LYSINE BIOSYNTHESIS IN BACTERIA – AN UNCHARTERED PATHWAY FOR NOVEL ANTIBIOTIC DESIGN

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Summary

Antibiotics act by killing or inhibiting the growth of disease causing microorganisms. Their mechanism of action often targets important microbial cellular processes, including protein and cell wall synthesis. However, the effectiveness of many frontline antimicrobials has become compromised through the emergence of antibiotic resistant strains. Accordingly, there is a pressing need to develop novel antibiotics that target previously unaffected biosynthetic pathways. This review describes lysine biosynthesis in bacteria, an unchartered pathway for novel antibiotic design.

1. Introduction

The discovery of penicillin in 1929 by Alexander Fleming and subsequent development by Howard Florey was one of the great achievements of the 20th century. The development of other classes of antibiotics during the 1950s to 1970s allowed for the treatment of many previously fatal diseases. Much recent scientific and media attention has focused on the emerging resistance of bacteria to even our most powerful 'antibiotics-of-last-resort', such as vancomycin. Although vancomycin had been used for about 40 years without bacterial resistance, the rapidly increasing reports of resistant strains, such as vancomycin-resistant *enterococci* (VR*E*) and vancomycin-resistant *Staphylococcus aureus* (VR*SA*) highlight the need for continual development of new antibacterial agents, ideally with a novel mode of action. Despite this dilemma, only a small number of new antibiotics, such as linezolid and daptomycin, have been approved in recent years, following a period of over two decades in which no new antibiotics were developed. Accordingly, there is an urgent need to develop novel antimicrobials and an equally urgent need to discover new antibiotic targets. One target of antibacterial agents that has yet to be exploited clinically is the biosynthesis of the amino acid lysine, and its immediate precursor *meso*-diaminopimelate (*meso*-DAP). Several publications over the past decade have covered our increasing understanding of the enzymes of the lysine biosynthetic pathway as antibiotic targets, and this review discusses progress since 2003. The lysine biosynthetic pathway in plants and bacteria yields the *de novo* synthesis of lysine for utilisation in protein synthesis. More importantly, lysine and *meso*-DAP are vital constituents of the bacterial peptidoglycan cell wall in Gram-positive and Gram-negative bacteria, respectively. Hence, inhibitors of the lysine biosynthetic pathway may provide a new class of antibacterial agents through inhibition of cell wall and/or protein synthesis. Additionally, mammals lack the ability to biosynthesise lysine and it is therefore one of the 9 essential amino acids that must be provided through a dietary source. The occurrence of the lysine biosynthetic pathway in microorganisms and plants, but not in mammals, suggests that specific inhibitors of this biosynthetic pathway may display novel antibacterial activity with low mammalian toxicity.

2. Lysine Biosynthesis Pathway in Bacteria.

The biosynthetic pathway leading to *meso*-DAP and lysine production in bacteria is known as the DAP-pathway (Figure 1). The initial steps toward the DAP-pathway involve the conversion of aspartate **1** to aspartyl phosphate **2**, with subsequent reduction to aspartate semialdehyde (ASA) **3**. These steps are common to lysine, threonine,

