Cloning, expression and characterisation of the starter module from indanomycin biosynthesis

Sasha Rebecca Derrington

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Abstract

Nonribosomal peptides and polyketides form important classes of pharmaceutical agents. Several architectures of biosynthetic machinery exist for the construction of these structurally diverse molecules. Many attempts to engineer these proteins have concentrated on the multimodular type I polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS). Here, work was carried out to express the NRPS module responsible for starter unit formation from indanomycin biosynthesis in *E. coli*. Three synthetic genes, for IdmI, IdmJ and IdmK, were cloned and subsequently used in expression trials. Despite multiple attempts, IdmI was always expressed as an insoluble protein. IdmJ was expressed as a soluble protein, and an unexpected post-translational modification was found and investigated. Mutagenesis studies suggested that the unknown post-translational modification was occurring at a cysteine 127. IdmK, the carrier protein, was expressed in a soluble form with good yield. Analysis by mass spectrometry showed that, surprisingly, *E. coli* was able to phosphopantetheinylate IdmK, which is required for a functional module. Preliminary structure determination was carried out by X-ray crystallography. A complete 3D structure was obtained using NMR spectroscopy of the [\(^{15}\)N] and [\(^{13}\)C, \(^{15}\)N] labelled protein. Structure determination was performed using CS-ROSETTA, which only uses chemical shift data, and ARIA, which assigns ambiguous NOE distance restraints. Both structure calculations produced comparable structures for IdmK. The structure showed a 3 \(\alpha\)-helix bundle, with the same topology, lengths and locations of helices as other carrier proteins. Initial investigations into holo-IdmK suggest that the phosphopantetheine co-factor is directed into the hydrophobic core of the protein. This research has set up the system for future studies to engineer the pathway for novel product biosynthesis.
## Contents

**Acknowledgments** ........................................................................................................... i

**Abstract** ................................................................................................................................. v

**Table of contents** ...................................................................................................................... vii

**List of figures** ............................................................................................................................ xiii

**Abbreviations** .......................................................................................................................... xvii

1. **Introduction** ......................................................................................................................... 1
   1.1 The Golden Age of Antibiotics .......................................................................................... 1
   1.2 Natural products as antibiotics .......................................................................................... 3
      1.2.1 Polyketides .................................................................................................................. 4
      1.2.2 Nonribosomal peptides ............................................................................................... 6
      1.2.3 Polyketide-nonribosomal peptide hybrid products ....................................................... 8
   1.3 Polyketide synthases ............................................................................................................ 9
      1.3.1 PKS biosynthetic gene clusters ...................................................................................... 10
      1.3.2 PKS module organisation ............................................................................................. 11
         1.3.2.1 A cyclotransferase (AT) domain .............................................................................. 12
         1.3.2.2 Ketosynthase (KS) domain .................................................................................... 13
         1.3.2.3 A cetyl carrier protein ............................................................................................ 13
         1.3.2.4 Chain initiation and termination ............................................................................. 14
         1.3.2.5 Linker regions ........................................................................................................... 15
         1.3.2.6 Accessory domains: KR, DH and ER ..................................................................... 15
         1.3.2.7 Biosynthetic pathways of polyketide synthases and fatty acid synthases .......... 15
         1.3.2.8 A model system ...................................................................................................... 17
      1.3.3 Structure determination of PKSs ................................................................................... 19
      1.3.4 Engineering of PKSs ..................................................................................................... 24
   1.4 Nonribosomal peptide synthetases ..................................................................................... 28
      1.4.1 NRPS biosynthetic gene clusters ................................................................................... 28
      1.4.2 Biosynthesis of nonribosomal peptides and module organisation ......................... 29
         1.4.2.1 Adenylation domain ................................................................................................. 30
         1.4.2.2 Condensation domain .............................................................................................. 32
         1.4.2.3 Peptidyl carrier protein ........................................................................................... 32
         1.4.2.4 Chain termination and macrocyclisation ................................................................. 33
         1.4.2.5 Epimerase domain .................................................................................................. 33
2.2.2.6 DNA quantification.................................................................53
2.2.2.7 DNA digests ........................................................................53
2.2.2.8 Ligation reactions ...............................................................54
2.2.2.9 Ligation independent cloning ...............................................54
2.2.2.10 Site directed mutagenesis ...................................................54
2.2.2.11 Transformation into E. coli cells .........................................55
2.2.2.12 DNA sequencing .................................................................55

2.2.3 Protein methods ......................................................................55
2.2.3.1 Small scale protein expression trials .....................................55
2.2.3.2 Sample preparation for analysis of protein expression ..........55
2.2.3.3 Detection of the N-terminal His\textsubscript{6}-tag ......................56
2.2.3.4 Nickel affinity purification of His\textsubscript{6}-tagged proteins ........57
2.2.3.5 Dialysis ..............................................................................58
2.2.3.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis 58
2.2.3.7 Determination of protein concentration ................................60
2.2.3.8 Increasing protein concentration .........................................61
2.2.3.9 Sample preparation for mass spectrometry .........................61
2.2.3.10 In-gel tryptic digestion and analysis by LC-MS/MS ............61
2.2.3.11 Cysteine alkylation ............................................................63
2.2.3.12 Mass spectrometry .............................................................63
2.2.3.13 Protein purification by size exclusion chromatography .......64

2.2.4 Crystallographic methods .......................................................64
2.2.4.1 Sample preparation ..............................................................64
2.2.4.2 Screening conditions and crystal trays ................................65
2.2.4.3 Data acquisition and processing ............................................65

2.2.5 Nuclear magnetic resonance (NMR) spectroscopy methods ....66
2.2.5.1 Sample preparation ..............................................................66
2.2.5.2 Data acquisition .................................................................66
2.2.5.3 Data processing and analysis ...............................................66
2.2.5.4 Structure generation ...........................................................69
2.2.5.5 RDC alignment media and measurements .........................70
2.2.5.6 Initial structural investigations of holo-IdmK ......................71
3 Cloning and recombinant protein expression of nonribosomal peptide synthetase proteins required for starter unit biosynthesis of the polyether ionophore indanomycin

3.1 Design of synthetic genes for recombinant protein expression in E. coli ...

3.1.1 Initial cloning strategy for NRPS starter module domains ...

3.1.2 Amplification of vector and target gene plasmids ...

3.2 The adenylyltransferase (IdmJ) ...

3.2.1 Cloning the adenylyltransferase (idmJ) into pET23a ...

3.2.2 Cloning the adenylyltransferase (idmJ) into pKK223-3 ...

3.2.3 Cloning the adenylyltransferase (idmJ) into pETDUET ...

3.2.4 Recombinant protein expression of the adenylyltransferase (IdmJ) ...

3.2.5 Purification of IdmJ and analysis ...

3.2.6 Characterisation of IdmJ† ...

3.2.7 Summary and conclusions ...

3.3 The prolyl dehydrogenase (IdmI) ...

3.3.1 Cloning the prolyl dehydrogenase (idmI) into pET23a ...

3.3.2 Cloning the prolyl dehydrogenase (idmI) into pKK223-3 ...

3.3.3 Recombinant protein expression of the prolyl dehydrogenase (IdmI) ...

3.3.4 Cloning the prolyl dehydrogenase (idmI) into pMAL-c5X ...

3.3.5 Expression of the MBP/prolyl dehydrogenase fusion protein ...

3.3.6 Cloning genes responsible for starter unit biosynthesis into pETDUET ...

3.3.7 Recombinant tandem expression of IdmJ, IdmI and IdmK ...

3.3.8 Summary and conclusions ...

3.4 Discussion ...

4 Cloning, expression and characterisation of the peptidyl carrier protein from the starter module in indanomycin biosynthesis ...

4.1 Cloning the peptidyl carrier protein (idmK) ...

4.1.1 Cloning the peptidyl carrier protein (idmK) into pET23a ...

4.1.2 Cloning the peptidyl carrier protein (idmK) into pKK223-3 ...

4.2 Expression of the prolyl carrier protein (IdmK) ...

4.3 Purification of the prolyl carrier protein (IdmK) ...

4.3.1 Confirmation of the site of phosphopantetheinylation ...

4.4 Structural characterisation of the prolyl carrier protein (IdmK) ...
List of figures

Figure 1.1- A timeline of the discovery of antibiotics that are still in use today. ........................................... 2
Figure 1.2- Examples of the secondary metabolites, polyketides ................................................................. 5
Figure 1.3- Examples of nonribosomal peptides .............................................................................................. 7
Figure 1.4- Examples of hybrid polyketide-nonribosomal peptides .............................................................. 9
Figure 1.5- Minimal PKS module illustrating the mechanism by which chain elongation occurs .............. 12
Figure 1.6- Mechanism by the ACP becomes phosphopantetheinylated ....................................................... 14
Figure 1.7- The biosynthetic pathways of FAS and PKS ............................................................................... 16
Figure 1.8- Biosynthetic gene cluster of the 6-deoxyerythronalide producing PKS, DEBS .................. 18
Figure 1.9- The structure of the KS-AT didomain of module 5 from DEBS ........................................... 20
Figure 1.10- Proposed structure of PKS and mFAS ....................................................................................... 22
Figure 1.11- Structure and chain elongation mechanism of a module of PikAIII ...................................... 23
Figure 1.12- Domain organisation within an NRPS minimal module ......................................................... 29
Figure 1.13- Amino acid activation and loading of substrates by adenylation domains in NRPS .......... 31
Figure 1.14- Epimerisation of the peptidyl chain mediated by an epimerase (E) domain .................... 34
Figure 1.15- Mechanism of heterocyclisation within NRPs ............................................................. 35
Figure 1.16- Biosynthetic gene cluster of the tyrocidine NRPS ............................................................... 19
Figure 1.17- Crystal structure of the PheA adenylation domain ................................................................. 39
Figure 1.18- Crystal structure of the condensation domain, VibH ........................................................ 40
Figure 1.19- Structure of Indanomycin .......................................................................................................... 44
Figure 1.20- NRPS initiation module in indanomycin biosynthesis ......................................................... 45
Figure 3.1- Indanomycin starter unit biosynthesis ......................................................................................... 75
Figure 3.2- Schematic of the design for synthetic genes ............................................................................ 76
Figure 3.3- “Cut and paste” cloning strategy for cloning idmI, idmJ and idmK into pET23a .................. 78
Figure 3.4- Agarose gel of a restriction digest of pUC7-gene constructs ................................................. 79
Figure 3.5- Agarose gel of the isolated plasmid DNA from the 10 colonies after digestion with
SacI-HF® and SalI-HF® restriction enzymes ............................................................................................ 81
Figure 3.6- Schematic for the method used to clone idmJ into pKK223-3 ............................................. 83
Figure 3.7- Agarose gel of restriction digest of the pKnanA construct using EcoRI-HF and
HindIII-HF .................................................................................................................................................. 84
Figure 3.8- Agarose gel of restriction digests of the 10 isolated plasmids from a ligation reaction
between pKK223-3 and idmJ ................................................................................................................. 84
Figure 3.9- Agarose gel showing restriction digests with SacI-HF® and SalI-HF® of plasmids
isolated from a ligation between pETDUET and idmJ ........................................................................... 86
Figure 3.10- Reducing SDS-PAGE gel showing initial overexpression of IdmJ ........................................ 87
Figure 3.11- pET(2)idmJ expression trials ................................................................................................ 89
Figure 3.12- N-terminal His6-tag detection in IdmJ expression trials ...................................................... 90
Figure 3.13- Reducing SDS-PAGE gel showing stages of the nickel affinity purification of IdmJ ....... 92
Figure 3.14- ESI-MS of eluted protein from the purification of IdmJ ....................................................... 93
Figure 3.15- Schematic of method for an in-gel tryptic digest followed by LC-MS/MS for protein identification.......................................................................................................................................................................................... 94
Figure 3.16- HPLC trace of purified IdmJ† digested with trypsin. ......................................................... 95
Figure 3.17- Summary of the peptide coverage of the IdmJ synthetic gene amino acid sequence from the tryptic digest ........................................................................................................................................................................................................ 96
Figure 3.18- ESI-MS analysis of alkylated IdmJ .......................................................................................... 97
Figure 3.19- ESI-MS analysis of IdmJ variants ........................................................................................... 100
Figure 3.20- Schematic of the expression of IdmJ/IdmJ† ........................................................................... 102
Figure 3.21- Agarose gel of plasmid DNA purified from colonies to screen for the pETidmI plasmid. ........................................................................................................................................................................................................ 104
Figure 3.22- Agarose gel of the PCR reaction to amplify the idmI gene .................................................. 106
Figure 3.23- Agarose gel of plasmid DNA purified ten colonies from the ligation between idmI and pKK223-3 after being digested with EcoRI-HF® and PsI-HF® ......................................................... 107
Figure 3.24- Reducing SDS-PAGE analysis of soluble protein produced in the expression conditions trialled for pKidmI .................................................................................................................. 108
Figure 3.25- N-terminal Hisc-tag detection in IdmI expression trials ..................................................... 109
Figure 3.26- Agarose gel of restriction digests with NdeI and EcoRI restriction enzymes of purified plasmid DNA from the pMAL-CSX vector and idmI ligation ............................................... 110
Figure 3.27- Reducing SDS-PAGE analysis of expression trials of the pMALidmI fusion protein ........................................................................................................................................................................... 111
Figure 3.28- Detection of the N-terminal Hisc-tag MBP-IdmI fusion protein ........................................ 112
Figure 3.29- Proposed strategy for cloning idmI, idmI and idmK into pETDUET. ................................... 114
Figure 3.30- Agarose gels displaying the results of steps taken to clone idmI into pETDUET ............. 115
Figure 3.31- Agarose gel of restriction digests with SacI-HF® and SalI-HF® of the pET(2)idmI idmI plasmid ........................................................................................................................................................................................................ 116
Figure 3.32- PCR amplification of pET(2)idmI idmI and idmK ................................................................ 118
Figure 3.33- Agarose gel of the pET(2)idmI idmK idmI plasmid digested with SacI-HF® and XhoI ........................................................................................................................................................................ 119
Figure 3.34- Reducing SDS-PAGE gel analysing soluble cell fractions from IdmI, IdmI and IdmK tandem expression ........................................................................................................................................... 120
Figure 3.35- Western blot probing for soluble protein expression ......................................................... 121
Figure 4.1- Agarose gel of isolated plasmid DNA from the ligation between pET23a and idmK ........................................................................................................................................................................... 127
Figure 4.2- Agarose gel showing restriction digests of purified plasmid DNA from the pKK223-3 idmK ligation ........................................................................................................................................................................................................ 129
Figure 4.3- Reducing SDS-PAGE analysis of expression of IdmK ................................................................ 130
Figure 4.4- Reducing SDS-PAGE of the purification of IdmK by nickel affinity chromatography .................................................................................................................................................................... 131
Figure 4.5- ESI-MS of the eluted protein from the purification of IdmK .................................................. 132
Figure 4.6- Purification of IdmK by size exclusion chromatography ....................................................... 133
Figure 4.7- ESI-MS analysis of the reduced IdmK dimer .............................................................. 134
Figure 4.8- ESI-MS analysis of the purified S44A IdmK variant .................................................. 136
Figure 4.9- Apo-IdmK crystals grown when screening for a crystallisation condition ............ 139
Figure 4.10- Reducing SDS-PAGE analysis of the small scale expression trial of IdmK in minimal media .................................................................................................................. 140
Figure 4.11- Reducing SDS-PAGE gel showing the stages of nickel affinity chromatography of IdmK grown in minimal media containing $^{15}$NH$_4$Cl ........................................................................ 141
Figure 4.12- ESI-MS spectrum to analyse the extent of isotopic labelling of IdmK .............. 142
Figure 4.13- $^1$H-$^{15}$N HSQC spectrum of apo-IdmK ................................................................. 144
Figure 4.14- Reducing SDS-PAGE to analyse the effects of changing the concentration of glucose in minimal media on protein expression ........................................................................ 145
Figure 4.15- Reducing SDS-PAGE gel of [$^{13}$C, $^{15}$N] IdmK purification .................................. 146
Figure 4.16- ESI-MS spectrum to analyse the extent of $^{13}$C and $^{15}$N isotopic labelling of IdmK .......... 147
Figure 5.1- Precession of nuclear magnetic moment, $\mu$, with a spin $\frac{1}{2}$ or $-\frac{1}{2}$ .................... 153
Figure 5.2- Bulk magnetisation of nuclei in an applied magnetic field ($B_0$) at equilibrium ........ 154
Figure 5.3- Behaviour of nuclear spins upon excitation by a radiofrequency pulse of 90° on the y axis ........................................................................................................................................ 154
Figure 5.4- Timescales to illustrate the molecular dynamics that can be measured by NMR experiments ........................................................................................................................................ 156
Figure 5.5- Canonical fold of carrier proteins ................................................................................ 157
Figure 5.6- Schematic of the canonical fold of carrier proteins .................................................. 158
Figure 5.7- Scalar couplings observed in the protein backbone ................................................ 159
Figure 5.8- Strip plot of the last 10 residues of the backbone of IdmK ....................................... 164
Figure 5.9- Assigned $^1$H-$^{15}$N HSQC of apo-IdmK ................................................................. 165
Figure 5.10- Secondary structure predictions by +TALOS ....................................................... 167
Figure 5.11- Secondary structure alignments of carrier proteins by Dali ................................ 168
Figure 5.12- Chemical shift structure of IdmK using CS-ROSETTA ........................................ 170
Figure 5.13- Dipole-dipole interaction between two magnetic moments ............................... 171
Figure 5.14- Partial alignment of a protein in an anisotropic solution ....................................... 172
Figure 5.15- Measuring residual dipolar couplings in an isotropic and anisotropic solution .... 173
Figure 5.16- Raw RDC data of three residues from IdmK ........................................................ 174
Figure 5.17- Measured RDCs for IdmK ...................................................................................... 175
Figure 5.18- Three CS-ROSETTA models selected by RDCs .................................................. 177
Figure 5.19- Comparison of the best CS-ROSETTA model with an ACP ................................ 178
Figure 5.20- Overlay of ROSETTA structures generated with RDCs as an additional restraint .... 179
Figure 5.21- hCCH TOCSY assignment of Leu 85 ............................................................... 181
Figure 5.22- hCCH TOCSY assignment of Leu 85 ............................................................... 182
Figure 5.23- Structure ensemble of IdmK calculated by ARIA and validation ............................ 186
Figure 5.24- Comparison of the 3D structures of acyl carrier protein and IdmK .................... 187
Figure 5.25- Plot showing short range NOEs assigned by ARIA .............................................. 188
Figure 5.26- An overlay of $^1$H-$^{15}$N HSQC spectra of apo- and holo-IdmK ........................................ 189
Figure 5.27- Chemical shift perturbations of apo- and holo- IdmK...................................................... 190
Figure 5.28- Conformational states of the TycC3-PCP ......................................................................... 193
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>ACP</td>
<td>Acyl carrier protein</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>CS/δ</td>
<td>Chemical shift</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNAse</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray ionisation mass spectrometry</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>TB</td>
<td>Terrific broth</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear single quantum coherence</td>
</tr>
<tr>
<td>idmI</td>
<td>Prolyl dehydrogenase gene</td>
</tr>
<tr>
<td>IdmI</td>
<td>Prolyl dehydrogenase protein</td>
</tr>
<tr>
<td>idmJ</td>
<td>Adenylyltransferase gene</td>
</tr>
<tr>
<td>IdmJ</td>
<td>Adenylyltransferase protein</td>
</tr>
<tr>
<td>idmK</td>
<td>Prolyl carrier protein gene</td>
</tr>
<tr>
<td>IdmK</td>
<td>Prolyl carrier protein protein</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-galactosidase</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>MBP</td>
<td>Maltose binding protein</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOE</td>
<td>Nuclear Overhauser Effect</td>
</tr>
<tr>
<td>NOESY</td>
<td>Nuclear Overhauser Effect spectroscopy</td>
</tr>
<tr>
<td>NRPS</td>
<td>Nonribosomal peptide synthase</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PCP</td>
<td>Peptidyl carrier protein</td>
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</table>
PCR  Polymerase chain reaction
PDB  Protein data bank
PKS  Polyketide synthase
PPant  Phosphopantetheine
ppm  Parts per million
PPTase  Phosphopantetheinyl transferase
RDC  Residual dipolar coupling
RMSD  Root-mean-square deviation
RNA  Ribonucleic acid
rRNA  Ribosomal RNA
BSA  Bovine serum albumin
S. antibioticus  Streptomyces antibioticus
SDS-PAGE  Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNAC  N-acetylcysteamine thioesters
TCEP  Tris-(Carboxyethyl)phosphine, Hydrochloride
TEMED  N,N,N′,N′′-tetramethylethylenediamine
TOCSY  Total correlation spectroscopy
Tris  Tris(hydroxymethyl)aminomethane
tRNA  Transfer RNA
MS  Mass spectrometry/mass spectrum
1. Introduction

1.1 The Golden Age of Antibiotics

In 1928 Alexander Fleming made the serendipitous discovery of penicillin (Fleming, 1929). Following this, in the 1940’s, Howard Florey and Ernst Chain started exploring the pharmaceutical benefits of penicillin, discovering its antibacterial properties (Chain et al., 1940). Penicillin targets peptidoglycan cell wall biosynthesis of bacteria and is therefore most active against Gram positive bacteria (Yocum et al., 1980). Between the discovery and development of penicillin, in 1935, scientists at the German company IG Farben discovered, developed and commercialised a synthetic sulfonamide drug named prontosil (4-[2,4-diaminophenyl]azo] benzenesulfonamide) for the treatment of Gram-positive bacteria by acting in a bacteriostatic manner and targeting folate synthesis (Domagk, 1935). These drugs sparked a cascade of discovery of a variety of pharmaceutically beneficial compounds; this is referred to as the “golden age” of antibiotics. The “golden age” of antibiotics was really in full stride between the 1950s and 1960s with a large percentage of antibiotics still in use today being discovered in this period (Davies, 2006). Figure 1 shows a timeline of antibiotics in use today, the majority of which were discovered during this era.

Since the discovery of antibiotics such as penicillin, overuse and misuse in the field of medicine and in intensive animal farming has caused a large increase in resistant strains of bacteria, rendering some of these well-established treatments generally ineffective. A combination of horizontal gene transfer and mutation has armed bacteria with an arsenal of mechanisms for antibiotic resistance (Davies, 2006). Some examples of mechanisms of resistance include: (1) efflux pumps which will remove the drug from the cell, this occurs in fluoroquinolone resistance (Hooper, 1999; Davies and Davies, 2010); (2) modification of the drug itself rendering it inactive, which plays a key role in penicillin resistance (Fisher et al., 2005; Wright, 2007); and (3) alteration of cellular metabolic pathways, bypassing the process the drug has inhibited, this occurs in sulfonamide resistance (Skold, 2000).
Figure 1.1- A timeline of the discovery of antibiotics that are still in use today. Drug classes (normal) and examples (bold) with chemical structures are shown above. Drugs highlighted in purple boxes are synthesised by non-ribosomal peptide synthetases (NRPS) and Type I modular polyketide synthases (PKS) (Section 1.4 and 1.5), those highlighted in blue boxes are partially synthesised by NRPS or PKS and those not highlighted are synthesised by other methods. Figure adapted from Davies (2006).
Some bacterial strains, such as *Staphylococcus aureus*, are known to be resistant to a number of antibacterial drugs and are therefore called multidrug resistant organisms (MDRO). Multidrug resistance (MDR) leaves only a very limited range of treatments available, such as vancomycin (Figure 1.1) for the treatment of the methicillin-resistant *Staphylococcus aureus* (MRSA) superbug (Alekshun and Levy, 2007). Now, with the “golden age” of antibiotics well and truly over, we have seen a decrease in the number of new antibiotics discovered and a combination of the financial drain and strict Food and Drug Administration requirements has caused a plummet in the investment the pharmaceutical industry (Davies, 2006). In 1990 there were 18 pharmaceutical companies researching antibiotic treatments; today, there are just four; AstraZeneca and GlaxoSmithKline (United Kingdom), Novartis (Switzerland) and finally Sanofi-Aventis (France) (Cooper and Shlaes, 2011).

Antibiotics are a vitally important element in the fight against infectious diseases and maintenance of a good quality of life. Natural products provide a wealthy source of antimicrobials and research into the production and engineering of novel antimicrobials may aid in the fight against, not only drug resistant bacteria, but also a whole host of life threatening conditions. Throughout this introduction I will be presenting examples of these antimicrobials and the reasoning and strategies behind engineering their producers for novel pharmaceutical compound biosynthesis.

### 1.2 Natural products as antibiotics

Analysis of the literature and public pharmaceutical documentation has highlighted 16 natural product derivatives across stages I, II and III of clinical trials (Butler *et al.*, 2013). Since 2000, over 50% of the drugs released onto the market were either natural products, or derived from natural products. From these 12 natural and natural based products, 9 are produced by actinomycetes and 3 are produced by fungi, both are prolific sources of natural products with pharmaceutical benefits (Butler *et al.*, 2013; Newman and Cragg, 2012).

Statistical analysis suggested that typically, pharmaceutical companies are able to screen tens of thousands of actinomycetes in one year; however undiscovered
antibiotics are likely to occur ≤1 in $10^7$ from randomly screened soil actinomycetes. Additionally, only a microscopic percentage of the soil on earth has been screened for actinomycetes, which incidentally is not the only environment in which these pharmaceutically beneficial compounds are found (Baltz, 2008; Clardy et al., 2006).

Efforts are being made to discover new antibiotics produced by Nature, identifying natural products that have otherwise been inaccessible or missed beforehand. Although previously the main source of antibiotics has been from *Streptomyces*, continuation of the traditional culturing methods are still aiding in the discovery of new actinomycete strains not only from soil, but also marine sediment (Clardy et al., 2006; Rocha-Martin et al., 2014). Ribosomal RNA (rRNA) sequencing has been exploited for investigating uncultivable bacteria. 16S rRNA is ubiquitous in bacteria and contains conserved regions that can be probed and variable regions, which enable identification of new bacterial strains. rRNA sequencing has highlighted the fact that only a small fraction of bacteria can be cultured using traditional methods (Clarridge, 2004; Relman, 1999; Clardy et al., 2006). This indicates that other approaches are required to access this untapped resource (Clardy et al., 2006).

Strategies for discovering new natural products for antibiotics include heterologous expression of genes in a culturable host, metagenomics in combination with heterologous expression, typically using 16s rRNA, and manipulation of currently known producers of natural products, also known as combinatorial biosynthesis (Clardy et al., 2006).

There are three classes of natural products with pharmaceutical benefits of particular interest: polyketides (PK), non-ribosomal peptides (NRP), and hybrid PK-NRP compounds which will be discussed further.

### 1.2.1 Polyketides

Polyketides are secondary metabolites known to possess a wealth of pharmaceutically beneficial activities such as antibacterial, antifungal, antiparasitic, antihelminthic and insecticidal properties (Kevin Ii et al., 2009; Dutton et al., 1995). The main producers of these biologically active molecules are the actinomycetes especially *Streptomyces* and *Saccharopolyspora* (Weissman and Leadlay, 2005).
Introduction

Figure 1.2 shows example structures of polyketides (and rifamycin in Figure 1.1) illustrating the structural diversity seen across these compounds (Staunton and Weissman, 2001).

Polyketides are composed mainly of three basic units; acetate, propionate and butyrate, derived from their CoA equivalents. These units are also utilised by fatty acid synthases for the synthesis of fatty acids and so are readily available in the cell (Figure 1.5) (Staunton and Weissman, 2001; Meier and Burkart, 2009). The structural diversity, wealth of stereochemistry and functional groups observed in polyketides can partly be attributed to the various starter and extender units that can be incorporated (Chan et al., 2009; Smith and Tsai, 2007; Cane et al., 1983).

Polyketides can be classed by their structure and how they were made, and can be divided into unreduced, reduced and unclassified polyketides. Reduced polyketides can be further divided into two different classes; the macrocycles which have large lactone or lactam rings (such as erythromycin A) or polyether antibiotics, these contain between 2 and 5 ether oxygen atoms (for example monensin A) (Figure 1.2) (Staunton and Wilkinson, 1997; Oliynyk et al., 2003; Weissman and Leadlay, 2005). The biological actions carried out by polyketides vary and are dependent on their
structure. Polyether ionophores, such as monensin, have the ability to chelate metal ions and disrupt ion balance in order to kill an organism. This involves transporting metal ions across a membrane either by: (1) binding to an ion in a polar cavity and concealing the charge, ferrying it across the plasma membrane, (2) forming a channel allowing ions to flow more freely into the cell, and finally (3) neutral ionophores aid in the diffusion of ions into a cell (Pressman, 1976; Pressman and Fahim, 1982). Macrolides such as erythromycin, which are constrained by macrocyclisation, work by inhibiting protein synthesis by premature chain termination (Menninger and Otto, 1982; Kohli and Walsh, 2003).

Total synthesis, a well-studied area, makes use of well-known and studied reactions such as the carbon-carbon bond forming aldol reactions to produce fragments of polyketide (Ireland et al., 1985; Faul and Huff, 2000; Schetter and Mahrwald, 2006). Challenges arise in linking such fragments with the correct stereochemistry thus making production of more diverse analogues difficult. Polyketides and specifically ladder polyethers become cyclic by epoxide-opening cascades, and, for total synthesis, work needs to be done on selective activation for these cascades to occur. Additionally efforts need to be made towards facile modifications of epoxide groups including being amenable to methyl substitutions (Ireland et al., 1985; Vilotijevic and Jamison, 2009; Faul and Huff, 2000). In Nature polyketides are produced by giant multimodular proteins termed polyketide synthases (PKS) (Gallimore, 2009; Liu et al., 2009). These macromolecular structures have the capacity to synthesise polyketides and modify them accordingly to efficiently produce complex structures with the correct stereochemistry (Staunton and Weissman, 2001; Weissman and Leadlay, 2005) (Section 1.4).

1.2.2 Nonribosomal peptides

Nonribosomal peptides (NRPs) are another class of natural products produced by bacteria, filamentous fungi and more generally actinomycetes (Perlman and Bodanszky, 1971). NRPs, as implied by the name, are synthesised independently of the ribosome and a nucleic-acid template (Finking and Marahiel, 2004; Grunewald
Introduction

and Marahiel, 2006). As with polyketides, nonribosomal peptides are a group of structurally diverse compounds with a large repertoire of activities such as antibacterial, immunosuppressive and cytostatic (Schwarzer et al., 2003).

NRPs are known to have several different modes of action; polymyxin antibiotics (Figure 1.1) target and bind to lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria, using their hydrophobic tails to disrupt inner and outer membranes (Velkov et al., 2010). Gramicidin S (Figure 1.3) is a cationic compound and the suggested mode of action is that it is taken up into the cell and disrupts the cytoplasmic membrane; polymyxins are also suggested to do this (Katsu et al., 1986; Velkov et al., 2010). Vancomycin (Figure 1.1) targets cell wall biosynthesis (Watanakunakorn, 1981). Siderophores, such as enterobactin (Figure 1.3), are essential for host survival and are released by bacteria in environments where iron is scarce as it has a picomolar affinity for the metal ion (Raymond et al., 2003).

NRPs are formed by oligomerisation through peptide bond formation of between 3 and 22 proteogenic, non-proteogenic amino acids and aryl acids (Sieber and Marahiel, 2003; Schwarzer et al., 2003). NRPs can undergo macrocyclisation such as

Figure 1.3- Examples of nonribosomal peptides. (a) Gramicidin S, produced by Bacillus brevis, has been shown to have antibacterial properties (Gause and Brazhnikova, 1944). (b) ACV tripeptide, is the precursor to penicillin and cephalosporin antibiotics (Martin et al., 1994; Keeler, 2002). (c) Enterobactin is a siderophore which chelates iron, it is found in some Gram-negative bacteria such as E. coli (Raymond et al., 2003; Fischbach and Walsh, 2006).
gramicidin S, or be formed from multiple copies of identical NRP chains like enterobactin (Figure 1.3) (Rusnak et al., 1991; Walsh et al., 1990). The macrocyclisation and oxidative cross-linking seen in NRPs aids in their biological functions. NRPs can be further modified by introducing small heterocyclic rings, N-methylation, N-formylation, glycosylation or by the addition of fatty acids (Walsh et al., 2001; Sieber and Marahiel, 2005). These structurally elaborate compounds are synthesised by the multimodular enzymes, nonribosomal peptide synthetases (NRPS) (Section 1.5).

1.2.3 Polyketide-nonribosomal peptide hybrid products

Nonribosomal peptides and polyketides are synthesised in a very similar way, by multimodular enzymes in an assembly line-like manner utilising an enzyme template-directed mechanism (Lipmann, 1980; Hopwood, 1997; Marahiel et al., 1997). Hybrid polyketide-polypeptide products (Figure 1.4) are composed of a combination of monomeric units utilised in both polyketides and nonribosomal peptides, again creating structurally diverse biologically active compounds (Du et al., 2001).

Nonribosomal peptide-polyketide hybrid products are synthesised by a combination of nonribosomal peptide synthetase modules and polyketide synthase modules. These products can be synthesised in one of two ways; by individual NRPS and PKS modules, coupling the NRP and PK moieties using a ligase, for example coronatine biosynthesis (Bender et al., 1999; Rangaswamy et al., 1998) or by a hybrid NRPS-PKS enzyme, as with epothilone biosynthesis (Molnar et al., 2000). The latter will be discussed below.
1.3 Polyketide synthases

There are three classes of polyketide synthases; I, II and III. Type I PKS can be subdivided into two types, iterative and modular. Type I modular polyketide synthases (PKS) are large multimodular enzymes composed of discrete domains covalently linked into modules often on a MDa scale. These enzymes, encoded either by a single operon or gene clusters in bacteria, fungi, plants and more infrequently animals, are responsible for the production of polyketides as secondary metabolites as mentioned previously (1.2.1). In type I modular PKSs a module describes a group of covalently linked discrete domains, with specific roles, responsible for incorporation, and in some cases modification, of a single extender unit to a growing polyketide chain (Staunton and Weissman, 2001). Iterative type I PKS have a similar architecture to a single module in type I modular PKS that is used in continuous rounds of elongation. Examples of type I iterative PKS include the PKS responsible for lovastatin synthesis (Figure 1.2) (Staunton and Weissman, 2001; Campbell and...
Type II PKS are composed mainly of monofunctional proteins i.e. two ketosynthase domains (KS) and an acyl carrier protein (ACP), chain elongation occurs in iterative cycles producing aromatic polyketides with additional tailoring domain such as oxygenases and methyl transferases increasing structural diversity in compounds produced. Type II PKS are the source of important pharmaceutical compounds such as tetracyclines (Hertweck et al., 2007; McDaniel et al., 2005). Type III PKS are composed of a KS homodimer that works in an iterative fashion to condense together monomeric units. Type III PKS are usually found in plants and produce compounds such as chalcones (Abe and Morita, 2010; Katsuyama and Ohnishi, 2012).

This research is intended to focus solely on the modular nature of proteins and how they construct such diverse products, with the hopes of engineering the modular enzymes to manufacture novel compounds. Therefore the rest of the introduction will focus on type I modular PKS and NRPS (section 1.4).

1.3.1 PKS biosynthetic gene clusters

In 1990 Peter Leadlay’s group cloned a 10 kb gene fragment from *Saccharopolyspora erythaea* encoding the modular polyketide synthase responsible for erythromycin biosynthesis. This was achieved by identifying the gene which encoded erythromycin resistance and sequencing from that point using chromosome walking, making comparisons with sequences of homologous proteins. The method used was based on the fact that the genes which encode the proteins that make up bacterial and fungal PKSs are clustered together within the genome, along with transcriptional regulators and self-resistance genes (Cortes et al., 1990). At approximately the same time Leonard Katz and his group also cloned the DEBS biosynthetic gene cluster using the same principles as Leadlay but working from a mutation within a gene which disabled erythromycin production (Tuan et al., 1990). Figure 1.8 illustrates the biosynthetic gene cluster of 6-deoxyerythronolide synthase (DEBS). The catalytic domains of DEBS were determined by comparison with homologous domains from FAS with identical activities (Bevitt et al., 1992; Cortes et al., 1990; Tuan et al., 1990; Donadio et al., 1991). Characterisation of the
biosynthetic gene cluster of DEBS has provided some insight into gene organisation in PKS.

With decreasing costs of genome sequencing and advances in *in silico* analysis, more than 50 PKS gene clusters have been elucidated (Weissman and Leadlay, 2005). Currently, common techniques used to identify PKS gene clusters include entire genome sequencing and screening cosmid or bacterial artificial chromosome libraries with probes based on homologous sequences, such as the KS domain, using hybridisation or PCR based methods (Weissman and Leadlay, 2005; Wang *et al.*, 2011).

Entire genome sequencing has highlighted an unexpected prevalence of potential genes for antibiotic synthesis in bacteria, entire genome sequencing of the *Bacillus subtilis* genome revealed more than 4% is suspected to encode for gene clusters responsible for polyketide and bacteriocin synthesis (Kunst *et al.*, 1997). Sequencing of certain *Streptomyces* strains have shown that the abundance of PKS and NRPS gene clusters is not unique to *B. subtilis* (Bentley *et al.*, 2002; Ikeda *et al.*, 2003). The elucidation of increasing numbers of PKS gene clusters is providing ever more options for engineering to produce novel compounds.

### 1.3.2 PKS module organisation

PKSs are composed minimally of three essential domains, an acyltransferase domain (AT), responsible for the substrate selected as the extender unit; a ketosynthase domain (KS) which catalyses the condensation between the selected extender unit and the growing polyketide chain, and an acyl carrier protein (ACP) which houses the growing chain (Staunton and Weissman, 2001; Weissman and Leadlay, 2005; Menzella *et al.*, 2005; Fischbach and Walsh, 2006). Polyketides are synthesised in an assembly line-like fashion with each individual module responsible for incorporating a single unit, and the number of modules dictating the number and specificity of units incorporated (Chan *et al.*, 2009; Staunton and Weissman, 2001; Fischbach and Walsh, 2006). Figure 1.6 illustrates the composition of the minimal module and mechanism by which chain elongation occurs.
Figure 1.5- Minimal PKS module illustrating the mechanism by which chain elongation occurs. (a) The acyltransferase (AT) domain selects an acyl CoA extender unit and catalyses its transfer onto the acyl carrier protein (ACP). (b) Carbon-carbon bond formation by decarboxylative condensation then occurs between the extender unit and growing polyketide chain catalysed by the ketosynthase (KS) domain (Fischbach and Walsh, 2006).

1.3.2.1 Acyltransferase (AT) domain
The acyltransferase (AT) domain, originally identified due to its homology with the malonyl acyltransferase (MAT) in FAS, is responsible for substrate selection and is suggested to possess high substrate specificity (Khosla et al., 1999; Walsh, 2008; Liou et al., 2003). The reaction catalysed by an AT domain is shown in Figure 1.5. The site of acylation is a conserved serine (Dunn and Khosla, 2013). There are two types of AT domains known to be utilised by PKS, a cis AT domain which associates with a single specific ACP, as illustrated in the DEBS PKS (Figure 1.8) (Dunn et al., 2014). Alternatively, there are trans acting AT domains, these domains are stand-alone and associate with multiple ACPs for loading of the extender unit as occurs with leinamycin biosynthesis (Piel, 2010; Cheng et al., 2003).

A highly conserved arginine at position 222, or a tryptophan in the DEBS and avermectin PKS, has been identified as controlling substrate specificity (Liou et al., 2003; Rangan and Smith, 1997). Substrate specificity of AT domains within
initiating modules varies; in contrast AT domains in the extending modules are more substrate specific acting as a gate-keepers, stopping chain elongation in the absence of the correct extender unit (Keating and Walsh, 1999; Khosla et al., 1999). The AT domain provides an excellent opportunity for engineering to introduce different extender units for novel product biosynthesis.

1.3.2.2 Ketosynthase (KS) domain
The ketosynthase (KS) domain, approximately 45 kDa in mass, catalyses the decarboxylative condensation of the extender unit and growing chain. A conserved cysteine in the active site enables the extender unit to be briefly transferred onto the KS domain before being added to the growing polyketide chain and contributes to the substrate specificity of the module in some way (Fischbach and Walsh, 2006; Chen et al., 2006). The KS domain is either inactive or missing completely in the initiating modules (Chen et al., 2011). The KS domain has also been pinpointed to be responsible for the wealth of stereochemistry observed in polyketides (Lau et al., 1999).

1.3.2.3 Acyl carrier protein
Acyl carrier proteins (ACP) are essential non-catalytic domains of less than 100 amino acids in length. They are the site of chain elongation and are known to interact with more than 30 partners (Fischbach and Walsh, 2006; Gully and Bouveret, 2006; Gully et al., 2003; Butland et al., 2005). Phosphopantetheinylation of carrier proteins is unconditionally required for biosynthesis of not only polyketides, but nonribosomal peptides and fatty acids. This post translational modification is mediated by a phosphopantetheinyl transferase (PPTase). Each enzyme complex; PKS, NRPS and FAS, is associated with a PPTase capable of this modification. Phosphopantetheinylation occurs on a conserved serine within the ACP, with the phosphopantetheine group from Coenzyme A being covalently attached by a phosphodiester link (Figure 1.6). The phosphopantetheine modification creates a flexible arm of approximately 20 Å in order to pass the growing chain from one module to another (Walsh et al., 1997; Lambalot et al., 1996; Quadri et al., 1998; Staunton and Weissman, 2001).
Figure 1.6- Mechanism by the ACP becomes phosphopantetheinylated. The hydroxyl group of a conserved serine of an acyl carrier protein (ACP) becomes phosphopantetheinylated by a phosphopantetheinyltransferase mediating the formation of a phosphodiester link, converting apo-ACP to holo-ACP (Walsh et al., 1997; Lambalot et al., 1996; Fischbach and Walsh, 2006).

