Institution: University of Nottingham

Unit of Assessment: 5 - School of Life Sciences

Title of case study:

Development of genetic modification tools to enable bioengineering of clostridial species for improved healthcare, chemical commodity and biofuel manufacture.

1. Summary of the impact

Bacteria of the *Clostridium* genus are of pathogenic, medical and industrial importance. Development by University of Nottingham School of Life Science researchers of three patented methods for genetic manipulation of clostridial species has led to licensing agreements for commercial exploitation of the methodology to enhance strains for chemical commodity and biofuel production and for targeted cancer therapy. These methods are providing significant world-wide impact by facilitating commercial R&D investment and technology developments in fields ranging from healthcare, through chemicals manufacture, to the environment.

2. Underpinning research

Historically, the bacterial genus *Clostridium* is most often associated with debilitating and life-threatening diseases such as botulism, tetanus, gangrene, antibiotic associated diarrhoea and food poisoning. However, the vast majority of clostridia are entirely benign, and far from being the scourge of humankind, may well be its saviour. Clostridia occupy many specialised biological niches and have evolved a plethora of bio-catalytic abilities that can be exploited for humankind's benefit. Many species are being pursued as cell factories for production of biofuels and chemicals through alternative processes to traditional petro-chemical routes. Yet others are being explored as tumour delivery vehicles for anti-cancer drugs.

Tools for manipulating clostridial genomes (for gene knock-out and knock-in) are essential for generating the functional knowledge needed to combat clostridial pathogens, and for genetic enhancement of strains used in chemical production and cancer therapy. Without those tools, in the 20 years prior to breakthrough research at Nottingham, just five mutants had ever been made in the biobutanol organism *C.acetobutylicum*, only one in the pathogen *C.difficile* and none in the pathogens *C.botulinum or C.sporogenes*. The majority of these mutants were made by single cross-over plasmid insertion, and consequently were genetically unstable due to plasmid excision.

The problem of achieving stable integration was solved in 2006 by Professor Nigel Minton's Clostridia Research Group, under a combination of BBSRC, MRC and industrial funding, by developing an intron retargeting system, the ClosTron[1,2]. The engineered plasmids (eg. pMTL007C-E2) encode a mobile group II intron that can be directed to insert into virtually any target DNA substrate by simply making a handful of nucleotide changes to the intron encoding region. Through the use of a Retrotransposition-Activated Marker (RAM) based on the *ermB* gene, successful insertion is selected by acquisition of erythromycin or lincomycin resistance. The necessary intron changes are designed using a 'free to access' online re-targeting algorithm at a purpose built website (www.clostron.com). Sequences can be ordered through this website with DNA2.0 (www.dna20.com) for both synthesis of the retargeted region and its cloning into the ClosTron vector. The paper describing the ClosTron[1] is the most cited paper in the Journal of Microbiology Methods since its publication in 2007. ClosTron has revolutionised clostridial molecular biology[3] and it is now the most widely used gene tool within the clostridial community. A patent protecting the system was filed in 2006[9].

However, the ClosTron tool has a significant drawback: it is an insertional mutagen and can therefore cause detrimental genetic effects. To overcome this limitation, in 2009-2010, new recombination-based methods were devised. Of most significance is Allele-Coupled Exchange (ACE) which allows the insertion of DNA fragments of any size or complexity into clostridial genomes[4]. Following integration of the ACE plasmid by single-crossover recombination, the system is designed so that the second recombination event leads to a plasmid borne allele becoming 'coupled' to a genome located allele, generating a new selectable allele for isolation of

double-crossover cells. Use of highly asymmetric homology arms dictates the order of recombination events. Moreover, ACE generates mutants of a heterologous pyrE gene[5] or another marker, CodA[7] subsequently developed at Nottingham, that can be used as negative selection markers in classical allelic exchange strategies. ACE and CodA patents were filed prior to publication, in 2009[10] and 2010[11]. To exemplify the ease, rapidity and iterative capability of ACE to insert and extend large operon sequences, the entire lambda genome (48.5 kb) was inserted into the *C. acetobutylicum* genome through the sequential delivery of three overlapping fragments of 28, 18 and 6.5 kb[4].