isoleucine and methionine biosynthesis. The first committed step towards lysine biosynthesis is the conversion of ASA and pyruvate to dihydrodipicolinate (DHDP) **5**, catalysed by DHDP synthase (DHDPS). DHDP is then reduced to tetrahydrodipicolinate (THDP) **6** by DHDP reductase (DHDPR). The majority of bacterial species convert THDP to *N*-succinyl-2-amino-6-ketopimelate **7a**, a process catalysed by an *N*-succinyltransferase (succinylase pathway). Some *Bacillus* species, including *B. subtilis*, incorporate an *N*-acetyl group rather than an *N*-succinyl group (acetylase pathway). Yet another small group of species (again including some *Bacillus* species) possess the enzyme *meso*-DAP dehydrogenase, which catalyses the direct conversion of THDP **6** to *meso*-DAP **10**, thereby shortcutting the predominant route by several steps (dehydrogenase pathway). Some bacterial species can use multiple pathways to synthesise lysine. For example, *Corynebacterium glutamicium* can synthesise lysine by either the dehydrogenase or succinylase pathway, whilst *Bacillus macerans* can employ enzymes of the dehydrogenase or acetylase pathways. However, most species, including *Escherichia coli*, only utilise the succinyl pathway: *N*-succinyl-2-amino-6-ketopimelate **7a** is converted to *N*-succinyl-L,L-DAP **8a** by an aminotransferase, with subsequent deacylation and epimerisation then providing *meso*-DAP **10**, which is converted to lysine **11** by DAP decarboxylase. Interestingly, while plants had been assumed to follow the same biosynthetic pathway as the majority of bacteria, only recently has it been discovered that both plants and bacteria of the *Chlamydiales* order lack the genes encoding for the *dap*D, *dap*C and *dap*E enzymes, which catalyze reactions central in the pathway. In fact, plants and *Chlamydia* possess the enzyme diaminopimelate aminotransferase that directly converts THDP **6** to diaminopimelate **9**, and hence synthesise lysine via a variant of the DAP pathway.

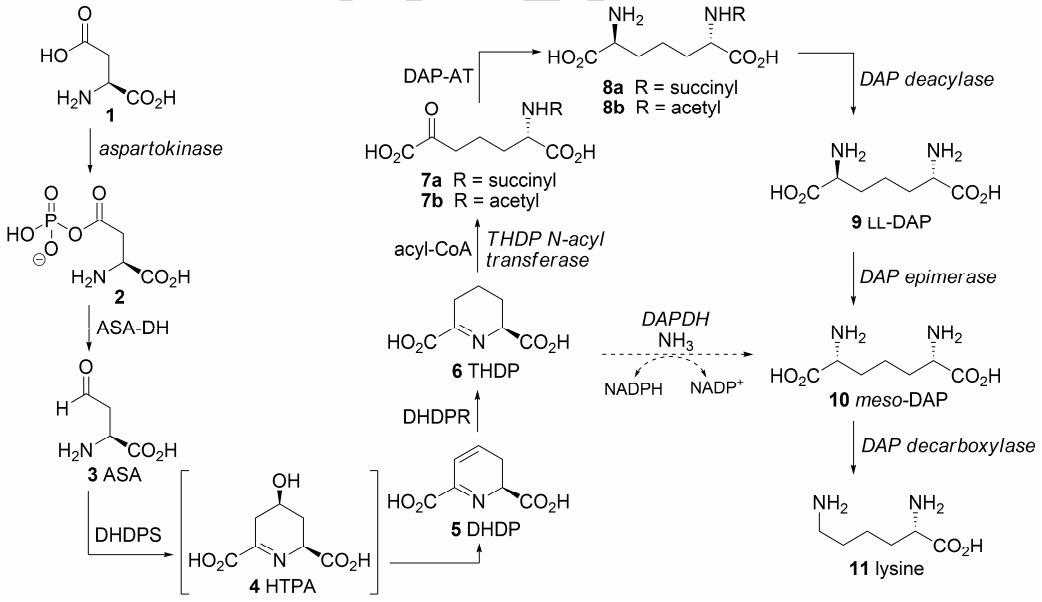

Figure 1 - Diaminopimelate pathway in bacteria.

