as, a negative regulator of toxin gene expression. qPCR data supports this, however further investigation is required. We wish to investigate Sig to determine if it is acting as a transcriptional repressor or an anti-sigma factor.

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PlcR- type Quorum Sensing in *Clostridium acetobutylicum*

ANN-KATHRIN KOTTE, KATRIN SCHWARZ, NIGEL P. MINTON
and KLAUS WINZER

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

The genome of *Clostridium acetobutylicum* features ten homologues of the *Bacillus cereus* response regulator plcR. In *Bacillus*, PlcR controls most known virulence factors. Knockout of the ten PlcR homologues in *C. acetobutylicum* indicates their role in the regulation of metabolism. The most prominent mutant abolished sporulation, butanol production and granulose accumulation. Further mutants show an elevated acetone production and increased slime production upon entry into stationary phase.

Characterization of Surface-associated Proteins of *Clostridium difficile* and Their Role in Disease

ANDREA KOVACS-SIMON, RICHARD W. TITBALL, STEPHEN L. MICHELL

Bacterial Pathogenicity Research Group, School of Biosciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD, UK

Intestinal diseases caused by the Gram-positive bacterium *Clostridium difficile* range from mild diarrhoea to life-threatening and sometimes fatal pseudomembranous colitis (PMC). Infection is almost always associated with administration of antibiotics. *C. difficile* is one of the most important causative agents of health care-acquired infections and are most commonly seen in the hospitalised elderly. Thus, identifying virulence factors of *C. difficile* apart from the toxins is an emerging issue. This study was aimed to investigate whether lipoproteins of *C. difficile* are involved in pathogenesis.

Gram positive bacterial lipoproteins are cell surface associated molecules that have been shown to contribute towards many cellular processes. Several lipoproteins are recognised by immune cells suggesting that they may play a role in the virulence of certain pathogens. Similar to many other Gram positive bacteria, *C. difficile* possesses the enzymes necessary for the biosynthesis and processing of bacterial lipoproteins. Lipoprotein diacylglycerol transferase (Lgt) is responsible for attaching the protein to the extracellular side of the cytoplasmic membrane using membrane lipid components as an anchor.

We used the ClosTron system to knock-out the *lgt* gene of *Clostridium difficile* 630 and performed experiments to compare the protein profiles of the mutant and the wild-type strain. Our findings show that the loss of *lgt* has an effect on the expression profile of a subset of *C. difficile* lipoproteins. Preliminary data of adhesion assay by using Caco-2 cells reveal that lipoproteins may have a role in adhesion. Identification of lipoproteins recognised by the immune system is in progress. And further characterisation of the mechanisms of *C. difficile* protein acylation is being undertaken.

Establishing a Markerless Deletion System for Comparative Analysis of Solventogenic *Clostridia*.

JUSTYNA M. LESIAK

Department of Microbiology, Technische Universität München, Freising, Germany

Metabolic engineered solventogenic *Clostridia* are considered as a big hope for more efficient and less expensive biotechnological solvent and fuel production. Increasing costs of crude oil as well as the problem of carbon dioxide emission make biofuels an attractive alternative. In order to optimize strains, more detailed information on biochemistry and regulation is needed. Developing an universal clean deletion system is crucial for the comparative analysis of solventogenic *Clostridia*.

Here we propose a clean deletion system based on the counter selection by the toxic base analogue 5-fluorouracil (5-FU) in the *upp* negative strains. Its removal causes resistance to 5-FU and therefore provides a counter selection strategy for plasmids containing the *upp* gene. In order to obtain an *upp* mutant we created a plasmid bearing homologous regions for the upstream and downstream region of the *upp* gene. Resulting mutants should be 5-FU resistant.

The strategy based on the counter selection by 5-FU in *upp* negative strains proved to be very efficient in other microorganisms, such as *Enterococcus faecalis*, *Bacillus subtilis* and *Gluconobacter oxydans*.

