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Uminho | 2013

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Master Thesis

Master in Molecular Genetics

Work done under supervision of:

Doutor Tony Collins

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Abril 2013

DECLARAÇÃO

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Título da tese: Thiol-disulphide oxidoreductases: production, purification and structural analysis of a cold adapted DsbA.
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Ano de conclusão: 2013
Designação do Mestrado: Genética Molecular

Universidade do Minho, ___/___/_____

Assinatura: _____

ACKNOWLEDGEMENTS/AGRADECIMENTOS

'Serei prosa serei verso?
Instrumento útil neste Universo.
Guiada num controverso caminhar,
tendo em missão seu guião findar.

Dia de hoje, que será sempre um só,
Produto palpável dos sonhos passados.
Desses, tantos se desmoronaram num pó,
e a tantos outros permanecem meus quereres abraçados.'

Catherine Ferreira

De um jeito poético começo por descrever a forma como encaro cada desafio. Tendo em mente que tudo se resume à descoberta incansável do nosso caminho, do nosso lugar. Este caminho de linhas tortas que se constrói das nossas vontades, das nossas opções mas que nunca é resultado de um só 'eu'. Nesta viagem beneficiamos da ajuda, dos conselhos e da força daqueles com que a vida nos quis cruzar. Começo assim por agradecer a quem permitiu que este passo fosse possível, a quem tão bem me recebeu e me integrou. Agradeço imenso aos meus orientadores pela indispensável presença que tanto me auxiliou neste percurso. À Professora Doutora Margarida Casal pelas palavras amigas que sempre reservou para mim, e pelo contagiante sorriso com que sempre me recebeu. Não dispenso valorizar-lhe a boa disposição, a serenidade e os certeiros conselhos que tanto me auxiliaram. Ao Professor Doutor Tony Collins pela incansável paciência, pela constante disponibilidade e pelo esforço acrescido que sempre demonstrou em contornar todas as situações inesperadas tão comuns de qualquer percurso. Agradeço também a todos que floriram os meus dias no laboratório. Assim, agradeço do fundo do coração aos que me arrancaram sorrisos em dias mais difíceis, aos que me presentearam com a sua presença e cuja personalidade de cada um fez daquele laboratório um lugar tão bom e tão melhor. À Joana Sá Pessoa, ao Raul Machado, ao João Silva, ao André Costa, ao Pedro Castro e ao Fernando Branca. De uma forma geral agradeço a forma tão especial como fui recebida, integrada e tratada. Enchem-me de alegria, antecipada saudade e da certeza de que todos os momentos estarão sempre carinhosamente guardados na minha memória.

Agradeço com um especial carinho a todas as pessoas que de uma forma ou de outra contribuíram no sentido de me ajudar neste percurso. Á Joana Tulha, à Filipa Pereira, à Andreia Pacheco, à Dulce Cunha, à Sara Alves, ao Flávio Azevedo, à

Helena Pereira e à Catarina Carneiro. Sem esquecer todos os técnicos que fizeram parte desta jornada, reconhecendo-lhes o tão importante trabalho que sempre tiveram e com o qual nada disto seria possível.

À Professora Doutora Maria João Sousa pela recepção no Mestrado em Genética Molecular.

'Quando o sonho abraça a fé, a coragem é véstia mais bela. Atributo invejado por quem vive só da razão. De quem vê e não entende, de quem toca e não sente, de quem passa sem viver. Pois se o sonho é meu e a fé minha loucura… a coragem enaltece o brilho de quem tem morada em mim. Dos que me vêm de verdade, dos que me sentem no infinito e me entendem sem perguntar. Nesse olhar, nesse abraço, nessas palavras plenas de silêncio. Na promessa de alinhar na primeira fila de cada batalha vossa, sei que vos terei em cada luta minha. Pois é desse brilho que vivo. E quanto mais invejado e incompreendido mais intenso e desejado será o seu brilho. Selvagens almas, misteriosos seres… sem vocês não seria mais eu.'