3. Identification and validation of targets

While enzymes of the lysine biosynthetic pathway have been extensively studied and

reviewed, only recently have rapid advances in genomics allowed for validation of this pathway as a viable target for antibiotics. The complete sequencing of numerous bacterial genomes has allowed the identification and validation of specific antibiotic targets in these species. Most essential genes are present throughout a wide range of bacteria, and analyses of essential genes from various strains show remarkable similarity. In the *Salmonella* genome 219 out of ~2200 genes have been shown to be essential, and in the *Bacillus subtilis* genome 271 of ~4100 genes have been shown or predicted to be essential. In the *E. coli* and *S. aureus* genomes, 620 from 4291 genes and 658 from 2588 genes are predicted to be essential, respectively. Most enzymes of the lysine biosynthetic pathway are amongst those shown to be essential or necessary for growth under normal conditions. The *asd* gene, encoding for aspartate semialdehyde dehydrogenase (ASA-DH) has been shown to be essential in all strains analyzed. The *dap*A, *dap*B, *dap*D, and *dap*E genes have been shown indirectly to be essential, or predicted to be so, in all strains. These genes code for DHDPS, DHDPR, THDP *N*-succinyl transferase, and LL-DAP desuccinylase, respectively. Additionally, the *dap*F gene, encoding for DAP-epimerase, is essential or predicted to be essential in most bacteria.

The *lys*A gene, encoding for DAP decarboxylase, is also essential under certain conditions, with auxotrophs exhibiting attenuated growth, but is not absolutely essential as most growth media contain sufficient lysine for growth. Genes in the DAP pathway shown to be non-essential include *dap*C, encoding for N-succinyl-L,L-diaminopimelate aminotransferase, and *dap*G/*lys*C, encoding for aspartokinase. The nonessential nature of the *dap*C gene is consistent with the recent finding that N-acetylornithine aminotransferase (*Arg*D, NAcO-AT) possesses significant DAP-aminotransferase activity, such that *dap*C auxotrophs are still viable. Additionally, many bacterial strains produce multiple isozymes capable of catalyzing the production of aspartyl phosphate. Finally, the *asd*, *dap*A and *dap*B genes have been found to be essential and preserved in over 80% of a diverse range of bacterial genomes.

Recent investigations of enzymes of the lysine biosynthetic pathway and progress towards the development of inhibitors of these enzymes will now be discussed further, focusing on three well characterized enzymes that have been validated as antibiotics targets, namely dihydrodipicolinate synthase (DHDPS), dihydrodipicolinate reductase (DHDPR), and diaminopimelate (DAP) epimerase.

4. Dihydrodipicolinate Synthase (DHDPS)

4.1 Function of DHDPS

DHDPS was first purified in 1965 from *E. coli* extracts. The enzyme catalyzes the condensation of pyruvate and aspartate semialdehyde (ASA) to form HTPA. It was first suggested that the product released by DHDPS was DHDP, but studies using ^{13}C-labelled pyruvate support the view that the product is the unstable heterocycle HTPA. Rapid decomposition of the ^{13}C-NMR signals of HTPA following its formation indicate that formation of DHDP occurs *via* a nonenzymatic step.

In all cases examined the DHDPS-catalyzed reaction proceeds *via* a ping-pong kinetic

mechanism in which pyruvate binds the active site, followed by the release of a protonated water molecule. ASA then binds to the active site and the product, HTPA, is released.

In the first step of the mechanism, the active site lysine, (Lys161 in *E. coli* DHDPS) forms a Schiff base with pyruvate (Figure 2). Formation of the Schiff base proceeds through a tetrahedral intermediate. It is proposed that a catalytic triad of 3 residues - Tyr133, Thr44 and Tyr107 - act as a proton relay to transfer protons to and from the active site via a water-filled channel leading to bulk solvent. The Schiff base (imine) is converted to its enamine form, which then adds to the aldehyde group of ASA. In aqueous solution, ASA is known to exist in the hydrated form rather than the aldehyde, but the biologically relevant form of the substrate remains to be determined. HTPA is then formed by nucleophilic attack of the amino group of ASA onto the intermediate imine, leading to cyclisation and detachment of the product from the enzyme, with release of the active site lysine residue.

