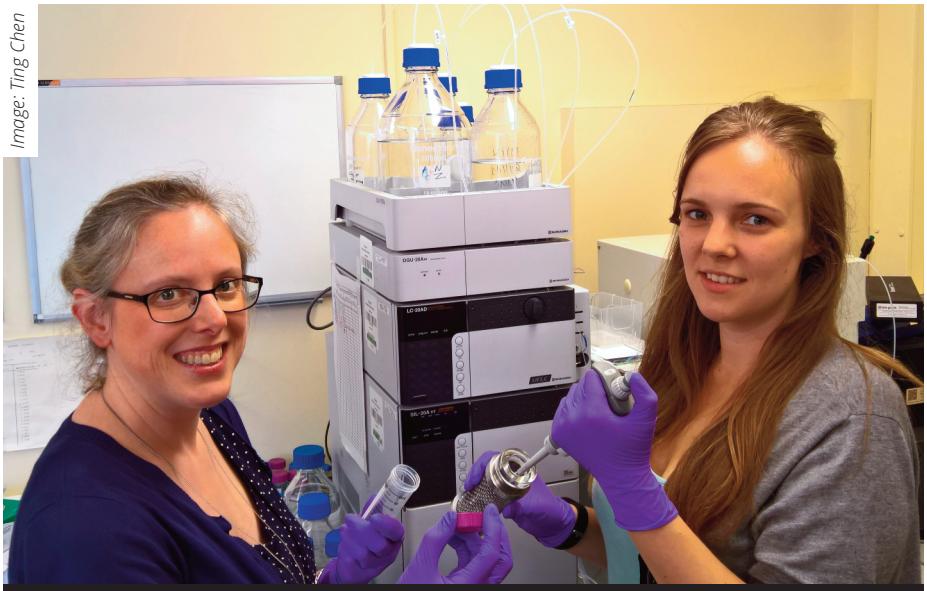
A collaborative journey to greener chemicals

Kylie Vincent, Holly Reeve and colleagues at Oxford University are working on ways to make greener chemicals. With government funding, the technology could soon be used in industry...

We are working on greener ways to make chemicals that are used in medicines, food and cosmetics by using enzymes that are found in nature – an area of research known as industrial biotechnology. Our group came to work in industrial biocatalysis in a roundabout way. We were studying types of bacteria that use the very small amounts of hydrogen gas found in the environment as 'food' or energy. To do so, these bacteria contain enzymes – hydrogenases – in their cells which use metal ions to split apart the hydrogen molecule. We were interested in how nature uses metal ions to enable, or catalyse, the split of the hydrogen molecule to better understand how we could make use of this reaction in the lab.

In the course of this work, we carried out collaborative experiments with Dr Oliver Lenz's group at the Technical University of Berlin into a special hydrogenase that uses hydrogen as a fuel to drive the production of a biological molecule called nicotinamide adenine dinucleotide hydride (NADH) [1]. NADH is an important energy currency inside cells, and is required for the function of many types of enzymes. In particular, NADH is essential to the types of enzymes that are gaining attention for their ability to make complicated chemicals such as pharmaceuticals, or flavour or fragrance chemicals in greener ways than in the past.

Image: Ting Chen



Kylie Vincent and Holly Reeve

Although the use of enzymes as biocatalysts in chemical synthesis is really taking off, a key challenge is developing an efficient supply of NADH. We quickly realised that we could make use of components from bacterial cells to assemble a system for recycling NADH that could be used outside of the bacterial cell by incorporating some of the cell components onto cheap carbon beads. The use of hydrogen gas as the energy source to drive the chemistry gives a much cleaner way of supplying the NADH for enzyme-catalysed chemical synthesis [2].

We developed this into a technology that we call HydRegen (short for hydrogen-driven regeneration). We were able to show quite quickly that our HydRegen beads work with a wide range of NADH-dependent enzyme reactions. This was a key finding, as it increases the potential applications of the technology. We filed a patent application with support from Oxford University's Technology Transfer company, Oxford University Innovation, and began talking to some of the companies that are interested in using NADH-dependent enzymes to make complex chemicals.

One of the questions we were frequently asked was whether we could extend to the related biological cofactor NADPH (nicotinamide adenine dinucleotide phosphate), as this is essential to many other industrially relevant enzymes. However, NADPH presents even more of a headache in industrial applications of enzymes as it is more expensive and less stable than NADH. After winning the prestigious Royal Society of Chemistry's Emerging Technologies Competition

in 2013, we were mentored by Dr Ian Churcher from the pharmaceutical company GlaxoSmithKline, who helped to advise us on development of the technology.

GlaxoSmithKline were natural partners as we started to explore the scope for extending our HydRegen beads to NADPH in an industrial setting. Together, we secured short-term funding in the form of a Business Interaction Voucher from the Metals in Biology BBSRC NIBB, to test whether we could produce enough NADPH to drive some NADPH-dependent enzymes that GlaxoSmithKline are interested in. The project was successful, and provided real proof of the versatility of the HydRegen technology.

However, major research challenges remain. The two enzymes we use are excellent biocatalysts – the hydrogenase enzyme splits the hydrogen molecule, causing electron transfer through the electronically conductive carbon beads to support the recycling of NADH (or NADPH) at the other enzyme. But they are complex metalloenzymes that can only be produced in bacterial cells.

Although making these enzymes for use in the lab is now relatively straightforward, making them on an industrial scale remains unchartered territory. We still need to find the best ways to make the bacteria produce these enzymes in high quantities, the minimal steps needed to isolate the enzymes, and the best ways to handle them once they are separated from the cells. To address these research challenges, we applied to a government funding scheme called the Industrial Biotechnology Catalyst scheme (run through the BBSRC/EPSRC/Innovate UK), and in January 2016 we were awarded £2.9 million of funding for a major research programme to explore and de-risk the scalability of the HydRegen technology.

We have assembled an industrial advisory board, taking advantage of industry contacts we have made along the way at Oxford University-led meetings, at Metals in Biology BBSRC NIBB meetings, and with help from Oxford's technology transfer team. The advisory board is helping to keep the project focussed on addressing real industry challenges in biotechnology.

"A Business Interaction Voucher from the Metals in Biology BBSRC NIBB, to test whether we could produce enough NADPH to drive some NADPH-dependent enzymes that GlaxoSmithKline are interested in provided real proof of the versatility of the HydRegen technology."

