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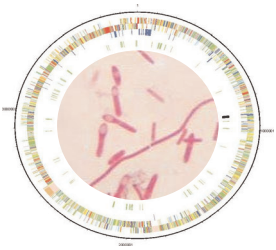
C L  S T P A T H 2006

Programme and Abstracts

5th International Meeting on the Molecular Biology and Pathogenesis of the Clostridia.

Nottingham UK

June 21-25, 2006

www.clostridia.net/clostpath2006

Secretariat

Jacque Minton
Email: Jacqueline.minton@nottingham.ac.uk
Tel. +44 (0)115 84 68003
Fax. +44 (0)115 84 84 67951

Diane Mitchell
E-mail: Diane.Mitchell@nottingham.ac.uk
Tel. +44 (0)115 8467950
Fax. +44 (0)115 84 84 67951

Institute of Infection, Immunity and Inflammation
School of Molecular Medical Sciences
Centre for Biomolecular Science
University of Nottingham
University Park
Nottingham
NG7 2RD
UNITED KINGDOM

Dear Colleagues

It is with great pleasure that we welcome you to the 5th International Meeting on the Molecular Genetics and Pathogenesis of Clostridia.

This is by far the largest gathering to have taken place in the ClostPath conference series to date, and reflects the growing recognition of the importance of the genus *Clostridium.* In attendance are many leading experts from around the world and, thanks to our principal sponsors, a high proportion of young researchers. Encouragement of the latter is essential if we are to enlarge the clostridial community to meet the challenges ahead.

The conference venue is the University of Nottingham Campus. Set in extensive woodland, parks and playing fields, the 330-acre University Park is one of the largest and most attractive campuses in the UK. We hope this setting will provide the perfect environment for stimulating interactions and discussions between established and new researchers alike.

The Organising Committee

ORGANISING COMMITTEE

Nigel P Minton (*University of Nottingham, UK*)
Neil Fairweather (*Imperial College London, UK*)
Maja Rupnik (*Institute for Public Health, Maribor, Slovenia*)
Miia Lindström (*University of Helsinki, Finland*)
Rick Titball (*DSTL, Porton Down, UK*)
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Thanks to Fischer Scientific (UK), Operon Biotechnologies GmbH (Germany) and Qiagen Ltd (UK) for donations to Conference materials

CLOSTPATH 2006 PROGRAMME

Wednesday, June 21st 2006

15:00-18:00	**Registration**	*Law and Social Sciences Building, B-Floor Atrium*
18.00-19:00	**Keynote Address** Bruce McClane (University of Pittsburgh School of Medicine, USA) *Clostridium perfringens* Enterotoxin: Foe and Friend?	
19:30-24:00	**Welcome Reception**	*University Staff Club*

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Thursday, June 22nd 2006

07:00-08:30	**Breakfast**	*Hugh Stewart & Cripps Hall*

08:30-10:30 Session I: Epidemiology and Diagnosis (Jon Brazier)

08.30-09.00	Dale Gerding (VA Hospital, USA) Clinical aspects of *Clostridium difficile*
09.00-09.30	Maja Rupnik (Institute for Public Health, Maribor, Slovenia) Epidemiology of *Clostridium difficile*
09.30-09.50	Jim McLauchlin (HPA Centre for Infections, London, UK) Wound botulism in injecting drug users in the UK
09.50-10.10	Oliver Hasselmeyer (University of Mainz, Germany) Use of the CdISt1 IStron for typing of *Clostridium difficile o*utbreaks
10.10-10.30	Denise Drudy (University College Dublin, Ireland) Fluoroquinolone resistance in toxin A negative toxin B positive *Clostridium difficile a*ssociated with a novel mutation in *gyrB*

10:30-11:00	**Coffee break**	*Law & Social Sciences Atrium*

11:00-13:00	**Session II: Entertoxins & Membrane Active Toxins - Part I (Maja Rupnik)**	
11.00-11.30	Ingo Just (Hannover Medical School, Germany) *Clostridium difficile* toxin A mode of action	
11.30-12.00	Jim Ballard (University of Oklahoma, USA) Decreased Akt-signaling and related events during early stages of cellular intoxication by *Clostridium sordellii* TcsL	
12.00-12.30	Klaus Aktories (University of Freiburg, Germany) The crystal structure of *Clostridium difficile* toxin B	
12.30-13.00	Michel Popoff and Blandine Geny (Institute Pasteur, France) *In vitro and in vivo* effects of *Clostridium sordellii* lethal toxin on intercellular junctions	
13:00-14:30	**Lunch**	*Hugh Stewart Hall*
14.30-16.30	**Session III: Entertoxins & Membrane Active Toxins - Part II (Julian Rood)**	
14.30-15.00	Ajit Basak (Birkbeck College, UK) Structure of *C.perfringens* epsilon toxin	
15.00-15.30	Rick Titball (DSTL, UK) Vaccines against *C. perfringens* alpha-toxin	
15.30-15.50	James Smedley (University of Pittsburgh, USA) Investigating post-binding steps in the mechanism of action of *Clostridium perfringens* enterotoxin	
15.50-16.10	Mariano Fernandez-Miyakawa (University of California, Davis, USA) *Clostridium perfringens* epsilon toxin increases small intestine permeability	
16.10-16.30	Johannes Huelsenbeck (Hannover Medical School, Germany) Upregulation of *rhoB* by clostridial cytotoxins	

16:30-18:00	**Posters I**	*B-62, Law & Social Sciences*
20:00-22.00	**Dinner**	*Cripps Hall*

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Friday, June 23rd 2006

07:00-08:30	**Breakfast**	*Hugh Stewart & Cripps Hall*

08:30-10:30 Session IV: Neurotoxins (Ornella Rossetto)

08.30-09.00	Eric Johnson (University of Wisconsin, USA) *Clostridium botulinum* neurotoxin subtypes and their biological significance
09.00-09.30	Thomas Binz (University of Hannover, Germany) The sugar binding domain of clostridial neurotoxins
09.30-09.50	Miia Lindstrom (University of Helsinki, Finland) Genomic comparison of group I (proteolytic) *Clostridium botulinum* Type B
09.50-10.10	Andreas Rummel (Hannover Medical School, Germany) Interaction with one ganglioside and one protein receptor mediates the neurotoxicity of botulinum neurotoxins
10.10-10.30	Frank Lebeda (USAMRIID, USA.) Botulinum neurotoxin: a toxicokinetic study

10:30-11:00	**Coffee break**	*Law & Social Sciences Atrium*

11:00-13:00 Session V: Treatment & Exploitation (Glen Songer)

11:00-11:30	Jan Theys (University of Maastricht, Holland) Clostridial spores and cancer therapy
11:30-12:00	Neil Green (Vanderbilt University, USA) Inhibitors of botulinum neurotoxin

12.00-12.20	Yue Chen (University of Pittsburgh, USA) Sequence specific mutagenesis by Targetron: disrupt endogenous genes and introduce foreign gene into *Clostridium perfringens* chromosome	
12.20-12.40	Ian Cheong (Howard Hughes Medical Institute, USA) *Clostridium novyi* can generate a potent therapeutic immune response against experimental tumours	
12.40-13.00	Kristin Nagaro (Hines V.A. Hospital, Illinois, USA.) Non-Toxic *Clostridium difficile* (CD) protects hamsters against historic and epidemic toxigenic "BI" strains	
13:00-14:30	**Lunch**	*Hugh Stewart Hall*
14:30-16:30	**Session VI: Veterinary Disease (Marietta Flores-Díaz)**	
14.30-15.00	Glenn Songer (University of Arizona, USA) Clostridial enteritis in domestic animal species	
15.00-15.30	Francisco Uzal (University of California, USA) A mouse model for *Clostridium perfringens* type D infection	
15.30-15.50	Luis Arroyo (University of Guelph, Canda) *Clostridium difficile*: potential causes of duodenitis proximal jejunitis in horses	
15.50-16.10	Filip Van Immerseel (Ghent University, Belgium) Clinical isolates of *C. perfringens* in poultry are not superior alpha toxin producers *in vitro*	
16.10-16.30	Anthony Keyburn (University of Monash, Australia) The alpha toxin of *Clostridium perfringens* is not an essential virulence factor in necrotic enteritis in chickens	
16:30-18:00	**Posters II**	*B-62, Law & Social Sciences*
20:00-22.00	**Dinner**	*Cripps Hall*

Saturday, June 24th 2006

07:00-08:30 **Breakfast** *Hugh Stewart & Cripps Hall*

08:30-10:30 **Session VII: Host-Pathogen Interactions (Paola Mastrantonio)**

08.30-09.00 Steve Melville
(VPI, USA) The interaction of *C. perfringens* toxins with macrophages

09.00-09.30 Marietta Flores-Díaz
(University of Costa Rica, Costa Rica) The cytotoxic effects of *Clostridium perfringens* α-toxin is mediated by endogenous mediators

09.30-09.50 Clair Janoir
(Université Paris-Sud, France) The CWP84 surface associated protein of *Clostridium difficile* is a cysteine protease with degrading activity against
extracellular matrix proteins

09.50-10.10 Elaine Hamm
(University of Oklahoma, USA.) Characterisation of the systemic effects of *Clostridium difficile* TcdB using developing Zebrafish embryos

10.10-10.30 Derek Fisher
(University of Pittsburgh School of Medicine, U.S.A.) The role of toxins from *Clostridium perfringens* type C in the mouse intravenous injection model

10:30-11:00 **Coffee break** *Law and Social Sciences Building Atrium*

11:00-13:00 **Session VIII: Genetics and Physiology (Nigel Minton)**

11.00-11.30 Mahfuz Sarker
(Oregon State University, USA) *Clostridium perfringens* sporulation

11.30-12.00 Trudi Bannam
(Monash University, Australia) The mechanism of conjugation in *Clostridium perfringens*

12.00-12.20	Jennifer O'Connor (Monash University, Australia) Construction and transcriptional analysis of *Clostridium difficile r*esponse regulator mutants	
12.20-12.40	Hubert Bahl (University of Rostock, Germany) Analysis of proteins involved in the oxidative stress response of *Clostridium acetobutylicum*	
12.40-13.00	Kazauki Miyamoto (Wakayama Medical University, Japan) Sequencing and diversity analysis of the enterotoxin-encoding plasmids in *Clostridium perfringens* type a nonfoodborne human gastrointestinal disease isolates	
13:00-14:30	**Lunch**	*Hugh Stewart Hall*
14:30-16:30	**Session IX: Genomics, proteomics & transcriptomics (Peter Mullany)**	
14.30-15.00	Mike Peck (IFR Norwich, UK) Exploitation of the *Clostridium botulinum* genome sequence	
15.00-15.30	Richard Stabler (LSTMH, London, UK) Comparative phylogenomics of *Clostridium difficile* reveals clade specificity and microevolution of hypervirulent strains	
15.30-15.50	Yoshihiko Sakaguchi (Okayama University, Japan) The Genome sequence of *Clostridium botulinum* Type C neurotoxin-converting phage and the molecular mechanisms of unstable lysogeny	
15.50-16.10	Katrin Schwarz (University of Rostock, Germany) The intra and extracellular proteome of *Clostridium acetobutylicum* under phosphate limitation	
16.10-16.30	Kaori Ohtani (Kanazawa University, Japan) Biological signaling to gene expression in *Clostridium perfringens*	
16:30-17:00	**Coffee break**	*Law & Social Sciences Atrium*

17:00-19:00 **Session X: Regulation of Virulence Genes (Anne Collignon)**

17.00-17.30 Bruno Dupuy
(Institute Pasteur, France) Regulation of clostridial toxins by alternative sigma factors

17.30-18.00 Akinobu Okabe
(University of Kagawa, Japan) DNA curvature and gene regulation in *Clostridium perfringens*

18.00-18.20 Susana Matamouros
(Institute Pasteur, France) Toxin synthesis regulation in *Clostridium difficile*

18.20-18.40 Sean Dineen
(Tufts University School of Medicine, USA) Regulation of *Clostridium difficile* toxin synthesis

18.40-19.00 Farida Siddiqui
(Hines V.A. Hospital, Illinois, USA.) The *tcdC* gene of *Clostridium difficile* variants including the epidemic BI strain contains stop codons and deletions.

20.00-24:00 **Medieval Banquet** *Nottingham - Tales of Robin Hood*

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Sunday, June 25th 2006

07:00-08:30 **Breakfast** *Hugh Stewart & Cripps Hall*

DEPART

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SPECIAL PUBLIC SESSION

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Sunday 25th June 2006

Lecture theatre B-63, Law and Social Sciences Building

University of Nottingham

10.00 am - 12.00 pm

Refreshments will be provided

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Clostridia: The Good, the Bad and the Beautiful

10.00-10.05	Professor Nigel P Minton, (University of Nottingham) Introduction
10.05-10.30	Dr Wilf Mitchell (University of Heriot-Watt, Edinburgh) Solvent production - not to be sniffed at!
10.30-10.55	Dr Adam Roberts (University College, London) *Clostridium difficile:* a new superbug?
10.55-11.20	Dr Keith Foster (Syntaxin Ltd., Salisbury) Botulinum toxin: more than just a pretty face!

ORAL PRESENTATIONS

B-63

CLOSTRIDIUM PERFRINGENS ENTEROTOXIN: FOE AND FRIEND?

B.A. McClane

Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261 USA

Clostridium perfringens enterotoxin (CPE) causes the gastrointestinal (GI) symptoms of *C. perfringens* type A food poisoning and also contributes to some cases of nonfoodborne human GI disease. CPE acts by binding to claudins to form a series of complexes, including a pore that allows Ca^{2+} influx to trigger cell death by either oncosis or apoptosis. CPE can also induce internalization of tight junction proteins, possibly affecting paracellular permeability. CPE causes these effects by segregating binding and cytotoxic activities to its C- and N-terminal halves, respectively. Intestinal proteases can activate CPE by removing extreme N-terminal sequences. The enterotoxin gene (*cpe*) is typically chromosomal in food poisoning isolates, where it may be part of an integrated transposon. Food poisoning isolates are exceptionally heat and cold resistant, which favors their food poisoning potential. In nonfoodborne human GI disease isolates, the *cpe* gene is present on plasmids. Unlike food poisoning isolates, nonfoodborne GI disease isolates typically express both CPE and beta2 toxin, which may serve as an accessory enterotoxin. Despite its disease association, CPE is now being explored as a therapeutic agent, particularly as a potential antitumor agent. This interest is based upon observations that many pancreatic, breast, prostate and ovarian tumors overproduce certain claudins that can function as CPE receptors, rendering them highly sensitive to CPE-induced cytotoxicity.

CLINICAL ASPECTS OF CLOSTRIDIUM DIFFICILE DISEASE

D. N. Gerding[1,2]

[1]Hines Veterans Affairs Hospital, Hines, IL 60141 USA, and [2]Loyola University Chicago Stritch School of Medicine, Maywood, IL USA

The clinical presentation of *Clostridium difficile*-associated disease (CDAD) has changed dramatically since the last ClosPath meeting. Rates of CDAD have increased markedly in US, Canadian, and many EU hospitals in association with identification of a newly epidemic toxinotype III strain of *C. difficile* identified as REA group BI, pulse field type NAP1, and PCR Ribotype 027. Mortality and morbidity (particularly colectomy, the need to surgically resect the colon to save the life of the patient) have both increased to levels never previously seen. Directly attributed mortality in Quebec hospitals was 6.9%, more than 4 times higher than previously observed. Incidence of CDAD in Quebec hospitals increased fourfold to 22.5 cases per 1000 hospital admissions. Patients frequently present with fulminant CDAD which is both sudden in onset and very severe in terms of symptoms. Shock, sepsis, ileus (gut paralysis), toxic megacolon, and colonic perforation are common complications. Management is exceedingly difficult because of problems of delivery of antibiotics to the colon and systemic toxicity. Colectomy may be the only intervention that will save the life of the patient. The elderly in hospitals have by far the highest risk of CDAD and the highest mortality, and those in the 7^{th}, 8^{th}, and 9^{th} decades are progressively at higher risk. In addition, recurrence of disease is increasing to rates of over 30%. Efficacy of metronidazole may not be as high as in the past resulting in greater treatment with oral vancomycin. In addition, anecdotal reports suggest that there may be increases in the number of community-acquired CDAD cases, particularly among younger people and pregnant women. New risk factors may be present such as use of proton pump inhibitor medications, however, these observations are not consistent. CDAD is clinically more common and more dangerous than ever.

EPIDEMIOLOGY OF VARIANT *Clostridium difficile* STRAINS

M. Rupnik[1]

[1]Institute for Public Health Maribor and Medical Faculty, University of Maribor; Maribor, Slovenia

Clostridium difficile produces at least three toxins, toxin A (enterotoxin, TcdA), toxin B (cytotoxin, TcdB) and binary toxin CDT. Until early 90-ties the majority of clinically important strains produced TcdA and TcdB. Therefore such A^+B^+ strains are considered as ordinary ones, while variant *C. difficile* strains include strains with different pattern of produced toxins (toxin-variant strains) and/or strains with changes in the PaLoc region encoding TcdA and TcdB (variant toxinotypes).

Among toxin-variant strains only toxin B-positive forms ($AflB^+$) were the first to be recognized. Despite lacking the production of one of the toxins, they were still highly pathogenic and were associated with several outbreaks. Only recently the long expected only toxin A-positive strains (A^+Bfl) seem to emerged. The production of binary toxin CDT can be associated with any combination of TcdA and TcdB production ($A^+B^+CDT^+$, $AflB^+CDT^+$, $A^+BflCDT^+$) and is also found in a low percentage of TcdA and TcdB negative strains ($AflBflCDT^+$). Binary toxin positive strains used to be rare among human isolates (less than 5%) but prevalent in strains isolated from animals (from 40 to 100%). However, the prevalence in human population is rising and is often associated with severe or with non-hospital infection.

Almost all of the toxin-variant strains as well as some of the ordinary A^+B^+ strains can have variant forms of *tcdA* and *tcdB* toxin genes and could be grouped into 24 different toxinotypes (from I to XXIV). Distribution of variant toxinotypes was studied within large *C. difficile* collections, in single hospitals during longer time interval and in studies including several hospitals within shorter time interval. Results suggest that prevalence of different variant toxinotypes can vary between different geographic regions and between hospitals. Toxinotype VIII and recently emerged subtype of toxinotype III are two toxinotypes that were so far found in large outbreaks.

WOUND BOTULISM IN INJECTING DRUG USERS IN THE UK

J McLauchlin, K Grant, V Hope, S Huntington, V Mithani, O Mpamugo, F Ncube.

HPA Centre for Infections, 61 Colindale Ave, London, NW9 5HT, UK

Clostridial infections amongst injecting drug users (IDUs) first came to prominence in the UK in 2000 when there was a large outbreak (108 cases including 44 deaths) probably due to *Clostridium novyi* infections. Clusters of *Clostridium tetani* and *Clostridium histolyticum* infections amongst IDUs also occurred in 2003 and 2004 respectively. The infections coincided with the recognition of wound botulism for the first time in the UK. Between 2000 and 2005, 120 cases of suspect wound botulism amongst IDUs were reported in the UK and Republic of Ireland with 5 cases in 2000, 4 in 2001, 20 in 2002, 15 in 2003, 40 in 2004 and 32 in 2005. 98 (82%) of all the cases occurred in England. For those whom detailed information was available all reported injecting heroine, 64% reported also injecting methadone. All used either vitamin C or citric acid for dissolving the drug. The duration of injecting ranged from 1 year to 30 years with an average of 10 years. All reported injection into skin (skin popping) or muscle (muscle popping) and that in the previous month they had noticed swelling and tenderness of an area of skin where they inject. The majority (75%) required ventilation during their hospital admission. Amongst all of the 120 cases, 45 were laboratory confirmed by either the detection of neurotoxin in serum or wound material, or by the isolation of *C.botulinum* from infected wounds. Amongst the laboratory confirmed cases, 39 were due to *C.botulinum* type A, 4 to type B and two to both types A and B. Wound botulism amongst IDUs is now the most common presentation of this disease in the UK, and despite drug using communities throughout the UK (as well as in other European countries) is largely confined to England.

USE OF THE CdI*St*1 IStron FOR TYPING OF *Clostridium difficile* OUTBREAKS

O. Hasselmayer, C. von Tilly, C. Nitsche, J. Reineke and Chr. von Eichel-Streiber

Institut f r Medizinische Mikrobiology und Hygiene, Johannes Gutenberg-Universit t Mainz, 55131 Mainz, Germany

The genetic element Cd*ISt*1 was originally characterized as an insertion into the *tcdA* gene of the clinical isolate *Clostridium difficile* C34. Cd*ISt*1 combines structural and functional properties of group I introns and IS*605* homologous insertion elements (V. Braun et al., 2000, Mol. Microbiol., 36(6): 1447-1459). Analysing *C. difficile* isolates from different sources (clinics, countries, continents) we identified four types of Cd*ISt*1 called type 0 to type III. Furthermore, every *C. difficile* strain without exception harboured at least one copy of Cd*ISt*1. In comparison search for the element in other bacterial species remained unsuccessful, indicating that Cd*ISt*1 is specifically found only in *C. difficile*. Comparing *C. difficile* isolates, Cd*ISt*1 copies were exclusively located downstream of a sequence TTGAT within open reading frames. Analysis of 26 Cd*ISt*1 integration sites within genomes of seven different *C. difficile* isolates showed considerable variation of sites and copy numbers between different strains. To prove our assumption that such variations might be useful for differentiation and typing of isolates, we performed Southern Analysis of genomic DNA of several strains restricted with HindIII and hybridised to a Cd*ISt*1-specific probe. While analysis of seven strains following serial passages over a two years period showed no changes of their particular Cd*ISt*1 integration profile at all, strains of different origin again displayed well defined differences of the number of CdIST1 integrations and their genomic location. We further used the approach to classify strains from a local outbreak in a mid size hospital of the northern part of Germany. The analysis of 63 probes yielded 18 clinical specimens positive for *C. difficile* (PCR for toxin genes, PCR for Cd*ISt*1, growth on agar). Southern Analysis of their genomic DNA (restricted with HindIII to completion) probed with Cd*ISt*1 we identified 16 identical and only two different hybridisation pattern. We took this as a proof of the local outbreak and here propose the element Cd*ISt*1 as a straight forward and efficient genetic marker for typing of *C. difficile* strains especially in such outbreaks situations.

FLUOROQUINOLONE RESISTANCE IN TOXIN A NEGATIVE TOXIN B POSITIVE *CLOSTRIDIUM DIFFICILE* ASSOCIATED WITH A NOVEL MUTATION IN *GYRB*

Denise Drudy [1], Teresa Quinn [1], Rebecca O Mahony[1], Lorraine Kyne [2] Fanning S amus [2]

[1] Centre for Food Safety; School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Dublin 4, Ireland.[2] Department of Medicine for the Older Person, Mater Misericordiae University Hospital Dublin 7

Clostridium difficile is a major cause of infectious diarrhoea in hospitalised patients. We have previously described an outbreak of Toxin A negative B positive (A^-B^+) *C. difficile* diarrhoea where *C. difficile* isolates demonstrated universal high-level resistance to fluoroquinolones. The aim of this study was to determine the mechanism of fluoroquinolone resistance in these A^-B^+ *C. difficile* isolates.

C. difficile isolates (n=90) were cultured on CCFA agar. 16S-23S PCR ribotyping was used to determine genomic relatedness between *C. difficile* isolates. Antimicrobial *ibitors reserpine (20 mg/ ml), PAbN (20 mg/ml) and verapamil 100 (mg/ml). PCR was used to amplify regions from the quinolone-resistance-determining-region (QRDR) of gyrA* and *gyrB* genes and the PCR amplicons were sequenced.

PCR ribotyping profiles identified one major cluster of A^-B^+ *C. difficile* which were universally resistant to all fluoroquinolones (Ofloxacin Ciprofloxacin Levofloxacin, Moxifloxacin and Gatifloxacin, MIC >32 mg/ml respectively). Non-clonal isolates were sensitive to Levofloxacin, Moxifloxacin and Gatifloxacin; MIC 4, 0.3 and 0.2 mg/me *agar. There were no amino acid substitutions found in the QRDR of gyrA*

CLOSTRIDIUM DIFFICILE TOXINS A AND B: NEW INSIGHTS INTO THEIR MODE OF ACTION

I Just, H Isenbeck, H Genth, R Gerhard

Dept. of Toxicology, Hannover Medical School, 30625 Hannover, Germany

Toxin A (TcdA) and B (TcdB) from *Clostridium difficile* are homologous single-chained exotoxins which act intracellularly onto small GTPases of the Rho subfamily. Based on their three functional domains they get access to their target cells by receptor-mediated endocytosis and escape from acidified endosomes. Only the proteolytically processed catalytic domain reaches the cytoplasm to catalyse mono-glucosylation of Rho subfamily GTPases. The glucose moiety resides at a pivotal threonine of the effector region of the Rho GTPase and alters the GTPase functions: The GTP-hydrolysing and the spatial cycling of Rho is blocked; functionally most important is the inhibition of the effector protein coupling resulting in an arrest of Rho downstream signalling.

Although the target GTPases (RhoA/B/C, Rac, Cdc42, RhoG and TC10) are inactivated by glucosylation, one toxin target — RhoB - is strongly up-regulated and in fact activated. Increase in RhoB level is a *de novo* synthesis but not stabilisation of RhoB protein. The newly formed RhoB is at least partially activated and thus, signalling competent as shown by pull-down experiments. Up-regulation clearly depends on the transferase activity of the toxins as enzyme-deficient TcdA or prevention of cell entry of TcdA/TcdB by bafilomycin completely have no effect on RhoB. Although glucosylation of Rho GTPases results in reorganisation of the actin cytoskeleton this reorganisation is not the basis of RhoB upregulation; direct depolymerisation of the actin cytoskeleton by latrunculin or C2 toxin is without effect on RhoB. RhoB is involved in vesicle trafficking and regulation of apoptosis.These cellular functions may be altered by *Clostridium difficile* toxins.

In sum, toxin-catalysed glucosylation of Rho GTPases results in direct inhibition of Rho downstream signalling; the mechanistically not yet understood up-regulation and activation of RhoB is a hallmark of intracellular activity of TcdA/TcdB and may contribute to the pro-inflammatory effects in the toxin-triggered pseudomembranous colitis.

DECREASED AKT-SIGNALING AND RELATED EVENTS DURING EARLY STAGES OF CELLULAR INTOXICATION BY *CLOSTRIDIUM SORDELLII* TCSL

Jimmy D. Ballard* and Daniel E. Voth
Department of Microbiology and Immunology, The University of Oklahoma Health Sciences Center, Oklahoma City, OK

Clostridium sordellii lethal toxin (TcsL) is a bacterial glucosyltransferase that inactivates the small GTPases Ras, Rac, Rap, Ral, and Cdc42, and induces apoptosis in mammalian cells. In late stages of TcsL intoxication, caspases are activated and cells exhibit classic signs of apoptosis. However, the early stage events, occurring between GPTase inactivation and induction of apoptosis remain poorly understood. In the current study, immediate downstream effects of Ras inactivation by TcsL were investigated by following changes in events related to signaling through Akt, a serine/threonine kinase involved in multiple cell survival pathways. Within one hour following treatment with TcsL, a rapid decline in the levels of phosphorylated (activated) Akt was observed, suggesting an inhibition of the pro-survival response known to be modulated by this kinase. Enzymatically-inactive TcsL did not alter the levels of phosphorylated Akt, indicating that the toxin s glucosyltransferase activity is required for modulating this pathway. Additionally, *Clostridium difficile* TcdB, a closely related glucosyltransferase, did not affect Akt levels, suggesting a TcsL-specific mechanism of action. Downstream of Akt inactivation, dephosphorylation of GSK-3b was observed, along with a decline in total GSK-3b, both of which are pro-apoptotic conditions. Interestingly, treatment with wortmannin reduced Akt phosphorylation, but did not alter levels of GSK-3b, suggesting other processes may also contribute to loss of GSK-3b following intoxication with TcsL. This temporal analysis of the early events in TcsL intoxication indicate a series of changes

STRUCTURE AND FUNCTION OF THE CATALYTIC DOMAIN OF CLOSTRIDIAL GLUCOSYLATING CYTOTOXINS

K. Aktories

Institut f r Experimentelle und Klinische Pharmakologie und Toxikologie der Universit t Freiburg, D79104 Freiburg, Germany

Clostridium difficile toxins A and B are the causative agents of the antibiotic-associated diarrhoea and the pseudomembranous colitis. Both toxins glucosylate and inactivate Rho GTPases, which are molecular switches involved in a large array of cellular processes including regulation of the cytoskeleton, cell adhesion and migration, gene transcription, cell cycle progression and transformation. Recently, the crystal structure of the catalytic domain of *C. difficile* toxin B has been solved (Reinert et al., JMB 351, 973 (2005)). These studies showed that the catalytic core covers 234 residues and shares a folding with members of the family of type A glycosyltransferases (GT-A family). In addition to the catalytic core, the enzyme domain of toxin B possesses 309 amino acid residues of mainly helical structure, which flank the catalytic centre. Because the crystal structure of the catalytic domain of toxin B contains the divalent cation Mn^{2+} and the hydrolyzed cosubstrate UDP and glucose, insights into the catalytic mechanism of the enzyme are possible. The structure suggests an S_{Ni}-like reaction of glucosylation, which is characterized by retention of the -anomeric configuration of the glucose. Crucial functions of recently identified essential amino acid residues e.g., Asp286, Asp288 and Trp102, can be explained by their role in Mn^{2+} coordination and interaction with the uracile ring of UDP-glucose, respectively. Deduced from the crystal structure, it was possible to change the co-substrate specificity of toxin B by site-directed mutagenesis from UDP-glucose to UDP-N-acetylglucosamine.
The data indicate that elucidation of the 3D-structure of toxin B allows novel insights into structure-function relationships of clostridial glucosylating cytotoxins.

IN VITRO AND *IN VIVO* EFFECTS OF *CLOSTRIDIUM SORDELLII* LETHAL TOXIN ON INTERCELLULAR JUNCTIONS

Catherine BOEHM, Maryse GIBERT, Blandine GENY, Michel R. POPOFF,
Unit_ des Bact_ries ana_robies et Toxines, Institut Pasteur, 28 rue du Dr Roux, 75724, Paris cedex 15, France.

C. sordellii lethal toxin (LT) is a glucosyltransferase which inactivates small GTPases from the Rho and Ras families. We have studied I/ the respective effects of two variants, LT82 and LT9048, which interact with different sets of small GTPases, on the integrity of epithelial cell barrier using polarized MCCD and II/ how intraperitoneally-injected LT82 induced the death in mouse.

I/ Both toxins modified the paracellular permeability concomitantly with a disorganization of basolateral actin filaments, without modifying apical actin. LTs mainly altered adherens junctions by removing E-cadherin-catenin complexes from the membrane to the cytosol, whereas tight junctions showed no or only limited perturbations. Rac, a common substrate of both LTs, might play a central role in LT-dependent adherens junction alteration. We showed that adherens junction perturbation induced by LTs neither results from a direct effect of toxins on adherens junction proteins nor from an actin-independent Rac pathway, but rather from a Rac-dependent disorganization of basolateral actin cytoskeleton.

II/ We observed that after i.p. injection, LT82 induced massive extravasation of blood fluid. Consequently, the increase in vascular permeability generated profound modifications such as animal dehydration, increase in hematocrit, hypoxia as assessed by the increase in serum erythropoietin and finally a cardio-respiratory distress. Increase in vascular permeability appeared to essentially result from modifications of lung endothelial cells as evidenced by electro-microscopical studies. No inflammation process appeared to be responsible for animal death. As the main effect of the LT82 known so far, is the glucosylation and, thus, the inactivation of small GTPases, in particular Rac involved in the actin polymerization, we concluded that when the toxin is i. p. injected the most susceptible cells for LT82 appear to be the lung endothelial cells.

STRUCTURES OF PHOSPHOLIPASE CS AND THEIR FUNCTIONAL MUTANT.

A. K. Basak[1] and R. W. Titball[2].

[1]School of Crystallography, Birkbeck College, London WC1E 7HX,
[2]DSTL, Porton Down, Salisbury, Wiltshire SP4 0JQ, UK.