Catherine Ferreira

Agradeço o que as palavras não conseguem descrever, à minha família, especialmente aos meus pais, pois o seu apoio a todos os níveis foi sem dúvida essencial. Aos meus irmãos pois não teria o mesmo sentido sem eles. Aos meus avós que sempre me apoiaram também. Dedico todo este esforço a eles e em especial à minha avó e à minha bisavó, esperando que se orgulhem desta batalha.

Aos meus amigos que em todos os momentos estiveram presentes, que foram incansáveis na ajuda, que albergaram a maior paciência e mesmo assim se mantiveram firmes ao meu lado. Por todos os momentos bons e menos bons. Agradeço assim à Rita Cunha, ao Pedro Castro, ao António Rego e à Sara Peixoto.

Um agradecimento especial ao Pedro Martinez que no momento certo surgiu, que do seu jeito único me estendeu a mão e que desde então se mantém fielmente ao meu lado. Para o que der e vier sei que estarás sempre lá.

Ao João digo que são poucas as palavras capazes de descrever a minha gratidão. Que nada seria possível se nos dias mais difíceis eu não encaminhasse a minha mente para um futuro melhor. E acredito tanto nele. É nesse futuro que descanso e vejo a paz e a felicidade de que preciso.

ABSTRACT

Thiol-disulphide oxidoreductases (DsbAs) are bacterial extra-cytoplasmic enzymes which catalyse oxidative disulphide bond formation (S-S) between the thiol sulphurs (-SH) of cysteine side chains in newly synthesised proteins (Shouldice, et al., 2011). In medicine, the key role of DsbA in catalysing the correct folding of many essential proteins that enable pathogenesis has led to suggestions for this enzyme as a potential antimicrobial drug target (Heras, et al., 2009). DsbA catalyses the correct folding of virulence factors associated with cell adhesion, bacterial mobility and host cell manipulation and hence its inhibition would reduce or impede bacterial pathogenesis. Indeed bacterial infections are a major cause of death in the world and this, in addition to current high levels of antibiotic resistance in many pathogenic bacteria, highlights the urgent need for new validated targets and for the design of new antibacterial agents against these targets. Due to its role in pathogenesis DsbA offers such a target for a new therapeutic approach and a better understanding of this enzyme and its function is of much importance.

In the present study a cold adapted DsbA from *Pseudoalteromonas haloplanktis* TAC125 was studied with the long term aim of better understanding its structure and function relationship. Previous studies of this enzyme made use of non-optimised production and purification procedures and production levels were found to be poor (approximately 50 mg/L) with large losses being noted during purification. Therefore the present study was focused on optimising the shake-flask batch production in *E. coli* and simplifying and improving the purification protocol for this protein. Furthermore, as an initial step in our quest for a better understanding of this enzyme, a comparative structural analysis (with homologous enzymes) was carried out to identify structural factors which may be important for the low temperature activity of cold-adapted DsbAs. Mutants were then designed and prepared in an attempt to investigate the roles of the observed structural differences.

We have shown that the rich medium Terrific broth (TB) with induction during the stationary phase of growth allowed for optimum DsbA production. Interestingly, high production levels were attained even in the absence of induction with IPTG. Optimisation of the purification protocol allowed for the development of a simplified procedure yielding 250 mg of purified protein per litre of production culture (a 5-fold increase on that previously reported) with a reduced DsbA loss during the process. Structural comparisons allowed for the identification of two loop insertions in the cold-adapted enzyme as compared to homologs adapted to higher temperatures and four deletion mutants investigating these insertions have been prepared.

