Final Report Summary - HYPERDIFF (The Physiological Basis of Hypervirulence in Clostridium difficile: a Prerequisite for Effective Infection Control)

Executive Summary:
Clostridium difficile infection (CDI) wreaks havoc in European health systems due to the emergence of hypervirulent strains. However, the physiological basis of pathogenesis is poorly understood. Here we have used ClosTron technology to systematically inactivate genes hypothesised to be involved in pathogenesis, and assessed the consequences in vitro and in vivo. In parallel, epidemiological studies have been undertaken on both human and animal populations. Main outcomes are:

Correlation between Hypervirulence and Toxins:
We re-established the importance of toxin A in CDI by showing that isogenic C. difficile mutants producing toxin A alone can cause disease in the hamster. Consequently, the development of diagnostics, vaccines and therapeutics should focus on both toxin A and B. Our data indicates that binary toxin (CDT) may also contribute to disease, and therefore hypervirulence. However, this contrasts with epidemiological data. Further research is required to determine the role of CDT but it may be prudent to test for its presence in clinical isolates through appropriate diagnostic tests. Toxin A and B genes can also be transferred between strains. This has implications for treatment plans that involve the use of non-toxinogenic strains.

Correlation between Hypervirulence and Factors other than Toxins:
Type 027 strain R20291 adhered more strongly to epithelial cells than the non-hypervirulent strain 630, more effectively colonized mouse intestines and adhered more strongly to caeca. Inactivation of adherence genes showed considerable variation in their roles between R20291 and 630, which may contribute to virulence. Our inability to inactivate certain genes (SplA) led to the development of an assay of essentiality, and demonstrated that gldA is essential. Drugs that interfere with the activity of GldA could provide a novel treatment therapy. Compelling evidence was amassed against the current dogma, that hypervirulent/epidemic strains are more prolific in terms of spore formation. Differences are more likely in the timing of sporulation. The majority of C. difficile mobile genetic elements were shown to be transferrable. Encoded genes may allow recipient strains to rapidly exploit a new niche and cause disease.

Correlation between Hypervirulence and Virulence Factor Regulation:
Transcriptomics showed that surface protein genes were differentially regulated in R20291 compared to 630 and specific
genes were early up-regulated in vivo. Consequently, R20291 may possess ecological advantages that enhance GI tract colonisation. Contrary to current dogma, we show that the tcdC genotype does not affect the amount of toxin A/B produced. It seems most likely that TcdC may act like a ‘safety catch’ to safeguard against inappropriate toxin expression, rather than having a quantitative effect on toxin production. In view of these findings, the use of diagnostic tests based on the detection of tcdC variant sequences may not be appropriate. More important in terms of elevated toxin production, maybe a specific Agr Quorum Sensing locus which is widely distributed in hypervirulent and epidemic strains. Alternative sigma factors carried by mobile elements may also have effects on any host acquiring the element.

The Prevalence and Significance of Hypervirulence Traits in Human and Animal Populations:

A database of human and animal C. difficile isolates has been assembled and used to both develop (MALDI-TOFF) and improve (MVLA) typing systems. Our analysis has shown that assumed virulence markers, such as hyperproduction of toxins and presence of binary toxin genes, are not associated with severity and outcome of the disease in humans. C. difficile isolates obtained from human infections in the community were shown to be different to those in hospitals, but significantly resemble isolates obtained from pet and farm animals indicating that they act as a reservoir for human infection, at least in the community.

Project Context and Objectives:

PROJECT CONCEPT AND MAIN OBJECTIVES

BACKGROUND

C. difficile infection induces severe diarrhoea in patients compromised by antibiotic usage and underlying disease conditions. Elderly patients are especially at risk of Clostridium difficile-associated disease (CDAD). Clinical severity ranges from a mild, self-limiting diarrhoea to the potentially fatal pseudomembranous colitis (PMC). Mortality rates can be as high as 30%. Although generally regarded as a healthcare-associated disease, it is increasingly recognised as a community-associated disease, eg., of 9.3% of 703 patients with diarrhoea visiting German general practitioners over a 5 month period were diagnosed with CDAD (presented at ECCMID 2007). C. difficile is also increasingly being recognised as an important cause of enteric disease in animals.

Of particular concern is the emergence of a highly virulent strain throughout Europe. These strains belong to ribotype 027, but are also characterised as toxinotype III, North American pulsed field gel electrophoresis type 1 (NAP1) and restriction endonuclease analysis group Bl. The occurrence of this strain is associated with an excessive use of quinolone antibiotics, and in N. America has been responsible for a 5-fold increase in the historical average of CDAD, more severe disease (complications increased from 7.1% to 18.2%), higher relapse rates (from 20.8% to 47.2%), increased mortality (from 4.7% to 13.8%), and greater resistance to fluoroquinolone antibiotics. Since 2005, C. difficile type 027 has caused outbreaks in England and Wales, Ireland, the Netherlands, Belgium, Luxembourg, Germany and France, and has also been detected in Austria, Scotland,
Switzerland, Poland and Denmark. The impact of CDAD in healthcare settings is considerable. Patients require isolation, revised supportive therapy for underlying disease and for CDAD, specific therapy to eliminate C. difficile, scrupulous hygiene in nursing, environmental decontamination, and (in outbreaks) ward closure. The financial impact of CDAD on the healthcare system is substantial (5-15,000 euro/case in England and $1.1 billion/year in the USA). Assuming a European Union population of 457 million, the potential cost of CDAD can be estimated to be 3000 million euro/year, and is expected to almost double over the next four decades.

The emergence of fluoroquinolone resistant hypervirulent strains has highlighted the possible dire consequences of a change in antibiotic susceptibility. However, the emergence of more catastrophic resistant profiles remains a serious possibility. The organism has long been recognised as multiply antibiotic resistance (eg., erythromycin, clindamycin, lincomycin, tetracycline, chloramphenicol), principally due to the presence of a large proportion of mobile elements in the genome carrying antibiotic resistance genes. As a consequence, just two antibiotics are available for treatment, metronidazole and vancomycin. Whilst the latter has fewer side-effects and is more effective, metronidazole has until now been preferred due to its lower price and concerns over using a last resort antibiotic such as vancomycin. However, metronidazole is becoming increasingly ineffective and reports on the isolation of less susceptible strains are beginning to appear. This has led to moves towards adopting vancomycin as the front line antibiotic, eg., in the UK. This is extremely worrying, as there are numerous examples of gene transfer between Enterococcus and Clostridium, either demonstrated or inferred by isolation of conjugative transposons common to both genera. As the major source of the vancomycin resistance gene vanA is Enterococcus faecium and it lives in the same niche as C. difficile, transfer of vanA to C.difficile would appear to be just a matter of time. The emergence of metronidazole resistance strains carrying vanA would have catastrophic consequences.

The physiological factors responsible for the rapid emergence of hypervirulent 027 strains remain unclear. Indeed, despite the medical importance of the organism, its pathogenesis is little understood. Thus, aside from the involvement of the two toxins, toxin A & B, in disease, no other virulence factors have been definitively identified. Hypervirulent 027 strains produce a binary toxin (CDT) in addition to Toxins A & B (encoded by tcdA and tcdB, respectively), have a deletion in a regulatory gene, tcdC, involved in repression of tcdA and tcdB, are resistant to fluoroquinolones due to mutations in gyrA, and are resistant to erythromycin by an unknown mechanism. Preliminary data suggests that representative 027 strains produce higher levels of Toxin A & B when grown in a particular media in the 'laboratory flask'. Whether such a finding is generally true, or relevant to the in vivo situation, remains to be tested. Higher toxin production may in part be due to the deletion in tcdC. Hypervirulent strains also appear to exhibit a greater sporulation rate than non-epidemic strains, and most recently it was report at the 2nd International Meeting on C. difficile (June 2007) that 027 strains demonstrate increased adherence to human intestinal epithelial cells due to altered surface proteins.

CONCEPT

The identification of those physiological factors responsible for hypervirulence of Clostridium difficile would have significance consequences for infection control and disease prevention. The identity of the principle factors involved would allow the derivation of diagnostic tests directed specifically at those factors (genes or their products) directly responsible for their
spread within the healthcare setting. Such tests would alert health authorities to the presence of hypervirulent strains and ensure implementation of appropriate infection control measures. They would also allow more appropriate and effective epidemiological analysis, enabling the source and spread of the strains involved to be more accurately monitored. Moreover, elucidation of the pathogenic factors involved should allow the derivation of crucial therapeutic countermeasures that could be deployed to both prevent and treat disease outbreaks.

If we are to understand the physiological basis of hypervirulence, then we need to identify those factors responsible. Our strategy will be to establish the major C. difficile virulence factors involved in the disease process, and then to look for differences that cause enhancement of virulence in 027 strains.

Our strategy is made possible by the developments made in the laboratories of partner 1 and 7, who have pioneered C. difficile gene transfer systems. In particular, partner 1 has recently solved the problem of making directional mutants through the development of ClosTron technology. Prior to the ClosTron, only 3 directed mutants had ever been reported in C. difficile. Based on the Sigma Aldrich Targetron system, the ClosTron allows the direct selection of mutants based on the acquisition of erythromycin resistance (or lineomycin resistance in the case of erythromycin resistant 027 strains). In a few short months, this ClosTron system has been used at Nottingham and in European collaborators laboratories to generate over 80 stable mutants in C. difficile, C. acetobutylicum, C. sporogenes and C. botulinum. The procedure is extremely rapid (8-14 days per mutant), highly efficient (100s of retargeted clones are obtained per experiment) and reproducible (100% success rate), and is revolutionizing functional genomic studies in clostridia. Moreover, since its publication, derivatives have been made in which the retargeted ermB gene can be flipped-out using FRT/FLP recombinase technology. This reduces polar effects and paves the way for the creation of multiple mutations using the same ermB gene.

At the outset of this project, little was known of the molecular basis of virulence/ hypervirulence in C. difficile. The only certainty is that the two toxins (TcdA and TcdB) play a central role in disease. The participation of all other factors, known or inferred, remained conjecture. Our baseline data was thus:

1. TcdA and TcdB are the only definitive virulence factors, but the absence of isogenic mutants has until now prevented the determination of their relative roles in disease

2. Certain strains, and in particular type 027 strains, also produce CDT toxin, but its relative contribution to disease is unknown

3. Variation in toxin sequence is known, but any contribution to hypervirulence has not been determined

4. Colonisation/adherence factors are presumed to be important in virulence/ hypervirulence, but direct evidence for their
involvement in the disease process is lacking

5. Hypervirulent strains are reported to be more prolific in terms of sporulation, but the evidence to support this assumption is scant

6. Great variation in surface layer proteins is evident, but any contribution to virulence/hypervirulence is unknown

7. Hypervirulent strains appear to carry genes that are either unique compared to, or significantly diverged from, genes present in the sequenced non-hypervirulent strain, but their contribution to hypervirulence is unknown

8. Virulence strains are known to carry different complements of mobile elements, but their relative contribution to hypervirulence is not known

9. Regulation of virulence factors is presumed to be important, but the relative contribution to virulence/hypervirulence is unknown

10. There are no currently identified hypervirulence traits that can be used in meaningful epidemiological studies

11. The relationship between strains responsible for community acquired and healthcare associated disease is not understood

12. C. difficile is a growing problem in animals, but the relationship between these strains, and their virulence traits, with strains that cause human disease is unclear

OBJECTIVES

The overall objective of this proposal was to determine the physiological factors that cause hypervirulence in Clostridium
difficile, to provide crucial information for both the development of more informed tests for diagnosis and epidemiological studies, and the formulation of more effective countermeasures for infection control and disease management.

To establish the basis of hypervirulence, our basic strategy was to systematically inactivate those chromosomal genes (WP2-WP8) which encode products hypothesised to be involved in pathogenesis, and to assess the effects on virulence using animal and cell culture models. Genes targeted were guided by comparative genomic studies, in which hypervirulent strains were compared to 'standard' strains. Until relatively recently, the strategy to be adopted here was inconceivable due to the recalcitrance of C. difficile to mutational analysis. Our approach is now possible due to the development of the ClosTron, 'a universal gene knock-out system for clostridia'.

To ascertain the prevalence of the identified hypervirulence traits in the human and animal population, epidemiological studies were undertaken (WP9 & WP10). Special attention was be paid to the wider human population to gauge the prevalence of community acquired C. difficile infections. C. difficile can infect a wide range of animal species, including household pets, food producing animals, zoo animals, laboratory animals and wildlife. A high prevalence of binary toxin positive strains among food animals has been noted, and in one study ribotype 027 represented 31.4% of isolates from beef and pork. Broader studies of C. difficile in animals and incorporation of animal and food contact into community based human CDAD studies is required to better understand this potential problem.

The principal specific objectives of HYPERDIFF are:

Objective 1:

To determine any correlation between hypervirulence and toxins, by the:

* purification of toxin B (TcdB) from a non-hypervirulent and hypervirulent strain;

* generation of mutants in tcdA, tcdB and cdtAB of a non-hypervirulent and hypervirulent strain;

* determination of the relative contributions of TcdA, TcdB and CDT toxin to virulence in the in vivo model;

* identification of epitopes specific to 027 TcdB toxin, and the;

* identification of any TcdB-027 specific epitopes which are correlated to hypervirulence of C. difficile 027 strains.
Objective 2:

To determine any correlation between hypervirulence and factors other than toxin, by the:

* generation of mutants in genes encoding putative colonization/surface factors;
* generation of mutants in genes unique/divergent in 027 strains;
* measurement of the degree of attenuation of putative colonization/surface factor mutants;
* measurement of the degree of attenuation of mutants in genes unique/divergent in 027 genes, and;
* determination of the role of S-layer proteins in immune modulation.

Objective 3:

To determine the correlation between hypervirulence and virulence factor regulation, by the:

* the establishment of the effect of antibiotic pressure on virulence factor regulation using in vivo transcriptomics;
* definitive establishment of the role of TcdC in toxin production and hypervirulence;
* determination of the role of the Agr system in virulence and hypervirulence;
* identification of any role mobile elements may play in hypervirulence;
* identification of environmental factors and regulatory genes that control virulence factor production, both in vitro and in vivo, and the;
* identification of potential vaccine candidates and or therapeutic targets.
Objective 4:

To determine, through epidemiological studies, the prevalence of the identified hypervirulence traits in human and animal populations, by the:

* establishment of a pan-European collection of C. difficile human strains derived from cases of both healthcare-associated disease and community-acquired disease;

* establishment of a pan-European collection of C. difficile strains of animal origin;

* comparison of the various typing systems available using the human strain collection;

* characterisation (molecular typing & colonisation properties) of the animal-derived strains, and the;

* refinement of available typing systems to specifically target hypervirulence markers and their subsequent use to determine the prevalence of these traits in both human and animal strains.

These objectives were pursued through 8 interrelated scientific work packages (WP2-WP9) which deployed a range of multidisciplinary approaches and technologies, including ClosTron mutant generation, transcriptome profiling, the application of various in vivo models, epitope mapping, MALDI-TOFF MS, MLVA, WAVE and epidemiological approaches.

Project Results:
WP2: TOXINS AND HYPERVIRULENCE

Objectives:

To establish the relative role of toxins in virulence. Specifically, to:
1) generate combinations of mutants in tcdA, tcdB and cdtA/B

2) assess their comparative virulence in the animal model

Task 1 - Mutant Generation

Using the ClosTron system, we targeted insertions to tcdA and tcdB in both the hypervirulent strain (R20291) and an erythromycin sensitive strain CD630-delta-erm, derived from the genome strain, CD630. The binary toxin (cdtAB) was additionally inactivated in strain R20291. Various combinations of isogenic mutants were made. The tcdB knock-out was made using a standard ClosTron based on the erythromycin resistance determinant, ermB. The tcdA gene was targeted with an intron which carried the chloramphenicol/thiamphenicol resistance gene catP in place or ermB, whereas, cdtA was targeted with an intron carrying the spectinomycin resistance gene aad9 instead of ermB. The use of these different antibiotic resistance markers allowed the creation of the requisite double and triple mutants. The genotype of each toxin mutant was characterised by PCR and DNA sequence analysis to confirm the exact location of each intron insertion made. Southern blot analysis of EcoRV-digested genomic DNA samples, using an intron-specific probe, confirmed that each single mutant only contained 1 intron insertion, double mutants contained just two insertions, whereas the triple mutant had three independent insertions of an intron. In every case the absence of the expected toxin was confirmed by Western blot and toxin specific antibodies. Cytotoxicity, measured using HT29 and Vero cell assays, was only ablated in those strains in which both toxin A and B were inactivated. Toxin specific antibody was used to show neutralization of cytotoxicity with appropriate single mutants.