The Industrial Biotechnology Catalyst-funded project is now up and running successfully, and one of the most exciting parts of the first six months has been seeing the project generate new fundamental insight into the enzyme systems and new patentable discoveries, as well as advances in the core HydRegen technology itself. We have also continued to make industrial contacts and develop our commercialisation strategy in conjunction with Oxford University Innovation.

We have always enjoyed the interplay between detailed studies of enzyme function and development of applications of enzyme biocatalysis. In parallel to the Industrial Biotechnology Catalyst project, we continue to work on fundamental studies into hydrogenase enzymes, and to develop new tools for studying metalloenzymes, supported by BBSRC responsive mode grants on which we are co-investigators [3]. Our strengthened understanding of how hydrogenase enzymes work, and the accumulation of related know-how in the group continues to feed very productively into our development of the HydRegen technology.

Indeed, the Industrial Biotechnology Catalyst funding should help this technology to cross the so-called 'valley of death' that often impedes early-stage technologies. With this funding, and our additional grants, we hope to bring the HydRegen system to market in the next 3-4 years.

1 Lauterbach, L., Idris, Z., Vincent, K.A., Lenz, O. 'Catalytic properties of the isolated diaphorase fragment of the NAD^+-reducing [NiFe]-hydrogenase from *Ralstonia eutropha*' PLoS ONE, 2011, 6, (10): e25939.

2 Reeve, H.A., Lauterbach, L., Lenz, O., Vincent, K.A. 'Enzyme-modified particles for selective bio-catalytic hydrogenation via H_2-driven NADH recycling' ChemCatChem, 2015, 7, 3480-3487.

3 Hidalgo, R., Ash, P.A., Healy, A.J., Vincent, K.A. 'Infrared spectroscopy during electrocatalytic turnover reveals the Ni-L active site state during H_2 oxidation by a NiFe hydrogenase' Angew. Chemie. Int. Ed. 2015, 54, 7110-7113.

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Adding value to galactomannan polysaccharides with copper enzymes

"By working with industrialists to address a specific question, we have gained unique insights from those with first-hand experience of running a commercial process." Julea Butt

Julea Butt, University of East Anglia
Seth Hartshorne, Schlumberger Gould Research

Guar beans, from which guar gum is made

OUTCOMES: Guar gum is a galactomannan that is comprised of covalently linked sugar molecules, namely mannose and galactose. In this project, polymer hydrolysis resulting in chain shortening was demonstrated using a commercially available enzyme, cellulase. Oxidation of the galactose sidechains within the polymer in aqueous solution was shown using another commercially available enzyme, galactose oxidase. In addition, electrochemical oxidation of the galactose and mannose sugars was demonstrated by oxidation of aqueous guar suspensions at a graphite electrode. The results demonstrate that commercially available enzymes offer routes to controlled modification of guar in aqueous solution and this offers prospects for the development of more sustainable routes to industrial-scale galactomannan modification.

INITIAL AIMS: Concerns over fuel security are frequent headline news and the rising costs of fuel are a daily reminder of the challenges faced by a global society with ever- increasing energy demands. Medium- to long-term solutions to these challenges will require effective access to renewable energy alongside the development of infrastructures that enable such energy to be delivered to the point of need with the same ease as fossil fuels. Improved technologies to increase the recovery of natural gas presents an attractive option for the short to medium term. Here we aim to investigate opportunities to develop improved stimulation technologies through the use of copper-containing enzymes that can modify the rheological properties of a natural biopolymer guar.

- Commercially available enzymes might provide a more sustainable route to industrial-scale guar gum modification
- Modified polysaccharides such as guar could improve hydrocarbon recovery technologies

Biotechnology and Biological Sciences Research Council

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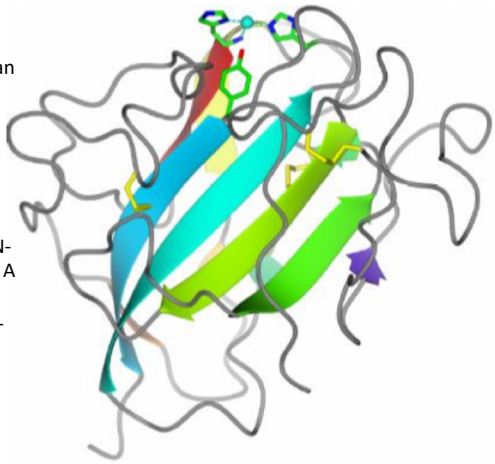
Arginine-terminated LPMOs: a new face in biomass breakdown? Follow-on studies

"The new form of LPMO enzymes is intriguing as it does not contain the usual amino acids at its active site, suggesting that it could be active on a new range of biomass components." Paul Walton, University of York

Paul Walton and Gideon Davies, University of York; Jens Erik Nielsen, Novozymes, Denmark



The overall structure of a typical LPMO has an extended flat face in the middle of which lies the enzyme's active site, containing copper ion coordinated by a N-terminal histidine. A new type of LPMO has a conserved N-terminal arginine instead of an N-terminal histidine.

OUTCOMES: In this project we examined the activity of this new LPMO using ultraviolet–visible spectroscopy and electron paramagnetic resonance. We also determined the structure of the enzyme, gaining particular insight into how it interacts with its substrates. This finding opens up the possibility of tailoring the enzyme to carry out new types of reactions which are of importance to biomass degradation. While we cannot report the details of the findings due to IP reasons, the project was successful in that we developed a new type of assay for this class of enzymes and we also demonstrated that the LMPOs had a new type of enzymatic activity on a range of substrates.

INITIAL AIMS: The generation of fuels and commodity chemicals from sustainable biomass hinges on a single key issue: that biomass (e.g. wood, plant matter) is very hard to break down in a controlled manner. The use of copper-containing lytic polysaccharide monooxygenases (LPMOs) — natural enzymes that are highly efficient at breaking down cellulose — could help circumvent this. In this project, we seek to maximise the ability of a new type of LPMO to break down woody biomass. Our previous Metals in Biology-funded studies showed that this new LPMO can bind metal ions, and we have also obtained a full molecular structure of the enzyme using X-ray diffraction. The structure of this new class of LPMOs suggests that they could be active on lignin and/or lignin components and that, indeed, these LPMOs use metal ions as part of their catalytic cycle. If true, this would represent a wholly new activity for LPMOs and be an exciting addition to the field of biomass degradation.