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

CAROLYN MEANEY

*Unilever, Safety and Environmental Assurance Centre, Colworth Science Park,
Sharnbrook, Bedford, England, MK44 1LQ, UK*

Is a bacterial spore like a “diamond in a mine?”.... robust, unique, inspires awe, capable of surviving a harsh environment, limited understanding of its ability to survive/persist.....

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. It is this spore formation that enables it to remain viable in the environment for long periods of time, 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. This is required for pathogenic spores in order to be able to initiate growth, toxin production and sometimes infection.

During germination, there is dipicolinic (DPA) acid release, cation excretion, cortex degradation, core hydration, small acid-soluble proteins (SASPs) degradation, loss of spore refractility, resistance and dormancy and initiation of RNA and protein synthesis. During the process of spore outgrowth, there is synthesis of amino acids, continued RNA and protein synthesis, cell wall synthesis and DNA replication.

Ultimately it is the aim of food industries, pharmaceutical industries and healthcare settings alike to gain an understanding of these germination and outgrowth processes in order to eradicate these spore forming bacteria as causes of food poisoning, food spoilage, contaminants of pharmaceuticals and to eradicate nosocomial infections.

By exploiting the current molecular tools available, it is the aim of this project to attempt to expand our current understanding of germination and outgrowth processes, to focus on the area of spore cortex degradation and cortex lytic enzymes and to examine and compare the gene expression profiles of both germinated spores and vegetative cells.

The Problematic of the Unknown Mechanisms of Antibiotic Resistance in *Clostridium difficile*

INES MOURA

Department of Infectious, Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità, Rome, Italy

Clostridium difficile is the main cause of infectious nosocomial diarrhea and colitis in adult patients during or following an antibiotic treatment. Since 2003, severe infections in hospitalized patients have increased in North America and in several European countries.

Broad-spectrum antibiotics, such as clindamycin, aminopenicillins, cephalosporins and fluoroquinolones, possess a high propensity to induce *C. difficile* infection (CDI) once they disturb the normal intestinal microbiota and its barrier effect, allowing subsequent *C. difficile* colonization and CDI. Many resistant *C. difficile* isolates show the absence of resistance mechanisms already known and well characterized.

It is known that the resistance to macrolides, lincosamides and streptogramins involves specific dimethylation of the 23S rRNA. The genes that code for these rRNA methylases are called *erm* genes and the predominant class in anaerobes is the *erm*(B). Currently, an increasing number of resistant *C. difficile* strains are *erm*(B)-negative and no other *erm* genes or efflux pumps seem to be involved.

The first-line drugs for the therapy of CDI are metronidazole and vancomycin, however, epidemiology of metronidazole susceptibility in *C. difficile* is changing, and *C. difficile* strains resistant or with reduced susceptibility to metronidazole have been reported, constituting a challenge to its use. Since *nim* genes, commonly associated with this resistance in other anaerobes, have not been detected in these *C. difficile* strains, the mechanism responsible is still unknown.

To understand the mechanisms that might be involved with the above mentioned resistances, experiments will be done to induce a resistant behavior *in vitro*. Phenotypic analyses and molecular approaches will be performed to characterize strains with induced resistance and to detect the determinants and/or mechanisms involved.

Endosporation: Origin and Evolution of a Prokaryotic Cell Differentiation Program

ABECASIS, A.¹, ALVES, R.^{1,2}, SERRANO, M.², QUINTAIS, L.¹, FERNANDES, C.²,
ISIDRO, A.L.², PEREIRA, F.², VULTOS, T.², HENRIQUES, A.O.²,
PEREIRA-LEAL, J.B.¹

¹Computational Genomics Laboratory, Instituto Gulbenkian de Ciência, Oeiras Portugal, ²Microbial Development Laboratory, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal

Bacterial endospores are the most resistance cell type known to man, able to withstand extremes of physical and chemical injury, and time. They are also the infective particles in a variety of human diseases, as is the case for *Clostridium difficile* spores, and are emerging bio-terror agents. On the other hand, sporeformers are part of the normal intestinal flora, and are gaining relevance in biomedical and biotechnological applications. Endosporation is characterized by the morphogenesis of a specialized dormant and highly resistant endospore within a mother cell. The process involves a modified cell division, and the engulfment of the prespore compartment (the future spore) by the mother cell. The spore will later be released upon lysis of the mother cell, and will germinate once appropriate environmental cues are sensed. Little is known about the origins and evolution of the differentiation program that controls endosporation. Starting with the developmental program of as defined in the model organism *Bacillus subtilis* we have defined a minimal core for endosporation. We further show that endosporation was likely invented once, at the base of the Firmicutes phylum (c.a., 3 Billion years ago), and that it involved the invention of new genes and functions, as well as the co-option of ancestral, house keeping functions. Predictions related to conservancy of function of the main transcriptional and morphogenetic regulatory proteins are currently being tested. These studies will be illustrated by gene inactivation studies of the genes for the cell type-specific sigma factors σ^F , σ^E , σ^G and σ^K in *C. difficile*.

Other than offering new insights into the genetic orchestration of spore morphogenesis, our analysis also opens novel possibilities for spore identification and inactivation in the case of *C. difficile* and other pathogenic sporeformers.

***Clostridium pasteurianum*: an unfamiliar spoilage organism**

CARLO ROTTA

*Safety/ Environmental Assurance, Colworth Science Park, Unilever,
Sharnbrook (Bedfordshire), MK44 1LQ, UK*

One of the main goals of the food industry is to produce good quality food, avoiding contamination by and multiplication of bacteria that cause human disease or spoilage of the product. Often overlooked as an organism of concern, *Clostridium pasteurianum* is considered an issue because of food spoilage. *Clostridium pasteurianum* spores are able to resist mild heat treatment and are relatively tolerant to low pH, and germination and growth can occur, especially in canned acid foods. Through well-described metabolic processes, the organism can produce many by-products, like butyric acid, that make the food undesirable and unpalatable for the consumer.

This species of *Clostridium* is not so well characterised, even though it was detected and described more than 60 years ago. Previous studies describe the behaviour of this bacterium in food, detailing the effects of factors such as pH and temperature on growth, but more detailed molecular investigations to understand and explain physiological responses have not been undertaken so far. Of the few studies that have been carried out, there is one that shows the homology between two genes *gerA* and *gerB*, operons that are present in many *Bacillus* and *Clostridium* spp., suggesting a common receptor system.

Although the receptor operon seems to be comparable to other clostridia, the germination signals have not yet been determined and may be different. The primary aims of this research work will be to better understand the germination mechanism(s), characterizing the signals from a cellular point of view and trying to clone and sequence the germinant receptors and operons (also using the new cutting edge technique ClosTron) from a molecular perspective, checking homologies with other spore-forming bacteria.

C. pasteurianum strains proposed for this studied are ATCC 6013, which is the most studied strains in the literature and NRRL B-598, mainly used in research related to biofuels (the other important area which involve this species).

Regulation of Alcohologenesis in *Clostridium acetobutylicum*

THAO NGUYHEN and PHILIPPE SOUCAILLE

Metabolic Explorer, Biopole Clermont-Limagne, 63360 Saint-Beauzire, France

Clostridium acetobutylicum is a gram-positive, spore-forming, anaerobic bacterium capable of converting different sugars and polysaccharides to organic acids (acetate and butyrate) and solvents (acetone, butanol, and ethanol). Three different stable metabolic states are observed in chemostat cultures of *C. acetobutylicum* according to the pH and availability of NAD(P)H: i) an acidogenic state (production of acetic and butyric acids) when cells are grown at neutral pH on glucose; (1) ii) a solventogenic state (production of acetone, butanol, and ethanol) when cells are grown at a low pH on glucose (1); and iii) an alcohologenic state (formation of butanol and ethanol but not acetone) when cells are grown at neutral pH with high NAD(P)H availability (2, 3).