Task 2 - Comparative Assessment of Virulence in The Animal Model

Hamsters (in groups of eight) were each challenged with 100 - 1000 spores of a single C. difficile strain, 5 days after an oral dose of clindamycin (30 mg/kg). Animals were followed over a 14 day interval, and euthanized if they passed a threshold in terms of CDI symptoms. Bacteriological and PCR analysis of caecum samples taken from each hamster post mortem confirmed that the only infecting strain of C. difficile was, indeed, the strain administered in every case, thus ruling out any possibility of cross-contamination between cages or contamination from the environment.

In the case of 630: whilst the double toxin mutant (A-B-) was avirulent, hamsters which received the A+B+ parental strain, the A-B+ mutant or the A+B- mutant all developed symptoms of C. difficile infection, which resulted in a mean time to endpoint of 1.0 day, 1.3 days and 4.0 days, respectively. These data confirmed that both toxin A and B play a role in disease. However, during the course of the study, an independent group published a similar investigation employing an independently derived erythromycin-sensitive derivative (630E) showing that an A+B- mutant was avirulent. As the two strains 630E and CD630-delta-erm were isolated independently through serial sub-culture, we hypothesised that either strain could have acquired one or more secondary mutations, which may affect the action of either one or both of the toxins - thus explaining the differences between the two studies. Phenotypic characterisation of the strains revealed that unlike CD630-delta-erm and the parent strain 630, strain 630E was non-motile, less virulent in the hamster model, had a tendency to flocculate, produced less toxin,
and was less prone to catabolite repression by glucose. Next Generation sequencing of the respective genomes revealed 9 non-synonymous SNPs (nSNP) compared to the GenBank sequence and common to both strains. In addition, strain 630E carried 12 additional unique nSNPs, whereas strain CD630-delta-erm had 9. However, whereas the SNPs of CD630-delta-erm seem relatively innocuous in nature (ie., conservative replacements in genes/proteins of little significance), the nSNPs of CD630E appear rather severe in nature. One single base deletion caused the fusion of two components of a putative glucose transport system, explaining the different response to glucose. Another caused a severe truncation of a chaperone involved in protein secretion, while another truncated topA (encoding topoisomerase I, the enzyme responsible for maintaining DNA supercoiling). The latter mutation is predicted to result in significant pleiotrophic effects. Indeed, in many organisms, topA mutations are lethal. A DNA inversion immediately before the flagella operon most likely explained the lack of motility. We intend to both introduce and correct nSNPs in the 2 strain lineages to either restore or ablate the virulence of the respective A+B- mutants - using our allele exchange technologies.

In the case of R20291 (hypervirulent): whilst it also produces CDT in addition to toxin A and B, as the primary goal of our study was to examine the relative roles of toxin A and B in CDI, we initially undertook all our experiments in a CDT knock-out strain. As expected all hamsters that were colonized with the wild type R20291 strain (9 out of 10) succumbed to the disease in a mean time of 3.7 days after infection. All 8 hamsters infected and colonized with the triple toxin mutant strain (A-B-C-) survived the entire duration of the experiment and showed no signs or symptoms of CDI. In contrast, all of the hamsters infected and then colonized with a strain making only toxin B (A-B+C-) (8 out of 8) succumbed to CDI with a mean of 2.3 days. Crucially, all hamsters infected and then colonized with a strain only making toxin A (A+B-C-) (7 out of 9) also developed terminal C. difficile disease with a mean of 5.9 days from infection to endpoint. These data are entirely consistent with the equivalent experiments previously conducted with mutants generated in CD630-delta-erm. That is to say, an isogenic strain producing toxin B alone is more virulent than an isogenic strains producing only toxin A, but that the latter is still able to cause disease in hamsters.

Not unsurprisingly, in the toxin A and B mutants in this CDT plus background (A-B-C+) were non toxigenic under the experimental conditions employed. The strains still expressing toxin B and CDT (A-B+C+) and expressing toxin A and CDT (A+B-C+), respectively, showed high levels of cytotoxicity, albeit slightly reduced compared to the wildtype in the latter case (statistically significant in a Vero cell assay). When tested in vivo, it was noted that all of the hamsters infected and colonized with the single toxin A (A-B+C+) and B (A+B-C+) mutants (8 out of 8) developed terminal CDI with means of 2.7 days and 3.0 days from infection to endpoint, respectively. Particularly striking was the apparent increased virulence of the A+B-C+ mutant (3.0 days average time to endpoint) compared to the A+B-C- mutant (5.9 days from infection to endpoint), suggesting that the presence of CDT accentuates the virulence of a toxin B mutant strain producing toxin A alone. Thus, CDT may act in concert with toxin A to increase virulence. Intriguingly, in the case of the (A-B-C+) mutant (ie., an isogenic mutant producing only CDT), 3 of the 9 animals succumbed to disease. However, these 3 animals did not show typical symptoms of C. difficile disease. Thus, whilst they exhibited wet tail, there was no evidence of the usually observed loose faeces or damage to the caecum. Rather, their small intestine was heavily damaged. This observation is in keeping with a previous suggestion that C. difficile can cause infection of the small intestine. Moreover, it has previously been shown binary toxin can cause enterotoxic effects in the ileal loop assay.

Conclusion:
Our studies have re-established the important of toxin A in CDI by showing that isogenic virulent derivatives of two different strains of C. difficile producing toxin A alone can cause disease in the hamster. Consequently, the development of diagnostics, vaccines and therapeutics alike should focus on both toxin A and B. Our data has also produced tantalizing evidence that CDT toxin may also be contributing to disease, and therefore hypervirulence. Others have previously suggested that the presence of binary toxin is linked to more severe disease outcomes. Moreover, the presence of CDT in all representatives of certain so-called hypervirulent strains (eg., PCR-Ribotype 027 and 078) provides further compelling evidence that it contributes to virulence. Further research will be necessary before the role of binary toxin in disease is fully understood, but in the meantime it may be prudent to test for its presence in clinical isolates through appropriate diagnostic tests.

Main Outputs:


WP3: IDENTIFICATION OF IMMUNOLOGICAL DIFFERENCES BETWEEN TCDB-027 AND TCDB OF STANDARD STRAINS (TCDB-10463)

Objectives

Identification of TcdB-027 specific epitopes which are correlated to hypervirulence of C. difficile 027 strains. Specifically, to:

1) purify TcdB from the 027 strain and the type strain

2) raise antibodies to both toxins
3) identify epitopes specific to 027 toxin

4) identify TcdB-027 specific epitopes which are correlated to hypervirulence of 027 strains.

Task 1 - Purification of TcdB-027 and TcdB-10463

Task 1 was started using a purification scheme established before the start of HYPERDIFF for isolation of TcdA-10463/630 and TcdB-10463/630. Since there is high sequence homology to the appropriate toxins of the genome-sequenced C. difficile 630 strain, all results obtained with toxins from 10463 will match to those of 630. Only strains 630 produces a low amount to toxin, which makes their purification difficult.

To purify ribotype 027 TcdA/B several strains were tested. The best producer was used with the purification scheme cited above. The yield of toxin is about 10-20% of that of strain 10463, purity and the absolute amount were reasonable to continue with the planned experiments. The HYPERDIFF project concentrated on the use of TcdB-630(10463) and TcdB-027 for comparative immunizations of mice and rabbits.

Task 2 - Producing the Vaccination Doses and Immunization

A major obstacle is the cytotoxic activity of TcdB (in vitro but more importantly in vivo). Several inactivation-methods were tried, the remaining cytotoxic activity of the TcdB preparations was tested on cultured cells. Only toxoids with a decrease to ~10% of the original cytotoxicity were used for further immunizations. None of the animals that were immunized suffered from the doses that were applied, showing that inactivation was efficient enough. Biologic active toxin B would have killed the animals immediately, again demonstrating the efficiency of our inactivation step.

Immunizations were done in Balb/c mice and in rabbits. All animal experiments were carried out as fee for service with licensed animal stables. The sera that were received during the period were tested on toxin coated plates and titers (of serial ten times dilutions) were determined to monitor the immune reaction. Epitope mapping is summarized below.

Task 3 - Mapping The Epitopes of TcdB-027 and TcdB-10463

TcdB-epitope slides were designed by tgcBIOMICS and produced as a fee for service by Intavis AG (Heidelberg). Two TcdB slides were generated, one based on the TcdB 630(10463) and the other on the TcdB-027 sequence. Both were used in the subsequent assays. Groups of four mice and three rabbits were vaccinated and thereafter tested individually. We observed that not only the response of mice versus rabbits varied, but also an intraspecies variation was observed between the
individual animals of one species. This overall variability made it difficult to identify sites (epitopes) that are major antigenic epitopes for the species (mice or rabbits), or that differ to a greater extend between TcdB-630 and ribotype TcdB-027 toxins.

One difference between TcdB-630 and TcdB-027 immunization was a more pronounced antibody response against several sides of the receptor-binding domain of TcdB-027 (as compared to TcdB-630. Additional evidence for a major difference in immune response of TcdB-630 vs TcdB-027 vaccinated animals, mice or rabbits, was not obtained.

A couple of reactive areas were subcloned, expressed, purified and used for immunization. The toxin-fragments (epitope-regions) were selected based on a common reaction pattern between most of the animals (a summary of all animals). Surprisingly, none of these epitope regions induced neutralizing antibody responses as judged in cell culture assays.

Task 4 - Mutagenesis of Immune Modulating Epitopes

As all polyclonal sera raised against TcdB-630(10463) and/or TcdB-027 were found to be cross-neutralizing, it would appear that there are no antigens present specific to hypervirulent toxin. Accomplishment of this task was, therefore, not possible.

Conclusion:

The impact of the HYPERDIFF-contribution of tgcBIOMICS GmbH to the present project was to clarify the antigenic effects of TcdB-630 versus TcdB-027. A good response against TcdB-630 versus a bad reaction of the animals against TcdB-027 would have given a hint for the contribution of the immune response, in hypervirulence. However, a clear difference was not observed. Moreover, antisera against TcdB630/10463 and TcdB-027 were mutually cross reactive and cross neutralizing. To switch to major antigenic regions (as deduced from the results with polyclonal sera against toxoids) we generated and used recombinant fragments for immunization. These subcloned fragments did induce positive sera, but these were not neutralizing. The use of mixtures of such fragments went beyond the given time frame of HYPERDIFF.

Main Outputs:
The results obtained by tgcBIOMICS under HYPERDIFF will contribute to vaccine development, a major social economic item that we see for the future of Europe.

WP 4: COLONIZATION FACTORS AND HYPERVIRULENCE

Objectives:

To determine the correlation between hypervirulence and factors other than toxins, specifically, to:

1) assess the role of surface proteins in the colonization process
2) assess the role of surface proteins in the virulence
3) evaluate the difference between strains to understand hypervirulence

Task 1 - Inactivation of Genes Encoding Colonization Factors

A number of putative colonisation factors were targeted with the ClosTron mutagenesis system in both CD630-delta-erm and the hypervirulent strain R20291. Successfully inactivated were the genes encoding FbpA (a surface protein, which binds fibronectin and vitronectin) and various genes encoding components of the flagella (FlIC, FlID, FlgE and MotB which respectively encode, flagellin, flagella cap protein, the hook protein the flagella motor component). It proved impossible to knock-out the Cwp84 protease suggested to be responsible for processing of adhesion factors and the genes encoding the major surface layer protein, SlpA.

To investigate the problem encountered with mutating the SlpA and Cwp84 genes, we developed an alternative method for mutant generation, which was successfully used to make an in-frame deletion of cwp84 in both CD630-delta-erm and R20291, but not splA. One likely reason for not being able to inactivate a gene is that the product is essential for growth under laboratory conditions. We therefore set out to develop a method which could demonstrate essentiality. In this method, a merodiploid cell is constructed carry two chromosomally located copies of the target gene. Crucially, the sequence of the second copy is altered, such that whilst still encoding the correct amino acid sequence, the nucleotide sequence is altered such that intron targeting site is removed. This strategy was made possible by a new method developed in the laboratory of partner 1, which allows genes to be easily inserted into the genome, termed Allele-Coupled Exchange (ACE) technology.

For proof of principle, we targeted the gldA (CD0274) gene which has previously been suggested to be essential. The central portion of this gene (289 bp), encompassed by a two Scal restriction sites was resynthesised such that a intron retargeting site at position 548 bp and 623 bp was removed through the use of degenerate codons. The parental portion of the gene between
the two Scal sites was then replaced by simple subcloning with the resynthesised fragment. The resultant gene (gldA*), therefore, still codes for GldA, but no longer contains the intron retargeting site. This was introduced into the genome of the host, which was then used with appropriate retargeted ClosTrons directed against position 548 and 623 of gldA. In both cases, mutants of gldA were readily obtained in the merodiploid strain, but not in the wildtype, i.e., containing only one copy of gldA. These data established the validity of the method.

Having established the method, we went on to attempt a similar approach with the slpA gene. However, time constraints meant that this work was not completed by the end of the award. A manuscript describing the new method has been drafted.

In parallel, and in order to further explore methods for determining essentiality, we pursued an approach termed, Transposon-Directed Insertion Site sequencing, or TraDIS. It utilizes nucleotide sequencing to prime from the transposon and sequence into the adjacent target DNA, simultaneously mapping the site of insertion of every transposon in a mutant pool. Genes that are under represented in terms of transposon insertion represent essential genes, and by implication therapeutic targets. TraDIS was undertaken by next generation sequencing. A total of 20 million mapped sequence tags of 40-50 bp were generated, representing approximately 15 bp of transposon sequence and 25-35bp DNA flanking Himar1 C9 insertion sites. On the first run, 2,600 genes were identified as non-essential. The results of a second and third run are still to be analysed, but potentially will identify genes essential for growth in the presence of bile acid.

Task 2 - In vitro Characterization of Colonization Factors Mutant Phenotypes

The two fbpA mutants obtained in strain CD630-delta-erm were shown to no longer produce the protein by Western blot and to no longer be able to bind fibronectin. It did not prove possible to obtain similar mutants in R20191. In the case of the flagella mutants, all were shown to be non-motile based on swarming and motility assays and to lack flagella under EM. These defects were returned to wildtype when each gene was complemented. Unexpectedly, R20291 proved to be less motile than strain CD630, and intriguingly possessed a single flagellum, as opposed to the multi-flagellated CD630. Additionally, a paralysed mutant was created in R20191 using our allelic exchange method, through the introduction of a single amino acid substitution of the Asp residue at position 23 of MotB with Ala. The mutant retained a single flagellum (visualised by EM) but was none motile.

The in vitro adherence of the two strains and their respective mutants was tested using various cell lines. Whilst the adherence of strains 630 and CD630-delta-erm was the same, the adherence of the hypervirulent strain R20291 to Caco-2 cells was significantly higher. The CD630-delta-erm FbpA mutant adhered more than the wild type strain to Caco-2 cells and HT 29 cells, as did the FliC, FliD and FigE mutants. In contrast, the mutants of R20291 showed no difference in adherence properties to the wildtype.
In parallel, in vitro proteolytic activity of the Cwp84 in-frame deletion mutants was compared to the wildtype isogenic and the complemented strains. The activity was tested on casein and synthetic substrates. The Cwp84 mutants either in 630 or 027 strains displayed a decrease caseinolytic activity (80% compared to WT strains). The complemented strains displayed 100% caseinolytic activity. Activity on the SlpA precursor. The profile obtained for the 630 WT, mutant and complemented strains are those expected, with a band corresponding to the SlpA precursor in the mutant strain and two bands corresponding to the cleaved S-layer proteins HMW and LMW, both in the WT and complemented strains. For the R20291 strains, a band corresponding to the precursor SlpA but also, the two bands corresponding to the HMW and LMW, although with a lower intensity, were found in the mutant strain. These results suggest that in 027 R20291 C. difficile strain another protease could be responsible for the cleavage of SlpA precursor.