- **The partners are in discussions on how to take the project forward**

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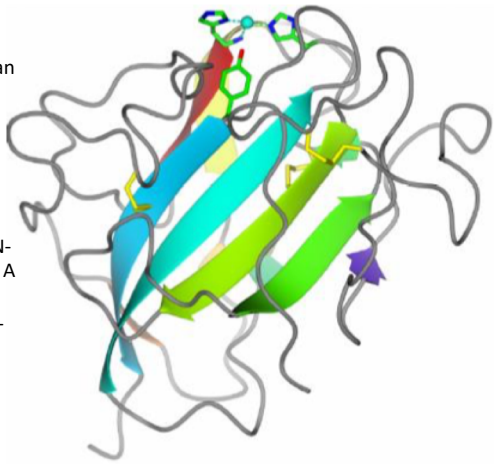

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Metals IN BIOLOGY

Biosynthesis of bimetallic nanoparticles for fine and specialty chemical production

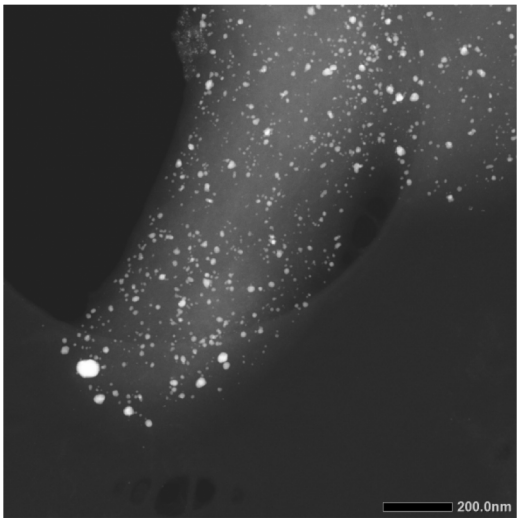
"This award provided a quick, convenient and effective route to bring together the Manchester group's expertise in bioproduction of metal particles with Johnson Matthey's catalysis know-how. We have begun to determine the potential of this technology for the production of novel catalysts." Nigel Powell, Johnson Matthey

MANCHESTER 1824 The University of Manchester

JM Johnson Matthey Inspiring science, enhancing life

Jon Lloyd and Richard Kimber, University of Manchester; Nigel Powell, Johnson Matthey plc

RESULTS: Building on from a successful Business Interaction Voucher project with Johnson Matthey, we continued to investigate the biosynthesis of novel bimetallic nanoparticles for fine and specialty chemical production. Electron microscopy revealed that different metals have varying affinities for forming bimetallic nanoparticles and that the bimetallic nature is also affected by the order the metals are supplied to the bacteria. In addition, we found that the pH buffer used during synthesis can exert some control over the formation of the bimetallic nanoparticles. This knowledge will help us tailor these products going forward. Several of the biosynthesised nanoparticles showed promising catalytic activity. Although they did not perform to the same level as a commercial catalyst, this project has provided us with valuable insights into the optimisation of these bionanocatalysts which we are continuing to explore.



Electron microscope image of bacteria that contain bimetallic nanoparticles. Image provided by G. Goodlet, Johnson Matthey Technology Centre.

INITIAL AIMS: Metal-reducing bacteria are able to recover a wide range of metals from process environments as catalytically active nanoparticles. This project will produce bimetallic nanocatalysts for use in fine and speciality chemical production. Bimetallic nanoparticles offer advantages over monometallic catalysts due to the properties that arise from the presence and synergy of the two metals, offering increased efficiency and specificity for speciality chemical production. This novel biotechnological process offers a simple, cost-effective, environmentally friendly synthesis route for bimetallic catalyst production.

- **The partners will continue to work together to optimise and tailor the catalytic activity of these materials**
- **We will then seek to identify potential avenues for further funding**

Durham University

University of Kent

UKRI Biotechnology and Biological Sciences Research Council

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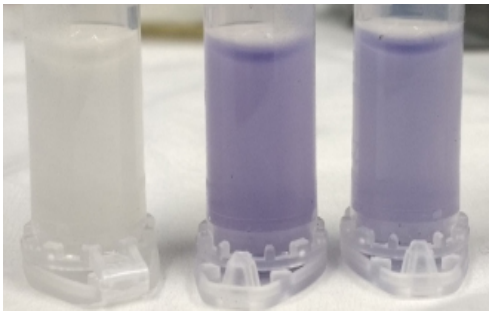
Cloning and metal analysis of recombinant aldehyde ferredoxin oxidoreductase

"We showed that a novel compartmentalisation strategy can be used to produce a biotechnologically important enzyme in bacteria. Our results are being used to help acquire further strategic investment in the partner company." Martin Warren, University of Kent

Martin Warren and Stephanie Frank, University of Kent; Michelle Gradley, ZuvaSyntha Ltd.



The activity of recombinant AOR in *E. coli*, as shown by an increase in the violet colour.

OUTCOMES: We first cloned two genes encoding for aldehyde ferredoxin oxidoreductase (AOR) from *Clostridium ljungdahlii* into *E. coli*. The purified proteins, termed AOR1 and AOR2, contained the predicted Fe-S centre and and a tungsten-pterin cofactor, which was produced by the *E.coli*. We next showed that the enzymes were active (but only when produced under strictly anaerobic conditions) in a whole cell assay (figure), with AOR2 displaying more activity than AOR1. We then investigated the targeting of AOR proteins to a bacterial microcompartment (BMC), by expressing the enzymes with a tag that directs them to the BMC. When the enzymes were co-produced with BMC shell-proteins, the AORs were indeed targeted to the BMC. Our work with the expression of AOR from the thermophile *Pyrococcus furiosus* proved more problematic, and is still under investigation. Our results demonstrate that recombinant AORs are active in *E. coli* if grown and kept under anaerobic conditions, and can be targeted to BMC. Our next steps are to see if the activity of AOR can be enhanced in acteogens, since this provides a powerful way in which acetate can be redirected for the production of commodity chemicals.