1.3.2.4 Chain initiation and termination

As well as individual modules responsible for extender unit incorporation, there are two additional features, a loading module composed of an AT domain and ACP responsible for initiation and a thioesterase domain responsible for chain termination.

More often than not the module responsible for polyketide chain initiation lacks the KS domain. In the initiation module, the AT domain is sufficient for loading the ACP with a starter unit before being handed off to the next module, this occurs in the DEBS PKS (Figure 1.8) (Keating and Walsh, 1999; Caffrey et al., 1992).

The thioesterase (TE) domains are responsible for chain termination and are located at the C-terminus of the PKS. Chain termination occurs using a Ser-His-Asp catalytic triad in an analogous manner to serine proteases. Depending on the nature of the TE domain, macrocyclisation can occur of the polyketide product being released. If the acyl-bound intermediate is attacked by an external nucleophile a linear product will be released, attack by a nucleophile within the polyketide chain will lead to a cyclised product (Du and Lou, 2010).
1.3.2.5 Linker regions
Assembly line synthesis by a multimodular protein requires the modules to be tethered together in some way, and additionally for loading and catalysing condensation between two acyl moieties, so will the globular domains. Therefore linker regions between both the individual domains and entire modules exist. Linkers between domains are up to 100 residues long and composed mainly of alanine, proline and charged residues, the composition of the linkers dictating their flexibility and thus the range of motion and interaction with other domains (Staunton and Wilkinson, 1997; Bevitt et al., 1992).

1.3.2.6 Accessory domains: KR, DH and ER
The structural diversity observed in polyketides is also achieved by the addition of accessory domains within a module. These domains control the level of reduction of the β-keto group of the extender unit added, and are termed a ketoreductase (KR), dehydratase (DH) and enoylreductase (ER). Unlike FAS, which have all three domains present achieving full reduction of all β-keto groups, PKS modules display varying levels of reduction at the β-keto group dependent on which of the accessory domains are present. Figure 1.7 illustrates how the β-keto carbon is processed in PKS and how it is comparable to FAS.

1.3.2.7 Biosynthetic pathways of polyketide synthases and fatty acid synthases
The idea of small monomeric units being condensed together by an enzyme template-directed mechanism is utilised by multimodular enzymes to produce structurally diverse compounds. This mechanism is common to fatty acid synthases (FAS), polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS) (Meier and Burkart, 2009). FAS and PKS utilise the same monomeric units and Figure 1.7 illustrates the units used by FAS and PKS and the differences in levels of reduction the acyl-CoA units undergo. FAS acyl-CoA monomers undergo reduction, dehydration and a second reduction; the acyl and malonyl CoA units are condensed together in a head-to-tail manner to create fatty acids. Polyketide acyl-CoA monomers in a polyketide chain can undergo varying levels of reduction contributing to the structural diversity observed in polyketides, dependent on the presence of the
accessory domains within a PKS module (Meier and Burkart, 2009; Staunton and Weissman, 2001).

In fatty acid biosynthesis (Figure 1.7 (2)) (as with PK biosynthesis) the FAS AT domain selects the extender unit, and a ketosynthase domain catalyses the carbon-carbon bond formation between the selected unit and the growing fatty acid. The β-keto carbon of the malonyl CoA or acetyl CoA unit added then undergoes reduction by a ketoreductase (KR) domain to a β-hydroxy group, this is followed by dehydration by a dehydratase (DH) yielding an α,β-enoxy product. Finally, a second round of reduction is carried out by an enoylreductase (ER) domain, to give a fully reduced product. In bacterial and mammalian FAS, more units are added in an

Figure 1.7- The biosynthetic pathways of FAS and PKS. FAS and PKS acyl-CoA monomers are condensed together in an identical manner. An extender unit is selected by acyltransferase (AT) domains, which catalyse the transfer onto an acyl carrier protein (ACP), the site of chain elongation. The ketosynthase (KS) domain then catalyses carbon-carbon bond formation between the extender unit and the growing acyl chain. FAS (2) then utilises a ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER), to fully reduce the acyl unit added. PKS acyl units undergo varying levels of reduction at the β-keto position (3); this is dependent on the presence of KR, DH and ER domains present. No reduction results in aromatic polyketides being formed (1) (Moore and Hertweck, 2002; Meier and Burkart, 2009).
iterative fashion giving full length fatty acids (Meier and Burkart, 2009; Staunton and Weissman, 2001). Type II PKS are iterative enzymes which produce aromatic PKs (Figure 1.7 (1)), this occurs when the accessory domains are absent (Das and Khosla, 2009). In type I modular PKSs differential levels of reduction occur dependent on which accessory domains are present. In addition to the essential domains within a module, in type I modular PKS, varying levels of accessory domains can exist (Figure 1.7 (3). The level of reduction the extender units undergo in each round of elongation is dependent within that module and is independent of preceding and subsequent modules, producing compounds composed of extender units differing at the β-keto position (keto, hydroxyl, enoyl and fully reduced) (Staunton and Weissman, 2001; Meier and Burkart, 2009). These domains provide another possible dimension for engineering PKS to produce novel compounds.

Figure 1.8 shows the biosynthetic gene cluster of the PKS DEBS and gives some idea as to the arrangement of domains within modules in PKSs, illustrating the combinations of accessory domains within different modules (Caffrey et al., 1992; Bevitt et al., 1992; Cortes et al., 1990; Tuan et al., 1990).

1.3.2.8 A model system

Extensive research has been carried out on the PKS responsible for erythromycin production, 6-deoxyerythronolide (DEBS). Both structural and mechanistic studies have provided great insight into how this PKS works and engineering has provided tantalising glimpses into the potential for novel compound production. DEBS has been the model system and so the biosynthetic gene cluster is shown in Figure 1.8 (Bevitt et al., 1992; Cortes et al., 1990; Caffrey et al., 1992; Staunton and Weissman, 2001; Staunton et al., 1996; Marsden et al., 1998; Oliynyk et al., 1996; Khosla et al., 2007; Khosla et al., 2014).
Figure 1.8- Biosynthetic gene cluster of the erythromycin precursor, 6-deoxyerythronalide, producing PKS, DEBS. (a) eryAI, II and III open reading frames encode three large polypeptides, DEBS 1, 2 and 3. DEBS 1 is composed of a loading didomain, module 1 and module 2, DEBS2 is composed of modules 3 and 4 and finally DEBS3 is composed of modules 5 and 6 and the thioesterase domain. (b) The thioesterase domain is not only involved in chain termination but macrocyclisation of the linear polyketide chain. Units incorporated are highlighted in bold on the structure on the left (Caffrey et al., 1992).
DEBS forms the macrolide intermediate 6-deoxyerythronolide B (6-dEB) (Figure 1.8). Three open reading frames, eryAI, eryAII and eryAIII, encode for the polypeptides DEBSI, DEBSII and DEBSIII respectively. DEBSI contains the loading module and two extender modules, DEBS II contains two extender modules and DEBS III contains two extender modules and the thioesterase. The molecular mass of this PKS is over 600 kDa (Cortes et al., 1990; Tuan et al., 1990; Kao et al., 1994; Caffrey et al., 1992). The macrolide intermediate is formed by 6 modules responsible for incorporation of ethyl malonyl CoA as extender units, a loading di-domain which lacks a KS domain and utilises propionyl CoA as the starter unit and finally a thioesterase domain which instigates chain release and aids in cyclisation of 6-dEB (Tuan et al., 1990; Cortes et al., 1990; Caffrey et al., 1992).

1.3.3 Structure determination of PKSs
The modularity of PKSs makes them an attractive target for combinatorial biosynthesis to produce novel compounds. Structural characterisation and mechanistic understanding will aid engineering of PKS to produce novel compounds.

There have been significant efforts to structurally characterise the domains within a PKS module, including di-domains and docking domains (between modules). Several structures exist of KS-AT didomains from DEBS, the KS-AT didomain from module 5 (PDB code 2HG4) (Figure 1.9) and the KS-AT didomain from module 3 (PDB code 2QO3). Structural information revealed well-structured inter- and intradomain linkers composed of highly conserved residues and a loop region in the KS domain homodimer interface which potentially plays a part in substrate specificity. Additionally, an 80 Å distance between the catalytic cysteine in the KS domain and the conserved serine in the AT domain was observed indicating that the DEBS PKS must undergo structural rearrangement for chain elongation to occur (Tang et al., 2007; Tang et al., 2006).
NMR and crystal structures of DEBS and pikromycin docking domain or linkers have been solved. Linkers between modules are structurally well defined and are composed of highly conserved residues which not only promote dimerisation of domains but create unfavourable interactions with other modules so as to prevent misdocking (Broadhurst et al., 2003; Buchholz et al., 2009).

The ketoreductase, dehydratase and thioesterase domains have been well characterised with crystal structures from a number of PKSs (Zheng et al., 2012; Keatinge-Clay, 2008; Keatinge-Clay, 2007; Keatinge-Clay and Stroud, 2006; Tsai et al., 2001; Akey et al., 2006; Scaglione et al., 2010; Zheng et al., 2010; Zheng and Keatinge-Clay, 2011; Gehret et al., 2011; Tsai et al., 2002). Less well characterised, the enoyl reductase, has been solved as a di-domain (ER-KR) (Zheng et al., 2012). Finally, a structure of an ACP from DEBS has been solved by NMR spectroscopy (Alekseyev et al., 2007). Details of the structure of carrier proteins will be covered later in this thesis. Even though the structures of individual domains are well documented there is no high resolution structure of an entire PKS module.
In 1996 Staunton, Leadlay and colleagues proposed an overall model for the DEBS PKS referred to as the “Cambridge model” (Figure 1.10) (Staunton et al., 1996). The foundations of this model were structural studies utilising limited proteolysis and analytical ultracentrifugation (AUC), which showed that KS-AT and TE domains are homodimeric whilst those domains involved in reduction are monomeric. In this model the PKS forms a “double-helical” structure with proteins intertwined forming a homodimer with both chains in the same orientation (“head-to-head”) and the essential domains (AT-KS-ACP) forming the core of the complex. The KS domain of one module was shown to have a favourable interaction with the ACP of the opposite module; however, all domains appear to have access to the ACP. The accessory domains then loop out from the main body of the PKS. These models satisfy the ability to vary the accessory domains within different modules, and allow communication between subunits but prevent cross-talk of ACPs with the incorrect domains. This model also supported the seemingly more successful engineering of accessory domains. A similar model was outlined two years later by Cane and Khosla (Cane et al., 1998).

Until recently structural studies involving entire PKS modules have been hindered by their sheer size and flexibility (Tsai and Ames, 2009; Staunton and Weissman, 2001). However, in 2008 advances in the field of X-ray crystallography enabled elucidation of the crystal structure of the mammalian FAS (mFAS) (PDB code 2V28). The mFAS displayed an intertwined homodimer in an X orientation with KS, DH and ER domains forming the dimer interface (Maier et al., 2008). The bottom portion of the X was composed of the essential catalytic domains (KS and MAT) and the top was composed of the domains responsible for reduction (KR, DH and ER) (Maier et al., 2008). As mentioned previously PKS are homologous to FAS and so this gave the best indication of the arrangement of domains within a module until recently (Dutta et al., 2014).
Figure 1.10- Proposed structure of PKS and mFAS (a) Schematic of modules 5 and 6 of DEBS illustrating the “Cambridge model” proposed by Staunton and Leadlay. The two chains are twisted together in a “head-to-head” orientation forming a helix. This helical conformation allows interactions between the KS domain and ACP from each chain. The KR domains loop out between the AT domain and ACP within the same monomer (red linkers) (Staunton et al., 1996; Staunton and Weissman, 2001). (b) Schematic of the structure of mammalian FAS. This iterative enzyme has a core formed of KS, DH and ER homodimers. The bottom of the mFAS is required for chain elongation and substrate selection and the top for reduction of the acyl-CoA. The ACP and TE domains do not form the body of the enzyme (Maier et al., 2008).

As mentioned previously, there had been no structures of an entire PKS module, however, recently electron cryo- microscopy (cryo-EM) was used to elucidate the structure of a dimer of module 5 of the pikromycin PKS (PiKAIII). The dimeric module was 328 kDa and had a fully functional holo-ACP associated. Figure 1.11 (a) shows the solid rendered map of the PKS domain solved to between 7.3 and 9.5 Å. The KS dimer is at the top, with the AT domain below supported through a large interaction surface area with the KS domain of the other chain. This is followed by the KR domains forming the base and interface with the next module. The relative orientations of the KS and AT domains observed in the EM structure are different to those in the di-domain crystal structures, having a larger interaction surface than originally shown, but supporting the “Cambridge model”. Finally the arch shape observed provides a “reaction chamber” for the ACP. (Dutta et al., 2014; Tang et al., 2007; Tang et al., 2006; Staunton et al., 1996). Recently, high resolution data has been combined with small-angle X-ray scattering (SAXS) to investigate the structure
of modules 5 and 6 from DEBS, providing further structural evidence (Edwards et al., 2014).

Figure 1.11- Structure and chain elongation mechanism of a module of PikAIII (a) Cryo-EM structure of module 5 of PikAIII composed of KS-AT-KR-ACP domains with a resolution of 7.3-9.5 Å. The KS forms a dimer at the top, stabilised by AT domains from the other monomer chain. The base of the structure is formed by the KR domains. (b) Dynamics of the module during chain elongation as observed in EM structures. The ACP domain resides within a cavity, then associates with the AT domain to be loaded with the extender unit. The ACP then moves to the KS domain and chain elongation is catalysed, the ACP then ferries the extended chain to the KR domain to undergo reduction. Finally, the ACP then passes the polyketide chain onto the KS domain of the following module (Dutta et al., 2014; Whicher et al., 2014).

The elucidation of the structure of module 5 of the pikromycin PKS by EM also provided information on how chain elongation occurs (Figure 1.11 (b)). The ACP appears to travel to each module for them to carry-out their jobs, specific protein-protein interactions with the catalytic domains are dependent on the substrate attached to the phosphopantetheine arm. There is evidence of domain cross-talk, with the KR domain flipping when the ACP interacts with the AT domain, to be in place for reduction of the growing chain. Conformational changes of the PKS module show the AT domain also restricts side substrate entry in the KS domain, the
route of the extending chain. The mechanism indicated by this data shows a cascade of conformational changes, dependent on the previous module, in an assembly line-like fashion (Whicher et al., 2014).

A combination of structure determination of individual domains, how they dock onto one another, and now a structure determination of an entire module and dynamics in combination with biochemical characterisation may aid in a more knowledge-based reasoning behind engineering PKS, and thus an increased chance in producing functional hybrid enzymes to produce novel compounds.

1.3.4 Engineering of PKS
Polyketide derived drugs are said to generate worldwide revenues of £10 billion per annum and account for 10% of the top selling drugs (Weissman and Leadlay, 2005). Traditionally, polyketide drugs have been isolated from natural sources such as the soil or by modifying existing drugs. More recently, the idea of creating novel polyketides by engineering polyketide synthases has become an attractive option for the production of novel compounds that have pharmaceutical properties (Weissman and Leadlay, 2005). It was this idea that has motivated decades of research to understanding the structure and function of PKSs.

Polyketide synthases provide a versatile system for engineering due to their modular nature. As described previously, each individual module is composed of domains with discrete active sites responsible for extender unit incorporation and catalysing chain extension. It was suggested that the structure and organisation of these multimodular enzymes would lend themselves to engineering by “cutting and pasting” domains and modules, leading to what is now termed “combinatorial biosynthesis” (Khosla and Zawada, 1996; Weissman and Leadlay, 2005). Combinatorial biosynthesis describes the act of creating novel products by mixing features of known structures, and utilising and manipulating the enzymes that naturally produce these features (Staunton and Weissman, 2001).

There are several approaches for engineering PKS to produce novel compounds, however engineering of PKS in the natural host is not amenable for a number of reasons, and therefore heterologous expression of PKS proteins is often the first
Introduction

obstacle in engineering PKSs. This is discussed in some depth in chapter 3. Strategies for engineering PKSs and their successes are covered below.

There are several strategies that can be considered for PKS engineering, focusing on either individual domains, whole modules, using synthetic molecules for incorporation and finally alteration of enzymes involved in modification of the final polyketide chain. The most practiced strategy of PKS engineering is engineering of domains within a PKS biosynthetic gene cluster (Staunton and Weissman, 2001).

Structural diversity observed in polyketides can, for some part, be attributed to the types of starter and extender units incorporated (Moore and Hertweck, 2002; Chan et al., 2009). One area of PKS engineering has considered the incorporation of alternative starter units. In 1998 Marsden et al. exchanged the promiscuous starter module of the avermectin PKS, able to incorporate C2 branched carboxylic acids, with the DEBS starter module. The resulting chimeric PKS was able to produce novel products. This success highlighted the self-sufficient nature of a PKS module and the ability for downstream modules to accept the new polyketide chain (Marsden et al., 1998; Dutton et al., 1991). Research into the incorporation of different starter units in the DEBS PKS has also included exchanging the DEBS loading di-domain with the oleandomycin and tylosin starter modules, creating truncated products with acetate or propionate as exclusive starter units (Long et al., 2002).

Additionally, a strategy called chemobiosynthesis has been used to incorporate novel unnatural starter units. To do this the loading KS domain of DEBS was inactivated, and the PKS was able to incorporate diketide-S-N-actetyl cysteamine (SNAC) or diketide-S-N-proionyl cysteamine (SNPC) to initiate novel product biosynthesis (Jacobsen et al., 1998; Frykman et al., 2001; Dutton et al., 1994). The use of SNACs and SNPCs is quite costly, however it has been shown that the DEBS can also use more cost efficient thioesters, such as methyl thioglycolate, as a means of chemobiosynthesis to create novel products (Murli et al., 2005).

In the same vein, engineering of AT domains within starter and extender modules has altered substrate specificity. There are two suggested ways to do this; the more commonplace method is AT domain exchange, selecting an AT domain with the desired substrate specificity or the less common way, by mutagenesis of amino acids...
determined to be involved in substrate specificity (Dunn and Khosla, 2013). The AT domains of both full length and truncated DEBS have been switched with AT domains from the rapamycin PKS, incorporating malonyl CoA as extender units as opposed to methylmalonyl CoA extender units, with varying levels of product yields observed (Lau et al., 1999; Oliynyk et al., 1996; Ruan et al., 1997). AT domain substitution has not been limited to rapamycin, with other PKS AT domains from the pikromycin PKS and the FK520 PKS being exchanged (Ruan et al., 1997; Kato et al., 2002).

The biggest pitfall of the production of novel polyketides by altering substrate selection by AT domain exchange appears to be the decrease in product titres, suggested to be due to the disruption of protein-protein interactions or the inability to process the extender chain downstream (Liou and Khosla, 2003; Hans et al., 2003). In addition to this there doesn’t appear to be any specific guidelines for successful AT domain substitutions, with some domains more difficult to replace, and in some cases being dependent on the location of the domain (Reeves et al., 2001; Lau et al., 1999; Ruan et al., 1997; Dunn and Khosla, 2013). To circumvent the issues related to decreased product titres, sections of AT domains responsible for substrate selectivity have been switched, creating hybrid AT domains (Lau et al., 1999). Mutagenesis of the YASH motif (key to methylmalonyl CoA specificity) to the HAFH motif (selective for malonyl CoA) has produced promiscuous DEBS AT domains with the ability to also incorporate unnatural extender units (Reeves et al., 2001; Sundermann et al., 2013; Del Vecchio et al., 2003).

There are two types of AT domains, cis- and trans- acting. Cis-acting AT domains reside within a particular module, trans-acting AT domains are detached single enzymes able to load multiple ACP with extender units. It has been suggested that an inactivated cis-AT domain could be substituted by a trans-acting AT domain (Piel, 2010; Dunn et al., 2014).

Combinatorial biosynthesis has also explored the possibilities of altering the β-keto carbon processing, by the introduction or removal/inactivation of the accessory domains. The bulk of this research has been carried out with the DEBS and rapamycin PKSs (Kao et al., 1997; Kao et al., 1998; McDaniel et al., 1997). Exchange of the KR domains has also shown effects on the stereochemistry of the
product. The KR domains located in the second and fifth modules of DEBS were exchanged with the KR domain from the rapamycin PKS altering the stereochemistry observed in the product (Kao et al., 1998).

The scope of combinatorial biosynthesis is not limited to single domain exchanges, McDaniel et al. reportedly substituted multiple AT domains and added the accessory β-processing domains in order to generate a novel macrolactone library, they otherwise claim would not be possible to create by chemical synthesis (McDaniel et al., 1999). Entire modules have been able to be linked together to create novel PKS products, this has been shown by fusing a module from the rifamycin PKS at the end of a truncated DEBS PKS and replacing module 2 in a full length DEBS PKS (Gokhale et al., 1999).

Functional hybrid PKSs have been expressed using multiplasmid approaches. One approach involved cloning native and variant (domain exchanges) DEBS genes into compatible vectors. These vectors were introduced into a Streptomyces strain consecutively, 70% of the strains containing a full PKS (3 plasmids) yielded products (Xue et al., 1999). This approach was repeated using pikromycin, erythromycin and oleandomycin PKSs, successfully producing hybrid PKSs (Tang et al., 2000). More recently, design of PKS genes for heterologous expression of PKS modules in E. coli has shown huge successes, with synthetic genes of 14 modules from 8 different PKS able to participate in bi-modular interactions in at least one combination (Menzella et al., 2005).

Engineering PKSs is not without its difficulties. As mentioned previously, PKS domains and modules are joined together by linkers and it is well-recognised that interference with these linkers can disrupt PK formation or skip the newly introduced module (Gokhale et al., 1999; Thomas et al., 2002). A greater understanding of linkers between domains and modules could enable design of universal linkers allowing any number of PKS domain and module combinations (Weissman, 2004). In addition to this, some PKSs, such as DEBS, are composed of multiple polypeptides, which need to associate for chain elongation. Highly structured docking domains are responsible for this (Broadhurst et al., 2003). Therefore, in order to create a hybrid PKS a greater understanding of the docking domains is
required. This may enable the design of an “orthogonal” pair of docking domains which could be utilised in engineering hybrid PKS (Weissman, 2004).

The modular nature of polyketide synthases and the applications for creating novel pharmaceutical products are mirrored in nonribosomal peptide synthetases described below.

1.4 Nonribosomal peptide synthetases

Nonribosomal peptide synthetases (NRPS), the producers of non-ribosomal peptides, are large multimodular enzymes on the scale of MDa. NRPS construct NRPs in an analogous manner to the way PKS produce PKs. Like PKSs, the modules within an NRPS are each responsible for the incorporation of a single extender unit, constructing a nonribosomal peptide in an assembly line-like fashion utilising the same enzyme-template mechanism as PKS and FAS. In an NRPS as module also describes a group of domains responsible for incorporation and modification a single extender unit to the growing peptidyl chain (Meier and Burkart, 2009). Also, as with PKSs, the number, type and order of modules within the NRPS dictates the product formed (Schwarzer et al., 2003). NRPS are also known to be able to utilise a vast number of substrates (Marahiel et al., 1997).

1.4.1 NRPS biosynthetic gene clusters

As with PKS, probing for NRPS biosynthetic gene clusters is carried out in a similar manner to PKSs, by exploiting the homologous nature of these multienzymes (Marahiel et al., 1997). Figure 1.16 shows the biosynthetic gene cluster of tyrocidine biosynthesis, the tyrocidine biosynthetic gene cluster was identified by cloning sections of 10-22 kb of B. brevis genomic DNA into a bacteriophage vector and screening for homologous DNA using polyclonal antibodies raised against Gramicidin S synthetase 2 (GrsB). This led to the discovery of TycA and TycB (Mittenhuber et al., 1989).

Identification of domains within the surfactin synthetase were identified using oligonucleotide probes based on highly conserved sequences within the gramicidin S, tyrocidine and ACV synthetases and carried out through hybridisation and in situ sequencing (Borchert et al., 1992). NRPS operons appear to be between 18 and 45 kb in length, with the operons containing anywhere between one and six modules,
such as TycA and TycC, respectively (Marahiel et al., 1997; Mootz and Marahiel, 1997). Other genes associated with NRPS are located at either the 5’ or 3’ ends of the operons, these proteins show high sequence homology to the type II fatty acid thioesterase (Mootz and Marahiel, 1997; Marahiel et al., 1997).

Comparison of NRPS DNA sequences and heterologous expression of DNA fragments revealed how NRPs were formed, with particular proteins involved in substrate recognition and activation. It also showed each module was involved in incorporation of a single unit and this dictated the length and size of the peptide synthetase, and *vice versa* (Marahiel et al., 1997). The greater the number of biosynthetic gene clusters that have been fully characterised could conceivably increase the molecular toolbox that could be used for engineering NRPS modules in order to produce novel pharmaceutical compounds.

### 1.4.2 Biosynthesis of nonribosomal peptides and module organisation

As with PKS’s, NRPS modules are composed minimally of three core domains, responsible for the substrate selection of the extender unit, catalysis and housing the growing chain, these are the condensation (C), adenylation (A) and peptidyl carrier protein (PCP) or thiolation (T) domains, respectively (Fischbach and Walsh, 2006). Figure 1.12 illustrates the domain organisation and chain elongation within a minimal module.

![Domain organisation within an NRPS minimal module](image)

**Figure 1.12** Domain organisation within an NRPS minimal module. The condensation domain catalyses the peptide bond formation between the growing peptidyl chain housed on the peptidyl carrier protein and an extender unit, covalently attached to the PCP domain in the next module (Fischbach and Walsh, 2006; Weber et al., 1990).
Synthesis of NRPs are carried out in a number of steps. The A domain is responsible for selection and activation of the correct substrate, converting a carboxylic acid to an aminoacyl adenylate (Figure 1.13) (Dieckmann et al., 1995). The activated amino acid is then transferred onto the terminal thiol of the phosphopantetheine co-factor of the PCP domain within the same module (Ehmann et al., 2000a; Stachelhaus et al., 1996). Formation of the peptide bond then occurs between the electrophilic thioester of the aminoacyl-S-PCP (extender unit) and the nucleophilic amine group of the upstream peptidyl-S-PCP (growing chain), the C domain catalyses this reaction. This continues, with each module adding a single extender unit before reaching the final module, which, more often than not contains a thioesterase domain for chain termination (Fischbach and Walsh, 2006).

Another factor contributing to the diversity of non-ribosomal peptides is the presence of accessory domains within the module; these can include an epimerase domain, an N-methyl transferase or a cyclase domain. The epimerase domain (section 1.4.2.5) catalyses the racemisation of the most recently added substrate to the growing peptidyl chain. The cyclase domain (section 1.4.2.6) catalyses the formation of small heterocyclic rings and N-methylation (section 1.4.2.7), often observed in NRPs produced by fungi, is carried out by a methyltransferase domain which uses an S-adenosylmethionine co-factor to add a methyl group to the N-terminus of the amino acids (Fischbach and Walsh, 2006).

### 1.4.2.1 Adenylation domain

The adenylation domain initiates the incorporation of a substrate, typically an amino acid, by selection and activation. The A domain, sometimes called “The Gatekeeper” enzyme, is specific for its cognate amino acid. A domains are usually approximately 550 amino acids and activates the amino acid through Mg$^{2+}$-dependent hydrolysis of ATP, producing an aminoacyl adenylate and pyrophosphate as a by-product. The aminoacyl adenylate can then undergo nucleophilic attack by the free thiol of the phosphopantetheine group attached to the PCP (Figure 1.13) (Sieber and Marahiel, 2005; Hur et al., 2012).
Amino acid activation in NRPSs by the adenylation domain is mechanistically similar to amino acid activation by aminoacyl-tRNA synthetases however there is little or no sequence and structural similarities between the two. Additionally, although the adenylate domain has substrate specificity, it is more promiscuous than its mechanistically similar counterpart aminoacyl-tRNA synthetases (Sieber and Marahiel, 2005).

A domains show approximately 30-60% sequence identity, and through sequence alignments an AMP binding domain has been identified. This site is conserved throughout the adenylate forming enzyme superfamily, including in acetyl CoA synthetases (Turgay et al., 1992; Starai and Escalante-Semerena, 2004). Sequence alignments of the PheA adenylation domain from the gramicidin S synthetase with 160 other A domains also highlighted 10 residues responsible for substrate specificity, in this case for phenylalanine (Stachelhaus et al., 1999).

Several A domains have been heterologously expressed in E. coli, purified and subsequently their activity and substrate specificity have been assayed in vitro by ATP-PPi exchange with radiolabelled substrates, for example the EntE and EntF adenylation domains from the enterobactin NRPS (Linne and Marahiel, 2004; Ehmann et al., 2000a). Several crystal structures of A domains have also been solved (section 1.4.3).
1.4.2.2 Condensation domain

Condensation (C) domains catalyse the formation of a peptide bond between the newly recruited extender unit and growing peptidyl chain housed on the PCP (Stachelhaus et al., 1998). Condensation domains also possess the ability to catalyse the formation of a bond between an acyl group from an ACP-bound PK from a PKS module and an aminoacyl group bound to a PCP. This occurs in PKS/NRPS hybrid enzymes (section 1.5), such as in the rapamycin PKS/NRPS (König et al., 1997; Hur et al., 2012). Some C domains also have the ability to catalyse the formation of ester bonds, in fact a C domain from an NRPS in S. globisporus is known to be able to do both (Lin et al., 2009; Hur et al., 2012). In an analogous manner to some PKSs, the domain responsible for catalysis, the condensation domain, is sometimes missing in the initiation module in some NRPS, such as in TycA (Figure 1.1) (Fischbach and Walsh, 2006; Mootz and Marahiel, 1997). Catalysis in C domains is mediated by electrostatic interactions, aided by the structure of the C domain (Keating et al., 2002; Hur et al., 2012).

It has been shown that in addition to the substrate specificity of the adenylation domain, the condensation domain also displays some level of substrate specificity for the aminoacyl adenylate to be added to the growing NRP chain, being selective for the D-enantiomer in addition to selectivity observed for particular side chains. This selectivity only seems to pertain to the aminoacyl adenylate and not the growing chain (Belshaw et al., 1999). This specificity of the C domain for the aminoacyl adenylate, once the peptide bond has been formed and the aminoacyl adenylate added to the growing chain, is lost and the peptidyl-S-PCP is no longer a substrate, this causes release of the growing chain. This mechanism has been suggested to prevent accidental initiation in an elongation module. This also explains the uni-directional nature of NRPS (Linne and Marahiel, 2000).

1.4.2.3 Peptidyl carrier protein

The peptidyl carrier protein (PCP) is homologous to the acyl carrier protein (ACP) in PKSs (Meier and Burkart, 2009; Crosby and Crump, 2012). As with the ACP, the
Introduction

PCP is a small non-catalytic protein essential for NRP chain elongation. The PCP is also phosphopantetheinylated on a conserved serine by an auxiliary phosphopantetheinyl transferase (PPTase) (Walsh et al., 1997; Lambalot et al., 1996). Elongation occurs in a synonymous fashion to PKS as illustrated in Figure 1.12. The structure of the PCP from module 3 of TycC has been elucidated by NMR (Weber et al., 2000), structural aspects of PCP domains will be discussed in depth in chapter 5.

1.4.2.4 Chain termination and macrocyclisation
The thioesterase (TE) domain resides at the C-terminus of the final module within the NRPS and is responsible for NRP chain termination (Schneider and Marahiel, 1998) using an active site serine in a Ser-Asp-His catalytic triad (Kohli and Walsh, 2003). Chain termination can have one of two outcomes; a linear NRP is observed when hydrolysis occurs, as occurs in vancomycin biosynthesis (Hubbard and Walsh, 2003; Keating and Walsh, 1999). Macrocyclisation occurs when a nucleophile within the NRP chain attacks itself, this occurs in daptomycin biosynthesis (Hur et al., 2012). The main difference between PKS and NRPS TE domains is the PKS TE domain is a homodimer, forming a hydrophobic channel, and the NRPS domain is a monomer. The NRPS TE domains of surfactin and fengycin have a hydrophobic cavity, with a lid region, which can accommodate the NRP chain and allow cyclisation (Grunewald and Marahiel, 2006; Bruner et al., 2002; Samel et al., 2006).

1.4.2.5 Epimerase domain
The structures of NRPs show the presence of D-amino acids to be ubiquitous, however in Nature, in the producing organisms, there is a predominant deficiency of D-amino acids. In most NRPSs a domain is present responsible for the epimerisation of the L-amino acids that are incorporated, residing at the C-terminus of the PCP within the same module as the newly added aminoacyl substrate. Epimerisation of amino acids occurs at the α-carbon of the newly added aminoacyl adenylate; in fact the amino acid must be tethered to the PCP. Figure 1.14 illustrates epimerisation of
an L-amino acid to a D-amino acid by an epimerase domain (Pfeifer et al., 1995; Stachelhaus and Walsh, 2000; Hur et al., 2012).

Studies were performed on modules 4 and 5 of the tyrocidine biosynthetic gene cluster (shown in Figure 1.16) to investigate substrate specificity of the C domain downstream for the L or D enantiomers. In module 4 of tyrocidine biosynthesis L-phenylalanine is incorporated into the peptidyl chain and is subsequently converted to D-phenylalanine by the E domain located at the C terminus of the PCP, between modules 4 and 5. The subsequent C domain (in module 5) is then responsible for the condensation of the new peptidyl growing chain and L-asparagine. This study showed that the C domain of module 5 is specific for the D-peptidyl growing chain, in addition to being specific for the L-aminoacyl adenylate (L-asparagine) to be added (Clugston et al., 2003). Although the E domain itself has been known to accept alternative substrates (Luo et al., 2001).

### 1.4.2.6 Cyclisation domain

Another domain that increases the diversity seen in NRPs is the cyclase (Cy) domain. Cy domains catalyse the formation of heterocyclic rings at cysteine, serine
Introduction

and threonine residues creating thiazoline or oxazoline rings (Hur et al., 2012). The Cy domains also catalyses the peptide bond formation between the extender unit and growing peptidyl chain (Fischbach and Walsh, 2006; Hur et al., 2012). Figure 1.15 illustrates the formation of the heterocyclic ring.

Figure 1.15 - Mechanism of heterocyclisation within NRPs. The cyclase (Cy) domain catalyses the formation of the peptide bond between the extender unit and growing peptide chain. The resulting peptide bond then undergoes intramolecular nucleophilic attack by the thiol/hydroxyl side chain, this is followed by dehydration, forming a thiazoline or oxazoline ring (Hur et al., 2012).

During heterocyclisation the thiol/hydroxyl groups of cysteine, serine and threonine residues added to the peptidyl chain can attack the peptidyl backbone creating a thiazoline or oxazoline ring. The newly formed heterocycle can then undergo further oxidation or reduction (Schneider et al., 2003; Reimmann et al., 2001).
**1.4.2.7 Additional NRP chain modifications**

*N*-methylation often aids in the biological activity of NRPs and is mediated by a methyltransferase (MT). The MT catalyses the transfer of a methyl group onto the NRP from a \((S)\)-adenosyl methionine (Hur et al., 2012). Methylation occurs when the amino acid is loaded onto the carrier protein. There are two types of MT domains, *cis*-MT domains which reside at the C-terminus of the A domains, and *trans*-acting MT domains which can methylate the amino acid extender unit or the growing peptidyl chain (Weber et al., 1994; O'Brien et al., 2000; Fischbach and Walsh, 2006).

Other modifications of NRPs can include formylation and halogenation. Formylation is mediated by a formylation (F) domain, utilising a \(N^{10}\)-formyltetrahydrofolate cofactor. Formylation is known to occur in the biosynthesis of linear gramicidins. This is the least studied accessory domain (Kessler et al., 2004; Schoenafinger et al., 2006; Hur et al., 2012). Finally, halogenation can occur on the heterocyclic rings introduced by Cy domains, this is mediated by a flavin dependent halogenase (Dorrestein et al., 2005; Hur et al., 2012).

**1.4.2.8 Linking modules in NRPS**

For efficient transfer of the peptide chain between modules, small (15-25 amino acid) domains exist between two different NRPS modules. The domains are called communication-mediating (COM) domains. COM domains reside at the C terminus of upstream module and N terminus of the downstream module; these are a cognate pair to ensure the modules are in the correct order for production of the target NRP (Hahn and Stachelhaus, 2004).

**1.4.2.9 A model system**

Tyrocidine, a cyclic NRP, is produced by *B. brevis*. Figure 1.16 illustrates the biosynthetic gene cluster of tyrocidine.
Figure 1.16- Biosynthetic gene cluster of the tyrocidine NRPS. Three polypeptides (TycA, B and C) contain a total of 10 modules. TycA contains module 1, the initiation module which incorporates L-Phe which is converted to D-Phe by an epimerase domain. Module 2 then incorporates L-Pro, followed by L-Phe in module 3. Module 4 then adds L-Phe which is converted to D-Phe. Modules 5 incorporates an L-Asn, followed by L-Gln by module 6. Modules 7 and 8 then incorporate L-Tyr and L-Val. Module 9 incorporates the unnatural amino acid ornithine (L-Orn). Finally, module 10 incorporates a L-Leu before product termination and cyclisation. The blue circle (*) on the tyrocidine structure indicates the join when the decapeptide is cyclised (Mittenhuber et al., 1989; Mootz and Marahiel, 1997).
The tyrocidine NRPS is encoded for by three open reading frames, producing three polypeptides; TycA, TycB and TycC. TycA is composed of the loading domain (module 1), TycB is composed of modules 2, 3 and 4. Finally, TycC, the largest of the polypeptides is composed of modules 5-10, modules 1 and 4 exhibit epimerase activity and module 9 incorporates the unnatural amino acid ornithine (Orn). Tyrocidine is composed of (2) D-Phe, (1) L-Phe, (1) L-Pro, (1) L-Asn, (1) L-Orn, (1) L-Gln, (1) L-Tyr, (1) L-Val and (1) L-Leu (Mootz and Marahiel, 1997). Chemical synthesis of analogues of tyrocidine, performing single amino acid substitutions has yielded analogues with improved antibacterial activities (Marques et al., 2007). It was determined that the starter unit D-Phe and L-Orn are required for cyclisation of tyrocidine (Kopp and Marahiel, 2007).

1.4.3 Structural and mechanistic studies of NRPSs

As with PKS, structural studies of entire NRPS modules are severly hindered by size and flexibility. However, structural understanding may enable combinatorial biosynthesis of these multimodular enzymes. Structure elucidation has been successful for individual domains and di-domains which has given insight into important structural characteristics, especially when considering engineering strategies.

X-ray crystallographic data shows that the adenylation domain is composed of two domains, a larger N-terminal subdomain and a smaller C-terminal subdomain (Marahiel and Essen, 2009). Crystal structures of A-domains responsible for activating 2, 3-dihydroxyxbenzoate (DhbE) from B. subtilis (May et al., 2002) and L-phenylalanine (PheA) from B. brevis (Conti et al., 1997) display highly conserved folds (Marahiel and Essen, 2009). In fact, although adenylation domains have really low sequence homology they appear to have a conserved, canonical fold (Sieber and Marahiel, 2005). Figure 1.17 shows the crystal structure of PheA, with specific residues for substrate recognition highlighted (Conti et al., 1997).

Figure 1.16 shows the crystal structure of the PheA domain. The conserved A domain structure is composed of two subdomains, a large N-terminal domain contributing 9 out of the 10 residues that compose the hydrophobic binding pocket of the substrate, phenylalanine. The C-terminal domain, a smaller domain of
Introduction

approximately 100 residues, contains K527, this residue is important for not only the coordination of Phe but also AMP (Conti et al., 1997; Stachelhaus et al., 1999).

Structural studies of DltA, the d-alanine protein ligase, a member of the adenylate activating family, provided some mechanistic insight into the adenylation domain. The small C-terminal sub-domain can be in an open state, allowing entry of the substrates, followed by a closed state, enabling adenylation of the amino acid and transfer onto the PCP (Yonus et al., 2008; Hur et al., 2012).

Structural studies of the condensation domain showed that it is also composed of N and C-terminal subdomains, forming a ‘V’ shape. Figure 1.17 shows the condensation domain VibH from Vibrio cholerae (PDB code 1L5A) (Keating et al., 2002). The ‘V’ structure of VibH revealed a solvent channel, in which the peptidyl growing chain and aminoacyl adenylate approach from each side and peptide bond formation catalysed (Keating et al., 2002).
The structure of a PCP domain has been elucidated by NMR, highlighting multiple conformations in the apo- and holo- states (Weber et al., 2000; Koglin et al., 2006), this will be further covered in chapter 5.

Crystal structures of the TycC5-6 PCP bi-domain (PDB code 2JGP) (Samel et al., 2007) from the tyrocidine NRPS and termination module from the surfactin NRPS (PDB code 2VSQ) have been solved (Tanovic et al., 2008). The bi-domain structure however did not show a functional interaction as the phosphopantetheine arm would not be able to reach the active site (Samel et al., 2007). The conformation of the PCP domain in the termination module however was in an appropriate orientation for an interaction with the C domain (Tanovic et al., 2008).

The overall module structure of mFAS, and more recently the EM structure of a PikAIII module showed these multienzymes forming a homodimer to function (Dutta et al., 2014; Asturias et al., 2005; Maier et al., 2008). In contrast in NRPSs, the TE domain and VibH C domain, suggested that NRPS are actually monomeric (Bruner et al., 2002; Tsai et al., 2001; Keating et al., 2002). Gel filtration, chemical
Introduction

cross-linking and AUC showed that the bimodule TycB was monomeric (Sieber et al., 2002; Glinski et al., 2002).

1.4.4 Engineering NRPSs

Engineering of NRPSs has been less extensive than its PKS counterparts. There are two approaches for the engineering of NRPS to yield novel products; editing the peptidyl chain or adding/removing groups on the peptidyl chain (Giessen and Marahiel, 2012). As with PKS however, heterologous expression can assist in easier manipulation for engineering novel product biosynthesis (Challis, 2006).