The possible role of flagella in cell signalling was also investigated. Our aim was to characterize the signalling pathways in epithelial cells elicited by the flagella of C. difficile 027 through Toll-Like Receptor-5 (TLR-5), which recognizes flagellin monomers. We focused our work on a potential activation of Mitogen Activated Protein Kinases (MAPK) and more specifically the activation of Extracellular signal-Regulated kinases 1 and 2 (ERK1/2).

In order to analyse the role of FliC of C. difficile 027 in cell signalling and in inflammatory response, the R20291 FliC mutant, the complemented flagella mutant and the R20291 wild type strain were tested on TLR-5 expressing cell lines.

TLR-5 expressing MDCK cell lines were obtained by transfection. The TLS-5 expressing cells and MDCK control cells were infected from 5 to 60 minutes by the different strains. In addition purified flagellin was used as a positive control. The ERK1/2 phosphorylation was followed on cell lysates by Western blotting.

The infection of MDCK-TLR5 by the flagellin expressing C. difficile hypervirulent strain triggered a time-dependent activation of MAPK ERK1/2 with a peak at 30 min while no response was observed with the R20291?FliC mutant. The Wildtype strain as well as the complemented strain activate ERK1/2 MAPK at 15 and 30 min via TLR-5. In contrast FliC mutant does not activate ERK1/2 MAPK during the first hours of infection. The ERK1/2 activation by the R20291 flagella might play a role in the initiation of an innate immune response and contribute to the inflammatory response of the intestinal mucosa.

Task 3 - In vivo Characterization of Colonization Factors Mutant Phenotypes

Three gnotoxenic mouse models developed by Partner 4 were used to further characterize the knockout mutants:

1) Monoxenic mouse model challenged by the mutant or by the wild type (WT) strain.
The intestinal implantation kinetics (faecal shedding) was followed during 7 days in faecal samples by C. difficile culture on selective media such as CCFA. In addition, at Day 7, the mice were euthanized and the caecal sampled in anaerobic conditions. Each caeca was rinsed by gently shaking in phosphate buffer pH7.2 and then crushed and diluted to obtain a concentration of 10mg/ml. Serial dilutions were seeded in duplicate on selective C. difficile media. The results correspond to adherent bacteria to caecal walls.

2) Dixenic mouse model challenged simultaneously by the mutant and the WT strain.

The intestinal kinetics of implantation (faecal shedding) was followed during 7 days in this competition model. Caecal adherence was determined at day seven. This was possible according to the differences in antibiotic susceptibility of the mutant and the WT strains.

3) Human Microbiota Associated mouse model

We have previously shown that the microbiota in this model is very similar to a human complex microbiota, with dominant anaerobic groups such as the Bacteroides and Eubacterium phyla. In this model, the microbiota displayed colonization resistance properties. Challenge with the various C. difficile strains was performed after disrupting the normal microbiota by amoxicillin-clavulanic acid. Intestinal implantation (faecal shedding) was followed by C. difficile culture in faecal samples. Caecal adherence was determined at day 7. All the animal experiments were performed in groups of at least 5 mice. The animals were bred in axenic or gnotoxenic conditions.

Study of C. difficile CD630-delta-erm and R20291 (027) regarding intestinal implantation:

This was performed in a dixenic mouse model by challenging axenic mice simultaneously with the 2 strains. In dixenic competition assay, intestinal implantation rate and caecal adherence were lower for CD 630?erm strain as compared to 027 strain. C. difficile 027 strain adheres more in in vitro model than the 630 strain. In vivo, in dixenic mouse model, the 027 strain had a greater intestinal implantation rate and caecal adherence than the 630 strain. These first results could in part explain the hypervirulence of 027 strain.

Fibronectin binding protein mutant:

In the monoxenic mouse models intestinal implantation of 630?erm FbpA mutant was similar to isogenic wildtype strain, whereas FbpA mutant adherence to caeca was significantly lower as compared to wildtype. In the dixenic competition assay, the implantation rate of the FbpA mutant significantly slower, but at Day 7, the colonisation rate was similar. In this model challenged simultaneously by 630?erm FbpA mutant and the isogenic wildtype strain, adherence to caeca (Day 7) was similar in the two groups of mice. In a human microbiota associated mouse model, which displays colonization resistance, faecal implantation after disruption of the barrier microbiota by amoxicillin-clavulanic acid was similar for the FbpA mutant and the parent strain. In this model, caecal adherence was also similar for the mutant 630?erm FbpA mutant and the isogenic wildtype strain.
Thus, in contrast to in vitro adherence assays, the FbpA knockout mutant displayed a trend to lower intestinal implantation and caecal adherence. This was observed mainly in monoxenic but not in human microbiota associated mouse model in which the microbiota is more complex.

Flagella mutants:

As in the in vitro adherence assays, the FliC knockout mutant of 630?erm strain had a trend to a better intestinal implantation and caecal adherence as compared to the isogenic wildtype strain. This was observed in dixenic mouse model but not in human microbiota associated mouse model in which the microbiota is more complex. In stark contrast, the FliC mutant of the hypervirulent strain R20291 proved lethal in the monoxenic model. In vivo comparative transcriptomic analysis in monoxenic mice challenged by R20291 on the one hand and on the other its FliC mutant have been performed (see WP8) in order to decipher the role of the absence of flagella in hypervirulence. In the dixenic mouse model, R20291 FliC mutant intestinal implantation was slower as compared to the wildtype strain suggesting a role of FliC in early intestinal colonization. However, after 7 days, gut colonization and caecum adherence were similar for the wildtype and mutant.

No difference in terms of intestinal implantation or caecal colonisation was observed between the paralysed MotB mutant and parent in the monoxenic model. The mutant could not be compared to the wildtype in the dixenic model due to the absence of a selectable marker. Therefore, dixenic assays used the marked FliC and MotB mutants. The former proved to exhibit reduced intestinal implantation and caecal colonization.

Task 4 - Study of the Role of S-layer Proteins in the Inflammatory Process

The presence and the conservation of SlpA were first confirmed in C.difficile PCR-ribotype 027 by comparing the sequences of the reference strains CD196 and R20291 with those of eight other epidemic 027 strains isolated in different geographic regions and years. The analysis was then extended to 15 clinical isolates belonging to different PCR-ribotypes (001, 012, 014, 017 and 078) in order to obtain, together with the sequences already deposited in GeneBank, at least four slpA sequences for each PCR-ribotype. All strains belonging to the same PCR-ribotype showed an identical SlpA sequence. In particular, strains 027 showed a SlpA sequence with an identity of 88% to that of the strain 001. Glycine extracts SlpAs from the different PCR-ribotypes were examined by SDS-PAGE. All strains showed two distinct S-layer proteins and the same pattern of proteins was observed for PCR-ribotypes 027 and 001. Ten areas with high sequence identity were identified between the SlpA variable regions (LMWs) of these two PCR-ribotypes. Eleven synthetic peptides were designed from the LMW proteins to perform dot blot analysis. Three peptides, two predicted to be surface exposed, were recognized by human sera of patients suffering from C.difficile ribotype 027 and 001 disease, and also by a rabbit polyclonal serum against the SLPs of PCR-ribotype 027. These results confirmed the antigenic role of the LMW SlpA proteins and the presence of variable regions support their role in evasion of the host immune response. Further analyses were performed on 13 C.difficile strains isolated from animals. An identity of 99-100% was found between the SlpAs of human and animal strains belonging to PCR-ribotype 012, 014/022, 027, 066 and 078, except for one SlpA variant of a strain 014 isolated from poultry that showed 53% identity with other strains of the same PCR-ribotype. This virulence factor seems to be highly conserved in each PCR-ribotype independently from the
strains origin.

In vitro adherence assays were performed using Caco-2 cells at 3 and 15 days of growth with a control strain C253 (PCR-ribotype 012), the parental strains CD630-delta-erm (PCR-ribotype 012), R20291 (PCR-ribotype 027) and the two mutants CD630-delta-erm?cwp84 and R20291?cwp84, obtained by Partner 1 deleting the cwp84, protein responsible for the activation of the S-layer precursor protein. The results demonstrated that the strain R20291 PCR-ribotype 027 adhered more strongly than the strain CD630-delta-erm, which did not show differences with its mutant CD630-delta-erm?cwp84. On the contrary, a statistically significant decrease in the adherence was observed in R20291?cwp84 compared to the wild type strain. So, the role of SlpA in adherence seems to be 1) strain-dependent, 2) partial, in agreement with the known multicomponent adhesive properties of the bacterial surface proteins. Experiments performed using the epithelial intestinal cell (Caco2) line have shown that either purified SLPs or the entire bacterium both derived from hypervirulent epidemic or non-hypervirulent epidemic C. difficile strains were not able to induce up regulation of ICAM-1 measured both by Western blot assay with anti ICAM-1 antiserum and by the mRNA expression.

SLPs from C. difficile strains belonging to the PCR-ribotypes 027 (R20291, AI13), 001 (001-3) and 012 (C253, 630) were tested in human purified monocytes and monocytes derived dendritic cells (MDDC) ex vivo models. Stimulation of monocytes with the different SLP preparations was followed by an increase in release of proinflammatory IL-1beta and IL-6 and IL-10, an anti-inflammatory/regulatory cytokine. SLPs induced significantly higher amount of all the cytokines tested compared to untreated cultures, while no statistically significant differences were found between SLPs from different from hypervirulent epidemic or non-hypervirulent epidemic strains. We tested the ability of SLPs to promote production by MDDC of IL-12p70 and IL-10, pro- or anti-inflammatory cytokines, respectively. High amounts of IL-10 were induced, without any statistically significant difference between the different SLP preparations. The levels of IL-12p70 were low or undetectable, the only exception were SLPs derived from the C253 strain, that induced substantial levels of IL-12p70, confirming our previous results (Ausiello et al., 2006). T-helper cell polarization studies showed that SLPs-treated MDDC drove the expansion of either IFNgamma-producing Th1 cells and IL-5-producing Th2 cells. Again, no differences were observed among the SLPs from hypervirulent epidemic or non-hypervirulent epidemic strains. Here again the C253 SLPs-treated MDDC induced a T cell polarization more oriented towards a Th1 profile respect to 630 SLPs. Overall our results suggested that SLPs are recognized by effectors of the innate immune system and possess very similar immunomodulatory activities. SLPs of hypervirulent strains trigger similar inflammatory processes as those extracted from non-hypervirulent PCR-ribotypes.

Conclusions:

Our studies have shown that it is not always possible to inactivate genes using the ClosTron, in some cases most likely due to the essential nature of the encoded protein, ie., SlpA. Accordingly we have developed a novel procedure which may be deployed to confirm essentiality, and used it to provide more compelling evidence that gldA is essential. Drugs that interfere with the activity of the encoding enzyme, could therefore provide a novel therapy for treating C. difficile disease. A manuscript describing this method has been drafted but cannot be submitted until the ACE method has been accepted for publication (currently under review).
We were also able to develop a novel procedure for in-frame deletions, and used it to create mutants of Cwp84 in both strains. Intriguingly, whilst the CD630-delta-erm mutant no longer processed SipA, residual activity was still detected in the R20291 mutant, raising the possibility that another protease activity may be present in the hypervirulent strain. Nonetheless, the R20291 Cwp84 mutant showed a significant decrease in its ability to adhere to cell lines. No such decrease was observed with the CD630-delta-erm mutants. Thus, the role of SipA in adherence seems to be 1) strain-dependent, 2) partial, in agreement with the known multicomponent adhesive properties of the bacterial surface proteins.

Our mutational analysis also identified clear differences between the role of FbpA and flagella proteins in strain 630 and the hypervirulent R20291. Notably, whilst the creation of non-motile flagella mutants in CD630-delta-erm reduced their capacity to adhere to cell lines, the same was not observed with the hypervirulent strain R20291. Differential behavior in the various in vivo models employed was also noted. Thus, in the dixenic competition assay, the intestinal implantation rate in mice was slower for the CD 630?erm strain as compared to hypervirulent R20291 strain. Furthermore, caecal adherence was significantly lower for CD 630?erm strain as compared to the hypervirulent R20291 strain. The greater intestinal implantation rate and stronger caecal adherence of the hypervirulent strains compared to the non-hypervirulent strains may in part explain its enhanced virulence. In the in vitro adherence assays, the Fbp knockout mutant exhibited a lower rate of transplantation and caecal adherence than the wildtype organism. In contrast, the flIC CD630-delta-erm mutant demonstrated an increased rate of transplantation and caecal adherence compared to the progenitor strain.

In general, our data suggests considerable variation in the roles of the various proteins studied between the hypervirulent strain and strain 630, which may contribute to pathogenesis.

Main Outputs:


WP5: OTHER GENES AND HYPERVIRULENCE

Objectives:

To assess the contribution of genes, other than toxins and adhesins, to hypervirulence, specifically to:

1) inactivate genes that are unique to 027 strains using ClosTron technology

2) inactivate genes that show significant variation in 027 strains, compared to the genome strain

3) assess the virulence of the mutants generated in vivo

Task 1 - Inactivation of Genes Unique to/ Divergent in 027

As reported at our Mid-Term review point, since submission of the proposal further characterization of the identity of genes unique to type 027 hypervirulent strains was undertaken through the examination of 94 clinical strains of the most common PCR-ribotypes isolated in Europe and the UK by array comparative genomic hybridization. Our analysis showed that the PCR-
ribotype 027 markers absent in the CD630 genome are not solely confined to PCR-ribotype 027 strains, but appear distributed amongst other PCR-ribotypes to varying degrees. In view of this finding, we elected to subtly change the direction of the project, by focusing on those observed phenotypic differences that are beginning to emerge between hypervirulent strains from both studies being undertaken within this project, as well as the wider scientific community. At the same time, we elected to develop a random mutagenesis system that would allow the isolation of mutants with reduced virulence using a 'forward genetics' approach.

Forward Genetics:

Pivotal to the molecular dissection of physiological traits is the ability to make mutations. In the absence of clues as to the nature of the genes and their products involved in any particular process, mutants of a desired phenotype may be generated randomly and thence the genotype responsible determined. This process of 'Forward Genetics' is reliant on the availability of effective random mutagens, most commonly transposons. Until now such elements were unavailable. Accordingly, we developed a novel mariner-based transposon system for in vivo random mutagenesis of C. difficile R20291, the BI/NAP1/027 epidemic strain at the centre of the CDI outbreaks in Stoke Mandeville, UK in 2003-4 and 2004-5. Transposition occurred at a frequency of 4.5(±0.4) × 10⁻⁴ per cell to give stable insertions at random genomic loci, which were only defined by the nucleotide sequence ‘TA’. Furthermore, mutants with just a single transposon insertion were generated in an overwhelming majority (98.3% in this study). Phenotypic screening of a C. difficile R20291 random mutant library yielded a sporulation/germination defective clone with an insertion in the germination specific protease gene cspBA, and an auxotroph with an insertion in the pyrimidine biosynthesis gene pyrB. These results validate our mariner-based transposon system for use in forward genetic studies of C. difficile.

An alternative system for forward genetics using the conjugative transposon Tn916 was developed by partner 7. The usefulness of this system was shown by the fact that we could obtain a mutation in a gene involved in flagella biosynthesis. Furthermore insertion sites of the transposon, using next generation sequencing, were determined in strains 630 and R20291. A consensus fifteen base pair Tn916 insertion sequence was identified which was similar in both strains although an extended consensus sequence was observed in R20291. A search of the C. difficile 630 genome showed that the Tn916 insertion motif was present 100,987 times, with approximately 63,000 of these motifs located in genes and 35,000 in intergenic regions. Therefore Tn916 is a useful alternative system for forward genetics in C. difficile.