RESUMO

As tiol-dissulfito oxidorredutases (DsbAs) são enzimas bacterianas extra citoplasmáticas que catalisam a formação oxidativa de pontes dissulfito (S-S) entre os grupos tióilicos (-SH) das cadeias laterais das cisteínas em proteínas recentemente sintetizadas (Shouldice, et al., 2011). Na medicina, o papel chave da DsbA prende-se com a catálise do correcto rearranjo de muitas proteínas essenciais na patogénese, têm assim surgido sugestões de que esta enzima possa ser um alvo de potenciais drogas antimicrobianas (Heras, et al., 2009). A DsbA é catalisadora do correcto rearranjo de factores de virulência associados à adesão celular, mobilidade bacteriana e manipulação das células hospedeiras. Na verdade, as infecções bacterianas são já a maior causa de morte no mundo e este facto, em junção com o actual alto nível de resistência a antibióticos por parte de várias bactérias patogénicas, enaltece a urgente necessidade tanto de validar novos alvos como de objectivar o desenho de novos agentes antibacterianos que actuem nesses alvos. Devido ao seu papel na patogénese, a DsbA, sendo um possível alvo apresenta-se assim uma nova abordagem terapêutica destacando-se a elevada importância de um melhor entendimento desta enzima e da sua função.

No presente estudo, foi estudada uma DsbA adaptada ao frio proveniente da bactéria *Pseudoalteromonas haloplanktis* TAC125 objectivando-se a longo prazo um melhor entendimento da relação entre a sua estrutura e função. Estudos anteriores centrados nesta enzima têm feito uso de processos de produção e purificação não optimizados tendo resultado em baixos níveis de produção (aproximadamente 50 mg/L), com grandes perdas observadas no processo de purificação. Assim sendo, o actual estudo centrou-se tanto na optimização da produção em batch em *E. coli* como em simplificar e melhorar o protocolo de purificação para esta proteína. Além disso, como parte inicial da nossa investigação focada na obtenção de um melhor entendimento desta enzima, uma comparativa análise estrutural (com enzimas homólogas) foi levada a cabo de modo a identificar os factores estruturais que possam ser importantes na actividade a baixas temperaturas desta DsbA naturalmente adaptada ao frio. Os mutantes foram então desenhados na tentativa de investigar o papel das diferenças estruturais observadas.

Demonstrou-se neste estudo que a junção do meio rico Terrific Broth (TB) com indução na fase estacionária de crescimento permitiu um nível óptimo de produção da DsbA. Interessantemente, elevados níveis de produção foram alcançados inclusive na ausência de indução. No entanto, altos níveis de produção foram também observados

aquando da indução com 1 mM de IPTG na fase de declínio exponencial. A optimização do protocolo de purificação permitiu o desenvolvimento de um simplificado procedimento, rendendo 250 mg de proteína purificada por litro de cultura d produção (5 vezes mais) com a reduzida perda de proteína ao longo do processo. Comparações estruturais permitiram a identificação de duas inserções em loop's da enzima adaptada ao frio quando comparada com os homólogos adaptados a temperaturas mais elevadas. Neste sentido, quatro mutantes centrados na investigação desses locais foram concebidos.

ABBREVIATIONS AND SYMBOLS

TRX – Thioredoxin

-SH – Thiol group of cysteine

S-S – Disulphide bond between cystines

E. coli – *Escherichia coli*

Cys – Cysteine

Val – Valine

Lys – Lysine

Leu – Leucine

Ala – Alanine

Ser – Serine

His – Histidine

Asp – Aspargine

pKa – Dissociation constant

NMR – Nuclear Magnetic Resonance

PhDsbA – DsbA protein from *Pseudoalteromonas haloplanktis*

PhDsbB - DsbB protein from *Pseudoalteromonas haloplanktis*

Tcp – Toxin co-regulated pilus

B. pertussis – *Bordetella pertussis*

k_{cat} – enzymatic reaction rate

A - Frequency factor related to the frequency of collision of the reactants and to the probability of the reactants being in the appropriate orientation to react