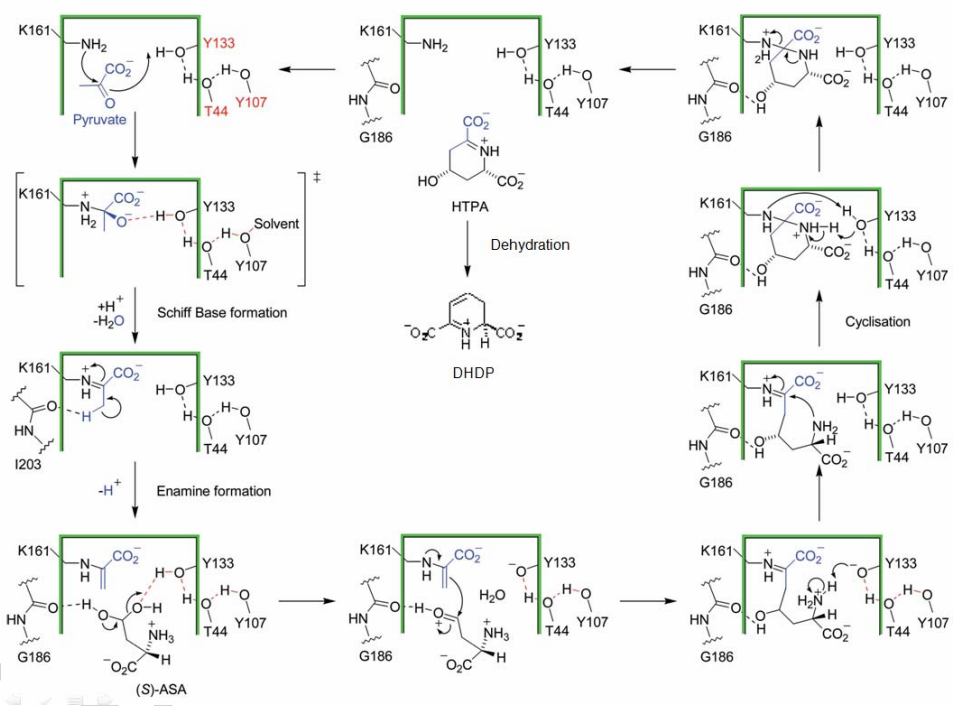

Figure 2 – The catalytic mechanism of DHDPS.

4.1.1 Regulation of DHDPS activity

The activity of DHDPS is regulated by lysine in some organisms. Regulation involves inhibition of the enzyme and has been investigated in several plant, Gram-negative and Gram-positive bacterial species. Studies involving *Triticum aestivium*, *Daucus carota sativa*, *Spinacia aloeracea*, *Zea mays* and *Pivus sativum* show that DHDPS from plant species are generally strongly inhibited by lysine (IC_{50} = 0.01-0.05 mM). In contrast, DHDPS from bacteria are significantly less sensitive to lysine inhibition than their plant counterparts. DHDPS from Gram-negative bacteria such as *E .coli*, *Bacillus sphaericus*

and *Methanobacterium thermoautotrophicum* display IC_{50} values that range from 0.25 mM to 1.0 mM. The enzyme from Gram-positive bacteria such as *Bacillus Lichenformis, Bacillus megaterium, Bacillus subtilis, Corynebacterium glutamicum, Bacillus cereus, Lactobacillus plantarum* and *Staphylococcus aureus* show little or no inhibition by lysine.

The crystal structure of DHDPS in complex with lysine from *E. coli* shows that the lysine binding site is situated in a crevice at the interface of the tight dimer, distal from the active site, but connected to the active site by a water channel. Two inhibitory lysine molecules are bound in close proximity in van der Waals contact to each other. Seven residues located within the allosteric site bind lysine, namely Ala49, His53, His56, Gly78, Asp80, Glu84, and Tyr106.