Sporulation:

Spores, formed during sporulation, play a pivotal role in disease transmission and it has been suggested that BI/NAP1/027 strains are more prolific in terms of sporulation in vitro than ‘non-epidemic’ C. difficile types. In this study, we analyzed the sporulation rates of 53 C. difficile strains, the largest sample size used to-date in such a study, including 28 BI/NAP1/027 isolates. Our data confirm that significant variation exists in the rate at which different C. difficile strains form spores. However, we clearly show that the sporulation rate of the BI/NAP1/027 type was no higher than that of non-BI/NAP1/027 strains. In addition, we observed substantial variation in sporulation characteristics within the BI/NAP1/027 type. This work highlights the danger of assuming that all strains of one type behave similarly without studying adequate sample sizes. Furthermore, we stress the need for a standard set of experimental procedures in order to quantify C. difficile sporulation more accurately in the future.
Germination:

Clostridium difficile spores play a pivotal role in the transmission of infectious diarrhoea, but in order to cause disease spores must complete germination and return to vegetative cell growth. While the mechanisms of spore germination are well understood in Bacillus, knowledge of C. difficile germination remains limited. Previous studies have shown that bile salts and amino acids play an important role in regulating the germination response of C. difficile spores. Taurocholate in combination with glycine, can stimulate germination, whereas chenodeoxycholate has been shown to inhibit spore germination in a C. difficile clinical isolate. We therefore investigated how the germination characteristics of different C. difficile isolates vary in response to bile salts. By analyzing 29 isolates, including 16 belonging to the BI/NAP1/027 type, we show that considerable diversity exists in both the rate and extent of C. difficile germination in response to rich medium containing both taurocholate and glycine. Strikingly, we also showed that although a potent inhibitor of germination for some isolates, chenodeoxycholate does not inhibit the germination, or outgrowth, of all C. difficile strains. Finally, obtained evidence that components of rich media may induce the germination of C. difficile spores, even in the absence of taurocholate. Taken together, these data suggest that the mechanisms of C. difficile spore germination in response to bile salts are complex and require further study. Furthermore, we stress the importance of studying multiple isolates in the future when analyzing the nutrients or chemicals that either stimulate or inhibit C. difficile spore germination.

Other genes inactivated:

A number of additional genes have been inactivated, including CD0153-55 (in collaboration with Prof Brendan Wren, LSTM) and the bile acid degradation gene CD0065. The involvement of CD0153-55 in p-cresol production has been confirmed, however, a phenotype associated with CD0065 has not been definitively established. Thus, the mutant exhibits a similar level of resistance to bile as the wildtype but shows a slightly reduced capability to form spores.

Task 2 - Inactivation of Unique Genes on Mobile Elements

ClosTron mutants have been made in the following mobile elements in 630; CTn1 was marked in the ABC-transporter gene CD0364 and a putative cell surface encoding protein CD0386, CTn7 was marked in a homologous cell surface protein CD3392. CTn2 and CTn4 were marked in transcriptional regulator genes, CD0428 and CD1099, respectively. CTn5 was marked in an ABC transporter CD1873. These insertions were verified by DNA sequencing. All the ClosTron-marked elements were capable of conjugal transfer from C. difficile 630 to C. difficile CD37 indicating that these genes were not essential for conjugal transfer.

The genome sequence of the ribotype 027 strain C. difficile R20291 has been subject to a bioinformatics search for conjugative transposons and prophage. Novel putative mobile elements have been identified in this strain, some of which are related to those in 630. A novel genetic element that contained a CTn5-like core but also contained putative mobilizable elements was marked with the ClosTron in ORF 1803, homolog to CD1873 of CTn5. The element could still excise form the genome but
transconjugants in C. difficile CD37 could not be detected.

The marked strains are now being investigated by transcriptomics analysis using RNAseq. At the time of completion, the data generated is still being analysed.

Task 3 - Assessment of Mutant Virulence in in vivo Models

Having undertaken a number of experiments with the mouse model, which measures colonisation/persistence as opposed to virulence, attention turned to the hamster model of infection, beginning with the fliC mutant of CD630-delta-erm. According to the previous experiments, two groups of 8 animals were challenged with spores prepared from either the mutant or wildtype strain. Unexpectedly, the fliC mutant proved more virulent than the control group, with hamsters succumbing to infection on average 2 days after challenge, compared to 3-4 days with the wildtype. In order to ascertain what could be causing this increased lethality, we measured toxin production in the two strains when growth in TY media in the 'laboratory flask'. From this analysis, it was clear that the supernatant of the fliC mutant of CD630-delta-erm contained much higher concentrations of toxin, by almost two orders of magnitude (Figure 28). A similar increase was evident in culture supernatants derived from the fliD mutant of CD630-delta-erm, but not from the flgE mutant. The levels of toxin obtained in the CD630-delta-erm fliC and fliD mutants were equivalent to that observed in culture supernatants derived from the wildtype R20291 strain. However, in the case of R20291, none of the flagellar mutants produced higher levels of toxin compared to the wildtype strain.

Complementation studies were undertaken to assess the ability of introduced wild-type genes to restore the toxin production levels to normal. It was observed that the fliD and fliC complemented strains completely restored toxin production to the wildtype level. However, toxin levels were only partially restored in the flgE mutant strain. This was consistent with the previous complementation studies, where flagellation, motility and in vitro cell adherence phenotypes were similarly only partially restored. One possible explanation for the increased amount of toxin in the mutants was that the level of expressed had been altered. Quantitative real-time RT-PCR was performed to compare the relative expression of the two major virulence factors, tcdA (toxin A) and tcdB (toxin B) genes in each flagellar mutant (fliD, fliC and flgE). Surprisingly the results of qRT-PCR analysis showed that the expression of the tcdA gene was strongly up-regulated in the fliC and fliD mutant strains. tcdA expression was on average 44.1-fold greater in the fliC mutant than in the wild-type strain. The fliD mutant expressed tcdA gene at an average of 7.4-fold higher level than the wild-type strain. These results confirmed that the high levels of toxin in the non-motile non-flagellated fliD and fliC mutant strains were indeed due to increased expression of the toxin A gene. In the case of flgE mutant, not unexpectedly, tcdA expression was an average of 10-fold lower compared to the wild-type strain.

Conclusions:
A major finding of this work package has been the provision of compelling evidence against the current dogma, that hypervirulent/epidemic strains are more prolific in terms of spore formation. This has clearly shown to be false. Previous studies have been guilty of using inappropriate experimental design and to have been reliant on small sample sizes. Moreover, it is likely that general assumptions being made about spore germination, particularly in terms of inhibition by chenodeoxycholate, will also turn out to be ill-founded. It may transpire that the differences in spore properties relevant to hypervirulence will be related either to the timing of spore production and/or the response to in vivo factors that control the process. It is notable that spore formation in the agr mutant is considerably brought forward in the growth phase. A naturally occurring strain that sporulates from very early exponential phase was discovered during the course of our experiments, which behaves in a very similar manner to the agr mutant. The genome sequence of this strain has been determined and is currently being annotated.

Regions on mobile elements that do not affect conjugative transfer have been identified. These regions have been marked by the antibiotic resistance gene contained within the clostron allowing the conjugal ability of these elements to be measured. Both C. difficile R20291 and contain a large number of integrated elements some of which were proven to be mobile in this study.

A further notable discovery in this work has been the unexpected consequences of certain mutants on toxin expression, most notably the fliC mutants of CD630-delta-erm. Given that the level of toxin produced in the CD630-delta-erm mutant now mirrors that of R20291, it raises the intriguing possibility that part of the reason for the higher levels of toxin seen in hypervirulent strains is that the mechanisms in place to control toxin expression have been deregulated. This may explain why mutation of the flagella apparatus in R20291 has no apparent effect on toxin levels in the supernatant. However, the mechanism underlying this regulatory relationship between synthesis of flagella locomotion apparatus and regulation of toxin production in this organism is not clear.

Main Outputs:


Burns DA, Heeg D, Cartman ST, Minton NP.(2011) Reconsidering the sporulation characteristics of hypervirulent Clostridium


WP 6: DEFINING THE ROLE OF MOBILE ELEMENTS IN HYPERVIRULENCE OF 027

Objectives:

To determine the correlation between hypervirulence and mobile genetic elements. Specifically to:

1) genetically mark 027 mobile elements using the Clostron

2) establish conjugative transfer of 027 mobile elements

3) assess the effects of the inheritance of mobile elements on virulence.
Task 1 - Genetic ‘Marking’ of Mobile Elements

Strain 630 has 6 putative conjugative transposons: CTn1, CTn2, CTn4, CTn5, CTn6 and CTn7, of which, all, except CTn6, have been shown to excise from the genome. In order to determine whether the other putative elements are capable of excision, specific oligonucleotide pairs were used to PCR amplify the element-genome junctions, the joints of the element in a circular form (the transposition and conjugal intermediate) and the regenerated target site after excision. PCR products were produced with and sequencing showed the element-genome junction, the empty target site in the chromosome after excision and the joint sequence in the circular molecules. This analysis allowed the ends of the elements to be defined.

Strain R20291 (ribotype 027) contains two putative conjugative transposons that are variants of elements found in strain 630. One of these has a structure comparable to CTn1 in strain 630, the main difference being the accessory module of the elements. Excluding the accessory module, the remainder of the two elements show at least 82% nucleotide sequence identity. We could not detect excision of the element in R20291, possibly because of a deletion of three ORFs, including the putative xis gene, compared to CTn1 in 630. Furthermore, this element was found to have integrated into a different site within the genome of R20291 when compared to 630, integrating between ORFs 3452 and 3476 in R20291, genes encoding a hypothetical protein and a putative transcriptional regulator, respectively (ORFs 3452 and 3476 in R20291 are homologues of CD3614 and CD3615 in strain 630). In 630, CTn1 is integrated into CD0354, a gene encoding a hypothetical protein; an uninterrupted homologue of this gene is present in R20291.

The second putative conjugative transposon in R20291, has been previously reported as CTn027 and an insertion in the element was erroneously called the Stoke Mandeville phage island, SMPI. There is no evidence of a phage within this element or that the element itself is a prophage, and we renamed it Tn6103 as it fits the criteria for a conjugative transposon according to the transposon registry guidelines.

This element is similar to CTn5 in strain 630, having at least 85% nucleotide identity along most of its length; however, it contains three insertions which are probably mobilizable transposons (see below), two of which are inserted within ORF 1743 and one within ORF 1776. These elements have been named Tn6104, Tn6105, and Tn6106. All three elements contain a recombinase gene, however, excision and circularization has been demonstrated only for Tn6104 as well as the composite element itself, Tn6103.

To study the transfer of the putative conjugative transposons, the ClosTron system was retargeted to ORFs within the accessory module of each element.
Task 2. Mobility Experiments

The transposons marked with the ClosTron could still excise from the genome, as determined by PCR. Filter mating assays were performed using strains containing a marked element as donor and C. difficile CD37 as recipient. Transconjugants were screened by PCR for the presence of the inserted ClosTron, as well as the absence of the PaLoc (to confirm them as strain CD37). All six marked elements from strain 630 transferred into the recipient strain CD37 at frequencies between $10^{-4}$ and $10^{-9}$. Transfer of Tn6103 from strain R20291 to CD37 was not detected indicating either that the element cannot transfer into CD37, or it does so at a transfer frequency below the detection limit.

Southern blot confirmed integration in a single target site for CTn2, CTn4 and CTn5 and multiple target sites for CTn1 and CTn7. Additionally CTn5 was shown to be capable of transfer to Bacillus subtilis.

In order to determine if some of the mobile genetic elements are capable of co-transfer in the absence of direct selection, PCR was performed to look for the presence of other mobile elements in the transconjugants (note that these elements were not marked with the clostron). Tn5397, CTn2 and CTn5 were found to be present in some transconjugants even though selection for these elements had not been applied.

One transconjugant contains the PaLoc, CTn1, CTn5 and Tn5397 but not the binary toxin, CTn2 or Tn5398. This strongly indicates that the PaLoc has been transferred from strain 630 to CD37. The transconjugants and recipient strains are currently being sequenced to confirm co-transfer of mobile elements and the PaLoc. The sequence data may provide clues to the mechanism of transfer, whether by cell fusion, Hfr-like transfer or just transfer of specific mobile elements.

Task 3. Assessment of Effects on Virulence

The isolation of a transconjugant containing the PaLoc in a previously non-toxigenic strain will allow the contribution of this locus to virulence to be unequivocally determined. This work has also demonstrated that the PaLoc maybe a mobile genetic element although the mode of transfer is currently unknown. Furthermore experiments on expression of the PaLoc genes in the new host are being undertaken.

Bioinformatics analysis of the accessory regions on some of the conjugative transposons have indicated that there are a number of genes with a potential role in virulence of C. difficile and its ability to survive in the human host. Clostron mutants have been constructed in the genes listed above. In addition, mutants have been constructed in a gene encoding a predicted cell surface protein, CD0386, and we are also determining if the protein is surface located by electron microscopy.
ClosTron mutants of transcriptional regulators on CTn2 and CTn4 have been made and their effect on the global transcriptome is being determined by transcriptomics analysis. The number of reads for each sequence shows the level of expression for each gene.

The transcriptomes of mutants are being compared to the wild-type strains to determine the global contribution of mobile elements to gene expression in C. difficile.

Conclusions:

This work has shown that all C. difficile strains for which sequence information is available contain mobile genetic elements. These elements contain accessory genes (i.e. those not involved in mobile element maintenance and transfer) that potentially allow the organism to survive in particular ecological niches, e.g. antibiotic resistance genes. However the majority of accessory genes carried on CTns in C. difficile encode ABC transporters and efflux systems presumed to function in resistance to antimicrobial peptides, produced either by the host innate immune response, or by microbial competitors in the intestinal niche. In addition, we have shown that some elements carry genes with the potential to encode bile salt hydrolases which could contribute to the ability of the bacterium to adapt to the human host. A secreted protein which appears to have a stalk-like structure that projects it away from the cell surface is also worthy of further investigation since it is likely to be involved in the interaction with the human host. Some of these elements also contain sigma factors which may have an effect on the global transcriptome of the C. difficile host.

A striking finding of this study showed that the pathogenicity locus of a toxigenic strain (i.e. the locus containing the toxins, the major virulence factors of this organism) can be transferred to a non-toxigenic strain. The fact that non-toxigenic strains can acquire the toxins has implications for management of C. difficile outbreaks. Clearly there is now an urgent need to understand the factors that contribute to the spread of the toxin genes. It is essential to determine if this locus can transfer to other species of clostridia, and beyond and if so, whether the locus is expressed and can none pathogenic strains be rapidly converted to pathogens. This observation also has implications for the treatment of C. difficile, as one of the proposed methods for treatment is to out-compete toxigenic strains with non-toxigenic strains. Clearly the ability to transfer the PaLoc will have serious implications for such an approach.

It is highly likely that the toxin locus is not capable of transfer on its own, as it does not have any obvious mobilisation or conjugation genes, but most probably requires other mobile elements to facilitate transfer. It is now imperative that we identify such elements in order to establish if transfer of the toxins occurs in nature and determine the conditions that promote transfer.
The fact that there are so many mobile elements in C. difficile and that these often contain genes that give their host a survival advantage indicate that strategies aimed at slowing down gene transfer between these pathogenic strains could be worthwhile. Furthermore, the finding that some of the mobile elements are capable of transfer to distantly related bacteria such as Bacillus subtilis indicates that C. difficile can act as a reservoir of antibiotic resistance genes and genes that could give bacteria other than C. difficile a survival advantage in particular ecological niches, such as the human gut. This underlines the needs to monitor the rapid evolution of bacteria as they acquire antibiotic resistance-, niche exploiting- and virulence genes. A major outcome from this work is that C. difficile is particularly adept at spreading these genes.