INITIAL AIMS: The aim of the project is to enhance the recombinant production of a key enzyme of biotechnological importance: aldehyde ferredoxin oxidoreductase (AOR). This enzyme allows the transformation of carboxylic acids into aldehydes, which could have use in the sustainable production of 1,3-butadiene – a key commodity chemical for the rubber and tyre market. We intend to explore the recombinant production of AORs in *E. coli* and to determine conditions that allow for the successful incorporation of its unusual metal complement, which includes a tungsten-molybdopterin cofactor and a 4Fe-4S centre. Moreover, we also want to see if this protein can be targeted to bacterial microcompartments (BMC) — proteinaceous organelles that can be used to encase metabolic process to protect a cell from toxic products — and to determine if AORs retain their metal complement once inside the BMC.

- The results this project have secured two further grants: Early Stage Catalyst Funding from BBSRC and Proof of Concept Funding from C1Net
- ZuvaSyntha and the University of Kent are seeking IP projection for aspects of this work

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Embedding technical expertise in the optimisation of trace metal supplementation strategies for successful biomethane production

"The work has been an eye-opener for the company in terms of the potential for optimisation of the plant, and a huge benefit in terms of skills transfer to our staff" Michael Mason, Tropical Power Ltd

Yue Zhang, University of Southampton and Michael Mason, Tropical Power Ltd

RESULTS: The project developed methods that are helping a UK company, which has built and is currently operating Africa's first grid-connected anaerobic digester, to determine more precisely the trace element requirements for optimum digestion of their novel agricultural waste feedstocks. We developed a method suitable for use in Africa that uses simple multi-purpose apparatus to test which trace elements are actually required. The methodology was made available to the industrial partner in the form of a training video and a detailed description of the procedures. In addition we helped our partner company interpret historical data from the digestion plant and provided them with a simple spreadsheet-based calculator to allow them to maintain steady state concentrations of essential elements in the digester in proportion to the feed added. The work also added tantalisingly to growing evidence that minor trace elements such as tungsten may play a critical role in the function of these microbial systems.

Tropical Power's grid-connected anaerobic digester in Kenya

INITIAL AIMS: Transformation of waste biomass into bioenergy is a key component in 21st century industrial biotechnology. It is increasingly clear that successful biomethanisation of mixed biomass requires complex enzyme systems that are produced by both natural and engineered synthetic microbial communities. Trace quantities of metals, which are required by certain essential metallo-enzymes, are needed to ensure that these microbial systems function in the most effective and productive way. There is a growing commercial market in trace metal supplements, but formulations of these are often generic rather than based on specific requirements. The current project will transfer knowledge and expertise in determining trace metal requirements to a UK company that uses novel waste feedstocks in Africa for renewable biomethane production. This will enable the company to formulate specific trace metal mixtures for optimum plant performance, and the scientific knowledge gained will contribute to the creation of future markets for UK suppliers of tailored supplements.

- **Collaboration broadened into new scientific areas help meet bioenergy needs of lower income countries**
- **Academic and industrial collaborators partnering on stage 1 GCRF application**

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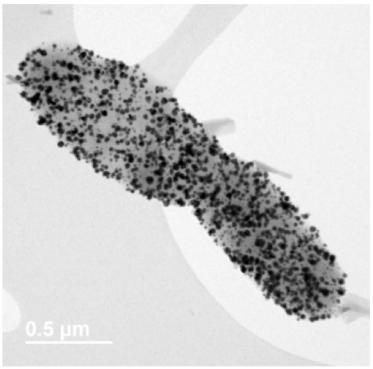
Exploiting the commercial potential of novel biometallic catalysts

"The BIV provided a quick, convenient and effective route for us to bring together the Manchester group's expertise in bioproduction of metal particles with Johnson Matthey's catalysis know-how. We have begun to determine the potential of this technology for the production of novel catalysts." Nigel Powell, Johnson Matthey.

Jon Lloyd, Nick Turner and Richard Kimber, University of Manchester; Nigel Powell, Johnson Matthey

OUTCOMES: Metal-reducing bacteria can accumulate metals from process environments in the form of catalytically active nanoparticles, offering a simple and green method for high-value nanoparticle production. These nanoparticles have many applications, including in the production of fine and speciality chemicals such as pharmaceutical intermediates, fats and oils, and upgrading of fuels and biorenewables. Bimetallic nanoparticles offer a number of advantages over their monometallic counterparts due to the combined properties of the two metals present, and through new properties created from the synergy between these metals.

We investigated the potential for a metal-reducing bacterium to produce bimetallic nanoparticles from metal solutions containing a range of metals supplied in combination. Metallic nanoparticles were biosynthesised at the University of Manchester and then Johnson Matthey's scanning transmission electron microscopy facilities were used to characterise the products. We found that the pattern of distribution of the metallic nanoparticles was highly dependent on the combination of metals supplied to the cells. Evidence was provided for the formation of bimetallic nanoparticles for some examples of metal combinations, and these nanomaterials are the focus of future work.



Bimetallic nanoparticles produced during the project

INITIAL AIMS: This project brings together biotechnologists from the University of Manchester and experts in industrial catalysis at Johnson Matthey, a leading multinational specialty chemicals and sustainable technologies company headquartered in the UK. This project will facilitate collaborative discussions required to underpin the development and exploitation of a new generation of 'biometallic' industrial catalysts. These are based on naturally occurring metal-reducing bacteria that are able to accumulate metals from process environments (as catalytically active nanoparticles), while also expressing enzymes that are able to extend the range and complexity of industrial reactions that can be produced from these novel microorganisms. This novel extension of synthetic biology has the potential to transform several sectors of UK industry, including those of industrial biotechnology and makers and users of catalysts, simplifying current processes, underpinning novel reactions and extending the range of available products.

- Obtained further funding through a BBSRC NIBB Proof of Concept award
- Industrial partner supported successful BBSRC Responsive Mode grant awarded to academic partner

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Improving biocatalytic processes by enzyme stability enhancement

"Increasing the stability of the enzyme catalyst in biocatalytic oxidation technology will improve the catalyst lifetime, leading to improved process productivity and broadening the range of products accessible to this technology platform." Luet Wong, University of Oxford

Stuart Ferguson and Luet Wong, University of Oxford; Jason King, Oxford Biotrans Ltd

RESULTS: To try to produce a P450BM3 enzyme containing a covalently bound heme, we first introduced a nucleophilic side chain close to the heme vinyl groups. Cysteine and histidine substitutions were introduced by site-directed mutagenesis to give a total of 20 single and double mutants. Expression trials showed that several mutants either did not produce holoprotein (the peptide and non-peptide parts of the enzyme) or that protein was produced at a low level. The nine mutants that formed holoprotein and the wild-type enzyme were tested for their ability to oxidise lauric acid (a natural substrate of the enzyme) and propylbenzene (an unnatural substrate). The wild type was the most active of the enzymes at oxidising lauric acid and each of the mutants had a similar profile to the wild type (63% to 90% of the activity). The activity profiles were also virtually identical for propylbenzene oxidation. Next, the wild type and mutant P450BM3 enzymes were treated with dithinoite, in expectation of form a covalently bound heme group. Encouragingly, the P450BM3 enzymes treated with dithinoite had a similar pattern of activity as the untreated enzymes in lauric acid and propylbenzene oxidation assays. Unexpectedly however, further tests showed that there was no covalent attachment of the heme vinyl groups to the introduced cysteine and histidine residues under the reaction conditions we used.