Studies show that lysine inhibition is cooperative; the second lysine molecule binds 10^5 times more tightly than the first. The mechanism by which lysine exerts regulatory control over bacterial DHDPS is not well understood, although kinetic and structural studies suggest that it is an allosteric inhibitor, causing partial inhibition (approximately 90%) at saturating concentrations. It has recently been suggested that lysine exerts some effect on the first half reaction, affecting the proton-relay as well as the function of Arg138, thought to be crucial for ASA binding. The crystal structure of the *E. coli* DHDPS-lysine complex was solved in the absence of substrate; however, thermodynamic studies have illustrated that the substrate pyruvate has a substantial effect on the nature of enzyme-inhibitor association.

The mechanism of lysine inhibition has been investigated in DHDPS from wheat and tobacco. Kinetic studies show that lysine is a non-competitive inhibitor relative to pyruvate and a competitive inhibitor relative ASA. A structural study of DHDPS from the plant species *Nicotiana sylvestris* shows that a significant change in conformation occurs upon binding of lysine. Several residues involved in mediating inter-subunit contact at the tight dimer interface are displaced when lysine is bound, thereby dislocating the dimers in relation to each other. This translocation displaces the active site residues Arg138 and Tyr133, which suggests inhibition may occur through hindering coordination of the carboxyl group of ASA, thus preventing cyclisation of the substrate. An equivalent conformational state is not observed in the lysine-bound structure of DHDPS from *E. coli*. The role of ASA in the regulation of *E. coli* DHDPS is not clearly defined. This is due to a number of conflicting studies reporting ASA either does or does not mediate inhibition. Early reports of substrate inhibition in *E. coli* DHDPS can be attributed to the quality of ASA preparations used in kinetic studies. However, inhibition of DHDPS activity from *Thermoanaerobacter tengcongensis, Neisseria meningitidis, Bacillus anthracis* and methicillin-resistant *Staphylococcus aureus* (MR*SA*) has been observed with pure preparations of ASA.

4.2 Structure of DHDPS

4.2.1 Subunit and quaternary structure of DHDPS

DHDPS from *E. coli, Mycobacterium tuberculosis, Thermotoga maritima, Thermoanaerobacter tengcongensis* and *Bacillus anthracis* and many other species is a

homotetramer in both crystal structure and solution (Figure 3). In *E. coli*, the monomer is 292 amino acids in length and is composed of two domains. The N-terminal domain is a $(\beta/\alpha)_8$ TIM-barrel (*E. coli* DHDPS: residues 1-224) with the active site located within the centre of the barrel (Figure 3). The C-terminal domain (residues 225-292) consists of three α-helices and contains several key residues that mediate contact at the weak dimer interface.

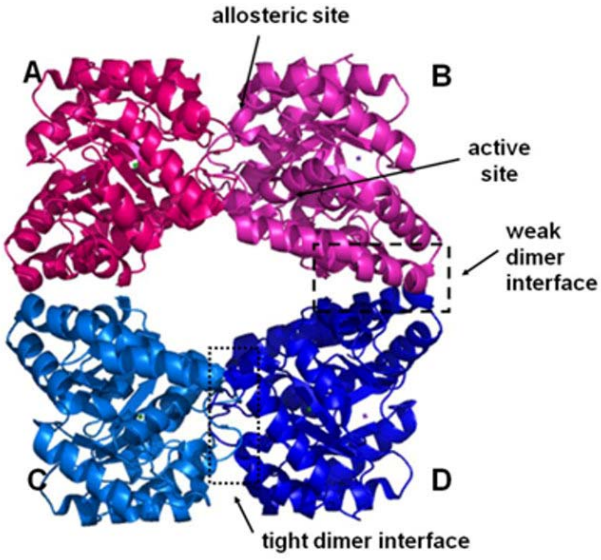

Figure 3 - *E. coli* DHDPS structure. The active sites, allosteric sites, dimerization interface (tight dimer interface) and tetramerization interface (weak dimer interface) are shown.