The genus Clostridium comprises several species of gram-positive, anaerobic, spore forming rod-shaped bacteria responsible for a wide variety of infections in humans and in their animals, usually by virtue of a myriad of protein exotoxins that they secrete. Of these, the a-toxin (phospholipase C) is the major virulence determinant in gas gangrene and has also been implicated in a number of other diseases of man and animals. The toxin is a Zn^{+2} dependent, Ca^{+2} activated phospholipase C (PLC), is haemolytic and able to interact with membrane-packed phospholipids. The ability to interact with eukaryotic cell membranes distinguishes the a-toxin from related enzymes, such as *C. bifermentans, C.barati, C. absonum, L. monocytogenes* and *B. cereus* PLC. We have determined a number of crystal structures of this enzyme from different Clostridial strains and sources have showed the structure is composed of two, an a-helical (N-terminal) and a b-sandwich (C-terminal) domains. Site directed mutagenesis study revealed that the substitution of a single residue, Thr74 with Ile (T74I), resulted in the loss of haemolytic, phospholipase C and the sphingomyelinase activities. Recently we have determined the crystal structure of T74I mutant in two different crystal forms, C222

VACCINES AGAINST *Clostridium perfringens* ALPHA-TOXIN

Richard W Titball

Defence Science and Technology Laboratory, Porton Down, Salisbury, Wilts, UK, SP4 0JQ

The alpha-toxin (phospholipase C) of *Clostridium perfringens* plays a major role in the pathogenesis of gas gangrene, a serious and usual fatal disease which is often consequence of a severe traumatic injury. During the first half of the 20th century several studies were carried out to evaluate the utility of alpha-toxin vaccines or alpha-toxin antisera for the prevention or treatment of gas gangrene in humans who sustained injuries during military campaigns. Whilst some of these studies demonstrated the value of these approaches, the potency of toxoids used to prepare vaccines or antisera appeared to be quite variable. Partly because of this antibiotics became the preferred method for treatment of disease. More recently the alpha-toxin has been implicated in the pathogenesis of several diseases of animals, including necrotic enteritis in fowl and enterotoxaemia in calves. There has been a renewed interest in vaccines against alpha-toxin for the prevention of these diseases. A genetic toxoid has previously been devised based on the membrane-binding domain of alpha-toxin. Immunisation of mice with this vaccine protects mice not only against *C. perfringens* alpha-toxin but also against the toxic effects of phospholipases C produced by other clostridial species. Mice immunised with this vaccine are protected against experimental gas gangrene caused by *C. perfringens*. The expression of this toxoid in vaccine strains of *Salmonella* provides an attractive prospect for protecting fowl from necrotic enteritis and calves from enterotoxaemia.

INVESTIGATING POST-BINDING STEPS IN THE MECHANISM OF ACTION OF C. PERFRINGENS ENTEROTOXIN

J. G. Smedley, III and B. A. McClane

University of Pittsburgh School of Medicine, MVM Graduate Program

The pore-forming C. perfringens enterotoxin (CPE) is a single polypeptide of 35 kDa. CPE s mechanism of action begins with receptor-mediated binding resulting in formation of an SDS-sensitive small complex . CPE then rapidly progresses to form large complexes which are resistant to denaturation by SDS, an event which correlates tightly with membrane disruption. The present study is focused on using structure-function analysis to examine the molecular interactions required for the post-binding steps in CPE s action. A region between amino acids 81-106 in CPE was identified as a putative transmembrane stem domain due to its distinctive pattern of alternating hydrophobic residues. When this region was deleted from CPE, the resulting rCPE-TM1 variant could form SDS-resistant large complex but was not cytotoxic. The rCPE-TM1 variant was also unable to elicit changes in membrane permeability in Caco-2 cells. Additionally, when associated with membranes, the large complex formed by rCPE-TM1 was over 20-fold more sensitive to proteolytic degradation than wild-type rCPE large complex, suggesting that the rCPE-TM1 large complex is trapped in a pre-pore state and unable to insert into the phospholipid bilayer. We then sought to determine whether this newly identified functional domain of CPE acts before or after the involvement of other previously recognized functional regions. To this end, the large complex-inhibitory D48A point mutation was introduced into the rCPE-TM1 variant. As expected, the resultant double variant was deficient at pore-formation, but failed to form the pre-pore large complex seen with rCPE-TM1. This finding indicates that sequences in CPE s Nterminal cytotoxicity region become engaged prior to the involvement of the putative transmembrane stem. Lastly, to ascertain whether or not CPE acts as a single copy or as an oligomer in the SDS-resistant large complex, we employed electrophoretic shift experiments with two CPE variants that differ in molecular weight by ~8 kDa. Using different ratios of the two variants on Caco-2 cells, we resolved several heteromeric large complexes which is indicative of CPE s oligomeric state within this large complex. Taken together, results from this study reveal a model for CPE s mechanism of action where after binding, CPE uses N-terminal sequences to form an oligomeric pre-pore, followed by a membrane insertion step requiring the putative transmembrane stem, ending ultimately pore formation.

CLOSTRDIUM PERFRINGENS EPSILON TOXIN INCREASES SMALL INTESTINAL PERMEABILITY

M.E. Fernandez-Miyakawa[1], S. Sayeed[2], J.I. Rood[3], B.A. McClane[2], F.A. Uzal[1].

[1]California Animal Health and Food Safety Laboratory System, San Bernardino Branch, School of Veterinary Medicine, University of California, Davis, USA [2]Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, USA. [3] Department of Microbiology, Monash University, Australia.

Epsilon toxin (ETX) produced by *Clostridium perfringens* types B and D is responsible for a highly fatal enterotoxemia in livestock. ETX is a potent neurotoxin that causes edema by damage to endothelial cell and produces physiological and/or morphological changes in the intestine. Although it is believed that ETX compromises the intestinal barrier and then enters the gut vasculature from which it is carried systemically, the mechanism of absorption in the intestine is unknown. The present study analyzes the effects of ETX on fluid transport and bioelectrical parameters of the small intestine of mice. The effects of epsilon toxin on the fluid balance in the small intestine were studied by *in vivo* techniques (ileal loops, single pass-perfussion and enteropooling). Short circuit current [SCC] and transepithelial resistance [Rt] were monitored in intestinal tissues treated with epsilon toxin *in vitro* as well as *ex vivo*. Changes induced by serosal theophyline and mucosal glucose were registered in *ex vivo* control and treated tissues. Potential dilutions were measured with modified Ringer s solution in serosal and mucosal sides. The results showed that, as observed in goats and sheep, ETX induces fluid accumulation in the small intestine of mice. *In vitro*, serosal ETX induced a reduction in Rt although mucosal ETX did not induce any significant change in Rt or SCC even when applied at relatively very high doses. *Ex vivo* analysis of the small intestine incubated with ETX resulted in reduction of Rt and increase of SCC. No significant changes were observed after *in vitro* treatment with theophylline or glucose or in potential dilutions. Binding of ETX to the mucosa of the small intestine, particularly to epithelial cells, was observed by immunoflourescence. The results suggest that fluid imbalance could be originated by decreased water absorption produced by an increase in the transepithelial permeability, which could also explain the finding that diarrhea or watery intestinal contents are observed in cases of enterotoxemia in sheep and goats. The increase in small intestinal permeability induced by epsilon toxin could explain the observation that epsilon toxin induces an augmented intestinal passage of macromolecules from the intestinal lumen to blood. Moreover, it could be the main mechanism of epsilon toxin absorption during enterotoxemia in sheep and goats.

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UPREGULATION OF RHOB BY CLOSTRIDIAL CYTOTOXINS

J. Huelsenbeck, R. Gerhard, I. Just, H. Genth Dept. of Toxicology, Hannover Medical School, 30625 Hannover, Germany

rhoB is an immediate early gene inducible by genotoxic stress (e.g. caused by DNA alkylating agents) as well as by cytotoxic stress (lovastatin). Its gene product, the RhoB GTPase, is involved in the regulation of vesicle trafficking, the actin-based cytoskeleton, and the cellular response to toxic stress, such as apoptosis. Clostridial protein toxins inactivate Rho- and Ras-GTPases by glucosylation or ADP-ribosylation, which results in re-organisation of the actin-based cytoskeleton (cytopathic effect). Toxin B from C. difficile (TcdB) and exoenzyme C3 from C. botulinum (C3), Rho inactivating toxins, both caused an increase of rhoB mRNA and GTPase (>10-fold) level in a time- and concentration-dependent manner in 3T3 fibroblasts. Lethal toxin from C. sordellii (TcsL) that glucosylates Rac and Ras but not Rho, caused a similar increase of rhoB mRNA and protein levels. Thus, inactivation of a distinct Rho GTPase was not necessary for RhoB up-regulation. Nevertheless, the up-regulation was based on the covalent modification of Rho, Rac, and Ras, as direct actin depolymerisation by C2 toxin from C. botulinum or latrunculin B did not induce RhoB. The sigmoid toxin-concentration versus cytopathic effect and RhoB up-regulation curves were fitted to determine the EC50-values of TcdB and TcsL. In both cases, the cytopathic effect (TcdB: 0.4 ng/ml; TcsL: 750 ng/ml) correlated with RhoB up-regulation (TcdB: 0.5 ng/ml; TcsL: 300 ng/ml). Pre-treatment of cells with cycloheximide, however, completely inhibited toxin-induced RhoB protein up-regulation without affecting the cytopathic effect, indicating that RhoB did not contribute to the toxin-induced morphological changes. The half life period of RhoB protein was higher in TcdB-treated (90 min.) than in TcsL-treated (48 min.) fibroblasts; thus, RhoB was stabilized by its glucosylation by TcdB. The RhoB protein level in toxin-treated cells was regulated by proteasome- and caspase-dependent degradation, as only pre-treatment with both proteasome- and caspase inhibitor protected RhoB from its degradation. In contrast to TcdB-treated cells, RhoB up-regulation was p38 MAPK-dependent in TcsL-treated cells. We suggest that RhoB is up-regulated as response to the cytotoxic stress caused by the toxins via a Rho (TcdB)- or a Ras/p38 MAPK (TcsL)-dependent pathway.

CLOSTPATH 2006 PROGRAMME CHANGE

Clostridium botulinum RESEARCH AT NOTTINGHAM: DEVELOPMENT OF A UNIVERSAL GENE KNOCK-OUT SYSTEM FOR CLOSTRIDIA

John Heap, Oliver Pennington, Ian Davis, Clare Cooksley, Stephen Cartman, Jamie Scott, Andrew Butcher and Nigel Minton
Institute for Infection, Immunity & Inflammation, School of Molecular medical Sciences, Centre for Biomolecular Sciences, University of Nottingham, Nottingham, NG7 2RD, UK

Completion of the genome sequence of *Clostridium botulinum* at the Sanger Institute, Cambridge, now provides the opportunity to begin to understand the physiological process that operate in this important human pathogen. The data generated also has relevance to the closely related organism *Clostridium sporogenes*, a leading candidate for use in the anticancer strategy, Clostridial-Directed Enzyme Prodrug Therapy (CDEPT). In our initial studies we have made use of this latter *Clostridium* as a surrogate for *C. botulinum.* A number of aspects are under investigation, including the role of homologues of the *Staphylococcus aureus* Agr quorum sensing system and CodY. Modulation of expression of key components of these systems using antisense (AS) RNA technology has been shown to affect the ability of the organism to sporulate. However, whilst AS RNA technology can provide valuable information, gene knock-out is preferred. Accordingly we have adapted the Sigma Aldrich TargeTron system to derive a clostridial plasmid which allows for the positive selection of gene knockouts in a variety of clostridial species. This is made possible through the use of an IPTG-inducible plasmid to regulate production of group II RNA, and the creation of a Retrotransposition-activated Marker (RAM) based on an erythromycin resistance gene. Successful retargeting of the modified group II intron to the gene of interest may therefore be selected on the basis of acquisition of erythromycin resistance. The system has been tested in 3 different clostridial species (*C. difficile, C. acetobutylicum* and *C. sporogenes*) with a 100% success rate. Typically, the number of mutants obtained per experiment number in the 100s, and from start (oligo design) to finish (confirmation of correct retargeting) are generated within ca. 8 days. These data suggest that this system may be universally applied to *Clostridium sp* and other Gram-positives, and open up the possibility of revolutionizing the assignment of function to clostridial genes identified by genome sequencing.

NEURAL CELL RECEPTORS OF BOTULINUM NEUROTOXINS

Binz T[1], Mahrhold S[1], Rummel A[2], Karnath T[1] Davletov B[3], and Bigalke H[2]

Institutes of [1]Biochemistry, OE 4310, and [2]Toxicology, OE 5340, Medical School Hannover, 30623 Hannover, Germany

[3]Medical Research Council, Laboratory of Molecular Biology, Cambridge CB2 2QH, U.K.

The seven serotypes of botulinum neurotoxins (BoNTs) cause botulism by cleaving synaptic proteins that constitute the core of the neurotransmitter release apparatus. Their extreme potency can largely be ascribed to the selective binding to nerve cells. It is assumed that BoNTs initially adhere to neurons via an interaction with the abundant polysialogangliosides of the outer membrane leaflet. Toxin entry appears to proceed in a neurotransmitter release-dependent fashion during which the protein receptor becomes transiently accessible on the cell surface. In accord with this idea, the synaptic vesicle protein Synaptotagmin has recently been demonstrated to mediate the entry of BoNT/B and BoNT/G. Following this line we systematically test other synaptic vesicle proteins on the ability to interact with any of the remaining BoNTs. We show that BoNT/A directly binds to the large intravesicular domain of the synaptic vesicle glycoprotein 2 (SV2) and provide evidence for the function of SV2 as the protein receptor of BoNT/A.

To learn more about the BoNT-receptor interaction mode we analyse binding and neurotoxicity of BoNT mutants. The present data suggest that the protein receptors interact with a shallow cavity in the proximity of the conserved ganglioside binding pocket which may facilitate capturing of the protein receptor during its surface retention period.

These studies are supported by a grant from the German research council.

GENOMIC COMPARISON OF GROUP I (PROTEOLYTIC) CLOSTRIDIUM BOTULINUM TYPE B

M. Lindstr m1, K. Kiviniemi1, P. Somervuo1,2, M. Nevas1, P. Auvinen2, H. Korkeala1
1Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki, Finland
2 Institute of Biotechnology, University of Helsinki, Finland

The group I Clostridium botulinum type B strains isolated from various sources in the Nordic countries possess a strikingly narrow genetic diversity. In a PFGE analysis with SacII of 243 type B isolates, two large clusters (I and II) with identical restriction patterns within a cluster have been observed. A total of 37 strains falling into cluster I or II were further characterized by genomic comparison with DNA microarrays (Institute of Food Research, Norwich, UK) based on the ATCC 3502 type A strain genome. Preliminary data analysis showed that the discriminatory power of the microarray analysis was similar to PFGE. Approximately 100 genes distinguished between the two clusters, and were present only in one of the two clusters but not in both of them. Most of these genes were present in cluster II strains only. A group of other type B strains of Nordic origin, not falling into the two PFGE types, closely resembled the cluster I and II strains. However, the international reference strains 126 B, CDC 7827 and McClung 133-4803 B were markedly different from all the Nordic strains. The results suggest that the Nordic group I C. botulinum type B strains form a distinct population that has a narrow genetic diversity. The population seems to possess two genetic lineages. The evolution of the two lineages warrants further investigation.

INTERACTION WITH ONE GANGLIOSIDE AND ONE PROTEIN RECEPTOR MEDIATES THE NEUROTOXICITY OF BOTULINUM NEUROTOXINS

A. Rummel1, T. Karnath2, T. Eichner1, T. Weil3, K. Sandhoff4, R. Proia5, T. Binz2, H. Bigalke1 1Toxikologie and 2Biochemie, Medizinische Hochschule Hannover, 3Merz Pharmaceuticals GmbH, Frankfurt/Main, 4Kekul -Institut, Bonn, 5NIDDK, NIH.

Bethesda Clostridial neurotoxins (CNTs) bind selectively to nerve cell membranes via a dual receptor mechanism. First, abundant complex polysialo gangliosides accumulate the CNTs at the nerve cell surface. Following exocytosis, a protein receptor appears on the plasma membrane, binds the CNTs with high affinity and is endocytosed. Recently, we have shown that botulinum neurotoxin (BoNT) A and B display a single ganglioside binding pocket in their HCC domains. Moreover, we could also assign the interaction of the protein receptors synaptotagmin I and II with BoNT/B and G to their HCC domains. Employing a site finder algorithm, putative synaptotagmin binding pockets within the HCC domain of BoNT/B were identified. Various BoNT/B HC-fragment mutants were generated by site directed mutagenesis to check their remaining binding to synaptotagmin I and II in GST-pull-down assays. Mutations in only one site interfered binding of the HC-fragments to synaptotagmin. Recombinant full length BoNT/B mutants containing the potent mutations were tested on the mouse phrenic hemidiaphragm preparation and correspondingly displayed reduced neurotoxicities. A homologous pocket within the HCC domain of the closely related BoNT/G could similarly be identified as synaptotagmin binding site. To verify the dual receptor mechanism, recombinant BoNT/B wild type and a mutant, which cannot bind to synaptotagmin I and II, were tested on mice hemidiaphragm organs derived from transgenic mice only synthesising GM3. The wild type BoNT/B showed a residual neurotoxicity of ¯2 % compared to neurons of wild type mice while the mutant was completely inactive in neurons of transgenic mice. Accordingly, another mutant of BoNT/B, which lacks both ganglioside and protein receptor binding, did not show any neurotoxicity in neurons of wild type mice. Thus, the residual neurotoxicity of BoNT/B in transgenic mice can be explained by binding to synaptotagmin I and II and proves the concept of dual receptors, at lleast as far as BoNT/B is concerned.

BOTULINUM NEUROTOXIN: A TOXICOKINETIC STUDY

Lebeda, F.J.[1], D. Bruining, U. Mudunuri[2] and R. Stephens[2]
[1]U.S. Army Medical Research Institute of Infectious Diseases; [2]National Cancer Institute at Frederick-Science Applications International Corp., Frederick, MD 21702
Frank.Lebeda@amedd.army.mil

A computational model has been developed that can simulate the acute onset of paralysis caused by type A1 botulinum neurotoxin at the mouse neuromuscular junction (NMJ). A set of coupled, first-order linear differential equations were represented using reaction schemes that were graphically constructed with JDesigner. These equations were then solved with Jarnac, an equation-solution engine that also provides a graphics output. Rate constants for the steps involved in the neuronal internalization of the neurotoxin and for describing its enzymatic activity have been gathered from the literature and archived at the clostridial neurotoxin database, BotDB, (Lebeda, 2004; http://botdb.abcc.ncifcrf.gov). Some published inhibitor studies, including those using monoclonal antibodies (Amersdorfer *et al.*, 1997), have also been incorporated into this database and simulated with the NMJ model. To understand more about its chronic paralytic effects, the same NMJ model was used to simulate the recovery-from-block seen clinically in patients treated with the neurotoxin (Dressler *et al.*, 2005). These results provide initial estimates of the neurotoxin s rate of elimination. Other results from these kinetic analyses will also be discussed. Supported by DTRA (D_X009_04_RD_B) and DARPA (05-0-DA-008) to FJL.

CLOSTRIDIAL SPORES AND CANCER THERAPY

J. Theys, B. Wouters, P. Lambin

University Maastricht, Maastro Lab, GROW Research institute, Maastricht, The Netherlands

Cancer is a disease with a high incidence in the western world. To increase treatment potential, substantial resources are currently being devoted to discovery and development of new anti-cancer therapies. We and others have been investigating the potential use of non-pathogenic strictly anaerobic bacteria such as *Clostridium*. Their tumor specificity is based upon the unique physiology of solid tumors which is often characterized by regions of hypoxia and necrosis. Although clostridia are strictly anaerobic, most of them can form spores allowing survival but not growth in oxic conditions. Paradoxically, it is the very ability to form spores that presents this genus with its potential for treating cancer. Indeed, intravenously injected clostridial spores in tumor-bearing animals localise and germinate only in hypoxic and necrotic regions of solid tumors. Furthermore, administration of genetically engineered clostridia to tumor-bearing animals leads to anti-tumor effects. A major breakthrough now allows genetic manipulation of these *Clostridium* strains that have been shown to have superior tumor colonizing properties. Until this development, these strains had proven refractory to transformation. We are now in position to express therapeutic anti-cancer genes in these hosts. The application of clostridia as a gene therapy delivery vehicle has proven to be highly feasible, safe and to achieve a high degree of tumor selectivity. Moreover, it targets specifically the hypoxic area of the tumor which has been shown to be a primary cause of treatment failure for conventional therapeutic agents. We believe that this approach will act in a complementary way to current radiotherapy and chemotherapy treatments of solid tumors and can be used to deliver virtually any conceivable gene to the tumor.

DEVELOPMENT AND APPLICATION OF HIGH-THROUGHPUT CELL-BASED ASSAYS FOR BoNT PROTEASES

Neil Green, Wentian Luo, and Hong Fang
Department of Microbiology & Immunology
Vanderbilt University Medical School
Nashville, TN USA 37232-2363

We have developed high-throughput cell-based assays for the light chain (LC) proteases of botulinum neurotoxin (BoNT), serotypes A, B, C, D & G. By utilizing the tools of yeast genetics, we engineered a series of yeast strains whose growth is inhibited by LC protease activity in a conditional manner. Pulse-labeling assays have also been established, providing a way to monitor SNARE substrate cleavage inside cells and the biochemical means to corroborate genetic data. To validate our assays, we growth-selected SNARE mutations that suppressed serotype B-LC growth defect. When mutations were sequenced and corresponding amino acid changes were identified, residues located near and distal to the cleavage site were found to inhibit SNARE substrate cleavage, including residues positioned near the SNARE transmembrane domain, an unexplored aspect of BoNT cell intoxication. Our yeast assays also offer a convenient cell-based system to select intracellular inhibitors of LC proteases. To date, we have identified proteins (i.e. intracellular antibodies) and small-molecules that inhibit serotype B & D proteases inside yeast cells. These findings will be presented at the meeting, and we will provide an update of our progress in finding additional intracellular inhibitors of BoNT/LC proteases.

SEQUENCE SPECIFIC MUTAGENESIS BY TARGETRON: DISRUPT ENDOGENOUS GENES AND INTRODUCE FOREIGN GENE INTO *C. PERFRINGENS* CHROMOSOME

Y Chen[1], B McClane[2], D Fisher[2], J Rood[3] and P Gupta[1]

Department of Infectious Diseases and Microbiology, Graduate School of Public Health[1], University of Pittsburgh School of medicine[2], University of Pittsburgh, Pennsylvania, USA and Department of Microbiology, Monash University, Victoria, Australia[3].

Clostridium perfringens type A strain ATCC3624 has been used as a delivery vector for HIV/SIV oral vaccine development. However, there are two potential problems with this delivery vector. First, ATCC3624 produces two extracellular toxins, alpha toxin (*plc*) and theta toxin (*pfoA*) which could cause gas gangrene and thus pose a potential danger for vaccinated people. Secondly, expression of foreign antigens from plasmids in ATCC3624 has the potential problems of instability and requiring plasmid-encoded antibiotic resistant gene which might be transferred to other bacteria in vivo. To construct a safe *C. perfringens* delivery vector that expresses a foreign gene inserted into the chromosome, we needed to first inactivate both the *plc* and *pfoA* genes in ATCC3624 and then introduce the foreign gene into the chromosome. Inactivating and inserting a gene in the *C. perfringens* chromosome by traditional mutagenesis methods is very difficult. Therefore, Group II intron based Targetron technology was used in this study to successfully disrupt the *plc* gene of ATCC 3624. Using the same technology, a SIV p27 gene cassette was successfully inserted into the *pfoA* open reading frame in the chromosome, which also resulted in *pfoA* inactivation. PCR amplification followed by sequencing of the expected insertion regions of *plc* and *pfoA* genes confirmed that the targetrons inserted into their target genes. Loss of both alpha and theta toxin activities were demonstrated by the lack of hemolysis surrounding colonies of these mutants grown on sheep blood agar plates. Advantages of this technology, such as site specificity, relatively high frequency of insertion and introduction of no antibiotic resistant genes into the chromosome, could facilitate construction of *C. perfringens* with multiple knockout mutations and for introduction of foreign genes into the chromosome.

C. NOVYI CAN GENERATE A POTENT THERAPEUTIC IMMUNE RESPONSE AGAINST EXPERIMENTAL TUMORS

I. Cheong [1,2], N. Agrawal [1,2,3], C. Bettegowda [1,2], J-F Geschwind [4], C.G. Drake [2], E.L. Hipkiss [2], M. Tatsumi [4], L.H. Dang [1,2], L.A. Diaz, Jr. [1,2], M. Pomper [4], M. Abusedera [4], R.L. Wahl [4], K.W. Kinzler [1,2], S. Zhou [1,2], D.L. Huso [5], and B. Vogelstein [1,2]

[1] Howard Hughes Medical Institute, [2] Sidney Kimmel Cancer Center, and [3] Departments of Otolaryngology-Head and Neck Surgery, [4] Radiology and Radiological Sciences, and [5] Comparative Medicine, Johns Hopkins Medical Institutions, Baltimore, MD 21231

Spores of the strict anaerobe *Clostridium novyi-NT* when systemically injected into animals will germinate exclusively within hypoxic regions of tumors. The germinated vegetative bacteria destroy tumor cells locally but leave a well-perfused rim of oxygenated tumor cells which continue to proliferate. We were therefore surprised to observe that approximately 30% of mice treated with *C. novyi-NT* spores were cured of their tumors despite the tumor rim initially remaining after spore germination. Cured animals rejected a subsequent challenge of the same tumor, suggesting that the mechanism underlying this effect is immune-mediated. This phenomenon was also observed in rabbits with intrahepatic tumors. It was especially notable that *C. novyi* was able to induced an immune response, which in combination with its endogenous bacteriolytic effect was able to eradicate large established tumors.

NON-TOXIGENIC *CLOSTRIDIUM DIFFICILE (CD)* PROTECTS HAMSTERS AGAINST HISTORIC AND EPIDEMIC TOXIGENIC BI STRAINS

KJ Nagaro[1], AK Cheknis[1], SP Sambol[1], WE Zukowski[1], DN Gerding[1, 2]

[1]Hines V.A. Hospital, Hines, IL USA, [2]Loyola University Chicago Stritch School of Medicine, Maywood, IL, USA

Epidemic CD [restriction endonuclease analysis (REA) group BI] has been associated with multiple hospital outbreaks and increased severity of CD-associated disease (CDAD). Non-toxigenic CD prevents CDAD in hamsters challenged with toxigenic CD strains. We tested the efficacy of two non-toxigenic types (REA types M3 & T7) in preventing disease in hamsters challenged with one of two toxigenic BI types (REA types BI6 and BI1), both previously shown to be 100% fatal in this model.

Group 1 consisted of 20 assay hamsters and 2 control hamsters challenged with epidemic type BI6. Clindamycin was given orally (30 mg/kg) to all 22 hamsters on Day 0 to render them susceptible to CD infection. Two groups of 10 hamsters were orally inoculated on Day 2 with 10^6 spores of T7 or M3. Control hamsters did not receive M3 or T7. All hamsters were challenged on Day 5 with an oral dose of 100 spores of epidemic toxigenic strain BI6.

Group 2 consisted of 20 assay hamsters and 2 control hamsters treated identically to those in Group 1, but challenged on Day 5 with 100 spores of historic type BI1. Colonization was confirmed by stool culture, and CD strains identified by REA typing.

In Group 1, the 2 BI6 control hamsters died on Days 7-8. Nine of the 10 M3-treated hamsters colonized with M3 and remained well until study end (Day 36). One M3-inoculated hamster failed to colonize with M3 and died on Day 7. All 10 T7-inoculated hamsters colonized with T7, and 5 remained well until study end. The other 5 became co-colonized with BI6, and died between Days 7 and 20. In Group 2, the 2 BI1 control hamsters died on Day 7. All 10 M3-inoculated hamsters and all 10 T7-inoculated hamsters colonized with M3 or T7 and remained well until study end (Day 36).

M3 prevented fatal CDAD in 9/10 hamsters challenged with BI6 ($P<.0003$) and 10/10 challenged with BI1 ($P<.00005$). T7 prevented fatal disease in 5/10 hamsters challenged with BI6 ($P<.02$), and 10/10 challenged with BI1 ($P<.00005$). Colonization with non-toxigenic CD (especially type M3) is highly effective in preventing CDAD in hamsters caused by REA group BI strains, and provides a novel approach with high potential to prevent CDAD caused by the new epidemic CD strains in humans.

CLOSTRIDIAL ENTERITIS IN DOMESTIC ANIMAL SPECIES.

JG Songer[1], HT Trinh[1], MK Keel[2], KC Cooper[1], MA Anderson[1], and M Yaeger[3].

[1]Department of Veterinary Science and Microbiology, University of Arizona, Tucson, AZ 85721

[2]Southeastern Cooperative Wildlife Disease Study, University of Georgia, Athens, GA 30602;

[3]Department of Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, IA 50010.

Vaccination of domestic animals yields substantial control over histotoxic, neurotoxic, and enteric disease, but clostridial infections continue to challenge producers, veterinary practitioners, and diagnosticians. Consumer demands on product quality and changes in production regimens have led to emergence of new problems and re-emergence of old ones. An example of the former is the appearance of *C. difficile* as a major cause of disease in piglets. Surveys in the US continue to reveal that as many as two-thirds of piglets submitted for diagnosis of enteritis are affected by *C. difficile*. Knowledge gained through study of this organism as a human pathogen serves as a basis for understanding pathogenesis of porcine CDAD and for development of control strategies. For example, evidence suggests that competitive exclusion can be an effective means for prevention. Enteric infection, with substantial toxin production, also occurs in beef and dairy calves. Data are limited, but now support a domestic animal source of infection for some cases of human disease. Poultry necrotic enteritis (NE) is re-emerging, as a consequence of withdrawal of ionophore coccidiostats and anticlostridial growth-promoting antimicrobials. Disease can be reproduced experimentally, and may serve as a model for study of aspects of other type A enteritides. Effective immunoprophylaxis of NE will require identification of appropriate immunogens and development of practical means for delivery. The next decade holds the promise of improved animal production and consumer safety through control of clostridial enteric diseases.

AN INTRAGASTRIC MODEL OF *CLOSTRIDIUM PERFRINGENS* TYPE D ENTEROTOXEMIA IN MICE

Uzal, F.A.,[1] Stephens, B.D.,[2] Berman, R.F., [2] Fernandez-Miyakawa, M.E.[1]

[1]California animal Health and Food Safety Laboratory, San Bernardino Branch, and [2]Department of Neurological Surgery, University of California, Davis.

Clostridium perfringens type D is responsible for enterotoxemia in sheep and goats. In these species, the major signs and lesions of the disease are caused by epsilon toxin, a major exotoxin produced by the types B and D of *C. perfringens*. This toxin has recently been included in the B list of Select Agents by the Center for Disease Control, which has renewed the interest in elucidating the pathogenesis of the diseases produced by *C. perfringens* type D and epsilon toxin. We present here an oral model for *C. perfringens* type D enterotoxemia in mice. Ten groups of 10, 20 gr Blab c mice each were inoculated by intragastric gavage with 10 different isolates of *C. perfringens* type D. Inocula consisted of washed cells at an approximate concentration of 10^9 CFU/ml, diluted in cooked meat medium. Seven out of 10 isolates produced lethality in mice, defined as spontaneous death or severe clinical signs necessitating euthanasia. Lethality of the different isolates varied from between 14 to 80%. Mice inoculated with the other 3 isolates remained asymptomatic during the 48 hr duration of the trial. Clinical signs consisted of seizures, convulsions, hyperexitability and/or depression. No gross abnormalities were observed at necropsy in any mice, while histology showed acute tubular necrosis in the kidney of most mice that developed clinical alterations. No histological abnormalities were observed in the brain of any mice, although sections stained with fluorojade showed positive staining in the hippocampus of mice developing clinical alterations. Histological and immunohistochemical (fluorojade) examination of the brain showed that neuronal damage occurs in mice with *C. perfringens* type D infection even when it is not detected in conventional histological sections. This study shows that epsilon toxin is readily produced and absorbed in the intestine of mice, a species which can provide a useful model for studying the pathogenesis of epsilon toxin producing *C. perfringens* type D infections.

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CLOSTRIDIUM DIFFICILE: POTENTIAL CAUSE OF DUODENITIS PROXIMAL JEJUNITIS IN HORSES

LG Arroyo; HR Staempfli; JS Weese.