INITIAL AIMS: The industry partner has licensed biocatalytic oxidation technology from the University of Oxford for the production of fine chemicals such as flavours, fragrances, agrochemicals and active pharmaceutical ingredients. This approach not only replaces classical, more energy-demanding and polluting chemical processes, it also enables the use of sustainable feedstocks that are not fossil fuels. Enhancing of the stability of the enzyme catalyst that lies at the heart of the technology will be a key improvement to the process, making it applicable to a wider range of products. In this project we aim to increase the stability of the cytochrome P450BM3 by introducing a covalently bound heme into the enzyme.

- **We were surprised not to be able to covalently attach heme onto the P450BM3 protein**
- **Systematically altering the conditions (time, concentration) for dithionite treatment should be explored as there is precedent for the attachment procedure being successful**

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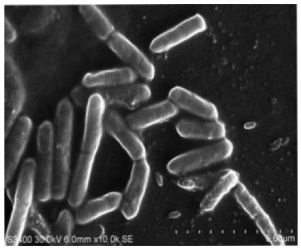
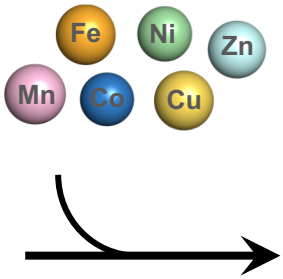
Investigating the link between metal homeostasis, sporulation, and solvent production in the *Clostridial* ABE fermentation process

"This study advanced understanding of metal requirements for commercial and research and development processes."

Peter Chivers, Durham University; Liz Jenkinson, GBL

OUTCOMES: Biomass samples were collected by GBL at 3-h intervals for metal content analysis and RNA isolation. An asporogenic mutant that did not show changes in metal content was sampled in parallel to understand the genetic requirements for changes in metal homeostasis. The project also provided key knowledge transfer – the metal content and RNA sample analysis were carried out at Durham by a GBL scientist. The informal communications during the 3-week visit will enhance future work at both sites.





AIMS: Solventogenic Clostridia were first used for fermentative acetone-butanol-ethanol (ABE) production roughly 100 years ago. The project focused on establishing a detailed picture of the changes in metal utilisation and homeostasis at the onset of sporulation using a combination of RNASeq and ICP-MS to correlate initiation of the sporulation genetic program and the role of metalloenzymes and proteins.

- **Understanding metal utilisation during sporulation could lead to improved control of fermentation processes**

Biotechnology and Biological Sciences Research Council

BIVMiB035 BB/L013711/1
1 April – 30 September 2017
Funded by a Metals in Biology BBSRC NIBB Business Interaction Voucher
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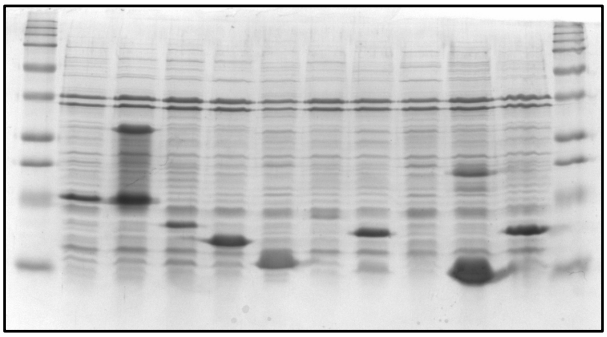
Metal utilisation in *Clostridium* microbial biocatalysts

"For GBL, this study highlighted the importance of metal requirements for commercial and research and development processes."

Peter Chivers, Durham University; Liz Jenkinson, Green Biologics

OUTCOMES: Metal contents were measured at different times during a GBL fermentation protocol used for Research and Development. The ICP-MS analysis revealed a large increase in metal content in *Clostridium,* coincident with the onset of butanol production. To complement the experimental data, bioinformatics analyses identified ten candidate metal sensor genes. These genes encode regulator proteins responsible for sensing and responding to changes in metal content. In several cases, their regulatory targets have also been identified. The proteins encoded by these genes have been overexpressed in *E. coli* to facilitate future studies of their metal selective transcriptional responses. These results will enable more detailed analysis of metal homeostasis networks in *Clostridium* to understand their link with butanol production or competing processes.



SDS-PAGE analysis of overexpression of *Clostridium* metal sensor proteins in *E. coli*

INITIAL AIMS: Little is known about the metal-demands of *Clostridium* strains during solvent production. This project will explore the metal requirements of solventogenic *Clostridium* during commercial and research and development processes used by Green Biologics. Metal content will be analysed by ICP-MS. A complementary aim is to identify the metal sensor genes responsible for maintaining metal homeostasis so that they may be overexpressed in *E. coli* to define the metal selective response of each protein.

- **Understanding metal demands could lead to improved control of fermentation processes**

BIVMiB008 BB/L013711/1
1 July – 30 April 2015
Funded by a Metals in Biology BBSRC NIBB Business Interaction Voucher
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Metallo-enzymes for production of nootkatol a potential new citrus flavour

"The project worked a lot better than even we expected. This BIV research program contributed to the scale-up and commercialisation of nootkatone (grapefruit flavour) production". **Oxford Biotrans Ltd**

Luet Wong ***University of Oxford***
Jason King ***Oxford Biotrans Ltd***

OUTCOMES: Samples of the pure nootkatol isomers were produced and delivered to the industry partner. The potential market of these novel flavours is being assessed. Enzyme variants that gave increased proportions of either nootkatol isomer were generated. Process optimisation also led to improved yields of the nootkatone production process. New strains from the NIBB MiB network can be applied to the systems and processes developed in this BiV project to benefit the UK industrial biotechnology sector.