The association of the four monomers leaves a large water-filled cavity in the centre of the tetramer, such that each monomer has contacts with two neighbouring monomers only. The tetramer is described as a dimer of dimers (monomers A and B and monomers C and D, Figure 3) with strong interactions between the monomers A and B at the so-called tight dimer interface and weaker interactions between the dimers at the weak dimer interface (Figure 3).

4.2.2 Active site

The active site is located in cavities formed by the two monomers of the dimer. A long solvent-accessible catalytic crevice with a depth of 10 Å is formed between β-strands 4 and 5 of the barrel. Lys161, involved in Schiff-base formation is situated in the β-barrel near the catalytic triad of three residues, namely Tyr133, Thr44 and Tyr107, which act as a proton shuttle transferring protons to and from the active site (Figure 4).
Thr44 is hydrogen bonded to both Tyr133 and Tyr107 and its position in the hydrogen-bonding network may play a role in Schiff base formation and cyclisation. The dihedral angles of Tyr107 fall in the disallowed region of the Ramachandran plot, suggesting an important role in the enzyme's function. It is believed to be involved in shuttling protons between the active site and solvent. In contrast, Tyr133 plays an important role in substrate binding, donating a proton to the Schiff base hydroxyl. It is also thought to

coordinate the attacking amino group of ASA, which requires the loss of a proton subsequent to cyclisation. A marked reduction in activity is observed in single substitution mutants, highlighting the importance of this catalytic triad.

Situated at the entrance to the active site, Arg138 is essential for ASA binding. In the *E. coli* DHDPS structure, a hydrogen bond is formed between Arg138 and Tyr107 and a water mediated hydrogen bond is formed between Arg138 and Tyr133. Arg138 is thus also important for stabilization of the catalytic triad, both of which are highly conserved in all DHDPS enzymes.

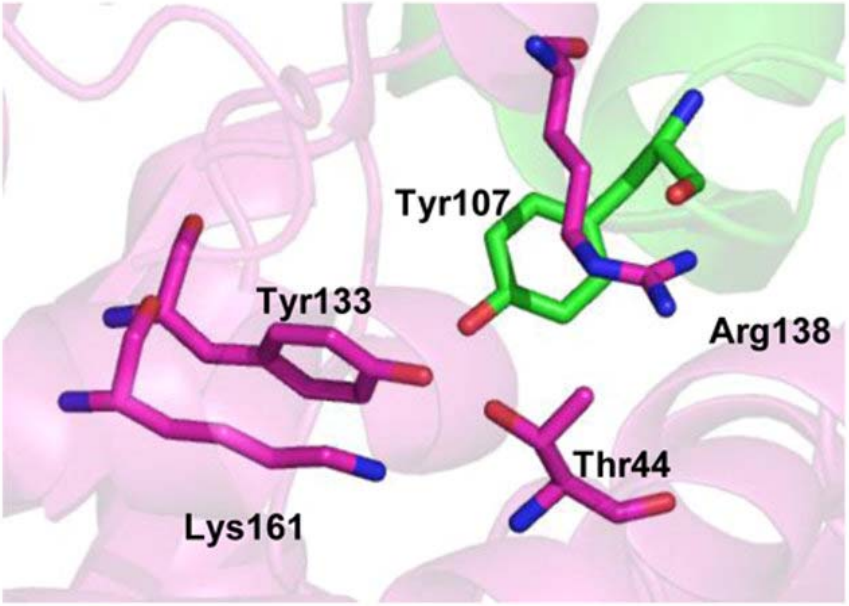

Figure 4 – *E. coli* DHDPS active site illustrating the catalytic triad Thr44, Tyr133 and Tyr107 interdigitating from the opposing monomer

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Biographical Sketches

Dr Con Dogovski received his Ph.D. after completing his graduate studies in the Department of Microbiology and Immunology, The University of Melbourne, Australia. During this period, his primary research interests centered on studying the structure and function of bacterial integral membrane proteins. Since then, he has worked within the Australian Department of Defence and private industry. Con is currently located at the Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne. His research covers a broad range of areas including, microbiology, molecular biology, protein chemistry, and biochemistry. More specifically, his interests involve studying enzymes involved in the synthesis of *meso* DAP and lysine and the development novel antimicrobials targeting several important human pathogens.