A target for NRPS engineering is the design of a hybrid NRPS, selecting modules depending on the desired product. This has been achieved with a two module system, creating a hybrid NRPS with a module from surfactin, selecting an aspartate residue and a second module from the tyrocidine NRPS which incorporates a phenylalanine. In addition to this, a termination module was present, this produced a functional NRPS (Doekel and Marahiel, 2000).

Originally, exchanges of A-PCP domains were made to alter the substrates incorporated; novel analogues of surfactin were made by exchanging the A-PCP didomain of module 7 with fungal A-PCP didomains, creating a hybrid NRPS. However, this yielded low product titres, potentially due to the substrate specificity of the downstream C domains (Stachelhaus et al., 1995; Belshaw et al., 1999; Ehmann et al., 2000b). The reduced product titres have also been suggested to be due to disruption of the COM domains, generating an orthogonal pair of COM domains may in fact aid in the creation of hybrid NRPS enzyme (Weissman, 2004). Identification of linkers more tolerant to forming new interactions between A and PCP domains has aided in construction of new hybrid PKS (Doekel and Marahiel, 2000).

The most promising aspect of NRPS engineering has been targeting specific residues within the active site of an adenylation domain, known as the specificity conferring code. This was originally carried out with the adenylation domain of L-Phe from the gramicidin NRPS, where mutagenesis of the PheA adenylation code led to the incorporation of L-leucine. This has also been done in the surfactin PKS, altering the
substrate specificity from L-Glu to L-Gln and L-Asp to L-Asn (Eppelmann et al., 2002). Genetic engineering of the adenylation domain in this way does seem to have some limitations, shape, size and hydrophobicity of the active site appears to control substrate specificity and thus the extent of engineering (Villiers and Hollfelder, 2009).

NRPS thioesterase modules could also be used to initiate macrocyclisation. Experiments carried out with the tyrocidine and surfactin TE domains showed that they were able to initiate cyclisation of synthetic peptides equivalent to the native peptides they cyclise (Tseng et al., 2002; Kohli et al., 2001; Trauger et al., 2000). The tyrocidine TE showed broad substrate tolerance, only really requiring the same N and C terminal residues in the peptidyl chain (Trauger et al., 2000).

The modular nature of these enzymes provides many aspects to engineer to produce novel compounds, some of these engineering strategies, such as the use of a thioesterase domain to initiate macrocyclisation can be used in conjunction with chemical synthesis to produce novel compounds (Sieber and Marahiel, 2003).

1.5 NRPS-PKS hybrid enzymes

Hybrid NRP/PK products can be found in Nature. There are two ways in which NRP/PK hybrid products are synthesised, either they are synthesised by separate enzymes and joined or they are synthesised by NRPS/PKS hybrid enzymes (Du et al., 2003).

Both NRPSs and PKSs work in an assembly line like fashion; NRPS and PKS modules are also found together in complexes in Nature, producing hybrid synthases. There are several well-characterised NRPS-PKS hybrids such as those the produce rapamycin and epothilone. The compounds that NRPS/PKS hybrid enzymes produce, just as with NRPS and PKS, can be predicted from the domains present. Hybrid assembly lines do not have to have a set ratio of NRPS to PKS modules (Schwarzer et al., 2003; Fischbach and Walsh, 2006). As usual growing chains are transferred between NRPS modules and PKS modules, switching from carbon-carbon bond formation to amide bond formation (Fischbach and Walsh, 2006; Schwarzer et al., 2003; Walsh et al., 2006). As mentioned previously, C domains in
NRPS are shown to perform both types of catalysis for bond formation (Hur et al., 2012).

Structurally, the dimeric nature of PKS modules, and the heavily implied monomeric structure of NRPS modules suggests that while the PKS portion of the enzyme dimerises, the NRPS portion remains a monomer (Sieber and Marahiel, 2005). However, investigation into the docking domains between NRPS and PKS modules suggests that NRPS potentially self-associate to aid in the interaction with their dimeric PKS partners (Sieber and Marahiel, 2005; Hur et al., 2012). Understanding the interactions between NRPS and PKS modules will theoretically increase the options when engineering NRPS and PKS modules.

1.5.1 The hybrid NRP/PK indanomycin

In Nature, pyrrole groups fulfil a number of roles, forming of hydrogen bonds, coordination of metal ions and taking part in stacking interactions (Walsh et al., 2006). Indanomycin is a pyrroloketoindane produced by an NRPS/PKS hybrid enzyme found in *Streptomyces antibioticus* NRRL 8167 (Li et al., 2009). Indanomycin displays a number of beneficial properties these include insecticidal, anti/protozoal and antibacterial properties (Liu et al., 1979; Zhang et al., 1997; Li et al., 2009) and works by complexing monovalent or divalent metal ions, using the carboxylic acid and ether oxygen to transport them across a cell membrane and disrupt ion balance (Roege and Kelly, 2009). Of the known pyrroloketoindanes, indanomycin is the only one whose biosynthetic gene cluster has been characterised.

1.5.1.1 Biosynthetic gene cluster and structure of indanomycin

Indanomycin is produced by an NRPS/PKS hybrid enzyme; this multimodular enzyme is composed of one NRPS module at the beginning of the biosynthetic gene cluster, followed by 11 PKS modules. Figure 1.19 illustrates the composition of indanomycin, composed of pyrrole-2-carboxylate, formed from the conversion of L-proline, six malonyl-CoA units, two methylmalonyl-CoA units and two ethylmalonyl-CoA units (Li et al., 2009; Roege and Kelly, 2009).
The biosynthetic gene cluster of indanomycin was discovered and characterised by the Kelly Group (Li et al., 2009). As with other NRPs and PKs, identification of the biosynthetic gene cluster involved probing for the presence of a gene suspected to be present. Knowing that indanomycin requires the incorporation of L-proline, primers were designed to probe for a proline adenylyltransferase (Li et al., 2009).

Interestingly, other NRPS such as those for tyrocidine biosynthesis (Figure 1.19) or vancomycin biosynthesis have adenylation domains which incorporate unnatural amino acids such as ornithine or β-hydroxytyrosine (Mootz and Marahiel, 1997; Smith et al., 1975) the NRPS module of indanomycin incorporates L-proline, and modifies when it is attached to the carrier protein as with biosynthesis of aminocoumarins (Garneau et al., 2005).

1.5.1.2 Starter unit biosynthesis
As mentioned previously, indanomycin is formed by an NRPS/PKS hybrid enzyme. The first module, the NRPS module, is responsible for the formation of the starter unit pyrrole-2-carboxylate instigating NRP/PK chain initiation. In starter unit biosynthesis L-Proline is activated and covalently attached to the phosphopantetheine arm of the carrier protein by the adenylyltransferase. The proline is then oxidised to a pyrrole by the flavin dependent prolyl-S-dehydrogenase to pyrrole-2-carboxylate. idmI encodes the dehydrogenase (IdmI), idmJ encodes the free standing adenylyltransferase (IdmJ), the activation domain, and idmK encodes the carrier protein (IdmK). The starter unit is synthesised then passed off into idmL, the first module of the PKS part of the NRPS/PKS (Li et al., 2009).
Introduction

It is interesting to note that the selection and activation of L-proline and the dehydrogenation to pyrrole-2-carboxylate are also observed in other biosynthetic clusters such as in aminocoumerin biosynthesis (Garneau et al., 2005). Proteins within the clorobiocin and coumermycin biosynthetic gene clusters have been shown to form pyrrole-2-carboxylate in the same manner as indanomycin, however adenylation and dehydrogenase proteins show lower sequence similarity (60-61%) to those found in indanomycin biosynthesis compared to between themselves (91-92%) (Garneau et al., 2005; Walsh et al., 2006). Genes responsible for the formation of the pyrrole group in clorobiocin and coumermycin biosynthetic gene cluster have been cloned and expressed in E. coli. ATP\(^{32}P\)PPi experiments were used to probe the substrate specificity of the adenylation domains, comparing L-proline to proline analogues and L-pipecolinic acid. This showed the adenylation domain already had the ability to accept alternative substrates (Garneau et al., 2005).

The aims of this project were to make some progress towards engineering modular enzymes to produce novel compounds with potential pharmaceutical benefits. The studies with the clorobiocin and coumermycin pyrrole forming enzymes indicated the potential for heterologous expression and expanding the substrate specificity of an L-proline activating adenylation domain.

Figure 1.20- NRPS initiation module in indanomycin biosynthesis. IdmJ, the adenylyltransferase selects L-proline as the starter unit and activates and loads it onto IdmK, the carrier protein. The prolyl-S-dehydrogenase then oxidises the proline to pyrrole-2-carboxylate (Li et al., 2009).
1.6 Project outlines

This thesis describes the experiments carried out to structurally and biochemically characterise proteins responsible for starter unit formation in indanomycin biosynthesis with the future aim to engineer these proteins as a source of novel polyketide/nonribosomal peptide production. The proposed work had three main goals:

- **Cloning and recombinant expression of proteins required for the conversion of L-proline in starter unit biosynthesis of indanomycin.** This involves cloning of the genes encoding for the adenylyltransferase (IdmJ), dehydrogenase (IdmI) and carrier protein (IdmK) into an appropriate expression vector and expression to be carried out in *E. coli*. Approaches for cloning and soluble protein expression are discussed in chapters 3 and 4.

- **Purification and biochemical characterisation of IdmJ, IdmI and IdmK.** Successful cloning and protein expression will be followed by purification by nickel affinity chromatography. Biochemical characterisation of the system will require phosphopantetheinylated IdmK, therefore investigation into exogenous PPTases with ability to modify IdmK may be required. Discussion of the expression of IdmK and obtaining a *holo*-IdmK species can be found in chapter 4. Characterisation of starter unit incorporation can be carried out *in vitro* with homogenous protein samples.

- **Structural characterisation of IdmJ, IdmI and IdmK.** After establishing a protocol to obtain a homogeneous protein sample, structural characterisation of individual proteins within the whole module can be carried out. A combination of biochemical and structural techniques can be used to investigate protein-protein interactions required for a functional NRPS module. Structural characterisation can provide insights into a structure function relationship, aiding in structure based engineering strategies. Structural characterisation of IdmK is discussed in chapter 5.
2. Materials and methods

2.1 Materials

2.1.1 Chemicals
Analytical grade chemicals and reagents were used throughout. Monobasic sodium phosphate, dibasic sodium phosphate, methanol, glycerol, glycine, sodium chloride, ampicillin, Tris base, glacial acetic acid, ethylenediaminetetraacetic (EDTA), isopropanol and ammonium acetate were purchased from Fisher Scientific Ltd. (Loughborough, UK). Agar, agarose, dithiothreitol (DTT) and isopropyl-β-D-1-thiogalactopyranoside were purchased from Melford Laboratories Ltd. (Suffolk, UK). Ethanol, bromophenol blue, N,N,N′,N′-tetramethylethylenediamine (TEMED), nickel (II) chloride, acrylamide:N,N'-methylenbisacrylamide 24:1, urea, tryptone and yeast extract were obtained from Sigma-Aldrich (Dorest, UK). Phosphate buffered saline (PBS) was purchased from Oxoid, part of Fisher Scientific Ltd. Hydrochloric acid, 2-mercaptoethanol and imidazole were purchased from Acros Organics, also part of Fisher Scientific. Ammonium persulfate was purchased from Amersham Biosciences Ltd. (Buckinghamshire, UK). Sodium dodecyl sulphate (SDS) was obtained from BDH Chemicals Ltd. (Poole, UK). SYBR® Safe was purchased from Invitrogen (Paisley, UK). 6 × Blue/Orange dye was purchased from Promega (Southampton, UK). Nickel (II) chloride hexahydrate was obtained from Alfa Aesar (Lancashire, UK). Lysozyme was purchased from MP Biomedicals (Cambridge, UK). $^{15}\text{NH}_4\text{Cl}$ and $^{13}\text{C}_6$-glucose were purchased from Cambridge Isotope Laboratories, Inc. (Leicestershire, UK).

2.1.2 Bacterial strains and plasmids
The bacterial strain used in transformations for DNA manipulation was E. coli XL10 Gold® Ultracompetent cells (TetΔ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F′ proAB lacIΔZAM15 Tn10(Tet') Amy Cam'])). Protein expression was achieved in the E. coli strain BL21-Gold (DE3) (B F−ompT hsdS(λrB mB) dcm+ Tet galX(DE3) endA Hte). The E. coli strains were both purchased from Agilent Technologies, Cheshire, UK.
Materials and methods

Both the pKK223-3 and pETDUET plasmids were provided by Prof. Alan Berry (Astbury Centre, University of Leeds, UK). The pMAL™ c5X vector was a kind gift from Dr James Ross (Astbury Centre, University of Leeds, UK). pET23a vectors were either a kind gift from Dr David Brockwell (Astbury Centre, University of Leeds, UK) or purchased from Merck chemical Ltd. (Formerly Novagen) (Nottingham, UK).

2.1.3 Enzymes
Restriction enzymes EcoRI-HF®, HindIII-HF®, KpnI-HF®, PstI-HF®, SacI-HF®, SalI-HF®, XhoI and NdeI were purchased from New England Biolabs® (Ipswich, MA, USA). PfuTurbo® was obtained from Promega (Southampton, UK). Deoxyribonuclease I from bovine pancreas was purchased from Sigma Aldrich (Dorset, UK).

2.1.4 Antibodies
The primary antibody used was the Monoclonal Anti-polyHistidine antibody produced in mouse at a 1: 3 000 dilution. The secondary antibody used was Anti-mouse IgG conjugated with horseradish peroxidase in a 1: 20 000 dilution, both were purchased from Sigma Aldrich (Dorset, UK).

2.1.5 Genes
Gene sequences from Streptomyces antibioticus NRRL 8167 encoding a prolyl-S-dehydrogenase (*idmI*), a proline adenylyltransferase (*idmJ*) and a proline carrier protein (*idmK*) were obtained from GenBank (Accession number FJ545274) (Li *et al.*, 2009) and genes were synthesised and purchased from Genscript (New Jersey, USA). Synthetic gene DNA and amino acid sequences can be found in the appendix.
2.1.6 Oligonucleotides

Oligonucleotides (primers) were synthesised and purchased from Sigma Aldrich (Dorset, UK) or Integrated DNA Technologies (IDT) (Glasgow, UK). Primer sequences can be found in the appendix.

2.1.7 Media

Bacterial cultures were grown in 2×TY medium unless stated otherwise stated. This medium contained 16 g tryptone, 10 g of yeast extract and 5 g of NaCl made up to 1 L with distilled water. Bacterial cultures of IdmJ were grown in LB medium, containing 10 g tryptone, 5 g yeast extract and 10 g NaCl made up to 1 L with distilled water. TB medium contained 12 g of tryptone, 24 g of yeast extract and 4 mL glycerol made up to 900 mL, after sterilisation by autoclaving at 121 °C for 20 min, 100 mL of sterile filtered 0.17 M KH$_2$PO$_4$ 0.72 M K$_2$HPO$_4$ was added prior to culturing. ZYP-5052 autoinduction medium contained 925 mL ZY (10 g tryptone, 5 g yeast extract made up to 925 mL with distilled water and 1 mL 1 M NaOH added, followed by sterilisation by autoclaving at 121 °C for 20 min), 1 mL 1 M MgSO$_4$ (filter sterilised), 20 mL 50 × 5052 (25 g glycerol, 73 mL distilled water, 2.5 g glucose and 10 g α-lactose filter sterilised) and 50 mL 20 × NPS (90 mL distilled water, 6.6 g (NH$_4$)$_2$SO$_4$, 13.6 g KH$_2$PO$_4$ and 14.2 g Na$_2$HPO$_4$ sterile filtered). Media was sterilised by autoclaving at 121 °C for 20 min. 1.5 % (w/v) agar was added to 2×TY for solid phase medium.

Isotopically labelled protein was produced by growing bacterial cultures in minimal medium containing 10 g K$_2$HPO$_4$, 10 g KH$_2$PO$_4$, 7.5 g Na$_2$PO$_4$, 9 g K$_2$SO$_4$ and 1 g NH$_4$Cl made up to 1 L with distilled water. Before culturing, 100 µL 1 M CaCl$_2$, 2 mL 1 M MgCl$_2$ and 20 mL 20 % (w/v) glucose were added. CaCl$_2$ was sterilised by autoclaving and MgCl$_2$ and glucose were sterile filtered. For $^{15}$N labelled protein NH$_4$Cl was substituted with $^{15}$NH$_4$Cl. For expression of $^{13}$C, $^{15}$N labelled protein the glucose was also substituted with 10 mL 20 % (w/v) D-glucose $^{13}$C$_6$. 
2.2 Methods

2.2.1 General methods

2.2.1.1 pH measurements
pH measurements were completed using a Jenway 3020 pH meter, calibrated according to the Manufacturer’s guidelines.

2.2.1.2 Centrifugation
Centrifugation was performed using an Avanti® J-26 XP centrifuge (Beckman Coulter®, Buckinghamshire, UK), at 4 °C unless otherwise stated. Samples of volumes ≤ 1.5 mL were centrifuged using a SANYO MSE Micro Centaur Centrifuge (MSE, London, UK) at room temperature unless otherwise stated.

2.2.1.3 As sterile technique
Standard aseptic techniques were employed throughout, media and heat resistant materials were sterilised by autoclaving at 121 °C for 20 min. Heat labile materials were sterilised by sterile filtering using a 0.22 µm Millex-GP syringe Tip filter purchased from EMD Millipore (Hertfordshire, UK) or wiped down with 70% (v/v) ethanol.

2.2.1.4 Antibiotic supplements
Ampicillin was made up as a 50 mg mL⁻¹ stock in distilled water, sterile filtered and used at a final concentration of 50 µg mL⁻¹ in solid phase and solution phase media.

2.2.1.5 Culture growth
Single colonies were picked from 2 × TY or LB agar plates supplemented with the correct antibiotic(s) and used to inoculate 5 mL 2 × TY or LB starter cultures in 50 mL Falcon tubes. These cultures were incubated for 16 hrs at 37 °C, shaking at 200 rpm in an orbital shaker. Unless stated otherwise, 50 mL day cultures were inoculated with 50 µL of the starter culture and incubated for 9 hrs at 37 °C, shaking
at 200 rpm in a 250 mL conical flask. 10 mL of the day culture was then used to inoculate 1 L 2 × TY medium in a 2.5 L conical flask. Protein expression was induced by the addition of IPTG, giving a final concentration of 0.1 mM unless stated otherwise. For optimal protein expression, the optical density (OD) at 600 nm was observed and the culture was induced when the cell density reached an OD of between 0.6 and 1.0.

2.2.1.6 Glycerol stocks
Glycerol stocks were used to streak out on 2 × TY or LB agar plates for culture growth (see 2.2.1.5). Glycerol stocks were made by adding 0.5 mL of starter culture to 0.5 mL sterile glycerol in a Nunc CryoTube™ (Thermo Fisher Scientific, Roskilde, Denmark), mixed well and stored at -20 °C.

2.2.2 DNA methods

2.2.2.1 Plasmid purification
Plasmid DNA was purified from 5 mL cultures using Wizard® Plus SV Minipreps DNA purification system (Promega, Southampton, UK) as instructed in the protocol provided. Plasmid DNA was purified from 50 mL cultures using HiSpeed® Plasmid Midi Kits (QIAGEN, West Sussex, UK) as per manufacturer’s guidelines.

2.2.2.2 Agarose gel electrophoresis
Unless stated otherwise agarose gels contained 0.7 % (w/v) agarose dissolved in 1 × TAE buffer and carried out according to Sambrook *et al.* (1989). A 1 kb ladder was used for determining the size of DNA fragments >2 kb, a 100 bp ladder was used for determining the size of DNA fragments <2 kb, DNA ladders were purchased from Promega (Southampton, UK).

10 × TAE buffer:

10 mM EDTA
200 mM Glacial acetic acid (17.5 M)
400 mM Tris base

2.2.2.3 Extraction of DNA from an agarose gel
DNA was purified from agarose gels by excising the bands and subsequently using a QIAquick® gel extraction kit (West Sussex, UK) according to the manufacturer’s guidelines.

2.2.2.4 Polymerase chain reaction (PCR) amplification of DNA
Different PCR extension times were used depending on the target DNA to be amplified. However, *Pfu* Turbo Polymerase was used consistently. All reactions were carried out in thin walled PCR tubes. The reaction mixture was as follows:

- 5 µL of 10 µM forward oligo
- 5 µL of 10 µM reverse oligo
- 5 µL of 0.1-50 ng/µL template DNA
- 5 µL of 2 mM dNTPs
- 5 µL of 10 × Cloned *Pfu* DNA polymerase reaction buffer
- 25 µL of sterile water

The reactions were then incubated at 95 °C for 5 mins, 0.5 µL of *Pfu* Turbo Polymerase (2.5 U µL⁻¹) was added to the reaction and the following cycling conditions were adjusted and used dependent on the length of the target DNA. A list of relevant primers and sequences can be found in the appendix.

Generally, the PCR program after the addition of *Pfu* Turbo polymerase used was as follows:

```
92 °C, 2 mins
30 × 92 °C, 30 secs
55-65 °C, 30 secs
72 °C, 1 min per kb of target
72 °C, 10 mins
```
Materials and methods

The annealing temperature was determined by subtracting 5 °C off of the primer melting temperature ($T_m$). The $T_m$ of the primer was calculated using the equation 2.1 below:

$$T_m = 64.9°C + 41.0°C \times (G + C - 16.4)/N$$

Equation 2.1- Equation to calculate the $T_m$ of the binding region of primers used in PCR amplification of DNA. Where G + C are the number of G and C bases and N is primer length.

The concentration of DNA used in the reactions ranged from 0.1 to 10 ng µL⁻¹. To assess the success of PCR amplification 5 µL of the reaction was run on an agarose gel (section 2.2.2.2).

2.2.2.5 PCR product purification

PCR products were purified using a QIAquick® PCR purification kit (West Sussex, UK) as per manufacturers guidelines, however sterile water was used to elute the DNA.

2.2.2.6 DNA quantification

DNA was quantified either spectrophotometrically at a wavelength of 260 nm, or by running a 0.7 % (w/v) agarose gel. To quantify DNA on an agarose gel densitometry was used. The intensity of the 500 bp band in the 100 bp ladder (Promega, Southampton, UK) which contains 150 ng per 5 µL of sample was compared to the target DNA band. This can be done using the GeneTools software from Syngene (Cambridge, UK).

2.2.2.7 DNA digests

DNA digests were performed on a 20 µL scale unless stated otherwise, incubated for between 2 and 16 hrs at 37 °C. The reaction set up was as follows:

2 µL compatible reaction buffer supplied
4-7 µL DNA (up to 50 ng)
1 µL of restriction enzyme 1 (20 units)
1 µL of restriction enzyme 2 (20 units)
Materials and methods

The reaction was made up to 20 µL with sterile deionised water. For single digests, one restriction enzyme was used and water adjusted accordingly. Where possible High-Fidelity (HF™) restriction enzymes were used.

2.2.2.8 Ligation reactions

DNA ligations were completed between a gene, termed the insert, and a target vector. Reactions were performed in thin-walled PCR tubes in a 10 µL reaction volume. Insert and vector DNA were incubated together in a molar ratio of 3:1 at 4 °C for 16 hrs in a reaction containing 1 µL T4 DNA ligase and 1 µL of 10 × T4 DNA ligase buffer. Between 2 and 4.5 µL of the reaction was then transformed into XL10 Gold® Ultracompetent cells (section 2.2.2.11).

2.2.2.9 Ligation independent cloning

Ligation independent cloning was carried out using the FastCloning method as described in Li et al. (2011). Primers were designed to amplify the target insert (gene) and vector creating complimentary 5’ and 3’ ends of DNA fragments (2.2.2.4). An agarose gel was used to confirm the presence of the PCR products (2.2.2.2), which were then mixed in a 1:1 volumetric ratio. The reactions were digested with DpnI for 1 hr and subsequently transformed into XL10 Gold® Ultracompetent cells (2.2.2.10). Figure 3.28 in section 3.3.6 illustrates the FastCloning strategy.

2.2.2.10 Site directed mutagenesis

Site-directed mutagenesis was performed using a QuikChange® Site-directed mutagenesis kit (Stratagene, Cambridge, UK) as per the manufacturer's instructions. Primers were designed as recommended by the manufacturer. Site-directed mutagenesis primers can be found in the appendix.
2.2.2.11 Transformation into E. coli cells
Plasmid DNA was transformed into E. coli XL10 Gold® Ultracompetent cells and/or E. coli BL21-Gold (DE3) cells (Section 1.1.2) using the heat shock method according to the manufacturer’s guidelines.

2.2.2.12 DNA sequencing
DNA sequencing was carried out by Beckman Coulter Genomics (Essex, UK). Sequencing primers used can be found in the appendix.

2.2.3 Protein methods
2.2.3.1 Small scale protein expression trials
Small scale protein expression was performed on a 5 mL scale. Glycerol stocks of E. coli BL21-Gold (DE3) cells containing the target plasmid were plated onto 2 × TY agar plates supplemented with ampicillin at a final concentration of 50 μg mL⁻¹ (section 2.2.1.4), plates were incubated at 37 °C for 16 hrs. A single colony was then used to inoculate 5 mL 2 × TY starter culture supplemented with ampicillin (section 2.2.1.4), this was incubated at 37 °C for 16 hrs. 5 μL of the starter culture was then used to inoculate a 5 mL culture of TB, 2 × TY, LB or autoinduction medium containing ampicillin (section 2.2.1.4). TB, 2 × TY and LB cultures were incubated at 37 °C until the optical density of the culture at 600 nm reached between 0.6 and 1.0, cells were then induced by the addition of IPTG giving a final concentration of 60 μM. Cultures were then incubated for 16 hr at 37, 21 or 18 °C. Cells were then harvested for analysis of protein expression.

2.2.3.2 Sample preparation for analysis of protein expression
Cells were harvested from 5 mL expression cultures by centrifugation of 1.5 mL of the culture in the microfuge at 13 300 × g for 5 min. Harvested cells were then resuspended and incubated with 400 μL of CelLytic™ B lysis reagent for 10 min, followed by centrifugation at 13 300 × g for 5 min to separate the soluble and insoluble cellular components. Insoluble cellular components were resuspended in
Materials and methods

100 μL of PBS. Samples were then prepared for analysis by SDS-PAGE and N-terminal His$_6$-tag detection by blotting with an anti-polyHistidine antibody (sections 2.2.3.3 and 2.2.3.6). Samples for SDS-PAGE were diluted 1:4 with sterile H$_2$O, samples for His$_6$-tag detection were not diluted.

2.2.3.3 Detection of the N-terminal His$_6$-tag

Detection of the N-terminal His$_6$-tag was carried out by blotting 2 μL of soluble or insoluble cellular samples onto a nitrocellulose membrane. The blotted membrane was left to dry for 20 mins, then incubated for 1 hr at room temperature in blocking buffer. The blocking buffer was then removed and the membrane incubated with 10 mL of primary antibody solution for 1 hr. The membrane was then washed with PBS-T for 10 mins; this was repeated 2 more times. The membrane was then incubated with 10 mL of secondary antibody solution for 45 mins at room temperature. The membrane was washed with PBS-T for 10 mins (3 times), followed by a 10 min wash in PBS. The membrane was then visualised by incubation with ECL Prime Western blotting detection reagent (VWR International Ltd, Leicestershire, UK) for 1 min, with increasing levels of exposure using a GeneGnome® system (Syngene, Cambridge, UK).

For detection of the N-terminal His$_6$-tag by western blot an SDS-PAGE gel was run as described in section 2.2.3.6. The SDS-PAGE gel was then placed in transfer buffer for 15 mins. Transfer of proteins from the SDS-PAGE gel to a nitrocellulose membrane was performed using a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad, Hertfordshire, UK). The transfer stack was assembled as instructed by the manufacturer, transfer was carried out at 100 V for 1 hr. Blocking was performed as described above, however the blocking was carried out at 4 °C and the blocking buffer contained 3 % BSA as opposed to 5 % skimmed dried milk powder. The antibody solution incubations, washing and visualisation was carried out as described above.

PBS-T:

$1 \times$ PBS

0.05 % (v/v) Tween-20
Materials and methods

Blocking buffer:
PBS-T
5 % skimmed dried milk powder or 3 % BSA

Antibody solution:
Blocking buffer
Antibody at appropriate dilution (section 2.1.4)

Transfer Buffer:
24 mM Tris base
194 mM Glycine
20 % (v/v) methanol

2.2.3.4 Nickel affinity purification of His₆-tagged proteins

E. coli cells were cultured, as described in section 2.2.1.5, containing the plasmid with the target gene for protein expression. Protein purification was performed in batch using Chelating Sepharose™ fast flow resin chelated with nickel ions. E. coli cells were harvested by centrifugation (2.2.1.2) at 12 000 g for 20 mins. The supernatant was discarded and the pellet re-suspended using a homogeniser in washing buffer, 10 g of cells in 50 mL buffer. The cells were then lysed using a cell disrupter from Constant Systems Ltd (Northamptonshire, UK) at 20 KPSI. The insoluble cell debris was then removed by centrifugation at 40 000 g for 45 mins. The supernatant containing the soluble target protein was then subjected to purification by nickel affinity chromatography.

The supernatant was incubated with the 5 mL of nickel resin in a 50 mL Falcon tube for 30 mins with mild agitation at 4 °C. The resin was then centrifuged at 4 000 g for 10 mins, the supernatant was discarded. The resin was then washed by the addition of washing buffer to a final volume of 50 mL, re-suspended thoroughly, then centrifuged at 4 000 g for 10 mins and the supernatant removed. This wash step was repeated 4 times. Bound protein was eluted by the addition of 15 mL of elution buffer to the resin and incubated for 1 hr at 4 °C with mild agitation; the resin was centrifuged at 4 000 g for 10 mins and the supernatant, containing the protein, was
Materials and methods

collected. The eluate was then dialysed into 50 mM Tris.HCl pH 7.4 as described in section 2.2.3.5. After dialysis protein was sterile filtered using 0.22 µm Millex-GP syringe tip filters and stored at 4 °C. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (2.2.3.6) and mass spectrometry (2.2.3.7) was used to confirm the presence of the correct protein.

Protein purification washing buffer:

50 mM Tris.HCl pH 7.5
20 mM Imidazole
0.5 M NaCl

Protein purification elution Buffer:

50 mM Tris.HCl pH 7.5
0.5 M Imidazole
0.5 M NaCl

2.2.3.5 Dialysis
Dialysis was performed at 4 °C in volumes 50-100 times larger than the sample, with mild stirring. For proteins smaller than 14 000 Da dialysis tubing with a molecular weight cut off of 3 000 Da was used, otherwise tubing with a molecular weight cut off of 14 000 Da was used. Dialysis tubing was purchased from Fisher Scientific Ltd. (Loughborough, UK). Briefly, samples for dialysis were placed in dialysis bags in the dialysis buffer. Samples were left to equilibrate for 4 hrs, and then the dialysis bag was transferred to freshly made dialysis buffer and again left for 4 hrs to equilibrate. After equilibration, the sample was sterile filtered using 0.22 µm Millex-GP syringe tip filters.

2.2.3.6 Reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis
Reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the standard method (Laemmli, 1970). For approximate molecular weight determination the Prestained Protein Marker, Broad Range (7-175 kDa) ladder was used (NEB, Ipswich, MA, USA). These contain the protein
Materials and methods

markers: *E. coli* MBP-β-galactosidase (175 kDa), *E. coli* MBP-paramyosin (80 kDa), *E. coli* MBP-CBD (58 kDa), *E. coli* CBD-Mxe Intein-2CBD (46 kDa), *E. coli* CBD-Mxe Intein (30 kDa), *E. coli* CBD-BmFKBP3 (25 kDa), chicken egg white lysozyme (17 kDa) and bovine lung aprotinin (7 kDa). SDS-polyacrylamide gel electrophoresis was carried out as described in Sambrook *et al.* (2001) using a 15% acrylamide resolving gel and 3.75% acrylamide stacking gel (recipe listed below). Ammonium persulfate (APS) was made up freshly for usage. The two polymerisation initiation components, APS and TEMED, were added to the gel solutions last. Gels were set between two glass plates as per manufacturer’s guidelines. The resolving gel was poured immediately followed by an isopropanol layer and allowed to set. The isopropanol was removed and the stacking gel poured and a comb inserted. Once the stacking gel was set, gels could be used immediately or wrapped in damp laboratory roll and covered in cling film. Wrapped gels could be stored at 4 °C for up to 1 week.

Resolving gel:

7.5 mL 30 % (w/v) acrylamide
3.75 mL 1.5 M Tris.HCl pH 8.8
3.5 mL H₂O
150 µL 10 % (w/v) SDS
50 µL 25 % (w/v) APS
5 µL TEMED

Stacking gel:

625 µL 30 % (w/v) acrylamide
625 µL 1 M Tris.HCl pH 6.9
3.65 mL H₂O
50 µL 10 % (w/v) SDS
50 µL 25 % (w/v) APS
5 µL TEMED

2 × loading buffer:

154 mg DTT
2 mL 10 % (w/v) SDS
Materials and methods

1 mL glycerol
170 µL 1 M Tris.HCl pH 6.9
163 µL H₂O
200 µL 0.2 % (w/v) bromophenol blue in ethanol

Running buffer:
3 g Tris Base
14.4 g glycine
1 g SDS
140 µL β-mercaptoethanol
Made up to 1 L with H₂O

Protein samples were prepared by adding 2 × loading buffer in a 1:1 ratio and boiled at 100 °C for 5 mins. Electrophoresis was performed at 30-60 mA for approximately 1-2 hrs. SDS-PAGE gels were then stained in a methanol/ acetic acid/ water (5/1/1; v/v/v) solution containing 0.1 % (w/v) Coomassie Brilliant Blue for 2 hrs and subsequently destained using a methanol/acetic acid/water (5/1/1; v/v/v) solution. Alternatively, for rapid determination of results, gels were stained using InstantBlue (Expedeon, Cambridgeshire, UK) or Quick Blue Coomassie Stain (Triple Red, Buckinghamshire, UK). InstantBlue was used for in-gel tryptic digests (section 2.2.3.10).

2.2.3.7 Determination of protein concentration
Protein concentration was determined spectrophotometrically at 280 nm and calculated using the Beer-Lambert law (Equation 2.2).

\[ A = \varepsilon cl \]

Equation 2.2- Beer-Lambert law, where A is the absorbance, \( \varepsilon \) is the molar extinction coefficient, \( c \) is the concentration of the protein and \( l \) is the path length.

The molar extinction coefficient for each protein was calculated using their amino acid sequence and the ExPASy ProtParam tool. The calculated molar extinction coefficient was used throughout this research. The molar extinction coefficient calculated by ExPASy ProtParam for IdmJ was 51130 M⁻¹ cm⁻¹, for IdmI was 25940 M⁻¹ cm⁻¹ and for IdmK was 5500 M⁻¹ cm⁻¹.
Materials and methods

2.2.3.8 Increasing protein concentration
Protein samples required to be at a higher concentration were concentrated by centrifugation through using a centrifugal concentrator. For sample volumes larger than 0.5 mL Sartorius Stedium Vivaspin® 6 or 20 centrifugal concentrators with a molecular weight cut-off of either 3 kDa or 10 kDa depending on the proteins’ molecular weight. For samples smaller than 0.5 mL Sartorius Stedium Vivaspin® 500 centrifugal concentrators were used, with a molecular weight cut-off of 5 kDa (Goettingen, Germany). Concentrators were used in accordance with the manufacturer’s guidelines.

2.2.3.9 Sample preparation for mass spectrometry
Buffer exchange or desalting of protein samples was carried out using either Zeba™ Spin Desalting Columns purchased from Thermo Fisher Scientific (Loughborough, UK) or Bio-Spin Exclusion Columns from Bio-Rad (Hertfordshire, UK) according to the manufacturer’s guidelines. For analysis by mass spectrometry, protein samples were exchanged into 50 mM ammonium acetate pH 7.4.

2.2.3.10 In-gel tryptic digestion and analysis by LC-MS/MS
In-gel tryptic digestion and analysis LC-MS/MS was carried out by Dr James Ault (Astbury Centre, University of Leeds, UK). Proteins to be analysed by in-gel tryptic digestion were run on a 15 % acrylamide gel as described in section 2.2.3.6. The gel was stained with InstantBlue (Expedeon, Cambridge, UK) to identify the target band. The band/s were excised and split into ~ 1 mm pieces. To remove the InstantBlue stain, gel pieces were immersed in 30 % ethanol in a 0.5 mL Eppendorf tube then incubated at 60 °C for 30 mins with shaking. Gel pieces were incubated in 100 % acetonitrile for 5 mins, followed by 40 µL 20 mM DTT in 25 mM ammonium bicarbonate at 56 °C for 1 hr with shaking. The supernatant was removed, substituted with 40 µL of 55 mM iodoacetamide in 25 mM ammonium bicarbonate, which was then incubated 37 °C for 45 min shaking, in the dark. The supernatant was removed and gel slices washed with 25 mM ammonium bicarbonate for 10 mins at room temperature, this was followed by a 5 min incubation in 100 % acetonitrile. Gel slices were then removed from the acetonitrile and left to dry completely for 1 hr.
Materials and methods

under a laminar flow hood. Once the gel pieces were dry they were cooled on ice and subsequently incubated with an ice-cold solution of 20 ng/µL trypsin in 25 mM ammonium bicarbonate for 10 mins, on ice. The supernatant was removed and 40 µL of 25 mM ammonium bicarbonate added to the rehydrated gel slices. The gel slices were briefly vortexed and centrifuged then incubated at 37 °C for 18 hrs. The digested peptides were recovered by vortexing and centrifugation of the solution, incubated for 18 hrs and the supernatant transferred to a fresh 0.5 mL Eppendorf tube. 5 µL of wash solution (acetonitrile/water/formic acid (60/35/5; v/v)) was added to the peptide solution. Additional peptide recovery from the gel pieces was performed by incubation of the gel slices with 40 µL of wash solution for 10 mins at 37 °C, the resulting supernatant was combined with the original peptide recovery solution; this was repeated a second time. The peptide solution was then dried by vacuum centrifugation. In preparation for separation by liquid chromatography (LC) and analysis by MS/MS, the dried peptides were resuspended in 20 µL acetonitrile/water/formic acid (2/97.9/0.1; v/v/v).

In order to separate the peptides, LC was carried out on the peptide mixture using an Ultimate 3000 nano LC system (Dionex, Amsterdam, Netherlands). 2 µL of the peptide mixture was loaded onto a C18 guard column. The column was then washed with 2 % acetonitrile/0.1 % formic acid for 5 min at 25 µL min⁻¹. The peptides were then separated by performing a gradient of 2-40% of 0.1% formic acid in acetonitrile with 0.1% formic acid in water over 60 min at 0.35 µL min⁻¹, on a PepMap C18, 100 µm i.d. x 15 cm analytical column (Dionex, Amsterdam, NL).

The peptides separated on the column were then analysed directly by a quadrupole-ion mobility - orthogonal time of flight mass spectrometer (Synapt G2-S, Waters UK, Manchester, UK) via Nano Flow electrospray ionisation (ESI). The mass spectrometer (MS) was operated in positive mode time of flight (TOF), using a capillary voltage of 3.8 kV and a sampling cone voltage of 20 V, source offset of 15 V and a trap bias of 5 V and backing pressure of 8.47 mbar. The buffer gas utilised was argon at a pressure of 5.0 × 10⁻⁴ mbar in both the trap and transfer regions. The source temperature was 80°C. Mass calibration was carried out through a separate injection of sodium iodide (2 µg/µL). A 1 µM solution of the peptide mass standard Glu-1- Fibronopeptide B (Glu-Fib) in acetonitrile/water/formic acid (50/49/1; v/v/v) was introduced as the lock mass calibrant at a flow rate of 1 µL min⁻¹ with a one sec
Materials and methods

lock-spray scan collected every 30 sec during data acquisition. To determine the lock mass calibration correction factor ten scans were averaged. Data acquisition was carried out using a data dependent analysis. A one sec MS over \( m/\epsilon \) 350-2000 was collected followed by three one sec MS/MS performed over \( m/\epsilon \) 100-2000 of the three most abundant ions in the MS spectrum. Capillary electrophoresis applied to the ion was dependent on its charge state and mass. For increased sequence coverage dynamic exclusion (60 sec) was used.

Data was processed using the MassLynx v4.1 suite of software supplied with the mass spectrometer. Peptide MS and MS/MS data were processed with ProteinLynx Global Server v3.0.2 software (Waters UK, Manchester, UK) and searched against reviewed entries of the UniProtKB/SwissProt database (release 2014_03), the database was modified to include the amino acid sequence of the synthetic IdmJ protein (amino acid sequence located in the appendix).

2.2.3.11 Cysteine alkylation

Alkylation of cysteines was performed using a 20-30 \( \mu \)M protein sample in 50 mM Tris.HCl pH 7.4 containing 8 M urea on a 500 \( \mu \)L scale. To reduce samples, DTT was added to a final concentration of 5 mM from a 0.5 M stock and incubate at 55 °C for 25-45 mins. The protein was allowed to cool to room temperature. 500 mM iodoacetamide was then added to a final concentration of 14 mM and incubated for 30 mins at room temperature in the dark. The reaction was then quenched by either adding DTT to give a final concentration of 10 mM or by preparing the samples for analysis by mass spectrometry by exchanging the buffer (2.2.3.9).