INITIAL AIMS: The sought-after grapefruit flavour compound nootkatone is biosynthesised by air oxidation of valencene catalysed by a haem enzyme, firstly to nootkatol and then nootkatone. Haem enzymes are involved in the biosynthesis of numerous natural products, including flavours such as menthol and nootkatone but also medicinal compounds such as antibiotics, the antimalarial artemisinin and the anticancer drug taxol. The industry partner is developing commercial scale biocatalytic synthesis of nootkatone from valencene. The academic partner will modify the haem enzyme used in this process to produce nootkatol, which is found in minute quantities in grapefruit, to explore its potential as a novel flavour. This primer-project and the nootkatone process will underpin future collaborative work within the metals-in-biology community (1) to test strains optimised (by others) in haem production to further enhance nootkatone/nootkatol synthesis, (2) to manipulate the primary and secondary coordination sphere of the haem moiety to further enhance nootkatol synthesis.

- Nootkatol and nootkatone produced
- Products introduced in Europe & Asia
- Relevant to GCRF-related initiatives on antimicrobial natural products

Biotechnology and Biological Sciences Research Council

BIVMiB002 BB/L013711/1
5 January – 4 July 2015
Funded by a Metals in Biology BBSRC NIBB Business Interaction Voucher
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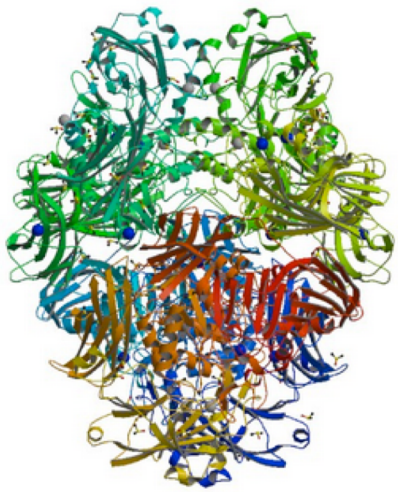
Optimizing metal acquisition by commercial metalloenzymes

"The BBSRC NIBB has brought me into contact with diverse researchers in the industrial sector, people who I would probably have never encountered through other channels." Kevin Waldron, Newcastle University

Kevin Waldron, Newcastle University
Stuart West, Biocatalysts Ltd

OUTCOMES: Three variants of magnesium-dependent galactosidase were selected for analysis. Because of the relative instability of complexes formed by magnesium compared to other transition metals, we hypothesised that it was likely that these enzymes could wrongly acquire a non-native, more stable complex-forming metal ion, either during heterologous expression or during sample preparation. Such association with the non-native metal cofactor is likely to lead to inhibition of the enzyme activity.
Our studies showed that small amounts of copper, zinc and nickel (but not iron) were present on the target enzyme in the preparations. The quantity of contaminating metals varied slightly between the longitudinal samples, and interestingly also varied between the three enzyme variants, suggesting possible differences in their metal-binding properties. Importantly, although the absolute quantity of these contaminating metals was low when expressed as a percentage of the total enzyme present (ranging from 3.8 — 12.8% occupancy), suggesting they would make only a minor diminution of the total enzyme activity of the enzyme preparations; a potential increase in enzyme activity of 4-12% would impact on profitability of commercial products.



Crystal structure of beta-galactosidase, PDB ID: 3SEP

INITIAL AIMS: Biocatalysts Ltd produce a number of metalloenzymes of commercial value through expression in bacteria and fungi. However, metal supply to these 'foreign' enzymes may not be optimal in these protein production hosts, so that a proportion of the commercial product is either bound to the 'wrong' metal, or lacks a bound metal ion altogether. Increasing the proportion of the enzyme that is correctly metal-loaded can directly increase profitability of metalloenzyme products. We will analyse the metal content of samples of the metalloenzymes produced by Biocatalysts.

- Although only a small amount of contaminating metals were detected, they reduce enzymatic activity – eliminating these contaminants would increase activity and thus profitability of these commercial enzyme preparations.

POCMiB016 BB/L013711/1
12 November 2015 – 11 November 2016
Funded by a Metals in Biology BBSRC NIBB Proof of Concept funding
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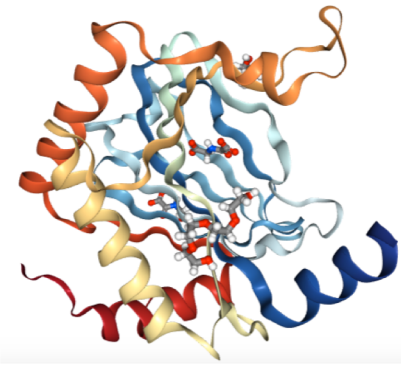
Proline hydroxylases for use in biocatalysis

"The project has revealed the substrate selectivity of oxygenases is much wider than we had expected, further highlighting their potential in biocatalysis for medicinal chemistry." Christopher Schofield, University of Oxford

Christopher Schofield & Michael McDonough, University of Oxford; Daniel Brookings, UCB Celltech

OUTCOMES: Our work concerned studies on a family of metal-dependent enzymes that add oxygen (or sometimes chlorine or bromine) atoms to drug-like small molecules and proteins. We focused on proline hydrolases, which in nature catalyse hydroxylation of the 5-membered ring amino acid proline. When we investigated the selectivity of the proline hydroxylases for different ring sizes and substitutions, we found that they can catalyses the hydroxylation of an unexpectedly wide range of rings, including bicyclic ring structures; some of these products are precursors for conversion into potential antibiotics. We also explored how these hydroxylases bind iron, using both assays for product formation and by X-ray crystallographic analyses of 'mutant' enzyme structures. Interestingly, we found that the proline hydroxylases can work with only two — rather than the normal three — points of attachment (ligand) of iron to the protein. These results inspired us to study metal binding by enzymes in cells (using mass spectrometry) and to study variations on iron binding by other types of hydroxylases. In one case we found the hydroxylase can work with only one protein ligand.