Department of Clinical Studies, Ontario Veterinary College, University of Guelph, Guelph, ON, CANADA

Duodenitis proximal enteritis (DPJ) is an acute sporadic syndrome in horses characterized by inflammation and edema of the duodenum and jejunum. The cause of this disease remains unknown, however, clostridia, *Salmonella* spp and mycotoxins have been suggested as being the initiating cause. *Clostridium difficile* is an important cause of enterocolitis in horses and while colitis in the most commonly reported presentation, this organism is known to have an affinity for the small intestine and its role in DPJ requires further scrutiny.

This preliminary study evaluated the role of *C. difficile* in DPJ. Nasogastric reflux was collected at the time of hospitalization from 10 horses with clinically diagnosed DPJ and 16 horses with nasogastric reflux due to other small intestinal lesions. Selective enrichment culture was performed by inoculating 2 ml of nasogastric reflux into test tubes with cycloserine-cefoxitin-fructose broth and incubating at 37 C for one week. Broth cultures were subcultured onto blood agar plates and incubated at 37C in an anaerobic chamber for 48 hours.

Clostridium difficile was isolated from 10/10 horses with DPJ but only 1/16 horses with nasogastric reflux of other etiologies (*P*0.0001*)*. Genes encoding for toxins A and B were detected in 8/10 isolates. The two remaining isolates carried the gene for toxin B but not toxin A. One isolate also carried the gene (*cdtB*) for binary toxin. Three horses with DPJ were euthanized due to the severity of the disease while the remaining cases fully recovered. Histological changes in the small intestine of horses succumbing to DPJ included sloughing of the villus epithelium, submucosal edema, fibrinoid necrosis of vessels walls and thrombosis of blood vessels within the submucosa, muscularis and subserosa.

These changes, while somewhat non-specific, are consistent with lesions present in the small intestine of horses with *C. difficile* enterocolitis. Further investigation into the role of *C. difficile* in DPJ is indicated.

CLINICAL ISOLATES OF CLOSTRIDIUM PERFRINGENS IN POULTRY ARE NOT SUPERIOR ALPHA TOXIN PRODUCERS IN VITRO

A.R. Gholamiandehkordi, L. Timbermont, A. Lanckriet, F. Haesebrouck, R. Ducatelle and F. Van Immerseel
Ghent University, Faculty of Veterinary Medicine, Department of Pathology, Bacteriology and Avian Diseases, Salisburylaan 133, 9820 Merelbeke, Belgium.

Due to the diminished use of growth-promoting antibiotics in the European Union, Clostridium perfringens induced necrotic enteritis and subclinical disease have become important threats to poultry health. A study was set up to evaluate whether C. perfringens isolates from animals with clinical disease differ in the ability to produce alpha toxin in vitro, compared with random isolates. Animals from healthy flocks were sampled by cloacal swabs, while intestinal and liver samples of animals suffering from necrotic enteritis were analyzed. A total of 27 isolates was obtained from 23 broiler flocks without clinical problems and 36 isolates were obtained from 8 flocks with clinical problems. Using PFGE typing, high genetic diversity was detected between isolates from different flocks. Isolates derived from flocks where disease outbreaks occurred were clonal within each flock, but each flock harboured a different clone. All isolates were of toxin type A. Isolates from 5 out of 35 PFGE types carried the cpb2 gene, encoding the beta2 toxin, and isolates from 2 out of 35 PFGE types harboured the cpe gene, encoding the enterotoxin. In vitro alpha toxin production for all isolates was quantified by enzyme-linked immunosorbent assay after growth of the isolates for 24h. A wide range of alpha toxin concentration values was detected. It was shown that in vitro alpha toxin production of C. perfringens isolates from diseased flocks was not higher than in vitro alpha toxin production from isolates derived from healthy flocks. Isolates producing high and low alpha toxin concentrations in vitro were injected in intestinal loops of anaesthetized laying hens. It was shown that injection of isolates producing high alpha toxin concentrations in vitro resulted in the appearance of a high amount of cell debris and protein material in the intestinal lumen, to which the bacteria were adhering and in which the bacteria were clearly multiplying. Inoculation of 3 weeks old broilers with high doses of an alpha toxin producing strain, after orally inoculating a tenfold dose of a coccidial live vaccine, resulted in similar microscopical observations in some of the animals a few days post-challenge as observed in the intestinal loop model.

THE ALPHA TOXIN OF *Clostridium perfringens* IS NOT AN ESSENTIAL VIRULENCE FACTOR IN NECROTIC ENTERITIS IN CHICKENS.

A.L. Keyburn[1,2], S.A. Sheedy[1,2], M.E. Ford[1], M.M. Williamson[3], M.M. Awad[2], J.I. Rood[2] & R.J. Moore[1,2]

[1]Commonwealth Scientific and Industrial Research Organisation, Livestock Industries, Australian Animal Health Laboratory, Geelong 3220,[2]Australian Research Council Centre of Excellence in Structural and Functional Microbial Genomics, Department of Microbiology, Monash University, Geelong 3800, Australia, [3]Gribbles Veterinary Pathology, Clayton 3168, Australia.

Clostridium perfringens is the causative agent of necrotic enteritis in chickens and is an increasing threat to poultry health. Alpha-toxin, a zinc-metallophospholipase C, has for the last 30 years been implicated as the major virulence factor in causing necrotic enteritis in chickens, although definitive proof of this hypothesis has not been reported. We began to question the involvement of alpha-toxin when a survey of local strains showed that the *in vitro* levels of alpha-toxin produced by virulent isolates was quite low. It is also clear that the cellular pathology associated with disease is not typical of that seen in diseases such as gas gangrene, in which alpha-toxin is clearly shown to be a major and essential virulence factor. To determine the role of alpha-toxin in necrotic enteritis, an alpha-toxin structural gene (*plc*) mutant was constructed in a virulent chicken isolate and its virulence assessed in a chicken disease model. Virulence testing of isogenic strains consisting of the wild-type and two independently derived *plc* mutants revealed that the development of necrotic enteritis in chickens was not dependent on the ability to produce a functional alpha-toxin. The *plc* mutants did not produce any alpha-toxin yet produced the same disease, at both the macroscopic and microscopic level, as the wild type strain This study represents the first definitive evidence that alpha-toxin is not essential virulence factor in the pathogenesis of necrotic enteritis in chickens and opens the way for further studies that will identify other virulence factors produced by *C. perfringens* and that are essential for this important emerging disease.

CLOSTRIDIUM PERFRINGENS TYPE IV PILI-DEPENDENT MOTILITY

S. B. Melville, J. Varga, V. Nguyen, D. O Brien, Richard Walker
Department of Biological Sciences, 2119 Derring Hall, Virginia Tech,
Blacksburg, VA, USA

Bacteria can move through liquid via flagella-mediated swimming and on surfaces by a variety of mechanisms called gliding or twitching motility. One type of gliding motility depends on the extension and retraction of type IV pili, and this form of motility has been observed in many Gram-negative bacteria but has not been clearly shown in Gram-positive bacteria. Clostridium perfringens is a Gram-positive anaerobic pathogen that causes gas gangrene and food poisoning. C. perfringens has always been classified as a non-motile bacterium but genome sequencing of three strains of C. perfringens revealed genes that code for components of type IV pili. We were able to demonstrate by video microscopy that the three sequenced strains of the bacterium are capable of surface movement and that such movement typically involves large groups of bacteria forming curvilinear flares that extend away from the colony. A faster moving variant of one strain was isolated in which bacteria formed thin columns of cells that created a latticework appearance at the colony edge. Electron microscopy demonstrated that cells from each of the three sequenced strains contained pili on the cell surface. pilT and pilC mutants were nonmotile and did not produce pili on the bacterial surface, indicating type IV pili were needed for gliding motility. Unlike wild-type C. perfringens, neither mutant was capable of forming an effective biofilm on a glass surface, indicating that gliding motility is essential for biofilm formation and may also play a role in pathogenesis in gangrene infections.

THE CITOTOXIC EFFECTS OF CLOSTRIDIUM PERFRINGENS £\-TOXIN IS MEDIATED BY ENDOGENOUS MEDIATORS.

M. Flores-D az1, L. Monturiol-Gross 1, L. F. Alvarado-Salazar 1, G. C. Clark2, R. W. Titball2, M. Thelestam3, and A. Alape-Gir n1, 4 1 Instituto Clodomiro Picado, Facultad de Microbiolog a, Universidad de Costa Rica, San Jos , Costa Rica 2 Defence Science and Technology Laboratory, Porton Down, Salisbury, Wilts, UK, SP4 0JQ 3 Microbiology and Tumorbiology Center, Karolinska Institute, Stockholm, Sweden 4 Departamento de Bioqu mica, Facultad de Medicina, Universidad de Costa Rica, San Jos , Costa Rica

C. perfringens phospholipase C, also called £\-toxin, the major virulence factor in the pathogenesis of gas gangrene, is a Zn2+ metalloenzyme with lecithinase and sphingomyelinase activities. Since £\-toxin causes hydrolysis of phosphatidylcholine and sphingomyelin, it could generate diacylglycerol and ceramide in an uncontrolled manner, dysregulating signal transduction processes in the target cells. We used two hypersensitive cell lines to study the mechanism of action of £\ toxin at concentrations which induce cell death without directly disrupting the plasma membrane. It was found that at these doses £\-toxin induces morphological and biochemical changes characteristic of apoptosis. The data demonstrate that £\-toxin causes oxidative stress and that the cytotoxic effect of £\-toxin is mediated by reactive oxygen species, involves the formation of O2 -, H2O2 and occurs in an iron dependent way. Thus, this work reveals the hitherto unrecognized mechanism by which C. perfringens £\-toxin causes cytotoxicity.

THE CWP84 SURFACE-ASSOCIATED PROTEIN OF *CLOSTRIDIUM DIFFICILE* IS A CYSTEINE PROTEASE WITH DEGRADING ACTIVITY AGAINST EXTRACELLULAR MATRIX PROTEINS.

Claire Janoir, SØverine PØchinØ and Anne Collignon.
FacultØ de Pharmacie, UniversitØ Paris-Sud, UnitØ EA 35-34, 92296 Ch tenay-Malabry Cedex, France.

Clostridium difficile is the etiological agent of pseudomembranous colitis and of many cases of nosocomial diarrhea. Its pathogenicity is mainly mediated by the two exotoxins A and B, both of which damage the human colonic mucosa by actin desorganization. Colonization of the host by *C. difficile* takes place after disturbance of the normal colonic flora, following antibiotic treatment. As in other enteric pathogens, this preliminary step involved adhesins and presumably hydrolytic enzymes. Some adhesins have already been characterized by us and others, but nothing is known about proteolytic enzymes of *C. difficile*. However, strains known to be highly virulent in the hamster model displayed the greatest proteolytic activity. We already characterized the gene *cwp84*, coding for a conserved putative 84 kDa-cysteine protease, highly immunogenic in patients with *C. difficile* associated-disease. In this work, the Cwp84 has been purified as a recombinant His-tag protein and specific antibodies were generated in rabbits. Purified fractions contained 3 major bands of 70, 55 and 47 kDa, suggesting an autocatalytic process of the Cwp84, as it has been well documented for other cysteine proteases. The 55 kDa fraction was shown to have a caseinolytic activity by zymogram analysis. The purified fractions showed concentration-dependant proteolytic activity on azocaseine and on the synthetic substrate BAPNA, and this activity was maximum at pH 7.5. The amidolytic activity was inhibited by various cysteine protease inhibitors, such as the specific inhibitor E64, and by the anti-Cwp84 specific antibodies. Fractionation experiments following by an immunoblot detection have localized the native protease as a membrane-bound protein which seemed to be associated with the S-layer proteins. To assess a putative role for Cwp84 in the pathogenicity of *C. difficile*, proteolytic assays were performed on physiologic substrates. No degrading activity on collagen IV has been detected. In contrast, Cwp84 has a degrading activity on the extracellular matrix proteins, fibronectin, vitronectin and laminin, which is neutralized by the E64 and specific antibodies. *In vivo*, this proteolytic activity could contribute to the degradation of the host tissue integrity and the dissemination of the infection.

CHARACTERIZATION OF THE SYSTEMIC EFFECTS OF *CLOSTRIDIUM DIFFICILE* TcdB USING DEVELOPING ZEBRAFISH EMBRYOS

EE Hamm, DE Voth, JD Ballard

University of Oklahoma Health Sciences Center, Department of Microbiology and Immunology, Oklahoma City, OK 73104 USA

Despite extensive studies *in vitro*, the *in vivo* effects of many bacterial toxins are poorly defined. As an example, *Clostridium difficile* toxin B (TcdB) is cytotoxic to a broad range of cell types in culture; yet, the organs targeted by this toxin during disease have not been identified. To address this problem, current experiments utilized *Danio rerio* (zebrafish) embryos for *in vivo*, real-time, direct visualization of toxin localization and organ-specific damage. Using fluorescently-labeled TcdB, the protein was found to localize in the frontal ventral region of the animal, with distinct foci of toxin detected within the pericardial sac. Corresponding to the observed toxin localization, TcdB treatment reduced zebrafish heart-rate, ventricle contraction, blood-flow, and caused pericardial edema. During the final stages of intoxication, zebrafish hearts became elongated and did not contract. In line with the zebrafish studies, TcdB altered sarcomere morphology and contractility in cultured cardiomyocytes. Correlating with TcdB s known pro-apoptotic effects, treatment of zebrafish with a caspase-3 inhibitor protected against cardiovascular damage. These findings provide the first insight into the *in vivo* tropism of TcdB, and indicate that the zebrafish embryo is a useful model for characterizing systemic intoxication and testing candidate therapeutics.

THE ROLE OF TOXINS FROM *CLOSTRIDIUM PERFRINGENS* TYPE C IN THE MOUSE INTRAVENOUS INJECTION MODEL

D.J. Fisher[1], M.E. Fernandez Miyakawa[2], S. Sayeed[1], R. Poon[3], V. Adams[3], J.I. Rood[3], F.A. Uzal[2], B.A. McClane[1]

[1]Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA. [2]California Animal Health and Food Safety Laboratory System, School of Veterinary Medicine, University of California, Davis, CA. [3]Department of Microbiology, Monash University, Victoria, Australia.

The Gram-positive anaerobe *Clostridium perfringens* produces a large arsenal of toxins that are responsible for histotoxic and enteric infections, including enterotoxaemias, in humans and domestic animals. *C. perfringens* type C isolates cause rapidly fatal diseases in domestic animals and enteritis necroticans in humans and carry the structural genes for alpha toxin (*plc*), perfringolysin O (*pfoA*), beta-toxin (*cpb*), and sometimes beta2 toxin (*cpb2*) and/or enterotoxin *(cpe)*. Due to the economic impact of type C-induced diseases, domestic animals are commonly vaccinated with crude type C toxoid (prepared from inactivated culture supernatants) or bacterin/toxoid vaccines making it unclear which toxin(s) present in these vaccines actually elicits the protective immune response. To improve type C vaccines, it would be helpful to assess the lethality contribution of each toxin present in type C supernatants. To address this issue, we surveyed a large collection of type C isolates for their toxin-producing ability. When late log-phase supernatants were analyzed by quantitative Western blotting or functional biological assays, most type C isolates produced at least three lethal toxins, i.e., alpha toxin, beta toxin and perfringolysin O, but several isolates also produced beta2 toxin. In the mouse intravenous injection model, beta-toxin was identified as the main lethal factor present in type C late log-phase culture supernatants. This conclusion was based upon monoclonal antibody neutralization studies, trypsin treatment of culture supernatants, and regression analyses comparing alpha, beta, perfringolysin O, and beta2 toxin production levels versus lethal activity. Collectively, these results highlight the importance of beta toxin for type C-induced toxemia.

Spo0A-REGULATED SYNTHESIS OF *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN

M. Sarker, I. Huang and D. Raju

Department of Biomedical Sciences and Microbiology, Oregon State University, Corvallis, Oregon, USA

C. perfringens enterotoxin (CPE) is an important virulence factor for food poisoning and non-food-borne gastrointestinal diseases in humans. Although CPE production is strongly regulated by sporulation, it is still entirely unclear at the molecular and genetic level how the process of sporulation regulates CPE synthesis. The initiation of sporulation in *Bacillus subtilis* is controlled by the phosphorylation state of the sporulation transcription factor, Spo0A. *B. subtilis* Spo0A has been shown to bind to specific sequences (0A box) in the DNA upstream of the promoters it positively regulates and downstream of the promoters it negatively regulates. Our recent *spo0A* knock-out studies indicated that Spo0A is essential for spore formation and CPE production in *C. perfringens*. Since a putative 0A box is present in the upstream of *cpe*, in this study we examined whether *C. perfringens* Spo0A (*Cp*-Spo0A) can bind to *cpe* promoter DNA *in vitro*. Electrophoretic mobility shift assay, using purified C-terminal domain of *Cp*-Spo0A, demonstrated that Spo0A can bind specifically to the promoter DNA of *cpe*, suggesting that *Cp*-Spo0A can play a direct role in CPE synthesis by activating the transcription of *cpe*. The presence of Spo0A-regulated sigma E (s^E)-or s^K-dependent promoters in the upstream of *cpe* also suggest that s^E and s^K might have a direct role in regulating *cpe* transcription. To test this hypothesis, in this study we constructed a knock-out mutant of s^E encoding gene (*sigE*) in *C. perfringens*. When the sporulation and CPE production capabilities of *sigE* mutant were compared with that of its parent strain, *sigE* mutant was completely deficient in sporulation and CPE production. The lack of CPE production in *sigE* mutant was due to the lack of *cpe* transcription. Collectively, our results suggest that Spo0A can regulate CPE synthesis in two ways i) by directly activating the transcription of *cpe* and ii) by transcriptionally activating the genes encoding sporulation-specific s factors, which, in turn, initiate the transcription of *cpe*. Further research on localization of Spo0A-binding region in the upstream of *cpe*, and isolation and characterization of *sigK* mutant are currently underway to fully understand the mechanism Spo0A-regulated CPE synthesis in *C. perfringens*.

THE MECHANISM OF CONJUGATION IN *C. perfringens*

T L Bannam, W L Teng, J A Parsons, D Bulach, D Lyras, R Poon, M Hughes, V Adams, R J Devenish and J I Rood

Australian Research Council Centre of Excellence in Structural and Functional Microbial Genomics and Victorian Bioinformatics Consortium, Department of Microbiology, Monash University, Victoria 3800, Australia.

Detailed molecular analysis of the tetracycline resistance plasmid pCW3 from *C. perfringens* has shown that the 47,263 bp plasmid encodes 51 putative genes and represents the prototype of a unique family of conjugative antibiotic resistance and virulence plasmids. Deletion and transposon mutagenesis studies identified a unique *rep* gene that was required for plasmid maintenance in *C. perfringens*. A conjugation-related region on pCW3 was identified and designated as the *t*ransfer *c*lostridial *p*lasmid (*tcp)* locus. It contained eleven putative genes, five of which encoded proteins with low level similarity to proteins from the conjugation region of the integrative conjugative element Tn*916* from *Enterococcus faecalis,* although the arrangement of these genes was different. Functional genetic studies demonstrated that three of the genes in the pCW3 *tcp* locus, *tcpA, tcpF* and *tcpH*, were essential for the conjugative transfer of pCW3. Studies on the putative transmembrane protein TcpH, which is a potential component of the transfer pore, have shown that the first 581 amino acids of the 832 amino acid protein are required for its function. In addition, protein-protein interaction studies using the bacterial two-hybrid system have suggested that TcpH interacts both with itself and with TcpC. Comparative analysis confirmed that the *tcp* locus was not confined to pCW3. The complete locus with only minor variation was also present on other large plasmids from *C. perfringens*, including other resistance plasmids, the enterotoxin plasmids, one of which has been shown to be conjugative, and the major lethal toxin plasmids from type C and type D strains. Further studies have shown that two -toxin plasmids are also conjugative. These results have significant implications for plasmid biology as they provide evidence that *C perfringens* conjugative plasmids encoding either major virulence genes or antibiotic resistance determinants utilise the same conjugation machinery.

CONSTRUCTION AND TRANSCRIPTIONAL ANALYSIS OF *Clostridium difficile* RESPONSE REGULATOR MUTANTS

J. R. O Connor[1], D. Lyras[1], V. Adams[1], D. Powell[2], J. K. Cheung[1], J. Hinds[3], K. A. Farrow[1] and J. I. Rood[1,2].

[1]Australian Bacterial Pathogenesis Program, Department of Microbiology, Monash University, Vic. 3800, Australia, [2]Victorian Bioinformatics Consortium, Monash University, Vic. 3800, Australia, [3]B GS Group, St. George's Hospital Medical School, London, United Kingdom.

Clostridium difficile is responsible for nosocomial diseases such as antibiotic associated diarrhoea, and the potentially fatal pseudomembranous colitis. Recently new, epidemic-causing strains with an increased ability to cause serious illness have emerged. Factors affecting the onset, development and severity of these diseases are not well understood because the use of conventional genetic techniques to study *C. difficile* has proven to be extremely difficult. Although the organism is not amenable to transformation, some mobilisable plasmids can be introduced, at low frequency, by conjugation from an appropriate *E. coli* donor strain. Until now, no reproducible method existed for the targeted disruption of genes of interest. In this study, a reproducible method for the construction of chromosomal mutants by homologous recombination was developed and used to mutate two response regulator genes, *virR* and *virT*. Microarray and quantitative real time RT-PCR analysis of the resultant mutants showed that the *C. difficile* VirR protein directly and positively regulates the expression of at least four genes in *C. difficile*, including putative genes that may be involved in quorum sensing. Further investigation revealed that *C. difficile* VirR behaves in a very similar manner to *C. perfringens* VirR (a transcriptional regulator of virulence and house keeping genes) at the molecular level. These response regulator mutants are the first such mutants to be constructed in a virulent strain of *C. difficile*. The development of this methodology will have major implications for genetic studies in *C. difficile* as it will significantly enhance our ability to use molecular approaches to develop a greater understanding of the processes involved in the ability of *C. difficile* to cause human disease.

ANALYSIS OF PROTEINS INVOLVED IN THE OXIDATIVE STRESS RESPONSE OF *CLOSTRIDIUM ACETOBUTYLICUM*

H. Bahl, O. Riebe, and F. Hillmann

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

The strict anaerobe *Clostridium acetobutylicum* survives short periods of aeration despite the lacking of the functional superoxide-dismutase/catalase system of aerobes. This could be confirmed by live/dead staining experiments. Therefore, an alternative system must exist for detoxification of reactive oxygen species (ROS). All genes for a superoxide-reductase dependent pathway proposed for *Desulfovibrio vulgaris* are also present in *C. acetobutylicum*. Using purified desulfoferrodoxin and rubredoxin of *C. acetobutylicum* we were able to reconstitute a NAD(P)H-dependent reduction of superoxide to H_2O_2 *in vitro*. The involvement of one of the several rubrerythrins of *C. acetobutylicum* (two normal Rbrs and a Rbr in which the order of the rubredoxin and the ferritin-like domains is reversed) in the detoxification of H_2O_2 is not clear yet. The revRbr is encoded by a duplicated gene (*rbr3A*, *rbr3B*) and is identical to the heat shock protein Hsp21. In agreement with the proposed function of rubrerythrins in ROS detoxification increased transcript levels of *rbr3AB* have been found not only after a heat shock but also after oxidative stress. Northern Blot analysis indicated that the transcription of the *rbr3AB* operon is transiently increased also in the presence of various environmental stress conditions. Therefore, the reverse rubrerythrin can be regarded as a member of a general stress regulon. By primer extension analysis, a stress inducible transcription start point was identified upstream of *rbr3A*. The promoter region has only limited similarity to the consensus promoter sequence of Gram-positive bacteria. Between this promoter and the initiation codon of *rbr3A* an inverted repeat was found which is present also in front of several other genes in the genome of *C. acetobutylicum,* involved either in iron homeostasis or in the defence against oxidative stress. The regulatory role of this conserved element and the promoter are being currently analyzed.

SEQUENCING AND DIVERSITY ANALYSIS OF THE ENTEROTOXIN-ENCODING PLASMIDS IN CLOSTRIDIUM PERFRINGENS TYPE A NONFOODBORNE HUMAN GASTROINTESTINAL DISEASE ISOLATES

Kazauki Miyamoto1, Derek J. Fisher2, Jihong Li2, Sameera Sayeed2, Shigeru Akimoto1, Bruce A. McClane2
1Department of Microbiology, Wakayama Medical University School of Medicine, Wakayama, Kimiidera811-1, Japan.
2Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA.

Enterotoxin-producing C. perfringens type A isolates are an important cause of food poisoning and non-food-borne human gastrointestinal diseases, e.g., sporadic diarrhea (SPOR) and antibiotic-associated diarrhea (AAD). The enterotoxin gene (cpe) is usually chromosomal in food poisoning isolates but plasmid-borne in AAD/SPOR isolates. Previous studies determined that type A SPOR isolate F5603 has a plasmid (pCPF5603) carrying cpe, IS1151, and the beta2 toxin gene (cpb2), while type A SPOR isolate F4969 has a plasmid (pCPF4969) lacking cpb2 and IS1151 but carrying cpe and IS1470-like sequences. By completely sequencing these two cpe plasmids, the current study identified pCPF5603 as a 75.3-kb plasmid carrying 73 open reading frames (ORFs) and pCPF4969 as a 70.5-kb plasmid carrying 62 ORFs. These plasmids share an ~35-kb conserved region that potentially encodes virulence factors and carrying ORFs found on the conjugative transposon Tn916. the 34.5-kb pCPF4969 variable region contains ORFs that putatively encode two bacteriocins and a two-component regulator similar to VirR/VirS, while the ~43.6-kb pCPF5603 variable region contains a functional cpb2 gene and several metabolic genes. Diversity studies indicated that other type A plasmid cpe+/IS1151 SPOR/AAD isolates carrying a pCPF5603-like plasmid, while other type A plasmid cpe+/IS1470-like SPOR/AAD isolates carrying a pCPF4969-like plasmid. Furthermore, Tn916-related ORFs similar to those in pCPF4969 (known to transfer conjugatively) were detected in the cpe plasmids of other type A SPOR/AAD isolates, as well as in representative C. perfringens type B to E isolates carrying other virulence plasmids, possibly suggesting that most or all C. perfringens virulence plasmids transfer conjugatively.

EXPLOITATION OF THE GENOME SEQUENCE OF PROTEOLYTIC *CLOSTRIDIUM BOTULINUM*

M.W. Peck, D.R. Mason, C. Pin and A.T. Carter

Institute of Food Research, Colney, Norwich, NR4 7UA, UK.

The complete genome sequence of the Hall A strain of proteolytic *Clostridium botulinum* (ATCC 3502) was determined at the Sanger Institute, and is available from their website (). Within the 3.9Mb genome there were 3648 predicted open reading frames. There was also a 16.3kb plasmid that encoded a bacteriocin (boticin). The annotated version of the genome sequence was used to produce a microarray of PCR products. These ranged in size from 100-500 base pairs. Due to repeat sequences and the very low G+C content (28.2%) not all ORFs were suitable for PCR amplification. The current microarray contains 3433 DNA species, corresponding to approximately 94% of all chromosomal ORFs, in addition to 18 probes from the plasmid. The array includes extra features that will facilitate the genotyping of new strains isolated from, for example, disease outbreaks. These include probes for all neurotoxin genes and neurotoxin-associated genes (e.g. haemagglutinin, NTNH, cntR, p47). Comparative genomic indexing studies showed that the sequenced Hall A strain contains two prophages not found in other strains. It was apparent that other strains of proteolytic *C. botulinum* and *C. sporogenes* were closely related (92% for other type A strains, 89% for type B strains, 90% for type F strains, and 84% for strains of *C. sporogenes*). DNA from strains of other clostridia (non-proteolytic *C. botulinum* and *C. difficile*) hybridised more weakly confirming that they are members of evolutionarily distinct groups.

We are grateful for funding from the competitive strategic grant of the BBSRC.

COMPARATIVE PHYLOGENOMICS OF *CLOSTRIDIUM DIFFICILE* REVEALS CLADE SPECIFICITY AND MICROEVOLUTION OF HYPERVIRULENT STRAINS

R. A. Stabler[1] B. W. Wren[1]

[1]Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London, WC1E 7HT. UK

Clostridium difficile is the most frequent cause of nosocomial diarrhoea worldwide and recent reports have suggested the emergence of a hypervirulent strain (epidemic BI/NAP1/027 strains) in North America and Europe. A *C. difficile* PCR product microarray based on the *C. difficile* 630 strain was designed and manufactured and a collection of 75 well-characterised isolates comprising hypervirulent, toxin variable and animal strains were analysed. Comparative phylogenomics using a Bayesian algorithm was used to model the phylogeny of *C. difficile*. The analysis identified four distinct statistically supported clusters comprising a hypervirulent clade, a toxin A-B+ clade, and two clades with human and animal isolates. Genetic differences among clades revealed several genetic islands relating to virulence and niche adaptation, including antibiotic resistance, motility, adhesion and enteric metabolism. The data has provided insight into the possible origins of *C. difficile* and its evolution plus potential virulence determinants that may have implications in disease control strategies.

THE GENOME SEQUENCE OF *CLOSTRIDIUM BOTULINUM* TYPE C NEUROTOXIN-CONVERTING PHAGE AND THE MOLECULAR MECHANISMS OF UNSTABLE LYSOGENY

Y. Sakaguchi[1], T. Hayashi [2], K. Kurokawa[3], K. Nakayama[2], M. Ohnishi[2], J-C. Lee[1], J-h. Cui[1], H-J. Hwang[1], Y. Yamamoto[1], and K. Oguma[1]

[1]Department of Bacteriology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan
[2]Division of Microbiology, Department of Infectious Diseases, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan
[3]Laboratory of Comparative Genomics, Graduate School of Information Science, Nara Institute of Science and Technology, Nara, Japan

Botulinum neurotoxins (BoNTXs) produced by *Clostridium botulinum* are one of the most poisonous-substance known. BoNTXs are classified into seven groups (A, B, C1, D, E, F, and G; BoNTX/A to BoNTX/G) based on the antigenicity of their BoNTs. Among the seven groups of BoNTXs, genes for type C1 and D toxins (BoNTX/C1 and D) are carried by host specific bacteriophages. The gene for exoenzyme C3 also resides on these phages. Very little information is available about toxin production and genomic structures of type C1 and D strains. However, some previous studies have reported that BoNT/C1 and D molecules are antigenically related. In this study, we determined the complete genome sequence of c-st obtained from type C strain, C-Stockholm. The c-st genome has characteristics such as a linear double-stranded DNA of ~186 kbp in size with 404-bp terminal direct repeats and a G+C content of 26.2%. We identified 198 potential protein-cording regions on the genome of c-st, including genes for BoNTX/C1, C3 exoenzyme and a number of genes homologous to those of a *Bacillus subtilis* bacteriophage SPβ. Very exceptionally, as a viable bacteriophage, a unique feature of the c-st phage is that it encodes many insertion sequence elements. In addition, molecular structure of the phage genome in the c-st lysogen revealed that c-st is lysogenized as a circular plasmid prophage. These features are likely responsible for the unstable lysogeny of BoNTX-transducing phages which has historically been called as pseudolusogeny . Furthermore, PCR scanning analysis of other BoNTX/C1 and D phages based on the c-st sequence further revealed that BoNTX phages comprise a divergent phage family, probably generated by exchanging genomic segments among BoNTX phages and their relatives.

THE INTRA- AND EXTRACELLULAR PROTEOME OF *CLOSTRIDIUM ACETOBUTYLICUM* UNDER PHOSPHATE LIMITATION

Schwarz, K.[1], Fiedler, T. [1], Mikkat, S. [2], Bahl, H. [1], Glocker, M. [2], Fischer, R.-J. [1]

[1] Institute of Biological Science/ Microbiology, University of Rostock, Albert-Einstein-Stra§e 3, 18051 Rostock

[2] Proteome Center Rostock, Institute of Immunology, University of Rostock, Schillingallee 69, 18055 Rostock

In *Clostridium acetobutylicum* phosphate limitation is known to be one of the signals triggering a radical physiological alteration of the metabolism yielding into a stable production of solvents instead of acids («solvent shift‘). Furthermore, sporulation, morpholigical changes like mobility and cell shape, as well as synthesis of granulose seem to be tightly connected to phosphate limitaion. To analyze the overall regulation pattern of this complex network two-dimensional polyacrylamide gel electrophoresis (2D PAGE) of the intra- and extracellular proteins were carried out. A Standard Operating Procedure (SOP) for sample preparation and 2D PAGE was developed. Spots were identified using MALDI/TOF analysis. Out of the 342 detected extracellular proteins 242 could be identified. They mainly belong to groups like proteases, hydrolases, cell wall associated, and substrate binding proteins. Intracellularly 736 spots were detected, about 500 were identified so far. As expected lots of them can be referred to metabolic pathways, protein biosynthesis, and protein folding. Comparisons of samples obtained from acid and solvent cells under phosphate limitation as well as surplus of Pi give information about differentially expressed proteins. Up to now only very few extracellular proteins like PstS and GlpQ were found to be induced under phosphate limitation.