2.2.3.12 Mass spectrometry

Samples to be analysed by mass spectrometry were desalted into 50 mM ammonium acetate as described in section 2.2.3.9. Samples were then analysed by nano-ESI-MS by a quadrupole-ion mobility spectrometry-orthogonal TOF MS (Synapt, HDMS Waters UK Ltd., Manchester, UK) operating in positive TOF ‘V’ mode. Samples were analysed in acetonitrile : 1% aqueous formic acid (50:50; v/v). The MS was operated with a capillary voltage of 1.2 kV and a cone voltage of 50 V, the
nanoelectrospray nitrogen pressure was set to 0.1 mbar with a backing pressure of 1.78 mbar. Nitrogen was used as a buffer gas at a pressure of $8.0 \times 10^{-3}$ mbar in the trap and transfer regions and $3.6 \times 10^{-4}$ in the ion mobility cell. The source temperature was 80 °C and desolvation temperature 150 °C. Mass calibration was carried out through a separate injection of sodium iodide (2 µg/µL) in 50:50 (v/v) water: acetonitrile. Data was processed using the MassLynx v4.1 suite of software supplied with the mass spectrometer. Analysis of protein samples by mass spectrometry were carried out by Dr James Ault (Astbury Centre, University of Leeds, UK).

2.2.3.13 Protein purification by size exclusion chromatography
For increased homogeneity, protein samples purified by nickel affinity chromatography (section 2.2.3.4) were subject to further purification by size exclusion chromatography. This was performed using an ÄKTA Prime Purifier System and a HiLoad™ Superdex™ S75 prep grade column (GE Healthcare Life Sciences, Buckinghamshire, UK). The column was equilibrated with a selected buffer which had been filtered and degassed. 3 mL of protein purified by nickel affinity chromatography at a concentration of 8 mg/mL was loaded onto the column using a 5 mL sample loop (GE Healthcare Life Sciences). The column was run at a flow rate of 2.0 mL/min. 2 mL fractions were collected and analysed by SDS-PAGE (2.2.3.3), fractions containing the purified protein were pooled and concentrated (2.2.3.5).

2.2.4 Crystallographic methods

2.2.4.1 Sample preparation
A homogeneous protein sample was prepared through purification by nickel affinity chromatography (2.2.3.4) and subsequent size exclusion chromatography (2.2.3.13). The protein sample was sterile filtered using 0.22 µm Millex-GP syringe tip filters purchased from EMD Millipore (Hertfordshire, UK) and concentrated (2.2.3.8) to 12 mg/mL.
2.2.4.2 Screening conditions and crystal trays
Crystallisation condition screens were carried out using 96 well CrystalQuick™ RW sitting drop trays purchased from Greiner Bio-One (Gloucestershire, UK) with a 10 mg/mL protein sample. The commercially available screens used were Crystal screen 1 and 2, Index 1 and 2, Salt RX (Hampton Research, Californiia, USA) and Wizard 3 and 4 (Emerald BioStructure, Washington, USA).

Crystallisation conditions identified for the prolyl carrier protein were pH 8.2, 0.056 M sodium phosphate monobasic monohydrate, 1.344 M potassium phosphate dibasic. Crystals were grown in 24 well plates by hanging drop vapour diffusion. Drops were dispensed in a 1:1, 2:1 and 1:2 crystallisation buffer to protein ratio. Protein samples were of a concentration 10 mg/mL.

2.2.4.3 Data acquisition and processing
Data acquisition and processing was carried out by Dr Chi Trinh (Astbury Centre, University of Leeds, UK). For data acquisition crystals were prepared by immersing in 25% (v/v) glycerol as a cryo-protectant, prior to flash cooling in liquid nitrogen. High resolution diffraction data was collected at 100 K on the I04 beamline Diamond Light Source Ltd (DLS) (Oxfordshire, UK). The data were reduced using the automated processing suit Xia2 at DLS (Winter, 2010). Phasing by molecular replacement was attempted using both Molrep (Vagin and Teplyakov, 1997) and Phaser (McCoy et al., 2007) with different homologous carrier proteins as the initial search model (PDB codes: 2FAE (Roujeinikova et al., 2007), 2LJU (Busche et al., 2012), 2LKI (Srisailam et al., 2006), 2XZ1 (Guy et al., 2011), 3GZL (Gallagher and Prigge, 2010), 2LPK (Ramelot et al., 2012), 1X3O (unpublished, Riken Structural Genomics/Proteomics), 2EHT (unpublished, Riken Structural Genomics/Proteomics), 2QNW (unpublished, Structural Genomics Consortium) and 2L4B (unpublished, Seattle Structural Genomics Center for Infectious Disease). SWISS-MODELLER (Arnold et al., 2006; Biasini et al., 2014; Guex et al., 2009; Kiefer et al., 2009) and ITASSER (Roy et al., 2010; Zhang, 2008) were also used to generate models from the IdmK amino acid sequence.
2.2.5 Nuclear magnetic resonance (NMR) spectroscopy methods

2.2.5.1 Sample preparation
Uniformly \( ^{15}\text{N} \) and \( ^{13}\text{C}, ^{15}\text{N} \) labelled protein was prepared by growing the bacterial cultures in minimal medium (2.1.7) using \(^{13}\text{C}_6\) glucose and \(^{15}\text{NH}_4\text{Cl} \) as the sole carbon and nitrogen sources. Labelled protein was expressed and purified as described in sections 2.2.1.5, 2.2.3.4 and 2.2.3.13. Mass spectrometry (section 2.2.3.12) was used to determine the extent of isotopic labelling. Protein samples were dialysed into 50 mM Tris.HCl pH 7.4, 0.02% (w/v) sodium azide (section 2.2.3.5). Samples to be used for NMR spectroscopy were concentrated to 0.5 mM and 10% (v/v) D\(_2\)O was added. Shegemi NMR tubes were used for data acquisition with samples containing 300 µL of the protein sample.

2.2.5.2 Data acquisition
2D and 3D NMR experiments were carried out at 25°C on Varian INOVA spectrometers (Varian Inc., California, USA) at 500 or 600 MHz equipped with room temperature probes or at 750 MHz with a cryogenically cooled probe. Table 2.1 shows the experimental details used to collect each spectrum.

2.2.5.3 Data processing and analysis
All data collected was processed with NMRPipe software (Delaglio et al., 1995). Typical data processing included multiplication by a cosine bell, followed by zero filling to increase the number of points at least by a factor of 2 followed by rounding. Fourier transformation was performed in all dimensions. In the case of the final \(^{15}\text{N} \) or \(^{13}\text{C} \) dimension, to double the number of data points, mirror image linear prediction was carried out. Phase corrections in the direct dimension were manually adjusted using NMRDraw and NMRPipe (Delaglio et al., 1995). In most cases phase corrections were not required in the indirect dimension due to use of an optimal initial delay time, however, when required they were adjusted in the same manner as the direct dimension. Data were converted to NMRView format by NMRPipe for use with CCPNmr analysis (Vranken et al., 2005).
Materials and methods

An initial $^1$H-$^{15}$N heteronuclear single quantum coherence spectrum (HSQC) was collected to assess the suitability of NMR for structural determination and further HSQC spectra were collected for sample quality control before or after collection of datasets. Backbone assignments were completed using the following spectra: HNCA, HNcoCA, HNcaCO, HNCO, HNCACB and HNcoCACB. Aromatic residue assignments were completed using 2D aromatic filtered $^1$H-$^{13}$C-NOESY-HSQC and 2D hbCBgcdHDceHE spectra (Marion et al., 1989a; Marion et al., 1989b; Zuiderweg and Fesik, 1989; Yamazaki et al., 1993; Kalverda et al., 2009). A 2D aromatic filtered $^1$H-$^{13}$C-NOESY-HSQC spectrum was acquired by using a standard $^1$H-$^{13}$C NOESY-HSQC pulse sequence (Biopack) with no increments for $^{13}$C and the $^1$H-$^{13}$C one-bond transfer time as 14.5 ms. Assignments of aliphatic amino acid side chain were completed using $^1$H-$^{13}$C HSQC, HcCH-TOCSY and hCCH TOCSY spectra. Finally distance restraints were measured using $^{15}$N-NOESY-HSQC and $^{13}$C-NOESY-HSQC spectra.

Backbone assignments were completed by a semi-automated method using MARS (Jung and Zweckstetter, 2004) and checked manually using strip plots. Secondary structure predictions for use with MARS were calculated using the PSIPRED server at http://bioinf.cs.ac.uk/psipred (Buchan et al., 2013; Jones, 1999). TALOS was used to predict $\Phi$ and $\Psi$ torsion angles and assigned from $^1$H$\alpha$, $^{13}$C$\alpha$, $^{13}$C$\beta$, CO and $^{15}$N nuclei from backbone assignments to assign secondary structural preferences (Shen et al., 2009a).

Chemical shift referencing was derived from the chemical shift of the water line assuming a temperature of 25°C and a pH of 7.4 (Cavanagh et al., 2007). Following backbone assignment the chemical shift referencing was further validated using the program CheckShift (Ginzinger et al., 2007). Chemical shift referencing with the referencing compound DSS is still required.
### Materials and methods

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Spectral frequency ((^1)H) MHz</th>
<th>SW (Hz)</th>
<th>Number of (complex) data points</th>
<th>nt</th>
<th>t\text{mix} (s)</th>
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<th>Experiment</th>
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<th>SW (Hz)</th>
<th>Number of (complex) data points</th>
<th>nt</th>
<th>t\text{mix} (s)</th>
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<td>300</td>
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Table 2.1- Experimental parameters of spectra acquired on the Varian INOVA spectrometer (Varian Inc., California, USA). SW-spectral width, nt- number of transients and t\text{mix}-mixing time (s).


2.2.5.4 Structure generation

Structure determination by CS-ROSETTA was aided by Dr Gary Thompson. An initial structure ensemble was generated using CS-ROSETTA 3.2 (Shen et al., 2008; Shen et al., 2009b), using H, HN, CA, CB and CO chemical shifts from the backbone assignments and the amino acid sequence of IdmK. A total of 5000 structures were calculated on a cluster of 7-14 CPU processors. Residual dipolar coupling (RDC) Q factor and combined chemical shift and ROSETTA energy scores were used to score the ensemble of structures. RDC Q values were calculated using PALES as described in section 2.2.5.5 and 5.3. Further validation was carried by comparison with known canonical acyl carrier protein folds (PDB codes: 1HY8, 1T8K, 1VKU, 2AVA, 2CGQ, 2GDW, 2KOO, 2KWL and 2L0Q) (Xu et al., 2001; Qiu and Janson, 2004; Zornetzer et al., 2006; Koglin et al., 2006; Ploskon et al., 2010; Barnwal et al., 2011; Chan et al., 2010) and structure homology searches were completed using the Dali server (http://ekhidna.biocenter.helsinki.fi/dali_server/) (Holm and Rosenstrom, 2010).

To validate the ROSETTA fold and generate a high resolution NOE (nuclear Overhauser effect) derived structure of IdmK, refinement of an ensemble of structures from the ROSETTA calculation was carried out using ARIA/CNS (Rieping et al., 2007). The structure ensemble was selected by taking the structure with the best fit for secondary structure elements to the measured $^1$H-$^{15}$N RDC data as measured by the RDC Q factor. A set of 20 structures were selected with the lowest RMSD for the backbone secondary structure elements when compared to this structure. ROSETTA structures were regularised using the xplor-nih script (AddAtoms.py) (Schwieters et al., 2006) with all backbone atoms fixed during minimisation (Schwieters et al., 2003; Schwieters et al., 2006).

ARIA structure calculations were carried out by Dr Gary Thompson. ARIA structures were calculated using the best fitting ROSETTA structures as a seed for structure calculation, replacing those generated by ARIA in the first of eight rounds of calculations (it0). The hot and cooling/annealing phases in the ARIA structure calculation were extended by a factor of 4 in length (Fossi et al., 2005b). The final structures were refined in a box of water. Chemical shift restraints were included as $\Phi$ and $\Psi$ dihedral restraints for regions of secondary structure showing consistent
Materials and methods

TALOS predictions (Rieping et al., 2007). RDC Q values were calculated using Equation 2.3 (Cornilescu et al., 1998).

\[ Q = \frac{\sqrt{\sum_i (RDC_{i}^{obs} - RDC_{i}^{calc})^2}}{\sqrt{\sum_i (RDC_{i}^{obs})^2}} \]

Equation 2.3 - Equation to calculate the agreement between experimental and calculated RDCs (Q factor), where i is the residue (Zweckstetter, 2000).

2.2.5.5 RDC alignment media and measurements

Residual dipolar couplings were measured in two different liquid crystalline alignment media, PF1 filamentous bacteriophage and an alkyl-poly (ethylene-glycol)/n-alkyl alcohol mixture.

For alignment of IdmK in Pf1 bacteriophage (ASLA Biotech, Estonia), an initial \(^1\)H-\(^15\)N HSQC was collected (isotropic) then Pf1 bacteriophage was added to a final concentration of 8 mg/mL. A \(^1\)H-\(^15\)N HSQC was collected and processed as described in section 2.2.5.3. This spectrum was used to determine the suitability of Pf1 bacteriophage as an alignment medium.

A second alignment medium was trialled. The alkyl-poly (ethylene-glycol)/n-alkyl alcohol mixture was used. Hexanol, the n-alkyl alcohol selected, and C12E6 alkyl-poly (ethylene-glycol) (PEG) alignment medium was made at a (2x concentration) by titrating hexanol into a solution of 10% C12E6 using 5\(\mu\)L and 0.5 \(\mu\)L MicroVolume syringes (SGE Analytical Science, Milton Keynes, UK). The magnitude of alignment of the dipolar coupling medium was monitored using the quadrupolar splitting of HDO peak in the D\(^2\)O lock solvent. For the Hexanol/PEG medium the quality of the alignment was followed by monitoring the lineshape in the sample, which showed a clean doublet with a splitting of 29 Hz when complete alignment was achieved at a PEG concentration of 10%. Final samples were prepared by adding 50% of the hexanol/PEG solution to the NMR sample. After alignment of the protein the deuterium splitting was checked again and adjustments to achieve a clean spectrum with a deuterium splitting of \(~25\)Hz were made with hexanol or PEG solution. The quality of the data and deterioration of the spectral quality due to over alignment and
increases in the rotational correlation time from the aligned samples was also monitored using $^1$H-$^{15}$N HSQC spectra and $^1$H coupled $^1$H-$^{15}$N HSQC spectra. RDC Measurements were made using $^1$H-$^{15}$N J modulated HSQC spectra (Tjandra et al., 1996) with $^1$J$_{HN}$ evolution delays ($\Delta$) of 0.001 ($\times 2$), 0.003, 0.005, 0.007, 0.009, 0.011, 0.013, 0.015 ($\times 2$), 0.017, 0.019, 0.021 and 0.023 s. Data were processed with NMRpipe and peak intensities were measured using NMRView 5.2. Fitting of peak intensity data was achieved using the in house python script fitR_bs (Dr Gary S Thompson and Dr Christopher MacRaild) which carries out non-linear least squares fitting starting with initial fitting parameters estimated using a number of heuristics. Statistical error analysis was carried out via 1000 rounds of Monte-Carlo simulations, with noise derived from differences in peak intensities from the 2 data sets measured with duplicate delays. The data was fitted to the equation 2.3.

$$I_{2\Delta} = I_0[-A + \cos(2\pi^2)_{NH\Delta}] \exp\left(-\frac{2\Delta}{T^*_2}\right)$$

Equation 2.4- With $I_0$ being the initial peak amplitude, $\Delta(t)$ the time delay (s), $A$ the unmodulated component of the peak intensity, $^1$J$_{NH}$ the measured one-bond coupling (with or without the dipolar component) and $T^*_2$ the effective transverse relaxation rate (Tjandra et al., 1996).

Tensor frames, predicted RDCs, magnitude of alignment ($D_a$), rhombicity ($R$) and quality of fit ($Q$) for RDCs were calculated using PALES (Zweckstetter and Bax, 2000). PALES fits the observed dipolar couplings to the alignment tensor frame, $D_a$ and $R$; values from which predicted dipolar couplings can be back calculated using the Equation 2.4.

$$D = D_a \left[\left(3 \cos^2 \theta - 1\right) + \frac{3}{2} R \sin^2 \theta \cos(2\varphi)\right]$$

Equation 2.5-Where $\theta$ and $\varphi$ are polar angles with respect to the alignment tensor frame ($D_a$ and $R$) (Schwieters et al., 2003; Schwieters et al., 2006).

2.2.5.6 Initial structural investigations of holo-IdmK

To investigate structural differences of holo-IdmK and apo-IdmK experiments were run using $^{15}$N holo-IdmK. To ensure monomeric-labelled holo-IdmK was present experiments were run in the presence of 1 mM DTT. An initial $^1$H-$^{15}$N HSQC was
Materials and methods

collected of $^{15}\text{N }$holo-IdmK. Conservative chemical shift perturbation mapping was used in order to identify sites of interaction of the post-translational modification without assignment of the holo state (Williamson et al., 1997). Combined $^1\text{H},$ $^{15}\text{N}$ chemical shift differences were calculated Equation 2.5.

$$\Delta = \left[ \left( \delta^{15}\text{N}_{\text{apo/ho}} \right)^{1/2} + \left( 5 \times \delta^1\text{H}_{\text{apo/ho}} \right)^2 \right]^{1/2}$$

Equation 2.6- Calculation of the differences in chemical shift from the $^1\text{H}-^{15}\text{N}$ HSQC spectra of holo- and apo- IdmK (Williamson et al., 1997).
3. Cloning and recombinant protein expression of nonribosomal peptide synthetase proteins required for starter unit biosynthesis of the polyether ionophore indanomycin

The native producers of natural products such as nonribosomal peptides and polyketides include organisms like gram-positive bacteria and fungi (Stachelhaus and Marahiel, 1995). In this thesis the target compound, indanomycin, is produced by a strain of <i>Streptomyces, Streptomyces antibioticus</i> NRRL 8167 (Liu et al., 1979). Over-expression of NRPS proteins, required for structural and biochemical characterisation, is problematic in <i>Streptomyces</i>. Issues which prevent the over-expression of proteins in the native system include slow bacterial growth during incubation and low product titres (Fujii, 2009; Peirú et al., 2010). An additional disadvantage is the genetic limitations of such an organism, including the occurrence of native PKS/NRPS gene clusters which may contaminate target protein PKS/NRPS expression and purification, the complexity of DNA manipulation with slow growing bacteria, and a lack of tools for genetic engineering (Fujii et al., 2009; Peirú et al., 2010; Cane et al., 1998; Weissman and Leadlay, 2005).

Over-expression of NRPS and PKS proteins has been carried out in some <i>Streptomyces</i> strains. Expression of these multimodular enzymes has been preferable due to the already existing abilities of these organisms to produce such macromolecules and the substrates required (Weissman and Leadlay, 2005). Expression is usually carried out in strains that are fully sequenced such as <i>S. coelicolor</i> A3 (2) (Bentley et al., 2002) or <i>S. avermilitis</i> (Ikeda et al., 2003).

Alternative strategies explored for the expression of NRPS or PKS proteins in <i>Streptomyces</i> includes genetic engineering to reduce contamination by native PKS/NRPS gene clusters, by targeted deletion of the native NRPS/PKS gene cluster (such as the deletion of the actinorhodin gene cluster in its producing strain, <i>S. coelicolor</i> CH999) (McDaniel et al., 1993). Another approach to reduce the background genetic noise was to perform systematic deletions of the genome to create a genome-minimised bacterial strain. A genome minimised <i>S. avermilitis</i> bacterial strain was used to produce the antibiotic streptomycin, at levels above those.
found in the native host *S. griseus* (Komatsu *et al.*, 2010; Waksman *et al.*, 2010). However more recently *E. coli* has become a popular choice of host system for recombinant protein expression due to its rapid life cycle, well characterised metabolic pathways, culture times, cost and ease of DNA manipulation (Fujii *et al.*, 1999; Rosano and Ceccarelli, 2014).

Recombinant protein expression of NRPS and PKS modules in *E. coli* is not without its pitfalls, one particular difficulty being the expression of post-translationally modified proteins, which is required for the expression of the PKS/NRPS carrier protein. This issue was addressed by genetic engineering of *E. coli* to introduce the gene encoding the *B. subtilis* phosphopantetheinyl transferase, *Sfp*, to enable production of functional 6-deoxyerythronolide B synthase modules (Pfeifer *et al.*, 2001).

In principle, producing recombinant PKS and NRPS proteins only involves a few, seemingly simple, steps; obtain the gene of interest, clone into a target vector under the control of an inducible promoter, express the target protein in a suitable host followed by protein purification (which can be aided by introduction of an affinity tag when obtaining the target gene). As expected there are a number of viable options at each stage of recombinant protein expression in order to attempt to produce soluble protein, including host, vector, promoter and affinity tag and this is another advantage as multiple combinations of these things can be trialled in *E. coli* (Rosano and Ceccarelli, 2014). The target host selected for recombinant gene expression in this case was *E. coli*. This chapter reviews work carried out to clone the indanomycin NRPS genes, *idmJ* and *idmI* and express soluble proteins.

The initial aim of the project was to clone the genes involved in conversion of L-proline to pyrrole-2-carboxylate (Figures 1.20 and 3.1), the starter unit for the polyether ionophore antibiotic indanomycin, into *E. coli* for recombinant gene expression.
Prior to this work a number of NRPS proteins have been expressed in *E. coli*, including other NRPS proteins that convert L-proline to pyrrole-2-carboxylate in aminocoumarin biosynthesis (Garneau *et al.*, 2005); however those required for indanomycin starter unit biosynthesis; IdmI, IdmJ and IdmK, had not. In order to characterise these proteins and their enzymatic mechanism for starter unit biosynthesis, and to enable protein engineering experiments, recombinant protein expression was essential.

### 3.1 Design of synthetic genes for recombinant protein expression in *E. coli*

To express the *S. antibioticus* enzymes responsible for indanomycin starter unit biosynthesis in *E. coli*, genes were designed and subsequently synthesised and purchased from Genscript (New Jersey, USA). Design and purchase of a synthetic gene is now an attractive and accessible option for obtaining the target gene, and preferable to obtaining the gene by PCR from the genomic DNA, since the cost of gene synthesis has significantly decreased (Mueller *et al.*, 2009). Additionally, genes can be designed to contain, or omit, certain features and characteristics such as the inclusion of an affinity tag or codon optimisation for recombinant expression. Amino acid sequences for the adenylyltransferase (*idmJ*), prolyl dehydrogenase (*idmI*) and
Results one

prolyl carrier protein (idmK) were obtained from GenBank (accession number FJ545274) (Li et al., 2009). Genes were designed so they could be cloned and expressed individually or as a synthetic operon.

Figure 3.2- Schematic of the design for synthetic genes. (a) Synthetic idmJ, idmI and idmK were designed with unique restriction sites at the 5’ and 3’ ends to be cloned into pET23a individually or altogether. BamHI and EcoRI restriction sites were at the 5’ and 3’ termini of the idmI gene. SacI and SalI restriction sites were at the 5’ and 3’ termini of the idmJ gene respectively, and HindIII and XhoI restriction sites were at the 5’ and 3’ termini of the idmK gene. (b) Synthetic genes contained a ribosome binding site, initiating methionine residue followed by a glutamate, a His6-tag at the 5’ terminus of the gene and a double amber stop codon at the 3’ terminus of the gene. These features are flanked by the selected restriction sites.

As illustrated in Figure 3.2, all genes were designed with unique restriction sites at their 5’ and 3’ ends and the selected restriction sites did not occur within any of the genes. All genes contained ribosome binding sites to permit expression in any vector, and a His6-tag was included at the N-terminus of each protein to allow protein purification by nickel affinity chromatography. Additional features included a glutamate residue following the initiating methionine in order to prevent cleavage of the methionine therefore preventing premature protein degradation (Tobias et al., 1991; Hirel et al., 1989). A double amber stop codon at the 3’ end of the gene was included to prevent a stop codon read-through. As these genes are usually expressed in Streptomyces, a bacterial strain whose genome has a high GC content (Muto and Osawa, 1987), genes were designed to be codon optimised for expression of the
Results one

protein in *E. coli*. Synthesised genes were supplied in pUC57, an appropriate cloning vector.

3.1.1 Initial cloning strategy for NRPS starter module domains

The initial strategy was to clone the genes individually into pET23a for protein expression. Figure 3.3 illustrates the standard “cut and paste” strategy used for cloning *idm*J, *idm*I and *idm*K into pET23a. Vector and insert DNA were amplified by growth in *E. coli* XL10 Gold Ultracompetent cells. Once amplified, insert and vector DNA was prepared by restriction digestion creating complimentary “sticky ends” for ligation. The resulting construct(s) were transformed into *E. coli* XL10 Gold Ultracompetent cells. Successful pETidmI/J/K constructs were indicated by growth on 2 × TY agar plates containing ampicillin and resulting constructs were screened with relevant restriction enzymes, and success was confirmed by DNA sequencing. The correct construct could then be transformed into an *E. coli* expression strain.
3.1.2 Amplification of vector and target gene plasmids

The pET23a vector DNA was purchased from Merck4Biosciences (Nottingham, UK) (kindly gifted from Dr David Brockwell, Astbury Centre, University of Leeds, UK). The empty vector was transformed into *E. coli* XL10 Gold Ultracompetent cells (Section 2.2.2.11). A single colony was used to inoculate a 5 mL 2 × TY starter culture, 0.5 mL of this was subsequently used to make a glycerol stock of *E. coli* XL10 Gold ultracompetent cells containing the empty pET23a vector (Section 2.2.1.6).
pUC57 plasmids containing the individual genes arrived in the form of lyophilised DNA and were resuspended in sterile water as per the guidelines of the supplier of the synthetic genes. The three pUC57 plasmids containing the adenylyltransferase, dehydrogenase and carrier protein were designated pUCidmJ, pUCidmI and pUCidmK, respectively. Plasmid DNA was then transformed into Agilent Technologies E. coli XL10 Gold Ultracompetent cells (Section 2.2.2.11). Glycerol stocks were made of XL10 Gold cells containing pUCidmI/J/K plasmids for storage and DNA production (Section 1.2.1.6). Initial restriction digests of pUCidmJ with SacI and SalI, pUCidmI with BamHI and EcoRI and pUCidmK with HindIII and XhoI were performed for each plasmid for a preliminary product check and digested DNA was run on a 0.7 % (w/v) agarose gel as described in Sections 2.2.2.2 and 2.2.2.7.

Figure 3.4-0.7 % (w/v) agarose gel of a restriction digest of pUC57-gene constructs (a) pUCidmJ digested with SacI and SalI yielding a band at 1540 bp corresponding to the idmJ gene (b) pUCidmI digested with BamHI and EcoRI yielding a band at 1180 bp corresponding to the idmI gene and (c) pUCidmK digested with HindIII and XhoI yielding a band at 325 bp corresponding to the idmK gene. All agarose gels contain a band at 2790 bp corresponding to the pUC57 vector. All gels indicate the presence of the correct plasmids after transformation into XL10 Gold Ultracompetent cells.

Once the preliminary digest was analysed the genes were sub-cloned for protein expression as described below.

### 3.2 The adenylyltransferase (IdmJ)

Conversion of L-proline to pyrrole-2-carboxylate requires the activation of the amino acid for loading onto the prolyl carrier protein (IdmK). The adenylyltransferase (IdmJ) is responsible for selection of the correct substrate, in this case L-proline, and
its activation. The adenylyltransferase (IdmJ) is required to load the carrier protein with L-proline (Li et al., 2009), or any other substrate in an enzyme dependent manner, for future engineering studies.

3.2.1 Cloning the adenylyltransferase (idmJ) into pET23a

Originally individual genes were to be sub-cloned into pET23a. pET vectors contain T7 promoters for induction by IPTG (Studier et al., 1990) and a range of restriction sites for cloning. To obtain the target gene idmJ (the insert) the glycerol stock of E. coli XL10 Gold Ultracompetent cells containing the pUCidmJ plasmid was used to inoculate 5 mL starter culture in 2 × TY medium supplemented with ampicillin. This in turn was used to inoculate a 50 mL culture of 2 × TY medium (Section 2.2.1.5). Plasmid DNA was then purified using a QIAGEN Plasmid Midi kit (Section 2.2.2.1). Vector DNA was produced in a similar manner. The pUCidmJ and pET23a vector were then digested with the restriction enzymes SacI-HF® and SalI-HF®; high fidelity (HF™) enzymes were used due to reduced star activity (Kamps-Hughes et al., 2013). Restriction digests were performed as described in section 2.2.2.7 and run on a 0.7 % (w/v) agarose gel. Insert and vector DNA bands were then cut out of the agarose gel and a gel extraction performed (section 2.2.2.3). A ligation reaction was performed between pET23a and idmJ (2.2.2.8), 4 μL of the ligation reaction was subsequently transformed into E. coli XL10 Gold Ultracompetent cells (2.2.2.11).

Successful plasmid ligation was screened for by antibiotic selection: bacterial colonies that grew on the 2 × TY agar plates containing ampicillin indicated the presence of a pETidmJ construct. To confirm the presence of the pETidmJ construct 10 colonies were picked and used to inoculate 10 individual 5 mL starter cultures. Plasmid DNA from selected colonies was purified using the Promega Wizard® SV Minipreps Purification kit (section 2.2.2.1) and restriction digests using SacI-HF® and SalI-HF® were performed to screen for the correct plasmid. Figure 3.5 shows digested plasmid DNA from 10 colonies screened for the pETidmJ plasmid. The expected sizes of the DNA bands for a restriction digest using SacI-HF® and SalI-HF® of the pETidmJ construct are approximately 3.7 kb (3666 bp) and 1.5 kb (1540 bp) corresponding to the vector and insert respectively.
Figure 3.5- 0.7 % (w/v) agarose gel of the isolated plasmid DNA from the 10 colonies after digestion with SauI-HF® and Sall-HF® restriction enzymes. The grey arrow indicates the expected size of the vector DNA and the red arrow indicates the expected size of idmJ. Lanes 1 and 2 appear to be undigested plasmid DNA, plasmids in lanes 3-6, 9 and 10 appear to have yielded two DNA fragments, one at approximately 3700 bp corresponding to the pET23a vector and the second approximately 2000 bp in size, this is too large to be idmJ. The plasmid in lane 7 contains a DNA fragment at 1500 bp corresponding to idmJ the vector is 2700 bp in size, corresponding to pUC57. Finally the digested plasmid in lane 8 contains only one DNA fragment at the approximate size for pET23a.

Figure 3.5 indicates that the ligation reaction between pET23a and idmJ was unsuccessful. Plasmids 1 and 2 do not appear to be digested by SauI-HF® and Sall-HF® (lanes 1 and 2). Plasmids 3, 4, 5, 6, 9 and 10 are digested by SauI-HF® and Sall-HF® yielding a DNA fragment corresponding to pET23a and an unidentified insert too large to be idmJ (lanes 3-6, 9 and 10). Plasmid 7 (lane 7), when digested by SauI-HF® and Sall-HF® produced two DNA fragments, one corresponding to idmJ and the other pUC57 indicating pUCidmJ contamination during insert preparation. Finally, digestion of plasmid 8 (lane 8) yielded one DNA fragment corresponding to empty pET23a; this indicates incomplete digestion of pET23a in vector preparations.

Several attempts at cloning idmJ into pET23a were made. The restriction enzyme stocks were replaced with new enzymes, as well as new vector DNA purchased from Merck4Biosciences (Nottingham, UK), however this strategy was not successful. Inefficient digestion during vector and insert preparations using SauI-HF® and Sall-HF® resulted in plasmids that are incompletely digested which are able to simply “snap shut” in the presence of T4 DNA ligase and will transform efficiently.
contaminating the transformation plates with the incorrect plasmid. Although digests were performed for extended periods of time there may have been incompatibility with the two restriction sites selected. In addition to this, sites selected may have been in too close a proximity to one another in the vector, decreasing the efficiency of activity of the restriction enzyme.

3.2.2 Cloning the adenylyltransferase (idmJ) into pKK223-3

The next approach to sub-clone idmJ was to select an alternative vector. The vector selected was pKK223-3 (Pharmacia). This was obtained in the form of pKnanA (Timms et al., 2013) from Prof. A. Berry (Astbury Centre, University of Leeds, UK). Figure 3.6 illustrates the planned cloning strategy. The idmJ gene was amplified by culturing cells containing the pUCidmJ plasmid, the plasmid purified and insert cut out of the pUC57 vector using EcoRI-HF® and HindIII-HF® restriction enzymes. The pKnanA plasmid was propagated by culturing of E. coli XL10 Gold Ultracompetent cells containing the pKnanA plasmid, purifying the plasmid DNA, performing a restriction digest using EcoRI-HF® and HindIII-HF® restriction enzymes to cut out the nanA gene and create complimentary sticky ends to the idmJ insert. The insert and vector will be ligated together and transformed into E. coli XL10 Gold Ultracompetent cells. A successful ligation between pKK223-3 and idmJ will be indicated by colony growth on 2 × TY agar plates supplemented with ampicillin. Restriction digests with EcoRI-HF® and HindIII-HF® will be used to screen resulting plasmids from the ligation. Confirmation of the ligation between idmJ and pKK223-3 can then be confirmed by sequencing and the resulting construct designated pKidmJ. This strategy exploits the removal of a gene from the target vector to confirm the double digest of the vector has been successful.
Insert and vector DNA were prepared as previously described, using the restriction enzymes *EcoRI-HF*® and *HindIII-HF*® (3.2.1). Figure 3.7 shows the *nanA* gene has been successfully cut out of the pKK223-3 plasmid using the *EcoRI* and *HindIII* sites.
The ligation reaction and subsequent transformation were also performed as before (section 3.2.1). 10 colonies were screened for the correct plasmid by restriction digestion with EcoRI-HF® and HindIII-HF®; results are presented in Figure 3.8. The pKidmJ construct digested with EcoRI-HF® and HindIII-HF® should yield two DNA fragments, one at 4600 bp and the other at 1500 bp.
The agarose gel in Figure 3.8 indicates that the ligation between pKK223-3 and idmJ was unsuccessful. Restriction digests with EcoRI-HF\textsuperscript{®} and HindIII-HF\textsuperscript{®} of plasmids 1 and 3 (lanes 1 and 3) yielded two DNA fragments, one at 4600 bp and a second at 900 bp corresponding to pKK223-3 and the nanA gene respectively. Lane 2 shows a DNA band at 4600 bp corresponding to linearised pKK223-3 with no insert present. Restriction digestion of plasmid 4 (lane 4) yielded two bands, one at 2700 bp and a second at 1500 bp corresponding to pUC57 and idmJ. Lanes 5-9 show two bands, one at 2700 bp corresponding to the expected size of pUC57 and an unknown DNA fragment approximately 500 bp in length. Finally, there is no DNA present in lane 10 suggesting plasmid purification was unsuccessful. Again, contamination from insert and vector preparations were observed (lanes 1-4) in addition to contamination by an unknown insert (lanes 5-9). This approach did not yield any positive results and was repeated without any success.

### 3.2.3 Cloning the adenylyltransferase (idmJ) into pETDUET

After the unsuccessful attempts at cloning idmJ into pET23a and pKK223-3 a third vector was trialled for sub-cloning, pETDUET. pETDUET is a vector that contains two multiple cloning sites. idmJ was to be cloned into the first multiple cloning site using the SacI and SalI restriction sites. The SacI and SalI sites are separated by a few more base pairs in pETDUET vector than pET23a. The empty pETDUET vector was transformed into *E. coli* XL10 Gold Ultracompetent cells and a glycerol stock made (sections 2.2.2.11 and 2.2.1.6). Vector and insert DNA were prepared as described in section 3.2.1, using SacI-HF\textsuperscript{®} and SalI-HF\textsuperscript{®} restriction enzymes. A ligation reaction was performed between pETDUET and idmJ, to create a plasmid designated pET(2)idmJ, followed by a transformation. Six colonies were picked from the transformation plate and used to inoculate six 5 mL cultures of 2 × TY medium. Plasmid DNA was purified from 4.5 mL of the culture and the pET(2)idmJ plasmid screened for by restriction digests with SacI-HF\textsuperscript{®} and SalI-HF\textsuperscript{®}. Figure 3.9 shows the results from the restriction digests.
Figure 3.9-0.7 % agarose gel showing restriction digests with SacI-HF® and SalI-HF® of plasmids isolated from a ligation between pETDUET and idmJ. Lanes 1-6 all show two DNA fragments after digestions with SacI-HF® and SalI-HF®, one band at 5500 bp and a second at 1550 bp corresponding to the expected sizes of pETDUET (5420 bp) and idmJ (1540 bp).

Initial screening from the ligation reaction indicates the presence of the pET(2)idmJ plasmid. DNA fragments in lanes 1-6 all showed a band at 5500 bp and another at 1540 bp, corresponding to the correct sizes for vector and insert, respectively. This suggested that all plasmids screened were pET(2)idmJ. Plasmid 1 was selected for sequencing. A glycerol stock was made of E. coli XL10 Gold Ultracompetent cells containing plasmid 1. Purified plasmid DNA from this stock was subjected to DNA sequencing using the primers “pET UPSTREAM” and “DuetDOWN1” (see Appendix) (section 2.2.2.12). The sequencing revealed that pETDUET contained the correct sequence for the idmJ gene.

The purified plasmid DNA of the pET(2)idmJ plasmid was then transformed into E.coli BL21 (DE3) Gold cells, a single colony was picked and used to inoculate 5 mL of 2 × TY medium. 0.5 mL of this culture was used to make a glycerol stock, which was used for subsequent protein expression trials.

3.2.4 Recombinant protein expression of the adenylyltransferase (IdmJ)

Protein expression of IdmJ was attempted in E.coli BL21 (DE3) Gold cells containing the pET(2)idmJ plasmid. Initial small scale protein expression was attempted in 2 × TY medium as described in section 2.2.3.1. To determine if IdmJ had been expressed as a soluble protein, cells from 1.5 mL of the culture were
harvested, lysed and soluble and insoluble fractions separated for analysis by SDS-PAGE (sections 2.2.3.3 and 2.2.3.6).

The expected mass of IdmJ, as calculated from the amino acid sequence of the synthetic gene, is 53 522 Da. From the SDS-PAGE gel in Figure 3.10 it was unclear as to whether IdmJ was expressed as a soluble or insoluble protein, if expressed at all.

There are a number of properties in protein expression that can be adjusted in order to produce soluble protein; one aspect is changing the growth medium. Changing the growth medium may help with solubility as different media permit differential cell growth patterns, for example LB medium, promotes cell growth at an early log phase, however prevents high cell densities (Sezonov et al., 2007; Rosano and Ceccarelli, 2014). Other media, such as TB, encourage high cell densities which could mean increased expression; however more cells means less oxygen, which will prompt expression of a large number of proteins creating a metabolic burden, perhaps inhibiting soluble protein expression (Rosano and Ceccarelli, 2014; Unden et al., 1995). For this reason a range of media of varying degrees of richness were trialled. In addition to 2 × TY (the current growth medium), protein expression was
tested in LB, TB and autoinduction media (section 2.1.7). Autoinduction medium supplies glucose as a nutrition source for *E. coli*, protein expression is suppressed in the presence of glucose. When glucose is no longer present protein expression is initiated. Another easily adaptable variable is the temperature at which the induced cells are grown. The temperature can be reduced in an attempt to improve protein solubility. Therefore induced cells will be grown at 37°C, 21°C (room temperature) or 18°C. In order to reduce the rate of protein expression, allowing more time for accurate protein folding, the concentration of IPTG was reduced, giving a final concentration of 60 μM (Tolia and Joshua-Tor, 2006).

Expression trials were carried out on a 5 mL scale. The glycerol stock of *E. coli* BL21 (DE3) Gold cells containing the pET(2)idmJ plasmid was used to inoculate a 5 mL overnight starter culture, which in turn was used to inoculate 5 mL day cultures of TB, 2×TY and LB media which were incubated at 37°C. Once the OD$_{600}$ of cell cultures reached between 0.6-1.0 the cells were induced with IPTG to a final concentration of 60 μM. Upon induction the temperature of incubation was either kept at 37°C or reduced to 21°C or 18°C. This method for cell growth and induction was utilised for all media trialled, excluding autoinduction medium. In autoinduction medium, cells were incubated at the same temperature throughout the experiment: due to the slow cell growth at lower temperatures (21 and 18°C) these cultures were incubated for approximately 28 hours in total. Each medium was trialled at all three temperatures. Samples were taken from the soluble cell fraction and analysed by SDS-PAGE (Figure 3.11).
Results one

Figure 3.11- pET(2)idmJ expression trials. Reducing SDS-PAGE gel of the soluble cell fractions from the IdmJ expression trial. Expression of IdmJ was carried out by growing *E. coli* BL21 (DE3) Gold cells containing the pET(2)idmJ plasmid in four different media at three different temperatures. Lanes 1-3 show the results of protein expression in TB medium at 37 °C, 21 °C (room temperature) and 18 °C respectively. Lanes 4-6 show the results of expressing protein in 2×TY at 37 °C, 21 °C and 18 °C respectively. Lanes 7-9 show the results of expressing IdmJ in LB medium at 37 °C, 21 °C and 18 °C respectively. Finally lanes 10-12 show the results of expressing IdmJ in autoinduction medium at 37 °C, room 21 °C and 18 °C respectively. IdmJ is approximately 53 kDa, the red arrows indicate where a band is expected if soluble protein is expressed.