Main Outputs:


WP 7: REGULATION OF TOXIN PRODUCTION & OTHER FACTORS IN HYPERVIRULENT STRAINS

Objectives:

To identify the regulatory factors controlling toxin production, and other factors, in hypervirulent strains. Specifically, to;

1) definitively establish the role of TcdC in toxin repression

2) establish the role of the two agr quorum sensing loci in 027 strains

3) investigate the role of the LytTR family of response regulators in virulence factor regulation

4) investigate the role of response regulators of two-component systems unique to 027

Task 1 - Generation of Regulatory Mutants

TcdC:
Toxin A and toxin B are the principal virulence factors of Clostridium difficile. The alternative sigma-factor TcdR directs production of both toxins, and is thought to be negatively regulated by TcdC. The tcdC sequence of BI/NAP1/027 strains has a single nucleotide deletion at position 117, resulting in a truncated TcdC, and an 18-nucleotide deletion. This is widely postulated to result in increased toxin production which, in turn, has been linked with reports that BI/NAP1/027 strains cause a more severe disease. To test this, we set out to make defined mutations in tcdC. The small size of the tcdC gene has meant that there are a paucity of strong candidate target sites for inactivation of the gene using the ClosTron. This has necessitated the development and implementation of an alternative approach based on generation of mutants by allelic exchange. To accomplish this we investigated the use of codA as a negative selection marker. The product of the codA gene, cytosine deaminase, converts 5-Fluorocytosine (5FC) into the highly cytotoxic derivative, 5-Fluorouracil (5FU). Using the principle of pseudo-suicide vectors, we exemplified the use of codA as a negative selection marker by creating an in-frame deletion of a gene previously inactivated using the ClosTron, spo0A. A plasmid was created in which the deletion site within spo0A was flanked by 500 bp of contiguous sequence, and incorporated into the vector backbone, a copy of the E.coli codA gene under the control of the C. pasteurianum fdx promoter, in addition to the selective marker catP. This plasmid was introduced into R20291 and single crossover integrants selected as faster growing colonies on agar media containing thiamphenicol. The isolated integrant was then plated on minimal media supplemented with 5FC. Those colonies that grow are 5FCR and must, therefore, have lost the plasmid through homologous recombination mediated excision.

Having successfully exemplified the method, we turned our attention to TcdC. Accordingly, we used our newly developed codA-based allelic exchange system to generate isogenic strains of the BI/NAP1/027 strain of C. difficile, R20291, in which we 1) deleted tcdC, 2) repaired the single nucleotide deletion, 3) repaired both the single- and the 18-nucleotide deletion and 4) substituted the native tcdC sequence for that of C. difficile 630. Furthermore, we deleted tcdC in the ribotype 012 strain of C. difficile, 630, and by way of complementation, we then restored the tcdC open-reading-frame with a silent nucleotide signature, so that it could be distinguished from the wild-type.

Generation of other regulatory mutants:

ClosTron technology was successfully used to generate mutants of agrB1 in CD630 and agrB2 and agrA in R20291. Mutants were also generated in luxS, CD0160, CD0311, CD1743, CD2214, CD2215, CD2603, 3 X feoB mutants (CD1479, CD1517, CD3274), a range of genes involved in sporulation (CD3490, CD2644, CD1192, CD2629, CD2642, CD1464, CD16465, CD0668, Cd0669) a fur and ferretin mutant (CD1287 and CD1295) and a rsmA homologue, CD0234.

Conjugative Transposon encoded factors:

ClosTron mutants of transcriptional regulators on CTn2 and CTn4 have been made and their effect on the global transcriptome is being determined by RNAseq. The number of reads for each sequence shows the level of expression for each gene. The transcriptomes of mutants are being compared to the wild-type strains to determine the global contribution of mobile elements to gene expression in C. difficile.

Task 2 - Assessment of the Effect of tcdC Inactivation on Toxin Production
The various strains constructed during Task 1 in CD630-delta-erm and R20291 were cultivated in BHIS media and the effect on toxin production analysed by cytotoxicity assays. Surprisingly, we did not observe any differences in the amount of toxin produced by isogenic strains of C. difficile when they were cultured in TY medium. As no differences were detected the use of hamsters in in vivo studies could not be justified. These results suggest that tcdC genotype does not affect the amount of toxin A and B produced by C. difficile. At first, this finding seems to be at odds with qualitative functional genetic studies which have found that TcdC is a negative regulator of toxin production. However, our study was designed to qualitatively assess the effect of different tcdC genotypes on toxin production, not qualitatively assess the functional role of TcdC in C. difficile. Therefore, taking all the evidence together, it seems most likely that TcdC may act like a ‘safety catch’ to safeguard against inappropriate toxin expression, rather than having a quantitative effect on the amount of toxin a strain produces. This idea is consistent with the findings of this study and previous studies.

Purely based on our findings presented here, it is possible that TcdC does not have a role in toxin production at all. This hypothesis would be highly controversial as it is not consistent with the findings of previous work. However, it is interesting to note that C. botulinum, C. perfringens, C. sordellii and C. tetani all harbour a TcdR-like group 5 Extra-Cytoplasmic-Function (ECF) sigma factor which direct the RNA-polymerase holoenzyme to the promoter regions of genes encoding botulinum neurotoxin, a bacteriocin, sordellii lethal toxin and tetanus toxin, respectively, yet none of these organisms harbour a tcdC-like ORF. Furthermore, BLAST searches reveal several organisms that encode proteins with homology to TcdC, but not to TcdR. Notably, the greatest conservation across TcdC-like sequences is seen in the C-terminal region, which is proposed to harbour the active domain of TcdC. This begs the question, what is the role of a TcdC-like protein in these organisms? Of course, it is possible that a TcdC-like protein may regulate sigma factors other than those in the TcdR-like group 5 ECF family. Even so, it remains the case that C. difficile appears to be the only organism identified to date which harbours both a tcdR-like ORF and a tcdC-like ORF. This suggests that either regulation of a TcdR-like group 5 ECF sigma-factor by a TcdC-like protein is unique to toxigenic strains of C. difficile, or that TcdC may carry out another function in the cell altogether. However, as this line of reasoning is purely based on circumstantial (and not experimental) evidence, we choose to retain an open mind as to the role of TcdC in C. difficile.

Task 3 - Role of TcdC in Toxin Regulation

A soluble, biologically active form of TcdC has been cloned and was purified using Nickel-Histidine6 tag chromatography. Expression of full-length recombinant TcdC in E. coli yielded a non-soluble protein, whereas deletion of the N-terminal 50 amino acids (membrane anchoring domain) generated a soluble protein. Mutants of TcdC, which lack (part of) the putative dimerization domain, have been generated. Complete truncation of the dimerization domain resulted in a lack of dimerization, as evidenced by cross linking and gel filtration. These results were confirmed by gel filtration.

Through bio-informatics we have predicted that the C-terminal domain of TcdC adopts a so-called OB fold (oligonucleotide/oligosaccharide binding-fold). This type of OB fold is predicted to bind to single stranded DNA. Gel shift experiments using single stranded DNA and recombinant TcdC readily showed binding. Dimerization of TcdC is a prerequisite...
for binding, as evidenced by lack of binding with a truncated TcdC. We were able to generate a consensus sequence that is bound by TcdC with high affinity, using a site selection procedure (Selex). Further investigation learned that TcdC binds this sequence because the oligonucleotide is able to form a folded structure, a so called quadruplex structure. Subsequently, TcdC was shown to bind to other quadruplexes. We are currently assessing the role of TcdC binding to quadruplex structures in respect to biological functions, such as inhibition of transcription and binding to cross-over junctions.

Task 4. Assessment of the Affects of Inactivation of Agr

In Staphylococcus aureus, the agr quorum sensing locus (agrCABD) has been implicated as a key regulator of many virulence factors. In strain CD630 only homologues of agrD and agrB were present, respectively encoding a prepeptide of a secreted small autoinducer peptide and a transmembrane protein (AgrB) involved in AgrD processing. The homologous system in S. aureus also contains two further genes; agrC and agrA encoding a two-component system. Preliminary 454 sequencing of the PCR-ribotype 027 had shown that R20291 contained a second complete copy of an agr locus (agrCABD) in addition to the agrBD genes of strain CD630. In this task we set out to establish the role, if any, of this second copy of agr.

Each mutant was cultivated in BHIS media at 37°C and the growth rate measured at regular intervals until the cells reached stationary phase. No difference was observed between any of the mutants and wildtype (data not shown). The ability of the strains to make spores was then measured by subjecting cells grown for 72 h to heat shock (65°C for 20 min) and then determining the Colony Forming Units (CFU) when plated on BHIS agar supplemented with taurocholate. In the case of agrB1 from both CD630 and R20291, a reduction in spore counts was evident, although some variation occurred. These experiments were, therefore, repeated with samples taken at various time points.

These experiments demonstrated that the number of spores present in the agrB1 mutant was initially reduced (at 24 and 48 h), but the number had recovered to wild type levels by 72h. A much greater reduction in spore counts was observed in the equivalent agrB1 mutant in the hypervirulent strains R20291. Thus, the time at which spores could be detected was significantly delayed, and the final spore counts after 120 h was slightly reduced compared to the parent organism. In contrast, mutation of the agrB2 gene caused an entirely opposite effect. Thus, spores were detected after only 24 h, and the final spore counts achieved was higher than in the wild type. Inexplicably, the agrA mutant had little if any effect on sporulation.

Affects on Toxin production:

To assess any effects on toxin production, strains were incubated in YT media for 24 and 48 h and toxins assayed by cytotoxicity assay. In the case of the agrB1 mutant of CD630, no significant effect on toxin production was noted. A small reduction in toxin production was noted in the R20291 equivalent agrB1 mutant. However, a considerable reduction in toxin production was noted in both the agrB2 and agrA mutant, corresponding to a 90% and 82% reduction, respectively. Levels of production at 48 h were broadly equivalent to 24 h.
Prevalence of the agr2 locus:

At the time of submission of this work programme it was unclear as to whether the second copy of agr was unique to PCR-ribotype 027 strains. To clarify this, oligonucleotides corresponding to this extra agrCABD locus was incorporated on a tiling array at high density with an additional 25 probes. Hybridization against our array demonstrated that the extra agrCABD locus found in R20291 is entirely present in the genomes of 82 of the 94 (86%) strains tested, including two of four non-toxigenic strains. The hybridization to a few probes by DNA isolated from each of the remaining 12 strain implies that this region is divergent rather than absent. PCR primer walking was performed using primers that were designed to the region CDR3184-3190. These primers generated amplicons of the expected size when DNA was derived from the positive control, R20291. No such amplicons were generated when DNA was derived from the 12 test strains. The positive control primers designed to amplify CDR3190 produced an amplicon with DNA isolated from all strains. Overall, these results indicate that the absence of this additional agrCABD locus is the exception, rather than the rule.

Transcription studies:

To gain information concerning the transcriptional organisation of the two agr regions, RNA was prepared from the two strains and RT-PCR undertaken using primers that spanned the intervening regions between adjacent genes (see Figure 56a, for the position of primers). In both cases, evidence was obtained that both regions are transcribed from a single RNA transcript. Perhaps more significantly, the operon appeared to extend to both the 'up'- and 'down'-stream genes. A similar situation has recently been reported in both C.perfringens and C. botulinum. However, the proteins encoded by these four genes (e.g., agr1, CD2749 & 2751 and agr2; agr2, CDR2091_3190 and CDR2091_3186) show no homology to those proteins encoded by the genes that flank the agr loci in either C.perfringens and C. botulinum. These results need to be confirmed.

The wildtype R20291 strain plus the agrA mutant were grown in YT media and samples taken when the culture had reached an OD600 of 0.4 0.8 and 1.2+2h. Cells were harvested and RNA extracted using standard procedures and used in microarray studies using an oligonucleotide array composed of probes directed against both CD630 and R20291. Unexpectedly, little overall difference in transcriptome profiles were found. The reason for this are not known, but the experiments are planned to be repeated in collaboration with Brendan Wren at the LSTHM, London.

Task 5. Assessment of the affects of inactivation of other regulators

Whilst a considerable number of additional regulatory mutants were created in addition to those in the Agr genes, their analysis was not progressed as far as was originally intended, particularly in terms of in vivo analysis. The latter was principally caused by the difficulties encountered in the reproducibility of infection of hamsters by the hypervirulent strain R20291 (see below). Nonetheless, some in vitro data was generated, particularly with respect to any effect on toxin production. In this respect, the data obtained was disappointing, as none of those mutants tested were affected with respect to toxin production. These included, luxS, CD0160, CD0311, CD1743, CD2214, CD2215, CD2603, CD1479, CD1517, CD1287, CD1464, CD16465, CD0668, CD0669 and CD0234, plus a range of genes involved in sporulation (CD3490, CD2644, CD1192, CD2629, CD2642,
CD1464, CD16465, CD0668, CD0669) including spo0A. The results obtained with the later mutants were somewhat unexpected. A recent study by Underwood and colleagues reported that a spo0A knock-out mutant of strain CD630-delta-erm showed a one thousand-fold reduction in the amount of toxin secreted. However, we saw no increase at all with our CD630-delta-erm mutant. In contrast, in the hypervirulent strain R20291, Spo0A appears to have a negative effect on toxin production. We also found that R20291 but not 630?erm produced different amounts of toxin when grown in different complex media. The regulatory network governing toxin production has not been thoroughly elucidated, but it appears that differences exist between strains which can affect their response to nutrient availability, and perhaps other factors.

In vivo Studies:

As indicated above, proposed in vivo experiments were somewhat hampered by the repeated observation that the hypervirulent strain R20291 exhibited great variability in its ability to colonise the hamster. Thus, regardless of the number of spores used in infection studies, the successful colonization of the hamster was not reproducible, and when colonization did occur, the length of time to the endpoint was considerably longer (mean time of 3.7 days) than was the case with 630?erm (mean time of 2.6 days). These findings complicated the undertaking of task 5.

In contrast, ClosTron mutants made in R20291 were consistently able to colonize, and furthermore, resulted in higher virulence (depending on the mutant), as indicated by shorter times to the endpoint (typically a mean time of 2.7 days). ClosTron mutants are, of course, resistant to erythromycin (Em) due to insertion of the group I intron and associated ermB gene. Accordingly, the MIC of R20291 on various antibiotics, and in particular clindomycin (Cl) was determined and compared to 630?erm. This revealed that strain R20291 had an MIC for Cl 16 µg/ml. This compares to 18 µg/ml for 630?erm. This lead to the hypothesis that strain R20291 may be sensitive to Cl, and consequently struggles to establish itself following Cl administration to animals prior to challenge.

To overcome this we set about creating an ermB-derivative of R20191. This was achieved by taking the same ermB ¬gene derivative that is delivered by the ClosTron system, and then integrating into the genome using ACE technology. The locus chosen for insertion was downstream of the pyrE gene. Accordingly, R20291 was first made pyrE minus using an appropriate ACE vector, and then using a sister ACE vector, converted back to pyrE prototrophy. At the same time, the ermB gene was delivered downstream of the corrected pyrE gene.

The ability of the created strain to more effectively colonise hamsters was then tested. Thiese experiments demonstrated that colonization was now completely reproducible. Furthermore, the effective virulence of the strain was increased, such that the mean time to endpoint was equivalent to ClosTron mutants (2 days). The availability of this strain should considerably facilitate future studies in the hamster using R20291. An article describing its construction is currently being prepared. However, its availability came too late to use in the anticipated experiments of this task.
Conclusion:

Our studies have shown that the tcdC genotype does not affect the amount of toxin A and B produced by the C. difficile strains studied here. At first, this finding seems to be at odds with qualitative functional genetic studies which have found that TcdC is a negative regulator of toxin production. However, our study was designed to qualitatively assess the effect of different tcdC genotypes on toxin production, not qualitatively assess the functional role of TcdC in C. difficile. Therefore, taking all the evidence together, it seems most likely that TcdC may act like a 'safety catch' to safeguard against inappropriate toxin expression, rather than having a quantitative effect on the amount of toxin a strain produces. This idea is consistent with the findings of this study and previous studies. In view of these findings, the advisability of using diagnostic tests based on the detection of the presence of tcdC variant sequences may not be appropriate.