BIOLOGICAL SIGNALING TO GENE EXPRESSION IN CLOSTRIDIUM PERFRINGENS.

Kaori Ohtani, Kousei Tanaka, Kenkei Hasatani, Kazuhiro Miwa, Atsuko Isu, Tohru Shimizu
Grad. Sch.Med.Sci.,Kanazaw Univ.,Japan

Clostridium perfringens causes clostridial myonecrosis or gas gangrene in humans by producing numerous extracellular toxins and enzymes. So far, it is not clear when and how *C. perfringens* produces these toxins rapidly and effectively, especially in infectious condition. To examine the global regulatory network that governs pathogenicity, a transcriptome analysis was carried out using DNA microarray for *C. perfringens*. We have two main focuses. First, how the two-component VirR/VirS system regulates the toxin genes and what is the signal of the sensor protein VirS. We compared gene expression patterns of wild type strain, TS133(*virRS* mutant) and TS133/405 (*virRS* complemented strain), TS140 (VR-RNA mutant) and TS140/VR-RNA using DNA microarrays and identified the VirRS-VR-RNA regulons which was classified into 6 groups. Next, how *C. perfringens* recognizes human body from other environment. We examined the effects of various materials (such as blood, serum, or catecholamine) on the expression of toxin genes in *C. perfringens*. We added these materials to *C. perfringens* cultured in minimum medium and prepared RNA for microarray analysis. Expression of many genes including toxin genes was altered by adding blood or serum. And when we added the horse serum to the culture, there was difference in expression patterns between human serum and horse serum. Furthermore, other bacteria that were co-cultured with *C. perfringens* also stimulated the gene expression in *C.perfringens*. Now we are trying to examine how these materials stimulate the gene expression and/or regulatory networks in *C. perfringens*.

REGULATION OF CLOSTRIDIAL TOXINS BY ALTERNATIVE SIGMA FACTORS.

B. Dupuy[1], S. Raffestin[2], S. Matamouros[1], N. Mani[3], M. R. Popoff [2] and A. L. Sonenshein[3]

[1]Unit_ de G_n_tique Mol_culaire Bact_rienne, Institut Pasteur, Paris, France;

[2]Unit_ des Bact_ries Ana_robies et Toxines, Institut Pasteur, Paris, France;

[3]Department of Molecular Biology and Microbiology, Tufts University School of Medicine,Boston, MA 02111 USA.

Most of the diseases caused by pathogenic *Clostridium*, such as tetanus, botulism, gas gangrene and pseudomembranous colitis, are mainly due to the production of potent extracellular toxins. The biochemical mechanisms of action of *Clostridium* toxins have been extensively studied in the past ten years. However, detailed information about transcriptional expression of toxin genes has only recently emerged. One breakthrough in this study came from the analysis of toxin gene synthesis in *Clostridium difficile.* In this bacterium TcdR, a 22 kDa protein, acts by interacting with RNA polymerase core enzyme and directs transcription from the toxin promoters and therefore functions as an alternative sigma factor. Proteins similar to TcdR have been found in other *Clostridium* species, including BotR in *Clostridium botulinum*, TetR in *Clostridium tetani* and UviA in *C. perfringens*. It has recently been demonstrated that these proteins are required, as alternative sigma factors, for transcription of the neurotoxin genes in *C. botulinum* and *C. tetani* and a UV-inducible bacteriocin gene in *C. perfringens* respectively. The majority of the s factors in eubacteria belong to the s^{70} - family, which can be divided into four structurally and functionally related groups. The TcdR-related s factors have some similarities with members of the ECF sigma factors (Group 4), but they differ sufficiently in structure and function. Thus, it was suggested that TcdR-related s factors form a new group, distantly related to the s^{70} — family. In support of this idea, we showed that TcdR-related s factors are sufficiently similar that they are able to substitute for each other *in vivo* and *in vitro,* suggesting a high degree of functional conservation. This correlates with near-identity in the —35 recognition sequences of promoters recognized by all of these sigma factors and the conservation of the region 4.2 of the s factor proteins. Thus, it appears that a common molecular mechanism, involving a new subgroup of the s^{70} - family of RNA polymerase sigma factors (Group 5), controls the production of important toxins and a bacteriocin in several major pathogenic *Clostridium* species.

DNA CURVATURE AND GENE REGULATION IN C. PERFRINGENS

A. Okabe[1], S. Miyata[1], E. Tamai[1], S. Shimamoto[2], Y. Taniguchi[1] and O. Matsushita[1]

[1]Department of Microbiology, [2]Department of Signal Transduction Sciences, Faculty of Medicine, Kagawa University, 1750-1 Miki-cho, Kita-gun, Kagawa 761-0793, Japan.

DNA curvature, intrinsically bent DNA, is formed in regions where special sequence motifs such as A-tracts and GC-types elements are periodically repeated. Curved DNA sequences located in the vicinity of promoters have been implicated mostly as the pivotal DNA element stimulating the activity of the core promoter through their interaction with RNA polymerase (RNAP) or other factors. We found different types of DNA curvature in or around the core promoters of three *Clostridium perfringens* genes. We have conducted studies to elucidate the molecular mechanisms involved in the stimulatory effects of DNA curvature on expression of these genes. In the first example, three phased A-tracts locating immediately upstream of the core promoter of a phospholipase C gene stimulates the initial binding of RNAP in a low temperature-dependent manner. We show that the ? subunit of RNAP binds to the minor grooves of the phased A-tracts through its C-terminal domain with increased affinity at low temperature. Second, we demonstrate a strong stimulatory effect of DNA curvature on the transcription from a ferredoxin gene (*fdx*). The DNA curvature of the *fdx* gene is formed by five phased A-tracts extending from upstream to downstream of the -35 region and enhances the promoter activity possibly due to the combined effects of the three A-tracts upstream of the promoter and the two within the promoter. Finally, we show a compensatory effect of DNA curvature, which confers high levels of gene expression from a weak promoter of an epsilon-toxin gene (*etx*). Although there is no canonical $?^{70}$ -35 DNA sequence nor extended TG motif in the *etx* promoter, it can be transcribed efficiently possibly due to the function of DNA curvature residing at two different regions, one being between -19 and +7 and the other being between + 18 and +52 relative to the transcriptional start site. We show involvement of these two regions in the stimulation of the *etx* promoter by deletion experiments.

TCDC INHIBITS TOXIN SYNTHESIS IN *CLOSTRIDIUM DIFFICILE*

S. Matamouros[1], R. Govind[2] and B. Dupuy[1]

[1]Unit de G n tique Mol culaire Bact rienne, Institut Pasteur, 28 Rue du Dr. Roux, 75724 Paris Cedex 15, France

[2]Department of Microbiology and Immunology, School of Medicine, Texas Tech University Health Sciences Center, Lubbock, Texas 79430

Infection with *Clostridium difficile* can produce a wide spectrum of clinical manifestations ranging from antibiotic associated diarrhoea to pseudomembranous colitis, a potentially lethal disease. Pathogenic strains produce two large protein toxins (TcdA and TcdB) that have been identified as major virulence factors. In fact, the level of toxin production *in vivo* is one of the determinant factors in the severity of the disease.

The toxin genes (*tcdA* and *tcdB*) are encoded in a pathogenicity locus (PaLoc) which also includes three accessory genes: *tcdC*, *tcdE* and *tcdR*. TcdE is a holin-like protein. TcdR, an alternative sigma factor is able to interact with the RNA polymerase core enzyme and stimulate transcription of both toxins as well as itself. The gene at the right end of the PaLoc, *tcdC*, has an interesting expression pattern. It is expressed at high level during rapid exponential growth and is shut off as cells enter stationary phase. The fact that this pattern is the inverse of that for *tcdR* and the toxin genes has led to the suggestion that TcdC is a negative regulator of toxin gene expression. However, apart its transcription profil there lacked other relevant data to support this idea.
TcdC is a small acidic protein without any conserved DNA binding motif. It is able to form dimers and it presents on its N-terminal region a putative transmembrane domain.

The subcellular localization of TcdC was determined using specific anti-TcdC antibodies which confirmed it to be a membrane associated protein.
We show that TcdC negatively regulates *C. difficile* toxin synthesis by inhibiting transcription of *tcdA* and *tcdB* both *in vivo* as well as *in vitro*.
In addition, the protein was found to be mainly present during exponential growth which correlates well with the transcription pattern already observed by others and it strengthens the role of TcdC as a negative regulator of the toxin synthesis in *C. difficile*.

REGULATION OF *CLOSTRIDIUM DIFFICILE* TOXIN SYNTHESIS

S. S. Dineen and A. L. Sonenshein

Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Ave, Boston, MA 02111, USA.

Clostridium difficile is the causative agent of antibiotic-associated colitis, one of the most common infections acquired by hospital in-patients. In severe cases, this infection can lead to pseudomembranous colitis. *C. difficile* produces two toxins, TcdA and TcdB, that are primarily responsible for the intestinal injury seen in the disease. TcdR, an alternative sigma factor of RNA polymerase encoded within the pathogenicity locus of *C. difficile*, is the direct regulator of toxin gene expression. The mechanisms responsible for regulation of *tcdR* expression have yet to be discovered. *C. difficile* encodes a protein with strong similarity to *Bacillus subtilis* CodY, a global transcriptional regulator of early stationary phase genes. We have shown through gel shift and DNase I footprinting experiments that *C. difficile* CodY interacts strongly with the *tcdR* promoter region, and to a lesser extent with the *tcdA* and *tcdB* promoters. Binding was enhanced in the presence of GTP and/or a mixture of branched-chain amino acids, which are known positive effectors of CodY binding to target promoters in *B. subtilis*. CodY was also shown to interact with the promoter region of the putative negative regulator of toxin expression encoded within the *C. difficile* pathogenicity locus, *tcdC*. Our results suggest that CodY may act as both a repressor and an activator in regulating toxin synthesis.

THE *tcdC* GENE OF *C. DIFFICILE* VARIANTS INCLUDING THE EPIDEMIC BI STRAIN CONTAINS STOP CODONS AND DELETIONS

F.Siddiqui[1, 2], G.Vedantam [2], D. Gerding[1, 2], S. Johnson[1, 2]

[1]Hines V.A Hospital, Hines, Illinois 60141. USA
[2]Loyola University Medical Center, Maywood, Illinois 60153. USA

A recently described toxin variant strain of *Clostridium difficile* has caused multiple hospital outbreaks across the North America and Europe. This strain, designated REA Group BI is also characterized as toxinotype (ttype) III, and contains a deletion in the *tcdC* gene. Although the function of this gene is unknown, it may be involved in down regulation of toxins A & B expression. We screened our collection of toxigenic *C. difficile* isolates and representative BI strain isolates for *tcdC* gene deletions and other changes in comparison to the recently sequenced strain, 630.

A set of primers that covers the entire tcdC gene plus the flanking regions on either side was used for amplification in 3 epidemic BI strains and 5 other *C. difficile* strains. The 900 bp PCR product was sequenced and compared to the control *tcdC* sequence from isolate 630.

REA type	ttype	tcdC deletion	Early STOP codon
I1	III	18bp	YES (nt 213)
N1	0	18bp	NO
AH1	?	36 bp	YES (nt 203)
BI-1,BI-6, BI-8	III	18 bp	YES(nt 207)
BK1	V	39 bp	YES (nt 297)
BW1	XXII	18 bp	NO

All deleted regions occurred downstream of the stop codons. *tcdC* deletions among the strains studied did not correlate with REA typing or toxinotyping. If changes in *tcdC* result in increased toxin production and virulence in the epidemic BI strain or other strains, premature stop codons may be more important than the deletions. Further characterization of tcdC is required to elucidate its role in wild type *C. difficile* strains and the effects of its polymorphisms seen in the variant strains, especially the epidemic BI strains.

POSTER PRESENTATIONS

Session I

Thursday, June 22nd 2006

16:30-18:00

B-62

LOW-LEVEL VANCOMYCIN RESISTANCE IN CLOSTRIDIUM HASTIFORME (RECLASSIFIED AS TISSIERELLA PRAEACUTA) AND ITS RELATIVES

C. Alauzet[1], L. Lemee[2], A. Dhalluin[2], J.P. Carlier[3], A. Lozniewski[1], J.L. Pons[2] and F. Mory[1].

[1]Laboratoire de Bact_riologie, UMR CNRS 7565, Facult_ de M_decine, 54505 Vandoeuvre-les-Nancy Cedex, France, [2]Groupe de Recherche sur les Antimicrobiens et les Microorganismes (G.R.A.M.), U.F.R. M_decine-Pharmacie, 76183 Rouen Cedex, France, [3]Centre National de R_f_rence des Ana_robies, Institut Pasteur, 75724 Paris Cedex 15, France.

Clostridia are generally considered susceptible to glycopeptides, as most of Gram-positive bacteria. However, low-level intrinsic resistance to vancomycin has been reported in *Clostridium innocuum* and acquired *vanB* resistance has been recently documented in other *Clostridium* species. We observed that some clinical isolates phenotypically related to *C. hastiforme* (recently reclassified as *Tissierella praeacuta*) exhibited low-level resistance to vancomycin. This prompted us to investigate the susceptibility of 20 clinical isolates phenotypically related to *C. hastiforme* and two reference strains (*C. hastiforme* ATCC 33268 and *T. praeacuta* ATCC 25539) to vancomycin by the agar dilution method. Low-level vancomycin resistance (MICs = 8 to 16 mg/l) was observed for 16 clinical isolates and the two Type strains, whereas four clinical isolates remained susceptible (MICs = 1 mg/l). We further characterize the taxonomic position of these isolates and their phylogenetic relationships by sequencing of 16S rDNA and triosephosphate isomerase encoding gene (*tpi*). Clustering from 16S rDNA as from *tpi* sequences provided the delineation of two distinct lineages among clinical isolates, those with vancomycin MIC 8 mg/l, and those with MIC = 1 mg/l. These results suggest that the strains phenotypically related to *C. hastiforme* but susceptible to vancomycin (MICs = 1 mg/l) were phenotypically misidentified and clustered together with the *C. subterminale* Type strain. No amplification product was observed by PCR with primers specific for resistance genes *vanA* to *vanG* suggesting that the low-level vancomycin resistance may be due to genetic determinants other than those already identified. Moreover, these results highlight that molecular methods may aid to unambiguously identify certain strains belonging to this very heterogeneous genus.

RECOMBINANT ANTIBODIES AGAINST *Clostridium difficile* TOXIN A

M. A. Alkhalifah, R. Aitken.

Division of Infection and Immunity, IBLS, Glasgow University.

Clostridium difficile produces two potent toxins (Toxin A and Toxin B) that work synergistically to generate damage at the intestinal epithelium. Tissue injury is initiated when the carboxy-terminal region of Toxin A recognizes receptor(s) at the epithelial surface. The cascade of reactions that follow culminate in the destruction of the cell cytoskeleton and cell rounding. In order to learn more of the organisation and mode of action of the carboxyl-terminal region of Toxin A, we have isolated recombinant antibodies against this part of the protein using phage display. The ability of these antibodies to block toxin action has also been evaluated.

The carboxy-terminal region of Toxin A was expressed as a recombinant protein (rTA) and purified by affinity chromatography. The activity of the protein in ELISA, immunofluorescence and haemagglutination assays was consistent with its behaviour as the receptor-binding component of the toxin. rTA was then used to isolate scFv antibodies from the Tomlinson I and J libraries using conventional bio-panning methods. The progress of panning was monitored by ELISA, confirming the progressive enrichment of rTA-specific scFvs through 3 rounds of selection. Those scFvs that showed strongest reaction with rTA in phage ELISA were sequenced, expressed as soluble antibodies and purified by nickel chelation chromatography for further characterisation. Across the panel of anti-rTA scFvs, common amino acid motifs could be identified in the CDRs but no two sequences were identical. Threonine and serine residues occurred commonly in CDRs carried by the light chain components. Competitive ELISA suggested that each scFv recognized a unique epitope with only slight evidence of cross-reaction. These findings are being further developed through the creation of fusion proteins comprising defined regions of the rTA sequence to allow detailed mapping of epitopes recognized by the scFvs. The biological activity of the recombinant antibodies was assessed in challenge studies employing F9 cells in culture and native Toxin A. Pre-incubation of the toxin with purified scFv led to a consistent delay in the rounding response. However, individual or mixed scFvs were unable to protect against toxin-mediated morphological change.

DETECTION OF NOVEL GENETIC ARRANGEMENTS FOR ERYTHROMYCIN AND TETRACYCLINE RESISTANCE IN *CLOSTRIDIUM DIFFICILE* CLINICAL ISOLATES.

F.Barbanti,P.Spigaglia and P.Mastrantonio

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

The study of the resistance to erythromycin and tetracycline in *Clostridium difficile* is of interest to find out whether, as many other intestinal bacteria, this microorganism is able to exchange and acquire genetic determinants and elements competent for antibiotic resistance. In particular, an *erm*(B) gene carried by the transposon *Tn5398* and a *tet*(M) carried by the conjugative *Tn5397* have been described and detected in many isolates. Among a sample of one hundred *C.difficile* strains isolated in the years 1986-2002 we found that many strains of different clonal origin showed different erythromycin and tetracycline resistance determinants and/or different genetic arrangements of the elements carrying the genes. Among those showing different genetic arrangements of *erm*(B) determinant, one was able to transfer the erythromycin resistance to a recipient strain belonging to a different species, *Butyrivibrio fibrisolvens* isolated from cattle rumen. In strains of more recent isolation, we detected the presence of *Tn916*-like elements, never found before in *C.difficile* clinical isolates. The molecular analysis of these elements showed they may have different genetic structures and carry new *tet*(M) alleles. Moreover, analysing all the strains harbouring both *erm*(B) and *int*, the latter used as the marker for *Tn916*-like element, we found one strain with a linkage of these two genes in the same element, indicating for the first time that *C.difficile* has acquired the ability to cluster antibiotic resistance genes and that *Tn916*-like elements are involved in this process. Finally, the *tet*(W) gene, a tetracycline resistance gene recently identified in anaerobic commensal bacteria from animals and humans, was detected in a clinical isolate of *C.difficile* providing further evidence of the spread of resistance determinants among gastrointestinal bacteria.

EVALUATION OF DIAGNOSTIC METHODS FOR THE DETECTION OF *CLOSTRIDIUM DIFFICILE* IN STOOL SAMPLES

BE Citil,[1] B Levent,[1] R Kayali,[1] H Arslan,[2] T Togan,[2] F Aktas,[3] M Dizbay,[3] S Kacar,[4] A Azap,[5] B Dokuzoguz,[6] DN Ozucelik,[7] A Karademir,[8] A Gozalan,[1] B Esen[1]

[1]Refik Saydam National Hygiene Center, [2]Faculty of Medicine, Baskent University, [3]Faculty of Medicine, Gazi University, [4]Yuksek Ihtisas Hospital, [5]Faculty of Medicine, Ankara University, [6]Ankara Numune Educational and Research Hospital, [7]Faculty of Medicine, Hacettepe University, and [8]Bayindir Hospital, Ankara, Turkey

Clostridium difficile associated diarrhea (CDAD) is the most important cause of nosocomial diarrhea. The aim of this study was to detect *C. difficile* toxin and bacteria by using different diagnostic tests in stool samples obtained from the patients with diarrhea.

One hundred seventy seven stool samples were collected from the patients complaining of diarrhea shorter than 2 months after antibiotic or anti-neoplastic therapy, and tested by Premier toxin A/B ELISA (Meridian Bioscience, Ohio, USA), rapid Triage® *C. difficile* panel (Biosite, CA, USA), and culture methods. One hundred thirty one of the samples (74.0%) were negative by using all test methods. Forty-six of the samples (25.9%) were positive for *C. difficile* toxin by using toxin A/B ELISA. Of 46 ELISA positive samples, eight were toxin A positive and 14 common antigen positive by rapid Triage panel. *C. difficile* was isolated from 15 (8.5%) of the 177 stool samples. Bacterial isolates were analysed for toxin A and B detection by ELISA and all was toxin positive. Additionally, in three stool samples, *Shigella flexneri*, *Salmonella* Typhimurium and *Campylobacter jejuni* was isolated by aerobic culture.

Our results show that combination of different diagnostic methods for detection of *C. difficile* toxin and bacteria could be useful for accurate diagnosis and give more information. Toxin A/B test is suitable for diagnosis of CDAD and also detects infections developed with toxin A negative, toxin B positive strains.

CHARACTERISATION OF MUTANT TOXIN A (TCDA W101A) FROM CLOSTRIDIUM DIFFICILE

R Gerhard, M Teichert, H Tatge, and I Just

Dept. of Toxicology, Hannover Medical School, 30625 Hannover, Germany

Clostridium difficile Toxin A (TcdA) is a bacterial single-chained glucosyltransferase (GT) of 308 kDa. It is causative for severe inflammation and of pseudomembranous colitis. TcdA transferes a glucose-moiety onto Rho-GTPases of target cells, and thereby blocks all downstream signaling. However, inhibition of Rho GTPases is not in accordance with pro-inflammatory signal transduction. Currently, additional glucosyltransferase-independent effects of TcdA are discussed in literature.

To address this question and to distinguish between glucosyltransferase-dependent and -independent effects, we generated the recombinant holo-TcdA and its mutant TcdA W101A. From the homolougus TcdB and TcsL it is known that mutation of tryptophane 102 leads to a >2.000-fold reduction of the GT-activity. The *in vitro* GT-activity of TcdA W101A (0.034 mol/mol*min) was reduced 350-fold compared to wild type TcdA (11.8 mol/mol*min). The GT-activity of the N-terminal fragments consisting of amino acids 1-1065 were identical to that of the corresponding holotoxins. In contrast to *in vitro* assay (350-fold reduced activity compared to wild type), there was an only 50-fold reduced activity in cell culture experiments. The cytopathic effect was studied by morphological changes of 3T3 fibroblasts as well as by measurement of the transepithelial electrical resistance of Caco-2 monolayers. Sequential [^{14}C]glucosylation of lysates from toxin-treated 3T3 fibroblasts indicated the same kinetics of Rho and Rac glucosylation by TcdA W101A when applied in a 50-fold concentration, compared to TcdA. To investigate the difference of the *in vitro* and intracellular GT-activity, we delivered the N-terminal catalytic fragments of toxins (ΔTcdA) into cells by circumventing the active endocytotic up-take process. Deliovery of ΔTcdA and ΔTcdA W101A into Caco-2 cells by electroporation revealed that the intracellular conditions but not the up-take process is causative for the increased activity of TcdA W101A in cellular context compared to cell-free systems.

Furthermore, application of the characterized TcdA W101A in equimolar and equipotent concentrations compared to wild type TcdA showed that the previously described up-regulation of RhoB was a cellular response to the intracellular GT-activity of TcdA.

LOCATION OF THE ENTEROTOXIN GENE IN STRAINS OF CLOSTRIDIUM PERFRINGENS ASSOCIATED WITH GASTROENTERITIS

K A Grant[1], S Kenyon[1], R Halford-Maw[1], E Pettitt[2], J McLauchlin[1],

1, Food Safety Microbiology Laboratory, Health Protection Agency Centre for Infections, 61 Colindale Ave, London, NW9 5HT, UK. (kathie.grant@hpa.org.uk)
2, Department of Microbiology, Chesterfield & North Derbyshire Royal Hospital NHS, Chesterfield, Derbyshire, S44 5BL, UK

Clostridum perfringens type A is a common cause of food poisoning and is also associated with non-food borne gastroenteritis including antibiotic associated, infectious and sporadic diarrhoea. The disease symptoms are due to an enterotoxin produced when the organism sporulates in the human small intestine. The *C. perfringens* entertoxin gene (*cpe*) has been shown to be located either on the chromosome or on one of two large plasmids and it is generally accepted that *C. perfringens* strains associated with food poisoning have a chromosomal *cpe* gene whilst strains isolated from non-food borne diarrhoea have a plasmid encoded *cpe* gene.

A published multiplex PCR assay was used to determine the location of the *cpe* gene in 107 strains of *C. perfringens* isolates associated with food borne illness and 16 strains associated with non-food borne illness. Thirty five percent of *C. perfringens* strains associated with food borne outbreaks in the UK were found to have a plasmid encoded *cpe* gene and 65 % were found to have a chromosomal *cpe* gene. All strains associated with non-food borne illness had the *cpe* gene located on one of two plasmids, as anticipated.

A significant number of food borne outbreaks of *C. perfringens* food poisoning were found to be caused by strains of *C. perfringens* carrying a plasmid encoded *cpe* gene. Since strains of *C. perfringens* with a chromosomal *cpe* and plasmid *cpe* genes have different physiological characteristics this may have a profound impact on their mode of transmission.

DETECTION OF CLOSTRIDIAL TOXIN GENES BY REAL TIME PCR ASSAYS

K A Grant[1], J Hunter[1], M Peck[2], S Stringer[2], I Nwafor[1], C Ohai[1] and J McLauchlin[1].

1, HPA Centre for Infections, 61 Colindale Ave, London, NW9 5HT, UK () 2, Institute of Food Research, Colney, Norwich, Norfolk, NR4 7UA.

The genus *Clostridium* produce a diverse range of toxins; some of which are directly responsible for human illness such as botulism, tetanus, gas gangrene and diarrhoea. Toxin detection is important for diagnosis and can be applied to clinical samples and foods, whilst toxin detection in pure cultures may be used for species identification and assessing the ability of the organism to cause disease. Although immunoassays have been used successfully to detect various clostridial toxins it is not always possible to induce toxin production in cultures in the laboratory; assays are not available for all toxins and if they do exist may be insufficiently sensitive to detect low toxin levels. The gold standard for toxin detection for many clostridial toxins remains the bioassay. However, bioassays are difficult to perform, expensive and involve the use of animals. In order to assist in the confirmation of diagnosis and to enhance outbreak investigations we have developed, evaluated and implemented the use of real time 5 endonuclease PCR assays for the detection of specific toxin genes from three clostridial species associated with human illness. The genes targeted are the *Clostridium perfringens* alpha and enterotoxin genes, *Clostrdium botulinum* neurotoxin genes A, B, E and F and the *Clostridium tetani* neurotoxin gene. These PCR assays have been assessed using DNA extracted from bacterial colonies, enrichment broth cultures and, where appropriate, with DNA from clinical and food samples. The assays are reliable, reproducible, sensitive and robust and have assisted in reducing the use of bioassays. This presentation will describe how these assays have improved confirmation of diagnosis and outbreak investigation of specific clostridial diseases.

THE ROLE OF HEALTHY HUMAN AS A RESERVOIR FOR DIFFERENT GENOTYPES OF *CPE* GENE- CARRYING *CLOSTRIDIUM PERFRINGENS*

A. Heikinheimo[1], M. Lindstr m[1], P. E. Granum[2] and H. Korkeala[1].

[1]Department of Food and Environmental Hygiene, University of Helsinki, Helsinki, Finland.

[2]Department of Food Safety and Infection Biology, Norwegian School of Veterinary Science, Oslo, Norway.

We found 18% prevalence of enterotoxin gene carrying (*cpe*$^+$) *Clostridium perfringens* type A in the feces of healthy food handlers by PCR and isolated the organism from 11 out of 23 PCR-positive individuals using hydrophobic grid membrane-colony hybridization. Several different *cpe* genotypes were recovered, the prevalence being 3.7% (plasmidial IS*1151*-*cpe*), 2.9%, (plasmidial IS*1470*-like-*cpe*), 0.7% (chromosomal IS*1470*-*cpe*) and 1.5% (unknown *cpe* genotype). Lateral spread of *cpe* between *C. perfringens* strains was evidenced by strains from the same human carrying IS*1470*-like-*cpe* but sharing no genetic relatedness in pulsed field gel- electrophoresis analysis. Our findings suggest that healthy human serves as a rich reservoir for *cpe*$^+$ *C. perfringens* type A and may play an important role in the etiology of gastrointestinal diseases caused by this organism. The results also indicate that human should be considered as a risk for contamination when *C. perfringens* type A food poisoning is at issue.

RIFAXIMIN FOLLOWING STANDARD THERAPY FOR BREAKING THE CYCLE OF MULTIPLE *C. DIFFICILE* DIARRHEA RECURRENCES

S. Johnson[1,2], C. Schriever[1], M. Galang[1], C.P. Kelly[3], D.N. Gerding[1,2]

[1]Loyola University Med. Ctr. and [2]Hines VA Hospital, Chicago, IL, USA
[3]Beth Israel Deaconess Medical Center, Boston, MA, USA

Recurrent *C. difficile*-associated diarrhea (CDAD) after successful treatment is common, but some patients have multiple recurrences (mCDAD). As an empiric strategy to interrupt this repeated cycle of CDAD recurrences after treatment with vancomycin or metronidazole, we used rifaximin (Xifaxan, Salix Pharmaceuticals), a non-absorbed, semisynthetic rifamycin antibiotic, currently approved for the treatment of non-invasive travelers diarrhea. We postulated rifaximin may be effective based on its high *in vitro* activity against *C. difficile*, high fecal concentrations after oral administration, and very low relapse rates in the hamster model of CDAD.

Seven mCDAD patients in our clinical practices were offered and agreed to an empiric strategy that used rifaximin as a chaser following their last vancomycin course. Our patients were women, aged 44 to 82 years who had between 5 and 7 CDAD episodes over a 5 to 14 month period. They had received multiple courses of metronidazole (n=7), vancomycin (n=7), and vancomycin in combination with rifampin (n=3) or *S. boulardii* (n=3), sometimes in tapering or pulsed dose regimens. Oral rifaximin (400 to 800 mg/day in 2 or 3 divided doses for 2 weeks) was administered immediately after completing a suppressive course of vancomycin and before recurrence of symptoms.

During follow-up ranging from 2 to 6 months for each patient, 6 of 7 patients had no further CDAD episodes. One patient had a diarrhea episode 10 days after completing the rifaximin course, which responded to a second course of rifaximin without subsequent diarrhea. Despite diarrhea resolution, a *C. difficile* isolate was recovered from this patient that was identical to a pre-treatment isolate by restriction endonuclease analysis (REA) typing.

Multiple recurrent episodes of CDAD were interrupted in seven patients using rifaximin for an off-label indication following standard CDAD therapy. Further controlled studies of this approach are warranted as well as studies that might elucidate the mechanism of this effect, such as the relative effect of rifaximin on the indigenous bowel flora, an important host defense mechanism for *C. difficile*.

MULTILOCUS SEQUENCE ANALYSIS AND COMPARATIVE EVOLUTION OF VIRULENCE-ASSOCIATED GENES AND HOUSEKEEPING GENES OF *CLOSTRIDIUM DIFFICILE*.

Ludovic Lemee[1], Ingrid Bourgeois[1], Elodie Ruffin[1], Anne Collignon[2], Jean-Fran ois Lemeland[1], and Jean-louis Pons[1].

Groupe de Recherche sur les Antimicrobiens et les Microorganismes (G.R.A.M. EA 2656, I.F.R. 23), UniversitØ de Rouen, FacultØ de MØdecine-Pharmacie, Rouen, France[1] ; and DØpartement de Microbiologie-Immunologie, FacultØ de Pharmacie Paris XI, Ch tenay-Malabry, France[2] .

A multilocus sequence analysis of ten virulence-associated genes was performed to study the genetic relationships between twenty-nine C. difficile isolates from various origins, hosts, and clinical presentations and selected from the main lineages described by MLST of housekeeping genes. Colonization factor encoding genes (cwp66, cwp84, fbp68, fliC, fliD, groEL and slpA), toxin A and B genes (tcdA and tcdB) and the toxin A and B positive regulator gene (tcdD) were investigated. Binary toxin genes (cdtA and cdtB) were also detected and internal fragments were sequenced for positive isolates. Virulence-associated genes exhibited a moderate polymorphism, comparable to the polymorphism of housekeeping genes, whereas cwp66 and slpA genes appeared highly polymorphic. Isolates recovered from human pseudomembranous colitis cases did not define a specific lineage. The presence of binary toxin genes, detected in five of the twenty-nine isolates (17%), was also not linked to clinical presentations. Conversely, toxigenic A-B+ isolates defined a very homogenous lineage, which is distantly related to other isolates. Animal isolates were intermixed with human isolates through clustering analysis. Multilocus sequence analysis of virulence-associated genes is consistent with a clonal population structure of C. difficile and the lack of host specificity. The data suggest a co-evolution of several of the virulence-associated genes studied (including toxins A and B and binary toxin genes) with housekeeping genes reflecting the genetic background of C. difficile, whereas flagellin, cwp66 and slpA genes may undergo recombination events and/or environmental selective pressure.