Analysis of the SDS-PAGE gel of the soluble cell fractions in Figure 3.11 and comparison with untransformed *E. coli* BL21 (DE3) Gold cells (see appendix) suggests that IdmJ was not being expressed as a soluble protein due to a lack of a protein band at the expected mass (indicated by the red arrow). To check for low levels of soluble protein expression an anti His<sub>6</sub>-tag antibody was used to detect His<sub>6</sub>-tagged protein as a more sensitive method of detection. This was carried out by blotting 2 µL of the soluble fraction or 2 µL of the insoluble fraction resuspended in PBS on a nitrocellulose membrane and the His<sub>6</sub>-tag detected as described in section 2.2.3.2. Figure 3.12 below shows the results of His<sub>6</sub>-tag detection in samples taken from the pET(2)idmJ expression trial.
Detection of the His$_6$-tag utilising an anti-polyHistidine tag antibody identified a number of expression conditions which produced soluble protein. Figure 3.12 shows that His$_6$-tagged protein was detected in the insoluble fractions of cells grown at 37 °C when cells were cultured in LB and autoinduction medium (outlined in red), and no protein expression detected in TB or 2 × TY medium. When the temperature of the cultures were reduced to 21 °C after induction, soluble protein was detected in LB, 2 × TY and autoinduction medium (green). Low levels of His$_6$-tagged protein were also detected in the insoluble fractions of cells. When cells were incubated at 18 °C after induction soluble protein was detected in TB, 2 × TY and LB medium, lower levels of His$_6$-tagged protein were detected in the insoluble fraction. At lower incubation temperatures, cell growth was slow in autoinduction medium with low levels of protein expression; this therefore suggested that this medium was unsuitable for the expression of IdmJ. The results show that expression of IdmJ is somewhat temperature dependent, with soluble protein being expressed at lower temperature. Although the gene was codon optimised for expression in *E. coli*, fermentation of *S. antibioticus*, the native host, is at 28 °C for 72 hours (Li *et al.*, 2009). The most consistent medium for expression of soluble IdmJ was LB medium,
with soluble protein being detected at 21°C and 18°C. The next stage was to attempt protein expression on a larger, 1 L scale, followed by subsequent protein purification.

### 3.2.5 Purification of IdmJ and analysis

After establishing an expression protocol, the next step was to purify IdmJ for analysis by SDS-PAGE and mass spectrometry. When the genes were synthesised they were designed to contain an N-terminal His$_6$-tag to enable purification by nickel affinity chromatography. Briefly, for large-scale expression, a glycerol stock of *E.coli* BL21 (DE3) cells containing the pET$(2)idmJ$ plasmid was used to inoculate a 5 mL starter culture of 2 × TY medium. The starter culture was then used to inoculate 500 mL of LB medium in a 2 L flask. The cell OD was monitored at 600 nm, when cell growth reached an OD of 0.6 the temperature was reduced to 18°C and protein expression induced by the addition of IPTG at a final concentration of 60 μM. The cultures were then incubated for a further 16 hr at 18°C. IdmJ was subsequently purified using batch nickel affinity chromatography as described in section 2.2.3.4. Samples were taken at each stage of the purification and subjected to analysis by SDS-PAGE.
Results one

![Reducing SDS-PAGE gel showing stages of the nickel affinity purification of IdmJ.](image)

Figure 3.13- Reducing SDS-PAGE gel showing stages of the nickel affinity purification of IdmJ. Broad range pre-stained protein markers are in lane (M). Lane 1 shows the whole cell lysate of induced BL21 (DE3) Gold cells containing the pETidmJ construct expressed in LB media at 18 °C, cells were harvested by centrifugation and lysed, the soluble (lane 2) and insoluble fractions (lane 3) were then separated by centrifugation. The soluble fraction was then incubated with resin chelated with nickel chloride for 1 hour then the post-load supernatant (lane 4) was discarded. The resin was washed with a low imidazole washing buffer (5, 6 and 7) to remove non-specifically bound proteins. Lane 8 shows purified protein after elution with a high imidazole buffer. There appears to be two protein species eluted from the resin, these species are present in the whole cell lysate (1) soluble fraction (2) and in post-load (4), there are lower levels in the insoluble fraction (3).

Analysis of nickel affinity purification of IdmJ by SDS-PAGE (Figure 3.13) revealed two bands in lane 8, suggesting two His6-tagged protein species were purified. The approximate molecular masses of these protein species appear to be approximately 58 and 53 kDa. Approximately 15 mg of protein was purified from 1 L of culture. For a more in-depth and accurate analysis of the molecular mass, a 70 μL sample of eluted protein was desalted into 50 mM ammonium acetate pH 7.4 (section 2.2.3.9) and analysed by ESI-MS (section 2.2.3.12). Figure 3.14 shows the mass spectrum of the eluted proteins.
Figure 3.14- ESI-MS of eluted protein from the purification of IdmJ. (a) mass spectrum raw data (b) deconvoluted mass spectrum of raw data identifying two species with masses of 55 953 Da and 56 131 Da. The expected mass for IdmJ is 53 522 Da.

The mass calculated from the amino acid sequence encoded by the synthetic gene for IdmJ is 53 522 Da. Although SDS-PAGE analysis (Figure 3.13) showed two protein species eluted from the resin with approximate masses of 58 and 53 kDa, the mass spectrum above shows the mass of two protein species at 55 953 Da and 56 131 Da. The smaller of these masses (55 953 Da) shows a difference from the expected mass of IdmJ of 2 431 Da and is clearly outside the error margin for mass spectrometry, this protein will be denoted as IdmJ†. There is a second peak at 56 131 Da, 178 Da larger than that of IdmJ†. After considering known post-translational modifications and their masses a suggested source of this additional mass could be due to a spontaneous post-translational modification of the His6-tag, alpha-N-6-phosphogluconylation (Geoghegan et al., 1999), although no further evidence for this modification was obtained.
3.2.6  Characterisation of IdmJ†

In order to identify the root of the additional mass the first aspect examined was the DNA sequence. Plasmid DNA was purified from the *E.coli* BL21 (DE3) Gold cell glycerol stock used for expression of IdmJ and transformed back into *E. coli* XL10 Gold Ultracompetent cells for DNA sequencing using the sequencing primers “pET UPSTREAM” and “DuetDOWN1” (see Appendix) (section 2.2.2.12). Sequencing results showed no change in the original plasmid, the gene or vector, indicating that IdmJ should have the expected amino acid sequence and thus the correct mass.

The next step was to eliminate the possibility that additions or changes to the amino acid sequence had occurred, to do this LC-MS/MS was utilised to evaluate peptide fragments generated from an in-gel tryptic digest for protein identification (section 2.2.3.10) this was carried out by Dr James Ault (Astbury Centre, University of Leeds, UK). The schematic in Figure 3.15 below illustrates the in-gel tryptic digest method.

![Schematic](image)

**Figure 3.15**- Schematic of method for an in-gel tryptic digest followed by LC-MS/MS for protein identification. A sample of eluted protein from the IdmJ purification was run on an SDS-PAGE gel, after staining the gel, protein bands were excised. The protein was reduced and alkylated followed by a digest using trypsin. Peptides were separated by high performance liquid chromatography (HPLC). Identical pools of peptides were subject to ESI-MS. Peptides were then sequenced by collision-activated dissociation followed by MS/MS. Peptide fragments were identified using a database edited to contain the synthetic IdmJ amino acid sequence (Henkin et al., 2004; Fujii, 2009).
Results one

The in-gel tryptic digest followed by LC-MS/MS was carried out for both eluted species seen in lane 8 of the SDS-PAGE gel in figure 3.13. Both bands were separately excised from the SDS-PAGE gel, reduced and alkylated and digested with trypsin. Subsequently peptides were separated by high performance liquid chromatography (HPLC) (2.2.3.10). Trypsin cleaves on the C-terminus of either arginine or lysine residues, therefore the fragments produced from a known amino acid sequence can be predicted. Figure 3.16 shows the HPLC traces of both proteins that were purified in the adenylyltransferase purification (Figure 3.13, lane 8) digested with trypsin.

Figure 3.16 - HPLC trace of purified IdmJ† digested with trypsin. A comparison of peptides present of bands excised from lane 8 of the SDS-PAGE gel in figure 3.13. The top trace shows peptide fragments present in the protein species in the top band on the SDS-PAGE gel. The bottom trace shows the peptide fragments present in the protein species in the bottom band of the SDS-PAGE.

Figure 3.16 shows the HPLC traces of peptide fragments from the two protein species eluted during purification of IdmJ. Both the top band of approximately 58 kDa (top trace) and bottom band of approximately 53 kDa (bottom trace) in Figure 3.13 display identical HPLC traces in Figure 3.16. As mentioned previously trypsin will cleave the amino acid sequence on the C-terminal side of arginine and lysine residues. This will create a pool of peptides, which can be predicted from the amino acid sequence alone. Identical HPLC traces therefore indicate that the protein species in the top and bottom bands of the SDS-PAGE gel are composed of the same peptides suggesting they are identical proteins.
Results one

Collision induced dissociation (CID) of peptides caused further fragmentation of the peptides. This enables sequencing of individual populations of peptides. The peptides that were identified from the tryptic digest and sequenced accounts for approximately 75% of the amino acid sequence of IdmJ; sequence coverage is illustrated in Figure 3.17.

Purification by nickel affinity chromatography followed by an in-gel tryptic digest and LC-MS/MS identified peptide fragments that were expected from a tryptic digest of IdmJ. This suggests that both bands in lane 8 of Figure 3.13 were modifications of IdmJ. Identification of the C-terminal peptide fragment indicated the additional mass does not come from a stop codon read-through and purification by the His6-tag indicated that the N-terminus of IdmJ is correct. Additionally, peptide fragmentation can also highlight any amino acids that have been post-translationally modified.

During sample preparation, prior to the tryptic digest, the cysteine residues were alkylated to prevent disulphide bond formation as it may reduce peptide yields and hinder identification. Aside from cysteine alkylation, and other modifications such as oxidation that are caused by the method, the identity of the remaining post-translational modification could not be determined.

DNA sequencing and the in-gel tryptic digest followed by analysis using LC-MS/MS has indicated that the source of the additional mass observed for IdmJ† is not due to changes in the DNA or amino acid sequences. Peptide sequencing by MS/MS was also unable to identify the post-translational modification. Reduction and alkylation
Results one

of cysteine residues prior to the tryptic digest could remove a covalent post-translational modification at one or more cysteine residues, therefore removing the source of the additional mass.

To investigate if the cysteine residues were playing a role in acquiring the additional 2 431 Da observed for IdmJ†, the cysteine residues were alkylated and the protein mass obtained by analysis using ESI-MS. 25 μM of IdmJ†, purified by nickel affinity chromatography, was unfolded by 8 M urea. The protein was reduced by the addition of DTT and the cysteine residues alkylated by the addition of iodoaceticamide (section 2.2.3.11). The reaction was incubated in the dark for 30 min, and then quenched by buffer exchange into 50 mM ammonium acetate pH 7.4 for analysis by ESI-MS (section 2.2.3.12). Figure 3.18 below shows the mass spectrum of alkylated IdmJ†.

Figure 3.18- ESI-MS analysis of alkylated IdmJ. 25 μM of purified IdmJ was unfolded by the addition of urea to a final concentration of 8 M. The protein was then reduced with DTT and alkylated by the addition of iodoaceticamide. (Top) mass spectrum raw data (bottom) deconvoluted mass spectrum of raw data above. 3 peaks are can be seen in the spectrum, at 53 753 Da, 56 186 and 56 368 Da.
Results one

There are four cysteine residues in the amino acid sequence of IdmJ at positions 104, 127, 169 and 308, therefore the potential for alkylation at four positions. Alkylation with iodoacetamide introduces a carbaminomethyl group onto the cysteine residue, increasing the mass of the protein by 57 Da per group added. Table 3.1 below shows and compares the expected and observed masses for the alkylation of IdmJ and IdmJ†.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Expected mass (Da)</th>
<th>Observed mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IdmJ (Figure 3.14)</td>
<td>53 522</td>
<td>55 953 (IdmJ†)</td>
</tr>
<tr>
<td>Fully alkylated IdmJ</td>
<td>53 753</td>
<td>53754</td>
</tr>
<tr>
<td>Fully alkylated IdmJ†</td>
<td>56 181</td>
<td>56 186</td>
</tr>
<tr>
<td></td>
<td></td>
<td>56 365</td>
</tr>
</tbody>
</table>

Table 3.1- Table showing the expected masses for full alkylation of all cysteine residues in both IdmJ and IdmJ† compared to the masses observed by ESI-MS.

The mass spectrum in Figure 3.18 shows the alkylation of IdmJ† yielded three protein species; 53 574 Da, 56 186 Da and 56 365 Da. Table 3.1 shows the comparison of expected masses for fully alkylated IdmJ and IdmJ†, i.e. alkylation of all four cysteine residues. Table 3.1 shows IdmJ of the correct mass (53 522 Da) with all four cysteine residues alkylated has an expected mass of 53 753. The mass spectrum in Figure 3.18 shows a peak at 53 754, corresponding to IdmJ with the addition of four carbamidomethyl groups. The expected mass of IdmJ† with all four cysteine residues alkylated is 56 181 Da, a protein species was observed with as mass of 56 186 Da, this is indicative of IdmJ† alkylated on all four cysteine residues. Finally, the peak at 56 368 Da indicates the presence of 7 carbamidomethyl groups, suggesting IdmJ† has been alkylated at 7 positions. Although excess iodoacetamide can eventually cause non-specific alkylation of other groups such as the amine group of lysine residues, the thioester of methionine residues, imidazole groups of histadines and carboxylate groups of aspartate or glutamate (Yang and Attygalle, 2007), the source of the extra three carbamidomethyl groups in IdmJ† is unlikely to be a non-specific alkylation. If this were the case more alkylated species would be observed e.g. +5, +6, +7 carbamidomethyl group etc. This could indicate that the source of the additional mass in IdmJ† contains four thiol groups that are being
alkylated. These results show when IdmJ† is unfolded, reduced and the cysteine residues alkylated the expected mass of IdmJ with four cysteine residues alkylated is observed. This provides further evidence of the involvement of the cysteine residues in acquiring the additional mass.

To further investigate the role of the cysteine residues in the observed increase in mass, to potentially deduce the contributions of each cysteine residue, and in an attempt to express a protein of the expected mass, individual cysteine to alanine mutations were made at all four positions. The pET(2)idmJ plasmid DNA was purified from E. coli XL10 Gold Ultracompetent cells and site-directed mutagenesis carried out using primers designated “C104A For”, “C104A Rev”, “C127A For”, “C127A Rev”, “C169A For”, “C169A Rev”, “C308A For” and “C308A Rev” designed to mutate the cysteine residues to alanine (sequences can be found in the appendix). Mutagenesis was carried out using the QuikChange™ Lightning Site-Directed Mutagenesis kit as described in section 2.2.2.10. 3 colonies were picked from the transformation plates of the mutagenesis reaction and plasmid DNA purified. DNA sequencing was carried out using the sequencing primers “pET UPSTREAM” and “DuetDOWN1”. This confirmed the presence of the correct point mutations within the idmJ gene. Plasmids containing the genes with the single point mutations C104A, C127A, C169A and C308A were transformed into E. coli BL21 (DE3) Gold cells for protein expression.

Protein was expressed using the same method for wild-type IdmJ. Protein expression was carried out on a 5 mL scale and IdmJ variants were purified by nickel affinity chromatography. A 70 µL sample of each variant was desalted into 50 mM ammonium acetate pH 7.4 for analysis by ESI-MS (sections 2.2.3.9 and 2.2.3.12). Figure 3.19 below shows the deconvoluted mass spectra of IdmJ C104A, IdmJ C127A, IdmJ C169A and IdmJ C308A.
Results one

Table 3.2- Expected masses for IdmJ and IdmJ† containing the cysteine to alanine mutation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Expected mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>IdmJ (C104A, C127A, C169A and C308A)</td>
<td>53 492 Da</td>
</tr>
<tr>
<td>IdmJ† (C104A, C127A, C169A and C308A)</td>
<td>55 923 Da</td>
</tr>
</tbody>
</table>

Figure 3.19- ESI-MS analysis of IdmJ variants. From top to bottom deconvoluted mass spectra of IdmJ C104A, IdmJ C127A, IdmJ C169A and IdmJ C308A. A number of protein species can be observed in each spectrum. Peaks suggested to be due to the same species are highlighted in the same colour. Table 3.2 shows the expected masses of IdmJ and IdmJ† containing the cysteine to alanine mutation.
Analysis of IdmJ variants by mass spectrometry (Figure 3.19) identified potential, consistent, degradation products seen in the four variants (masses ~ 43 200, 28 947 and 26 971 Da (purple, green and yellow)). Analysis indicated that when C104A, C169A and C308A were expressed both IdmJ and IdmJ† were present (red and blue highlighted peaks). MS analysis of these three species also show the ~178 Da adduct seen in Figure 3.14 present (light green). Expression, purification and MS analysis of C127A shows a peak at 53 944 Da (pink), this is a mass difference of 452 Da, compared to IdmJ; this does not correspond to any known particular modification. C127A is the only variant where the larger species, IdmJ†, is not observed. This may indicate that the source of the additional mass may be modification at position C127.

Figure 3.20 illustrates what may be occurring in expression of IdmJ in *E. coli*.

Figure 3.20 summarises the results of the cysteine alkylation and mutagenesis experiments and a suggested theory behind what may be occurring. During alkylation (Figure 3.20 (b)) of the cysteine residues IdmJ is fully alkylated (four positions). IdmJ† is still present, however it has been alkylated four and seven times. This may indicate that the post-translational modification is occurring at one position and contains one thiol group and three slightly less reactive groups (marked x) that are able to be alkylated, accounting for the extra masses. Identification of the cysteine residue being post-translationally modified was carried out by creating alanine variants C104A, C127A, C169A and C308A (Figure 3.20 (c)). Expression of C104A, C169A and C308A showed the continued presence of the post-translational modification, as opposed to C127A suggesting this as the residue that becomes modified, potentially during expression. Removal of the modification may create an active enzyme and the additional mass (452 Da) seen in the MS of C127A could be attributed to phosphopantetheine loaded with proline (452 Da) catalysed by IdmJ.
3.2.7 Summary and conclusions

Original difficulties in cloning idmJ into pET23a may have arisen from the two restriction sites selected for cloning idmJ, SacI and SalI, being too close in proximity to one another in the multiple cloning site (11 bp). Attempts at cloning idmJ into pKK223-3 were also unsuccessful. idmJ was finally successfully cloned into...
Results one

pETDUET. Expression trials of the pET(2)idmJ plasmid was carried out in E. coli BL21 (DE3) Gold cells. Soluble protein was not expressed at 37°C. Soluble protein expression was however seen at 25°C and 18°C in TB, LB and autoinduction medium (Figure 3.12). Soluble protein expression was then carried out at 18°C and in LB medium.

Protein purification was carried out using nickel affinity chromatography. Analysis by SDS-PAGE (Figure 3.13) showed purification of two protein species, and analysis by MS (Figure 3.14) showed the protein purified was 2431 Da larger than the expected mass of IdmJ believed to be a post-translational modification, this protein was designated IdmJ†. DNA sequencing and an in-gel tryptic digest indicated that the protein purified was IdmJ (Figure 3.17).

To probe whether a post-translational modification was occurring at one of the cysteine residues the protein was treated with iodoacetamide in order to alkylate the cysteine residues. Mass spectrometry results (Figure 3.18) showed masses equivalent to IdmJ being alkylated four times, equivalent to the number of cysteine residues in the protein, and IdmJ† alkylated four and seven times. Figure 3.20 shows a possible explanation behind how IdmJ† is alkylated in four and seven positions. Potentially, the modification of IdmJ† contains groups that can react with iodoacetamide.

Finally, in order to identify the site at which a potential modification could occur, the cysteine residues in IdmJ were mutated to alanine residues, the variants were then expressed, purified and analysed by MS (Figure 3.19). Purification of C104A, C169A and C308A and analysis by MS showed peaks corresponding to IdmJ and IdmJ† containing the cysteine to alanine mutation. Purification and MS analysis of C127A showed a peak at 53 944 Da, 452 Da larger than expected for IdmJ, while IdmJ† was absent. These results suggest C127 is the site of a 2431 Da modification.

3.3 The prolyl dehydrogenase (IdmI)

The formation of the starter unit, pyrrole-2-carboxylate, requires an enzyme to oxidise the prolyl group (Figure 3.1); this is the role of the prolyl dehydrogenase, IdmI. IdmI carries out the oxidisation of the prolyl group in a flavin-dependent
manner. To successfully reconstitute this system in *E. coli* cloning and expression of IdmI was required.

### 3.3.1 Cloning the prolyl dehydrogenase (*idmI*) into pET23a

As mentioned previously, the original strategy was to clone each individual gene into the expression vector pET23a. The *idmI* gene was engineered to have *Bam*HI and *Eco*RI sites at the 5’ and 3’ ends of the DNA respectively. The cut and paste method illustrated in Figure 3.3 was the initial method selected for sub-cloning *idmI* from pUCidmI into pET23a, creating a pETidmI plasmid. Insert and vector DNA were prepared by digesting the pUCidmI plasmid and pET23a vector with *Bam*HI-HF® and *Eco*RI-HF® restriction enzymes for 16 hr. The restriction digestes were run on a 0.7 % (w/v) agarose gel and the insert and vector DNA were purified from the agarose gel (section 2.2.2.3). Insert and vector DNA were ligated as described in section 2.2.2.8. Ligated DNA was transformed into *E. coli* XL10 Gold Ultracompetent cells and plated onto 2 × TY agar plates containing ampicillin to select for the pETidmI construct. Plasmid DNA was purified from 10 individual colonies and restriction digests performed using *Bam*HI-HF® and *Eco*RI-HF® restriction enzymes to screen for the correct construct. When digested with *Bam*HI and *Eco*RI the correct construct should yield bands at approximately 3.6 kb (3666 bp) and 1.2 kb (1180 bp) corresponding to the vector and insert respectively.

![Figure 3.21-0.7 % (w/v) agarose gel of plasmid DNA purified from colonies to screen for the pETidmI plasmid. Purified plasmid DNA digested with *Bam*HI-HF® and *Eco*RI-HF®. Plasmid DNA in lane 1 was undigested; lane 2 shows a single DNA band at approximately 3000 bp. Digests of plasmids in lanes 3 and 4 yielded two DNA fragments, one at 2700 bp corresponding to pUC57 and the other an unidentified band at approximately 300 bp. Digestion of plasmid DNA in lanes 5-10 yielded two DNA fragments, one 2700 bp in length and a second 1180 bp this corresponds to pUC57 and *idmI* respectively.](image)
Figure 3.21 indicates that the ligation between pET23a and idmI was unsuccessful. Plasmid 1 does not appear to have been digested with *Bam*HI-HF® and *Eco*RI-HF®. There is a single DNA band in lane 2 of approximately 3000 bp, there was no insert of 1180 bp corresponding to idmI. Plasmid DNA in lanes 3 and 4 yielded two DNA fragments when digested with *Bam*HI-HF® and *Eco*RI-HF®, one 2700 bp in length and a second 300 bp in length, corresponding to pUC57 and an unidentified insert. Plasmid DNA in lanes 5-10 yielded two DNA fragments when digested with *Bam*HI-HF® and *Eco*RI-HF®, one 2700 bp in length and the other 1180 bp in length corresponding to pUC57 and idmI. These results indicated that the ligation reaction was contaminated by the pUCidmI plasmid, as well as other unknown contaminants. The preparation of insert and vector DNA was repeated as was the ligation, with no success. Potentially, as with the pET23a digest for idmI, the proximity of the two restriction sites being utilised in pET23a may have prevented efficient digestion, even with an extended incubation period. Additionally incomplete digestion of the pUCidmI plasmid was contaminating the transformation reaction. An alternative approach of cloning idmI into pKK223-3 was then taken.

### 3.3.2 Cloning the prolyl dehydrogenase (idmI) into pKK223-3

In order to clone idmI into pKK223-3, creating the plasmid pK(idmI) plasmid, a similar strategy was used as illustrated in Figure 3.6. However, PCR had to be utilised to alter the restriction sites at the 5’ and 3’ ends amplifying the insert idmI. idmI was originally designed to have an *Bam*HI site at the 5’ end of the gene and *Eco*RI site at the 3’ end of the gene, however to use the pKK223-3 vector from the pKnanA precursor the nanA gene must be cut out using an *Eco*RI site at the 5’ end and *Pst*I site at the 3’ end of the nanA gene.

Primers designated “IdmI *Eco*RI For” and “IdmI *Pst*I Rev” were designed to change the 5’ BamHI site to an EcoRI site and the 3’ EcoRI site to a PstI site (Sequences can be found in the appendix). A PCR reaction was performed using Pfu Turbo DNA polymerase (section 2.2.2.4). Figure 3.22 shows samples taken from PCR reactions set-up to contain 0.1, 1 and 10 ng of template DNA.
Insert DNA from the 10 ng template PCR reaction (Figure 3.22, lane 3) was purified using a QIAquick® PCR purification kit (section 2.2.2.5) and digested with EcoRI-HF® and PstI-HF®. The subsequent product, the insert, was purified in the same manner as the PCR product. A ligation reaction was then performed between the insert and the pKnanA vector digested with EcoRI-HF® and PstI-HF®. Subsequent constructs from the ligation reaction were transformed into E.coli XL10 Gold cells and the correct plasmid screened for by restriction digests using EcoRI-HF® and PstI-HF®. Figure 3.23 shows purified plasmid DNA from the pKK223-3 ligation with idmI digested with EcoRI-HF® and PstI-HF®.
A pKidmI plasmid digested with EcoRI-HF® and PstI-HF® should yield two bands, one at approximately 4.6 kb (4586 bp) and a second at 1.2 kb (1180 bp). Plasmid DNA in lanes 2, 4, 5 and 10 show two DNA fragments, one at 4.6 kb and the other at 1.2 kb. Plasmid DNA in lanes 3 and 6 digested with EcoRI-HF® and PstI-HF® yielded one DNA fragment approximately 4.6 kb in length corresponding to pKK223-3. Plasmid DNA in lanes 7 and 9 yielded two bands, one at 1.2 kb corresponding to pUC57 and the second unknown DNA fragment approximately 1.5 kb in length. Plasmid DNA digested in lanes 1 and 8 has yielded two fragments, one at 4.6 kb and the other at 1.2 kb corresponding to pKK223-3 and idmI respectively.

Figure 3.23-0.7 % (w/v) agarose gel of plasmid DNA purified ten colonies from the ligation between idmI and pKK223-3 after being digested with EcoRI-HF® and PstI-HF®. A pKidmI plasmid digested with EcoRI and PstI should yield two DNA fragments, one at 4.6 kb and the other at 1.2 kb. Plasmid in lanes 2, 4, 5 and 10 show two DNA fragments, one fragment 2700 bp in length corresponding the correct length of pUC57, and a second unknown DNA fragment approximately 300 bp. Plasmid DNA in lanes 3 and 6 show a single band at approximately 4.6 kb corresponding to pKK223-3. Plasmid DNA in lanes 7 and 9 yielded two bands, one at 2.7 kb corresponding to pUC57 and the second unknown DNA fragment approximately 1.5 kb in length. Plasmid DNA digested in lanes 1 and 8 has yielded two fragments, one at 4.6 kb and the other at 1.2 kb corresponding to pKK223-3 and idmI respectively.

3.3.3 Recombinant protein expression of the prolyl dehydrogenase (IdmI)
After successful sub-cloning of idmI into pKK223-3, the pKidmI construct was transformed into E. coli BL21 (DE3) Gold cells for protein expression and a glycerol stock made. Small-scale protein expression trials were attempted on a 5 mL culture...
Results one

scale in TB, 2 × TY, LB and autoinduction medium at 37 °C, 21 °C and 18 °C as described for the pETidmJ plasmid (section 3.2.4). The soluble cellular components were subject to analysis by SDS-PAGE in order to identify protein expression. The expected mass for IdmI as calculated from the amino acid sequence of the synthetic gene is 41 191 Da. The blue arrow in Figure 3.24 indicates the expected mass of IdmI.

![SDS-PAGE gel](image)

**Figure 3.24** Reducing SDS-PAGE analysis of soluble protein produced in the expression conditions trialled for pKidmI. Expression conditions for pKidmI. Lanes 1-3 is soluble protein expressed in TB medium at 37 °C, room temperature and 18 °C respectively. Lanes 4-6 is soluble protein expressed in 2 × TY medium 37 °C, room temperature and 18 °C respectively. Lanes 7-9 is soluble protein expressed in LB medium at 37 °C, room temperature and 18 °C respectively. Finally lanes 10-12 is soluble protein expressed in autoinduction medium at 37 °C, room temperature and 18 °C respectively. The blue arrow indicates where a protein band for IdmI would occur.

The SDS-PAGE gel in Figure 3.24 shows the soluble protein expressed in *E.coli* BL21 (DE3) Gold cells containing the pKidmI plasmid in TB, 2 × TY, LB and autoinduction medium at 37°C, 21°C and 18°C. There does not appear to be a protein band at the expected mass for IdmI in the SDS-PAGE gel. This suggests that IdmI was not expressed as a soluble protein, therefore as with the pET(2)idmJ plasmid, an anti polyHistidine antibody was used as a more sensitive probe for
Results one

protein expression in the soluble and insoluble cell fractions. Figure 3.25 shows the results for probing for the His₆-tag of IdmI in the soluble and insoluble fractions.

Figure 3.25- N-terminal His₆-tag detection in IdmI expression trials. Soluble (top row) and insoluble (bottom row) fractions were blotted onto nitrocellulose membrane and an anti His₆-tag antibody was used to detect the N terminal His₆-tag of IdmI, probing for protein expression. Blots are of *E. coli* BL21 (DE3) Gold cells containing the pKidmI plasmid grown in TB, 2 × TY, LB and autoinduction media at 37°C, 21°C and 18°C. The blot indicates that there was little, if any His₆-tagged protein expressed from the pKidmI construct. This is a negative display image for clarity.

In order to identify expression conditions that produced soluble protein an anti polyHistidine antibody was used to detect the N terminal His₆-tag of IdmI from the soluble and insoluble fractions of the expression trial. Figure 3.25 shows no reactivity patches indicating there was too little or no His₆-tagged protein present.

Expression of IdmI from the pKidmI plasmid in *E. coli* BL21 (DE3) Gold cells was unsuccessful in TB, 2 × TY, LB and autoinduction medium at 37°C, 21°C and 18°C. This therefore led to exploring alternative methods for protein expression.

3.3.4 Cloning the prolyl dehydrogenase (idmI) into pMAL-c5X

Since soluble and insoluble IdmI was not expressed from the pKidmI plasmid, it was decided to clone IdmI into a pMAL vector, creating pMALidmI. Cloning IdmI into a pMAL vector will create a fusion protein with a maltose binding protein (MBP) tag. Although a clear explanation of why MBP helps with protein solubility does not exist, it has been hypothesised that a large soluble affinity tag will encourage the otherwise insoluble protein to be more soluble (Rosano and Ceccarelli, 2014).

To create an MBP-IdmI fusion protein the restriction sites for idmI were changed to NdeI and EcoRI restriction sites at the 5’ and 3’ of the gene respectively using the
“IdmI NdeI For” and “IdmI EcoRI Rev” in a similar manner to the pKidmI plasmid, using the same PCR conditions (section 3.3.2) (primer sequences are located in the appendix). The pMAL vector, pMAL-c5X, was purchased from New England Biolabs® (Ipswich, MA, USA) (kindly gifted by Dr James Ross, Astbury Centre, University of Leeds). The insert and vector DNA were prepared by digesting with NdeI and EcoRI-HF® and a ligation reaction performed as described previously for the pKidmI construct (section 3.3.2). Plasmid DNA was purified from 8 individual colonies selected from the pMAL-C5X and idmI ligation transformation plate and the pMALidmI plasmid screened for by restriction digest with NdeI and EcoRI-HF®.

Figure 3.26 below shows the results from the restriction digest.

![Figure 3.26: 0.7% agarose gel of restriction digests with NdeI and EcoRI restriction enzymes of purified plasmid DNA from the pMAL-C5X vector and idmI ligation.](image)

When digested with NdeI and EcoRI a pMALidmI construct would yield two DNA fragments, one 5.6 kb in length (vector) and the other 1.2 kb in length (idmI). Plasmid DNA in lanes 1-3 and 5, when digested with NdeI and EcoRI, yielded a single band at approximately 5.6 kb corresponding to pMAL-C5X. Restriction digests of Plasmid DNA in lanes 4 and 8 shows two bands, one corresponding to the vector (5.6 kb), the other approximately 2 kb in length, too large to be idmI. Finally, plasmid DNA in lanes 6 and 7 shows two bands, one at 5.6 kb corresponding to the vector pMAL-C5X and the other the correct length for idmI (1.2 kb).

Lanes 6 and 7 in Figure 3.26 suggests the successful sub-cloning of idmI into the pMAL-c5X. Plasmid 6 (lane 6) was transformed into E. coli XL10 Gold cells and was confirmed to be the pMALidmI construct by DNA sequencing (section 2.2.2.12). The pMALidmI plasmid was also transformed into E. coli BL21 (DE3) Gold cells for protein expression and a glycerol stock made of these cells containing this plasmid too.
3.3.5 Expression of the MBP/prolyl dehydrogenase fusion protein

A small scale protein expression trial of *E. coli* BL21 (DE3) Gold cells containing the pMALIdmI plasmid was carried out as previously described for the pKidmI construct, screening TB, 2 × TY, LB and autoinduction medium at 37 °C, room temperature and 18 °C. The soluble cellular components were analysed by SDS-PAGE. As mentioned previously, the expected mass for IdmI is 41 191 Da, however the MBP-IdmI fusion protein will have a total mass of approximately 83 kDa indicated by the blue arrow in Figure 3.27.

![Figure 3.27](image)

**Figure 3.27**- Reducing SDS-PAGE analysis of expression trials of the pMALIdmI fusion protein. Protein expression trials of MBP-IdmI were carried out with *E. coli* BL21 (DE3) Gold cells containing the plasmid pMALIdmI being grown and induced in TB, 2 × TY, LB and autoinduction media at 37 °C, room temperature and 18 °C. Lanes 1-3 is soluble protein expressed in TB media at 37 °C, room temperature and 18 °C respectively. Lanes 4-6 is soluble protein expressed in 2 × TY media 37 °C, room temperature and 18 °C respectively. Lanes 7-9 is soluble protein expressed in LB medium at 37 °C, room temperature and 18 °C respectively. Finally lanes 10-12 is soluble protein expressed in autoinduction medium at 37 °C, room temperature and 18 °C respectively. The blue arrow indicates where a protein band for MBP-IdmI fusion protein would be expected to occur, at approximately 83 kDa in mass.

The expected mass for the MBP-IdmI fusion protein is approximately 83 kDa. There is no clear overexpression of soluble MBP-IdmI in TB, 2 × TY, LB or autoinduction
media in the range of temperatures trialled as illustrated in the SDS-PAGE gel in Figure 3.27 by comparison with uninduced *E. coli* BL21 (DE3) Gold cells (see appendix). In order to determine if the MBP-IdmI fusion protein was being expressed as a soluble or insoluble protein, blotting for the His$_6$-tag using an anti polyHistidine antibody was performed on both the soluble and insoluble cellular components as carried out for the pET(2)idmI and pKidmI constructs (sections 3.2.4 and 3.3.3). Figure 3.28 below shows results when blotting for the His$_6$-tag in soluble and insoluble fractions of the expression of pMALidmI in TB, 2 × TY, LB and autoinduction media at 37°C, 21°C and 18°C.

![Figure 3.28- Detection of the N-terminal His$_6$-tag MBP-IdmI fusion protein. Soluble (top row) and insoluble (bottom row) fractions from *E. coli* BL21 (DE3) Gold cells containing the pMALidmI construct being expressed in TB, 2 × TY, LB and autoinduction medium at three different temperatures. The His$_6$-tag was detected using an anti His$_6$-tag antibody. The blot above indicates that extremely low levels of MBP-IdmI fusion protein were in the insoluble fractions of cells grown at 21°C and 37 °C. This is a negative display image for clarity.](image)

The MBP-IdmI fusion protein was mainly expressed as an insoluble protein, extremely low levels of His$_6$-tagged protein is present in the insoluble fractions of cells grown in 2 × TY, LB and autoinduction medium at 37°C and 21°C as illustrated by Figure 3.28 (red boxes). Soluble expression of the MBP-IdmI fusion protein was not achieved.

Thus far the expression of soluble IdmI had been unsuccessful, expressing the protein individually and as a fusion protein, however there were still a number of avenues to explore. The next strategy was to clone all three genes into the same vector and express them together. In the natural host all three proteins would be
expressed together and expression of IdmI may dependent upon expression of the other proteins within the same module (Figure 3.1).

3.3.6 Cloning genes responsible for starter unit biosynthesis into pETDUET
In an effort to express the prolyl dehydrogenase (IdmI) as a soluble protein all three protein were to be cloned into the same vector and expressed simultaneously. Figure 3.29 illustrates the strategy for cloning the adenylyltransferase (IdmJ) and proline carrier protein (IdmK) into the same vector as IdmI creating a plasmid designated pET(2)idmJ/idmK/idmI.
Figure 3.29- Proposed strategy for cloning \textit{idm}J, \textit{idm}I and \textit{idm}K into pETDUET. (a) Step one involves cloning \textit{idm}I into multiple cloning site 2 in pETDUET. PCR amplification will be used to amplify \textit{idm}I from the pUC\textit{idm}I plasmid altering the 5’ and 5’ restriction sites to NdeI and XhoI. pETDUET will be amplified by growth of the plasmid in \textit{E. coli} XL10 Gold cells. Both the insert and vector will be digested with \textit{NdeI} and \textit{XhoI} restriction enzymes, and are subsequently ligated together. The resulting constructs will be transformed into \textit{E. coli} XL10 Gold cells and a potential pET(2)\textit{idm}I plasmid screened for by restriction digests with \textit{NdeI} and \textit{XhoI}. Sequencing will confirm the pET(2)\textit{idm}I plasmid. (b) The next step is to clone \textit{idm}J into multiple cloning site 1 of pETDUET. The pET(2)\textit{idm}I plasmid and pUC\textit{idm}J plasmid were amplified by growth in \textit{E. coli} XL10 Gold cells. Both the vector and insert DNA were prepared by digestion with \textit{SacI} and \textit{SalI}. A ligation reaction between the insert and vector DNA followed by subsequent transformation into \textit{E. coli} XL10 Gold cells will be carried out. Resulting constructs will be screened by restriction digestion with \textit{SacI} and \textit{SalI}, the resulting plasmid pET(2)\textit{idm}J/\textit{idm}I will be confirmed by sequencing. (c) The final step, cloning \textit{idm}K into pET(2)\textit{idm}J/\textit{idm}I, was to be cloned in a ligation independent manner between \textit{idm}J and \textit{idm}I. PCR primers with complimentary ends to each other are to be used to amplify both the insert and vector. Insert and vector DNA can then be digested by \textit{DpnI} in a 1:1 vector to insert (v:v) ratio and then transformed into \textit{E. coli} XL10 Gold cells. Subsequent constructs can be screened by restriction digestion and DNA sequencing used to confirm construction of the pET(2)\textit{idm}J/\textit{idm}K/\textit{idm}I plasmid.

The first step to create the pET(2)\textit{idm}J/\textit{idm}K/\textit{idm}I is to clone \textit{idm}I into the multiple cloning site 2 of pETDUET. To do this, PCR primers were designed to alter the 5’ and 3’ restriction sites to \textit{NdeI} and \textit{XhoI}. Insert DNA was amplified by PCR which
simultaneously changed the 5’ and 3’ end restriction sites, pETDUET was amplified by growth of *E. coli* XL10 Gold cells containing the pETDUET plasmid. Both insert and vector DNA were digested with *NdeI* and *XhoI* and subsequently ligated together. A transformation was performed of resulting constructs, colony growth on 2 × TY agar plates containing ampicillin indicated the presence of the pET(2)*idmI* plasmid. The plasmid was screened for by digestion with NdeI and XhoI restriction enzymes. Figure 3.30 (a) shows an agarose gel of the PCR reaction carried out to amplify *idmI* from the pUCidmI using “*NdeI IdmI For*” and “*XhoI IdmI Rev*” primers (primer sequences located in the appendix). Figure 3.30 (b) shows the agarose gel of four individual plasmids purified from four individual colonies picked from the transformation plates of resulting constructs from the ligation between pETDUET and *idmI*, creating a pET(2)*idmI* plasmid.

The PCR amplification of *idmI* was successful (Figure 3.30 (a)). A positive pET(2)*idmI* clone would yield two bands on an agarose gel, one at approximately 5.5 kb (5420 bp) corresponding to pETDUET, a second 1.2 kb (1180 bp) corresponding to *idmI*. Lanes 1 and 2 in the agarose gel in Figure 3.30 (b) show DNA bands at these expected sizes, suggesting the ligation between pETDUET and *idmI* was successful. Plasmid DNA in lane 1 was subsequently transformed into *E. coli* XL10.
Gold cells (section 2.2.2.11), a glycerol stock made and subject to DNA sequencing to confirm this plasmid was the pET(2)idiMI construct (section 2.2.1.6 and 2.2.2.12).

The next stage was to clone idmJ into multiple cloning site one of the pET(2)idiMI plasmid. Figure 3.29 (b) illustrates the strategy to be used to create the pET(2)idiMI/idiMJ plasmid. In brief, the standard cut and paste method was used to cut idmJ out of the pUCidiMJ plasmid using the SacI and SalI sites and ligated into the pET(2)idiMI plasmid, also digested with SacI-HF® and SalI-HF® restriction enzymes. A ligation reaction was carried out as described in section 2.2.2.8. 8 colonies were picked from the transformation plate from the ligation reaction between pET(2)idiMI and idmJ and the pET(2)idiMI/idiMJ plasmid screened for by digestion with SacI-HF® and SalI-HF® restriction enzymes. A pET(2)idiMI/idiMJ plasmid digested with SacI-HF® and SalI-HF® would yield two DNA fragments, one at approximately 6.7 kb corresponding to pETDUET (5420 bp) and idiMI (1180 bp) and a second DNA fragment at 1.5 kb corresponding to idmJ (1540 bp).