ACE has most recently been used within the BBSRC Sustainable BioEnergy Centre (BSBEC) to introduce genes encoding components (scaffold & hydrolases) capable of degrading cellulose into the genome of the biobutanol organism, *C. acetobutylicum*[6]. Significantly, ACE has now been extended to [text removed from publication] *Geobacillus*, allowing [text removed from publication] strain to be recreated in just 30 days by 3 simple genetic manipulations. These technologies, therefore, translate beyond *Clostridium* species.

3. References to the research

Publications (UoN authors in bold, key author(s) underlined)

1. **Heap JT**, **Pennington OJ**, **Cartman ST**, **Carter GP**, **Minton NP** (2007). The ClosTron: A universal gene knock-out system for the genus *Clostridium*. *J Microbiol Methods*. **70:** 452-64. doi: 10.1016/j.mimet.2007.05.021
2. **Heap JT**, **Kuehne SA**, **Ehsaan M**, **Cartman ST**, **Cooksley CM**, **Scott JC**, **Minton NP**. (2010) The ClosTron: Mutagenesis in *Clostridium* refined and streamlined. *J Microbiol Methods*. **78:** 79-85. doi: 10.1016/j.mimet.2009.10.018
3. **Kuehne SA**, **Cartman ST**, **Heap JT**, **Kelly M**, **Cockayne A** and **Minton NP** (2010) The role of toxin A and toxin B in *Clostridium difficile* infection. Nature **467**(7316): 711-713. doi:10.1038/nature09397. ***Included in REF2.***
4. **Heap JT**, **Ehsaan M**, **Cooksley CM**, **Ng Y-K**, **Cartman ST**, **Winzer K**, **Minton NP** (2012) Integration of DNA into bacterial chromosomes from plasmids without a counter-selection marker. Nucleic Acids Research **40**(8) e59; doi:10.1093/nar/gkr1321. ***Included in REF2.***
5. **Ng YK, Ehsaan M, Philip S, Collery MM,** Janoir C, Collignon A, **Cartman ST, Minton NP.** (2013) Expanding the repertoire of gene tools for precise manipulation of the *Clostridium difficile* genome: allelic exchange using *pyrE* alleles. PLoS One **8**(2):e56051. doi: 10.1371/journal.pone.0056051
6. **Kovács K, Willson BJ, Schwarz K, Heap JT**, Jackson A, Bolam DN, **Winzer K, Minton NP** (2013) Secretion and assembly of functional mini-cellulosomes from synthetic chromosomal operons in *Clostridium acetobutylicum* ATCC 824. Biotechnol Biofuels **6**(1):117-130. doi:10.1186/1754-6834-6-117
7. **CartmanST**, **Kelly ML**, **Heeg D**, **Heap JT**, **Minton NP**. (2012) Precise manipulation of the *Clostridium difficile* chromosome reveals a lack of association between the tcdC genotype and toxin production. Appl Environ Microbiol **78**(13):4683-4690. doi: 10.1128/AEM.00249-12.
8. Bradshaw M, Marshall KM, **Heap JT**, Tepp WH, **Minton NP**, Johnson EA (2010) Construction of a nontoxigenic *Clostridium botulinum* strain for food challenge studies. Appl Environ Microbiol **76**(2) 387–393. doi:10.1128/AEM.02005-09

Patents:

9. **Heap JT** and **Minton NP** (2007) DNA Molecules and Methods; WO2007148091; Morvus Technology Ltd, published 27 Dec 2007.
10. **Heap JT**, **Minton NP** (2009) Methods; WO/2009/101400; University of Nottingham; Published 20 Aug 2009
11. **Cartman ST**, **Minton NP** (2010) Method of double crossover homologous recombination in Clostridia; WO/2010/084349; University of Nottingham; published 29 July 2010.