More important in terms of elevated toxin production, maybe the agr system. The introduction of the agrCABD present in the R20291 hypervirulent strain into CD630-delta-erm, resulted in a substantial increase in toxin production. Conversely its inactivation in R20291 resulted in a reduction in toxin production. However, the possession of this second copy is not restricted to PCR-ribotype 027 strains, but appears widely distributed in other PCR-ribotypes commonly found in Europe. This does not rule out the possibility that agrCABD may be indicative of increased virulence. Thus, PCR-ribotype 001 is one of the commonest types in Europe, and frequently associated with outbreaks, PCR-ribotype 106 was until recently the epidemic strain in England and Wales, whilst PCR-ribotype 078 strains are increasing recognized as being as equally aggressive as PCR-ribotype 027 strains. The presence of a system in these epidemic strains which contributes to virulence common to 027 would, therefore, not be unexpected.

Main Outputs:

Cartman ST, Kelly ML, Heap JT, Minton NP (2011) Precise manipulation of the Clostridium difficile chromosome reveals that tcdC genotype has no quantitative effect on toxin production PLoS Pathogens (submitted)

Manisha Patel (2011) "Characterisation of a putative Quorum Sensing system in Clostridium difficile". University of Nottingham, PhD Degree (awarded Sept 2011),
Objectives:

To determine the correlation of between hypervirulence and virulence factors regulation. Specifically, to:

1) assess the role of antibiotic on regulation of genes encoding virulence factors

2) assess the kinetic of intestinal colonization and the factors involved

3) assess differences between strains

4) select new vaccine candidates to be combined with toxins.

Task 1 - Role of Antibiotic Pressure on Colonization Factors

We investigated by real-time RT-PCR the impact of sub inhibitory concentrations of ampicillin, clindamycin, ofloxacin and moxifloxacin on the expression of genes encoding colonization factors: the protease Cwp84, the high molecular weight S-layer protein and the fibronectin-binding protein Fbp68. We analyzed the expression of these genes in four NAP1/027 strains, one moxifloxacin-susceptible and three moxifloxacin-resistant strains. Two in vitro selected moxifloxacin-resistant mutants were also analyzed. Moxifloxacin resistance was associated to the Thr82?Ile substitution in GyrA in all moxifloxacin-resistant strains but one. The expression of cwp84 and slpA was strongly increased after culture with ampicillin or clindamycin in NAP1/027 strains. Interestingly, after culture with fluoroquinolones, the expression of cwp84 and slpA was only increased in four moxifloxacin-resistant strains, including the NAP1/027 strains and one of the in vitro selected mutants. The cwp84 over expression was correlated with an increased production of the protease Cwp84.

Task 2 - Transcriptomic in vivo Studies in C. difficile Monoxenic Mouse Model

The objectives of this particular task were to:

- improve the understanding of C. difficile pathogenesis by the analysis of the genome wide temporal expression of C. difficile genes during the first hours of infection.

- identify new putative colonisation factors as genes encoding surface proteins over expressed at the early stage of infection

- compare in vivo virulence factor genes regulation of epidemic and hypervirulent 027 strain and non-epidemic strains such as the 630.
Genome-wide temporal expression of the C. difficile 630 strain in monoxenic mice:

Three groups of 4 axenic mice each were challenged by the 630 C. difficile strain, and sacrificed at 8, 14, and 38 hours post-infection. Pure prokaryotic RNA was obtained from caecal bacteria. Comparative hybridizations on 630 microarrays were done using a cDNA issued from an 8-hours in vitro culture as a control, with a dye swap protocol for each sample. Normalisation of the data and statistical analysis were done thanks to the "Linear model for Microarray data" package.

Overall, a total of 550 genes exhibited a significantly differential expression during the course of host infection. Among these 550 genes, 201 were down regulated and 349 genes were up regulated; among them, 200 genes were specifically regulated within the host as compared to their in vitro expression. The results strongly support a two-step infection model, since during the course of infection, a significant increase in the toxin expression contrasted with a decreased expression of most of the putative colonization factors. Several paralogs of the HMW S-layer protein were also down regulated, some of which could be strong candidates for colonisation factors. Bacterial adaptation to the host microenvironment was characterized by the regulation of numerous metabolic pathways, such as the up regulation of the ethanolamine catabolic operon or the modulation of several PTS systems. Inactivation of putative virulence factors identified by this methodology completed this analysis.

Genome-wide temporal expression of a C. difficile 027 hypervirulent strain in monoxenic mice:

We analyzed the C. difficile 027 transcriptome during the very early stages of intestinal colonization in our monoxenic mouse model. Bacterial mRNA was extracted from the caeca at 4, 6, 8, 14 and 38 h post-infection, then transcribed into cDNA by RT-PCR and finally labelled for hybridization to DNA microarrays (Agilent™). The overall analysis of data (by software Ma2HTLM) showed a differential expression of 707 genes in the early stage (4-6 h) and late stage (14-38h) of colonization. 285 genes were significantly up regulated, while 456 were down-regulated. Similarly to the results obtained for the 630 strain, genes encoding known virulence factors such as the toxins were up-regulated in the late phase (38 h), whereas genes encoding surface proteins likely involved in the colonization process showed little variation. Genes involved in sporulation, membrane transport, metabolism and fermentation, underwent significant changes during the infection process suggesting a potential role for the encoded proteins in the early stages of C. difficile intestinal colonization. This analysis is being completed by RT-qPCR on the regulated genes.

Genome-wide expression of a C. difficile FliC mutant in monoxenic mice:

The FliC mutant showed: 1) inability to synthesize FliC and flagella, 2) a greater adherence on human colonic cell line Caco-2 as compared to the parental strain, and 3) ability to colonize the mouse intestine either in a monoxenic or a dixenic model in competition with the wild type strain. Surprisingly, the fliC mutant was more virulent than the wild type strain. Actually, all monoxenic mice challenged with the FliC mutant died 48 h post-infection in contrast to mice colonized with the wild type strain, which all survived. Thus, the absence of FliC increases the virulence of the C. difficile 027 strain suggesting that FliC could play a role in virulence.
We then analyzed and compared the bacterial transcriptomes of the FliC mutant and the parental strains at the early stage of intestinal colonization in the monoxenic mouse model. The global analysis of the fliC mutant data (by Ma2HTLM) showed a differential expression of 310 genes. High up-regulation were particularly striking in genes involved in motility, membrane transport systems (PTS, ABC transporters), carbon metabolism, regulation (Agr-2, s54, PadR system) and sporulation. Significant regulation was also observed for genes involved in the synthesis of toxins (tcdC down-regulated), the cell wall (cwp66 up-regulated), cell growth, fermentation, metabolism (AA, nucleic acids, lipids), stress (antibiotic resistance) and anaerobic respiration.

**Task 3 - Transcriptomic in vivo Studies in the Hamster Model**

The use of the hamster model was abandoned as it proved impossible, even after disruption of the microbiota by large spectrum antibiotics, to obtain sufficient C. difficile mRNA. The in vivo transcriptomic studies were therefore only possible in axenic animals, such as the mouse model. Consequently, we have performed all the transcriptomic studies in the mouse model (3 different transcriptomic studies see Task2).

**Conclusion:**

The historical NAP1/027 moxifloxacin-susceptible strain and its mutant appear to be differentially regulated by fluoroquinolones. Overall, fluoroquinolones appear to favour the expression of some colonization factors encoding genes in resistant C. difficile strains. The fluoroquinolone resistance of the 027 epidemic strains could be considered as an ecological advantage. This could also increase their colonization fitness and promote infection rates.

Our in vivo transcriptome data should help to elucidate the mechanisms of intestinal colonization by C. difficile and adaptation of the bacterium to its host. Concerning in vivo transcriptomic of C.difficile 630 strains, our results showed that genes involved in metabolic pathways were highly upregulated in vivo as compared to in vitro. In addition genes encoding unknown proteins were also upregulated.

Comparing in vivo transcriptomic analyses of the different strains has provided tantalising clues concerning the hypervirulence of C. difficile 027 strain. Genes absent in the 630 strain and also in the historic 027 strain but present in the epidemic hypervirulent 027 strain were upregulated in the early steps of the colonisation process. These up-regulations can favour the implantation of the strain and the colonisation of the digestive tract and constitute another ecological advantage.
Genes present in the epidemic hypervirulent 027 strain and absent from the 630 and regulated during the infection process are interesting clues to explain a better adaptation of 027 strain to its host. Some of these genes will be further studied to check their potential as new vaccine antigens or therapeutic targets.

Taken together the results of in vitro and in vivo studies demonstrate that the epidemic hypervirulent 027 strain which is resistant to fluoroquinolone displays ecological advantages which facilitate the intestinal colonization, the first step of the pathogenic process.

Main Outputs:

Publication in peer review journals


A. Barketi-Klai, M. Monot, S. Hoys, S. Lambert, B. Dupuy, I. Kansau and A. Collignon.

Analysis of the genome-wide temporal expression of a Clostridium difficile 027 hypervirulent strain in monoxenic mice (in preparation).

Objectives:

To identify the prevalence of hypervirulence traits in human C. difficile strains. Specifically, to:

1) establish a collection of strains derived from endemic cases of hospital-associated CDAD, from outbreaks of hospital-associated CDAD and from patients with community-associated CDAD

2) optimize MLVA and MALDI-TOF for subtyping of the assembled C. difficile strains

3) refine the typing system to target newly identified (WP2 - WP8) hypervirulence traits

4) To assess the prevalence of identified hypervirulence traits through epidemiological studies on human (healthcare & community) and animal strains

Task 1 - Collection of Strains from Community and Healthcare Facilities

Using support of the ECDC, we organised a network of 106 laboratories capable of isolating C. difficile strains in 34 European countries and obtained detailed information on 509 patients with CDI. In total, 395 isolates of CDI patients are available for characterization of which 56 strains derived from community-acquired cases, 316 from hospitals (outbreak and endemic cases) and 23 from an unknown origin. A substudy was performed in The Netherlands to the prevalence, characteristics and risk factors of community-onset CDI. In total, 2443 stool samples were tested, and 37 patients (1.5%) with positive toxin test results were identified. Among 31 patients with toxin-positive stool samples for whom information was available, 20 (65%) had not been admitted to a healthcare institution in the year before, 13 (42%) had not used antibiotics during the 6 months before, and eight (26%) had neither risk factor.

Task 2 - Initial Molecular Characterization of the Strain Collection

In total, 395 strains from the European study have been collected; 66 different ribotypes were found, including 6 new PCR ribotypes. Dominant PCR ribotypes were 014/022, 001 and 078, whereas 015, 018 and 056 were associated with a complicated disease course. Of 395 isolates studied, toxintype 0 was most prevalent, representing 248 (65%) of 383 isolates. The substudy to community-acquired CDI performed in The Netherlands resulted in 37 cultured C. difficile isolates which belonged to 13 different PCR ribotypes. Twenty-four percent of the isolates were non-typeable (rare or new) PCR ribotypes. Several PCR ribotypes not encountered in hospital-associated outbreaks were found, suggesting the absence of a direct link between outbreaks and community-onset. Characterization of available isolates revealed that most strains associated with community-acquired CDI (40%) belonged to Type 078. It was concluded that a significant overlap exists between Type 078 derived from
community-acquired CDT with Type 078 strains found in pigs and other animals. Further investigations by MLVA revealed that Type 078 from human and animal sources are indistinguishable and zoonotical transmission should be considered.

MALDI-TOF MS was optimized for recognition and typing of C. difficile. The most important issue was to obtain spectra of good quality. Several matrices, pretreatment methods and amounts of bacterial material have been used. This has resulted in an optimized protocol for MALDI-TOF analysis of C. difficile which enables us to do a thorough comparative analysis of numerous C. difficile types. A new database was established using reference strains of C. difficile belonging to the 4 most frequently found types; 012, 027, 078 and 001. It was clear that specific MS spectra specific peaks for Type 027 can be observed. In addition, the spectra obtained with Type 078 strains seem to be quite different than other types. To further study and to be able to compare the obtained spectra in an automated fashion, we tested new software (ClinProtocols) and also applied MALDI-TOF MS from another company (Shimadzu, Biomerieux. France). Using a standard set of 25 different C. difficile PCR ribotypes a database was made by different mass spectra recorded in the SARAMIS software (AnagnosTec, Zossen, Germany). The database was validated with 355 C. difficile strains belonging to 29 different PCR ribotypes collected prospectively from all submitted feces samples in 2009. The most frequent PCR ribotypes were type 001 (70%), 027 (4.8%) and 078/126 (4.7%). All three types were recognized by MALDI-TOF MS. We conclude that an extended MALDI-TOF system was capable to recognize specific markers for ribotypes 001, 027 and 078/126 allowing an effective identification of these strains.

Task 3 - Refinement of Typing Systems to Target Newly Identified Traits

We applied multilocus sequence typing for recognition and classification of hypervirulent Clostridium difficile PCR ribotypes, using the MLST as described by Griffiths et al. In total, 94 C. difficile isolates belonging to 75 different PCR ribotypes were investigated using MLST. In addition to the already described five evident clades of C. difficile, a potential sixth clade was discovered. This clade comprises of one sequence type, ST-122 associated with RT131. This type is found in humans, although infrequently. Furthermore, it was observed that the hypervirulent PCR ribotypes 027 and 078 are present within two distinct clades, which also harbour several other PCR ribotypes that are highly related to RT027 and RT078. Analysis of the posed controversy showed that C. difficile RT122 with the 027 marker is typed as a different sequence type located in a divergent clade as RT122 not containing the marker. Conclusion: PCR ribotyping is a less effective typing method for studying close phylogenetic relations. MLST can accurately link groups of isolates and thereby appears to be an appropriate method for recognition and classification of potential hypervirulent strains. Furthermore, a new sixth clade is identified, of which the clinical and epidemiological relevance is under study.

Task 4 - Prevalence of Hypervirulence Traits in the Community and Healthcare Facilities

Based on sequence comparisons between C. difficile strains, we detected two gene insertions into the genome of hypervirulent PCR ribotypes 078 and 027. Analysis of these regions, of 1.7 and 4.2 kb, respectively, revealed that they contain several interesting ORFs. The 078 region is inserted intergenically and introduces an enzyme that is involved in the biosynthesis of several antibiotics. The 027 insert disrupts the thymidylate synthetase (thyX) gene and replaces it with an equivalent, catalytically more efficient, thyA gene. Both gene insertions were used to develop ribotype-specific PCRs, which were
validated by screening a large strain collection consisting of 68 different PCR ribotypes supplemented with diverse 078 and 027 strains derived from different geographical locations and individual outbreaks. The genetic markers were stably present in the hypervirulent PCR ribotypes 078 and 027, but were also found in several other PCR ribotypes. Comparative analysis of amplified fragment length polymorphisms, PCR ribotype banding patterns and toxin profiles showed that all PCR ribotypes sharing the same insert from phylogenetically coherent clusters. The identified loci are unique to these clusters, to which the hypervirulent ribotypes 078 and 027 belong. This provides valuable information on strains belonging to two distinct lineages within C. difficile that are highly related to hypervirulent strains.

Conclusions:

This workpackage has enabled the assembly of a large collection of human C. difficile isolates which not provides an invaluable resource for future studies directed at the development and testing of new virulence markers, as well as for typing system evaluation. In this study, the collection has been used to test a recently developed MALDI-TOF typing technique, which was shown to be capable of recognizing the 5 most frequently found PCR ribotypes in Europe. In parallel, improvements were made to the MLVA procedure, which has resulted in the most discriminatory typing method for C. difficile. Our analysis has shown that assumed virulence markers, such as hyperproduction of toxins and presence of binary toxin genes, are not associated with severity and outcome of the disease in humans.

Using the developed procedures, our analysis has also demonstrated that Clostridum difficile isolates obtained from human infections in the community differ from those isolates obtained in hospitals but significantly resemble isolates obtained from pet and farm animals. These data provide compelling evidence that animals may act as a reservoir for human infection, at least in the community.