Figure 3.31-0.7% agarose gel of restriction digests with SacI-HF® and SalI-HF® of plasmid DNA from 8 colonies screening for the pET(2)idiMI/idiMJ plasmid. 8 individual colonies were selected from the resulting constructs from the pET(2)idiMI and idmJ ligation transformation. Digestion of plasmid DNA in lane 1 yielded two DNA fragments, both unknown fragments, one fragment >10 kb and the second approximately 4.5 kb. Digestion of plasmid DNA in lanes 2, 7 and 8 yielded two DNA fragments, one at approximately 6.7 kb and a second at 1.5 kb corresponding to pET(2)idiMI and idmJ respectively. Plasmid DNA in lane 3, digested with SacI-HF® and SalI-HF® yielded at single band at approximately 5.5 kb, corresponding to the pET(2)idiMI plasmid. Plasmid DNA in lanes 4 and 6 also yielded a single band when digested with SacI-HF® and SalI-HF®, this time an unknown DNA fragment at approximately at 8 kb. Finally restriction digestion of plasmid DNA in lane 5 yielded two DNA fragments one at ~5 kb and a second at 2 kb from an unknown origin.
To create the pET(2)idmJ/idmI plasmid, idmJ had to be cloned into the first multiple cloning site of pETDUET using the SacI and SalI restriction sites. Figure 3.31 is the agarose gel of the resulting constructs from a ligation between pET(2)idmI and idmJ digested with SacI-HF® and SalI-HF®. Plasmid DNA in lanes 1, 4, 5 and 6 yielded DNA fragments of varying sizes that were unidentified. Plasmid DNA in lane 3 corresponded to the pET(2)idmI plasmid, idmJ was not present. Although the plasmid in lane 8 appears to display bands corresponding to pET(2)idmI and idmJ there are additional bands above the band corresponding to pET(2)idmI, therefore indicating the presence of DNA fragments of unknown origin. Plasmid DNA in lanes 2 and 7 however did yield two DNA fragments, one at 6.7 kb and a second at 1.5 kb corresponding to pET(2)idmI and idmJ respectively. This indicates that the pET(2)idmJ/idmI construct has been made. Plasmid DNA from lane 2 was transformed into E. coli XL10 Gold cells, a glycerol stock made. DNA sequencing with sequencing primers “pET UPSTREAM” and “DUETDOWN1” was used to confirm that idmJ was cloned into multiple cloning site one of pETDUET (primer sequences are located in the appendix).

The final stage in creating the pET(2)idmJ/idmK/idmI clone was to utilise a ligation independent cloning method called FastCloning (Li et al., 2011) to sub-clone idmK into the pET(2)idmJ/idmI plasmid to express all three proteins together. Figure 3.28 (c) illustrates the FastCloning strategy to be used. In short, insert DNA from pUCidmK was amplified using primers designated “IdmK Triple Clone For” and “IdmK Triple Clone Rev”. The PCR primers used to amplify the pET(2)idmJ/idmI plasmid were designated “pET(2)idmJ/idmI Triple Clone For” and “pET(2)idmJ/idmI Triple Clone Rev”. Amplification with these primers created overlapping complimentary ends between “IdmK Triple Clone For” and “pET(2)idmJ/idmI Triple Clone For” and between “IdmK Triple Clone Rev” and “pET(2)idmJ/idmI Triple Clone For” (primer sequences are located in the appendix). Results from the PCR reactions can be seen in Figure 3.32.
The agarose gel in Figure 3.32 shows the successful amplification of idmK (lanes 3 and 4) and pET(2)idmJ/idmI (lanes 1 and 2). idmK and pET(2)idmJ/idmI were mixed in a 1:1 volumetric ratio and digested with DpnI for 1 hour. DpnI digests the template DNA from the reaction but is also said to possess a small amount of non-specific exonuclease activity and randomly removes a few DNA bases off the ends of the insert and vector DNA, thus creating complimentary sticky ends. The DpnI digested DNA was then transformed into E. coli XL10 Gold cells. Subsequent colony growth on 2 × TY agar plates containing ampicillin indicated the presence of the pET(2)idmJ/idmK/plasmid. Restriction digests were performed with SacI-HF® and XhoI. Digestion of the pET(2)idmJ/idmK/plasmid with these restriction enzymes expected to yield three DNA fragments, one 1.8 kb in length corresponding to idmJ and idmK as one fragment, one 1.2 kb in length corresponding to idmJ and finally a band at 5.5 kb corresponding to pETDUET. Figure 3.33 below shows the restriction digest of the pET(2)idmJ/idmK/idmI plasmids purified from a colony from the transformation of DpnI digested DNA.
Figure 3.32 illustrates the potential pET(2)idmJ/idmK/idmI plasmid created using the FastCloning strategy. This plasmid was subsequently transformed into *E. coli* XL10 Gold cells and the plasmid sequenced with the “pETUPSTREAM” and “DUETDOWN1” sequencing primers. This confirmed the presence of *idmK* in the pET(2)idmJ/idmK/idmI plasmid from Figure 3.32. The next stage was to attempt protein expression.

### 3.3.7 Recombinant tandem expression of IdmJ, IdmI and IdmK

The pET(2)idmJ/idmK/idmI plasmid was transformed into *E. coli* BL21 (DE3) Gold cells for the purposes of protein expression. Small scale protein expression trials were carried out, testing soluble protein expression in TB, 2 × TY, LB and autoinduction medium at 37°C, 21°C and 18°C as described for IdmJ in section 3.2.4. The soluble cellular fractions for *E. coli* BL21 (DE3) Gold cells containing the pET(2)idmJ/idmK/idmI plasmid were analysed by SDS-PAGE (Figure 3.33). The expected masses of IdmJ, IdmI and IdmK are approximately 53 kDa, 41 kDa and 11 kDa respectively, indicated by the red, blue and green arrows in Figure 3.34.
Analysis of the SDS-PAGE gel (Figure 3.34) of soluble cell fractions from the IdmJ, IdmI and IdmK tandem expression trials, in comparison to uninduced E. coli BL21 (DE3) Gold cells (See appendix), suggests that IdmK may have been expressed as soluble protein in all media barring autoinduction medium and at all three expression temperatures. It is unclear as to whether IdmI and IdmJ had been overexpressed in any of these conditions due to a lack of obvious protein bands at the expected masses.

In order to probe the tandem expression of IdmJ and IdmI with IdmK a western blot was performed (section 2.2.3.3). Figure 3.34 shows that IdmK was expressed as soluble protein in all conditions apart from autoinduction medium. Probing for the His$_6$-tag in the soluble fraction may well give a positive result solely due to the
presence of IdmK and will provide no further information as to whether IdmJ or IdmI were being expressed as soluble proteins. A western blot enabled discrimination of which proteins were being expressed as soluble proteins as they are separated by size on an SDS-PAGE gel. Figure 3.35 shows the results of the western blot performed of expression of pET(2)/idmJ/idmK/idmI plasmid in TB and LB medium at 21°C, which indicated there was soluble protein expression in a prior His$_6$-tag screen.

The western blot (Figure 3.35) showed that when the pET(2)/idmJ/idmK/idmI plasmid was expressed in E. coli BL21 (DE3) Gold cells, soluble protein was expressed at 21°C in TB (Figure 3.35 Lane 1) and LB (Figure 3.35 Lane 2) medium. However, the approximate size of the protein expressed was comparable to that seen when expressing the pET(2)/idmJ plasmid, indicating that only soluble IdmJ was expressed (SDS-PAGE in Figure 3.13). This was also true for expression the pET(2)/idmJ/idmK/idmI plasmid at 18°C.
3.3.8 Summary and conclusions

After initial difficulties in cloning idmI into pET23a, the gene was successfully cloned into pKK223-3. Expression trials of the pKidmI plasmid however did not yield any soluble protein (Figure 3.25). In order to express soluble protein idmI was then cloned into a pMAL-c5X vector to create a pMALIdmI construct, expressing IdmI as a fusion construct with the maltose binding protein (MBP). Again, expression of a soluble fusion protein was not achieved. Probing for the His6-tag indicated that the expression of IdmI may be somewhat temperature dependent (Figure 3.28). Tandem expression of IdmI with IdmJ and IdmK from the pET(2)idmJidmKidmI plasmid did not yield soluble IdmI either (Figure 3.35). The fact that protein is continually expressed as an insoluble aggregate may indicate that IdmI is not being folded correctly. This may be due to E. coli’s inability to form disulphide bonds in the cytoplasm. A cell line like Origami2 (DE3) could be used in attempts to express soluble protein (Bessette et al., 1999). An additional option includes unfolding insoluble aggregates in 8 M urea followed by refolding to resolubilise IdmI. The latter of these two options will require screening for a refolding buffer and optimisation. No biochemical or structural characterisation could be carried out as no soluble protein was purified.

3.4 Discussion

Attempts at heterologous expression of the proteins responsible for starter unit construction in indanomycin biosynthesis began with cloning and expression of IdmJ and IdmI, the adenylyltransferase and dehydrogenase. IdmJ was, to some extent, successfully expressed heterologously, but contains an unidentified post-translational modification. IdmI was unable to be successfully, heterologously expressed in E. coli as a soluble protein.

Figure 3.20 shows a schematic to explain what may be occurring when expressing IdmJ and the IdmJ variants. It suggests that mutating C127A could be creating an active adenylyltransferase that can load phosphopantetheine, which may be present in the cell, with proline. Mechanistic determination of a PKS module by Whicher et al. (2014) showed that for successful chain elongation and β-keto processing, specific protein-protein interactions with the carrier protein and corresponding
enzyme within the module was required. This may indicate that C127A was able to load phosphopantetheine with proline but unable to release the subsequent product as the system lacked the cognate carrier protein. Further experimental evidence for this theory could be provided using traditional feeding as carried out with PKS, using radiolabelled proline (Birch et al., 1955; Day and Mantle, 1982; Staunton and Weissman, 2001). Optimisation and purification of cysteine variants, especially C127A, may also allow biochemical characterisation, and determine if the variants were expressing as fully folded and functional enzymes.

As mentioned previously, other enzymes required for pyrrole formation in the same manner have been reconstituted in E. coli. The genes of pyrrole forming enzymes in clorobiocin and coumermycin biosynthesis were obtained by PCR amplification from cosmids containing genomic DNA, cloned into pET vectors and expressed from E. coli BL21 (DE3) cells (Garneau et al., 2005). It is interesting to note that expression and MS analysis of these proteins also showed the +178 Da species seen when expressing IdmJ (Garneau et al., 2005; Geoghegan et al., 1999).

There are a number of strategies still available to produce soluble IdmI. One such strategy includes obtaining the gene from genomic DNA and introducing a C-terminal His\textsubscript{6}-tag as with the dehydrogenase found in clorobiocin biosynthesis. Expression could be carried out in E. coli cells which aid in disulphide bond formation such as E. coli Origami (DE3) cells. Finally, another option is to unfold the IdmI insoluble aggregate and screen for a refolding condition. Biochemical and structural characterisation using techniques such as circular dichroism may provide evidence a fully folded functional enzyme has been obtained. Formation of a pyrrole by an NRPS in an NRPS/PKS hybrid enzyme, specifically as a starter unit, may require the rest of the enzyme, the downstream PKS modules, to dock onto and fold. This may have been why difficulties in soluble expression of individual domains were encountered. Reconstitution of the starter module with a downstream PKS module may provide evidence for this.

The next chapter in this thesis will cover cloning and expression of the third protein in this module, the prolyl carrier protein, IdmK.
Results one
4. Cloning, expression and characterisation of the peptidyl carrier protein from the starter module in indanomycin biosynthesis

As described in the introduction (section 1.4), nonribosomal peptide synthetases (NRPS) are large multimodular proteins, with a minimal module being composed of an adenylation domain, condensation domain and finally a peptidyl carrier protein. Within the NRPS modules, the carrier protein (CP), a non-catalytic protein, is essential for function. The phosphopantetheinylated CP houses the growing nonribosomal peptide (NRP) chain, receiving it from the CP within the preceding module. The CP then “passes” the extending chain to the CP in the following the module. The carrier proteins are post-translationally modified by an auxiliary enzyme, a phosphopantetheinyl transferase (PPTase), which modifies a conserved serine, adding a 4'-phosphopantetheine, providing a thiol for attachment of the extending chain (Lambalot et al., 1996). Chain elongation occurs in an assembly-line like fashion with each module responsible for adding a single unit to the extending chain. The extending unit selected by the AT/A domain residing on the ACP/PCP undergoes condensation with the extending chain catalysed by the KS/C domain (Meier and Burkart, 2009; Fischbach and Walsh, 2006).

The prolyl carrier protein (IdmK) is the post-translationally modified non-catalytic essential domain in the starter module of the indanomycin biosynthetic gene cluster. After receiving the activated L-proline from the adenylyltransferase (IdmJ) (section 3.2) the IdmK bound L-proline becomes a substrate for further modification by IdmI (section 3.3) before the starter unit, pyrrole-2-carboxylate, is handed onto the next module in the NRPS-PKS multienzyme (Li et al., 2009). Figure 3.1 shows the role of IdmK in starter unit biosynthesis in the indanomycin biosynthetic gene cluster.

In a number of previous studies, various NRPS and PKS carrier proteins have been recombinantly expressed in E. coli, not only as individual domains but also as part of reconstituted modules and extensive structural studies have been carried out (Crosby and Crump, 2012; Chan and Vogel, 2010; Meier and Burkart, 2009). Studies have been conducted attempting to phosphopantetheinylate the conserved serine with
exogenous PPTases, exploring not only the carrier protein but the PPTases tolerance for modification (Beld et al., 2014).

The carrier protein, a small protein of less than 100 amino acids, is an essential part of the modular enzymes. The carrier protein must have the ability to “communicate” with other domains within its own module to receive substrates and with domains in adjacent modules in order to pass and receive the extending chain (Tran et al., 2010; Weissman and Muller, 2008; Crosby and Crump, 2012). Structural and dynamic information including protein-protein interactions could provide insight into how this protein functions, in the hope of engineering it for the production of novel compounds. Figure 1.10 shows the proposed mechanism (from cryo-EM data) by which acyl carrier proteins interact with each domain involved in substrate selection, catalysis and modification meting chain elongation (Dutta et al., 2014; Whicher et al., 2014). These mechanistic findings show that chain elongation is very much carrier protein centric, with the carrier protein being essential for chain elongation and modification, and further emphasises the importance in understanding a carrier protein and its interactions with other domains within its own, and subsequent modules (Dutta et al., 2014; Whicher et al., 2014; Nguyen et al., 2014).

Cloning, protein expression and purification followed by structural characterisation of IdmK will hopefully enable mechanistic insights into how chain elongation occurs during indanomycin biosynthesis as well as allowing the fundamental protein-protein interactions of the carrier protein to be determined.

4.1 Cloning the peptidyl carrier protein (idmK)

4.1.1 Cloning the peptidyl carrier protein (idmK) into pET23a

The synthetic idmK gene was designed with HindIII and XhoI sites at the 5’ and 3’ ends of the gene, respectively. As with the two previous genes (Chapter 3), the sites were selected specifically for sub-cloning idmK into pET23a using the cut-and-paste method (as illustrated in Figure 3.3). Insert DNA was prepared in an identical manner to idmJ and idmI however the restriction enzymes HindIII-HF® and XhoI were used to digest the pUCidmK plasmid. The pET23a vector was digested with the same restriction enzymes, creating complimentary sticky ends. Vector and insert
Results two

DNA were then run on a 0.7 % agarose gel and purified as described in section 2.2.2.3. Ligation reactions between pET23a and idmK were performed as described in section 2.2.2.8. 4 µL of this ligation reaction was then transformed into *E.coli* XL10 Gold Ultracompetent cells and cells were subsequently plated onto 2 × TY agar plates containing ampicillin. Colony growth on the transformation plates suggested the presence of the desired plasmid, pETidmK. To screen for the correct plasmid, DNA from 10 colonies was purified and digested with *HindIII*-HF® and *XhoI* restriction enzymes (section 2.2.2.7). Resulting constructs were analysed by running the digested DNA on an agarose gel. Figure 4.1 shows the results of the restriction digests of plasmid DNA purified from the 10 colonies selected from the ligation between pET23a and idmK.

![Figure 4.1](image)

**Figure 4.1**-0.7 % agarose gel of isolated plasmid DNA from the 10 colonies selected from the transformation plate of the ligation reaction between pET23a and idmK. Plasmid DNA was digested with *HindIII*-HF® and *XhoI* to screen for the pETidmK plasmid. The grey arrow indicates the expected size of the pET23a vector (3666 bp) and the green arrow indicates the expected size of idmK (325 bp). Plasmid DNA in lane 1 appears to remain undigested plasmid DNA. In lanes 2, 4 and 6 plasmid DNA digested with *HindIII*-HF® and *XhoI* yielded two DNA fragments at approximately 2700 bp and 325 bp corresponding to the original vector pUC57 and idmK. Plasmid DNA in lane 3 digested with *HindIII*-HF® and *XhoI* yielded a single band at approximately 3000 bp, this corresponds to the pUCidmK plasmid digested at a single point. Plasmid DNA in lane 5 appears to be 2700 bp corresponding to empty pUC57. The plasmid purification in lane 7 appears to have been unsuccessful. Finally plasmid DNA in lanes 8, 9 and 10 digested with *HindIII*-HF® and *XhoI* show a single band at approximately the correct size for pET23a alone (3666 bp).

A pETidmK plasmid digested with *HindIII* and *XhoI* should yield two bands on a DNA gel; a band at 3666 bp corresponding to pET23a and a second band at 325 bp corresponding to idmK. Figure 4.1 indicates the ligation between pET23a and idmK.
Results two

was unsuccessful. Plasmid DNA in lane 1 appears to have remained undigested by HindIII-HF® and XhoI, indicating it is not the pETidmK plasmid. Plasmid DNA in lanes 2, 4 and 6 show DNA fragments 2700 bp and 325 bp in length corresponding to pUC57 and idmK, this is indicative of pUCidmK contamination of the ligation reaction. Plasmid DNA in lanes 3, 5, 8, 9 and 10 digested with HindIII-HF® and XhoI show single DNA fragments of varying sizes, none of these plasmids appear to contain the expected 325 bp insert corresponding to idmK. Further attempts were made to clone idmK into pET23a however this approach was not successful. The next strategy was to clone IdmK into pKK223-3 plasmid as attempted with idmJ and idmI.

4.1.2 Cloning the peptidyl carrier protein (idmK) into pKK223-3

As with idmJ and idmI the next vector selected for sub-cloning idmK was pKK223-3 (as illustrated in Figure 3.6), creating a plasmid designated pKidmK. To prepare the insert, restriction digests were carried out with the pUCidmK plasmid and pKnanaA plasmid using EcoRI-HF® and PstI-HF® creating complimentary sticky ends for ligation (section 2.2.2.7). Digested plasmids were run on a 0.7 % agarose gel and insert and vector DNA extracted from the gel (section 2.2.2.3). A ligation reaction was then carried out between pKK223-3 and idmK digested with EcoRI-HF® and PstI-HF®. 4 µL of the ligation reaction was transformed into E. coli XL10 Gold Ultracompetent cells. Successful ligation of idmK and pKK223-3 was indicated by cell growth on 2 x TY agar plates containing ampicillin. 10 colonies were selected from the transformation plates, plasmid DNA purified, and the pKidmK plasmid screened by restriction digestion with EcoRI-HF® and PstI-HF®. A pKidmK plasmid digested with EcoRI-HF® and PstI-HF® restriction enzymes should yield two DNA fragments, one at 4600 bp and a second at 325 bp corresponding to pKK223-3 and idmK respectively. Figure 4.2 below shows the results from the restriction digests screening for the pKidmK plasmid.
Figure 4.2 - 0.7% agarose gel showing restriction digests of purified plasmid DNA from the pKK223-3 idmK ligation. Plasmid DNA was purified from 10 colonies and digested with EcoRI-HF® and PstI-HF® to screen for the pKidmK plasmid. Plasmid DNA in lanes 1 and 2 digested with EcoRI-HF® and PstI-HF® have yielded a single band approximately 3000 bp in length, corresponding to incomplete digestion of pUCidmK. Digestion of plasmid DNA in lane 3 yielded two DNA fragments, one 2700 bp in length corresponding to pUC57 and a second 325 bp corresponding to idmK. Plasmid DNA digested by EcoRI-HF® and PstI-HF® in lanes 4 and 7 both yielded DNA fragments of approximately 4600 bp and 325 bp corresponding to pKK223-3 and idmK. Digestion of plasmid DNA in lane 5, 8 and 10 yielded two DNA fragments, one approximately 2700 bp (pUC57) and an DNA fragment approximately 1200 bp of unknown origin. Finally digestion of plasmid DNA in lanes 6 and 9 yielded a single band at approximately 4600 bp corresponding to pKK223-3.

Restriction digestion of plasmid DNA in lanes 4 and 9 in Figure 4.2 shows successful ligation between idmK and pKK223-3, creating the pKidmK plasmid, exhibiting DNA bands 4.6 kb and 325 bp in length corresponding to the correct sizes for the pKK223-3 vector and idmK insert respectively. A glycerol stock was made of E. coli XL10 Gold Ultracompetent cells transformed with the plasmid from lane 4. Confirmation of the pKidmK plasmid was obtained by DNA sequencing of plasmid 4 with the sequencing primers “PTRC-99A-FOR” and “PTRC-99A-REV” (sequences can be located in the appendix). Sequencing showed that the pKK223-3 vector contained the correct insert. The sequenced pKidmK plasmid was then transformed into E. coli BL21 Gold (DE3) cells for protein expression.

4.2 Expression of the prolyl carrier protein (IdmK)
Expression of the prolyl carrier protein was carried out using E.coli BL21 Gold (DE3) cells transformed with the pKidmK plasmid. A small scale protein expression
of the putative *S. antibioticus* prolyl carrier protein (IdmK) was tested on a 5 mL scale, in 2 × TY medium at 37°C as with IdmJ (section 3.2.4). Soluble and insoluble cellular components of lysed cells were analysed by SDS-PAGE in order to evaluate whether IdmK was expressed as a soluble protein (Figure 4.3).

![Reducing SDS-PAGE analysis of insoluble (lane 1) and soluble (lane 2) fractions suggested that IdmK was successfully expressed as soluble protein (indicated by the green arrow) in 2 × TY media at 37 °C.](image)

The expected mass of IdmK, as calculated from the amino acid sequence of the synthetic gene, is approximately 11044 Da, as indicated by the green arrow in Figure 4.3. The SDS-PAGE gel in Figure 4.3 suggests that IdmK has been expressed as a soluble protein, indicated by the presence of a band at the expected mass in the soluble fraction of the *E. coli* BL21 (DE3) Gold cells transformed with the pKidmK plasmid (lane 2). This protein band is absent in the insoluble fraction providing validation that IdmK is expressed as a soluble protein.

### 4.3 Purification of the prolyl carrier protein (IdmK)

Protein expression was repeated on a larger (1 L) scale. IdmK was then purified by nickel affinity chromatography as described in section 2.2.3.4. Samples were taken at each stage of the purification and analysed by SDS-PAGE (Figure 4.4).
Figure 4.4- Reducing SDS-PAGE analysis of the purification of IdmK by nickel affinity chromatography. IdmK was expressed in *E. coli* BL21 (DE3) gold cells induced by IPTG (1), cells were harvested, lysed and the soluble (2) and insoluble (3) fractions separated by centrifugation. The soluble supernatant was then incubated with nickel resin for one hour, the supernatant (4) was discarded and the resin was washed with a low imidazole buffer (20 mM) (5-8) to remove non-specifically bound proteins. IdmK was then eluted from the resin using a high imidazole elution buffer (0.5 M) (9). Two dominant protein species were eluted from the resin, one approximately 11 kDa (dark green arrow) possibly corresponding to IdmK, the second (light green arrow) with an approximate mass of 22 kDa.

Figure 4.4 (lane 9) suggests that IdmK has successfully been purified using nickel affinity chromatography although two bands were seen. To provide further evidence IdmK had been successfully purified and to determine more accurate masses of the two species, a 70 µL sample of eluted protein was desalted into 50 mM ammonium acetate pH 7.4 (section 2.2.3.9) for analysis by ESI-MS (section 2.2.3.12). Figure 4.5 shows the results of ESI-MS analysis of the purification of IdmK.
Results two

Figure 4.5- ESI-MS of the eluted protein from the purification of IdmK. A sample of eluted protein (Figure 4.4, lane 9) was desalted into 50 mM ammonium acetate pH 7.4 and analysed by ESI-MS to obtain a molecular mass of the species present. (Top) Mass spectrum raw data; (bottom) deconvoluted mass spectrum of raw data showing the masses of proteins purified. Two species were detected, one at 11 044 Da, and a second at 22 768 Da.

The expected mass of IdmK as calculated from the amino acid sequence of the synthetic gene is 11045 Da (Table 4.1). This mass of \textit{apo-}IdmK does not include post-translational modification of the carrier protein by phosphopantetheinylation. After modification the mass expected for phosphopantetheinylated IdmK, \textit{holo-}IdmK, should be 11 386 Da (Table 4.1). The mass spectrum (Figure 4.5) shows that \textit{apo-}IdmK, with a mass of 11 044 Da, was purified as one of the species, while the mass of the second protein species eluted during purification (Figure 4.4 lane 9) (22 768 Da) corresponds to the mass that would be expected for dimeric \textit{holo-}IdmK. A possibility is that dimerisation might occur through the terminal thiol of the phosphopantetheine group. This dimerisation would create a non-functional covalent dimer with an expected mass of 22 770 Da, compared to the 22 768 Da.
Results two

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nomenclature</th>
<th>Expected mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>IdmK</td>
<td>apo-IdmK</td>
<td>11 045</td>
</tr>
<tr>
<td>Phosphopantetheinylated</td>
<td>holo-IdmK</td>
<td>11 386</td>
</tr>
</tbody>
</table>

Table 4.1- Table showing the expected masses of IdmK as calculated from the amino acid sequence.

In order to test the idea that a covalent dimer was being formed through disulphide bond formation between the terminal thiol groups of the phosphopantetheine modification of holo-IdmK the two protein species were further purified and characterised. The protein eluted from the nickel affinity purification was subject to further purification by size exclusion chromatography, thus separating dimeric and monomeric IdmK by size. Figure 4.6 shows the size exclusion chromatography trace for IdmK. SDS-PAGE analysis was used to confirm the separation of the two species.

![Size exclusion chromatography trace](image)

Figure 4.6- Purification of IdmK by size exclusion chromatography. Size exclusion chromatography trace, the Y axis shows the absorbance of the eluent. The X axis shows the volume. Fractions from peak 1 and peak 2 were run on SDS-PAGE gels (inset). The light green arrow indicates the potentially dimeric form of IdmK and the dark green arrow indicates the monomeric form of IdmK.
Results two

IdmK was gel filtered using a HiLoad Superdex 75 prep grade (S75) column (Figure 4.6). The dimeric species (Figure 4.6 peak 1) eluted from the S75 column between 160 and 180 mL, while monomeric apo-IdmK (Figure 4.6 peak 2) was eluted between 190 mL and 210 mL. Samples from the peak fractions were analysed by SDS-PAGE (Figure 4.6).

To provide evidence that IdmK was assembling as a dimer through the formation a disulphide bind between the terminal thiol of the phosphopantetheine modifications, Tris (2-carboxyethyl) phosphine (TCEP) was used as a reducing agent to break the suspected disulphide bond. A 70 µL sample was taken from peak one of the size exclusion chromatography trace shown in Figure 4.6, indicated to be dimeric IdmK, and was desalted into 50 mM ammonium acetate pH 7.4. TCEP was added to the sample to a final concentration of 5 mM and the reduced protein sample was then analysed by ESI-MS (Figure 4.7).

![Figure 4.7- ESI-MS analysis of the reduced IdmK dimer. A sample from peak one of size exclusion chromatography was reduced by the addition of TCEP and analysed by ESI-MS. A single major protein species at 11 384 Da was observed, this corresponds to phosphopantetheinyalted IdmK (holo-IdmK).](image-url)
The mass spectrum measured after reduction (Figure 4.7) corresponds to the expected mass of the monomeric holo-IdmK, with a mass of 11 384 Da (Table 4.1), confirming the possibility that IdmK could be oxidised to form a covalent dimer during the growth, cell breakage or purification. The fortuitous discovery of holo-IdmK forming a homodimer can be utilised to purify homogeneous samples of both apo- and holo-IdmK. Incidentally, this result demonstrated that *E. coli* possesses the ability to phosphopantetheinylate the recombinantly expressed carrier protein.

### 4.3.1 Confirmation of the site of phosphopantetheinylaition

The phosphopantetheinylation of IdmK, or any carrier protein, is required for function. The site of post-translational modification is a highly conserved serine residue (Quadri *et al.*, 1998; Walsh *et al.*, 1997; Staunton and Weissman, 2001). To confirm the presence of the correct post-translational modification, the conserved serine residue in IdmK was identified. A BLAST search followed by a multiple sequence alignment of similar amino acid sequences was performed identifying Ser44 to be conserved (appendix figure 7.3). To confirm that this was the site of phosphopantetheinylation, site-directed mutagenesis was used to mutate the conserved serine 44 to an alanine (S44A), thereby preventing the modification from occurring. To create the S44A variant the pKidmK plasmid DNA was purified from *E. coli* XL10 Gold Ultracompetent cells and site-directed mutagenesis performed using the Stratagene QuikChange® Site-Directed Mutagenesis kit (section 2.2.2.10) using the mutagenic primers “S44A For” and “S44A Rev” (sequences located in the appendix). DNA sequencing using the “PTRC-99A-FOR” and “PTRC-99A-REV” sequencing primers confirmed the presence of the targeted serine to alanine mutation (sequences located in the appendix). The pKidmK mutant plasmid was transformed into *E. coli* BL21 (DE3) Gold cells for subsequent protein expression. The IdmK variant was expressed and purified in an identical manner to wild-type IdmK and analysed by ESI-MS.
Figure 4.8 above shows the mass spectrum of the purified S44A IdmK variant. Only a single peak was observed (at 11 028 Da) corresponding to the expected mass of the apo-S44A variant. The lack of any covalent dimeric species along with the lack of any phosphopantetheinylated species confirmed the site of post-translational modification to be Ser44.

Figure 4.8- ESI-MS analysis of the purified S44A IdmK variant. The IdmK S52A variant was expressed in *E. coli* BL21 (DE3) Gold cells in an identical manner to wild-type IdmK, eluted protein was desalted into 50 mM ammonium acetate pH 7.4 and analysed by ESI-MS. (Top) mass spectrum raw data (bottom) deconvoluted mass spectrum of raw data showing the mass of the purified protein. The expected mass of the IdmK variant was 11 028 Da, this was observed, in addition there is another species identified, 57 Da larger than the IdmK variant, the potential adduct being a nickel ion.
4.4 Structural characterisation of the prolyl carrier protein (IdmK)

Prior to this work a number of carrier protein structures from FAS, PKS and NRPSs existed in the protein data bank (PDB) the structures of which were resolved by a number of biophysical techniques. Table 4.2 shows examples of carrier proteins with structures resolved deposited in the PDB.

<table>
<thead>
<tr>
<th>PDB code</th>
<th>Structure</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2JU1</td>
<td>Apo-ACP from module 2 of 6-deoxyerythronalide B</td>
<td>NMR spectroscopy</td>
<td>Alekseyev et al., 2007</td>
</tr>
<tr>
<td>1T8K</td>
<td>Apo-ACP from FAS in <em>E. coli</em></td>
<td>X-Ray crystallography (Multi-wavelength anomalous dispersion)</td>
<td>Qiu and Janson, 2004</td>
</tr>
<tr>
<td>1L0H</td>
<td>Butyryl-ACP from FAS in <em>E. coli</em></td>
<td>X-Ray crystallography (Multi-wavelength anomalous dispersion)</td>
<td>Roujeinikova et al., 2002</td>
</tr>
<tr>
<td>1DNY</td>
<td>Apo-PCP from module 3 of tyrocidine synthetase</td>
<td>NMR spectroscopy</td>
<td>Weber et al., 2000</td>
</tr>
<tr>
<td>2LIU</td>
<td>Holo-ACP from the Curacin A PKS</td>
<td>NMR spectroscopy</td>
<td>Busche et al., 2012</td>
</tr>
<tr>
<td>3GZM</td>
<td>Holo-ACP from FAS in <em>Plasmodium Falciparum</em></td>
<td>X-Ray crystallography (molecular replacement)</td>
<td>Gallagher and Prigge, 2010</td>
</tr>
<tr>
<td>2LPK/2LL8</td>
<td>Holo/apo-ACP from FAS in <em>Rhodopseudomonas palustris</em></td>
<td>NMR spectroscopy</td>
<td>Ramelot et al., 2012</td>
</tr>
<tr>
<td>1AF8</td>
<td>Apo-ACP from the actinorhodin PKS in <em>S. coelicolor</em> A3(2)</td>
<td>NMR spectroscopy</td>
<td>Crump et al., 1997</td>
</tr>
<tr>
<td>1NQ4</td>
<td>Apo-ACP from oxytetracycline PKS</td>
<td>NMR spectroscopy</td>
<td>Findlow et al., 2003</td>
</tr>
<tr>
<td>2PNG</td>
<td>Apo-ACP from rat FAS</td>
<td>NMR spectroscopy</td>
<td>Ploskon et al., 2008</td>
</tr>
<tr>
<td>1HY8</td>
<td>Apo/Holo-ACP from FAS in <em>B. subtilis</em></td>
<td>NMR spectroscopy</td>
<td>Xu et al., 2001</td>
</tr>
</tbody>
</table>

Table 4.2- Examples of carrier protein structures found in the PDB.

A comprehensive review of carrier protein structures can be found in Crosby and Crump (2012).

4.4.1 Sample preparation and screening crystallisation conditions

For structural studies of IdmK homogeneous protein samples were required. Due to the dimerisation that occurs via the terminal thiol of the phosphopantetheine post-translational modification, homogenous samples of both *apo* and *holo* IdmK
could be obtained by subjecting IdmK to further purification by size exclusion chromatography as in Figure 4.6. Fractions collected of apo-IdmK (Figure 4.6, peak 2) were pooled and concentrated to between 10 mg/mL for the screening of crystallisation conditions.

Due to the considerable differences between the amino acid sequence of IdmK and the previous carrier proteins available in the PDB, as well as the disparity in crystallisation conditions described, a set of commercially available crystallisation screens were set up (Hampton Research (USA) screens; Crystal screen 1 and 2, Index 1 and 2, Salt RX and from Emerald BioStructure (Germany) Wizard 3 and 4). Crystallisation was attempted by vapour diffusion of 2 µL sitting drops (1:1 protein to mother liquor ratio) in 96 well-plates at 18°C. For crystallisation trials apo-IdmK, in 50 mM Tris/HCl pH 7.4, was used at a concentration of 10 mg/mL. A high percentage of drops showed protein precipitation, indicating the protein concentration was close to supersaturation. Nevertheless, 11 initial crystal hits were observed after one week, crystal growth appears to be encouraged in conditions containing PEG 3350 and a higher pH (8.5). Figure 4.9 shows some of the crystal forms observed.

Crystals were cryoprotected in 25 % (v/v) glycerol, flash cooled in liquid nitrogen and screened for diffraction at 100 K on beamline I04-1 at Diamond Light Source Ltd (DLS). High-resolution diffraction data were collected from crystals grown in pH 8.2 0.056 M sodium phosphate monobasic monohydrate, 1.344 M potassium phosphate dibasic. Further optimisation of the crystallisation conditions was attempted by varying protein concentration (6-12 mg/mL), protein to mother liquor ratio (1:1, 1:2 and 2:1) and pH (7.8-8.2). Subsequent crystal trays were set up using these conditions in a hanging drop format. The best crystal growth was observed at pH 8.0 and 8.2 with protein concentrations above 9 mg/mL.
Results two

X-ray data were collected at I04 beamline at DLS. Data collection and processing was carried out by Dr Chi Trinh (Astbury Centre, University of Leeds, UK). The data were reduced using the automated processing suit Xia2 at DLS. Data reduction statistics and unit cell parameters can be located in the appendix. Phasing by molecular replacement (using both Molrep (Vagin and Teplyakov, 1997) and Phaser (McCoy et al., 2007)) was unsuccessful, mainly due to the large number of monomers present in the asymmetric unit cell. An estimated 7 or 8 monomers were predicted from Matthews co-efficient (Matthews, 1968). Since molecular replacement failed for structural characterisation of IdmK there are number of alternatives that could be considered, including screening for a different crystal form, use anomalous diffraction using selenomethionine labelled protein, or use of another biophysical technique. Structural characterisation of IdmK by X-ray crystallography was not continued.

4.5 Structure determination using NMR spectroscopy

As mentioned in section 4.2 a number of carrier protein structures exist in the PDB, solved by a number of biophysical techniques. Nuclear magnetic resonance has been used to solve a number of apo- and holo- carrier protein structures including the peptidyl carrier protein from the third module of the tyrocidine biosynthetic gene cluster (Figure 1.16) from Brevibacillus brevis (PDB codes 1DNY, 2GDW, 2GDX, 2GDY) (Weber et al., 2000; Koglin et al., 2006) and of the acyl carrier protein from B. subtilis (PDB code 1HY8) (Xu et al., 2001). These structures illustrate the
potential for using NMR to structurally characterise IdmK, and potentially make comparisons between the apo- and holo- protein species. The first step in studying IdmK by NMR was to express and purify isotopically labelled protein.

4.5.1 Initial analysis of the suitability of IdmK for structural studies by NMR spectroscopy

Previous results in this chapter have shown that IdmK had successfully been expressed and purified from *E. coli* BL21 (DE3) Gold cells transformed with the pKidmK construct. In order to assess the suitability of the use of NMR for structural studies, expression of IdmK in minimal medium was required. Expression of IdmK in minimal medium was carried out in M9 minimal medium (section 2.1.7) and the standard expression protocol used (section 2.2.1.5 and 2.2.3.4). The inoculation procedure of the minimal medium was augmented to prevent transfer of 2 × TY medium from the day culture: cells from the 10 mL day culture were harvested by centrifugation and resuspended in 2 mL of minimal medium, and this was used to inoculate 500 mL expression cultures. To determine if IdmK could be expressed in minimal medium small-scale expression was trialled in minimal medium in the absence of labelled medium components. To analyse the expression of IdmK, *E.coli* BL21 (DE3) Gold whole cell lysate was run on a SDS-PAGE (Figure 4.10).

![Figure 4.10 - Reducing SDS-PAGE analysis of the small scale expression trial of IdmK in minimal media. Lane 1 is the whole cell lysate from *E. coli* BL21 (DE3) Gold cells containing the pKidmK plasmid.](image)
As can be seen in the SDS-PAGE gel in Figure 4.10 protein over-expression was observed when expressing IdmK in minimal medium, as indicated by the protein band at approximately 11 kDa (green arrow).

Due to the observed over-expression of IdmK in minimal medium, IdmK was then expressed in minimal medium containing $^{15}$NH$_4$Cl as the sole nitrogen source. Protein expression and purification were carried out by nickel affinity chromatography as previously described (section 2.2.3.4). Samples were taken at each stage of the purification and analysed by SDS-PAGE (Figure 4.11).

![Graph showing stages of nickel affinity chromatography](image)

**Figure 4.11-** Reducing SDS-PAGE gel showing the stages of nickel affinity chromatography of IdmK grown in minimal media containing $^{15}$NH$_4$Cl. Broad range protein size markers are displayed (M). Cells expressing IdmK (1) were harvested by centrifugation, lysed and the soluble (2) and insoluble (3) fractions separated. Although there is a small band at approximately 11 kDa, indicating some insoluble material, the majority of protein appears to be expressed as soluble protein. After binding the soluble protein to the nickel resin (4), four washes were performed to remove any non-specifically bound protein (5-8). The bound protein was eluted by washing with 0.5 M imidazole, purified $^{15}$N labelled IdmK is shown in lane (9). The dark green arrow indicates monomeric apo-IdmK and the light green arrow indicates the presence of dimeric holo-IdmK.

Figure 4.11 shows the successful purification of IdmK when expressed in minimal medium. As mentioned beforehand, both monomeric apo- and dimeric holo- species of IdmK are present upon purification by nickel affinity chromatography (Figure 4.11); therefore further purification by size exclusion chromatography was used to obtain a homogeneous protein sample of apo-IdmK for structural studies (Figure 4.12).
Results two

Before collecting any NMR spectra, the extent of \([^{15}N]\) labelling of IdmK was established. A sample of purified apo-IdmK was desalted into 50 mM ammonium acetate pH 7.4 (section 2.2.3.9) and the mass determined by ESI-MS. The amino acid sequence of the synthetic gene and the Internet tool Protein Calculator v3.4 (http://protcalc.sourceforge.net) were used to calculate the mass of uniformly labelled monoisotopic IdmK. The expected mass for uniform \([^{15}N]\) labelled IdmK was 11 191 Da. Figure 4.12 shows the result of attempting to express \([^{15}N]\) labelled IdmK.

Figure 4.12- ESI-MS spectrum to analyse the extent of isotopic labelling of IdmK. E. coli BL21 (DE3) Gold cells containing the plasmid pKidmK were used for expression of IdmK in minimal media containing \(^{15}\text{NH}_4\text{Cl}\) as the sole nitrogen source. Apo-IdmK was purified by nickel affinity chromatography followed by size exclusion chromatography. The labelled protein was then desalted into 50 mM ammonium acetate pH 7.4 and analysed by ESI-MS. A peak is observed at 11 188 Da, corresponding to fully \(^{15}\text{N}\)-labelled IdmK. (Inset) Reducing SDS-PAGE of a sample of \(^{15}\text{N}\)-labelled IdmK from size exclusion chromatography used in further NMR spectroscopy experiments.
Figure 4.12 shows that when IdmK is expressed in minimal medium containing $^{15}$NH$_4$Cl as the sole nitrogen source it has an observed mass of 11 188 kDa, which is in excellent agreement with the calculated molecular mass of fully $^{15}$N labelled apo-IdmK. The next stage in determining the suitability of IdmK for structural studies using NMR spectroscopy was to collect a 2D $^1$H-$^{15}$N Heteronuclear Single Quantum Coherence ($^1$H-$^{15}$N HSQC) spectrum.