T – Temperature

R - Universal gas constant

Ea - Activation energy necessary for the reaction

IPTG - isopropyl β-D-1-thiogalactopyranoside

kDa – Atomic mass unit

LB – Lysogeny broth

TB – Terrific broth

SB – Super broth

HPLC – High-performance liquid chromatography

FPLC – Fast protein liquid chromatography

DTNB - 5,5'–dithiobis–2–nitrobenzoic acid

TNB – 2-nitro-5-thiobenzoic acid

NaCl – Sodium chloride

pI – Isoelectric point

HIC – Hydrophobic interaction chromatography

IEX – Ion exchange chromatography

WT – Wild type

$K_2HPO_4.3H_2O$ – dipotassium phosphate trihydrate

KH_2PO4 – potassium dihydrogen phosphate

UV – Ultra violet

Vis – Visible

EDTA - Ethylenediamine tetraacetic acid

DTT – Dithiothreitol

MOPS - 3-(N-morpholino)propanesulfonic acid

BSA – Bovine serum albumin

DNA – deoxyribonucleic acid

RPM – Rotations per minute

CH_3COOK – Potassium acetate

$CaCl_2$ – Calcium chloride

$RbCl_2$ – Rubidium chloride

PNK - T4 polynucleotide kinase

PCR – Polymerase chain reaction

$MnCl_2$ – Magnesium chloride

G – Guanidine

C – Cytosine

H_2O – Water

Tris-HCl – Tris-hydrochloride

DNTP's – deoxyribonucleotides

Tm – Melting temperature

$MgSO_4$ – Magnesium sulphate

GSSG – Oxidised glutathione

GSH – Reduced glutathione

DSC – Differential Scanning Calorimetry

BLAST - Basic Local Alignment Search Tool

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Chapter 1: State of the art

THIOL-DISULPHIDE OXIDOREDUCTASE (EC 1.8.4.-)

1. 1. Thiol-disulphide oxidoreductases (DsbAs) are bacterial extra-cytoplasmic enzymes which catalyse disulphide bond formation in newly synthesised proteins. Of small size (typically around 21 kDa), they belong to the thioredoxin (TRX) superfamily of structurally related proteins (Collet & Bardwell, 2002) and have been mainly isolated from Gram-negative bacteria, with those from *Escherichia coli* and *Vibrio cholerae* being the most studied (Shouldice, et al., 2011; Ruddock, et al., 1996). DsbA homologs have also been identified in Gram-positive organisms, but, in contrast, these have been poorly studied.

The first report of DsbA was made when the enzyme's gene sequence was identified in *E. coli* and its function determined through the analysis of *dsbA*$^-$ mutants (Bardwell, et al., 1991). These mutants showed a defect in the construction of disulphide bonds in newly synthesized periplasmic proteins and hence the function of DsbA was correlated to the oxidative formation of disulphide bonds, i.e. covalent chemical bond formation between the thiol sulphurs of cysteine side chains (Figure 1) (Shouldice, et al., 2011; Fabianek, et al., 2000). Here, the reduced thiols (-SH) of a proteins cysteine residues are oxidized to give the disulphide derivative cystines (S-S). This is a key step in the folding and stability of many secreted proteins and forms part of a complex cycle involving numerous other intervenient enzymes (Madonna, et al., 2006). Indeed DsbA activity plays a key role in cell survival as the activity, stability (chemical and thermal) and resistance to proteases of many essential proteins are dependent on correct disulphide bond formation (Dutton, et al., 2010).

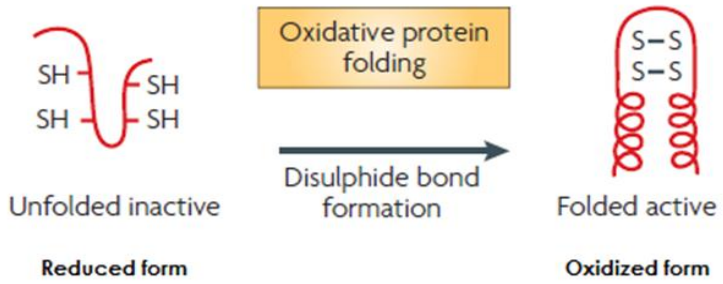


Figure 1: Representative scheme for disulphide bond formation. In: (Heras, et al., 2009).