The objective of this work is to determine how alcohologenesis is regulated in *C. acetobutylicum*. Alcohol production during alcohologenesis specifically involves adhE2 (4). We will determine how adhE2 is regulated by: (i) identifying its promoter operator sequence; (ii) isolating the regulatory protein that binds to the isolated operator using magnetic bead technology; (iii) cloning of the regulatory gene using reverse genetics, and; assessing the physiological effect of its inactivation using the

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Iron Homeostasis and Oxidative Stress Response in the Obligately Anaerobic Bacterium *Clostridium acetobutylicum* ATCC 824

DELYANA VASILEVA AND HUBERT BAHL

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

Iron is an important nutrient which is indispensable for almost all bacteria. It is required for a great variety of cellular functions. Nonetheless, excess levels of iron could catalyze the Fenton reaction, which generates toxic levels of hydroxyl radicals that can damage DNA, proteins and lipids. Therefore, the intracellular iron concentrations must be tightly regulated to meet the metabolic needs of the cell.

A recent transcriptomic analysis of the O₂ stimulon in the gram-positive, obligate anaerobe *Clostridium acetobutylicum* ATCC 824 has revealed that several genes involved in the uptake and metabolism of iron are upregulated during oxidative stress.

Numerous studies indicate the function of the ferric uptake regulator (Fur) protein as a milestone in maintaining of the cellular iron homeostasis in bacteria as well as its role in oxidative stress response. Two genes encoding Fur-homologues, potentially involved in the regulation of intracellular levels of iron, have recently been identified in the genome of *Clostridium acetobutylicum*. Thus, it is of particular interest to us to investigate their physiological role in this bacterium.

Fitness Cost of Antibiotic Resistance in *Clostridium difficile*

FRANÇOIS WASELS

*Department of Infectious, Parasitic and Immune-mediated Diseases,
Istituto Superiore di Sanità, 299 Viale Regina Elena, Rome, Italy*

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 (CDI), as this pathogen can only colonise the gut if the normal intestinal microbiota is disturbed or absent. The resistance of *C. difficile* to antibiotics such as erythromycin or fluoroquinolones correlates with emergence of hyper-virulent strains and outbreaks.

Most antibiotics resistance mechanisms are associated with a fitness cost that is typically observed as a reduced growth rate, and modifications of survival and/or virulence capacities. The magnitude of this cost is the main biological parameter that influences the rate of development of resistance, the stability of the resistance and the rate at which the resistance might decrease if antibiotic use was reduced.

A better understanding of fitness costs and of its impact on the emergence and spread of resistant bacteria should allow us to make better quantitative predictions about the rate and trajectory of the evolution of resistance towards new and old drugs and, by interference, should also give us possibilities to prevent this evolution.

For this purpose, we will determine whether the acquisition of macrolides or fluoroquinolones resistance, through mutation or acquisition of exogenous genes, could have a biological cost in *C. difficile*. Fitness of the parent and mutants strains will be assessed by using non-competitive growth, pair-wise competitive growth and by the analysis of the expression of genes encoding for virulence factors.

Analysis of Metabolic Mutants of *Clostridium acetobutylicum*

MINYEONG YOO and PHILIPPE SOUCAILLE

Metabolic Explorer, Biopole Clermont-Limagne, 63360 Saint-Beauzire, France

The ability to form mixtures of organic acids and/or solvents represents a defining feature of *C. acetobutylicum*. An understanding of the regulation of solvent formation will be key for the exploitation of *C. acetobutylicum* for the production of chemicals and biofuels. Currently little is known of the molecular mechanisms involved in the induction of solventogenesis in non-pathogenic *C. acetobutylicum*.