### 4.5.2 Collection of a 2D heteronuclear single quantum coherence (HSQC) spectrum

Collection of a $^1$H-$^{15}$N HSQC spectrum allows initial analysis as to whether the target protein is suitable for further studies by NMR spectroscopy and therefore is the first spectra collected (Cavanagh et al., 2007; Rehm et al., 2002). Spectra were collected of apo-IdmK at a concentration of 0.5 mM in 50 mM Tris.HCl pH 7.4, 0.2% (w/v) sodium azide. D$_2$O was added to the sample to a final concentration of 10% (v/v). The $^1$H-$^{15}$N HSQC spectrum was collected at 25°C at a field strength of 500 MHz (Table 2.1 shows experimental parameters). Table 4.3 shows the coherence transfer or the path of magnetisation in the $^1$H-$^{15}$N HSQC experiment.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Correlation observed</th>
<th>Magnetisation transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^1$H-$^{15}$N HSQC</td>
<td>$^1$H$_i$-$^{15}$N$_i$</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.3**- Coherence transfer of the 2D Heteronuclear experiments $^1$H-$^{15}$N HSQC. The path of magnetisation is indicated by the double-headed red arrow, indicating the out-and-back nature of this experiment. The nuclei detected are highlighted in red circles (Cavanagh et al., 2007).

Figure 4.13 shows the $^1$H-$^{15}$N HSQC spectrum collected of $^{15}$N labelled apo-IdmK. In a $^1$H-$^{15}$N HSQC each peak corresponds to a proton coupled to nitrogen, therefore in this experiment each amide in the protein should have a representative peak in the $^1$H-$^{15}$N HSQC spectrum.
Results two

![Figure 4.13- $^1$H-$^{15}$N HSQC spectrum of apo-IdmK. The spectrum was measured in 50 mM Tris.HCl pH 7.4, 0.2% (w/v) sodium azide, at a protein concentration of 0.5 mM recorded at 500 MHz at 25 °C.](image)

The good peak dispersion in the spectrum (Figure 4.13) and sharp line intensities indicate that the protein is folded and stable in these conditions. IdmK is a 98-residue protein including the His$_6$-tag; approximately 90% of the peaks, representative of the amides, are present in the $^1$H-$^{15}$N HSQC. The number and equal intensities of the peaks indicates that there is little or no protein dynamics on a microsecond-millisecond timescale that will hinder structure determination by NMR. This spectrum indicates that NMR may be able to be used to elucidate an overall fold for IdmK.

As each peak represents an amide in the protein the $^1$H-$^{15}$N HSQC will provide the HN resonances, or ‘root’ resonances, to aid in the assignment of the triple resonance spectra and consequently structure determination of IdmK (described in chapter 5). The next requirement for structure determination by NMR is the expression and purification of doubly labelled [$^{13}$C, $^{15}$N] labelled IdmK.
4.5.3 Expression and purification of $[^{13}\text{C}, \text{N}^{15}] \text{IdmK}$

Initial experiments showed that IdmK could be expressed in minimal medium containing 4 g/L of glucose; however for cost efficiency when using $^{13}\text{C}$-glucose as the carbon source, the concentration of glucose required for protein over expression was investigated. Expression trials with minimal medium containing 2, 3 and 4 g of glucose per litre were tested. Protein was expressed as previously described (section 4.3.1), 3 separate cultures were grown with the varying glucose concentrations. Cells were harvested by centrifugation and whole cell lysate analysed by SDS-PAGE (Figure 4.14).

![Reducing SDS-PAGE to analyse the effects of changing the concentration of glucose in minimal media on protein expression. Lane 1 is the whole cell lysate of IdmK grown in minimal media containing 2 g/L of glucose, lane 2 contains 3 g/L of glucose and lane 3 contains 4 g/L of glucose.](image)

The expression trial showed that 2 g of glucose per litre was sufficient for protein expression. Subsequent protein expression for double labelled protein contained 10 mL 20 % (w/v) d-glucose-$^{13}\text{C}_6$ purchased from Cambridge Isotope Laboratories in 1 L. To produce $[^{13}\text{C}, \text{N}^{15}]$ labelled protein IdmK was expressed in medium containing $^{15}\text{NH}_4\text{Cl}$ and d-glucose-$^{13}\text{C}_6$ as the sole nitrogen and carbon sources (section 2.1.7). IdmK was purified by nickel affinity chromatography. Samples from each stage of purification were run on a SDS-PAGE gel for analysis (Figure 4.15).
As before, a homogeneous sample of apo-IdmK was obtained by size exclusion chromatography. The protein sample was then concentrated to 0.5 mM and the extent of labelling analysed by mass spectrometry (Figure 4.16).
Figure 4.16- ESI-MS spectrum to analyse the extent of $^{13}$C and $^{15}$N isotopic labelling of IdmK. *E. coli* BL21 (DE3) Gold cells containing the plasmid pKidmK were used for expression of IdmK in minimal medium containing $^{15}$NH$_4$Cl as the sole nitrogen source and D-Glucose-$^{13}$C$_6$ as the sole carbon source. Apo-IdmK was purified by nickel affinity chromatography followed by size exclusion chromatography. The labelled protein was then desalted into 50 mM ammonium acetate pH 7.4 and analysed by ESI-MS. A peak is observed at 11 663 Da, corresponding to fully $^{13}$C, $^{15}$N- labelled IdmK. (Inset) Reducing SDS-PAGE of $^{13}$C, $^{15}$N- labelled IdmK purified by size exclusion chromatography used in further NMR spectroscopy experiments.

The expected mass for $^{13}$C, $^{15}$N labelled IdmK as calculated by Protein Calculator v3.4 (http://protcalc.sourceforge.net) using the amino acid sequence of the synthetic gene was 11 668 Da. The observed mass for IdmK expressed in minimal medium containing $^{15}$NH$_4$Cl and D-glucose-$^{13}$C$_6$ as the sole nitrogen and carbon sources was 11 663 Da indicating that 99.9 % of the protein was isotopically labelled.
Results two

Having successfully labelled apo-IdmK ($^{15}$N and $^{13}$C) the next step in structure determination is assignment of the protein backbone, determining the chemical shifts of not only the $^1$H and $^{15}$N nuclei of the amide bond but also the Cα, Cβ and carbonyl nuclei. To do this 3D NMR spectra need to be collected. Chapter 5 will discuss the stages of structure determination by NMR spectroscopy.

4.6 Summary

IdmK was successfully cloned into pK223-3 and protein expressed and purified, as confirmed by SDS-PAGE and ESI-MS (Figures 4.4 and 4.5). Expression of IdmK produced both monomeric and dimeric species. Treatment of the dimeric IdmK species with TCEP, a reducing agent, showed that during expression of IdmK a PPTase endogenous to *E. coli* was able to phosphopantetheinylate IdmK. The dimeric species occurs due to the formation of a disulphide bond between the terminal thiol groups of two phosphopantetheine modifications. Contrary to expectations, both apo- and holo species were expressed in *E. coli*. BLAST searches in combination with multiple sequence alignments identified the potential serine, which becomes phosphopantetheinylated; this was confirmed by making a single point mutation of the identified serine and analysis by SDS-PAGE and ESI-MS. This showed only monomeric apo-IdmK was expressed when serine 44 was mutated to an alanine. The dimerisation of holo-IdmK allows purification of the apo- or holo-IdmK thus enabling a homogenous protein sample to be obtained. IdmK is an essential non-catalytic protein; it requires IdmJ to activate the amino acid it is to be loaded with. Initial attempts at structural characterisation of IdmK identified protein crystallisation conditions which produced crystals used for high resolution data collection. Subsequent analysis of the data collected showed that there were a large number of IdmK monomers within the asymmetric unit cell which meant molecular replacement could not be used for structure elucidation. Of the alternative options for structure determination NMR spectroscopy seemed a promising alternative, with a number of carrier protein structures having previously being solved by multidimensional NMR spectroscopy. Expression of $^{15}$N labelled IdmK followed by analysis by collecting a $^1$H-$^{15}$N HSQC spectrum suggested that IdmK was suitable
for structural studies by NMR and thus $[^{13}\text{C},^{15}\text{N}]$ labelled IdmK was expressed and purified for backbone assignments.

### 4.7 Discussion
Successful cloning and expression of IdmK has allowed characterisation of the site of post-translational modification. The unexpected post-translational modification, carried out by a PPTase endogenous to *E. coli* such as EntD (Lambalot *et al.*, 1996; Gehring *et al.*, 1997), enables purification of homogeneous *apo*- and *holo*- IdmK samples for biochemical and structural characterisation. Although structure determination using X-ray crystallography was problematic, screening for a new crystallisation condition, could open up this avenue for further structural characterisation. Expression of IdmK in minimal medium with $^{15}\text{NH}_4\text{Cl}$ and D-glucose-$^{13}\text{C}_6$ enabled expression of single, $[^{15}\text{N}]$, labelled IdmK and double, $[^{13}\text{C},^{15}\text{N}]$, labelled IdmK. An initial $^1\text{H}$.$^{15}\text{N}$-HSQC showed that IdmK was suitable for structural studies using NMR. Chapter 5 describes the use of NMR for structure determination and how this was carried out for determining an overall fold for IdmK.
Results two
5. Structural characterisation of IdmK by nuclear magnetic resonance spectroscopy

The previous chapter presented the results of cloning and expressing the putative proline carrier protein from *S. antibioticus* NRRL 8167 in *E. coli*. Although structural characterisation by X-ray crystallography began, difficulties in solving the phase problem due to the high number of monomers in the asymmetric unit cell hindered the continued use of this method. Initial investigation into the suitability of IdmK for structural studies by NMR revealed that IdmK could be isotopically labelled, and collection and analysis of an $^1$H-$^{15}$N HSQC spectrum showed that IdmK was in a folded and a single monomeric conformer, thus suitable for structure determination by NMR.

NMR as a tool for structure determination is particularly suitable for proteins of approximately 30 kDa or less, and is possible due to the intrinsic spin properties of particular nuclei (Cavanagh *et al.*, 2007). Below, a brief introduction to NMR spectroscopy will be given.

In 1946 the occurrence of nuclear magnetism was reported when Purcell *et al.* (1946) and Bloch *et al.* (1946) measured the NMR spectra of paraffin and water, respectively. This was possible due to nuclei possessing nuclear spin angular momentum. Nuclear spin angular momentum is a quantum mechanical property, characterised by the nuclear spin quantum number, \( I \) (Cavanagh *et al.*, 2007; Teng, 2005). Any nuclei with odd numbers of either neutrons or protons have a spin quantum number >0, some nuclei have half integer numbers if the neutrons and protons are not both odd e.g. $^1$H has a spin quantum number of a $\frac{1}{2}$; and $^{12}$C has a spin quantum number of 0. Nuclei with a spin quantum number of 0 are NMR inactive (Cavanagh *et al.*, 2007; Teng, 2005). Table 5.1 shows the properties of nuclei used in biological NMR experiments.
Nuclei with spin angular momentum, i.e. NMR active nuclei with nonzero spin quantum numbers, also have nuclear magnetic moments (μ) (Cavanagh et al., 2007). The nuclear magnetic moment of each nucleus is characterised by an individual unique constant, the gyromagnetic ratio (γ), which indicates how large its magnetic field is and is related to how receptive that particular nucleus is in NMR spectroscopy (shown for each isotope in Table 5.1) (Cavanagh et al., 2007; Teng, 2005). It is the interaction of the nuclear magnetic moment with an applied, external magnetic field (B₀), which gives rise to populations of nuclei with measurable differences in energy states. The NMR signal or resonance is produced as a result of the nuclear magnetic moment dictating the nuclear rotation frequency in relation to the external magnetic field. It is the differences between the energy states which leads to different resonance frequencies. The volume of the signal from the resonance is proportional to the population difference of the states. When the spin quantum number is half an integral, for example ¹H, (Table 5.1) the nuclear magnetic moment can occupy one of two states due to the quantisation of angular momentum, either pointing with the main magnetic field B₀, which is typically on the Z axis (low energy, α) or against it (low energy, β). Spins in the α and β states precess around the magnetic field at their Larmor frequency (ω₀), and are randomly distributed with respect to the x-y plane (Cavanagh et al., 2007; Keeler, 2002; Teng, 2005). Figure 5.1 illustrates the behaviour of nuclei in an applied magnetic field (B₀).

### Results three

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>Spin quantum number, I</th>
<th>Gyromagnetic ratio, γ (T⁻¹·s⁻¹)</th>
<th>Natural abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>¹H</td>
<td>½</td>
<td>2.6752 × 10⁸</td>
<td>99.99</td>
</tr>
<tr>
<td>²H</td>
<td>1</td>
<td>4.107 × 10⁷</td>
<td>0.012</td>
</tr>
<tr>
<td>¹³C</td>
<td>½</td>
<td>6.728 × 10⁷</td>
<td>1.07</td>
</tr>
<tr>
<td>¹⁵N</td>
<td>½</td>
<td>-2.713 × 10⁷</td>
<td>0.37</td>
</tr>
<tr>
<td>¹⁹F</td>
<td>½</td>
<td>2.518 × 10⁷</td>
<td>100.0</td>
</tr>
<tr>
<td>³¹P</td>
<td>½</td>
<td>1.0839 × 10⁸</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Table 5.1- Properties of biologically relevant nuclei in NMR spectroscopy. I is the nuclear spin quantum number, γ is the gyromagnetic ratio. The natural abundance for each particular isotope is given (%) (Cavanagh et al., 2007).
The Larmor frequency describes the frequency of the precession (Cavanagh et al., 2007; Teng, 2005) and is defined by:

$$\omega_x = -\gamma_x B_0$$

Equation 5.1 - Equation defining the Larmor frequency. Where \(\gamma\) is the gyromagnetic ratio and \(B_0\) is the strength of the magnetic field, \(x\) is the nucleus (Cavanagh et al., 2007; Teng, 2005).

As the Larmor frequency is dependent on the susceptibility of a nuclei to a magnetic field (\(\gamma\)) and also to the strength of the magnetic field, the frequency of precession increases in a linear manner when \(B_0\) increases (Equation 5.1). When \(B_0\) increases the energy difference between the two spin states that the nuclei can occupy increases, and the population difference between the two states increases due to the Boltzmann distribution. This results in a 1 in 10 000 population difference for protons at a magnetic field strength of 14.1 tesla. An NMR signal can only be observed when there is a difference in the populations of the two levels. At equilibrium, spins in the two states can be viewed as precessing as two cones pointing with and against the magnetic field \((B_0)\) (Cavanagh et al., 2007; Keeler, 2002; Teng, 2005). Summation of these spins gives a bulk magnetisation aligned with the field (Figure 5.2).
The field strength of an NMR magnet is measured in Tesla, however the strength of an NMR magnet is more often referred to by the proton Larmor frequency, for example a magnet with a field strength of 14.1 Tesla has a proton Larmor frequency of 600 MHz (Teng, 2005).

In the course of an NMR experiment, NMR active nuclei are excited by short radiofrequency pulse of defined power and time, which pushes the spin state away from equilibrium. Figure 5.3 shows the effects of applying a 90° pulse on the y axis ($90^\circ_y$), which excites the bulk magnetisation from the z axis to the xy plane where it can be detected (Teng, 2005; Keeler, 2002).
After excitation nuclei take time to return to equilibrium, this is known as relaxation. T₁, or longitudinal relaxation describes one process for a return to equilibrium, and occurs by the nuclei exchanging energy with the environment over time leading to random spin flips from α to β and vice versa. The nuclei can undergo a second form of relaxation, T₂, spin-spin relaxation. This describes the loss of magnetisation on the xy plane due to local loss of magnetic field homogeneity in a molecule (Teng, 2005).

An NMR signal is observed in the form of Free Induction Decay (FID), which is produced by the oscillating magnetic field of all excited nuclei in the sample following a radio frequency pulse, The FID is measured by a receiver coil in the xy plane and is measured over time (typically < 1s). The FID signal is Fourier transformed to convert it into frequencies suitable for analysis, resulting in a NMR spectrum (Cavanagh et al., 2007; Teng, 2005).

Different signals arise from nuclei within a molecule, including those from nuclei of the same isotope, due to the dependence of the magnetic field at the nucleus on the environment it is in. This dependence can be described as a chemical shift and moves the position of an NMR signal (Cavanagh et al., 2007; Teng, 2005). The two main causes of the chemical shift are bonding electrons and aromatic rings, double bonds which contain pi orbitals (Cavanagh et al., 2007; Teng, 2005). Bonding electrons in a molecule produces a magnetic field which opposes the induced bulk magnetisation. Therefore the greater the density of electrons, the smaller the local magnetic field and a lower frequency for the NMR transition is recorded. Aromatic rings and double bonds contain circular regions of pi electrons which can also induce large orientation dependant magnetic fields which are often described as ring current shifts and provide much of the dispersion of chemical shifts for proteins. The origin of the name chemical shift is due to its great dependence on the chemical environment, and it therefore provides a unique fingerprint of signals for a particular protein (Cavanagh et al., 2007; Teng, 2005).

The normalisation of chemical shifts between different samples is achieved by referencing with compounds with chemical shifts defined as zero, such as tetramethylsilane (TMS), which is soluble in organic solvents, and the water soluble
Results three

form of TMS, DSS (4,4-demethyl-4-silapentane-1-sulfonic acid) (Teng, 2005). Chemical shift differences measured in ppm of the main magnetic field strength are not influenced by magnetic field strength though the frequencies that determine them are; therefore the same sample will produce identical spectra on the chemical shift scale when using any NMR spectrometers (Teng, 2005).

In structure determination and assignment, scalar (J) couplings provide information about the two atoms separated by one or more bonds due to interactions between bonded electrons. This is exploited for chemical shift assignments of atoms (Teng, 2005; Cavanagh et al., 2007; Bax and Grzesiek, 1993), how scalar couplings are used for chemical shift assignments is described in section 5.2.

Dipolar couplings describe through space interactions of two nuclei, and are described in structure validation (section 5.3). Nuclear Overhauser effects (NOEs) arise from the transfer of energy from one spin to another spin through space by dipole-dipole interactions. NOEs can typically be observed between two protons which a distance of <6 Å and will produce NOE cross peaks in $^1$H-$^1$H NOESY spectra which can be used in structure determination (section 5.4) (Cavanagh et al., 2007; Teng, 2005).

While X-ray crystallography gives a static picture of the structure of proteins, NMR has the ability to provide dynamic information about the target protein. Figure 5.4 below illustrates the range of timescales of NMR spectroscopy can monitor via a range of techniques (Markwick et al., 2008).

![Figure 5.4](image)

Figure 5.4- Timescales to illustrate the molecular dynamics (dark blue) that can be measured by NMR experiments (light blue) giving measurable data (green), Adapted from Markwick et al. (2008).
As mentioned in chapter 4, IdmK is the carrier protein in the first NRPS module of the indanomycin biosynthetic gene cluster. As with all carrier proteins, IdmK is a non-catalytic protein, phosphopantetheinylated for function. Structure determination of apo-carrier proteins shows a conserved canonical fold (Crosby and Crump, 2012; Li et al., 2003). Figure 5.5 and 5.6 illustrate an alignment of an acyl carrier protein structures from FAS, PKS and an NRPS and a schematic of the canonical fold observed.

Figure 5.5- Canonical fold of carrier proteins. (Top) Structure of ACP from a fatty acid synthase (pdb code 2AVA) (Zornetzer et al., 2006). (Bottom) An overlay of carrier proteins from a FAS (red, PDB code: 2AVA), a PKS (yellow, PDB code: 2JU2) and an NRPS (blue, PDB code 2GDW) (Zornetzer et al., 2006; Alekseyev et al., 2007; Koglin et al., 2006).
Figure 5.6 shows a schematic of the secondary structural elements of the canonical fold of the homologous carrier protein structure. Helix 1 and helix 2 are antiparallel to each other, and helix 3 runs parallel to helix 2. Although slight deviations in helix lengths are seen in carrier proteins, generally they all appear to adopt the same fold (Crosby and Crump, 2012).

Figure 5.6- Schematic of the canonical fold of carrier proteins. Slight variations are seen in helix length. A fourth small pseudo helix is also sometimes observed between helix 2 and 3, this is more variable.

Structural studies by NMR spectroscopy of the TycC3 peptidyl carrier protein revealed conformational changes are required for access to the pantetheine co-factor, showing significant conformational changes between what is described as the A/H state, an equilibrium state adopted by both apo- and holo- conformers of the PCP, and the holo- and apo- states. These conformational changes are suggested to bring the terminal thiol, where the NRP chain is located in proximity with the adenylation and condensation domains (Weber et al., 2000; Koglin et al., 2006). Structure determination of IdmK by NMR spectroscopy will provide both a structure and basic dynamic information for the protein, providing a foundation for investigation into the dynamics and mechanisms of starter unit construction in indanomycin biosynthesis. This chapter describes the experiments and stages required for structure determination and validation of the initial structure calculation of the apo-state of IdmK.
5.1 Backbone assignments of IdmK

5.1.1 Triple resonance experiments

Prior to committing to structure determination of IdmK using NMR spectroscopy a $^1$H-$^{15}$N HSQC spectrum was collected. This spectrum provided information on the conformational state of the protein and if it is folded in solution, thus indicating the suitability for further NMR experiments (Cavanagh et al., 2007; Rehm et al., 2002). The $^1$H-$^{15}$N HSQC spectrum of IdmK (Chapter 4, Figure 4.13) indicated that the protein was suitable for structural studies by NMR as it contained the correct number of peaks and was well resolved and sharp. The next step was to collect triple resonance spectra for the sequential assignment of the protein backbone. Triple resonance experiments, originally described in 1990 for the backbone assignment of calmodulin (Ikura et al., 1990; Kay et al., 1990), are an alternative means of backbone assignments from the previous use of short range NOEs (Wuthrich et al., 1982). Unlike the short range NOEs which use distances between protons for sequential backbone assignments, triple resonance experiments use one-bond and two-bond scalar couplings, or J couplings, which are easier to measure and interpret once the molecule has been uniformly labelled with $^{13}$C and $^{15}$N isotopes (Cavanagh et al., 2007). Figure 5.7 illustrates the one-bond scalar couplings exploited in triple resonance experiments.

![Figure 5.7- Scalar couplings observed in the protein backbone. The red arrows indicate the one and two bond scalar couplings used in triple resonance experiments (Teng, 2005).](image)

Exploitation of these one-bond couplings (and two-bond in the case of the HN and Cα of the preceding residue) has led to the design of several sets of triple resonance experiments which correlate the spins of NMR active nuclei in the protein backbone (Bax and Grzesiek, 1993; Grzesiek and Bax, 1992; Ikura et al., 1990; Cavanagh et
Running triple resonance experiments; HNCA, HNcoCA, HNCO, HNcaCO, HNCACB CBCAcoNH experiments gives chemical shift information about the $^1H$, $^{15}N$, $^{13}C_\alpha$, $^{13}CO$ and $^{13}C_\beta$ of either the nuclei within the same residue as the H and N resonances observed (i) or the nuclei i-1 to the H and N resonances being measured and are used for sequential backbone assignments (Cavanagh et al., 2007; Kay et al., 1990; Grzesiek and Bax, 1992; Farmer et al., 1992; Bax and Ikura, 1991; Clubb et al., 1992; Bax and Grzesiek, 1993; Ikura et al., 1990). Table 5.2 illustrates the transfer path used for each of the triple resonance experiments.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Correlation observed</th>
<th>Magnetisation transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNCO</td>
<td>$^1H^N_i-^{15}N_i-^{13}CO_{i-1}$</td>
<td>![HNCO diagram]</td>
</tr>
<tr>
<td>HNcaCO</td>
<td>$^1H^N_i-^{15}N_i-(^{13}C_\alpha_{i-1})-^{13}CO_{i-1}$ $^1H^N_i-^{15}N_i-(^{13}C_\alpha_{i})-^{13}CO_i$</td>
<td>![HNcaCO diagram]</td>
</tr>
<tr>
<td>HNCA</td>
<td>$^1H^N_i-^{15}N_i-^{13}C_\alpha_i$ $^1H^N_i-^{15}N_i-^{13}C_\alpha_{i-1}$</td>
<td>![HNCA diagram]</td>
</tr>
</tbody>
</table>

160
Results three

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Correlation observed</th>
<th>Magnetisation transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNcoCA</td>
<td>$^1H^N_i-^15N_i-(^{13}CO_{i-1})$</td>
<td><img src="image" alt="HNcoCA" /></td>
</tr>
<tr>
<td></td>
<td>$^{13}C^\alpha_{i-1}$</td>
<td></td>
</tr>
<tr>
<td>HNCA CB</td>
<td>$^{13}C^\beta_{i-1}/^{13}C^\alpha_{i-1}-^{15}N_i-^{1}H^N_i$</td>
<td><img src="image" alt="HNCA CB" /></td>
</tr>
<tr>
<td></td>
<td>$^{13}C^\beta_{i+1}/^{13}C^\alpha_{i+1}-^{15}N_{i+1}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$^{1}H^N_{i+1}$</td>
<td></td>
</tr>
<tr>
<td>CBCAcoNH</td>
<td>$^{13}C^\beta_{i-1}/^{13}C^\alpha_{i-1}-^{15}N_{i+1-}$</td>
<td><img src="image" alt="CBCAcoNH" /></td>
</tr>
<tr>
<td></td>
<td>$^{1}H^N_{i+1}$</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.2: The coherence transfer in triple resonance experiments used for sequential backbone assignments. The arrows indicate the path of magnetisation; double headed arrows indicate transfer of an “out-and-back” nature. Red circles indicate nuclei that were detected, blue circles are those that were on the pathway but no chemical shift information was collected. Figure adapted from Cavanagh et al. (2007).

The triple resonance spectra HNCA, HNcoCA, HNCO, HNcaCO, HNCA CB CBCAcoNH were collected for apo-IdmK, at a concentration of 0.5 mM, 50 mM Tris.HCl pH 7.4, 0.02% sodium azide containing 10 % (v/v) D$_2$O. Data were collected at 25 °C at a field strength of 600 MHz. Spectra were then processed as described in section 2.2.5.2, and imported into CCPNmr Analysis (Vranken et al., 2005). CCPNmr analysis was used to peak pick the $^1H-^{15}N$ HSQC and all triple resonance spectra. Analysis was also used to assign anonymous spin systems in the triple resonance spectra to their root resonance in the $^1H-^{15}N$ HSQC. Once this was completed, semi-automated sequential backbone assignments were carried out.
5.1.2 Semi-automated backbone assignments using MARS

Sequential protein backbone assignments rely on the correlation of an NH group with the Cα, Cβ and carbonyl (CO) nuclei within the same residue and the preceding residue (i and i-1). Table 5.2 illustrates the correlation of nuclei within each experiment, and indicates whether they are used to collect chemical shift information from nuclei within the same or preceding residue. Collection of the HNCA spectra provides chemical shifts for the Cα peaks of residues (i) and (i-1) and collection of HNcoCA spectra provides chemical shift information for just the preceding residue (i-1). Superimposition of these two spectra enables the i and i-1 peaks in the HNCA spectra to be distinguished. The i-1 peak can then be matched to an i peak with an identical chemical shift in another residue, linking two residues together, sequentially. The HNCO and HNcaCO can be used to sequentially link the CO, and the HNCACB and CBCAcoNH can be used to sequentially assign residues using the Cα and Cβ nuclei in a similar manner and are important for overcoming degeneracies. Strip plots can be used to perform the sequential assignments. An example strip plot can be seen in Figure 5.8.

In order to speed up the complete backbone assignments of IdmK an automated assignment program called MARS was used (Jung and Zweckstetter, 2004). MARS works by trying to optimise local and global assignments in order to reduce the carry through of initial errors to final assignments, is conservative in the assignments it makes and is able to complete assignments with missing chemical shift data (Jung and Zweckstetter, 2004). Prior to the use of MARS both the $^1$H-$^{15}$N HSQC and triple resonance spectra had been peak picked and the triple resonance peaks had been assigned to the same spin system as their root resonances in the $^1$H-$^{15}$N HSQC as described above. Peaks in the same spin system are referred to as pseudosidues (PR) prior to their assignments. Once PRs are linked into segments and then mapped onto the amino acid sequence of the protein, MARS identifies the amino acid types by characteristic chemical shifts, such as the Cβ chemical shifts of serine and threonine residues and detects sequential connectivities of the PR (Moseley and Montelione, 1999; Jung and Zweckstetter, 2004). MARS evaluates the assignments and its output indicates the reliability of assignments, classing the confidence of the assignment as High (H), Medium (M) or Low (L) (Jung and Zweckstetter, 2004). All assignments by MARS were also manually checked using strip plots (Figure 5.8).
Further analysis of side chain assignments also showed all MARS assignments used subsequently were correct. A total of 92% of backbone nuclei were assigned using this method. Chemical shifts can be found in a table in the appendix.
Figure 5.8: Strip plot of the last 10 residues of the backbone of IdmK. Strip plots were used to manually check the automated assignments completed by MARS. (Top) HNCA (red) and HNcoCA (blue) spectra were used to assign the Cα chemical shifts. (Bottom) HNCO (pink) and HNcaCO (purple) spectra were used to assign the carbonyl chemical shifts. Triple resonance spectra were measured in 50 mM Tris.HCl pH 7.4, 0.2% (w/v) sodium azide, at a concentration of 0.5 mM recorded at 600 MHz at 25 °C.
The successful assignment of the $^{1}$H-$^{15}$N HSQC spectrum is shown in Figure 5.9. The triple resonance spectra provided the chemical shift information for the following nuclei of IdmK: N, HN, Ca, Cβ and CO which are given in table the chemical shift table in the appendix.

As discussed above, the good peak dispersion and sharp lines indicate a well-folded and stable protein in solution. Recent developments in NMR structure calculations now allow the use of chemical shifts alone to determine an overall protein backbone fold (Shen et al., 2008; Shen et al., 2009b). The chemical shifts for HN, N, Ha, Ca, Cβ and CO generated by backbone assignments were subsequently used in secondary and tertiary structure calculations.
5.2 Structure determination using chemical shifts

The unique chemical shifts for the backbone nuclei in particular secondary structure shifts and the effect of the ring current can be used to determine secondary, and more recently, tertiary chemical structures using +TALOS and CS-ROSETTA respectively (Shen et al., 2009a; Shen et al., 2008; Shen et al., 2009b) and this approach was adopted to determine a preliminary overall fold of IdmK.

5.2.1 Determination of secondary structure

To predict the secondary structural elements of IdmK +TALOS was used (Shen et al., 2009a). TALOS, Torsion Angle Likelihood Obtained from Shifts and sequence similarity, uses experimentally determined chemical shifts of HN, N, Hα, Cα, Cβ and CO nuclei to predict the phi and psi torsion angles of the protein backbone using a database of chemical shifts and the random coil shifts predicted by the primary sequence (Cornilescu et al., 1999; Shen et al., 2009a). TALOS is also able to predict how dynamic the residue is, quantified by an approximate value for the order parameter, $S^2$ (Figure 5.10 - bottom panel) (Berjanskii and Wishart, 2007).
The secondary chemical shifts depend on the orientation of the Cα-Hα bond in relation to each adjacent carbonyl (Tjandra and Bax, 1997b; Shen et al., 2009a; Teng, 2005). Figure 5.10 shows the results of the secondary structure predictions for IdmK using +TALOS (Shen et al. 2009a). The top panel shows that the secondary chemical shifts of the Cα are positive, indicating that IdmK is an α-helical protein. This is reinforced by the fact that the Cβ chemical shifts show no overall pattern possibly due to slightly incorrect chemical shift referencing of the spectra. Chemical shift referencing will be completed with an internal reference compound (DSS). The positive ΔCα values indicated that IdmK has 3 helices, with helix 1 composed of...
residues 6-20, helix 2 composed of residues 45-57 and helix 3 composed of residues 75-85. To compare the predicted secondary structure elements of IdmK with those of other carrier proteins the Dali server was used (Holm and Rosenstrom, 2010). Dali performs a 3D structure alignment of a target protein with those deposited in the PDB. Figure 5.11 shows the results of the alignment by Dali.

This analysis revealed that carrier proteins consist of: helix 1, centred around residue 10, with a length of 14 residues (+/- 2), helix 2, centred around residue 46, with a length of 11 residues (+/- 4), this is followed by a small “pseudo” helix which is not always present and helix 3, centred around residue 74, which is 13 residues long (+/- 3). Exact locations of the helices vary slightly due to differences in the lengths of the carrier proteins. IdmK shows comparable helix lengths and relative locations in the sequence.

An order parameter of 0 indicates a completely disordered residue and an order parameter of 1 is a completely rigid protein (Berjanskii and Wishart, 2007; Engelke and Rüterjans, 1999). The order parameters shown in Figure 5.10 indicated that IdmK is a well-ordered protein, apart from the extreme N- and C-terminal residues. These results provide the first evidence that IdmK may have the canonical carrier protein fold. Phi and psi angles predicted by TALOS can be used as restraints in structure calculations (Section 5.2.2). The chemical shifts were then used to determine the overall fold of IdmK using CS-ROSETTA.

Figure 5.11- Secondary structure alignments of carrier proteins by Dali. H indicates the residue resides within a helix and L indicates the residue is in a loop. The PDB codes refer to carrier protein structures with comparable secondary structure selected by DALI deposited in the PDB.
5.2.2 Determination of the overall fold of IdmK using CS-ROSETTA

Until recently, it was not possible to define the fold of a protein structure by use of the torsion angles determined by TALOS (Cornilescu et al., 1999; Shen et al., 2009a), and structure determination from assigned side chain proton requires the addition of large numbers of standard NOE distance restraints. The past seven years has seen the emergence of programs which determine a protein fold solely from the chemical shifts of backbone and Cβ nuclei; CHESHIRE, CS-ROSETTA and CS23D (Cavalli et al., 2007; Shen et al., 2008; Wishart et al., 2008). CS-ROSETTA, a program developed by the Baker lab (Shen et al., 2008; Shen et al., 2009b), utilises the experimentally determined chemical shifts to determine an overall protein fold de novo. The ROSETTA protein structure prediction algorithm is a software package that allows the determination of a protein fold based only on the amino acid sequence and chemical shifts determined in backbone assignments. The general strategy involves the selection of fragments based on the target amino acid sequence. 9 and 3 residue fragments are selected from known structures in a database, which are then assembled using a Monte Carlo simulated annealing approach. CS-ROSETTA uses the $^{13}\text{C}_\alpha$, $^{13}\text{C}_\beta$, $^{13}\text{CO}$, $^{1}\text{HA}$, $^{1}\text{HN}$ and $^{15}\text{N}$ chemical shifts to bias the fragment selection, so as that the selected fragments have chemical shifts that agree with the experimentally measured ones and so occupy the correct part of Ramachandran space. The lack of NMR assignments in structural databases may have previously hindered searches, however development of SPARTA (which is based on TALOS (Shen and Bax, 2013)), a program that can predict the chemical shifts during ROSETTA modelling and thus allows correctly folded structures to be selected. CS-ROSETTA has been evaluated using a number of proteins up to 15 kDa in size adopting a range of conformations (Shen et al., 2009b), indicating that this program may be appropriate for determination of an overall fold of IdmK. Dr Gary Thompson aided in running the CS-ROSETTA calculations.

5000 ROSETTA models were calculated using the experimentally determined $^{13}\text{C}_\alpha$, $^{13}\text{C}_\beta$, $^{13}\text{CO}$, $^{1}\text{HA}$, $^{1}\text{HN}$ and $^{15}\text{N}$ chemical shifts, and were ranked by their CS-ROSETTA scores (Shen et al., 2008; Shen et al., 2009b). The results showed that the five best ROSETTA structures show an overall similar fold, with helix 1 antiparallel to helix 2 and 3 which are parallel to each other, as expected for a canonical carrier protein fold (Figure 5.12 and 5.6).
Figure 5.12 shows a chevron plot with the best ROSETTA scored model highlighted. The models are analysed by plotting their all atom energy against the Cα RMSD indicates how similar the other models are to the lowest energy model. Although the CS-ROSETTA models appear to show the same overall orientation, i.e. helix 1 antiparallel to helix 2 and 3, which are parallel to each other, the exact angles between the helices deviate somewhat from the known canonical fold and between the models themselves. Though the observed structures don’t show the clearest deviation in the energy vs Cα RMSD this may be attributed to the large content of loops or long meanders between helices. Figure 5.18 shows a comparison of the CS-ROSETTA model of IdmK with an acyl carrier protein from FAS.

Although CS-ROSETTA is able to predict the correct overall fold of IdmK, further experimental validation is required to assess the quality of the models. An easy experimental restraint to determine, that can be used to validate the models, are residual dipolar couplings (RDCs).
5.3 Structure validation using residual dipolar couplings

To validate the calculated CS-ROSETTA structures of IdmK, residual dipolar couplings (RDCs) were measured. RDCs depend on the distance between two NMR active nuclei \((i\) and \(j\)) and the angle between them. As the distance between the nuclei which are directly bonded to one another is a known constant based on classical chemistry (bond lengths), RDCs \((D_{ij})\) provide accurate relative angular information between bonds \((\theta_{ij})\) that is difficult to determine by other NMR methodologies (Bax, 2003; Ottiger et al., 1998; Lipsitz and Tjandra, 2004; Teng, 2005).

\[
D_{ij} = -\frac{\gamma_i \gamma_j \mu_0 h}{8\pi^3} \left( \frac{3\cos^2 \theta_{ij}(t) - 1}{2r_{ij}^3(t)} \right) = 0
\]

Equation 5.2- Determination of the RDC between two nuclei. Where \(\gamma_i\) and \(\gamma_j\) are the gyromagnetic ratios of the two NMR active nuclei, \(\mu_0\) is the vacuum permeability, \(h\) is Planks constant, \(r\) is the distance between the two nuclei and \(\theta\) is the angle between them and \(t\) is time (Bax, 2003; Ottiger et al., 1998).

Figure 5.12 shows the relationship between two nuclei within an external magnetic field, \((B_0)\) defining the distance and angle between them.

Figure 5.13- Dipole-dipole interaction between two magnetic moments. \(B_0\) is the external magnetic field, \(r\) is the distance between the two nuclei and \(\theta\) is the angle between them (Teng, 2005; Blackledge, M., personal communication).

In an isotropic solution, when a molecule is tumbling freely is solution, the RDCs are averaged to close to zero. However, in an anisotropic solution the molecular orientation is slightly restricted and this introduces measurable dipolar couplings (Equation 5.2 is no longer equal to zero).
As mentioned previously, partial alignment of the protein is required to introduce measureable dipolar couplings (Fleming and Matthews, 2004; de Alba and Tjandra, 2004). Figure 5.14 shows the partial alignment of molecules in an anisotropic solution, in order to introduce measurable dipolar couplings.

Figure 5.14- Partial alignment of a protein in an anisotropic solution. In an isotropic solution (a) the protein is tumbling freely in solution and RDCs are averaged to zero. In an anisotropic solution (b) the range of motion is partially restricted and this introduces measurable dipolar couplings (Blackledge M, personal communication).

Figure 5.15 shows a simple method by which RDCs can be directly measured by comparison of a coupled spectrum in anisotropic and isotropic solutions. It shows how the residual dipolar coupling can be measured by subtraction of the known one-bond J coupling from the coupling measured in the experiment performed in an anisotropic solution. However, for IdmK instead of measuring the difference in peak positions in an undecoupled HSQC as shown in Figure 5.14, a series of decoupled $^1$H-$^{15}$N HSQC J-modulated spectra (Tjandra and Bax, 1997c) were collected with different time intervals ($\Delta$) during which the coupling is encoded in the intensities of the peaks. This approach was used to accurately measure the RDCs in IdmK.
Partial alignment of the protein is often achieved using a liquid crystalline medium which can introduce measurable dipolar couplings (Tjandra and Bax, 1997a). A variety of different media can be used to partially align protein molecules such as bicelles, filamentous bacteriophage and SDS-PAGE gels (Fleming and Matthews, 2004; de Alba and Tjandra, 2004). The first liquid crystalline medium trialled for partial alignment of IdmK was Pf1 bacteriophage. Pf1 bacteriophage is a filamentous bacteriophage which aligns in parallel with the magnetic field due to being naturally susceptible to the magnetic field, thus creating a liquid crystalline medium and partially aligning the phage and the protein (Hansen et al., 1998; Torbet and Maret, 1979). A $^1$H-$^{15}$N HSQC spectrum was collected of IdmK at 0.5 mM in Tris.HCl pH 7.4, i.e. in an isotropic solution. Pf1 bacteriophage was then added to the isotropic solution at a final concentration of 8 mg mL$^{-1}$ and a second $^1$H-$^{15}$N HSQC spectrum was collected. Analysis of the anisotropic $^1$H-$^{15}$N HSQC spectrum showed significant peak broadening also occurring, indicating an interaction of the Pf1 bacteriophage with IdmK, as indicated by a decreased $T_2$. Calculation of the theoretical isoelectric point (pI) of IdmK using ExPASy ProtParam (Gasteiger et al., 2003) gives a pI of 5.82. At pH 7.4 IdmK would have an overall positive charge and the Pf1 bacteriophage has an overall negative charge, explaining the interaction between the two molecules. This alignment medium was therefore unsuitable for determination of the RDCs and a new alignment medium was required.