DsbA forms part of the disulphide bond formation system (DSB), with the first oxidative event of this system being the oxidized form of DsbA interacting with reduced substrates (nascent proteins translocated to the periplasm) to catalyze the oxidation of the cysteine residues and form disulphide bonds (Heras, et al., 2009; Fabianek, et al., 2000; Madonna, et al., 2006). During the reaction, DsbA becomes reduced on receiving two electrons from the substrate protein and is thereafter reactivated via re-oxidation by a membrane bound partner known as DsbB (Figure 2). This latter then transfers the two electrons from DsbA to membrane-bound quinones (Dutton, et al., 2010; Horne, et al., 2007). Further enzymes are involved in the DSB system, including DsbC, a disulphide isomerase that proofreads and reshuffles incorrectly formed disulphides and DsbD a partner of DsbC that maintains this in its active reduced form (Heras, et al., 2009; Horne, et al., 2007). Indeed all proteins of the DSB system are essential and act together in ensuring correct disulphide bond formation, DsbAs directly act on the substrate cysteines, DsbBs are essential for re-oxidation of DsbA (Figure 2) and DsbC and DsbD are essential for correcting improperly formed disulphide bonds (Fabianek & Thöny-Meyer, 2000). In the present study we will focus only on DsbA and in particular on a DsbA isolated from the Gram negative cold adapted bacterium *Pseudoalteromonas haloplanktis* TAC125.

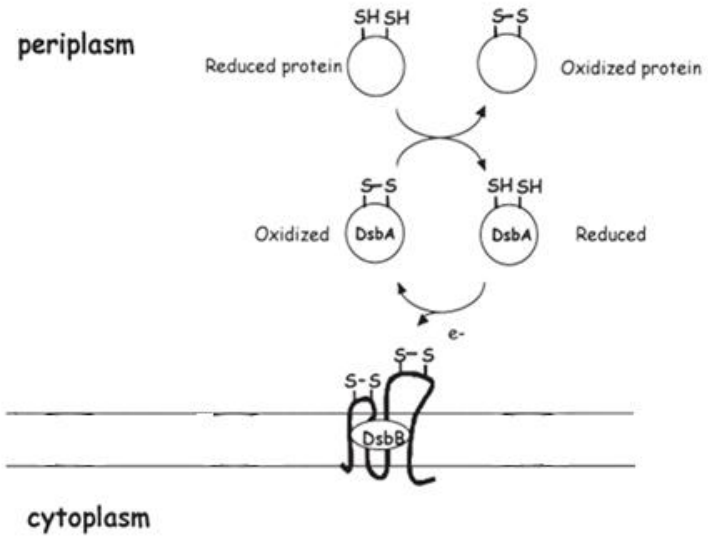


Figure 2: Schematic representation of the disulphide bond formation cycle in *Escherichia coli*. In: **(Collet & Bardwell, 2002)**

All proteins of the TRX superfamily share the structural characteristic of an α-helical domain juxtaposed with β-strands and with a pair of redox active cysteines

(Cys-X-X-Cys) located at the N-terminal end of the first helix at the active site of the enzyme (Figure 3) (Heras, et al., 2009; Guddat, et al., 1997; Ruddock, et al., 1996).