Main Outputs:


WP10: PREVALENCE OF HYPERVIRULENCE TRAITS IN ANIMAL POPULATIONS

Objectives:

To determine whether animal strains represent a reservoir of hypervirulent traits responsible for increases in CDAD in human populations. Specifically to:

1) establishing the first representative pan-European collection of animal C. difficile isolates

2) characterize the animal strains through molecular typing and colonization properties

3) to investigate the presence and prevalence of hypervirulent traits associated with human strains

Task 1 - Collection of Representative C. difficile Strains of Animal Origin

Collection of animal C. difficile strains was established and is curated at Institute of Public Health Maribor. Collection data is accessible at web page http://164.8.68.130/mikro/animalcdiff/

Strains were included in the collection by criterion single ribotype per species/ per country. Strains were selected either by contacting laboratories in EU and world wide or by studies performed by partner P2. In contrast to pigs where single genotype (or low number of genotypes) is present in the single farm, calf and poultry farms are likely to have a greater diversity of genotypes. Isolations were therefore performed at poultry farm in Slovenia and another such study was done at single
Belgium calf farm with the aim to isolate C. difficile strains in controlled fashion.

A total of 300 samples were collected from same 50 animals on six different occasions (at animal age 14 to 194 days). Of all 300 tested samples, 21 (7.0 %) were C. difficile culture positive and 38 C. difficile strains were isolated. During the first 46 days of sampling, prevalence of C. difficile ranged from 16% to 2%. A total of 5 different PCR ribotypes (078/126, 066, 033, 012, SLO 010) were identified. Of these, 4 PCR ribotypes were toxigenic and represented 95% of all isolates. Variant toxinotype V/ PCR ribotype 078/126, comprising some 26 (68, 4 %) isolates predominated, whereas only 3 (7, 9%) isolates belonged to toxinotype XI (PCR ribotype 033), and 3 (7, 9%) to non-variant toxinotype 0 (PCR ribotype 012). Strains differed in antibiotic genetic determinants (done in collaboration with partner P7) and in sporulation properties.

Number of strains in the HYPERDIFF animal collection is constantly increasing. Currently there are more than 60 strains from 8 countries included, belonging to 10 species, 31 ribotypes (only types 078 and 014/020 have more than 5 representatives) and 8 toxinotypes with toxinotype V being most prevalent.

Task 2 - Characterization of Animal Strains

Within this task we have focused on characterization of colonization properties of animal strains as this is one of the important steps in pathogenesis. Experiments are distributed into two parts: one is comparison of colonization properties of C. difficile strains of same ribotype in the mouse model (collaboration of partners P2, P3 and P4) and the second are studies of animal/human gut microbiota.

In comparative study of colonization properties of human and animal strains the objective was to select ribotype(s) commonly found in animals and in humans and i) compare spl sequences to define possible differences between animal and human strains of the same ribotype and ii) compare two strains of the same ribotype with different slp in mouse model.

Slp sequencing was done in collaboration of Partner 2 (Inst Publ Health Maribor) and Partner 3 (Instit Super di Sanita, Rome). For the analysis we have selected 12 strains of ribotypes 014/020, 066, 012, 078. No major differences in SlpA sequences were found amongst animal and human strains within same ribotype, except for a single poultry isolate. This strain did not group together with other ribotype 014/020 slp sequences. From the human 014/020 ribotype we selected strains with different antibiotic resistances than detected in poultry strain so that differentiation was possible after colonization experiment of mouse. Colonization experiment was done by partner P4:

Three C. difficile strains of ribotype 014/020 from different origin (human and animal) and with different Slp type were compared, respectively: strain P30 (poultry origin, Slp type V4) compared to strain 4684/08 (human, Slp type V2) and strain
4684/08 (human, Slp type V2) compared to strain 1064 (human, Slp type V5). For each pair in vivo and in vitro experiments were performed as described in WP4 task 3.

Comparison P30/4684/08: In dixenic competition assay in axenic mice, intestinal colonization rate was highly faster for P30 strain compared to 4684/08 strain. At day 7, P30 intestinal colonization reached 10^8 cfu/g compared to 0 cfu/g for 4684/08. Similarly adherence to caeca at day 7 was significantly higher for animal strain P30.

Comparison 1064/4684/08: In dixenic competition assay in mice, intestinal implantation rate was similar for the two strains. However at day 7, a small advantage in colonization was observed for strain 1064. At day 7, adherence to caeca was greater for strain 1064. The poultry strain with a Slp type V4 had a trend to a better intestinal colonization and caecal adherence as compared to two human strains, Slp type V2 and V5. These first results need to be controlled.

In studies of gut microbiota we have used a new molecular high-throughput method for DNA fragment analysis called denaturing high performance liquid chromatography (DHPLC; Wave system, Transgenomic). The level of the analysis is comparable to the gradient gels with the advantage of high automation and repeatability. So far only few studies on C. difficile associated changes in gut microbiota are published and all of them describe only bacterial populations and humans. Within this project we have established DHPLC protocol for analysis of bacterial, archaea and fungal complex populations and have used it for studies in humans and in animals. The method includes amplification of marker gene (16S ribosomal gene for Eubacteria and Archea and ITS regions for fungi), DHPLC separation, collection of peaks, identification of each peak by sequencing and comparison to Genebank and RDP database. Additionally, several novel approaches were designed for statistical analysis of chromatograms.

We have analysed 200 human and 140 chicken faecal samples from subjects colonised and non-colonised with C. difficile. In humans the main differences were observed in the species diversity. The results show that the C. difficile colonised subjects have a simpler bacterial and archaeal microbiota and more complex fungal microbiota. The colonisation status is connected to the presence/absence of certain bacterial groups like clostridia, bacteroides and bifidobacteria; high abundance of archaea which seems to be the trait of the C. difficile non-colonised patients and a proliferation of opportunistic fungi in C. difficile colonised patients.

In chickens the colonisation status was associated to the presence/absence of certain enterococci. While the archaeal microbiota developed between 2 and 4 months of age, the fungal microbiota seemed to be relatively diverse from the beginning. (This work was presented on several poster presentations and two manuscripts are currently in preparation)

Task 3 - Prevalence of Hypervirulence Traits in Animal Strains

From our laboratory C. difficile culture collection we have selected 927 isolates. Strains were grouped into 103 different PCR ribotypes; human isolates in 89 ribotypes, animal isolates in 35 ribotypes and environmental isolates in 36 ribotypes. There was a considerable overlap between C. difficile ribotypes isolated from humans, animals and environment. Fourteen PCR
Isolates of most common PCR ribotypes (e.g. 014/020, 010, 023, 029, 002) found in humans and animals were further characterized with PFGE and antimicrobial susceptibility testing.

Human and animal isolates of the same PCR ribotype clustered together with PFGE but are not identical and had mostly also similar MIC values for all antibiotics tested. This indicates transmission of given genotype from one reservoir to the other and also confirms that antibiotic resistance is more likely to appear/is retained in hospital environment. (Manuscript submitted.

Conclusions:

Characterization of animal strains from large number of species and geographic locations has revealed that variability of animal-associated genotypes is greater than previously assumed. Most of the previous publications have described prevalence of single type within a farm/species. We have shown that with increased number of animal samples variability of the strains in animal host will also increase.

During this project the first international collection of animal strains was established and represents a platform for scientists working on epidemiology, molecular typing methods and on veterinary aspects of CDI.

Some of the virulence associated traits were studied in animal strains including antibiotic resistance and adhesion. With respect to host factors, we have studied the differences in the gut microbiota changes during C. difficile infection in animals and humans. We were able to show that animal and human strains belonging to the same ribotype can have different slpA genes and differed in their ability to colonize mice gut. Also, antibiotic resistance is higher in human isolates as in animal isolates.

Gut microbiota in humans and animals have different groups that are present or absent during C. difficile colonization, suggesting that different groups have protective potential.

The results obtained have implications in understanding of possible transfers between animals and humans, in understanding
of the importance of the animal reservoir for the human infection and in the future development of probiotics.

Main Outputs:

Janezic S., Ocepek M., Zidaric V. and Rupnik M. Clostridium difficile genotypes other than ribotype 078 that are prevalent among human, animal and environmental isolates, submitted, BMC Microbiology


ETHICAL ISSUES

Throughout the programme the individual relevant members of the HYPERDIFF continued to address the ethical, legal, social, and safety issues raised by their research activities. These issues were mainly related to bio-safety, use of animals and clinical research. Project management will ensured that the activities of each partner in the consortium were in agreement with local, national and international regulations. All animal work was approved by the appropriate National Licensing Authority, subject to local ethical review and undertaken by appropriately trained and qualified personnel in accordance with local guidelines to ensure appropriate standards of animal welfare were maintained at all stages of the procedures. Group sizes for each study have been calculated to minimise total animal usage without compromising the statistical validity of data output. All animals were humanely killed by approved methods at the end of the experiment.

Peripheral blood monocytes and dendritic cells for immunological studies were isolated from surplus donated blood provided by the Italian Red Cross. Donors provided informed consent to the donation of blood or blood components and to their
subsequent (legitimate) use. The surveillance study of the incidence of CDAD in humans in several European countries, only used demographical patient data (such as date of birth, gender, living place and date of the positive test), and were supplied by participating laboratories.

All other laboratory work conformed to the national regulations of the host establishment, and was consistent with the EC directives: 93/88 (pathogens) 90/394 & 97/42 (carcinogens), 92/69 & 93/21 (labelling and packaging), 89/655 & 95/63 (equipment), 1986 Article 130R(2) on Environmental Protection, 90/219/EEC on contained use of genetically modified microorganisms, and its amendment 94/51/EC. All procedures, including GM modification, were approved by the local safety committees.

Potential Impact:

POTENTIAL IMPACT

There have been a number of significant impacts of the project:

1. The Importance of Toxin A in CDI

The role of the two large toxins in disease has been the subject of intense debate since the initial discovery of the association between enterocolitis and Clostridium difficile. For many years, Toxin A was assumed to be the principle vehicle of disease symptoms. The emergence of A-B+ virulent strains then led to the notion that Toxin B was the most important. This appeared to be confirmed in 2009 with the publication of a paper that demonstrated that an isogenic mutant of a toxin A+B- strain caused disease in hamsters, whereas an A-B+ strain did. This lead to the assertion that only Toxin B was essential to disease, and that Toxin A alone could not cause disease.

One outcome was that pharmaceutical and diagnostic companies questioned the need to include countermeasures directed at both toxins in tests and therapies under development. Indeed a number of manufacturers of PCR-based diagnostic kits made the decision to only target toxin B alone.
The studies undertaken as part of HYPERDIFF demonstrated that equivalent mutants to those described above, created in two different C. difficile strains, did indeed cause disease in hamsters. These findings re-established the importance of Toxin A in CDI, and should encourage pharmaceutical and diagnostic companies to continue to target both toxins in developed counter measures.

2. The Importance of Variant TcdC Alleles in Hypervirulence

Toxin A and toxin B are the principal virulence factors of Clostridium difficile. The alternative sigma-factor TcdR directs production of both toxins, and is thought to be negatively regulated by TcdC. The tcdC sequence of BI/NAP1/027 strains has a single nucleotide deletion at position 117, resulting in a truncated TcdC, and an 18-nucleotide deletion. This is widely postulated to result in increased toxin production which, in turn, has been linked with reports that BI/NAP1/027 strains cause a more severe disease. Consequently, the presence of these TcdC is taken as synoymous to hypervirulence and is deliberately targeted in diagnostic tests by many manufacturers.

Here we have developed the cytosine deaminase gene (codA) of Escherichia coli as a negative selection marker, and used it to carry out allele exchange in C. difficile for the first time. We generated isogenic strains of the BI/NAP1/027 strain of C. difficile, R20291, in which we 1) deleted tcdC, 2) repaired the single nucleotide deletion, 3) repaired both the single- and the 18-nucleotide deletion and 4) substituted the native tcdC sequence for that of C. difficile 630. Furthermore, we deleted tcdC in the ribotype 012 strain of C. difficile, 630, and by way of complementation, we then restored the tcdC open-reading-frame with a silent nucleotide signature, so that it could be distinguished from the wild-type. Surprisingly, we did not observe any differences in the amount of toxin produced by isogenic strains of C. difficile when they were cultured in TY medium.

These results suggest that tcdC genotype does not affect the amount of toxin A and B produced by C. difficile. Thus, TcdC may simply act as a ‘safety catch’ to guard against inappropriate toxin expression, or it may have another role altogether. This calls into the question the value of targeting TcdC variants in diagnostic tests as a indicator of hypervirulence.

3. CDT Binary Toxin and Hypervirulence

Our data has also produced tantalizing evidence that CDT toxin may also be contributing to disease, and therefore hypervirulence. Thus, it was noted that the presence of the binary toxin in a mutant strain producing only Toxin A led to increased virulence over an equivalent strain that was not producing CDT. The A+B-C+ mutant exhibited a 3.0 day average...
time to endpoint in the hamster model compared to 5.9 days for the A+B-C- mutant. This suggests that CDT may act in concert with toxin A to increase virulence. Intriguingly, in the case of the (A-B-C+) mutant (i.e., an isogenic mutant producing only CDT), 3 of the 9 animals succumbed to disease. However, these 3 animals did not show typical symptoms of C. difficile disease. Thus, whilst they exhibited wet tail, there was no evidence of the usually observed loose faeces or damage to the caecum. Rather, their small intestine was heavily damaged. This observation is in keeping with a previous suggestion that C. difficile can cause infection of the small intestine.

Others have previously suggested that the presence of binary toxin is linked to more severe disease outcomes. Moreover, the presence of CDT in all representatives of certain so-called hypervirulent strains (e.g., PCR-Ribotype 027 and 078) provides further compelling evidence that it contributes to virulence. Further research will be necessary before the role of binary toxin in disease is fully understood, but in the meantime it may be prudent to test for its presence in clinical isolates through appropriate diagnostic tests.

4. The Spread of C. difficile Toxin Potential in The GI Tract

A striking finding of this study showed that the pathogenicity locus of a toxigenic strain (i.e. the locus containing the toxins, the major virulence factors of this organism) can be transferred to a non-toxigenic strain. The fact that non-toxigenic strains can acquire the toxins has implications for management of C. difficile outbreaks. Clearly there is now an urgent need to understand the factors that contribute to the spread of the toxin genes. It is essential to determine if this locus can transfer to other species of clostridia, and beyond and if so, whether the locus is expressed and can none pathogenic strains be rapidly converted to pathogens. This observation also has implications for the treatment of C. difficile, as one of the proposed methods for treatment is to out-compete toxigenic strains with non-toxigenic strains. Clearly the ability to transfer the PaLoc will have serious implications for such an approach.

It is highly likely that the toxin locus is not capable of transfer on its own, as it does not have any obvious mobilisation or conjugation genes, but most probably requires other mobile elements to facilitate transfer. It is now imperative that we identify such elements in order to establish if transfer of the toxins occurs in nature and determine the conditions that promote transfer.

The fact that there are so many mobile elements in C. difficile and that these often contain genes that give their host a survival advantage indicate that strategies aimed at slowing down gene transfer between these pathogenic strains could be worthwhile. Furthermore, the finding that some of the mobile elements are capable of transfer to distantly related bacteria such as Bacillus subtilis indicates that C. difficile can act as a reservoir of antibiotic resistance genes and genes that could give bacteria other than C. difficile a survival advantage in particular ecological niches, such as the human gut. This underlines the needs to monitor the rapid evolution of bacteria as they acquire antibiotic resistance-, niche exploiting- and virulence genes. A major outcome from this work is that C. difficile is particularly adept at spreading these genes.
5. The Relevance of S-Layer Proteins to Hypervirulence

Overall, our results suggest that S-layer proteins (SLPs) are recognized by effectors of the innate and adaptive cell immune system and possess immunomodulatory activities. SLPs of hypervirulent and epidemic strains trigger inflammatory processes similar to those obtained from PCR ribotypes usually associated with non-hypervirulent and epidemic sporadic cases. Therefore, SLP-mediated immunity does not seem to be linked to a specific ribotype and does not appear to contribute to the recently emerged hypervirulence phenomenon. However, the results of these studies show that C. difficile SLPs may contribute to the pathogenicity of the microorganism by perturbing the fine balance of inflammatory and regulatory cytokines. Moreover, due to the ability to induce a Th1/Th2 response, C. difficile SLPs may be considered for their adjuvant properties and included in future studies in vivo mouse models to confirm these adjuvant properties. Another relevant implication of SLPs due to their antigenic role may be their inclusion as subunits in a multicomponent vaccine against C. difficile infections for high risk patients.