The second alignment medium tried was a $n$-alkyl-poly(ethylene glycol)/$n$-alkyl alcohol mixture (Section 2.2.5.5) (Rückert, 2000), if these molecules are mixed in
the appropriate concentration they create a liquid crystalline medium that partially aligns the protein. This liquid crystalline medium is not charged, and is relatively insensitive to pH and applicable over a range of temperatures (Rückert, 2000). The nomenclature for the \( n \)-alkyl-poly(ethylene glycol)/\( n \)-alkyl alcohol mixture is \( CmEn \), denoting the number of carbons in the alkyl group (\( m \)) and the glycol groups in the PEG (\( n \)) (Rückert, 2000). A C12E6, hexanol mixture (0.64 molar ratio) containing 5\% PEG was selected for the alignment of IdmK. An initial \( ^1 \)H,\( ^15 \)N HSQC spectrum was collected of IdmK in anisotropic conditions and no significant chemical shift differences were observed in the in this alignment medium. A J modulated series was then carried out. Figure 5.15 shows the how the raw data are fitted to calculate the RDCs from the change in peak intensity (Section 2.2.5.5, Equation 2.3) (Tjandra et al., 1996).

![Figure 5.16- Raw RDC data of three residues from IdmK. The Raw RDC data shows a change in the peak intensities in the J modulated series as a function of time. A cosine function is fitted to extract 2 × the dipolar coupling for a residue. Error bars represent propagated errors calculated from duplicate measurements for each peak.](image)

The RDC value extracted from the raw data by fitting a modified cosine function (see materials and methods (2.2.5.5) using the in-house python script FitR_bs (Dr Gary Thompson) which uses appropriate heuristics to determine initial values for fitting. Statistical errors were analysed using duplicate values and Monte Carlo simulations (Tjandra et al., 1996). RDCs can then be plotted against residue number, for comparison with RDC values calculated from models. Figure 5.17 shows the plot of all RDCs measured for IdmK for each residue.
PALES, Prediction of alignment from structure, is a program that can fit the RDCs to a known structure (Zweckstetter, 2000), in this case the ROSETTA models. The measured RDCs define an average alignment of the molecule in three orthogonal directions, defining the alignment tensor frame (Zweckstetter, 2000). To do this the structures from ROSETTA calculation were used. To validate the CS-ROSETTA models generated, the 10 best CS-ROSETTA models were analysed using PALES in order to compare the experimentally determined RDCs with those predicted from the models. For validation of the models generated by CS-ROSETTA only RDCs for the secondary structure elements as determined by TALOS+ were used. This is because residues in loop regions tend to be less well defined and do not have well defined angles in relation to the rest of the molecule and the aligning medium. The RDC waves in figure 5.18 indicate the presence of α-helices (Mesleh et al., 2002).

Figure 5.17- Measured RDCs for IdmK collected in 50 mM Tris.HCl pH 7.4, 0.2% (w/v) sodium azide in a C12E6, hexanol mixture (0.64 molar ratio) containing 5% PEG, at a concentration of 0.5 mM apo-IdmK recorded at 500 MHz at 25 °C. RDC data collected for IdmK. The grey boxes designate secondary structure elements. Error bars represent propagated errors calculated from duplicate measurements for each peak.
Results three

Figure 5.18 shows the three models that agree the best with the experimentally determined RDCs for secondary structure. All structures show the canonical fold of a carrier protein (Figure 5.5 and 5.6). Each model is ranked by their Q factor, which indicates the quality of the fit of the experimental RDCs to the model. Equation 2.3 illustrates how the Q factor is calculated to determine the overall quality of the fit of the data. A low Q factor indicates a better fit (Zweckstetter, 2000).

The structure with the lowest Q factor (Figure 5.18, top panel) was not the lowest energy ROSETTA model. Although the 2nd model selected by RDCs (middle panel) has a higher Q factor, it is clear from the RDC plots (right) that the secondary structure elements agree better with the experimentally determined data (red and blue lines overlay).

Table 5.3 shows the Q factors calculated for the 10 best CS-ROSETTA, including those in Figure 5.18.

<table>
<thead>
<tr>
<th>Model</th>
<th>Q factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>0.479</td>
</tr>
<tr>
<td>1.2</td>
<td>0.527</td>
</tr>
<tr>
<td>1.3</td>
<td>0.528</td>
</tr>
<tr>
<td>1.4</td>
<td>0.544</td>
</tr>
<tr>
<td>1.5</td>
<td>0.561</td>
</tr>
</tbody>
</table>

Table 5.3- Q factors of five best CS-ROSETTA models. The best Q factor can be calculated from the experimental errors, in this case the best Q factor for the dataset is 0.1 (Tang et al., 2008; Karamanos et al., 2014).

The best Q factor as calculated from the experimental data set for IdmK is 0.1, the Q factors states in Table 5.3 are 0.479 and above. Clearly, the Q factor calculated for illustrates that these models still require refinement. However, the pattern of the RDCs is comparable.
Figure 5.18- Three CS-ROSETTA models selected by RDCs. (Top panel) model 1.1, (middle panel) model 1.2 and (bottom panel) model 1.3 are the three models with calculated RDCs that agree the best with experimentally determined RDCs, as indicated by the Q factor. Dotted lines represent the residues in loops that were not used in fitting. Blue line is the expected RDCs calculated from the models, Red lines are the experimentally determined RDCs. Error bars represent propagated errors calculated from duplicate measurements for each peak.
Results three

The agreement of RDCs predicted from the models with the experimentally determined RDCs, and the overall fold predicted by ROSETTA are strong indications that IdmK probably has the canonical fold as seen for other carrier proteins. Figure 5.19 illustrates the differences in angles of the helices, comparing model 1.2 (Figure 5.17) with a carrier protein from a FAS. While helix 2 and 3 appear to be in the correct, same, orientation as the canonical fold, helix 1 appears to be rotated by 90°.

Figure 5.19- Comparison of the best CS-ROSETTA model with an ACP. Overlay of model 1.2 of IdmK (pink), selected as the best CS-ROSETTA module, of the 5 lowest energy models, this was selected due to the agreement with experimentally determined RDCs (Figure 5.12, Table 5.3) with an ACP from FAS (yellow) (PDB code: 2AVA) (Zornetzer et al., 2006).

To guide the ROSETTA structure calculation, and taking advantage of further features of the CS-ROSETTA software package, instead of scoring the structures that were produced by CS-ROSETTA with chemical shifts alone the HN RDCs were also incorporated as additional restraints in the calculation. Figure 5.20 shows an overlay of the five new CS-ROSETTA structures that agreed the best with the RDC restraints for secondary structure regions. Table 5.4 shows the Q factors for these structures.
Results three

Figure 5.20-Overlay of ROSETTA structures generated with RDCs as an additional restraint. 5 models selected that best fit the RDC data as well as sharing the same secondary structure, as judged by backbone RMSD Table 5.4 shows Q factors related to RDC fits.

<table>
<thead>
<tr>
<th>Model</th>
<th>Q factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 (Green)</td>
<td>0.411</td>
</tr>
<tr>
<td>2.2 (Blue)</td>
<td>0.460</td>
</tr>
<tr>
<td>2.3 (Pink)</td>
<td>0.487</td>
</tr>
<tr>
<td>2.4 (Yellow)</td>
<td>0.503</td>
</tr>
<tr>
<td>2.5 (Orange)</td>
<td>0.533</td>
</tr>
</tbody>
</table>

Table 5.4- CS-ROSETTA structure Q factors. Q factors for secondary structure elements of CS-ROSETTA structures generated with RDCs as a restraint in the calculation. The best Q factor can be calculated from the experimental errors, in this case the best Q factor for the dataset is 0.1 (Tang et al., 2008; Karamanos et al., 2014).

This round of structure calculations by CS-ROSETTA produced models comparable to the initial CS-ROSETTA calculation, as illustrated by the Q factors (Figure 5.17). As expected the fit to the RDC data is improved. However, within these ensembles the exact angles of the helices still appears to deviate from the canonical fold, and from each other somewhat. The 20 structures that showed the best agreement with the structure selected using RDCs were then taken forward to seed an ARIA NOE based structure calculation (section 5.4).
Results three

5.4 NOE restraints and ARIA structure calculation

In order to generate a higher resolution structure $^1$H-$^1$H NOE distance restraints were required. In order to assign the NOE distance restraints in NOESY spectra, full amino acid side chain assignments were required. HccoNH and HBHAcbcacoNH spectra of IdmK were collected. These spectra provide a link between the $^1$H-$^1$5N HSQC and and $^1$H shifts in the $^1$H-$^1$3C HSQC, and were used in combination with the HNCA and HNcoCA to assign cross peaks in the $^1$H-$^1$3C HSQC, this provided a starting point for side chain assignments. Structure calculations by ARIA were carried out by Dr Gary Thompson.

5.4.1 Aliphatic side chain assignments

To assign the aliphatic side chains, especially the methyl bearing side chains such as leucine, isoleucine, valine, threonine and alanine, $^{13}$C-HSQC (Cavanagh et al., 2007), hCCH-TOCSY and HcCH-TOCSY spectra were collected (Bax et al., 1990; Olejniczak et al., 1992). A $^{13}$C HSQC spectrum detects all of the carbon nuclei in the protein through one bond H-C correlations and is the carbon equivalent to the $^1$H-$^1$5N spectrum. This spectrum is assigned through the use of hCCH-TOCSY and HcCH-TOCSY spectra and provides the basis for assigning NOE peaks in $^1$H-$^1$3C-NOESY-HSQC spectra during structure determination. An HcCH-TOCSY spectrum provides chemical shifts for the side chain protons by correlating them with their corresponding $^{13}$C nuclei. By identification of the carbon chemical shifts in an hCCH-TOCSY spectrum, navigation to the carbon plane in the HcCH-TOCSY spectrum can match the side chain protons to the corresponding carbons. The Cα and Cβ chemical shifts assigned in the triple resonance experiments can be used as a starting place to identify the rest of the carbon nuclei in the side chain using this approach. Figure 5.21 and 5.22 below show the hCCH-TOCSY and HCCH-TOCSY spectra assignment of Leu85, respectively.

Currently, this assignment process has been carried out for assignment of approximately 77% of all amino acid side chain protons of IdmK, and 60% of heavy side chain atoms of IdmK, including all of the methyl bearing side chains. A chemical shift table can be located in the appendix.
Figure 5.21 - hCCH TOCSY assignment of Leu 85. The hCCH TOCSY was used to assign the Cβ, Cδ¹ and Cδ² carbon nuclei in Leu 85. The carbon planes for Cβ, Cδ¹ and Cδ² were identified in strip 1, where the peaks marked are the Cα, Cβ, Cδ¹ and Cδ² (bottom to top) identified by characteristic chemical shifts. The dotted lines indicate corresponding carbon peaks in another carbon plane. Spectrum was collected in 50 mM Tris.HCl pH 7.4, 0.2% (w/v) sodium azide with apo-IdmK at a concentration of 0.5 mM.
Figure 5.22: HcCH TOCSY assignment of Leu 85. The HCCH TOCSY was used to assign protons in the amino acid side chain. The hCCH-TOCSY was used to identify the carbon chemical shifts for the $\text{C}_\alpha$, $\text{C}_\beta$, $\text{C}_\delta^1$ and $\text{C}_\delta^2$. Navigation to these carbon planes in the HCCH-TOCSY enabled identification of the corresponding protons. The dotted lines indicate the same protons in another carbon plane. Spectrum was collected in 50 mM Tris.HCl pH 7.4, 0.2% (w/v) sodium azide with apo-IdmK at a concentration of 0.5 mM.
5.4.2 Aromatic side chain assignments
The amino acid sequence of IdmK contains four aromatic residues, not including the His\textsubscript{6}-tag, three phenylalanine residues and one tryptophan. Aromatic residues usually form part of the hydrophobic core of a protein and provide useful NOE restraints for structure determination (Prompers et al., 1998). The methodology used for assignment of aromatic residue side chains differed from assignment of the other amino acid side chains. To assign the phenylalanine residues in IdmK a combination of a 2D \textit{hbCBcgcdHdeHE} spectrum and a 2D aromatic $^{13}$C filtered $^1$H-$^1$H NOE spectrum were used (Yamazaki et al., 1993; Marion et al., 1989a; Marion et al., 1989b; Cavanagh et al., 2007). The \textit{hbCBcgcdHdeHE} exploits the chain of one bond (J coupling) between the C\textbeta chemical shifts and the H\delta and H\epsilon in the connected ring making it possible to assign the H\delta protons and H\epsilon protons of the aromatic side chains. However, this spectrum has relatively low resolution in the $^1$H dimension due to the use of constant time and power limits imposed by the pulse sequence and spectrometer (Yamazaki et al., 1993). In addition to this the peaks in this spectrum can also be masked by those generated by the mobile His\textsubscript{6}-tag and the overlap of H\beta chemical shifts in IdmK. To combat these problems a $^1$H-$^1$H-aromatic $^{13}$C filtered NOE spectrum was also collected, this experiment uses the $^1$H-$^1$H NOEs observed between the H\beta and H\delta protons for assignment. The H\beta and H\delta NOE cross peaks are always, at least weakly, present due to the restraints of the covalent geometry of the ring and side chain. To assign the tryptophan residue in IdmK, short $^1$H-$^1$H NOE distance restraints were used in addition to assignment of the characteristic chemical shifts in the $^1$H-$^{13}$C aromatic optimised HSQC. This resulted in assignment of all four of the non-histidine aromatic side chains.

After assignment of of the amino acid side chains of IdmK had begun, once a good proportion had been assigned, especially those suspected to form the hydrophobic core, were used to peak pick the $^{13}$C-NOESY-HSQC. In addition the $^1$H-$^{15}$N HSQC was used to peak pick the $^{15}$N-NOESY-HSQC (Marion et al., 1989a; Marion et al., 1989b; Zuiderweg and Fesik, 1989).

5.4.3 Progress towards a high resolution solution structure of IdmK
To calculate a higher resolution structure of IdmK the program ARIA, Ambiguous restraints for iterative assignment, was used (Rieping, 2007). Historically, NMR structure calculations were performed using unambiguously assigned NOE cross
peaks, converting them into distance restraints. However, the manual unambiguous assignment of cross peaks in NOESY spectra is time consuming and inaccurate (one peak may contain several distances due to resolution limitations). To deal with this issue, structure calculations have relied on iterative process where an initial 3D structure model created from the unambiguous NOEs is used to seed further NOE assignments which can be obtained by quantifying the agreement of the ambiguous NOEs with a model (Nilges, 1995; Teng, 2005). ARIA uses an automated iterative version of this strategy, however all possible assignments from an ambiguous NOE are considered for the initial structure calculation. The correct assignments of NOEs is achieved through several rounds of simulated annealing energy minimisation interspersed with automated assignment. Typically calculations start with a structure with random coordinates i.e. an unfolded polypeptide chain. During each round following an increasingly rigorous selection of assignments based on the current model the improved restraint set is used to calculate further structures, trying to satisfy as many distance restraints as possible. Over 8 rounds, only NOEs that agree with the structure generated in the previous round are selected and therefore the population of incorrect NOE assignments decreases leading to convergence (Rieping, 2007; Fossi et al., 2005a).

The ARIA structure calculation of IdmK was carried out using $^1$H-$^1$H NOEs from the filtered $^{13}$C-NOESY-HSQC and $^{15}$N-NOESY-HSQC spectra. The $^1$H-$^{13}$C-HSQC, assigned by the HCCH- and hCCH-TOCSY experiments, was used as the root spectrum for the $^{13}$C-HSQC shifts of NOE peaks used in the ARIA calculation. $^1$H NOE chemical shifts were assigned using the ARIA protocol during the structure calculation. A total of 1123 distance restraints were obtained for IdmK, this includes long and short range NOEs as summarised in Table 5.5.
Results three

<table>
<thead>
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<th>Distance constraints</th>
<th>IdmK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total NOE</td>
<td>1123</td>
</tr>
<tr>
<td>Intraresidue</td>
<td>449</td>
</tr>
<tr>
<td>Interresidue</td>
<td></td>
</tr>
<tr>
<td>i+1</td>
<td>317</td>
</tr>
<tr>
<td>i+2-4</td>
<td>184</td>
</tr>
<tr>
<td>Long range</td>
<td>173</td>
</tr>
<tr>
<td>Violations</td>
<td>28</td>
</tr>
</tbody>
</table>

Table 5.5- Unambiguous distance restraints assigned by ARIA during the structure calculation of IdmK.

To assist the convergence of the structures generated by ARIA rather than starting from a random structure, the first round of the calculation (it0) was seeded with the CS-ROSETTA structures generated when RDCs were included as a restraint (Figure 5.20). 20 structures were used to seed the first round of the calculation, and 20 structures were carried through to the next round of the calculations (it1-8). Refinement in a bath of TIP3 water was carried out for 10 structures from it8 to give the final ensemble. During the calculation, the hot and slow cooling stages were extended by a factor of four as described in (Fossi et al., 2005c) in an effort to aid convergence. As mentioned previously, +TALOS phi and psi angles derived from the Cα and Cβ chemical shifts for residues shown to form the α-helices were included in the calculations.

To further improve convergence in the first round of structure calculations only the HN chemical shifts were used and the $^1$H-$^{15}$N NOEs initially assigned. In this round of calculations a structure composed of three α-helices was generated, however there was no consistent overall tertiary fold. The $^1$H-$^{15}$N-$^1$H NOEs assigned in this round of calculations for the secondary structure elements were fixed in subsequent calculations, this included 64 unambiguous and 3 ambiguous distance restraints, each of which had two ambiguous assignments (Fossi et al., 2005a).

The second round of calculations used a subset of assignments, specifically those of the methyl groups from Ile, Leu Thr and Val methyl protons and shifts Hα and Hβ protons in order to separate the spectrum into two sub-spectra for which clear and confident assignments could be made. Any other assignments within the two sub-
spectra were assigned to prevent miss-assignment of chemical shifts. The regions used were 6-3.5ppm and 1.3-1ppm.

Finally, for the calculations initial weak NOEs restraints of those identified by the CS-ROSETTA models, between secondary structure elements, were introduced into the calculation and used with their upper bounds extended by 5 Å. Any restraints between the secondary structure elements that were assigned and violated by greater than 10 Å were removed and flagged for further investigation. RDCs were not used in the second round of calculations and so were able to be used for subsequent structure validation. Figure 5.23 shows the ensemble of IdmK structures generated by ARIA.

![Figure 5.23- Structure ensemble of IdmK calculated by ARIA and validation. (a) Overlay of the 10 lowest energy structures generated by ARIA through unambiguous assignment of NOE distance restraints. (b) Structure ensemble was validated by comparison of the calculated RDCs against experimentally determined RDCs.](image)

The ARIA structure ensemble shows a three α-helical bundle, with helix one (residues 6-19) running antiparallel to helix two (residues 44-54) and three (74-86). The conserved serine required for post-translational modification is located at the top of helix 2. Contrary to the CS-ROSETTA structure, the ARIA structure does not have the small-helix (or turn) seen in the long loop between helices 2 and 3. In carrier proteins this is termed as a pseudo helix, and is variable between structures,
this may be because of the limited restraint set used. This fold shows the same
topology of the helices and loops as the canonical fold; however the meanders are
not well ordered between the helices and relative angles of the helices to each other
vary somewhat from those seen in the canonical fold of the carrier proteins (Figure
5.5). Figure 5.24 shows a comparison of the IdmK structure generated by ARIA in
comparison with an acyl carrier protein from FAS.

Figure 5.24- Comparison of the 3D structures of acyl carrier protein and IdmK.
View of the 3D structures of 2AVA (yellow) with the best ARIA structure (blue)
showing to compare orientation and positioning of helices (Zornetzer et al.,
2006).

Although the Q factor for the ARIA ensemble indicates that the agreement with the
measured RDCs is slightly worse than that seen for the CS-ROSETTA structures,
ARIA has been able to improve the convergence of the ensemble structures
generated. This is shown by a lower RMSD of the secondary structure elements, an
average of 0.66 Å compared with 3.43 Å of the RDC restrained CS-ROSETTA
structures. The initial structure calculated with ARIA provides a solid starting point
for further refinement of the structure and introduction of further NOEs to achieve a
complete structural model of IdmK. Figure 5.25 shows the short range NOE
assigned by ARIA. The resulting pattern of short range NOEs are in good agreement
with the location and lengths of the secondary structure elements.
Figure 5.25- Plot showing short range NOEs assigned by ARIA. Comparison of the short range NOEs assigned by ARIA with the secondary structural elements predicted from the chemical shifts by TALOS show good agreement for the location and lengths of helices.
5.5 Initial phosphopantetheine characterisation

As with other carrier proteins, phosphopantetheinylation of IdmK is required for function. Chapter 4 showed that when IdmK is expressed in *E. coli* it is phosphopantetheinylated by an endogenous PPTase (Chapter 4, Figure 4.7). Furthermore, it was shown that it was possible to purify *holo*-IdmK (Chapter 4, Figure 4.6 and 4.7). In order to elucidate the mechanism of this module, the next step taken was to investigate structural changes observed in *holo*-IdmK, and investigate the location of the modification. Chemical shifts provide a sensitive monitor of changes in structure and dynamics (Teng, 2005).

![Figure 5.26](image)

Figure 5.26- An overlay of $^1$H-$^15$N HSQC spectra collected in 50 mM Tris.HCl pH 7.4, 0.2\% (w/v) sodium azide of *apo-* and *holo*-IdmK. Chemical shift differences of the *apo-* (black) and *holo-* (red) spectra are shown by peak shifts, which indicates changes in the local environment of the nuclei. Green arrows identify peaks that have altered chemical shifts in the *holo-* spectrum. The blue arrows identify two new peaks observed in the *holo-* spectrum. Insert illustrates peak shifts observed in the overlay of the two spectra.
Results three

Superimposition of the $^1$H-$^{15}$N HSQC spectra of the apo- and holo- states of IdmK identified two new peaks (blue arrows), indicating the presence of two new amides in the protein, comparable to the structure of phosphopantetheine though two amides are also missing from the apo spectrum. Chemical shift differences, characterised by peaks shifts were also observed for some other residues. Figure 5.27 shows a graph of the differences of chemical shifts between the apo- and holo- IdmK, identified using conservative shift mapping (Williamson et al., 1997) (section 2.2.5.6 Equation 2.5) and those above 0.2 ppm were mapped onto the most recent, ARIA, structure for reference.

Figure 5.27- Chemical shift perturbations of apo- and holo- IdmK. The graph shows the chemical shift differences measured from the $^1$H-$^{15}$N HSQSC spectra of apo- and holo- IdmK. Chemical shift differences were then mapped onto the best ARIA structure. The red patches indicate residues with chemical shift differences ≥0.2 ppm. The residue highlighted in green is the conserved serine that gets phosphopantetheinylated.
When the chemical shift differences observed in the $^1$H-$^{15}$N HSQC spectra of apo- and holo-IdmK were mapped onto the apo- ARIA structure of IdmK, it indicated that the phosphopantetheine was lying down the side of helix 2, directed into the core of the protein. Chemical shift differences were also observed in residues within the loop between helix 1 and 2. For complete validation of the structure of IdmK with phosphopantetheine a full assignment of the phosphopantetheine moiety is required, and in addition a full set of NOE restraints need to be measured before the full holo state of the protein can be determined. However, this initial data suggests that the phosphopantetheine may be more occluded with a different orientation to those previously observed, unless major movements in the secondary structure elements occur (Johnson et al., 2014; Nguyen et al., 2014; Masoudi et al., 2014).

### 5.6 Summary

Structure determination by NMR spectroscopy began with collection of a $^1$H-$^{15}$N HSQC spectrum. This spectrum indicated that apo-IdmK would be suitable for structure determination by NMR spectroscopy. Backbone assignments of IdmK were completed by collection of triple resonance spectra, and using the semi-automated assignment program MARS (Jung and Zweckstetter, 2004). MARS was able to assign approximately 92% of the protein backbone; the MARS output was manually checked using strip plots (Figure 5.8). Assignment of the protein backbone provided chemical shifts for $^1$H$_N$, $^{15}$N, $^{13}$Ca, $^{13}$CO and $^{13}$Cβ nuclei. These chemical shifts were then used in a structure calculation by CS-ROSETTA to generate an overall fold for IdmK (Shen et al., 2008; Shen et al., 2009b). The models generated were validated by RDCs measured by collection of a J modulated series. CS-ROSETTA models were also generated using RDCs as a restraint in the calculation. Models generated by CS-ROSETTA showed the observed topology expected for a carrier protein (Figure 5.6), however the angles of the helices and relative positioning differed from the observed expected canonical fold of other carrier proteins (Figure 5.19). RDC data (Figure 5.18) indicated that the secondary structure elements in the models agreed with experimental data collected. In an effort to elucidate a higher resolution structure short and long range NOEs were used. Side chain assignments were completed for 60-77% of side chains atoms of residues, assisting in assignment of
Results three

NOE distance restraints by ARIA (Rieping et al., 2007). Structure calculations carried out by ARIA generated a converged ensemble. The structures showed a three-helix bundle with the expected topology for carrier proteins. The ARIA structures satisfy NOEs expected from the canonical fold, the orientations of the helices are comparable to the CS-ROSETTA structures. Although the fit of the RDC data is slightly worse than the CS-ROSETTA structures this data provides a strong foundation for determination of a high-resolution structure, using ARIA.

5.7 Discussion

Initial analysis of the solution structure ensemble of IdmK generated by ARIA with other carrier proteins from fatty acid synthases and polyketide synthases has inferred that IdmK adopts an almost canonical carrier protein fold differing in the orientations of the helices, although more work needs to be done to complete the models generated. Investigations into the protein dynamics of peptidyl carrier proteins has been carried out on the TycC3 PCP. Figure 5.27 shows the multiple conformers this carrier protein was seen to adopt in the apo- and holo- forms, the A/H state is seen in both apo- and holo- PCP states. The first solution structure (PDB code: 1DNY) of the TycC3 PCP is comparable to the A/H state elucidated some years later (Weber et al., 2000; Koglin et al., 2006). The PCP from the tyrocidine biosynthetic gene cluster appears to adopt different conformations, with the holo-state being the most expanded, and the A/H state being the most compact. Variations in helix angles with respect to each other differ between states, as does the length of helices. The small helix/turn between in the A/H state is also missing in the A and H states (Koglin et al., 2006). The dynamic nature and more open conformation of holo-TycC3-PCP may be to assist in protein-protein interactions and access of the phosphopantetheine modification to other domains and downstream modules to elongate the growing peptidyl chain.
So far, there has been no obvious evidence of IdmK being as dynamic in nature as the TycC3-PCP. However, collection of perhaps a $^{15}$N, $^1$H-TROSY spectrum and full characterisation of the phosphopantetheine co-factor and holo-state of IdmK may shed some light on whether IdmK is as dynamic, as the TycC3-PCP (Koglin et al., 2006). Characterisation of the phosphopantetheine modification and holo-state of the carrier protein is in its preliminary stages. Results so far indicate that the phosphopantetheine may be positioned down the side of helix 2, directed into the hydrophobic core of the protein. Further validation of the current ARIA models is required before assuming any definitive conformations of the phosphopantetheine modification. Assignment of the phosphopantetheine can be carried out using a standard triple resonance HNCA experiment that has already been collected, in addition to collection of $^1$H-$^{15}$N-NOESY data. Recently, vibrational spectroscopy and crystallography were able to probe the movements of the phosphopantetheine arm in a holo-ACP, showing that prior to being loaded with its substrate the
phosphopantetheine arm is solvent exposed, directed away from the core of the protein, upon acylation large conformational changes were observed in helix 2 and 3, causing dissociation from the loading enzyme, potentially in preparation for subsequent protein-protein interactions downstream (Johnson et al., 2014; Nguyen et al., 2014; Masoudi et al., 2014). It would be interesting to probe the conformational changes that may occur when loading holo-IdmK with L-proline, either enzymatically or chemically.

NMR structure calculations are difficult as they require the assignment of the entire protein backbone, amino acid side chains and NOE distance restraints (Teng, 2005). This chapter described the use of MARS to assign the backbone of IdmK. This program provided fast and reliable assignments. MARS is a powerful program for small proteins such as IdmK (Jung and Zweckstetter, 2004). The increasing number of assignments required for structure calculations can be difficult and laborious, especially as confidence in the assignment is important as mistakes can severely affect the outcome (Markwick et al., 2008; Nilges, 1995).

ROSETTA has been used successfully for ab initio protein structure prediction for proteins up to 20 kDa (Shen et al., 2008; Sgourakis et al., 2014; Bouvignies et al., 2011). A combination of unambiguous NOE assignments in the ROSETTA structure calculation could and has been used to help higher resolution structure prediction, even for larger proteins (Loquet et al., 2012; Raman et al., 2010; Sgourakis et al., 2011). In the structure determination of IdmK using CS-ROSETTA, RDCs were used as a restraint to aid the structure calculations, producing a carrier protein with the same topology as the canonical fold. The structures produced were equivalent to the CS-ROSETTA structure calculation without the RDCs, showing the high predictive power of the technique.

Assignment of NOESY spectra can be very tedious even for small proteins. ARIA performs an iterative assignment of ambiguous NOEs to determine long range distance restraints for structure determination (Rieping et al., 2007). Although this program has obviously sped up structure determination, it still requires the input of good quality NOESY spectra. Overlap and miss assignment of peaks will hinder the ARIA structure calculation and severely affect the outcome of the structure calculation. More recently, CS-ROSETTA structure calculations have been
completed using RDCs and sparse Ile, Leu and Val NOE restraints, making it possible to calculate a structure with an RMSD of 0.5 Å with respect to the crystal structure (Sgourakis et al., 2014).

Structure determination of IdmK using CS-ROSETTA provided a solid foundation for high resolution structure determination using ARIA. Seeding ARIA calculations with the CS-ROSETTA models, in addition to including some weak initial NOE restraints was able to produce a structure of IdmK in some agreement with other carrier proteins. Further structure refinement is required of IdmK. Once the high-resolution structure is complete, investigation into protein-protein interactions and dynamics of both apo- and holo- IdmK will provide some insight into peptidyl chain elongation and starter unit construction in indanomycin biosynthesis.
Results three
6. Summary, future work and perspectives

6.1 Summary

This thesis has described the successes and difficulties in heterologous expression of the indanomycin nonribosomal peptide synthetase (NRPS) proteins in *E. coli*. Attempts to clone and express the three proteins, IdmJ, IdmI and IdmK, from indanomycin starter unit biosynthesis are presented.

Chapter 1, the introduction to the focus of this thesis, outlined a huge foundation of research into NRPS and PKSs, from their biosynthetic gene clusters to production of novel compounds. It was decided that a heterologous expression of the target module would be the appropriate first step to take in engineering substrate specificity in the domains required for starter unit biosynthesis of indanomycin. This research began by designing and purchasing synthetic genes, codon optimised for expression in *E. coli*. These genes encoded an adenylyltransferase (IdmJ), responsible for selection of L-proline and activating it, a carrier protein (IdmK), responsible for transfer of the substrate to the downstream module, and the dehydrogenase (IdmI), which oxidises the L-proline to a pyrrole. Chapter three presented the issues encountered when attempting to express IdmJ and IdmI in *E. coli* outlining the approaches taken in order to produce soluble protein. Complications encountered with expression of IdmJ and investigation into a potential post-translational modification is outlined in this work. Unfortunately, the measures taken to express IdmI as a soluble protein did not solve the problem. Chapter four discussed the successes of cloning and expressing IdmK followed by the fortuitous discovery that IdmK was also post-translationally modified in *E. coli*. This chapter also described the first attempts at structural characterisation by crystallography, and why NMR was chosen as an alternative for structure determination. Chapter five describes the steps taken for structure determination by NMR and has presented a protein fold elucidated solely from chemical shifts in addition to a higher resolution structure of IdmK. The first steps in characterisation of *holo*-IdmK and the phosphopantetheine are also described. This data so far suggests that IdmK has canonical carrier protein fold, and the phosphopantetheine modification may be pointing into the core of the protein.

Structure determination of IdmK has provided a solid foundation for investigations into dynamics of the NRPS module, and potentially the carrier proteins interactions.
with an adjacent PKS module. Including refinement of the carrier protein structure and assignment of the phosphopantetheine modification, there are a number of directions for future work.

6.2 Extension of current work

6.2.1 Expression and purification of the dehydrogenase (IdmI)
Soluble protein expression of IdmI was not achieved; however there are still a number of strategies which could be trialled, including expression in Origami™ 2 (DE3) cells, to aid in disulphide bond formation in E. coli. Although genes were synthesised to be codon optimised for expression in E. coli, previous dehydrogenases from aminocoumarin biosynthetic gene clusters have been cloned from genomic DNA with a C-terminal His₆-tag (Garneau et al., 2005), therefore this could be attempted. Finally, insoluble aggregates could be unfolded, and refolded for purification.

6.2.2 Purification and characterisation of the adenylyltransferase
Expression and purification of IdmJ resulted in an unknown post-translational modification at C127. Site directed mutagenesis of the cysteine residues resulted in purification of IdmJ variants without the post-translational modification. Further purification by size exclusion chromatography of IdmJ alanine variants (C104A, C127A, C169A and C308A), characterisation of secondary structure by CD and assaying activity using an ATP/PPi exchange assay could provide insight into to whether the alanine variants are folded correctly and functional.

6.2.3 Structural characterisation of the carrier protein (IdmK)
The current solution structure of IdmK requires further refinement and validation; once this has been achieved it provides the basis for investigation into structural changes that may occur when the carrier protein is phosphopantetheinylated or loaded with its substrate. Exploration into protein-protein interactions with a variety of PPTases could be carried out, investigating the binding surface of the protein. In
Conclusions

lieu of the adenylyltransferase, investigation into the use of chemical modification using thiols as an alternative method to chemically load the carrier protein with the substrate, using the method described for conversion of cysteines into dehydroalanine followed by a reaction with a thiol, could be carried out (Chalker et al., 2011; Bernardes et al., 2008; Timms et al., 2013). NMR could be used to investigate structural changes that have occurred as a consequence of holo-IdmK being loaded with the appropriate and alternate substrates.

6.3 Future perspectives

Following any successes highlighted above structural and biochemical characterisation could be carried out. Alternatively, investigation into substrate specificity could be carried out by substituting IdmJ with the adenylation domains found in clorobiocin and coumermycin biosynthesis, known to be successfully cloned and expressed in E. coli (Garneau et al., 2005). Identification of residues which contribute to substrate specificity and mutagenesis could aid in engineering substrate specificity in the adenylation domains (Stachelhaus et al., 1999). In addition to biochemical assays such as ATP/PPi exchange, NMR could be used to follow the progress of loading the carrier protein with alternative substrates.

IdmK is a peptidyl carrier protein, located in an NRPS module, that is required for the transfer of the starter unit, pyrrole-2-carboxylate, to a downstream PKS module. Investigating the interaction of IdmK with PKS domains could provide insight into communication between NRPS and PKS modules in hybrid enzymes. However, this would require the simultaneous expression of PKS modules, such as the downstream PKS module in indanomycin biosynthesis IdmL (Li et al., 2009).

Combining chemical loading of the carrier proteins via a dehydroalanine or enzymatically through the use of an adenylation domain able to accept alternative substrates, with investigation of the interaction of IdmK with the downstream module IdmL could generate novel products.
6.4 Concluding remarks

This research has provided a basis for investigation into NRPS module dynamics based around the carrier protein using NMR spectroscopy as a tool. Although difficulties were experienced when attempting to clone and express the adenylyltransferase and dehydrogenase, characterisation of the carrier protein has been successful. Structure determination of a carrier protein involved in communication between an NRPS and PKS module could provide insight into this interaction, potentially highlighting differences between the interactions of carrier proteins in NRPS and PKS only multienzymes. This could aid in the efforts to create an NRPS/PKS toolbox for combinatorial synthesis of novel compounds.
Appendix

7. Appendix

7.1 Sequences

7.1.1 Adenylyltransferase *(idm*J*) DNA sequence

GAGCTCAAGGAGATATAACCATTAGGAACATCATATCATATCATCATACACAAACTCTGATCTGGTGTCA
TACGGCCGCTTAAAGAACGGATCTGTGGCTGCGTGGACCCGGCAGCAGCTGAGTGAACCCAGA
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CTGTTGGCTGTGACAGCCTGACACCCGACCTGGCCCAACCAGCAGAAGGCTGAAATGCTCTTGTTTC
CCGCAGGCTGGCGTGCTGGCCGGCTGGCACCGAGCACGTCTGGACGTACGCAGA
ACTGGATTCTACCGGAAAACGGCAGCTTGGCCTGGCGCCGGCTTGGTGTTGGTTCGCGGCTGCCAG
CTGTTGGCTGTGACAGCCTGACACCCGACCTGGCCCAACCAGCAGAAGGCTGAAATGCTCTTGTTTC
CCGCAGGCTGGCGTGCTGGCCGGCTGGCACCGAGCACGTCTGGACGTACGCAGA

7.1.2 Adenylyltransferase *(IdmJ)* amino acid sequence

MEHHHHNNLHQLVLDTAIAKPEPRLAVAGTAAARLYAEIDSTANALAHRLALGVGPGDRVVLWSD
KSPAVVAAMOQAIVRLAAYVPADAGLPIARVAAMAADDCAALLAAPRDLAPAVDLGPRCPADL
AQRPDPAAEPLNALVAPDDLAYLTYSTSGTAPGVCISHR NRARAFV DAVEELAPGPQDRFSNHAPFT
FDLSVLALAFSASAGVLHPSLEYAPEQLVEFHLHRQITVWYSVPALTLMMRDGGGLDRPALRPL
RTLVFAPEFPLPGVRALAGWDARLNLNYGPEPNVCTRHEVRPTDLGDRLPLIFGTAVSGDRAWE
GPDPGLALAPGEEGELLVDGGTVMLYWGGHGPTGPPYRTGDLVRLPGGFSMYLGRDDHMVKVRGRHV
ELGVESESVLALHPDVAEAAAVVVGSGMDGRLVAFVPEPDRPVGLSLVRHAQQLRPYMVADMERV
LPGLPRTRNGKVRDLRALRTDAEAPAGAAA

7.1.3 Prolyl dehydrogenase *(idm*I*) DNA sequence

GGATCCAAGGAGATATAACCATTAGGAACACCCACCAACCACACCAACCAGACTTTGAGCTGACCCAGAA
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CCGCAGGCTGGCGTGCTGGCCGGCTGGCACCGAGCACGTCTGGACGTACGCAGA
ACTGGATTCTACCGGAAAACGGCAGCTTGGCCTGGCGCCGGCTTGGTGTTGGTTCGCGGCTGCCAG
CTGTTGGCTGTGACAGCCTGACACCCGACCTGGCCCAACCAGCAGAAGGCTGAAATGCTCTTGTTTC
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201
7.1.4 Prolyl dehydrogenase (IdmI) amino acid sequence

```
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7.1.5 Prolyl carrier protein (idmK) DNA sequence

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7.1.6 Prolyl carrier protein (idmK) amino acid sequence

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AIQIFGGSYLSPAQIEQQLFVLPSTFSSQTDQIREIVAREIGL
```

7.2 Vector maps

7.2.1 pET23a
Figure 7.1- Vector map of the pET23a vector purchased from Merck4Biosciences (Nottingham, UK) highlighting key features such as the multiple cloning site (MCS), origin or replication, antibiotic resistance and promoter.
7.2.2 pKnanA plasmid map

Figure 7.2-Plasmid map of the pKnanA plasmid (Timms et al., 2013) The nanA gene (933 bp) resides between the EcoRI and PstI restriction sites. The origin of replication, antibiotic resistance and promoter are also highlighted.
7.2.3 pETDUET

Figure 7.3- Vector map of the pETDUET vector purchased from Merck4Biosciences (Nottingham, UK) highlighting key features such as the multiple cloning sites 1 and 2 (MCS1 and 2), origin or replication, antibiotic resistance and promoter for each multiple cloning site.
7.2.4 pMAL c5X

Figure 7.4- Vector map of pMAL c5X purchased from New England Biolabs (Ipswich, MA, USA) highlighting key features such as the multiple cloning site, promoter, maltose binding protein (MBP) and antibiotic resistance marker.
### 7.3 Oligonucleotide sequences

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<th>Oligonucleotide primers</th>
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<td>5’-GAT TAT GCG GCC GTG TAC AA-3’</td>
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**Table 7.1**: Table of sequences of oligonucleotide used.
7.4 PSI-BLAST search

Figure 7.4-Top 10 sequences from the PSI-BLAST search with IdmK as the target sequence. The conserved serine is highlighted in the sequence.

7.5 SDS-PAGE analysis of uninduced *E. coli* BL21 (DE3) Gold

Figure 7.5- Reducing SDS-PAGE of uninduced *E. coli* BL21 (DE3) Gold cells. Expected masses for IdmJ, IdmI, IdmI-MBP and IdmK are highlighted for comparison.
Table 7.2-Table of chemical shift assignments (ppm) of IdmK. The protons have not been stereospecifically assigned.

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7.7 Crystallography statistics

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Table 7.3- Table of crystallography statistics of IdmK. Values in parentheses correspond to the lowest resolution range. R<sub>pim</sub> indicates precision (multiplicity-weighted) R<sub>merge</sub>.
References


References


References

lipopeptide antibiotic surfactin by the thioesterase domain SrfTE. *Structure, 10*, 301-310.


References


References


References


References


Gehring, A. M., Bradley, K. A. & Walsh, C. T. (1997). Enterobactin biosynthesis in Escherichia coli: Isochorismate lyase (EntB) is a bifunctional enzyme that is phosphopantetheinylated by EntD and then acylated by EntE using ATP and 2,3-dihydroxybenzoate. Biochemistry, 36, 8495-8503.


References

National Academy of Sciences of the United States of America, **86**, 8247-8251.


References


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References


References