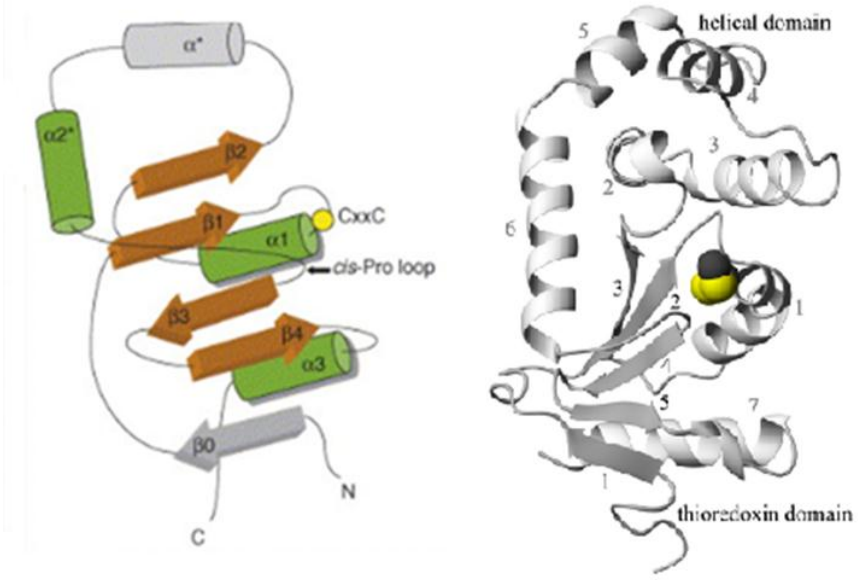


Figure 3: Common structural organization of DsbA fold. At left: Helices are illustrated in green and β sheets in brown. The locations of the catalytic CxxC motif (shown by a yellow sphere) and the *cis*-Pro loop (arrow) are also specified. Data from: (Gruber, et al., 2006). On the right: Crystal structure representation of oxidized *Vibrio cholerae* DsbA. The elements of secondary structure are sequentially numbered from the N terminus. Helices are presented with black numbers and the grey numbers denote strands. The active site is presented in a CPK representation. In: (Horne, et al., 2007).

DsbAs are the most oxidizing proteins known, probably as a result of the CXXC motif structure and, more precisely, due to an unusually low pK_a of the most N-terminal cysteine in the active site (Collet & Bardwell, 2002). The high reactivity of this Cys is due to an electrostatic interaction with a nearby His which stabilizes the Cys in its thiolate anion form (Guddat, et al., 1997). The redox potential of the enzyme also depends on the type of residues, XX, flanked by the two cysteines of the general motif (Ito & Inaba, 2008). Indeed the canonical sequence of the active site motif for TRX enzymes is C-P-H-C (Paxman, et al., 2009; Madonna, et al., 2006) but variations in the third residue (i.e the histidine) have been observed. In fact, Guddat and collaborators showed in 1997 that a mutation of the histidine residue of the active site motif leads to a significant decrease in the redox potential of these mutants (Guddat, et al., 1997).

As mentioned above, generally disulphide bonded proteins are more stable than their non-disulphide bonded forms yet it has been reported that the disulphide bond of DsbA is very unstable and that the stability of this protein is increased on disulphide

bond reduction (Zapun, et al., 1993). Furthermore, a previous study showed that oxidised DsbA from *E. coli* is more rapidly cleaved by proteases than its reduced form. These results suggest a lower stability and higher flexibility of the oxidized form of the enzyme which may have importance in its disulphide bond donation activity and in the the accommodation of substrate (Horne, et al., 2007).

1. 1. 1. WHY STUDY DsbA?

DsbAs have been the focus of much study over recent years, not only because of their fundamental interest, but also because of their potential applied importance. From a fundamental point of view, a better understanding of the implications of this enzyme in protein folding is of obvious importance. In relation to its applied interest, its potential for developments in the fields of biotechnology and medicine has led to a drastic increase in interest in this enzyme.

In biotechnology, the use of DsbA in catalysing the correct folding of disulphide bond containing proteins has led to suggestions for co-expression of this enzyme during recombinant protein production, for its use as an additive in cell free protein production systems (Kuroita, et al., 2007) and even for the refolding of misfolded proteins (Antonio-Pérez, et al., 2012). Indeed studies have shown that the use of this in cell free systems leads to higher production levels (approximately two fold higher) of active properly folded disulphide containing proteins (Kuroita, et al., 2007). Obviously, such increases in production levels warrants the use of DsbA as a tool in protein production procedures and the development of further more cheaply produced and more highly active DsbAs is called for.