Our aim is to: (i) carry differential transcriptomic and proteomic analysis of acidogenic chemostat culture versus solventogenic culture. (ii) associate the complete transcriptomic and proteomic analysis to metabolic flux analysis and (iii) based on these analyses, construct and characterise from a physiological point of view specific “metabolic mutants”. We envisage that these comparative transcriptomic, proteomic and metabolic studies coupled with the characterisation of mutants will help to identify the mechanism of induction of solventogenesis. This should also allow us to propose specific genetic modification for the metabolic engineering of *C. acetobutylicum* for the production of bulk chemicals and biofuels at high yield.

A Two-component System Negatively Regulates Botulinum Neurotoxin Expression

ZHEN ZHANG¹, HANNU KORKEALA¹, ELIAS DAHLSTEN¹, JOHN T. HEAP²,
NIGEL P. MINTON², MIIA LINDSTRÖM¹

¹*Department of Food Hygiene and Environmental Health, Centre of Excellence in Microbial Food Safety, Faculty of Veterinary Medicine, University of Helsinki, P. O. Box 66, 00014 University of Helsinki, Finland*

²*School of Molecular Medical Sciences, Centre for Biomolecular Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom*

Two-component signal transduction systems (TCSs) have been found to play an important regulatory role in virulence in many pathogenic bacteria. However, little is known about the role of TCSs in *Clostridium botulinum*. We identified a TCS involved in neurotoxin regulation in *C. botulinum* type A strain ATCC3502. Using the ClosTron mutagenesis system (Heap *et al.*, 2007), we inactivated *tcsR*, encoding a response regulator and *tcsK*, encoding a sensor histidine kinase. Inactivation of *tcsR* and *tcsK* resulted in significantly higher level of neurotoxin gene (*botA*) transcripts and neurotoxin production as measured with ELISA. Complementation of *tcsR* mutant cells with a plasmid (Heap *et al.* 2010) expressing *tcsKR* restored neurotoxin production to the wild-type level. Further experiments suggested that TcsKR also down-regulates the transcription of *botR*, the alternative sigma factor which activates *botA* transcription (Marvaud *et al.* 1998). Currently we are confirming the regulatory role of TcsKR by protein-DNA binding assays. To our knowledge, this is the first report on negative regulation of botulinum neurotoxin production and a role of TCS in *C. botulinum*. Profound understanding of the negative regulation of the neurotoxin production may provide tools to control the risk of botulism in foods.

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The Role of *adh E 2* and Ferredoxins in Solventogenic *Clostridium*

LIU ZIYONG

*Lehrstuhl für Mikrobiologie, Technische Universität München, Emil-Ramann-Str.4,
Freising, Germany*

As a promising biofuel producer, *Clostridium acetobutylicum* has a lot of complex changes which are not well understood in the life cycle. Regulation of solvents, gas and acids formation is embedded in a complex network, including sporulation, pH and redox control. What's more, there is a metabolism switch from acidogenic growth to solventogenic growth in batch fermentation of *C. acetobutylicum*. Now, clarify the metabolic mechanism of *C. acetobutylicum* is a hot research point.

The completion of *C. acetobutylicum* genome sequencing could pave the way for transcriptional profiling and thus allow comprehension of the coherent regulatory networks of solventogenesis and sporulation. We could obtain mutants using ClosTron which is an effective method for the directed inactivation of specific genes. Then, we could obtain overview of the transcript levels of the mutant and wild type. After comparative analysis, the important information could be obtained using MicroArray technique.

Five open reading frames (ORFs) are annotated as coding for putative ferredoxins in the *C. acetobutylicum*. They are CAC0075, CAC0105, CAC3527, CAC3621, and CAC0303. The *adhE2* gene is only expressed under the condition of a high NADH/NAD ratio (alcohologenesis). Previous studies have shown that these six genes are closely related to the solventogenesis in *C. acetobutylicum*. We choose these six genes to start our research. Two mutants have been obtained and we are carrying on the further studies.