Sarah Atkinson is a 2nd year Ph.D. student at the University of Melbourne, with interests in molecular biology, enzymology and structural biology. Her Ph.D. project focuses on understanding the structure-

function and inhibition of bacterial and plant DHDPS which is described in detail in this review. She also plans to adopt an *in silico* drug design approach to her project with the potential of yielding lead antimicrobial inhibitors targeting DHDPS. Sarah completed an undergraduate Bachelor of Science degree at the University of Melbourne 2004-2006, before embarking on a successful biochemistry Honours (4th year undergraduate) project in 2007. Her work focused on the DHDPS from the pathogen *Clostridium botulinum*. Upon completing of her graduate studies, Sarah aspires to stay in biochemical research, expanding her knowledge of structural biology.

Sudhir Dommaraju is a 3rd year Ph.D. student at the University of Melbourne, with interests in bioinformatics, protein chemistry and microbiology. His Ph.D. project focuses on structural and mechanistic characterization of bacterial DHDPR, a potential antimicrobial target.

Sudhir holds a Masters degree in medical microbiology from Manipal Academy of Higher Education, India and a post graduate Diploma in Bioinformatics. Prior to the commencement of his Ph.D project in 2006, he worked as a scientist at the Institute of Bioinformatics, India and was involved in the bioinformatic analysis and annotation of various human proteins, as part of a HUPO initiative project. Upon completion of his Ph.D., Sudhir aspires to work on drug discovery projects that utilise his skill base in protein chemistry and bioinformatics.

Lilian Hor completed her undergraduate Bachelor of Science (Honours) and Bachelor of Information Systems degrees at the University of Melbourne. She is currently a 2nd year Ph.D. student at the University of Melbourne with an interest in medicinal chemistry, enzymology and structural biology. Lilian's Ph.D. project focuses on understanding the structure-function relationships of bacterial DAP epimerase, which is described in detail in this review. She also plans to adopt a rational drug design approach to her project with the potential of yielding lead antimicrobial inhibitors targeting DAP epimerase.

Dr Craig Hutton undertook his undergraduate and Ph.D. degrees at the University of Adelaide (Prof. Christopher J. Easton, 1994) before completing postdoctoral studies at the University of California, Berkeley (Prof. Paul A. Bartlett) and The University of Melbourne (Australian Postdoctoral Fellow). He was appointed to his first independent academic position in 1999 in the School of Chemistry at The University of Sydney, before returning to The University of Melbourne in 2003 as a Senior Lecturer. In 2005 his laboratory relocated to the newly-opened Bio21 Molecular Science and Biotechnology Institute (Bio21 Institute), where he heads a group comprised of approximately a dozen research staff, graduate and undergraduate students. His research interests include the design and synthesis of inhibitors of enzymes in the lysine biosynthetic pathway, novel methods for the asymmetric synthesis of amino acids, and the construction of cross-linked amyloid peptides associated with Alzheimer's disease.

Dr Hutton also serves as an Associate Editor for the Australian Journal of Chemistry, and on a number of local committees including as secretary of the *Royal Australian Chemical Institute (RACI) Victorian branch (organic group)* and the *RACI/University of Melbourne Annual Synthesis Symposium* organizing committee.