MUTATIONS IN *GYR A* AND *GYR B* IN FLUOROQUINOLONE-RESISTANT *CLOSTRIDIUM DIFFICILE* (CD) NEW EPIDEMIC BI TYPES

SP Sambol[1], FS Siddiqui[1,2], S Johnson[1,2], DN Gerding [1,2]

[1]Hines VA Hospital, Hines, IL, USA, [2]Loyola University Chicago Stritch School of Medicine, Maywood, IL, USA.

Recent CD epidemics in North America with increased mortality and severity of symptoms have been linked to CD strains categorized as toxinotype III, restriction endonuclease analysis (REA) Group BI, and PCR ribotype 027. Subtypes of Group BI (REA types) have been isolated from outbreaks in the United States, Canada, and Europe. New REA BI epidemic types have been associated with fluoroquinolone (FQ) use in patients. REA types BI 1 through BI 5 were isolated prior to 1994 and are FQ susceptible (<0.5 ug/ml) to moxifloxacin (MX) and gatifloxacin (GAT), whereas the new outbreak types BI 6 through BI 20 are resistant (>32 ug/ml) to MX and GAT. FQ resistance in CD has been linked to mutations in the *gyrA* and *gyrB* genes of DNA gyrase. Primers for the quinolone resistance-determining regions of *gyrA* and *gyrB* (Dridi et al, AAC 2002; 46: 3418-3421) were used to amplify approximately 400 bp segments of *gyrA* and *gyrB* in historic REA types BI 1 and BI 5, and eight new epidemic REA types: BI 6 from Oregon and Illinois, BI 8 from Maine, BI 9 from Pennsylvania and New Jersey, BI 12 from Pennsylvania, BI 17 from Montreal, and BI 20 from Arizona.

Sequence comparison of *gyrA* amplicons to the genome of CD strain 630 (REA type R23 isolated 1996, susceptible to MX and GAT at ≤4 ug/ml) showed that the historic FQ susceptible BI 1 and BI 5 *gyrA* sequences were identical to strain 630 *gyrA*. All of the new epidemic types BI 6-20 showed the same single base substitution (ACT>ATT) that changed threonine (Thr) at position 82 to isoleucine. The *gyrB* sequences of all but one isolate were identical to the *gyrB* gene in strain 630. The *gyrB* gene of the BI 6 isolate from Illinois had a single base substitution (GAT>AAT) that changed aspartic acid at position 426 to asparagine, whereas the *gyrB* amplicon of the identical REA type BI 6 isolate from Oregon showed no mutations.

A single mutation in the *gyrA* gene of REA group BI epidemic CD types at position Thr82 (the same position associated with FQ resistance in *E. coli* and other bacterial species) is associated with MX and GAT resistance in CD. One epidemic REA BI 6 isolate had an additional mutation in the *gyrB* gene. Further studies are in progress to determine if topoisomerase IV genes and efflux pump mechanisms also play roles in the FQ resistance of these new epidemic REA BI types.

IMMUNE RESPONSE TO *CLOSTRIDIUM DIFFICILE* IN THE HOSPITALISED ELDERLY POPULATION IN EDINBURGH

K. Sanchez Hurtado[1], M. Corretge[2], J. Starr[2] and I.R. Poxton[1]

[1] Centre for Infectious Diseases, University of Edinburgh, Edinburgh
[2] Royal Victoria Hospital, Edinburgh

The incidence of *Clostridium difficile* in Scotland has reported a significant increase in the past few years being especially high in the city of Edinburgh and the Lothians. In 2005 more than 1,000 inpatients in Edinburgh hospitals were symptomatically colonized with *C. difficile*.

This study is a comparative study of the immune response of the elderly (over 65) population under antibiotic treatment in hospitals around Edinburgh. Some were colonized with *C. difficile* and some of those developed symptoms. The difference between those who develop symptoms and those who remain asymptomatic is hypothesized to be due to the inability of the former to mount a specific immune response to *C. difficile*.

We consented and sampled inpatients in all of the major hospitals in Edinburgh. The average age was 83 and the female:male ratio was 2:1, and we assigned them to one of the three groups: diagnosed symptomatic cases of *C. difficile*, asymptomatic carriers of *C. difficile* (culture positive in stool) and controls (culture and toxin negative in stool).

The levels of IgG against different antigens of *C. difficile*: toxins, a mixture of surface proteins, the surface layer proteins and the lipocarbohydrate of the cell wall were measured by ELISA. We observed that patients that were positive for *C. difficile* in stool but did not develop symptoms had higher levels of antibodies for every antigen compared to those who developed symptoms and to those who were not colonized by *C. difficile*. However, when antibodies were measured against general immunity indicators, such as *E. coli* LPS, it was observed that although the level in asymptomatic group remained higher than the symptomatic group, the levels of the non-colonized population were the highest of the three.

These findings suggest that people who are still able to produce specific antibodies against *C. difficile* even when their immune system is not at its best are protected against disease, and that the loss of that ability is linked to the development of disease.

SUMMARY OF THE EPIDEMIOLOGICAL INVESTIGATIONS OF HUNGARIAN *C. DIFFICILE* STRAINS

Gabriella Terhes[1], Edit UrbÆn[1], Jon S. Brazier[2], J zsef S ki[1], Elisabeth Nagy[1]

[1]Institute of Clinical Microbiology, Faculty of Medicine, University of Szeged, Hungary

[2]Anaerobe Reference Laboratory, Department of Medical Microbiology and Public Health Laboratory, University Hospital of Wales, Cardiff, United Kingdom

Objectives: The aim of this study was to analyse the results of previous epidemiological investigations, in which the prevalence of toxin-producing strains and the main ribotypes were determined in Hungary from 2002-2004.

Methods: 118 *C. difficile* strains were isolated from faecal specimens at different Hungarian laboratories. The presence of the toxin genes (*tcd*A, *tcd*B, *cdt*A, and *cdt*B) was detected by PCR. Cell cytotoxicity assay was carried out to determine the toxin production of these strains. The PCR ribotyping pattern (105 of 118 strains) was also determined by a PCR method. Western blotting and toxinotyping of binary toxin-positive strains were performed by Frederic Barbut (Laboratoire de BactØriologie, UniversitØ Paris).

Results: The PCR products of the toxin A and B genes were observed in 85 strains and cytotopathic effect were demonstrated on the HeLa cell line. 33 isolates were negative for both the toxin A and B genes; none of those 33 isolates caused morphologic damage to HeLa cells either. Toxin-positive isolates had no deletions or insertions in the repeating 3 end of the toxin A gene. Three isolates were carried *cdt*A and *cdt*B; at the same time, both the *tcd*A and *tcd*B genes were amplified. In all cases, the presence of the binding and enzymatic components of the binary toxin was justified.

A total of 31 ribotypes were detected among the 105 tested *C. difficile* strains: 5 ribotypes were distinct from all previously described types, suggesting that these are new types. PCR ribotyping revealed different distributions of *C. difficile* ribotypes in three Hungarian regions. Changes in the distribution of *C. difficile* ribotypes in time were also demonstrated.

ROLE OF LIPID RAFTS IN CLOSTRIDIUM PERFRINGENS ENTEROTOXIN ACTION

J.A. Caserta and B.A. McClane

Dept of Molecular Genetics and Biochemistry
Molecular Virology and Microbiology Graduate Program
University of Pittsburgh, School of Medicine

Clostridium perfringens enterotoxin (CPE) is a 35 kDa pore-forming toxin that targets enterocytes in the GI tract. Upon binding, CPE forms large, 155 kDa and 200 kDa SDS-resistant complexes which affect the membrane permeablility of host cells. The permeability changes induced by these pore-forming complexes cause an influx of Ca^{2+} that leads to cell death. Many bacteria, viruses, and parasites utilize sphingolipid/cholesterol-rich microdomains of the cell membrane, termed lipid rafts, to aid in their pathogenesis. Hijacking of host cell lipid rafts has been implicated in the action of many bacterial toxins. The activity of these toxins has been shown to rely on raft domains for clustering of toxin receptors in binding, oligomerization, and/or insertion. Lipid rafts are also characterized as being insoluble in non-ionic detergents such as Triton X-100 (TX-100) at 4 C. To study the role of lipid rafts in the action of CPE, the cholesterol perturbing drug, methyl- -cyclodextrin (M CD), has been used to disrupt the formation/stability of rafts and to determine if cytotoxicity and/or CPE complex formation are affected by removal of cholesterol. The current data show that the 155 kDa complex is present in both soluble and insoluble fractions after extraction in TX-100, while the 200 kDa complex is only found in the insoluble TX-100 fraction. This result suggests that the 200 kDa complex forms exclusively within raft domains while the 155 kDa complex can be formed both in and out of lipid rafts. However, in kinetic experiments, upon depletion of cholesterol in Caco-2 membranes, the formation of the 155 kDa complex is delayed by ~15 minutes. Additionally, lipid raft disruption is seen to protect Caco-2 cells from killing after CPE treatment in cytotoxicity assays. These data suggest lipid rafts are playing an early role in 155 kDa complex formation and insertion, and a later role in the formation of the 200 kDa complex.

DISTINCT DYNAMICS OF THE CYTOPATHIC AND CYTOTOXIC EFFECT INDUCED BY *CLOSTRIDIUM DIFFCILE* TOXIN B

Harald Genth, Stefanie Dreger, Johannes Huelsenbeck, and Ingo Just

Dept. of Toxicology, Hannover Medical School, D-30625 Hannover, Germany

Small Rho GTPases regulate cell motility by mediating re-organisation of the actin cytoskeleton and chemotaxis in rat basophilic leukemia (RBL) cells. Toxin B from *Clostridium difficile* (TcdB), inactivates Rho, Rac, and Cdc42, whereas lethal toxin from *Clostridium sordellii* (TcsL) inhibits Rac and Ras but not Rho. In this study we report on the up-regulation of RhoB, a GTPase involved in the regulation of apoptosis. TcdB and TcsL induced RhoB up-regulation in a time- and concentration-dependent manner in RBL cells. The concentration – RhoB up-regulation curves recorded after 3h were sigmoid; the EC_{50} values regarding RhoB up-regulation were: TcdB: EC_{50} = 12 ng/ml; TcsL: EC_{50} = 50 ng/ml. The toxin-induced change of actin re-organisation ("cytopathic effect" = CPE) is recorded in terms of rounded per total cells. The concentration - CPE curves recorded after 3 h were sigmoid with comparable EC_{50} values for both toxins (TcdB: EC_{50} = 25 ng/ml; TcsL: EC_{50} = 31 ng/ml). Thus, the CPE correlated with RhoB up-regulation. The toxin-induced cell death ("cytotoxic effect" = CTE) was measured in terms of caspase-3 activation and phosphatidyl serine (PS) externalisation. The CTE was quantified in terms of annexin V-positive per total cells. The concentration – CTE curves recorded after 24h were sigmoid; the EC_{50} values were: TcdB: EC_{50} = 12 ng/ml; TcsL: EC_{50} = 10 ng/ml. These EC_{50} values differed from those found for cytopathic effect (24h): TcdB: EC_{50} = 0.05 ng/ml; TcsL: EC_{50} = 0.1 ng/ml, showing that cytopathic and cytotoxic potency differed by about 2 orders of magnitude. In conclusion, inactivation of Rho GTPases by clostridial glucosylating toxins induced the CPE including changes of actin dynamics and RhoB up-regulation and CTE including caspase-3 activation and PS externalisation with distinct dynamics.

EC_{50} [ng/ml]	**TcdB**		**TcsL**	
	3 h	24 h	3 h	24 h
CPE	25	0.05	31	0.1
CTE	n.o.	12	n.o.	10

TCDB-INDUCED ACTIVATION OF HUMAN MAST CELLS

G.K.A. Meyer, I. Just, R. Gerhard Dept. of Toxicology, Hannover Medical School, Hannover, Germany

Clostridium difficile toxin B (TcdB) is a single chain protein toxin that mono-glucosylates and hereby inactivates Rho-GTPases, master regulators of the actin cytoskeleton. TcdB is one of the causative agents for antibiotic-associated pseudomembranous colitis in humans, mast cells playing a crucial role in the inflammatory processes underlying this disease. So far, detailed studies on stimulation of mast cells by Clostridium difficile toxins have been done on rat-derived mast cells. In this cell type TcdB inhibited the IgE-dependent degranulation, indicating rather an inhibitory effect. In order to reflect human pathophysiological conditions, we now studied the effects of TcdB on the human mast cell line HMC-1. Immunofluorescence stainining as well as electron microscopy studies revealed a TcdB-induced reorganisation of the actin cytoskeleton. In contrast to rat-derived mast cells TcdB time dependently increased the release of hexosaminidase as a marker of degranulation with a rate of 8% per hour compared to basal release of untreated cells. Similar kinetics were detected after mere disruption of the cytoskeleton by latrunculin B, an agent that directly induces depolymerisation of actin filaments. Focussing on the regulators supposed to be downstream of the Rho-GTPases Rho, Rac and Cdc42, treatment with TcdB or latrunculin B resulted in a strong activation of p38 MAPK and ERK1/2. Inhibition of p38 MAPK by SB202190 reduced TcdB- and latrunculin B-induced hexosaminidase release by HMC-1 cells. Furthermore, TcdB-treated mast cells showed a p38 MAPK-dependent increase in IL-8 release and in the release of PGD2 and PGE2. An autocrine stimulation of HMC-1 by prostaglandins contributed only marginally to degranulation. Latrunculin B alone was not able to induce IL-8 release, indicating additional effects induced by TcdB that are not due to mere disruption of the actin cytoskeleton. In conclusion, TcdB-dependent inactivation of Rho GTPases leads to reorganisation of the actin cytoskeleton and seems to contribute to a release of nflammatory mediators, p38 MAPK playing a crucial role. Differences in the mast cell response towards TcdB compared to latrunculin B is presumably due to the presence of functionally inactive Rho-GTPases in TcdB-treated cells.

INORGANIC PHOSPHATE, AN ENVIRONMENTAL SIGNAL THAT INDUCES SPORULATION AND ENTEROTOXIN PRODUCTION IN *CLOSTRIDIUM PERFRIGENS*

Valeria A. Philippe[1], Marcelo B. M_ndez[1], I-Hsiu Huang[2], Lelia M. Orsaria[1], Mahfuzur R. Sarker[2] and Roberto R. Grau[1]

[1]Departamento de Microbiolog_a, Facultad de Ciencias Bioqu_micas y Farmac_uticas, Universidad Nacional de Rosario - Instituto de Biolog_a Molecular y Celular de Rosario (IBR-CONICET), Rosario-Argentina

[2]Department of Biomedical Sciences, Collage of Veterinary Medicine, Oregon State University, Corvallis, OR 97331, Corvallis-USA

C. perfringens enterotoxin (CPE) is an important virulence factor for food poisoning and non-food borne gastrointestinal (GI) diseases in humans and animals. Although CPE production is strongly regulated by sporulation, the nature of the signal/s triggering sporulation remains unknown. Here, we demonstrate that millimolar amounts of inorganic phosphate (Pi), and not pH, constitutes an environmental and universal signal, probably found in the intestines of *C. perfringens* mammal hosts, inducing sporulation and CPE synthesis. In the absence of Pi-supplementation, *C. perfringens* displayed a *spo0A* phenotype, i.e., absence of polar septation and DNA partitioning, as determined by DAPI and FM-64 fluorescence staining, in cells that reached the stationary phase of growth. The final cellular yield and the rate of growth of *C. perfringens* cultures, in different media, were higher under non Pi-supplementation conditions than those in the presence of Pi-supplementation, suggesting that Pi acted as an environmental, but not nutritional, signal for sporulation. These results received support from Northern blot analyses which demonstrated that Pi was able to counteract the inhibitory effect of glucose at the onset of sporulation and induced *spo0A* expression, indicating that Pi acts as a key signal triggering spore morphogenesis. Besides to be the first study reporting the nature of a physiological signal triggering sporulation in clostridia and bacilli, these findings have relevance for the development of anti-sporulation drugs to prevent or treat CPE-mediated GI diseases in humans and domestic animals (***Philippe V. et al., Infection and Immunity, in press***).

EXCHANGE OF A SINGLE AMINO ACID SWITCHES THE SUBSTRATE PROPERTIES OF RHOA AND RHOD TOWARDS GLUCOSYLATING AND TRANSGLUTAMINATING TOXINS

T. Jank, U. Pack, T. Giesemann, G. Schmidt and K. Aktories

Institut f r Experimentelle und Klinische Pharmakologie und Toxikologie, Albert-Ludwigs-Universit t Freiburg, Germany

Rho GTPases are the preferred targets of various bacterial cytotoxins, including *Clostridium difficile* toxins A and B, *Clostridium sordellii* lethal toxin, the cytotoxic necrotizing factors (CNF1) from *E. coli* and the dermonecrotizing toxin (DNT) from *Bordetella* species. The toxins inactivate or activate specific sets of Rho GTPases by mono-O-glucosylation and deamidation/ transglutamination, respectively. Here we studied the structural basis of the recognition of RhoA, which is modified by toxin B, CNF1 and DNT, in comparison with RhoD, which is solely a substrate for lethal toxin. We found that a single amino acid residue in RhoA and RhoD define the substrate specificity for toxin B and lethal toxin, respectively. Change of a specific serine to phenylalanine in RhoA turned RhoA into a substrate for lethal toxin. Accordingly, change of the equivalently positioned phenylalanine in RhoD with serine allowed glucosylation by toxin B. Comparable results were achieved with the Rho-activating and transglutaminating enzymes CNF1 and DNT. Here, amino acid glutamate 64 of RhoA and the equivalent aspartate 76 of RhoD define substrate specificity for CNF1 and DNT, respectively. These data indicate that single amino acid residues located in the switch II region of Rho proteins determine enzyme specificity for diverse bacterial toxins.

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CHARACTERIZATION OF CLOSTRIDIUM PERFRINGENS ENTEROTOXIN INTERACTIONS WITH CLAUDINS IN NATURALLY-SENSITIVE CACO-2 CELLS AND TRANSFECTED FIBROBLASTS

S.L.Robertson[1], U. Singh[1,2], G. Chakrabarti [1,3], C. M. Van Itallie [4] J. M. Anderson[4], and B. A. McClane[1].

[1] Dept. of Mol. Gen. and Biochem., Univ. of Pittsburgh, Pittsburgh, PA.

[2] Rutgers University, New Brunswick, NJ.

[3] Zydus Research Center, Moraiya Ahmedabad, India.

[4] Dept. Cell & Mol. Phys., Univ. of N. Carolina-Chapel Hill, Chapel Hill, NC.

Clostridium perfringens Enterotoxin (CPE) is responsible for the GI symptoms of the third most common food poisoning in the USA. CPE has a unique action that begins with its binding to receptors to form a small (~90 kDa) complex. The toxin present in the small complex then moves into a larger CPE complex of ~155 kDa; formation of this ~155 kDa complex alters membrane permeability properties. With extended treatment time, CPE also becomes associated with a second large complex of ~200 kDa whose formation apparently removes occludin from tight junctions and may alter paracellular permeability properties of polarized monolayers. Fibroblast transfectant studies suggest that the CPE receptor may include certain claudins (~22 kDa proteins that play important roles in maintaining normal tight junction structure/function). However, CPE interactions with claudins have never been demonstrated in naturally CPE-sensitive cells. In the current study, we demonstrated by co-immunoprecipitation analysis that claudins -3 and -4, are present in the ~155 kDa complex formed in naturally CPE-sensitive CaCo-2 cells. Surprisingly, these studies revealed that claudin -1 (which cannot serve as a CPE receptor) is also present in the CaCo-2 cell ~155 kDa complex, i.e., this complex contains CPE and a mixture of both receptor and non-receptor claudins. Electroelution studies confirmed these findings for the ~155kDa complex and further established the presence of claudins -1, -3 and -4, as well as occludin and CPE, in the ~200 kDa CPE complex formed by the CaCo-2 cells. Studies are currently underway to evaluate the presence of claudins -1, -3 and -4 in the small (~90 kDa) CPE complex formed by CaCo-2 cells. Collectively, these studies suggest that CPE is a unique toxin that directly or indirectly interacts with several tight junction proteins in naturally CPE-sensitive, CaCo-2 cells, both when forming pores and when altering paracellular permeability properties.

RAC1 PHOSPHORYLATION OF RHO GTPASES ALTERS ITS GLUCOSYLATION BY CLOSTRIDIUM DIFFICILE TOXIN A

J. Sch ntaube, I. Just, and R. Gerhard

Dept. of Toxicology, Hannover Medical School, 30625 Hannover, Germany

Toxin A from *Clostridium difficile* is a single-chain protein, that monoglucosylates proteins of the Rho GTPase family. Glucosylation leads to functional inactivation of Rho GTPases and causes disruption of the actin cytoskeleton. In this study, we investigated the effect of Rac1 phosphorylation on the glucosylation by *Clostridium difficile* toxin A (TcdA).

Stimulation of CaCo-2 cells with epidermal growth factor (EGF) resulted in a maximal activation of Akt1 kinase as well as in a phosphorylation of Rac1 at Ser-71 and increased the glucosylation of Rho GTPases. GTPases from CaCo-2 cell lysates treated with alkaline phosphatase were less glucosylated.

In a recombinant system we sequentially phosphorylated and glucosylated the Rho GTPases Rac1, RhoA and RhoB. These Rho GTPases share the consensus motif ^{64}YD**R**L**R**PL**SY**P^{73} as recognition site for the Akt1 kinase. Radioactive signals of the [^{14}C]glucosylated GTPases showed that their phosphorylation by Akt enhanced the subsequent glucosylation by TcdA of all three GTPases compared to the non-phosphorylated state.

We conclude that phosphorylated Rho GTPases are more accessable to clostridial glucosyltransferases. To test this hypothesis the phosphomimetic Rac1 mutant (Rac1S71E), which resembles phosphorylated serine, is to be used for kinetic studies.

Mutational analysis of the functional domains of *Clostridium septicum* -toxin.

C. Kennedy[1], L. Daniel[1], J. Melton[2], J. Emmins[3], D. Lyras[1], R. Tweten[2] & J. I. Rood[1]. [1]Australian Bacterial Pathogenesis Program, Department of Microbiology, Monash University, Victoria, Australia,,[2]Department of Microbiology and Immunology, University of Oklahoma Health Sciences Centre, Oklahoma City, USA, .[3]Department of Immunology, Monash University, Victoria, Australia,

The -toxin of *Clostridium septicum* is a lethal pore-forming cytolysin that is essential for the development of fulminant myonecrosis. It is activated by cleavage of the protoxin and then binds GPI-anchored proteins and oligomerises into a heptameric complex before inserting into the cell membrane to form a pore. The objective of this work was to use site-directed mutagenesis to modify the domains of the -toxin and to examine the resultant effects in a mouse myonecrosis model. Separate toxin gene derivatives with mutations altering the protoxin cleavage recognition site or attenuating oligomerisation, receptor binding, and the ability to form a transmembrane domain (TMD) were constructed and introduced *in trans* into an -toxin negative *C. septicum* mutant. When these strains were tested for virulence in mice, all strains except the TMD deletion mutant were as virulent as the wild-type strain. Haemolysin assays indicated that the virulence phenotype correlated with the ability of the mutated toxin to lyse mouse red blood cells but not human or horse erythrocytes. These data suggest that the ability to form a transmembrane pore is essential for virulence and that the -toxin binds to different cell surface receptors in different host species, prompting further studies to clarify the nature of its host cell receptor.

eANALYSIS OF GERMINATION-SPECIFIC CORTEX-LYTIC ENZYMES, SLEC and SLEM, OF CLOSTRIDIUM PERFRINGENS SPORES

A. Masayama[1], T. Kumazawa[1], S. Fukuoka[2], S. Kato[1], H. Hemmi[1], T. Yoshimura[1], and R. Moriyama[3]

[1]Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Aichi, Japan; [2]National Institute of Advanced Industrial Science and Technology, AIST Shikoku, Takamatsu, Kagawa, Japan; [3]College of Bioscience and Biotechnology, Chubu University, Kasugai, Aichi, Japan.

Bacterial spore germination is triggered by specific germinants and causes the irreversible loss of spore dormancy. One of the key enzymes involved in spore germination is spore peptidoglycan (cortex) hydrolases. It has been suggested that SleC and SleM are putative cortex lytic enzymes of *Clostridium perfringens* spores, but little known about the details of the hydrolytic process by these enzymes during spore germination, except that SleM functions as a muramidase. In this study, we analysed the muropeptides derived from SleC-digested decoated spores of a *Bacillus subtilis* mutant, which lacks the cortex lytic enzymes, SleB, CwlJ and YaaH. The analysis of muropeptides by RP-HPLC and mass spectrometry suggested that the SleC enzyme is likely to be bifunctional, having both a lytic transglycosylase and an *N*-acetylmuramoyl-L-alanine amidase activity.

OLIGOMERIZATION OF CLOSTRIDIUM PERFRINGENS EPSILON-TOXIN IS DEPENDENT ON MEMBRANE FLUIDITY IN LIPOSOMES.

Masahiro Nagahama, Keiko Kobayashi, Hideki Hara, Mariano Fernandes Miyakawa Jun Sakurai

Department of microbiology, Faculty of pharmaceutical sciences, Tokushima Bunri University, Japan

Clostridium perfringens epsilon-toxin binds to receptors on MDCK cells and forms a heptamer in membranes. The mechanism behind the oligomerization of epsilon-toxin was studied using carboxyfluorescein (CF)-loaded liposomes composed of various phosphatidylcholines (PC). The toxin caused CF to leak from liposomes in a dose-dependent manner. The toxin-induced leakage of CF, binding of the toxin to liposomes, and formation of a functional oligomer increased as the phase transition temperature (*Tm*) of the PC used in the liposomes decreased. Surface plasmon resonance analysis using an HPA sensorchip (BIAcore) also revealed that the binding of the toxin to liposomes increased with a decrease in the *Tm* of the PC used in liposomes. The oligomer that was formed in 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID)-treated liposomes was @labeled, indicating that it inserts into a hydrophobic region. Furthermore, the rate of epsilon-toxin-induced CF leakage was enhanced by treatment with phosphatidylethanolamine or diacylglycerol, which is known to favor a lamellar-to-inverted hexagonal (L-H) phase transition. We show that membrane fluidity in the liposome plays an important role in the binding of the toxin to liposomes, insertion into the hydrophobic region in the bilayer of liposomes, and the assembly process in the bilayer.

CLOSTRIDIUM PERFRINGENS ALPHA-TOXIN INDUCES SUPEROXIDE ANION GENERATION IN RABBIT NEUTROPHILS

Masataka Oda, Syusuke Ikari, Takayuki Matsuno, Yuka Morimune, and Jun Sakurai.
Department of Microbiology, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Japan.

Clostridium perfringens alpha-toxin induces the generation of superoxide anion (O_2^-) via production of 1, 2-diacylglycerol (DG) in rabbit neutrophils. The mechanism of the generation, however, remains poorly understood. Here we report a novel mechanism for the toxin-induced production of O_2^- in rabbit neutrophils. Treatment of the cells with the toxin resulted in tyrosine phosphorylation of a protein of about 140 kDa. The protein reacted with anti-TrkA (nerve growth factor high affinity receptor) antibody and bound nerve growth factor. Anti-TrkA antibody inhibited the production of O_2^- and binding of the toxin to the protein. The toxin induced phosphorylation of 3-phosphoinositide-dependent protein kinase-1 (PDK1). K252a, inhibitor of TrkA receptor, and LY294002, inhibitor of PI3K, certainly reduced the toxin-induced production of O_2^- and phosphorylation of PDK1, but not the formation of DG. These inhibitors inhibited the toxin-induced phosphorylation of protein kinase C-q (PKCq). U73122, a phospholipase C inhibitor, and pertussis toxin (PT) inhibited the toxin-induced generation of O_2^- and formation of DG, but not the phosphorylation of PDK1. These observations show that the toxin independently induces production of DG through activation of endogenous PLC and phosphorylation of PDK1 via the TrkA receptor signaling pathway and that these events synergistically activate PKCq in stimulating an increase in O_2^-. In addition, we show the participation of mitogen-activated protein kinase (MAPK)-associated signaling events via activation of PKCq in the toxin-induced generation of O_2^-.

CORRELATING IN VITRO CYTOTOXICITY WITH IN VIVO ENTEROTOXIC EFFECTS OF THE CLOSTRIDIUM PERFRINGENS ENTEROTOXIN

J. G. Smedley, III1, J. Saputo2, F. A. Uzal2, and B. A. McClane1
1
University of Pittsburgh School of Medicine, MVM Graduate Program
2California Animal Health and Food Safety, University of California, Davis

The Clostridium perfringens enterotoxin, or CPE, is the toxin responsible for the second and third most common cause of food poisoning in the UK and US, respectively. CPE mediates its effects by forming oligomeric pores in the membranes of intestinal epithelial cells, leading to cell death and destruction of the intestinal epithelium. An in vitro cell culture model employing the human colon carcinoma Caco-2 cell line has been previously used to study CPE s molecular mechanism of action. However, CPE action in vitro has yet to be validated in an in vivo model of CPE-mediated intestinal disease. In efforts to correlate our in vitro cytotoxicity findings with an in vivo enterotoxicity system, a rabbit intestinal loop model was used to test recombinant CPE (rCPE) and two of its noncytotoxic variants. While rCPE was able to elicit significant fluid accumulation in these rabbit intestinal loops, the noncytotoxic (but binding-capable) point variant D48A was unable to cause a similar response. In addition, a C-terminal deletion fragment of rCPE, rCPE168-319, was also unable to elicit significant fluid accumulation in this in vivo model. Subsequent histopathological analysis of intestinal sections treated with rCPE demonstrated severe mucosal damage characterized by necrosis and loss of superficial epithelium and lamina propria, blunting and fusion of villi, transmural edema and hemorrhage, with minimal neutrophilic infiltrate. Treatment of similar tissues with each of the noncytotoxic rCPE variants resulted in effects comparable to control-treated loops only. In other preliminary findings, rCPE-containing large complexes could be resolved on Western immunoblotted SDS-PAGE gels loaded with tissue samples from rCPE-treated intestinal loops. These complexes resembled those seen previously in the Caco-2 cell culture model. Altogether, our results thus far from the rabbit intestinal loop model have correlated closely with our in vitro findings and help confirm our previous observations of CPE s molecular mechanism of action toward sensitive cells.

IDENTIFICATION OF THE PROTEIN RECEPTOR OF BOTULINUM NEUROTOXIN TYPE A AND CHARACTERIZATION OF ITS BINDING SITE

Mahrhold S[1] , Karnath T[1] , Rummel A[2] , Bigalke H[2] , Davletov B[3] , and Binz T[1]

[1]Institute of Biochemistry, OE 4310, Medical School Hannover, 30623 [2]Hannover, Germany, [2]Institute of Toxicology, OE 5340, Medical School 3Hannover, 30623 Hannover, Germany, and [3]Medical Research Council, Laboratory of Molecular Biology, Cambridge CB2 2QH, UK

The seven serotypes of botulinum neurotoxins (BoNTs) are extremely potent bacterial protein toxins that provoke the disease botulism. BoNTs bind with extraordinary specificity to nerve terminals at the neuromuscular junction. Polysialogangliosides, i.e. glycosphingolipids that are enriched in the outer leaflet of neural cell membranes, are essential for the adherence of BoNTs to neurons. However, protein receptors are regarded as crucial for toxin uptake. Synaptotagmin I and II, two integral membrane proteins of synaptic vesicles, have recently been shown to act as protein receptors for BoNT/B and BoNT/G. In the present study, we investigated whether intravesicular parts of other synaptic vesicle proteins could serve as protein receptors for the remaining BoNTs. Our results establish that BoNT/A directly interacts with the large intravesicular domain of the synaptic vesicle glycoprotein 2C (SV2C), but not with the corresponding region of the homologous SV2A and SV2B proteins. Preincubation of BoNT/A with the isolated large intravesicular domain of SV2C efficiently decreased its toxicity at mice phrenic nerve preparations demonstrating the physiological relevance of this major vesicular protein for the activity of BoNT/A. In order to identify the binding site of SV2C within the receptor binding fragment of BoNT/A we performed site directed mutagenesis. Prominent amino acid residues of potentially interacting cavities were replaced and the resulting BoNT mutants were tested for their ability to interact with SV2C in GST-pull-down assays. Effects on neurotoxicity were determined at mouse phrenic nerve preparations. The results suggest that the receptor binding pocket lies in the proximity of the ganglioside binding pocket, facilitating the simultaneous binding of ganglioside and protein receptor.