Cartoon of HIF prolyl hydroxylase 2 in complex with N-oxalylglycine (PDB-ID 5L9R)

INITIAL AIMS: Metal-dependent enzymes are incredibly powerful biological catalysts. In nature, biocatalysts can modify a common chemical scaffold to give several other products, which may have very different biological functions. Harnessing the power of such late-stage modification for drug discovery has potential to generate many molecules from a single drug candidate and could enable the efficient discovery of optimised molecules. There is a current lack of accessible libraries of oxygenase enzymes that are suitable for use in biocatalysts, and little information on how their activity is limited by metal binding in cells. We aim to develop proof of concept for the use of engineered proline hydroxylases for the stereoselective oxidation of substrates of choice.

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- Manuscript published: Zhang et al. (2017) PNAS 114: 4667- 4672
- Symposium on late-stage modification of pharmaceuticals
- Project expanded to include another potentially useful enzymes

Biotechnology and Biological Sciences Research Council

Workshops kickstart a *Rhodococcus* molecular toolkit

Workshops funded by Metals in Biology BBSRC NIBB have built a new community of scientists involved in *Rhodococcus* research and initiated a project to develop a community resource of molecular biology tools to enable the wider use of *Rhodococcus* bacteria in industrial biotechnology.

Rhodococcus species — the majority of which are non-pathogenic soil inhabitants — contain useful enzymes, pathways and systems that can be harnessed for industrial biotechnology applications. For example, they are able to breakdown environmental pollutants and explosives, aid the recycling of rubber tyres, be used in biofuel production systems, turn metal contaminants into useful products and synthesise complex chemical compounds.

A scoping workshop on "Metalloproteins in Biocatalysis and Bioenergy" was held at Durham University in July 2014; not long after the start of the Metals in Biology Network. At this meeting, Alison Parkin from the University of York and Colin Murrell from University of East Anglia, advocated the idea of holding a focused workshop on *Rhodococcus*. "We realised that there were a number of research groups in the UK who were working on *Rhodococcus* and related genera of actinomycetes, and that there was common interest in the biology, molecular genetics and the potential for biotechnological applications of this group of bacteria," says Colin.

Alison highlights that that *Rhodococcus* research is an area where the UK has both strength and breadth, "UK science in this area spans industrial applications, environmental science and understanding disease". A key consideration was the format of the focused meeting to help maximize potential output. "I wanted to have a meeting where attendees could have chance to get to know the main players in this field in a very focused, friendly and open manner that would let us discuss future directions and find new ways of working as a strong team," says Alison.

The meeting, held at the University of York in November 2015, had about 20 attendees (a summary of the meeting is available here). As well as allowing academic researchers and industry scientists to mix, the meeting initiated new projects. For example, as a result of the meeting, Alison's lab will now be able to start studying new bacteria and enzymes to find new biocatalysts that activate challenging chemical reactions.

One key theme that emerged at this meeting was the paucity of molecular biology tools (such as plasmids, vectors and mutagenesis protocols) to extract and manipulate *Rhodococcus* enzymes to enable their optimization for research and industrial biotech applications. To address this challenge, the attendees proposed the establishment of a molecular tool kit for *Rhodococcus*.

This idea was championed by Jon Marles-Wright and Louise Horsfall from the University of Edinburgh. "Building a molecular biology toolkit, based on established synthetic biology standards and containing well characterised parts for the control of gene expression and genome editing, will enable us to use *Rhodococcus* species more widely as an industrial host," says Jon. "It will also speed up research by making the tools available freely to anyone interested in working with these strains".

To initiate this project, Jon collated information related to *Rhodococcus* strains, genomes and genetic tools used in the attendees' research groups. The next phase of the project is moving into the lab; during the summer of 2016, Jon and Louise have a group of Masters students who are competing in the iGEM competition – an international synthetic biology completion for early career scientists.

"The iGEM competition has an ethos of openness and collaboration, so the idea of developing a set of community tools to enable *Rhodococcus* species to be easily engineered and used in research and industrial settings was something that really appealed to our students", says Jon. The plan is for part of the team to focus on a *Rhodococcus* toolkit, with the *Rhodococcus jostii* RHA1 strain as their model system. The students will also be involved in maintaining the community of *Rhodococcus* researchers.

So as a result of the workshops, the community-building that researchers hoped to achieve has indeed worked and they are now starting to construct a shared resource for molecular biology tools in *Rhodococcus*. "This has been a brilliant result", concludes Alison.

Attendees at the *Rhodococcus*-focused workshop

For more information please contact
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01 November – 31 December 2017
Funded by Metals in Biology BBSRC NIBB Business Interaction Voucher
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Evaluation of the potential of the molybdenum-containing enzyme DMSO reductase as an oxygenation catalyst

"This collaboration has allowed us to initiate a collaboration with Piramal that will hopefully lead to many other useful interactions," Gary Black, Northumbria University

Gary W Black and Justin J Perry, Northumbria University; Robert A Holt, Piramal Healthcare UK

Picture: pixabay.com

A chiral drug has a spatial arrangement of atoms that cannot be superimposed on its mirror image – rather like a pair of mittens.

OUTCOMES: In total six DMSO reductase enzyme preparations were produced from several bacterial strains from *Rhodobacter capsulatus, Rhodobacter sphaeroides, Cupriavidus metallidurans, Aeromonas hydrophila* and *Oceanithermus profundus,* and their capacity to perform complex oxidation chemistry was determined.

INITIAL AIMS: The enantiomers of a chiral drug — one that has a spatial arrangement of atoms that cannot be superimposed on its mirror image — can have different properties with respect to pharmacology, metabolism, immune response and so on. There has been significant effort to develop cost-effective and scalable methodology for the synthesis of enantiomerically pure compounds, but the development of processes involving oxidative reactions has lagged behind. Dimethylsulphoxide reductase (DMSOR) is a molybdenum-containing enzyme that can reduce sulphoxides to the corresponding sulphides. When the sulphoxide is a mixture of both enantiomers, DMSOR reduces one enantiomer much more rapidly, which leads to enantiomeric enrichment of the slower reacting enantiomer. This project will explore the potential of DMSOR to carry out complex oxidation chemistry

- Depending further results, the industrial and academic partners are considering an application to Innovate UK