In medicine, the key role of DsbA in catalysing the correct folding of many essential proteins, and in particular those that enable pathogenesis (i.e. the virulence factors), in pathogenic organisms has led to suggestions for this enzyme as a potential antimicrobial drug target (Lasica & Jagusztyn-Krynicka, 2007); Heras, et al., 2009; Shouldice, et al., 2011). DsbA has an essential role in pathogenesis as it catalyses the correct folding of virulence factors associated with adhesion (e.g. fimbriae, intimin), bacterial mobility (flagella) and host cell manipulation (e.g. toxins, such as the cholera and pertussis toxins) (see below for a more in-depth discussion) (Heras, et al., 2009; Shouldice, et al., 2011). Blocking DsbA activity would therefore interfere with the functioning of these virulence factors and hence impede the pathogens ability to cause disease. Indeed previous studies have demonstrated that hosts with defective DsbA

display reduced virulence in animal infection models (Bardwell, et al., 1991; Lin, et al., 2008; Heras, et al., 2009; Shouldice, et al., 2011). Therefore, better understanding DsbA and its inhibition opens up exciting new possibilities for novel antibacterial agents (Früh, et al., 2010). Indeed bacterial infections are a major cause of death in the world and this, in addition to current high levels of antibiotic resistance in many pathogenic bacteria, highlights the urgent need for new validated targets and for the design of new antibacterial agents against these targets. Due to its role in pathogenesis DsbA offers such a target for a new therapeutic approach. Nevertheless, it is important to note that drugs acting against DSB may not necessarily kill pathogens, but instead would impede or reduce bacterial pathogenesis by interfering with multiple essential virulence factors encoded by the pathogens (Heras, et al., 2009). However, this may not be a disadvantage as it may even result in less evolutionary pressure for bacteria to develop resistance (Heras, et al., 2009).

Some examples of virulence factors, how they intervene in the disease causing process and documented examples of the role of DsbA in their correct functioning will now be discussed.

1. 1. 2. 1. DsbA in cell adhesion

For a large number of bacterial pathogens the first and possibly the most important step is adhesion to the host cell. This process is essential for host colonisation and in establishing the disease. Adhesion is initially mediated by *pili* or fimbriae which are hair like structures typically made up of multiple protein subunits that propagate from the surface of the bacterium (Heras, et al., 2009). *E. coli* DsbA is reported to be important in the formation of disulphide bonds in the P fimbrial adhesion subunit protein PapG that recognises and binds to carbohydrates in the urinary tract surface (Heras, et al., 2009). DsbA has also been shown to be important in fimbriae construction in another urinary tract pathogen, *Proteus mirabilis* and plays a critical role in functional *pili* assembly for *Vibrio cholerae* colonisation mediated by the toxin co-regulated pilus (Tcp).

1. 1. 2. 2. DsbA in host cell manipulation

Following adhesion the success of bacterial colonisation is mainly dependent on the capacity to manipulate the hosts. Here, mass cell damage and destruction induced by secreted toxins and proteases occurs. Indeed, numerous secreted virulence factors and the secretion systems required for their discharge necessitates the DSB system to

catalyse their correct folding and function. This necessity shows their important role in this more advanced stage of bacterial pathogenesis.

Pathogens dispose of six different methods for secreting the toxins required for cell damage and DsbA activity is reported to be important in both type II and type III secretion systems (Lasica & Jagusztyn-Krynicka, 2007; Durand, et al., 2009). Type II secretion systems export proteins from the periplasm via a multimeric complex (Durand, et al., 2009) while type III secretion uses a multi-subunit molecular syringe like structure that directly injects the virulence proteins into the cytosol of eukaryotic cells (Heras, et al., 2009). In both of these cases DsbA is essential for correct folding of protein subunits which themselves are essential for the establishment of the correct structural conformation and function of the secretion apparatuses (Heras, et al., 2009). Furthermore, DsbA also acts as a catalyst in the structural assembly of many of the actual toxins to be secreted. Examples include functional assembly of: the cholera toxin of *Vibrio cholera,* the heat-labile enterotoxin assembly in *E. coli* and functional disulphide bond formation in almost the entire complex structure of the pertussis toxin of *B. pertussis.* Here, DsbA plays an important role in the structural assembly of a complex structure that includes six subunits and eleven intramolecular disulphide bonds (Heras, et al., 2009).