Grant Funding from 2006 to date:

BBSRC: BB/D522289/1, 2006-2009, £**199,304;** BB/D522797/1, 2006-2009, £**187,191;** BB/F003390/1, 2007-2010, £**364,436;** BB/E021271/1, 2007-2011, £**643,519;** BB/G017395/1, 2009-2013, £**74,410**; BB/G530341/1, 2009-2013, £**26,518;** BB/G016224/1, 2009-2014, £**2,127,704**; BB/I004475/1, 2010-2013, £**452,694;** KTP with Green Biologics, 2011-2013**, £47,514**; BB/L004356/1, TSB SynBio, 2013-2014, **£109,539**; BB/L000105/1, ERANET, 2013-2016,

£147,227; BB/K020358/1, RICEFUEL, 2013-2016, **£1,393,966**; BB/J020427/1, India, 2012-2016, **£13,692**; BB/L01081X/1, China, 2013-2017, **£26,518**; BB/K00283X/1, GASCHEM, 2013-2018, **£2,396,136**
MRC: Programme grant on *C. difficile* virulence (G0601176), 2007-2013; £**1,610,998**
EU FP7: HEALTH-F3-2008-223585; 2008-2011; **£632,229;** People Programme 2009 – 2013, **£867,641**; Marie Curie **£100,473**. CLOSTNET: **€5 million** with Minton as coordinator.

Industrial Funding:
£2.5 million from pharma, biotech, biofuel and chemical commodity companies; 2009 onwards.

4. Details of the impact

Impact 1: Technology Transfer, Out-Licensing and Commercial Exploitation

Since publication of the patents, Material Transfer Agreements have been established with over 250 research and industrial institutions worldwide[A] to allow distribution of ClosTron, ACE and CodA reagents. The technologies have been licensed through the university to 5 companies worldwide [A], with other companies accessing the technologies by collaborative research with Minton and the Clostridial Research Group. This has enabled further commercial investment and exploitation by the companies, bringing financial benefits to them and the university. In addition, patent search has identified 15 patents published by other research and development groups that cite use of ClosTron to achieve their inventive step(s)[B]. These include genetic manipulations for production of biofuels (including LanzaTech, Total S.A., Qteros, IFP Energies and TetraVitae) and vaccines (Wyeth/Pfizer, Altermune and London School of Hygiene and Tropical Medicine). All these businesses and other organisations are beneficiaries of the university's research.

Impact 2: Reducing clostridial infection and food contamination

Knowledge of *C.difficile* and *C.botulinum* virulence mechanisms is being enhanced through the generation by Minton's group, and others, of virulence mutants and their phenotypic characterisation. For instance, strains of *C.difficile* expressing recombinant immunogenic but non-toxigenic toxoids A and B, generated using ClosTron by Wyeth (subsequently acquired by Pfizer)[C] have led to development of a *C.difficile* vaccine to prevent hospital acquired infections (currently in Phase 1; http://www.clinicaltrials.gov/ct2/show/NCT01706367). Wyeth/Pfizer have benefitted from the university's research by being able to develop the vaccine. Further tangible benefits will arise worldwide for patients suffering from *C.difficile* associated infections (approximately 350,000 in the US[D] and 15,000 in the UK annually[E]). The technology will also help to reduce associated deaths (14,000 in the US[D] and 2,000 in the UK[F] annually), and reduce the estimated annual healthcare burden (~$5 billion USD annually). Similarly, generation of a non-toxigenic strain of *C.botulinum* for use by the food industry as a food processing challenge test strain[8] overcomes strict regulations on containment and use of highly toxigenic strains of *C.botulinum*, benefitting manufacturers by reducing costs of food testing, contamination and wastage, as well as reducing costs and improving food safety for consumers.