PRODUCTION OF THE TRANSLOCATION DOMAIN OF BoNT/A

S. Raffestin[1], C. Montagner[2], J. Barbier[1], S. Pichard[1], M. Galloux[1], M.R. Popoff[3], A. MØnez[1], V. Forge[2] and D. Gillet[1]

1 : DØpartement d IngØnierie et d Etudes des ProtØines, DIEP, Bat 152, CEA Saclay, 91191 Gif sur Yvette, France ; **2** : DØpartement RØponse et Dynamique Cellulaires, Laboratoire de Biophysique MolØculaire et Cellulaire, CEA Grenoble, 38054 Grenoble Cedex, France ; **3** : Institut Pasteur, UnitØ des BactØries AnaØrobies et Toxines, 28 rue du Dr Roux 75724 Paris cedex 15, France.

In order to study the function of the translocation domain (T domain or H_N fragment) of BoNT/A, we have produced a recombinant T domain in *E. coli*. A synthetic DNA sequence encoding residues K547 to S877 of T was made in which codons were optimized for expression in *E. coli*, codons for isolated Cys residues were mutated to Ser codons and potential alternative Shine-Dalgarno sequences were removed. Expression in *E. coli* under control of the phage T5 promoter allowed production of mg amounts of recombinant T domain per liter of culture. Varying the temperature of the culture and the concentration of inductor (IPTG) had marginal effects on production yields. The protein was recovered as inclusion bodies, purified under denaturing conditions by IMAC using an N-terminal histidine tag and refolded by dialysis. The protein could be lyophilized and solubilized in aqueous buffer. However, freezing and thawing of samples or further purification by ion exchange chromatography resulted in aggregation and loss of the protein. Studies of the conformational change of the T domain as a function of pH and in the presence of membranes are underway.

OPTIMISATION OF PRODRUG CONVERTING ENZYME GENE EXPRESSION FOR USE IN CLOSTRIDIAL DIRECTED ENZYME PRODRUG THERAPY (CDEPT)

O. J. Pennington[1], J. Theys[2], G. M. Anlezark[3], P. Lambin[2] and N. P. Minton[1].

[1] Institute of Infection, Immunity & Inflammation, Centre for Biomolecular Sciences, University Park, University of Nottingham, Nottingham, NG7 2RD, U.K. [2] Maastro Lab, Maastricht Radiation Oncology, University of Maastricht, Postbox 616, 6200 MD Maastricht, The Netherlands. [3] HPA Centre for Emergency Preparedness & Response, Porton Down, Salisbury, SP4 0JG, U.K.

A fundamental requirement of any new anti-cancer therapy, is the facility to subject tumour cells to a toxic agent, while at the same time excluding normal healthy tissues from such exposure. Despite the conceptual simplicity, the derivation of such therapies is proving extremely challenging. One strategy demonstrating particular promise is based on the use of clostridial spores, and during the past decade, several recombinant clostridial species have been generated able to produce biologically functional proteins which result in high local concentrations of anticancer drugs in tumours. Notable amongst these has been the *E. coli* B nitroreductase (NTR-B), which reduces the 4-nitro group of the prodrug CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) to a cytotoxic 4-hydroxylamine (4HX) derivative which on a dose-by-dose basis is 10^4-to 10^5-fold more cytotoxic than CB1954.

Although these reports established the feasibility and safety of the delivery system, the properties of the strains used were suboptimal. One required improvement relates to the effectiveness of enzyme:prodrug combination employed. We used *in silico* database mining to isolate a novel nitroreductase (NTR-H) able to turnover the prodrug CB1954 more effectively than NTR-B. This novel enzyme has an equivalent K_m to NTR-B, but a 10-fold higher K_{cat} for the prodrug. Moreover, whereas NTR-B converts only 50% of CB1954 to the cytotoxic 4HX, 100% of the NTR-H product is converted. To maximise production of recombinant NTR in clostridia, a number of parameters were optimised. These included; (i) stabilisation of the plasmid; (ii) selection of the strongest promoter from half a dozen candidates, and; (iii) re-synthesis of the gene to incorporate clostridial codon usage. As a result of these modifications, high level expression (equivalent to 10% of total cellular soluble protein) of NTR-H was achieved in *C. sporogenes*.

DISTRIBUTION AND EFFECTS OF -TOXIN-GFP IN MOUSE BRAIN AND KIDNEYS

J. Blasi[1], A. Soler[1], J. Dorca-ArØvalo[1], J. Saura[2], J.M. Tussell[2], M. Gibert[3], M. Poppof[3], J. Serratosa[2] and M. Mart n-SatuØ[1].

[1]Departament de Patologia i TerapŁutica Experimental. Universitat de Barcelona-IDIBELL. Feixa Llarga s/n, 08907 L Hospitalet Spain.

[2] Departament de Farmacologia i Toxicologia. IIBB-CSIC, IDIBAPS. Rossell , 161, 6“ - 08036 Barcelona

[3] CNR AnaØrobies, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris cedex 15, France.

- toxin, from *Clostridium perfringens* type B and D strains, is a protein of about 32 kDa that causes a very severe and often fatal form of enterotoxaemia in sheep, goats, and occasionally calves and other animals, resulting in heavy economic losses. In mice, intravenous injection of the toxin produces its accumulation in several defined organs, but mainly in kidneys and central nervous system. This accumulation correlates with the toxic effect at cellular level, producing selective death of epithelial cells from the distal tubule of the nephron and cerebral edema with occasionally neuronal death. Curiously, the accumulation of -toxin was specific in the brain but at least partially non-specific in the kidney (*). In this study -toxin-GFP was intravenous injected into mice in order to monitor the in vivo distribution of this protein in the brain and kidneys. The toxin was detected in kidney tubules, both in the apical or basolateral region of proximal and distal tubules respectively. When similar amounts of GFP were injected, only the apical region of proximal tubules was labeled suggesting that the non-specific binding previously found was due to the toxin accumulation in proximal renal tubules. After 5-10 minutes of the i.v. injection, -toxin-GFP was found lining vascular endothelium of brain microvasculature. This binding was no visible at longer times after injection, suggesting that the toxin could cross the endothelia (and blood brain barrier) and gain access to the nervous tissue. The possible effect of the toxin on glial cells and isolated nerve terminals (synaptosomes) as a further step in the intoxication pathway in the nervous system of the -toxin-GFP was studied. The permeabilizing effect of the toxin on vascular endothelia was also studied by co-injection of $Alexa_{688}$ coupled bovine serum albumin ($Alexa_{688}$-BSA). All together, these results show the in vivo differential distribution of -toxin-GFP after i.v. injection and the use of the fusion protein (-toxin-GFP) as a valuable tool for the study of the intoxication pathway.

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EXPERIMENTAL NECROTIC ENTERITIS IN POULTRY

K. Cooper, H. Trinh, and J.G. Songer

Veterinary Science and Microbiology, University of Arizona, Tucson, AZ

Clostridium perfringens type A is the most common cause of necrotic enteritis (NE), which is estimated to cost the world's poultry industry $2 billion annually. To develop a model for study of pathogenesis and immunoprophylaxis, newly-hatched Cornish x Rock male chicks were fed a low protein diet for one week, a high protein diet for a second week, and then challenged with log-phase *C. perfringens* type A cultures mixed with high protein feed. Small intestine was inflamed, friable, and distended in mild cases, but large sections contained a tightly adhered diphtheritic membrane in more severely affected birds. Microscopic lesions were characterized by sloughing of the epithelium, with abundant fibrin mixed with cellular debris. Areas of necrosis were surrounded by heterophils, creating a sharp line of demarcation between necrotic and healthy tissue. Lesions developed at villous apices, but extended into the submucosa and muscular layers. Strain JGS 4143 [genotype A, beta2 (CPB2) positive, from a chicken with NE] produced gross lesions compatible with field cases of NE in 95.1% of inoculated birds, with a mortality rate of 19.7%, and inoculated birds had an average gross weight of 303.17 g. Unchallenged birds had no gross lesions, no mortality, and an average gross weight of 391.1 grams. Challenge with strain JGS 1473 [genotype A, CPB2+, chicken normal flora] and non-avian strains of *C. perfringens* failed to produce gross lesions. Our results suggest that lesions of NE can be reproduced experimentally, with signs and lesions, which approximate those in natural disease.

BINDING OF *CLOSTRIDIUM PERFRINGENS* EPSILON TOXIN TO MOUSE CENTRAL AND PERIPHERAL NERVOUS SYSTEM

J. Dorca-ArØvalo[1], A. Soler[1], M. Gibert[2], M. Poppof[2], J. Blasi[1] and M. Mart n-SatuØ[1]

[1] Departament de Patologia i TerapŁutica Experimental. Universitat de Barcelona-IDIBELL. Feixa Llarga s/n, 08907 L Hospitalet Spain

[2] CNR AnaØrobies, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris cedex 15, France

Epsilon toxin (-toxin), produced by *Clostridium perfringens* strains B and D, is a powerful toxin that causes enterocolitis in goats and enterotoxemia in sheep, cows and occasionally in other species. -toxin is synthesized as an inactive protoxin which is activated by selective proteolysis at the N- and C- ends.

The most relevant effects are: an increase of vascular permeability causing generalized edema, a burden affectation of kidneys and different neurological effects leading to the death of intoxicated animals.

The lethal activity of the toxin has been related to its specific binding and accumulation in the brain, where it is supposed to bind to and cross the vascular endothelium entering to the brain parenquima, in close contact with nervous cells.

To further study the binding properties of -toxin, we have produced the recombinant -toxin as a fusion protein with the Green Fluorescent Protein (GFP) and used on histological slices of mouse tissues including brain, spinal cord, peripheral nerves and kidneys.

In the nervous system preparations, -toxin-GFP specifically binds to myelin enriched areas, either at the central or peripheral nervous system. Distal tubules of the kidney, a renal structure highly sensitive to the toxin, also were stained by -toxin-GFP.

According to these results, -toxin-GFP can be further used as a molecular tool to define potential targets of the toxin and to study its effects at a cellular and molecular level.

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TOXIGENIC CHARACTERISTICS OF *CLOSTRIDIUM PERFRINGENS* TYPE B

M.E. Fernandez-Miyakawa[1] D. J. Fisher[2], S. Sayeed[2], R. Poon[3], V. Adams[3], J. Rood[3], B.A. McClane[2], F.A. Uzal[1]

[1]California Animal Health and Food Safety Laboratory System, San Bernardino Branch, School of Veterinary Medicine, University of California, Davis, USA

[2]Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, USA. [3]Department of Microbiology, Monash University, Australia

Clostridium perfringens type B produces alpha, beta and epsilon toxins and it is associated with illness in animals characterized by sudden death or acute neurological signs with or without intestinal damage and/or hemorrhagic diarrhea. However, the pathogenesis of type B infections in ruminants is poorly understood. Although beta toxin is considered essential for the occurrence of diseases produced by type C strains and epsilon toxin is considered responsible for clinical signs and lesions of type D enterotoxemia, the roles of beta and epsilon toxin in the development of type B disease are not known. It is not known if the disease outcome is determined by the toxigenic characteristics of individual isolates of *C. perfringens* type B. In order to clarify this situation, a large collection of *C. perfringens* type B isolates were evaluated. Beta, epsilon and beta2 toxin production in late log-phase supernatants of type B isolates were quantified by Western blotting and alpha and perfringolysin-O toxins levels were estimated by biological activity assays. The intravenous mouse lethality of culture supernatants was evaluated under non-trypsinised and trypsinised conditions. Also, monoclonal antibody neutralization studies and regression analyses comparing alpha, beta, epsilon and perfringolysin-O toxins production levels versus lethality were performed. The results showed that although most supernatants of *C. perfringens* type B contained alpha or perfringolysin-O toxins, those toxins are not essential for intravenous lethality in mice. The only contributor to lethality in mice was beta toxin under non-trypsinised conditions and beta and epsilon toxins under trypsinised conditions, although epsilon toxin was the main contributor. Beta and epsilon toxin levels produced by type B isolates were comparable to the toxin levels produced by type C and type D isolates, respectively. The lethality of most type B supernatants was lowered after trypsin treatment. These results indicate that although both beta and epsilon toxin are produced at variable level by type B isolates, beta toxin seems to be the most important toxin produced by most type B isolates.

EVALUATION OF *CLOSTRIDIUM PERFRINGENS* TYPE D VACCINES IN GOATS

J.L.A. Veschi[1], M.E. Fernandez-Miyakawa[3], I.S. Dutra[1] , J.Bermudez[4] and F.A. Uzal[3]

[1]Departamento de Medicina Veterin_ria Preventiva, Universidade Estadual Paulista, SP, Brazil. [3]California Animal Health and Food Safety, School of Veterinary Medicine, University of California, Davis, San Bernardino, CA, USA , [4]Universidad de la Republica, Montevideo, Uruguay.

Clostridium perfringens type D enterotoxemia is considered to be the most important infectious cause of death of sheep and goats in many countries. Although the disease can be readily controlled by vaccination in sheep, the same is not completely true for goats because serum titers associated to protection fall fast. It is proposed that improvement of potency of the vaccines as well as the immunization schemes could reduce the number of doses and develop a long lasting protective response in goats. Two experimental vaccines containing epsilon toxoid (9UI/ml) in aqueous (A) or oily (B) adjuvant was tested in goats with different immunization approaches (see table). Control group consisted of non-vaccinated animals. Ten animals per treatment were used.

		Vaccination (days after birth)					
		7	42	45	80	150	270
Vaccine A (aluminium hydroxide)	G1	X	X				
	G2	X	X			X	X
	G3			X	X		
	G4			X	X	X	X
Vaccine B (oil)	G1	X	X				
	G2	X	X			X	X
	G3			X	X		
	G4			X	X	X	X

Blood was collected on day 0, 22, 42, 57, 72 and 97 days of age and every 30 days until day 390. IgG epsilon antitoxin levels were measured by an indirect ELISA. The site of vaccination was evaluated periodically for local reactions. Animals vaccinated for the first time at 45 days of age, presented higher antibody titers than those that received primo vaccination at 7 days of age. No differences in titers were observed between animals that received 2 doses of vaccine versus those that received 4 doses of the same vaccine. Although both vaccines induced protective immune responses in goats, oil-adjuvanted vaccines provided higher antibody titers that would presumably last longer than those produced by aluminium hydroxide vaccines. Local reactions, however, were an undesired effect of the oil-adjuvanted vaccines.

ISOLATION OF CLOSTRIDIUM DIFFICILE FROM DOMESTIC ANIMALS IN SLOVENIA

T. Pirs1, M. Ocepek1, B. Krt1, J. Micunovic1, T. Kotnik1, M. Vengust1, M. Rupnik2
1Veterinary Faculty, Ljubljana, Slovenia
2Institute for Public Health Maribor and Medical Faculty, Maribor, Slovenia

Clostridium difficile has emerged as an important cause of enteric disease in animals. It was isolated from animals receiving antimicrobial therapy as well as from healthy domestic animals and diarrheic animal with no history of antibiotic treatment. The published data suggest a difference between C. difficile populations in humans and animals.The strains isolated from animal hosts often produce the binary toxin (A+B+CDT+). Faecal samples collected from 119 pigs, 12 dogs, 4 calves, 5 horses and two cats were analyzed for the presence of C. difficile. A total of 55 C. difficile isolates were obtained, one from a dog and the majority from the piglets from two different breeding facilities in Slovenia. The samples were cultured onto standard selective medium with cefoxitin and cycloserine and incubated in anaerobic atmosphere at 37¡C for 48 hours. The isolates were identified based on morphological criteria, the characteristic odour and a commercial latex agglutination test. The identification was further confirmed by amplification of C. difficile specific gene cdd2 located downstream of PaLoc using the primer pair Tim6/Struppi6. The great majority of the piglet isolates were binary toxin-positive and belonged to toxinotype V. A high prevalence of variant toxinotypes among animal isolates is comparable to previous reports. Interestingly, the same toxinotype was reported also in piglets in USA.

CORRELATING NECROTIZING ENTERITIS TO TOXIGENIC CLOSTRIDIUM PERFRINGENS IN THE INTESTINE OF NEWBORN PIGLETS.

H. Posthaus[1], M. Jaeggi[1], C. Brachelente[1], M. Wyder[1], N. Wollschlaeger[2], S. Albini[3], E. Viley[3] and C. Abril[3]

[1]Institute of Animal Pathology, [2]Swine Clinic, [3]Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Bern, Switzerland

Clostridium perfringens is an important pathogen and the cause of different clinical diseases in a variety of hosts. Different strains are known to secrete a large array of potential virulence factors, however which of these are responsible for disease development is unresolved for many of the observed clinical syndromes. *Clostridium perfringens* Type A and C strains are the causative agents of a fatal necrotizing enteritis in neonatal piglets. Two toxins, - and 2, are likely to be key factors in disease development. Despite epidemiological evidence supporting this hypothesis, it is currently unknown whether secretion of these toxins occurs in the intestinal tract of affected piglets. Our project is designed to i) evaluate the prevalence of toxigenic *C. perfringens* strains in piglets, ii) investigate the expression of potentially pathogenic toxins in the intestine of diseased and non-diseased piglets and iii) correlate this to the presence of pathological lesions. Our approach is to perform systematic clinical, bacteriological, pathological, immunohistochemical, and molecular biological investigations on diseased and non diseased animals from selected pig herds. The goal is to define those clostridial exotoxins, which are secreted in the intestinal tract and are associated with pathologic lesions. This is important for the understanding of the basis of clostridial virulence and the development of preventive strategies.

DEVELOPMENT OF PROTECTIVE ANTIBODY AFTER IMMUNISATION OF CATTLE WITH A RECOMBINANT *Clostridium perfringens* a-TOXOID

P. Coppe[1], A. Ginter[1], A. Howells[2], and R. W. Titball[2]

[1] BioX Diagnostics, Site du complexe des postes, 49 Rue Joseph Wauters, B-5580, Jemelle, Belgium.

[2] Defence Science and Technology Laboratory, Porton Down, Salisbury SP4 OJQ, UK.

Twenty four calves were immunised with recombinant carboxy-terminal domain of *Clostridium perfringens* a-toxin ($Cpa_{247-370}$) fused to glutathione-S-transferase (GST) with Quil A adjuvant. Fourteen calves a subcutaneous dose of 350 g of recombinant fusion protein and ten other animals received a 700 g dose by the same route. Six animals were used as non-vaccinated controls. Twenty-one days after the primary immunisation, a second dose of vaccine was give by the same route. All of the immunised animals developed neutralising antibody against the a-toxin. The trial confirms that the recombinant truncate ($Cpa_{247-370}$-GST) is a candidate vaccine which is able to induce high level of protective antibodies against the *C. perfringens* a-toxin in cattle.

POSTER PRESENTATIONS

Session II

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THE MYOTOXIC EFFECT OF CLOSTRIDIUM PERFRINGENS £\-TOXIN IS MEDIATED BY REACTIVE OXYGEN SPECIES

M. Flores-D az1, Mar a Jos Pineda-Padilla1, G. C. Clark2,
R. W. Titball2, and A. Alape-Gir n1,3
1 Instituto Clodomiro Picado, Facultad de Microbiolog a, Universidad de Costa Rica, San Jos , Costa Rica
2 Defence Science and Technology Laboratory, Porton Down, Salisbury, Wilts, UK, SP4 0JQ
3 Departamento de Bioqu mica, Facultad de Medicina, Universidad de Costa Rica, San Jos , Costa Rica

Gas gangrene is a devastating infection characterized by massive local edema and severe myonecrosis. It is caused most frequently by Clostridium perfringens and several lines of evidence indicate that £\-toxin plays a key role in the pathogenesis of this disease. When injected intramuscularly in experimental animals £\-toxin promotes thrombotic events leading to extensive myonecrosis which reproduces the histopathological features of gas gangrene. This work demonstrates that free radical scavengers significantly reduce the myonecrosis caused by £\-toxin, demonstrating that its myotoxic effect is mediated by reactive oxygen species. These results strongly suggest that antioxidant based therapies could reduce myonecrosis during gas gangrene.

THE LARGE SIALIDASE, NANI, OF *CLOSTRIDIUM PERFRINGENS* IS NOT ESSENTIAL FOR VIRULENCE.

M. Chiarezza[1], L. Daniel[1], S. Pidot[1], J.J. Emmins[2], D. Lyras[1] and J.I. Rood[1]

[1]Australian Bacterial Pathogenesis Program, Department of Microbiology, Monash University, Victoria, Australia, [2]Department of Immunology, Monash University, Victoria, Australia.

C. perfringens encodes one to three sialidases, the intracellular NanH enzyme, the secreted NanI, and the putative extracellular sialidase NanJ. Most strains that cause clostridial myonecrosis produce the NanI enzyme. It has previously been shown that cell lines that lack surface associated sialic acid, either through endogenous mutations to the cell line or treatment of normally sialylated cells with sialidase, become hypersensitive to the effects of the lethal *C. perfringens* α-toxin. Injection of purified NanI and α-toxin into mice also results in more severe disease than when α-toxin is administered alone (Flores-Diaz *et al*, 2005, J. Biol. Chem., 280, 26680-9). The objective of our work was to determine if NanI has a similar synergistic effect with α-toxin during the infection process. A *nanI* mutant was constructed in a derivative of *C. perfringens* strain 13, which has the *nanI* and *nanJ* genes but not *nanH.* The mutant was analysed *in vitro* and *in vivo*. This strain was found to have lost almost all of its extracellular sialidase activity, a phenotype that was restored when the wild-type *nanI* gene was provided *in trans*. These results indicate that NanI is the major extracellular sialidase produced by strain 13. The mutant and appropriate control strains then were tested for their ability to cause disease in the mouse myonecrosis model of *C. perfringens* infection. No significant differences were observed in the gross pathology of animals infected with the wild type and mutant strains. Since the *C. perfringens nanI* mutant was still virulent, it was concluded that the large sialidase NanI is not an essential virulence factor in this infection model. However, these data do not rule out a role for NanI in the disease process since like perfringolysin O it still may have more subtle synergistic effects that enhance the *in vivo* activity of the essential -toxin.

REFINEMENT OF THE SYRIAN GOLDEN HAMSTER MODEL OF *CLOSTRIDIUM DIFFICILE.*

Gill Douce
Division of Infection and Immunity, GBRC, University of Glasgow, Glasgow G12 8QQ

Clostridium difficile is a spore forming, anaerobic bacteria that causes gastrointestinal infections in humans with symptoms that range from simple colonisation with no adverse effects to severe diarrhoea, pseudomembraneous colitis and death. Currently no vaccine exists and treatment is difficult, especially for susceptible groups, and the relapse rate is high (up to 55%) so improved treatments are desperately required.

The scientific community in the UK, who have an international reputation in this field, are working hard to develop new treatments for this disease, require the capacity to test such treatments in an appropriate animal model prior to clinical evaluation. As although in vitro models exist, it is clear that such cell-based models are limited in their usefulness. This is because outcome of infection in the whole animal is dependent on the dynamic interaction between the pathogen and its host, a situation that is virtually impossible to replicate in vitro.

Due to the potential severity of this disease in animals, it is essential that animal suffering is minimised. In order to refine the model that is used currently (in which a fatal outcome is frequently observed), hamsters have been challenged with pathogenic organisms and the progress of disease monitored by observation of behavioural changes (activity, response to stimulus, changes to state of the animals coat) and specific measurement of physical symptoms such as (loss of body weight, physical activity and change in body temperature). By correlating these symptoms with level of colonisation by the bacteria and alterations to the structure of the bowel, we hope to refine the model to provide a specific humane end-point for such experiments. Initial findings will be described.

EFFECT OF MUCOSAL IMMUNIZATION WITH CELL WALL EXTRACT OF *CLOSTRIDIUM DIFFICILE* ON THE COURSE OF CLOSTRIDIUM DIFFICILE INFECTION IN HAMSTERS

S. P chin , C. Janoir, H. Boureau, S. Hoys and A. Collignon
Facult de Pharmacie, Universit Paris Sud, EA 35-34, 92296 Ch tenay-Malabry Cedex, France

Clostridium difficile produces toxins that cause inflammation, necrosis and fluid accumulation in the intestine, and is the most important cause of nosocomial antibiotic-associated diarrhoea and colitis. The first step of pathogenesis is the colonization process. Different adhesins implicated in the colonization have been described particularly surface proteins. Blocking the primary stages of infection namely bacterial attachment to host cells and colonization of the mucosal surface, may be an effective strategy to prevent *C. difficile* infection. Because of the mucosal nature of this infection, vaccination aimed at providing prophylactic immune protection have included immunization by mucosal route.

In this study, using the hamster model of *C. difficile* infection, we assessed the protective effect of cell wall extracts of a toxigenic and a non toxigenic strain of *C. difficile* used as vaccine antigens and administered by rectal route.

After three immunizations with antigens combined with Cholera toxin as adjuvant, hamsters received clindamycin. Then, 5 days later, they were challenged by a toxigenic strain of *C. difficile*. Post-challenge survival was followed. In the two immunized groups, survival was prolonged as compared to the control group. These results suggest that mucosal immunization with surface proteins could at least partially protect the hamster against *C. difficile* infection. Studies are in progress to characterize the best vaccine antigens.

IDENTIFICATION AND CHARACTERISATION OF THE LINCOMYCIN RESISTANCE TRANSPOSON, Tn*4460*, FROM *Clostridium perfringens*

V. Adams[1], D. Lyras[1], S.A. Ballard[1], W.L. Teng[1], P. Howarth[1], P. K. Crellin[1], T.L. Bannam[1], J.G. Songer[2] and J.I. Rood[1].

[1] Australian Bacterial Pathogenesis Program, Department of Microbiology, Monash University, Vic 3800, Australia, [2] Department of Veterinary Science and Microbiology, University of Arizona, Tucson, AZ 85721, U.S.A.

Resistance to lincomycin in *C. perfringens* is usually mediated by *erm* genes, which also confer resistance to macrolides and streptogramins. However, analysis of multiply antibiotic resistant isolates of *C. perfringens* has revealed several porcine strains that are only resistant to lincomycin and not to erythromycin. The objectives of this study were to determine the mechanism of lincomycin resistance, to see if lincomycin resistance was transferable and to determine the genetic organisation of the resistance genes. The lincomycin resistance determinant, *lnuP*, from strain 95-949 was cloned and sequenced. Analysis indicated that *lnuP* was a homologue of *lnuA* genes from the staphylococci. LnuA-related proteins are lincosamide nucleotidyltransferases that mediate modification of lincomycin molecules, thereby inactivating them. Furthermore the *lnuP* gene was found to be located on a 1.9 kb transposon, designated Tn*4460*, movement of which leads to an 8 bp target site duplication. Mixed plate matings were carried out using genetically marked *C. perfringens* recipient strains and it was shown that Tn*4460* is located on a large conjugative plasmid, pJIR2774, which shows significant sequence similarity to pCW3, a conjugative tetracycline resistance plasmid from *C. perfringens*. This region of similarity includes the known pCW3 conjugation genes. In summary, a conjugative plasmid that confers lincomycin resistance has been identified in *C. perfringens*. Tn*4460* and its cloned *lnuP* gene provide another transposable element and resistance gene for use in the genetic manipulation of the clostridia. The newly identified pJIR2774 is the first conjugative antibiotic resistance plasmid that does not carry a tetracycline resistance determinant to be identified in *C. perfringens*.

CHARACTERISATION AND ROLE IN VIRULENCE OF A *Clostridium perfringens* AUTOLYSIN

Emilie Camiade, Ingrid Bourgeois, Martine Pestel-Caron and Jean-Louis Pons
emilie.camiade@etu.univ-rouen.fr, jean-louis.pons@univ-rouen.fr

University of Rouen, Groupe de Recherche sur les Antimicrobiens et les Microorganismes, IFR23,
76031 Rouen, France.

Autolysins, enzymes hydrolysing the bacterial peptidoglycan, are implicated in the physiology (growth and cellular division) of the bacteria. They could also contribute to the virulence of some pathogenic species, by mediating cellular adherence, toxin release or by generating peptidoglycan released peptides with proinflammatory activity. We have characterized *in silico* the sequence of an *acp* gene encoding a putative autolysin of *Clostridium perfringens*. We cloned this gene and expressed the corresponding protein in order to study the properties and functions of this putative autolysin.

The Acp protein is an autolysin with putative *N*-acetylglucosaminidase activity able to lyse the wall of several bacterial species. The study of the *acp* gene expression during various growth phases of *C. perfringens* shows an overexpression at the beginning of the stationary phase, which could suggest a role in the sporulation of *C. perfringens* (release of the mature spores after lysis of the peptidoglycan).

The hydrolytic specificity of Acp will be studied by analysis (RP-HPLC/mass spectrometry) of the degradation products of bacterial peptidoglycan. The implication of Acp in the virulence of *C. perfringens*, in particular its implication in adherence to the eucaryotic cells and in release of inflammatory mediators by peptidoglycan degradation products, will be further investigated.

INVESTIGATING THE ROLE OF CodY IN *Clostridium sporogenes* SPORULATION.

I. J. Davis[1], M. Bradshaw[2], E. A. Johnson[2] & N. P. Minton[1].

[1]Institute of Infection, Immunity & Inflammation, Centre for Biomolecular Sciences, University of Nottingham, Nottingham, NG7 2RD, UK.

[2] Dept. Food Microbiology & Toxicology, University of Wisconsin, Madison, WI 53706, USA.

The global regulator CodY is well characterised in *Bacillus subtilis* where it represses its regulon in nutrient rich conditions. In bacillus this repression is lifted at stationary phase when intracellular GTP and/ or isoleucine reserves are depleted. CodY has been implicated in repression of Spo0A and thence inhibition of sporulation in bacillus. The elimination of *Clostridium botulinum* spores is a major concern for the food industry and an understanding of environmental signals that inhibit sporulation is of key importance. *C. sporogenes,* due to its lack of toxicity and relatedness to *C. botulinum* can be used as a model organism for *C. botulinum* sporulation. In this work the *codY* of *C. sporogenes* has been identified and modulation of expression undertaken via asRNA technology. The affect of antisense-*codY* on *spo0A* transcription and sporulation has been studied and the interaction of CodY with *spo0A* promoter DNA investigated.

PHOSPHATE-DEPENDENT GENE REGULATION IN CLOSTRIDIUM ACETOBUTYLICUM

T. Fiedler1, M. Mix1, K. Schwarz1, R.-J. Fischer1, H. Bahl1
1 University of Rostock, Biological Sciences/Microbiology, A.-Einstein-Str. 3, 18051 Rostock, Germany

Phosphate as an essential bioelement with limited availability in most habitats often represents the growth-limiting factor for soil-borne microorganisms. Therefore, efficient phosphate-uptake and phosphate-sensing systems were developed. In Clostridium acetobutylicum phosphate limitation is one signal among others in a complex regulatory network leading to a radical physiological alteration of the metabolism yielding into stable production of solvents. Furthermore, sporulation seems to be tightly connected to the phosphate-starvation response. Thus, we investigate general mechanisms of phosphate-regulation, -uptake, and -storage. Two operons of C. acetobutylicum, pst and phoPR could be shown to be strictly regulated by phosphate. The pst operon, encoding for a high affinity phosphate-specific ABC-transporter system, might represent one of the most important members of a predicted PHO-regulon of C. acetobutylicum. Binding of PhoP to the promoter region of the pst operon could be shown indicating a key role of PhoP/R as main two-component regulatory system in phosphate starvation response. A Pho-box motif as binding site for PhoP in the pst promoter region shares similarities to Pho-boxes of B. subtilis as well as E. coli. Furthermore, it could be proved that phosphate is stored as volutin, that means as long chained, insoluble granula.