LIST OF PARTICIPANTS

SURNAME	NAME	INSTITUTE	EMAIL ADDRESS
Aboobaker	Aziz	University of Nottingham	Aziz.Aboobaker@nottingham.ac.uk
Baban	Soza	University of Nottingham	mrkstmb@nottingham.ac.uk
Bahl	Hubert	University of Rostock	hubert.bahl@uni-rostock.de
Bollard	Niall	Institute of Public Health, Maribor	snapdragon@hush.com
Bullough	Per	University of Sheffield	p.bullough@sheffield.ac.uk
Burns	David	University of Nottingham	david.a.burns@nottingham.ac.uk
Butala	Matej	University of Ljubljana	matej.butala@bf.uni-lj.si
Cartman	Stephen	University of Nottingham	stephen.cartman@nottingham.ac.uk
Celik	Hamza	CNRS, Marseille	hcelik@ifr88.cnrs-mrs.fr
Cockayne	Alan	University of Nottingham	Alan.Cockayne@nottingham.ac.uk
Croux	Christian	INSA, France	croux@insa-toulouse.fr
Dahlsten	Elias	University of Helsinki	elias.dahlsten@helsinki.fi
Dao Thi	Minh-Uyen	CNRS, Marseille	mdaothi@ifr88.cnrs-mrs.fr
Dapa	Tanja	Novartis, Italy	tanja.dapa@novartis.com
de Philip	Pascale	CNRS, Marseille	dephilip@ifr88.cnrs-mrs.fr
Desvaux	Mickaël	INRA, Clermont	mickael.desvaux@clermont.inra.fr
Du	Ran	University of Nottingham	mxrd@nottingham.ac.uk
Ehsaan	Muhammad	University of Nottingham	mxme2@nottingham.ac.uk
Espejo	Ana	University of Rostock	ana.corredera@uni-rostock.de
Faulds-Pain	Alex	University of Nottingham	alexandra.pain-faulds@nottingham.ac.uk
Fit	Magda	University of Nottingham	mxmf3@nottingham.ac.uk
Guentner	Bernard	Fraunhofer Institute, Aachen	bernhard.guentner@ime.fraunhofer.de
Harwood	Colin	Newcastle University	colin.harwood@ncl.ac.uk
Heap	John	University of Nottingham	john.heap@nottingham.ac.uk
Hecker	Michael	Ernst-Moritz-Arndt University, Greifswald	hecker@biologie.uni-greifswald.de
Heeg	Daniela	University of Nottingham	mxrdh@nottingham.ac.uk
Henriques	Adriano	University of Lisbon	aoh@itqb.unl.pt
Henstra	Anne	University of Nottingham	Am.Henstra@nottingham.ac.uk
Holden	Matt	Sanger Institute, UK	mh3@sanger.ac.uk
Janssen	Holger	University of Rostock	holger.janssen@uni-rostock.de
Joshi	Nimitray	University of Nottingham	mxrnj1@nottingham.ac.uk
Kasendra	Magdalena	Novartis, Italy	magdalena.kasendra@novartis.com
Kelly	Michelle	University of Nottingham	Michelle.Kelly@nottingham.ac.uk
Kirk	David	University of Helsinki	david.kirk@helsinki.fi
Kontinen	Vesa	University of Helsinki	Vesa.kontinen@thl.fi
Kotte	Ann-Kathrin	University of Nottingham	mxakk@nottingham.ac.uk
Kovacs Simon	Andrea	University of Exeter	A.Kovacs-Simon@exeter.ac.uk
Kubiak	Ola	University of Nottingham	mxak2@nottingham.ac.uk
Kühne	Sarah	University of Nottingham	sarah.kuehne@nottingham.ac.uk
Lesiak	Justyna	Technical University Munich	j.zaremba.lesiak@gmail.com
Liebl	Wolfgang	Technical University Munich	wliebl@wzw.tum.de
Lindström	Miia	University of Helsinki	miia.lindstrom@helsinki.fi
Little	Gareth	University of Nottingham	mxgl1@nottingham.ac.uk
Loveleen	Tina	Cardiff University	JoshiLT@cardiff.ac.uk
Mastrangelo	Sarah	University of Nottingham	mxsm15@nottingham.ac.uk