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23 October 2017 – 4 January 2018
Funded by Metals in Biology BBSRC NIBB Business Interaction Voucher
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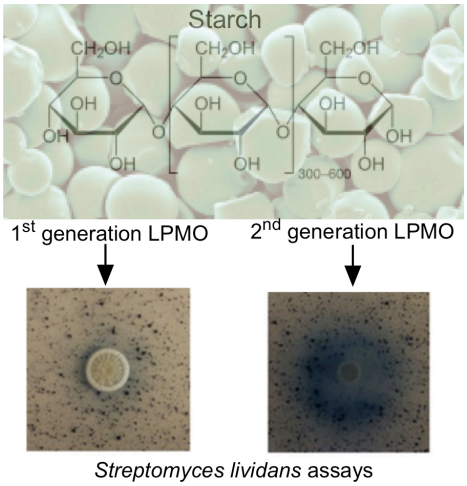
Creating new starch-active copper LPMOs through the generation of loop libraries

"We are pleased to be part of this research project as we believe that LPMOs will have significant role in starch hydrolysis and starch modification. So far the development of the LPMOs has progressed very swiftly and we are excited to see the next steps in this project." Johannes de Bie, WeissBioTech

Jonathan Worrall, University of Essex; Johannes de Bie, WeissBioTech

OUTCOMES: To create second-generation starch-active lytic polysaccharide monooxygenases (LPMOs), loops that form the active surface surrounding the catalytic copper ion were targeted. In total, there are five active surface loops that may be considered important for interaction and specificity with starch. Active surface loop libraries have been designed *in silico* with saturating mutations in selected amino acid positions in each loop based on sequence variability within the starch-active LPMO family. This business interaction voucher allowed for the synthesis — by combining site evaluation library and combinatorial library technology — of two out of the five loop libraries in a starch-active LPMO. These two loop libraries will be screened in *Streptomyces lividans* for enhanced activity relative to the wild-type LPMO. Following this, we will conduct *in vitro* characterisation of recombinantly produced proteins and then test the activity of the selected variants under industrial conditions.



Improving the degradation efficiency of insoluble starch granules through the creation of 2nd generation LPMOs.

INITIAL AIMS: The efficient deconstruction of plant biomass into biofuels and other chemicals is a key challenge to secure a low carbon economy. In nature, many microorganisms secrete enzymes that can break down recalcitrant biomass that is composed mostly of lignocellulose into soluble substrates. Harnessing the catalytic power of these enzymes to treat biomass outside of their natural habitats is challenging and a major goal of industrial biotechnology. Recently, a new class of enzyme that drastically increases the efficiency of biomass conversion has been identified. These enzymes contain a copper ion and are called lytic polysaccharide monooxygenases (LPMOs). The aim of this project is to assess whether second-generation LPMOs with enhanced substrate activities can be created. As a proof of principle, we will use a starch-degrading LPMO as a template to design and synthesize DNA libraries that will then be screened for substrate activity.

- Two loop libraries from a starch-active LPMO were synthesised
- Further project work between the academic and industrial partners is ongoing

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Funded by a Metals in Biology BBSRC NIBB Seeding Catalyst grant
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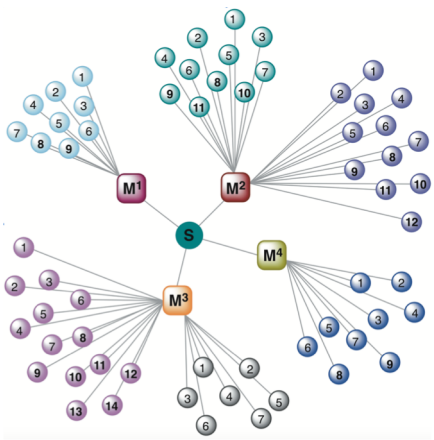
Adding value to biocatalytic hydroxylation products for synthesis and drug discovery

"We were pleased to see such encouraging results from the project, both in terms of the whole-cell bioconversions and the subsequent derivatisation of the metabolites." Jason King, Oxford Biotrans

Jeremy Robertson and Luet Wong, University of Oxford; Jason King, Oxford Biotrans



Engineered $P450_{BM3}$-mediated hydroxylation of fragment-sized unfunctionalised substrates (S) provides a series of metabolites (M) that may then be elaborated into a diversified set of new fragment molecules.

RESULTS: Following preliminary optimisation studies, engineered *E. coli* cells expressing cytochrome P450 mutants from *Bacillus megaterium* ($P450_{BM3}$) were used to produce two hydroxylated derivatives (termed **A** and **B**) of a nitrogen heterocyclic fragment molecule (*N*-Boc-8-azaspiro[4,5]decane). For **A**, 91% conversion of the starting material was achieved after 43 h; for **B**, 85% conversion was achieved after just 3 h and the reaction was complete within 20 h; the cells were still active and were re-used in an identical reaction. Both reactions scaled well to 340 mg of substrate. These proof-of-concept reactions provided synthetically useful outcomes, with reactions processing 1.70–3.4 g of substrate per litre of culture. The second phase of the project scoped a proposed method for elaborating the hydroxylated fragments into a variety of derivatives, based on tethered C–H insertion reactions. Two Rh(II)-catalysed variants were studied: (1) Du Bois' conditions for nitrenoid insertion; (2) Doyle–Lee carbenoid insertion. These reactions generated several derivatives that included three C–H amination products, currently assigned as two stereoisomers of the five-membered cyclic sulfamate (β-insertion) and the product of γ-insertion, a six-membered bridged cyclic sulfamate. This work provided insight into the likely outcomes of C–H insertion reactions in conformationally-biased molecules.

INITIAL AIMS: We intend to use iron-containing enzymes to produce compounds of value to the pharmaceutical industry. Drug discovery campaigns based on fragment-based screening combine the features of a number of 'fragment' compounds that are weakly-active at the desired target to identify promising 'lead' compounds. The highest chance of identifying leads that develop into successful drugs arises when the initial fragment collection is structurally diverse. Therefore, we aim to diversify compound collections in a two-stage process that mimics the biosynthesis of known medicines such as the anti-cancer drug paclitaxel (Taxol). Stage one employs engineered *E. coli* cells to enzymatically introduce a 'handle' (a hydroxyl group) onto a nitrogen heterocyclic fragment. In stage two, the chemical properties of this handle will be exploited to introduce features that promote favourable interactions with drug targets.

- Collaboration to continue to identify parameters that lead to shorter reaction times and higher conversions and substrate concentrations, and to improve product isolation
- Literature survey undertaken to identify alternatives to the Rh(II)-catalysed reactions
- Manuscript published: Syntrivanis et al. (2018) EUR J ORG CHEM.: https://onlinelibrary.wiley.com/doi/full/10.1002/ejoc.201801206