Professor Juliet Gerrard trained at Oxford University, where she completed an Honours degree in Chemistry and a DPhil in Biological Chemistry. In 1993, she was appointed as a research scientist at Crop & Food Research Ltd, NZ, where her multidisciplinary research portfolio included a substantial element of applied research in the food science area. She was appointed as a Lecturer in Biochemistry at the University of Canterbury in 1998, promoted to Senior Lecturer in 2000, Assoc. Professor in 2004, and Professor in 2006. Juliet's research is interdisciplinary, cutting across biochemistry, chemistry, health, agricultural and food science and biomaterial design. It also incorporates a full spectrum of applied and fundamental research. At present, a major focus is the understanding of the quaternary structure of proteins and the implications that this has for evolution of oligomeric proteins. This research has potential application in the design of novel therapeutic agents and also in the assembly of novel materials. Juliet has published over 90 papers in international refereed journals, several book chapters and three books, two of which have been translated into other languages. She won a National Teaching Award for Sustained Excellence in Tertiary Teaching in 2004 and is currently on the Editorial Board of Process Biochemistry and an Academic Editor for PLoSONE. As the Principal Investigator on several major research grants Juliet leads a productive research laboratory currently comprising 15 members; the team is

supplemented by national and international collaborators.

Dr Renwick Dobson is currently a C.R. Roper Senior Research Fellow in the Department of Biochemistry and Molecular Biology at the University of Melbourne. He received in Ph.D. at the University of Canterbury in 2004. His research is interdisciplinary, cutting across protein structural biology, chemistry, enzymology and biochemistry. His current field of interest is the relationship between protein structure and function, with a focus on the enzymology of lysine biosynthesis in bacteria. In particular, he is interested in understanding the mechanisms that underpin function of the enzyme DHDPS. This work is significant, since the enzyme is a potential drug target. Dr Dobson's goal is the rational design of inhibitors of DHDPS, which is possible *via* a thorough knowledge of enzyme's structure and function.

An active member of the scientific community, Dr Dobson is on the organising committee of the biannual Biomolecular Dynamics and Interactions 2009 (BDI2009) Symposium (Bio21, UOM). He is a current member of the Royal Society of New Zealand, New Zealand Institute of Chemistry (NZIC), New Zealand and Australian Societies of Biochemistry and Molecular Biology (NZSBMB & ASBMB), and the Society of Crystallographers in Australia and New Zealand (SCANZ). Dr Dobson regularly referees manuscripts for international journals, including *Acta Crystallogr. D*, *Acta Crystallogr. F*, *J. Agric. Food Chem.*, *Biochem, J.*, *Biochemistry* and *ChemBioChem.*

Dr Matt Perugini completed both his undergraduate *BSc(Hons)* degree in the 1990s and his Ph.D. at the University of Melbourne in the Department of Biochemistry and Molecular Biology in 2002.

In October 2004, he was awarded the Young Biophysicists Award (YBA) by the Australian Society for Biophysics, largely for his contribution to the field of analytical ultracentrifugation; and then in 2005, Dr Perugini was awarded an Australian Postdoctoral Fellowship (APD) by the Australian Research Council. In the same year, he moved to the new Bio21 Molecular Science and Biotechnology Institute (Bio21 Institute) in Parkville, where he heads a laboratory comprised of more than a dozen research staff, graduate students, and undergraduate (Honours) students. His research focuses on novel antibiotic design targeting key enzymes of the lysine biosynthesis pathway of pathogenic bacteria and the more esoteric aim of unravelling the molecular evolution in quaternary structure of these enzyme targets, including DHDPS. As a result of his recent research activities, Dr Perugini was awarded the prestigious 2007 Applied Biosystems Edman Award by the Australian Society for Biochemistry and Molecular Biology.

Dr Perugini also serves on a number of local committees, including the *Lorne Conference on Protein Structure and Function* and the *Australasian Proteomics Society* organization committees. In addition, he has forged strong collaborations with local and international scientists focusing on the characterization of protein structure and function, and the quantitation of protein-protein and protein-ligand interactions. These collaborations are yielding exciting results that have provided insight into the structure and function of several protein systems, including bacterial DHDPS, DHDPR and DAP epimerase, which are described in detail in this EOLSS publication.