Mastrantonio	Paola	Istituto Superiore di Sanità, Rome	paola.mastrantonio@iss.it
McClure	Peter	Unilever UK	Peter.McClure@unilever.com
McKee	Pete	University of Ulster	McKee-P@email.ulster.ac.uk
Meaney	Carolyn	Unilever, Sharnbrook	carolyn.meaney@unilever.com
Michell	Steve	University of Exeter	S.L.Michell@exeter.ac.uk
Minton	Nigel	University of Nottingham	nigel.minton@nottingham.ac.uk
Minton	Jacque	University of Nottingham	jacqueline.minton@nottingham.ac.uk
Moir	Anne	University of Sheffield	A.Moir@sheffield.ac.uk
Moura	Ines	Istituto Superiore di Sanità, Rome	ines.moura@iss.it
Mullany	Peter	Eastman Dental Institute, UCL	p.mullany@eastman.ucl.ac.uk
Ng	Rachel	University of Nottingham	mrzyn@exmail.nottingham.ac.uk
Pereira	Fátima	University of Lisbon	fpereira@itqb.unl.pt
Rotta	Carlo	Unilever, Sharnbrook	carlo.rotta@unilever.com
Rupnik	Maja	Institute of Public Health, Maribor	maja.rupnik@zzv-mb.si
Sanders	Jan-Willem	Unilever R&D, Vlaardingen	Jan-Willem.Sanders@unilever.com
Schwarz	Wolfgang	Technical University Munich	wschwarz@wzw.tum.de
Schwarz	Katrin	University of Nottingham	Katrin.Schwarz@nottingham.ac.uk
Serruto	Davide	Novartis, Italy	davide.serruto@novartis.com
Sheng	Lili	University of Nottingham	mrxls6@nottingham.ac.uk
Shirvan	Ali Nazari	University of Glasgow	a_nazari5@yahoo.com
Soriani	Marco	Novartis, Italy	marco.soriani@novartis.com
Soucaille	Philippe	Metabolic Explorer (INSA), France	psoucaille@metabolic-explorer.com
Tardif	Chantal	CNRS, Marseille	tardif@ibsm.cnrs-mrs.fr
Thao	Nguyen	Metabolic Explorer (INSA), France	Rubik85@gmail.com
Thauer	Rolf	Max Planck Institute, Marburg	thauer@mpi-marburg.mpg.de
Titball	Rick	University of Exeter	R.W.Titball@exeter.ac.uk
Vasileva	Delyana	University of Rostock	delyana.vasileva@uni-rostock.de
Verstraete	Isabelle	Institut Pasteur, Paris	isabelle.martin-verstraete@pasteur.fr
Walker	David	University of Nottingham	mrxdjw@exmail.nottingham.ac.uk
Walter	Beata	Institute of Public Health, Maribor	b.m.walter@hotmail.com
Wang	Hengzheng	University of Nottingham	mrxhw2@nottingham.ac.uk
Wasels	Francois	Istituto Superiore di Sanità, Rome	francois.wasels@iss.it
Wilson	Benjamin	University of Nottingham	mrxbjw@nottingham.ac.uk
Winzer	Klaus	University of Nottingham	Klaus.Winzer@nottingham.ac.uk
Yoo	Minyeong	Metabolic Explorer (INSA), France	minyeongyoo@gmail.com
Young	Mike	Aberystwyth University	miy@aber.ac.uk
Zhang	Zhen	University of Helsinki	zhen.zhang@helsinki.fi
Zhang	Ying	University of Nottingham	Ying.Zhang@nottingham.ac.uk
Ziyong	Liu	Technical University Munich	liuziyong1010@126.com



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