6. Better Measures of Hypervirulence?

The work performed by obtaining mutants and performing transcriptomic studies has highlighted the role of the colonization factors and metabolic pathways in addition to toxins in the pathogenesis. Especially concerning the specificity of the R20291 Stoke Mandeville 027 C. difficile strain, additional genes encoding either metabolic enzymes or mobile genetic elements or antibiotic resistance could be involved in the better adaptation of the bacterium to its host and consequently to its hypervirulence. Our results suggest that the genetic potential is more important than the PCR-ribotype 027 to determine the virulence potential of a strain.

7. Identification of Therapeutic Targets by Essentiality

Essential genes (genes that encode protein factors that are essential for the normal growth of the organism) represent potential therapeutic targets. Here we have developed a method that can determine whether a specific factor/gene is essential for growth and used it to demonstrate that GldA (CD0274) is essential. Inhibitors that interfere with the function of this enzyme would therefore have potential as anti-C. difficile drugs. In parallel, and in order to further explore methods for determining essentiality, we pursued an approach termed, Transopson-Directed Insertion Site sequencing, or TraDIS. It utilizes
nucleotide sequencing to prime from the transposon and sequence into the adjacent target DNA, simultaneously mapping the site of insertion of every transposon in a mutant pool. Genes that are under represented in terms of transposon insertion represent essential genes, and by implication therapeutic targets. TraDIS was undertaken by next generation sequencing. A total of 20 million mapped sequence tags of 40-50 bp were generated, representing approximately 15 bp of transposon sequence and 25-35 bp DNA flanking Himar1 C9 insertion sites. On the first run, 2,600 genes were identified as non-essential. The results of a second and third run are still to be analysed, but potentially will identify genes essential for growth in the presence of bile acid.

8. Improved Molecular Typing of C. difficile

LUMC developed and optimized molecular tests directed to specific DNA inserts in hypervirulent C. difficile isolates belonging to the 027 clade and 078 clade. These assays will undergo validation with one partner outside the consortium (Prof. Mark Wilcox, Leeds University). Simultaneously, we have been requested to patent the finding and found that several microbiological companies are interested to collaborate further on this. The knowledge obtained on this specific DNA insert during this study, has been used to apply for new grants on CDI diagnostics.

A network of 106 laboratories capable of isolating C. difficile strains in 34 European countries has been established and a CDI incidence survey has been performed in November, 2008. Using ECDC funded support (2011-2014), we will have the opportunity to enhance this network and collect new data on circulating C. difficile types.

The activities on molecular typing and typing by MALDI-TOF MS resulted in a new international collaborations to standardize C. difficile typing.

SOCIO-ECONOMIC IMPACT / WIDER SOCIETAL IMPLICATIONS

The socio-economic impacts of the project outside of the immediate employment of staff on the project, at least in the short to medium term are difficult to quantify. It is likely that the outputs of the project will lead to the development of new diagnostic tests and therapies, which in turn could lead to the creation of jobs to support their manufacture and distribution. These may not necessarily be located in the EU, but would still result in benefits through license fees, royalties, milestone payments etc.
1. Training/ Employment within the Project

The project enabled the employment and training of a cadre of early and late stage researchers, either directly on the project, or associated to it. Throughout, the regular communication between partners, and the attendance of dissemination events and conferences has provided opportunities for the non-English speaking team members to improve their communication skills in English. These opportunities have also pervaded all associated staff involved in HYPERDIFF, i.e., technicians and administrators at the respective institutes. Examples include:-

ZZV (Maribor, Slovenia)

At Institute of Public Health Maribor this project has enabled employment and training of early stage researchers for different duration of time. This employment has resulted in further enrolment of two of them (1 male, 1 female) into national system for financing of the training of PhD students, which means financing beyond the duration of the Hyperdiff project and hereby the sustainability of career of the early stage researchers. This is especially important as partner 2 is located in a region (Maribor/Slovenia) where employment options in scientific field are not numerous.

UCL (London, UK)

This work has allowed the UCL research fellow undertaking the work to gain an understanding the practical aspects of molecular microbiology. He is also writing up his work in the form of a PhD thesis which will allow him to gain the skills required for written communication and a deeper understanding of the scientific method. Throughout the period of the project he presented his work at our regular project meetings and at scientific meetings therefore gaining skills in written presentation. He also gained valuable networking skills hopes to use his newly gained skill set to undertake postdoctoral work in his native Holland underlining the mobility of labor this project has enabled.
Associated to the project were several early stage researchers undertaking PhD training. Two of them, Soza Baban (female) and David Walker (Male) were awarded their PhDs during September/November 2011. Both of them regularly took part in partnership meetings where they present their work on C. difficile flagella and gene essentiality, respectively, and were able to network and interact with consortium partners and their teams. Soza Baban additionally spent a number of months in the laboratory of Anne Collignon (partner 4). She will be returning to her home country, Iraq, in 2012 where she will establish a diagnostic laboratory.

UPS (Paris, France)

The project enabled the employment and training of a UPS PhD fellow. This work has allowed the PhD fellow to gain skills in in vitro and in vivo experiments and also in molecular microbiology such as transcriptomic studies. She presented her work at different HYPERDIFF meetings and international meetings as well. She is writing her work in order to pass her PhD at the beginning of 2012.

2. European Scientific Collaborations

This contract has strengthened the relations between European scientific teams and allowed the creation of new collaborations. Thus, young researchers had the opportunity to present and discuss their work in scientific sessions. These meetings also allowed them to create collaboration between young European researchers of several countries, both within the EU and wider.

Many of the partners involved strengthened their collaboration through the award of a Marie Curie ITN, CLOSTNET, which is training a total of 22 PhD students, many of them focussed on Clostridium difficile.

3. Engaging with Civil Society and Policy Makers
Some of the project partners are national reference laboratories for C. difficile and as such generally contribute to the national policy regarding the surveillance of CDI, specifically:

Development of Guidelines

A collaboration of Hyperdiff with ESGCD and the European Society for Clinical Microbiology and Infectious Disease (ESCMID), resulted in two specific guidelines for diagnostics of CDI and treatment of CDI that were published in 2009 in Clin Microbiol Infect Dis (December issue). The Hyperdiff consortium collaborated with ESGARAB (ESCMID Study Group on Antimicrobial Resistance in Anaerobic bacteria) and recommendations have been composed to standardize susceptibility tests and EUCAST cut-off values. These recommendations have been published at the Eucast website.

Reducing antibiotic prescriptions and improved hospital infection control

The European guidelines on treatment and prevention of CDI have focused on rapid diagnostics and on interventions to improve antibiotic prescribing to hospital inpatients. In some European countries, among which The Netherlands, the incidence of CDI due to hypervirulent strains clearly decreased.

Increasing awareness for CDI in animals and possible role of zoonotical transmission.

Characterization of animal strains from large number of species and geographic locations has revealed that variability of animal-associated genotypes is larger as previously assumed. Most of the previous publications have described prevalence of single type within a farm/species. We have shown that with increased number of animal samples variability of the strains in animal host will also increase. Moreover, the finding that an increase of C. difficile Type 078 in human CDI parallels Type 078-associated CDI in animals, has stimulated national authorities to study the possible role of feed animals and meat as a source for community-acquired CDI. For example, new studies have been initiated in The Netherlands and preliminary data confirm this hypothesis.

The results obtained have implications in understanding of possible transfers between animals to humans, in understanding of
the importance of the animal reservoir for the human infection and in the future development of probiotics.

DISSEMINATION ACTIVITIES

Results were disseminated to broader scientific and nonscientific community by several means.

1. Publication in Scientific Journals

A total of 19 peer reviewed publications were published as a direct result of HYPERDIFF outputs, with a further 6 papers being in preparation. An additional 5 articles were published as book chapters/conference proceedings. A total of 14 associated papers have also been published.

2. Presentations at Scientific Meetings

The HYPERDIFF consortia have been extremely active in presenting the outputs of the project at prestigious national and international meetings, both as invited and selected oral presentations, as well as poster presentations. These include:-

ICDS: "The International Clostridium difficile Symposia". This is the premier event for the scientific community working on Clostridium difficile. Organised by Maja Rupnik (Partner 2), the 3rd meeting of the series was held in Bled (Slovenia) in 2010. In total, the HYPERDIFF consortium were involved in 3 oral presentations and 10 poster presentations. Consortium members will also be well represented at the 4th meeting to be held in September 2012, again in Bled, Slovenia.

ClostPath: "International Conference on the Molecular Biology and Pathogenesis of the Clostridia". This is the largest conference devoted to the pathogenic Clostridia, and is dominated by research on Clostridium difficile. The 6th meeting was held in Rome in 2009, organised by partner 3 (Paola Mastrantonio), while the 7th meeting was held in Oct, 2011, in Ames, USA. In total, the HYPERDIFF consortium were involved in 2 oral presentations and 6 poster presentations at the Rome
meeting, and 4 oral presentations and 5 poster presentations at the Ames meeting. Consortium members will also be well represented at the 8th meeting to be held in Australia in 2013.

ECCMID: The yearly congress of the “European Society of Clinical Microbiology and Infectious Diseases”, a non-profit organisation whose mission is to improve the diagnosis, treatment and prevention of infection-related diseases. These congresses attracts 7000 to 8000 participants. HYPERDIFF members have been well represented at these congresses with 4 speakers in 2009, 2 speakers and 3 posters in 2010, and 1 speaker and 5 posters in 2011.

HYPERDIFF consortium members have also been speakers at other prestigious international events, including the 20th European Congress of Clinical Microbiology and Infectious Diseases (Vienna, Austria, 2010), Anaerobe 2010 (Philadelphia, USA, July 2010), Clostridium XI, (San Diego, USA, Oct 2010), 50th ICAAC (Boston, USA, Sept 2010) and the 4th International Spores Conference (Cortona, Italy, May 2010).

Consortium members have also regularly attended, and spoken at national meetings, as well as local meetings at their respective institutes.

3. Public Engagement and Media Activities

All of the partners have, wherever possible, taken part in Public Engagement in Science activities.

i) At the launch of Hyperdiff, the Project Coordinator, Nigel Minton, was interviewed on the topic of "Superbugs" by East Midlands Today, a BBC TV popular news bulletin programme with viewing figures of about 400,000.

ii) The same day he featured in the Nottingham local paper "The Nottingham Post" with the headline story "Nottingham Scientist to lead £3m C.diff Study", with a circulation of approx. 13000 copies. This feature was also published at the newspaper's website http://www.thisisnottingham.co.uk.

iii) The following day, he was interviewed by Smooth Radio, on "Superbugs", in a current affairs broadcast with listening figures of about 3 million.
iv) The workpackage leaders from UCL have been involved in public engagement activity such as Bright Club

v) The coordinator has been on Media Training courses at The Science Media Centre, London
(http://www.sciencemediacentre.org). The Science Media Centre is an independent venture working to promote the voices,
stories and views of the scientific community to the national news media when science is in the headlines.

vi) P Mullany (Partner 7) received training in stand-up comedy techniques to present part of this work to a general audience at
a comedy club run by UCL, Bright Club.

vii) Adam Roberts (from Partner 7 laboratory) was a member of the expert panel in a public microbiology based drama
focusing on "Stopping the spread of Superbugs" at the SGM Autumn Meeting, The University of Nottingham, 6-9 September
2010. This was attended by > 300 members of the public. A write up is attached from Microbiology today.

viii) Professor Maja Rupnik (Partner 2) gave a public lecture to an audience of 25 on "How do microorganisms cause the
disease?" at Hisa eksperimentov/Science centre, Ljubljana, Slovenia. http://www.he.si Video available at
http://vimeo.com/21053709

ix) A Special Public Session was held at the International ClostPath meeting in October 2009, in Rome on the final morning of
the event. Introduced by the local host Paola Mastrantonio (Partner 3), the session informed the public with 4 presentations, 2
in English (one with Italian subtitles), and 2 in Italian. There was a coffee break in the middle and the whole session finished
with a "Question and Answer" session.

The Talks:

- Clostridial Infections: Afflictions For All Seasons" - Sherwood Gorbach - Tufts University School of Medicine, Boston, USA -
described the range of clostridial infections.

- Training the Next Generation of European Clostridium Researchers - Nigel Minton (Project Coordinator)) - University of
Nottingham - explained the importance and future of EU (Marie Curie) funding of Clostridial research in Europe.
- Clostridium difficile in Italy - Paola Mastrantonio (Partner 3) - ISS, Rome - explained the national picture in Italian.

This session was advertised at the website www.clostrida.net through advertising posters in the ISS, by contacting local schools, through the local press and by contacting local special interest groups, e.g. hospitals (see public-dissemination attached). A total of 104 people attended this event: 27 students and 1 teacher from a local High School, 35 students from different institutions (University La Sapienza, National Research Council, Hospital Staff), 16 ClostPath delegates and 25 M.C.students.

x) Public interest in C. difficile in some countries, like Slovenia, is still low as compared to UK, for instance. Nevertheless, there was certain coverage of this topic in daily/weekly newspapers and Maja Rupnik (Partner 2) is often contacted by journalists and quoted in daily press. She has also given a general public lectures in Slovenia on microbes and disease for non-scientific audiences. e.g. She gave a public lecture to an audience of 25 on “How do microorganisms cause the disease?” at Hisa eksperimentov/Science centre, Ljubljana, Slovenia. http://www.he.si Video available at http://vimeo.com/21053709

xi) Following publication of their Nature paper, the UNOTT team received extensive media coverage, with articles in the local and national press, as well as on Radio and TV. A special press event was held at the UK Festival of Science in Birmingham, attended by the public and media representatives.

EXPLOITATION OF RESULTS

One of the outputs with long term impact is the establishment of international collection of animal strains that is currated at Institute of Public Health Maribor and accessible to scientific community via web page (http://164.8.68.130/mikro/animalcdiff/).

Many of the partners have been contacted by various commercial enterprises concerned with Diagnosis or therapy as a result of publication/presentation of their outputs. Many of these discussions are ongoing, whereas others have resulted in commercial contracts. Some examples are detailed below.
1. New Diagnostic Tests

The results found on new DNA markers for a 027-clade and 078-clade have been patented by partner 5 (Leiden, NL) and has received much interest from companies. Second, results have been used to successfully apply new national grants and an ECDC funded surveillance study in Europe. Finally, the association of community acquired CDI with an increase of CDI observed in food animals has resulted in a new hypothesis on the possible zoonotical transmission and will be studied in more detail in The Netherlands.

2. Vaccine Development

The results obtained by tgcBIOMICS under HYPERDIFF will contribute to vaccine development, a major social economic item that we see for the future of Europe. Similarly, the studies of UPS and UNOTT have also attracted considerable interest. The latter has received funding from a major US pharmaceutical company to take their work forward.

3. Novel Therapeutics

The work on spore germination and sporulation has led to a contract being awarded to UNOTT from major European pharmaceutical company to test the efficacy of their new product against these processes in hypervirulent strains.

4. Basic Science

As a result of their Nature paper, UNOTT has been funded by a MERCK investigators award to provide further evidence on the role of Toxins A and B in disease.

5. Strain Development
The gene technologies developed by UNOTT have been licensed by a major US company, and are currently being employed to develop new strains of clostridia useful in the manufacture of chemical commodities from renewables. Further licenses are under negotiation.

List of Websites:
http://www.clostridium-difficile.com/

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