VARIABILITY OF THE PALOC UPSTREAM REGION IN *Clostridium difficile*

B. Geric[1,3], M. Rupnik[1,2]

[1]University of Ljubljana, BF, Department of Biology, Vecna pot 111, SI-1000 Ljubljana, Slovenia; [2]Institute of Public Health Maribor and University of Maribor, Medical faculty; [3] Agricultural Institute of Slovenia, Hacquetova 17, SI-1000, Ljubljana, Slovenia

The region coding for toxins TcdA and TcdB in *C. difficile* is called PaLoc and according to deletions, insertions and changes in restriction sites in this region strains are divided into toxinotypes. Despite its variability is PaLoc always integrated in the chromosome at the same insertion site and its boundaries are conserved. The only exception is toxinotype XI. This group possesses functional genes for binary toxin CDT and nonfunctional remnant of the pathogenicity locus (PaLoc), bearing only part of the sequence for TcdA. Genes *tcd*R, B and E could not be amplified.

To characterize the extent of upstream deletion in toxinotype XI we have used six PCR reactions within 14 kb region adjacent to the PaLoc in representatives of toxigenic strains from different toxinotypes and in a nontoxigenic strain. Our results show that in different strains some parts of upstream regions are heterogeneus, whereas others are highly conserved. Several PCR fragments could not be amplified in toxinotype XI indicating that larger adjacent regions are missing in addition to 5 -end of PaLoc. Sequencing of 5 -end has confirmed the major genetic rearrangements in this toxinotype.

A TARGETRON SYSTEM FOR CLOSTRIDIA: POSITIVE SELECTION OF GENE KNOCKOUTS USING A CLOSTRIDIAL RETROTRANSPOSITION-ACTIVATED MARKER (RAM)

J. T. Heap, O. J. Pennington and N. P. Minton.

Institute of Infection, Immunity and Inflammation, School of Molecular Medical Sciences, Centre for Biomolecular Sciences, University of Nottingham, UK.

Despite the tremendous commercial and medical importance of the genus *Clostridium*, progress either towards their effective exploitation, or on the development of rational approaches to counter the diseases they cause, has been severely hindered by the lack of effective integration vectors for mutational studies. The general body of evidence amassed to date suggests that whilst recombination in clostridia is possible, it is a relatively inefficient process. An alternative mutagenesis approach, largely independent of host factors, has been developed using the mobile group II intron from the *Lactococcus lactis* gene *ltrB*. This intron integrates into its target gene by a mechanism termed retrohoming, using activities encoded within the intron, and with a target specificity determined largely by a region of the intron RNA. Changing the corresponding DNA sequence alters the intron s target specificity, allowing it to be re-targeted to almost any gene of interest.

The efficiency of re-targeted introns varies widely, and the integration events can be difficult to detect. To overcome this, a Retrotransposition-Activated selectable Marker (RAM) is inserted into the intron. The RAM consists of an antibiotic resistance marker interrupted by a group I intron, which renders the marker inactive. This nested group I intron splices out of the RNA intermediate, restoring the antibiotic resistance marker after integration of the group II intron into its target site. This allows positive selection for integration by acquisition of antibiotic resistance. This Targetron technology was originally optimised for use in *E. coli*. The RAM employed is derived from a kanamycin resistance marker, which cannot be used in clostridia.

We have therefore constructed a clostridial Targetron, in which group II intron RNA production is controlled by the IPTG-inducible *fac* promoter, and in which the kanamycin RAM has been replaced with a novel RAM, based on an antibiotic resistance gene that functions in clostridia. Here we demonstrate the use of this clostridial Targetron to positively select for gene knockout in clostridia.

GROWTH OF GROUP I (PROTEOLYTIC) *CLOSTRIDIUM BOTULINUM* AT DIFFERENT TEMPERATURES

K. Hinderink, M. Lindstr m, H. S derholm, H. Korkeala
Department of Food and Environmental Hygiene
Faculty of Veterinary Medicine
University of Helsinki
katja.hinderink@helsinki.fi

The maximum and minimum growth temperatures and the relative growth rates at different temperatures of group I *C. botulinum* strains are examined in this study. The *C. botulinum* strains have been isolated from different origins mainly in Finland, and they possess distinct PFGE and AFLP patterns. The temperature boundaries for growth and the relative growth rates at a low temperature are expected to reflect the genetic differences between these strains. A total of 20 group I strains are grown on anaerobic agar plates placed in a temperature gradient incubator under temperature gradients spanning the expected maximum and minimum growth temperatures for 3 and 45 days, respectively. Growth will be detected visually and microscopically. The relative growth rates are determined as a change of the optical density of the culture of *C. botulinum* strains anaerobically grown at a low temperature. The results will yield new information on the temperature tolerance of *C. botulinum*, providing tools to control the food safety hazard posed by this dangerous pathogen.

TRANSPOSON MUTAGENESIS OF A BILIRUBIN-REDUCING C. PERFRINGENS STRAIN

Jir skov , A.1,2, Phillips-Jones, M.K.3, V tek, L.2, Fevery, J.4, Branny, P.1
1 Institute of Microbiology AS CR, Prague, Czech Republic. 2 Institute of Clinical Biochemistry and Laboratory Diagnostics, 1st Medical Faculty, Charles University, Prague. 3 School of Biochemistry and Molecular Biology, University of Leeds, UK. 4 Department of Hepatology, University Hospital Gasthuisberg, Catholic University of Leuven, Belgium.

Background: In adults reduction of bilirubin to urobilinoids by intestinal bacterium C. perfringens represents the natural detoxification mechanism. Absence of such intestinal microflora during the first months of life contributes to elevations of serum bilirubin levels and development of neonatal hyperbilirubinaemia. Aim: Identification of C. perfringens gene(s) involved in bilirubin metabolism by transposon mutagenesis.
Results: A rapid and simple method was developed for the electroporation of C. perfringens P90.2.2., bilirubin-reducing strain, with plasmid DNA. The new improvements, harvesting cells early in the logarithmic stage of growth, keeping the cells at room temperature and the absence of post-shock incubation on ice resulted in the maximal transformation efficiency of 1.37 x 104 transformants/ g DNA (Jir skov at al. 2005). The protocol was further used for electroporation of two non-replicative plasmids pAM120 and pTV408 carrying transposons Tn916 and Tn917 respectively, into C. perfringens P90.2.2. Tn916 belongs to a group of conjugative transposons, thus conjugative transfer of pAM120 from E. coli S17-1 to C. perfringens P90.2.2 was performed. Unfortunately both approaches failed to introduce Tn916 and Tn917 into C. perfringens, and to isolate mutants defective in bilirubin metabolism. Usage of alternative approaches leading to identification of genes involved in bilirubin metabolism is discussed.

GENETIC ANALYSIS OF THE *tcdA* AND *tcdB* GENES FROM *Clostridium difficile*

D. Lyras, J. R. O Connor, P. Howarth, G. Carter, and J. I. Rood

Australian Bacterial Pathogenesis Program, Department of Microbiology, Monash University, Vic 3800, Australia.

Clostridium difficile is the causative agent of a spectrum of chronic gastrointestinal syndromes in humans, ranging from mild diarrhoea, through moderately severe disease with watery diarrhoea, abdominal pain and systemic upset, to often fatal pseudomembranous colitis. Pathogenesis primarily involves the action of the two large clostridial cytotoxins, Toxin A (308 kDa) and Toxin B (270 kDa), which are encoded by the *tcdA* and *tcdB* genes, respectively. Toxin A is an extremely potent enterotoxin and causes extensive damage to the intestine. Toxin B is a potent cytotoxin. These toxins have been purified and *in vitro* studies have been extensive. However, the precise role of these toxins in the disease process has not been determined, primarily because of the lack of tools for the genetic manipulation of *C. difficile*. Using a new genetic manipulation approach developed in our laboratory, we have constructed separate chromosomal *tcdA* and *tcdB* mutants derived from a virulent *C. difficile* strain. Genotypic characterisation that involved PCR analysis and Southern hybridisations confirmed that the mutants had the expected genetic organisation. Phenotypic analysis of the mutants has involved both Western blotting and *in vitro* cell culture assays. These mutants represent the first virulence gene mutants to be constructed in *C. difficile* and will prove to be invaluable for further studies on the pathogenesis of *C. difficile* infections.

GERMINATION-RELATED SERINE PROTEASES OF CLOSTRIDIUM PERFRINGENS

S. Kato[1], A. Masayama[1], T. Yoshimura[1], T. Kihara[2], H.i Henmi[1] and R. Moriyama[2]

[1]Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Japan 464-8601, [2]College of Bioscience & Biotechnology, Chubu University, Kasugai, Aichi, Japan 487-8501

The cortex, a thick layer of peptidoglycan unique to bacterial spores, is responsible for the maintenance of dormancy and heat resistance of spores, and cortex hydrolysis is one of the most crucial step during germination. We have identified and characterized the germination-specific cortex hydrolase SleC and the germination-specific protease GSP, which probably consists of CspA, CspB and CspC, from *C. perfringens* S40 spores. SleC exsits as an inactive precursor (ProSleC) which is processed by GSP during germination. Csp proteins are putative subtilisin-like serine proteases. Genome analyses of the various bacteria revealed that the set of *csp* and *sleC* genes are also conserved in *C. acetobutylicum*, *C. difficile*, and *C. tetani*, suggesting that the mechanisim of cortex hydrolysis might be conserved at least in *Clostridia*. However, the role of each Csp protein in ProSleC processing remains unknown.

In this study, we will represent the effects of the over expressed Csp proteins, which are site-directly mutated at their putative active sites, on the spore germination and the post-translational processing of the proteins during sporulation.

THE LARGE EPSILON TOXIN PLASMID OF *CLOSTRIDIUM PERFRINGENS* TYPE D IS CONJUGATIVE.

R. Poon[1], M. L. Hughes[1], V. Adams[1], F. A. Uzal[2], B.A. McClane[3] and J.I. Rood[1].

[1]ARC Centre of Excellence in Structural and Functional Microbial Genomics, Dept of Microbiology, Monash University, Victoria, Australia. [2]California Animal Health and Food Safety Laboratory System, San Bernardino Branch, School of Veterinary Medicine, University of California, Davis, San Bernardino, CA, USA. [3]Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA. USA.

Type D strains of *C. perfringens* are significant pathogens of sheep and goats, resulting in significant economic losses to the livestock industry. The major toxin produced by these strains is epsilon toxin but the role of this toxin in disease is not well understood. The epsilon toxin structural gene (*etx*) is carried on large plasmids in type D strains and it has been shown that these plasmids have a gene region that is required for conjugative transfer in the tetracycline resistance plasmid pCW3. The objective of this study was to determine if these *etx* plasmids were conjugative. The *etx* gene was insertionally inactivated in two type D strains using a chloramphenicol resistance cassette, *catP*. No epsilon toxin was detected in culture supernatants derived from the mutants when they were analysed by Western blotting with epsilon toxin specific monoclonal antibodies. These mutants were used as donors in mating experiments with JIR325, a type A recipient strain, selecting for transfer of chloramphenicol resistance. The results showed that conjugative transfer of the genetically marked type D plasmid occurred at frequencies comparable to that of pCW3. In addition, transconjugants isolated from these matings could act as donors in subsequent matings, confirming that these plasmids encoded their own conjugative transfer. Finally, we were able to transfer the native epsilon toxin plasmid into JIR325, without any prior selective pressure. The resultant transconjugants had the multiplex PCR profile expected of a type D strain, providing experimental evidence that the toxin type of *C. perfringens* strains is plasmid determined. The significance of the finding that the *etx* plasmids are conjugative is that in the animal population it may not be necessary for an invading *C. perfringens* type D isolate to have the ability to colonize the gastrointestinal tract. By conjugative transfer of its -toxin plasmid to an already adherent *C. perfringens* cell, the resident bacterium could acquire the potential to produce the relevant toxin and cause disease.

CHARACTERISATION OF THE CARBOHYDRATE MOIETIES OF THE SURFACE LAYER PROTEINS FROM *CLOSTRIDIUM DIFFICILE*

O. Qazi[1], P. Hitchen[2], S. Haslam[2], A. Dell[2] and N. Fairweather[1]

[1]Division of Cell and Molecular Biology and [2]Division of Molecular Biosciences, Faculty of Natural Sciences, South Kensington Campus, Imperial College London

Antibiotic-associated diarrhoea (AAD) is caused by infection with *Clostridium difficile*. *C. difficile* is an opportunistic, nosocomial bacterial pathogen, with tissue damage primarily induced by the action of two toxins (TcdA and Tcd B). The mechanism of gut colonisation is not well characterised and may involve bacterial surface associated proteins. Like many other bacteria, *C. difficile* displays a cell surface layer (S-layer). The surface layer of *C. difficile* is composed of two surface layer proteins (SLPs); one of ~ 45 kDa and the second of ~ 36 kDa. The roles of bacterial S-layers are in general poorly understood, but various functions have been proposed including host-cell interaction in pathogens.

Many bacterial species have been shown to exhibit glycosylated SLPs and, in some instances, the glycans have been extensively characterised. In this study we have extracted and analysed SLPs from strains of *C. difficile* for the presence of glycans by Western blotting. The LMW SLP protein from nine out of the twelve strains of *C. difficile* analysed was shown to be glycosylated. For each strain, the glycan was chemically released from the SLPs and derivatised prior to MALDI-ToF mass spectrometry and ES-Q-ToF tandem mass spectrometry. Through analysis of this data, the glycan was shown to be a trisaccharide with a composition of rhamnose1-2 rhamnose1-3 N-acetylglucosamine (Rha1-2Rha1-3GlcNAc), where each rhamnose residue may carry a native methyl group. Evidence from tandem mass spectrometry tentatively suggests that the trisaccharide may represent a core unit that is further decorated, based on the observation of at least one molecular component consistent with an additional hexose residue attached to this core glycan. We are currently utilising glycoproteomics in order to identify the site(s) of glycan occupancy and confirm which SLP protein bears this modification.

INSERTION OF A RECOMBINANT CONJUGATIVE TRANSPOSON BY HOMOLOGOUS RECOMBINATION INTO THE *CWP66* GENE OF *CLOSTRIDIUM DIFFICILE*

A. P. Roberts, H. Hussain, P. Mullany

Division of Microbial Diseases, UCL Eastman Dental Institute, University College London, London

We have been investigating the possibility of using homologous recombination to allow the generation of defined mutants in *Clostridium difficile*. A recombinant conjugative transposon carrying the 5 region of the adhesin gene *cwp66* enters the genome of two strains of *C. difficile* by homologous recombination with the wildtype gene in 60% of transconjugants tested; the remaining 40% enter by transposition. However, homologous recombination is only observed if the start of the gene is included in the recombinant fragment cloned into Tn*916.*

THE GENOME SEQUENCE OF CLOSTRIDIUM BOTULINUM TYPE C NEUROTOXIN-CONVERTING PHAGE AND THE MOLECULAR MECHANISMS OF UNSTABLE LYSOGENY

Y. Sakaguchi1, T. Hayashi 2, K. Kurokawa3, K. Nakayama2, M. Ohnishi2, J-C. Lee1, J-h. Cui1, H-J. Hwang1, Y. Yamamoto1, and K. Oguma1
1Department of Bacteriology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan
2Division of Microbiology, Department of Infectious Diseases, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan
3Laboratory of Comparative Genomics, Graduate School of Information Science, Nara Institute of Science and Technology, Nara, Japan

Botulinum neurotoxins (BoNTXs) produced by Clostridium botulinum are one of the most poisonous-substance known. BoNTXs are classified into seven groups (A, B, C1, D, E, F, and G; BoNTX/A to BoNTX/G) based on the antigenicity of their BoNTs. Among the seven groups of BoNTXs, genes for type C1 and D toxins (BoNTX/C1 and D) are carried by host specific bacteriophages. The gene for exoenzyme C3 also resides on these phages. Very little information is available about toxin production and genomic structures of type C1 and D strains. However, some previous studies have reported that BoNT/C1 and D molecules are antigenically related. In this study, we determined the complete genome sequence of cst obtained from type C strain, C-Stockholm. The c-st genome has characteristics such as a linear double-stranded DNA of ~186 kbp in size with 404-bp terminal direct repeats and a G+C content of 26.2%. We identified 198 potential protein-cording regions on the genome of c-st, including genes for BoNTX/C1, C3 exoenzyme and a number of genes homologous to those of a Bacillus subtilis bacteriophage SPb. Very exceptionally, as a viable bacteriophage, a unique feature of the c-st phage is that it encodes many insertion sequence elements. In addition, molecular structure of the phage genome in the c-st lysogen revealed that c-st is lysogenized as a circular plasmid prophage. These features are likely responsible for the unstable lysogeny of BoNTX-transducing phages which has historically been called as pseudolusogeny . Furthermore, PCR scanning analysis of other BoNTX/C1 and D phages based on the c-st sequence further revealed that BoNTX phages comprise a divergent phage family, probably generated by exchanging genomic segments among BoNTX phages and their relatives.

Spo0A IS ESSENTIAL FOR BIOFILM FORMATION & SWARMING MOTILITY IN THE ANAEROBIC PATHOGEN *CLOSTRIDIUM PERFRINGENS*

M. Sarker[1], I. Huang[1], R. Grau[2], M. M ndez[2], and D. Raju[1]

[1]Department of Biomedical Sciences and Microbiology, Oregon State University, Corvallis, Oregon, USA; and [2]Departamento de Microbiolog a, Facultad de Ciencias Bioqu micas y Farmac uticas e Instituto de Biolog a Molecular y Celular de Rosario, Rosario-Argentina.

The ability of bacteria to behave as a multicellular unit (i.e., cell-cell communication) can lead to the production of sophisticated physical structures of unique sedentary characteristics (i.e. fruiting bodies, colonies and biofilms) or develop in social and active communitarian behaviors such as swarming migration. This amazing capacity of bacteria to adapt and survive in different niches, including mammal hosts, is of particular importance in the case of pathogenic bacteria. During recent years, numerous studies have reported the significance of bacterial social behavior in diseases. Among gastrointestinal human pathogens, one important and novel model organism, to analyze the link between wild behaviors and virulence, is the Gram-positive spore-forming anaerobic bacterium *Clostridium perfringens (C.p)*. In this study, we explored the capacity of a sporulation-proficient *C.p* human food poisoning strain SM101 to express the social conducts of biofilm formation and swarming motility. Our results indicated that SM101 has the ability to exhibit biofilm formation and swarming motility. These social behaviors are under the control of the master regulatory protein Spo0A, as demonstrated by the observation that *C.p spo0A* mutant IH101 was unable to exhibit biofilm formation and swarming motility, and these effects could be fully restored by complementing IH101 with wild-type *spo0A*. Furthermore, the requirement of *C.p*-Spo0A for biofilm formation and swarming motility was independent of the process of spore formation since under our experimental conditions we could not detect any spore. This finding is in total agreement with previous findings for *Bacillus subtilis*, where the ability to form a biofilm (and other social behaviors) was Spo0A-dependent but completely sporulation-independent. Further detailed studies on Spo0A-regulated genes should help in understanding the mechanism of Spo0A-dependent biofilm formation and swarming ability in *C.p*. This study opens a new avenue of research to understand the ecology and physiological regulation of *C.p* diseases.

COMPLEMENTATION OF *CLOSTRIDIUM PERFRINGENS spo0A* MUTANT WITH WILD-TYPE *spo0A* FROM OTHER *CLOSTRIDIUM* SPECIES

M. Sarker and I. Huang.

Department of Biomedical Sciences and Microbiology, Oregon State University, Corvallis, Oregon, USA

The initiation of sporulation in *Bacillus subtilis* is controlled by the phosphorylation state of the sporulation transcription factor, Spo0A. The Spo0A homologue have been detected in the genome of all sequenced *Clostridium* species. Our recent *spo0A* knock-out studies presented evidences that expression of *spo0A* is essential for spore formation and enterotoxin (CPE) production in *C. perfringens (C.p)*. In this study, we hypothesized that if the mechanism of Spo0A-regulated sporulation in *C.p* is similar to that in other *Clostridium* species, the lack of spore formation in a *spo0A* mutant of *C.p* should be complemented by the wild-type *spo0A* from other *Clostridium* species. To evaluate this hypothesis, we constructed recombinant shuttle plasmids carrying wild-type *spo0A* from *C. acetobutylicum (C.a)*, *C. botulinum (C.b), C. difficile (C.d)* or *C. tetani (C.t)*; introduced these plasmids into *C.p spo0A* mutant IH101; and compared the sporulation capabilities of the complemented strains with that of their host IH101. Wild-type *spo0A* from *C.a* and *C.t* restored sporulation and CPE production in IH101. However, wild-type or chimeric *spo0A* (N-terminal domain of *spo0A* from *C.p* was fused to C-terminal domain of *spo0A* from *C.b* or *C.d*) from *C.b* and *C.d* was unable to complement the sporulation defects of IH101. The reason for the lack of spore formation in IH101 carrying wild-type or chimeric *spo0A* from *C.b* and *C.d* was further evaluated. *C.d spo0A* was expressed in IH101 using its own promoter while *C.b spo0A* was expressed only when it was fused with *C.p spo0A* promoter. IH101 carrying wild-type or chimeric *spo0A* from *C.b* and *C.d* showed significantly low-level Spo0A production compared to that of IH101 carrying *C.p spo0A*. These results are in agreement with Northern blot analyses which demonstrated at least 5-fold less *spo0A* mRNA synthesis in IH101 carrying wild-type or chimeric *spo0A* from *C.b* and *C.d* compared to that in IH101 carrying *C.p spo0A*. Collectively, our results suggest that the lack of spore formation in IH101 complemented with *spo0A* from *C.b* or *C.d* was, at least in part, due to the low level of *spo0A* expression and Spo0A production which was insufficient to initiate sporulation.

PLASMID DIVERSITY OF *CLOSTRIDIUM PERFRINGENS* TYPE D ISOLATES.

S. Sayeed[1], J. I. Rood[2], F. A. Uzal[3] and B. A. McClane[1]

[1]University of Pittsburgh School of Medicine Pittsburgh, PA 15261, [2] Department of Microbiology, Monash University, Victoria, Australia, [3]California Animal Health and Food Safety Laboratory System, San Bernardino Branch, School of Veterinary Medicine, University of California, Davis, San Bernardino, CA 92408.

C. perfringens, an anaerobic, Gram-positive bacterium, is classified as Type A to E on the basis of production of 4 typing exotoxins (alpha, beta, epsilon and iota). *C. perfringens* Type D isolates cause enterotoxaemia in sheep, goats and (occasionally) cattle. Epsilon-toxin, a CDC/USDA class B select toxin, is absorbed through the intestinal mucosa and then spreads via the circulation to internal organs, where it causes blood pressure elevation and fluid accumulation in body cavities, as well as edema in brain, heart, lungs, liver and kidney. In type D isolates, the gene encoding epsilon toxin is present on poorly characterized large plasmids. Type D isolates sometimes also produce other important plasmid-encoded toxins besides epsilon toxin, including enterotoxin (*cpe* gene) and beta2 toxin (*cbp2* gene). The objective of this study was to characterize the virulence plasmids in type D isolates at the molecular level to gain insights into the genetic organization and diversity of these plasmids. Using Pulse Field Gel Electrophoresis (PFGE) we found that the epsilon toxin gene (*etx*) in Type D isolates can be present on at least three different sized plasmids of 75, 95 and 97 kb. IS*1151* and an ORF (ORF16) found in the putative conjugative transfer region of the *cpe* plasmid of type A isolates also appear to be present on these *etx*-encoding plasmids. PCR amplification revealed differences between the conserved and variable regions of these *etx* plasmids and the *cpe* plasmid of type A isolates. PFGE Southern blots demonstrated that the plasmids in type D isolates carrying the *cpe* and *etx* genes does not always co-migrate. Those analyses also revealed that the *cpb2*-carrying plasmids of Type D isolates do not co-migrate with the plasmids carrying the *cpe* or *etx* genes. Collectively, these studies indicate that the virulence of type D isolates is heavily dependent on plasmids and that a single type D isolates can carry more than one virulence plasmid. Finally, the apparent presence of ORF16 on the *etx* plasmid suggests this plasmid may be capable of conjugative transfer.

HYPERVIRULENT EPIDEMIC STRAINS OF *Clostridium difficile* HAVE ALTERED HOST CELL ADHERENCE AND PROTEIN EXPRESSION

G. Vedantam1,2,3, M.M. Merrigan2, and D.N. Gerding1,3
Departments of Medicine1 and Microbiology/Immunology2, Loyola Medical Center, Maywood, IL and Hines VA Hospital, Hines, IL. USA3.

Introduction: Hypervirulent strains of Clostridium difficile (CD) are being identified from epidemics in the USA, Canada, and Europe. These isolates have been restriction endonuclease typed as group BI. BI strains produce 16-23 fold more toxin, can cause a fulminant colitis, and are associated with increased mortality. Because BI strains appear to rapidly cause epidemics when present in hospitals, we hypothesized that (a) BI strains may have increased ability to adhere to host intestinal epithelia and that (b) non-toxin proteins important for persistence may also be differentially expressed. Methods and Results: First, we developed an in vitro anaerobic adherence assay to assess the ability of the BI-6, BI-8 and BI-17 strains to attach to the Caco-2 human intestinal epithelial cell line. The non toxigenic CD strains M3 and T7, and the toxigenic but non-epidemic strains K14 and 630 were used as controls. M3 exhibited the highest adherence, followed by the BI strains. Both K14 and 630 had the lowest adherence. These results corroborate hamster studies where it was shown M3 conferred greater protection against BI strain challenge than T7. Hamster protection differences may thus be due to differential adherence abilities of the non-toxigenic strains. We also identified proteins dysregulated in the BI strains. Exponentially growing BI-6, BI-17, M3, 630 and K14 strains were fractionated to obtain soluble and membrane proteins. SDS-PAGE revealed that expression of BI strain proteins was altered, and that several proteins were distinctly up- or down-regulated. Using MALDI mass spectrometry, we determined that one over-expressed protein in BI-6 and BI-17 was the SlpA surface layer protein. Conclusions: Epidemic stains of CD have enhanced adherence to human intestinal epithelial cells, and this may be mediated by an increased expression of surface layer proteins. Our data may provide insights into how the BI strains predominate in their environment, and how they are able to cause more severe disease outcomes that are not solely toxin-related.

THE ROLE OF THE TRANSCRIPTIONAL REGULATOR CCPA IN *CLOSTRIDIUM PERFRINGENS* GLIDING MOTILITY

J. Varga and S. B. Melville

Department of Biological Sciences, Virginia Tech, Blacksburg VA, USA, 24061

Clostridium perfringens is a gram-positive, anaerobic, spore-forming bacterium that has been considered non-motile since it was initially isolated. Recently, our lab has shown that *C. perfringens* exhibited gliding motility in a type-four-pilus (TFP) dependent manner. Initial observations indicated that high glucose levels repressed motility, so the motility rate of *C. perfringens* on carbohydrates (glucose, galactose, lactose, mannose) of varying concentrations (0 mM, 1 mM, 5 mM, 10 mM, 50 mM, 100 mM) was measured in strain SM101, a derivative of an acute food poisoning isolate. Colonies exhibited maximum rates of expansion at 5-10 mM carbohydrate concentrations. To further investigate the role of carbohydrates in regulation of motility, assays were performed with *C. perfringens* SM101, and SM120, a *ccpA*- strain generated from SM101. During the first 48 hours, *C. perfringens* SM101 initiated colony expansion on all four carbohydrates, while SM120 showed a loss of motility. As galactose, lactose, and mannose concentrations were increased, the motility rate of SM101 decreased, while the rate of motility shown by SM120 increased. Glucose showed similar effects on *C. perfringens* SM101, however, increasing glucose concentrations completely inhibited motility of *C. perfringens* SM120. These results suggest that CcpA functions to activate motility, and that glucose possesses a CcpA-independent ability to repress motility.

Currently, promoter regions for several TFP structural genes are being characterized by examining -glucuronidase promoter fusion constructs. Several putative CcpA binding sites have been located near TFP structural genes, and electrophoretic mobility shift assays are being performed to determine if CcpA directly regulates TFP expression.

COMPARITIVE GENOMICS OF PATHOGENIC BACTERIA

H. Br ggemann

Institut Pasteur, UnitØ Genomics of Microbial Pathogens, Rue du Docteur Roux, Paris, France

The recently decoded genomes of the major clostridial toxin-producing pathogens *Clostridium perfringens*, *Clostridium tetani*, *Clostridium botulinum* and *Clostridium difficile* have provided a huge amount of new sequence data. The data revealed the genomic background of virulence genes, as well as the contribution of extrachromosomal elements to a pathogenic phenotype. Here, a comparative genome analysis is presented, separating common genes to all clostridial genomes from subsets of strain-specific genes. In addition, the genomes were scanned for signs of alien genes , which might have been acquired via horizontal gene transfer. In this respect, extrachromosomal elements were analysed in depths since plasmids and/or phages are present in all clostridial species sequenced to date, which underlines their importance as a sink for horizontally acquired genes that confer fitness functions to increase the survival of the clostridial species in the environment and to elevate their pathogenic potential.
It could be shown that the plasticity of clostridial chromosomes is low compared to chromosomes of other Gram-positive and, in particular, Gram-negative bacteria. Only few mobile genetic elements can be found and signs of horizontally acquired genes, such as aberrant codon usage statistics, are hardly detectable in clostridial genomes. By contrast, several toxin-encoding loci as well as loci of associated virulence factors show signatures of genetic mobilization in their genomic location, despite partial degeneration of these mobile elements by mutations and rearrangements. It seems probable that these toxin genes are, or were in the past, part of the flexible gene pool, and transfer of toxin genes among clostridial species appears to have occurred in their evolutionary history.

COMPARATIVE GENOMIC INDEXING OF STRAINS OF PROTEOLYTIC *CLOSTRIDIUM BOTULINUM* AND OTHER CLOSTRIDIA USING A NEW MICROARRAY

A.T. Carter[1], D.R. Mason[1], C. Pin[1], M.H.J. Wells-Bennik[1], J. Parkhill[2], A. Ivens[2], N. Nikolaidou-Katsaridou[2], C. J. Paul[3], J. W. Austin[3], and M. W. Peck[1]

[1] Institute of Food Research, Colney, Norwich, NR4 7UA, UK.
[2] The Wellcome Trust Sanger Institute, Hinxton, Cambridge, CB10 1SA, UK.
[3] Health Canada, Tunney's Pasture, Ottawa, Canada.

In collaboration with the Sanger Institute, Cambridge, UK, the complete sequence of the Hall A strain of proteolytic *Clostridium botulinum* was obtained. The 3.9Mb genome contained 3648 predicted coding sequences (CDS); a 16.3kb plasmid was also discovered. These data were used to produce a microarray. Due partly to the very low G+C content (28.2%) not all CDS were suitable for PCR amplification; the current array contains 3433 DNA species, corresponding to approximately 94% of all chromosomal ORFs, in addition to 18 features from the plasmid. The array includes extra features that will facilitate the genotyping of new strains isolated from, for example, disease outbreaks. These include the N- and C- termini of neurotoxin genes A-G, (with two probes designed to identify the type A3 toxin sub-type), plus 17 probes for toxin-associated genes. The latter include haemagglutinins, NTNHs, cntRs, together with p47, lycA, ORFX1, ORFX2 and ORFX3. The array has been used for comparative genomic indexing. Results show that the Hall A strain contains two prophages not found in other strains. It was apparent that other strains of proteolytic *C. botulinum* (toxin types A, B, F and mixed toxin types) and even *C. sporogenes* strains were closely related (approx 92% for other type A strains, 89% for type B strains, 90% for type F strains, and 84% for strains of *C. sporogenes*). However, DNA from a strain of non-proteolytic *C. botulinum* and the sequenced strain of *C. difficile* proved to be too evolutionarily distant to give meaningful results on the Hall A microarray.

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RESPONSE OF *CLOSTRIDIUM DIFFICILE* TO STRESS CONDITIONS

J Emerson[1], R Stabler[2], A Witney[3], K Gould[3], B Wren[2], J Hinds[3] and N Fairweather[1]

[1]CMMI, Exhibition Road, Imperial College London, SW7 2AZ
[2]London School of Hygiene and Tropical Medicine, London, EC1A 7HT
[3]Bacterial Microarray Group, St George s Hospital, London, SW17 0RE

Clostridium difficile is an emerging nosocomial pathogen of increasing clinical significance. *C. difficile* causes antiobiotic-associated diarrhoea after disruption of the normal gastrointestinal flora by processes such as antibiotic exposure. In order to be able to adapt to the intestinal environment, *C. difficile* must react to the many stresses involved with colonisation. In order to investigate the response if *C. difficile* to various stresses, the *C. difficile* 630 microarray developed by the Bacterial Microarray Group at St. George s, University of London (B G@S) was utilised. Upregulated genes are identified which are both common between and unique to different stresses, and it is hoped that this information will allow us to further understand expression of *C. difficile* genes within the gut.