Impact 3: Novel anti-cancer therapeutic approaches

The anaerobic requirement of *C.sporogenes* can be used to target the poorly vascularised centre of a solid tumour mass; early studies injecting unmodified spores induced partial tumour lysis but showed systemic toxicity. Development of non-toxigenic *C.novyi-NT* spores avoided this toxicity in Phase I trials in 2011 (http://www.clinicaltrials.gov/ct2/show/NCT01118819), but did not cause complete tumour lysis. Transfecting *C.sporogenes* with an *E.coli* enzyme (via single cross-over plasmid insertion) to locally activate a prodrug, enhanced tumour lysis (Lui et al. 2002; Gene Therapy 9: 291–296. doi: 10.1038/sj/gt/3301659). This novel anti-cancer treatment method, termed 'Clostridial-directed Enzyme Prodrug Therapy' (CDEPT) was patented in 2002 (US20030103952, with Minton as an inventor). It offers improved, targeted oncolytic efficacy and significantly reduced side effects[G]. However, Regulatory Authority requirements stipulate that genetic modifications of bacteria that encode therapeutic enzymes must be stably integrated to prevent gene loss in clinical use. The knock-in capability of ACE was used for the first time in 2012[7] to meet this requirement, opening up the pathway to achieve significant impacts in cancer therapy.

Impact 4: Bioengineering benefits

The capability of these *Clostridia* genetic manipulation tools is proving pivotal to gaining a greater understanding of metabolic networks present in clostridia that are useful in chemical commodity

production, as well as in metabolic engineering approaches to improve product yield. Industrial exploitation of ACE technology, under licence from the university, is most visible in development of second generation biofuels. TMO Renewables[H] are engineering *C.acetobutylicum* and *C.thermolyticum* to produce butanol and ethanol from cellulosic and lignocellulosic feedstock, whereas LanzaTech are developing a microbial fermentation process for conversion of carbon dioxide to ethanol. TMO Renewables has a current book value of £10.9 million[H] and has recently signed a $500 million order to build 15 factories across the US to produce ethanol from household waste[H]. Similarly, LanzaTech has received in excess of $70 million of government and private funding, and has recently joined a biofuels collaboration with the Indian government and the Indian Oil Corporation[I]. Both companies aim to address the worldwide demand for ethanol (principally as a petroleum blending additive) which is projected to be nearly 200 billion litres annually by 2022[J]. First generation production capacity (from maize and sugar cane fermentation) is currently only 50 billion litres. The impact of using clostridial genetic engineering tools to expand biofuel and chemicals production, without impinging on use of agricultural land, not only benefits the university through technology license fees, and the companies through access to the technology and revenues generated from it, but also has significant worldwide environmental benefit.

Summary

The beneficiaries of the clostridial bioengineering technology developed at Nottingham, as a consequence of its myriad of applications, are extremely diverse and its subsequent benefits far reaching. Its availability has revolutionised the genetic approaches being undertaken by academic and industrial researchers alike, aimed at understanding pathogenic and commercially-important clostridial species through mutational analysis. It has implications for disease prevention and treatment, cancer therapy and chemical commodity and biofuels production. It therefore has direct and consequential impacts in Health, Energy and, through the generation of sustainable biofuels, the Environment.

5. Sources to corroborate the impact

A. Numbers of MTAs assigned and licences awarded can be confirmed under confidentiality by Business Engagement and Innovation Services, University of Nottingham.
B. PCT Patent search performed on http://patentscope.wipo.int/search/en/search.jsf using Full Text search term: EN_ALLTXT:(ClosTron)
C. http://www.icds.si/docs/Speakers/O2_Jansen_et_al.pdf
D. *C.diff* infections and deaths in the US 2012: http://www.cdc.gov/hai/eip/pdf/Cdiff-factsheet.pdf
E. Hospital Acquired Infections in the UK 2012: http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1284473407318
F. *C. difficile* deaths, England & Wales 2011: http://www.ons.gov.uk/ons/dcp171778_276892.pdf
G. Wei et al (2008) Cancer Letters 259: 16–27; doi: 10.1016/j.canlet.2007.10.034
H. http://www.tmo-group.com/adeptt/
https://www.duedil.com/company/04405622/tmo-renewables-limited
http://www.bbsrc.ac.uk/news/industrial-biotechnology/2012/120127-f-british-biofuel-technology.aspx
I. http://www.lanzatech.com/content/media-releases-2013
http://www.lanzatech.com/content/media-releases-2012
J. Global Biofuels predicted markets: http://unctad.org/en/Docs/ditcbcc20091_en.pdf - in particular Chapter I, pp1-6 for projected ethanol demand and production capacities and Chapter III, pp29-50 for agricultural benefits of second generation biofuels.

Corroborative documents and copies of webpages are held on file and are available on request.