THE PHOSPHOTRANSFERASE COMPLEMENT OF *CLOSTRIDIUM BOTULINUM*

W. J. Mitchell, P. Tewatia and P. G. Meaden.

School of Life Sciences, Heriot-Watt University, Riccarton, Edinburgh EH14 4AS, UK.

Clostridium botulinum is capable of fermenting carbohydrates, but there have been no detailed studies of the uptake of sugars and related substrates. In obligate and facultative anaerobes, the predominant system of carbohydrate uptake is the phosphoenol-pyruvate (PEP)-dependent phosphotransferase system (PTS). This multi-protein complex catalyses a group translocation involving both uptake and phosphorylation of carbohydrates, and is also known to play an important role in environmental sensing and metabolic regulation. The genome of *C. botulinum* encodes 15 phosphotransferases which, based on phylogenetic relationships and analysis of gene clusters, appear to be involved in the uptake of hexoses, hexose derivatives and disaccharides. *C. botulinum* also contains the components of PTS-associated regulatory mechanisms which have been characterised in other bacteria. It therefore seems likely that the PTS plays a significant, and previously unrecognised, role in the physiology of this bacterium.

GENOMIC SEQUENCING OF *CLOSTRIDIUM SEPTICUM*

T. Shimizu, K. Tanaka, A. Isu, K. Miwa, K. Hasatani, K. Ohtani
Grad. Sch. of Med. Sci., Kanazawa Univ., Kanazawa, Japan

Some of Clostridial species cause gas gangrene (clostridial myonecrosis) in humans and animals. Among them, *Clostridium septicum* is known to be a causative agent of malignant edema in animals as well as gas gangrene in humans. The organism is known to produce α-toxin, β-toxin (DNase), δ-toxin (hyaluronidase), γ-toxin (hemolysin) and neuraminidase, some of which are thought to be responsible for its pathogenicity. To elucidate a precise genetic basis of the pathogenicity of *C. septicum*, we undertook the genomic sequencing of *C. septicum* using a whole genomic shot-gun method. At present, 381 contigs (total 3,321,170 bases) are assembled which is under gap closure work now. We found over 3,100 ORFs in these contigs using a glimmer program. The *C. septicum* 3,100 ORFs were compared with 2,660 ORFs of *Clostridium perfringens* strain 13, another causative agent of gas gangrene. 1628 ORFs are common to both bacteria, while 1062 and 707 ORFs were unique to *C. septicum* and *C. perfringens*, respectively. At present, we are searching candidate genes for the virulence factors *in C. septicum* genome.

CLOSTRIDIUM BOTULINUM GENE EXPRESSION AT LOW TEMPERATURE

H. S derholm[1], M. Lindstr m[1], P. Somervuo[2], K. Kiviniemi[1], K. Hinderink[1], P. Auvinen[2] and H. Korkeala[1]

[1]Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki, P.O. Box 66, 00014 University of Helsinki, Finland. [2]Institute of Biotechnology, University of Helsinki, P.O. Box 56, 00014 University of Helsinki, Finland.

Preservation of foods at refrigerator temperatures is becoming more and more important to maintain the good quality of foodstuffs. Therefore the ability of pathogenic bacteria to grow at low temperatures has become a serious risk for consumer s health. Knowledge of factors related to bacterial cold tolerance helps to understand and control these risks. The aim of this study is to obtain new information about the genetics related to cold tolerance in *C. botulinum*. The expression profile of *C. botulinum* strain ATCC 3502 at different growth temperatures is studied by using a DNA microarray (Institute of Food Research, Norwich, UK). Preliminary tests with *C. botulinum* ATCC 3502 grown at 30 C and 37 C showed that at 37 C the expression of genes encoding some phage proteins and toxin production was increased as opposed to the lower temperature. At 30 C a cluster of genes encoding proteins of unknown function was up-regulated in relation to the higher temperature. Further studies will show if these results are reproducible and if similar changes in gene expression occur also at lower temperatures. These studies help us to understand the mechanisms of cold tolerance in *C. botulinum*, providing tools to prevent the food safety risks posed by this pathogen.

COMPARATIVE PHYLOGENOMICS OF *CLOSTRIDIUM DIFFICILE* REVEALS CLADE SPECIFICITY AND MICROEVOLUTION OF HYPERVIRULENT STRAINS

R. A. Stabler[1] B. W. Wren[1]

[1]Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London, WC1E 7HT. UK

Clostridium difficile is the most frequent cause of nosocomial diarrhoea worldwide and recent reports have suggested the emergence of a hypervirulent strain (epidemic BI/NAP1/027 strains) in North America and Europe. A *C. difficile* PCR product microarray based on the *C. difficile* 630 strain was designed and manufactured and a collection of 75 well-characterised isolates comprising hypervirulent, toxin variable and animal strains were analysed. Comparative phylogenomics using a Bayesian algorithm was used to model the phylogeny of *C. difficile*. The analysis identified four distinct statistically supported clusters comprising a hypervirulent clade, a toxin A-B+ clade, and two clades with human and animal isolates. Genetic differences among clades revealed several genetic islands relating to virulence and niche adaptation, including antibiotic resistance, motility, adhesion and enteric metabolism. The data has provided insight into the possible origins of *C. difficile* and its evolution plus potential virulence determinants that may have implications in disease control strategies.

CATABOLITE REGULATION OF *CLOSTRIDIUM DIFFICILE* TOXIN SYNTHESIS

A. Antunes, S. Matamouros, B. Dupuy

Unit_ G_n_tique Mol_culaire Bact_rienne, Institut Pasteur,
28 rue du Dr. Roux, 75724 Paris CEDEX 15, FRANCE

Clostridium difficile is a major pathogen responsible for antibiotic-associated colitis (AAC), and pseudomembranous colitis (PMC), a potential lethal disease. Virulent strains of *C. difficile* produce two important toxins, toxin A (TcdA) and toxin B (TcdB), which are the major virulence factors. The level of toxin synthesis seems to be correlated with the disease severity. However, the regulation of toxin production is still poorly understood at the molecular level.

Multiple forms of environmental regulation control toxin gene expression. It is the case of carbon catabolite repression (CCR), in which the Catabolite control protein A (CcpA) plays a key role. CcpA is a member of the LacI/GalR family of transcription regulators that recognizes specific DNA sequences in the target genes called catabolic-responsive element (*cre*), thereby modulating the target gene expression. CcpA binding to the *cre* site is enhanced by the interaction with its cofactor, HPr-Ser46-P, a key element of the phosphotransferase system (PTS system).

Preliminary studies done by *in vitro* approaches show that *C. difficile* CcpA is capable to bind specifically to the *tcdB* promoter where a putative *cre* site can be found. Furthermore, *in vivo* studies performed in *B. subtilis* and *C. perfringens* surrogate hosts, suggest that CcpA is implicated in the CCR of the *C. difficile* toxin synthesis.

TRANSCRIPTIONAL ACTIVATION OF THE *pfoA* GENE FROM *Clostridium perfringens.*

J. K. Cheung[1], S. McGowan[1], M.M. Awad[1], S. Katayama[2] and J. I. Rood[1]

[1]Australian Bacterial Pathogenesis Program, Department of Microbiology, Monash University, Victoria 3800, Australia, [2]Department of Biochemistry, Faculty of Science, Okayama University of Science, Okayama-shi, Okayama, 700-0005, Japan.

Clostridium perfringens produces an array of extracellular toxins, of which α-toxin and perfringolysin O have been shown to be involved in the pathogenesis of gas gangrene. The production of these toxins is regulated by the VirS/R two-component signal transduction system at the transcriptional level. This regulatory network is initiated upon the detection of an as yet unidentified stimulus by the VirS sensor histidine kinase, and through a phosphorelay cascade, culminates in the regulation of target genes by the VirR response regulator; with the *pfoA* gene, which encodes perfringolysin O, being directly activated. To examine the phosphorelay process, a truncated form of VirS that consisted of the soluble C-terminal region (VirS~$_{2\text{-}214}$), was purified and used in autophosphorylation experiments. The results showed that this truncated protein was capable of undergoing autophosphorylation. By contrast, equivalent derivatives of the VirS H255I (proposed site of autophosphorylation), G400A and G402D (G2 box) mutants were unable to autophosphorylate. The analysis of a C335Y mutant (near N box) indicated that this cysteine residue was also important for autophosphorylation. *In vitro* phosphotransfer from purified VirS~$_{2\text{-}214}$, to purified wild-type VirR protein was also observed, which is the first time that phosphotransfer has been demonstrated in this system. In comparison, phosphotransfer was not observed between VirS~$_{2\text{-}214}$ and a VirR(D57N) mutant, which is consistent with our hypothesis that D57 is the phosphoacceptor site of VirR. To examine transcriptional activation of *pfoA*, *in vitro* transcription experiments using *C. perfringens* RNA polymerase and a combination of the purified VirR and VirS~$_{2\text{-}214}$ proteins were carried out. The results showed that the addition of VirR increased transcription, which was further enhanced by the addition of VirR and phosphorylated VirS~$_{2\text{-}214}$. These data provide evidence that phosphorylation plays a significant role in transcriptional activation. Finally, the importance of the VirR targets (two directly repeated VirR boxes) in the transcription process was demonstrated when mutation of these binding sites resulted in a significant decrease in transcriptional activation.

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

C M Cooksley[1], W C Chan[1], K Winzer[1], A Cockayne[1], I J Davis[1], M W Peck[2], N P Minton[1]

1 *Centre for Biomolecular Sciences, University of Nottingham, Nottingham NG7 2RD. UK*
2 Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA. UK

Botulinum neurotoxin induces a potentially fatal paralytic condition in humans and various animal species collectively known as "botulism". Traditionally, botulinogenic clostridial strains have been classified as *Clostridium botulinum,* which may be divided into distinct physiological groupings (Group I, II, II & IV). Each has a non-toxinogenic counterpart. In Group I, this counterpart is *Clostridium sporogenes.*

The genome sequence of the *C. botulinum* Group I strain ATCC 3502 has recently been determined. *In silico* analysis has revealed the presence of two distinct loci capable of encoding proteins with homology to AgrB and AgrD of the *Staphylococcus aureus* agr quorum sensing system. We have begun the functional characterisation of these genes in order to determine whether they play a role in quorum sensing.

To simplify laboratory procedures, we have initially focused on. *C. sporogenes*. The equivalent regions to those present in ATCC 3502 were shown to be present in this non-toxinogenic clostridium, and to be highly conserved. Regions of conservation are also apparent with similar loci in other clostridia and, to a lesser extent, with staphylococci. Transcriptional linkage assays have shown some of the genes of the *C. sporogenes agr* regions to be co-expressed, and to have higher expression during early exponential growth. Modulation of the expression of the identified *agr* genes is a prerequisite to determining their function. We have used antisense RNA expression for this purpose, and have shown that down regulation of the *agrB* gene affects sporulation.

COLONIZATION FACTOR GENE EXPRESSION OF *CLOSTRIDIUM DIFFICILE* IS MODIFIED BY STRESS CONDITIONS

C. Den ve, C. Janoir, C. Delom nie, A. Collignon

D partement de Microbiologie, EA 35-34, Facult de Pharmacie, Universit Paris Sud, 92296 Ch tenay-Malabry Cedex, France.

Clostridium difficile is recognized as the major causative agent of pseudomembranous and antibiotic associated-colitis. The pathogenicity is mainly due to toxins A and B ; nevertheless, other factors, mainly those which promote the bacterial colonization of the host, may play a fundamental role. This colonization process involves potentially various surface proteins of *C. difficile*, including P47, one of the two S-layer proteins, the two adhesins Cwp66 (Cell Wall Protein of 66 kDa) and Fbp68 (Fibronectin Binding Protein of 68 kDa), and the protease Cwp84 (Cell Wall Protein of 84 kDa). Our laboratory has previously shown that *in vitro* cell adherence of *C. difficile* was increased by environmental factors. In addition, it is known that antibiotics cβ *encoding colonization factors of C. difficile*. This analysis was performed on toxigenic and not toxigenic *C. difficile* clinical isolates by Northern-Blot and real-time RT-PCR. The expression of genes *cwp66*, *fbp68* and *cwp84* was increased in presence of subinhibitory concentrations of ampicillin or clindamycin, which are known to be usually involved in the apparition of *C. difficile* associated disease ; on the other hand, the presence of ofloxacin or kanamycin, rarely or never involved in *C. difficile* associated disease, did not significantly modify the colonization factor gene expression. Moreover, the increase rates of gene expression differed depending on the gene and on the toxigenic status of C. difficile strains ; these rates appeared the most important among the non toxigenic strain. Furthermore, investigations are underway to correlate these results with in vitro cell adherence.

ANALYSIS OF *CLOSTRIDUM PERFRINGENS* PROMOTERS UNDER VR-RNA CONTROL

Tanaka Kousei, Ohtani Kaori, Shimizu Tohru

Department of bacteriology graduate school of medical science Kanazawa University

Clostridium perfringens causes clostridial myonecrosis or gas gangrene in humans by producing numerous extracellular toxins and enzymes. A two-component regulatory system, VirR/VirS, has been reported to be involved in global regulation of the production of theta-toxin (or perfringolysin O), kappa-toxin(or collagenase, *colA*), alpha-toxin (or phospholipase C, *plc*), sialidase, and protease. Further studies of the mechanism of the genetic regulation revealed that VR-RNA (VirR-regulated RNA), which is positively regulated by the VirR/VirS system, plays an important role on the regulation at transcriptional level, although its mechanism is not known in detail.

To determine the *cis*-acting region necessary for the *colA* and *plc* regulation by VR-RNA, we performed promoter deletion experiments using the pJIR418 plasmid in VR-RNA^{+} and VR-RNA^{-} backgrounds. We found that the *colA* promoter activity was induced when 206 bp of sequence upstream of the initiation codon was retained, but a further deletion of 20 bp resulted in partial induction and the additional deletion of 31 bp completely abolished the *colA* promoter activity. Similarly, a full induction of the *plc* expression was observed in cells having at least 128 bp of sequence upstream of the *plc* initiation codon, while removal of an additional 25 bp abolished the promoter activity. These data suggested that VR-RNA itself or VR-RNA complex with other proteins and/or RNAs may interact with these regions.

MANIPULATION OF SPORULATION INITIATION IN *CLOSTRIDIUM DIFFICILE*

Underwood, S.[1], Wilcox, M. H.[2], Stephenson, K[1].

[1]IMCB, Leeds University, Leeds; [2]Leeds Teaching Hospital, Leeds.

Background: With over 35,500 cases of antibiotic-associated diarrhoea and pseudomembranous colitis reported per year in England, Wales and Northern Ireland, the causative agent *Clostridium difficile* is now recognised as a major nosocomial infection. One of the major contributory factors to the spread and persistence of this organism is its ability to form stable and resistant spores. Although a great deal of work has been done on the sporulation pathway in various organisms, the specific signals that induce sporulation in any species are still unknown.

Work published by Kojetin *et al* in 2006 showed that the presence of a divalent metal ion bound to one of the sporulation initiation response regulators is essential for efficient sporulation. Based on this evidence, the effect of several divalent metal ions and a chelating agent on *C. difficile* sporulation was investigated.

Method: The UK epidemic strain of *C. difficile* was cultured for 96 hours in Brain-Heart Infusion media with/without a range different divalent metal ions and EDTA, a chelating agent. Culture samples were then air-dried on glass slides. After staining with malachite green/carbol fuschin, spores were counted using light microscopy and expressed as a percentage of total cells. ANOVA was used to determine significant differences in sporulation ($P<0.05$)

Results: Most of the divalent metal ions investigated showed no significant effect on sporulation. However, Fe^{2+} caused a significant increase, whilst EDTA inhibited sporulation completely.

Discussion: This evidence suggests that Fe^{2+} may act as a signal for sporulation in *Clostridium difficile*. Furthermore, EDTA can inhibit sporulation completely. Therefore the sporulation capacity of *C. difficile* cultures can now be manipulated biochemically thereby offering the possibility of studying the regulation and expression of genes involved in sporulation.

Participants

PARTICIPANTS		
Adams	Vicki	University of Monash, Australia vicki.adams@med.monash.edu.au
Aktories	Klaus	University of Freiburg, Germany klaus.aktories@pharmakol.uni-freiburg.de
Alape-Giron	Alberto	University of Costa Rica aalape@cariari.ucr.ac.cr
Alauzet	Corentine	Nancy University Hospital, France c.alauzet@chu-nancy.fr
Alkhalifah	Mohammed	University of Glasgow, UK mohad1010@hotmail.com
Antunes	Ana	Institute Pasteur, France antunes@pasteur.fr
Arnon	Stephen	California Department of Health Services, USA sarnon@dhs.ca.gov
Arroyo	Luis	University of Guelph, Canada larroyo@uoguelph.c
Bahl	Hubert	University of Rostock, Germany hubert.bahl@uni-rostock.de
Ballard	Jimmy	University of Oklahoma, USA jimmy-ballard@ouhsc.edu
Bannam	Trudi	University of Monash, Australia trudi.bannam@med.monash.edu.au
Barbanti	Fabrizio	National Institute of Health, Rome, Italy sofia@iss.it
Barbier	Julien	CEA Saclay, France julien.barbier@cea.fr
Basak	Ajit Kumar	Birbeck College London, UK a.basak@mail.cryst.bbk.ac.uk
Binz	Thomas	Medical School Hannover, Germany binz.thomas@mh-hannover.de
Blasi	Juan	University of Barcelona, Spain blasi@ub.edu
Bradshaw	Marite	University of Wisconsin, USA mbradsha@wisc.edu
Brazier	Jon	University Hospital Cardiff, UK Brazier@cardiff.ac.uk

Bruggemann	Holger	Institute Pasteur, France hbruegg@pasteur.fr
Callus	Marion	Schering-Plough, New Zealand marion.callus@spcorp.com
Camiade	Emilie	University of Rouen, France emilie.camiade@etu.univ-rouen.fr
Carter	Glen	University of Monash, Australia glen.carter@med.monash.edu.au
Carter	Andy	Institute Food Research Norwich, UK andrew.carter@bbsrc.ac.uk
Caserta	Justin	University of Pittsburgh, USA jac86@pitt.edu
Chen	Yue	University of Pittsburgh, USA cheny@pitt.edu
Cheong	Ian	Howard Hughes Medical Institute, USA rod@jhmi.edu
Cheung	Jackie	University of Monash, Australia jackie.cheung@med.monash.edu.au
Chiarezza	Martina	University of Monash, Australia martina.chiarezza@med.monash.edu.au
Citil	Burak Ekrem	University of Baskent,Turkey burakcitil@yahoo.com
Collignon	Anne	Université Paris-Sud, France anne.collignon@cep.u-psud.fr
Cooksley	Clare	University of Nottingham, UK paxcmc1@nottingham.ac.uk
Cooper	Kerry	University of Arizona, USA kcooper@email.arizona.edu
Couesnon	Aurelie	Institute Pasteur, France couesnon@pasteur.fr
Davis	Ian	University of Nottingham, UK ian.davis@nottingham.ac.uk
Demuez	Marie	INSA Toulouse, France marie.demuez@insa-toulouse.fr
Deneve	Cecile	Université Paris-Sud, France cecile.deneve@cep.u-psud.fr

Dineen	Sean	Tufts University, USA sean.dineen@tufts.edu
Dorca-Arevalo	Jonatan	University of Barcelona, Spain cecile.deneve@cep.u-psud.fr
Douce	Gillian	University of Glasgow, UK g.douce@bio.gla.ac.uk
Drudy	Denise	University College Dublin, Ireland denise.drudy@ucd.ie
Duerre	Peter	University of Ulm, Germany peter.duerre@uni-ulm.de
Dupuy	Bruno	Institute Pasteur, France bdupuy@pasteur.fr
Emerson	Jenny	Imperial College London, UK jenny.emerson@imperial.ac.uk
Fagan	Robert	Imperial College London, UK r.fagan@imperial.ac.uk
Fairweather	Neil	Imperial College London, UK n.fairweather@imperial.ac.uk
Fernandez-Miyakawa	Mariano	University of California, USA mmiyakaw@cahfs.ucdavis.edu
Fiedler	Tomas	University of Rostock, Germany tomas.fiedler@uni-rostock.de
Fisher	Derek	University of Pittsburgh, USA djf46@pitt.edu
Flores-Diaz	Marietta	University of Costa Rica floresd@icp.ucr.ac.cr
Genth	Harald	Hannover Medical School, Germany genth.harald@mh-hannover.de
Geny	Blandine	Institute Pasteur, France geny@pasteur.fr
Gerding	Dale	Hines VA Hospital, USA dale.gerding2@med.va.gov
Gerhard	Ralf	Hannover Medical School, Germany gerhard.ralf@mh-hannover.de
Geric	Barbara	Agricultural Institute of Slovenia, Slovenia barbara.geric@kis.si

Gillet	Daniel	CEA Saclay, France daniel.gillet@cea.fr
Grant	Kathie	HPA Centre for Infections, UK kathie.grant@hpa.org.uk
Grau	Roberto	University of Rosario, Argentina robertograu@fulbrightweb.org
Green	Neil	Vanderbilt University, USA neil.green@vanderbilt.edu
Hamm	Elaine	University of Oklahoma, USA elaine-hamm@ouhsc.edu
Hasselmayer	Oliver	University of Mainz, Germany hasselm@uni-mainz.de
Heap	John	University of Nottingham, UK paxjth@nottingham.ac.uk
Heikinheimo	Annamari	University of Helsinki, Finland annamari.heikinheimo@helsinki.fi
Hinderink	Katja	University of Helsinki, Finland khinderi@mappi.helsinki.fi
Huelsenbeck	Johannes	Hannover Medical School, Germany johuelsenbeck@gmx.de
Jank	Thomas	University of Freiburg, Germany tjank@gmx.de
Janoir	Clair	Université Paris-Sud, France claire.janoir@cep.u-psud.fr
Jiraskova	Alena	Czech Academy of Sciences, Prague alena@biomed.cas.cz
Johnson	Stuart	Loyola University Chicago, USA sjohnson@lumc.edu
Johnson	Eric	University of Wisconsin, USA eajohnso@wisc.edu
Just	Ingo	Hannover Medical School, Germany just.ingo@mh-hannover.de
Kennedy	Catherine	University of Monash, Australia catherine.kennedy@med.monash.edu.au
Keyburn	Anthony	University of Monash, Australia anthony.keyburn@csiro.au

Kobayashi	Keiko	Tokushima University, Japan kobakei@ph.bunri-u.ac.jp
Korkeala	Hannu	University of Helsinki, Finland hannu.korkeala@helsinki.fi
Lebeda	Frank	USAMRIID, USA frank.lebeda@amedd.army.mil
Lemaire	Cédric	University of Liège, Belgium clemaire@ulg.ac.be
Lemee	Ludovic	University of Rouen, France ludovic.lemee@chu-rouen.fr
Lindstrom	Miia	University of Helsinki, Finland miia.lindstrom@helsinki.fi
Liu	Shie-Chau	University of Stanford, USA liusc@stanford.edu
Lotierzo	Manuela	CEA Saclay, France lotierzo@cea.fr
Lyras	Dena	University of Monash, Australia dena.lyras@med.monash.edu.au
Mahrhold	Stefan	Hannover Medical School, Germany mahrhold.stefan@mh-hannover.de
Martin-Satue	Mireia	University of Barcelona, Spain martinsatue@ub.edu
Masayama	Atsushi	Nagoya University, Japan masayama@nuagr1.agr.nagoya-u.ac.jp
Mastrantonio	Paola	National Institute of Health, Rome, Italy pmastran@iss.it
Matamouros	Susana	Institute Pasteur, France susanam@pasteur.fr
McCallan	Lyanne	Queens University, Belfast L.MCCALLAN@QUB.AC.UK
McClane	Bruce	University of Pittsburgh, USA bamcc@pitt.edu
McGlashan	Joanna	CEPR Porton Down, UK Joanna.MCGLASHAN@hpa.org.uk
McLauchlin	Jim	HPA Centre for Infections, UK jim.mclauchlin@hpa.org.uk

Meisohle	Dominik	University of Ulm, Germany dominik.meisohle@gmx.de
Melville	Stephen	Virginia Tech, USA melville@vt.edu
Minton	Nigel	University of Nottingham, UK nigel.minton@nottingham.ac.uk
Miyamoto	Kazuaki	Wakayama Medical University, Japan kazuaki@wakayama-med.ac.jp
Morbiato	Laura	University of Padova, Italy morbiato@bio.unipd.it
Moriyama	Ryuichi	Chubu University, Japan moriyama@isc.chubu.ac.jp
Mühlenstädt	Corinna	Hannover Medical School, Germany fredhofmann@web.de
Mullany	Peter	University College London, UK p.mullany@eastman.ucl.ac.uk
Nagahama	Masahiro	Tokushima Bunri University, Japan nagahama@ph.bunri-u.ac.jp
Nagaro	Kristin	Loyola University Chicago, USA ishakryssa@hotmail.com
Nölkes	Dagmar	University of Göttingen, Germany dnoelke@gwdg.d
Ocepek	Matjaz	University of Ljubljana, Slovenia matjaz.ocepek@vf.uni-lj.si
O'Connor	Jennifer	University of Monash, Australia jennifer.oconnor@med.monash.edu.au
Oda	Masataka	Tokushima Bunri University, Japan masa@ph.bunri-u.ac.jp
Ohtani	Kaori	Kanazaw University, Japan ohtanik@med.kanazawa-u.ac.jp
Okabe	Akinobu	Kagawa University, Japan microbio@kms.ac.jp
Pechine	Severine	Université Paris-Sud, France severine.pechine@cep.u-psud.fr
Peck	Mike	Institute Food Research Norwich, UK Mike.Peck@bbsrc.ac.uk

Phumoonna	Tongted	University of Monash, Australia tongted.phumoonna@med.monash.edu.au
Pirs	Tina	University of Ljubljana, Slovenia tina.pirs@vf.uni-lj.si
Pons	Jean-Louis	University of Rouen, France Jean-Louis.Pons@univ-rouen.fr
Poon	Rachel	University of Monash, Australia rachaelpoon@hotmail.com
Popoff	Michel R.	Institute Pasteur, France mpopoff@pasteur.fr
Posthaus	Horst	University of Bern, Switzerland horst.posthaus@itpa.unibe.ch
Qazi	Omar	Imperial College, UK omar.qazi@imperial.ac.uk
Raffestin	Stephanie	CEA Saclay, France stephanie.raffestin@cea.fr
Reineke	Jessica	University of Mainz, Germany reineke@uni-mainz.de
Reinmuth	Sibylle	University of Göttingen, UK sibylle.reinmuth@gmx.de
Riebe	Oliver	University of Rostock, Germany oliver.riebe@uni-rostock.de
Riley	Thomas	The University of Western Australia triley@cyllene.uwa.edu.au
Roberts	Adam	University College London, UK aroberts@eastman.ucl.ac.uk
Robertson	Susan	University of Pittsburgh, USA sur18@pitt.edu
Rood	Julian	University of Monash, Australia julian.rood@med.monash.edu.au
Rossetto	Ornella	University of Padova, Italy rossetto@bio.unipd.it
Rummel	Andreas	Hannover Medical School, Germany rummel.andreas@mh-hannover.de
Rupnik	Maja	Institute for Public Health, Maribor, Slovenia mrupnik1@gwdg.de

Sakaguchi	Yoshihiko	Okayama University, Japan ysakaguc@md.okayama-u.ac.jp
Sambol	Susan	Hines VA Hospital, USA nuidres@yahoo.com
Sanchez Hurtado	Karla	University of Edinburgh, Scotland k.sanchez@sms.ed.ac.uk
Sarker	Mahfuzur	Oregan State University, USA
Sayeed	Sameera	University of Pittsburgh, USA sarkerm@oregonstate.edu
Schiavo	Giampietro	Cancer Research UK, London giampietro.schiavo@cancer.org.uk
Scalfaro	Concetto	National Institute of Health, Rome, Italy scalfaro@iss.it
Schöntaube	Janett	Hannover Medical School, Germany Schoentaube.Janett@mh-hannover.de
Schwarz	Katrin	University of Rostock, Germany katrin.schwarz@uni-rostock.de
Sebaihia	Mohammed	Sanger Institute, UK ms5@sanger.ac.uk
Shimizu	Tohru	Kanazawa University, Japan tshimizu@med.kanazawa-u.ac.jp
Shone	Clifford	CEPR Porton Down, UK cliff.shone@hpa.org.uk
Siddiqui	Farida	Hines VA Hospital, USA farida.siddiqui@va.gov
Skipper	Philip	CEPR Porton Down, UK philip.skipper@hpa.org.uk
Smedley III	James G.	University of Pittsburgh, USA jgs44@pitt.edu
Soderholm	Henna	University of Helsinki, Finland henna.soderholm@helsinki.fi
Sonenshein	Abraham L	Tufts University, USA linc.sonenshein@tufts.edu
Songer	J Glenn	University of Arizona, USA gsonger@u.arizona.edu

Stabler	Richard	London School of Hygiene & Tropical Medicine, UK richard.stabler@lshtm.ac.uk
Steiner	Elisabeth	University of Aberystwyth, UK eis@aber.ac.uk
Stephenson	Keith	University of Leeds, UK bmbks@bmb.leeds.ac.uk
Stiles	Bradley	USAMRIID, USA bradley.stiles@amedd.army.mil
Swain	Gemma	Imperial College, UK gemma.swain@imperial.ac.uk
Taal	Makie	CEPR Porton Down, UK Makie.TAAL@hpa.org.uk
Tanaka	Kousei	Kanazawa University, Japan kotanaka@med.kanazawa-u.ac.jp
Tang	Hong	NIAID Bethesda, USA tanghon@niaid.nih.gov
Terhes	Gabriella	University of Szeged, Hungary terhesga@freemail.hu
Theys	Jan	University of Maastricht, The Netherlands jan.theys@maastro.unimaas.nl
Titball	Richard	DSTL Porton Down, UK rtitball@dstl.gov.uk
Trinh	Hien	University of Arizona, USA htt@u.arizona.edu
Underwood	Sarah	University of Leeds, UK bmbsu@leeds.ac.uk
Uzal	Francisco	University of California, USA fuzal@cahfs.ucdavis.edu
Van Immerseel	Filip	University of Ghent, Belgium filip.vanimmerseel@UGent.be
Vandersmissen	Liesbeth	University of Leuven, Belgium lies_beth@hotmail.com
Varga	John	Virginia Tech, USA jvarga@vt.edu
Vedantam	Gayatri	Loyola Medical Centre, USA gvedant@lumc.edu

Von Eichel Streiber	Christoph	University of Mainz, Germany veichel@uni-mainz.de
Wren	Brendan	London School of Hygiene & Tropical Medicine, UK Brendan.Wren@lshtm.ac.uk
Young	Mike	University of Aberystwyth, UK miy@aber.ac.uk
SPONSORS		
Broom	Colin	ViroPharm, USA colin.broom@viropharma.com
Carman	Bob	TechLab Inc., USA rjcarman@techlab.com
Ginter	Annita	BioX Diagnostics, Belgium a.ginter@biox.com
Kadra	Benaouda	Ceva Sante Animale, Hungary benaouda.kadra@ceva.com
Redhead	Keith	Intervet UK Ltd keith.redhead@intervet.com
Short	Gerard	Genzyme Europe Research, UK ged.short@genzyme.com
Van De Verg	Lilian	NIH, Bethesda, USA LVanDeVerg@niaid.nih.gov
PUBLIC SESSION		
Foster	Keith	Syntaxin Ltd, UK Keith.Foster@Syntaxin.com
Mitchell	Wilf	Heriot-Watt University, UK W.J.Mitchell@hw.ac.uk
LOCAL HELPERS		
Butcher	Andy	University of Nottingham, UK nixab@nottingham.ac.uk
Cartman	Steve	University of Nottingham, UK stephen.cartman@nottingham.ac.uk
Pennington	Oliver	University of Nottingham, UK Oliver.pennington@nottingham.ac.uk
Scott	Jim	University of Nottingham, UK paxjcs@nottingham.ac.uk