1. 1. 2. 3. DsbA in cellular spread and survival

Attachment through fimbriae permits bacteria to establish infections in cells. However mobility, which is the opposite phenotype, is also very important for virulence and bacterial fitness because it enables the bacteria to spread across the host cells (Heras, et al., 2009). Studies in several bacteria show that mutation of *dsbA* impedes functional flagella production and hence also bacterial mobility. As an example, in *E. coli,* DsbA is reported to be required for catalysing the formation of disulphide bonds in the FlgI protein that acts as the flagellar P-ring motor, for cell mobility (Dailey & Berg, 1993).

1. 1. 2. A COMPARATIVE STUDY: UNDERSTANDING DsbAs AND COLD-ADAPTATION

As part of a long term goal of obtaining a better understanding of DsbAs so as to enable the development of their use in protein production and, more importantly, in the design of novel antibacterial agents, a comparative study of a cold adapted and

mesophilic homolog has been initiated (Collins et al, 2010). To attain the objectives, the structures-stabilities-functions and also dynamics of both the oxidised and reduced states of the two DsbA homologs, adapted to low (~5°C) and moderate temperatures (~37°C), will be compared. The results obtained will give clues on Nature's strategies for modifying proteins to attain a desired catalytic rate within the environmental constraints and will help show how evolution optimises and balances dynamics, stability and activity (Tomatis, et al., 2008). Mutagenesis studies will then be carried out to better investigate these observations. DsbA is a well studied enzyme, both at the structural and biochemical levels (Heras, et al., 2009; Horne, et al., 2007; Schirra, et al., 1998) but most previous studies have focused on individual enzymes under specific conditions and we believe that an in-depth comparative approach should offer a more 'complete picture' and better pinpoint those regions important for function and stability. This, in turn, should aid in identifying the most appropriate regions for targeting by inhibitor.

The comparative studies should also allow for a better comprehension of life in the extremes and in particular of enzyme adaptation to various temperatures. More specifically, the study of a cold adapted DsbA should enable a better understanding of the molecular determinants of low temperature adaptation in enzymes. Furthermore, the expected high activity of the cold-adapted enzyme could allow for the development of a novel highly active tool for cell free protein synthesis of disulphide bond containing proteins.

A more in-depth discussion of the state of the art in cold-adaptation will now be presented.

1. 1. 3. UNDERSTANDING LIFE IN COLD ENVIRONMENTS

Life on Earth is ubiquitous, it is not restricted to those regions which we, as humans, classify as being normal but it is also found in those 'extreme regions' on Earth such as the deep seas, the polar regions, the volcanic regions or/and the saline pools. On the one hand, these regions constitute the major portion of the Earth's surface and are far from being sterile (Lonhienne, et al., 2000), but on the other hand, to survive these various extremes, these organisms had to adapt at all levels of organization; from structural to physiological adaptation. The present study is focused on adaptation to low temperatures and how organisms are able to not only tolerate, but to grow and maintain high enzyme activities in this permanently extreme condition.

Cold-adapted microorganisms capable of growing at 0ºC were identified by Forster as early as 1887 when he isolated them from fish (Zecchinon, et al., 2001). In fact, for some cold adapted organisms, low temperatures are not only optimal, but mandatory, for continued cell proliferation, with moderate to high temperatures (e.g., >12 °C (Xu, et al., 2003)) being inhibitory. These unique organisms, called psychrophiles, have effectively colonized cold environments thanks to successful adjustments which counteract the negative effects of low temperatures. These negative effects include a reduction of reaction rates, alterations in enzyme-substrate interaction strength, increase in solvent viscosity and a modified solubility of proteins, gases and salts and finally also, protein cold-denaturation (Georlette, et al., 2004). Psychrophiles have overcome all these challenges and reveal metabolic fluxes at low temperatures more or less comparable to those shown by mesophilic species living at moderate temperatures (Zecchinon, et al., 2001). Indeed, bacterial cell densities as high as 10^7 ml^{-1} have been found in the Antarctic oceans, similar to the densities of temperate waters (Gerday, et al., 2000).

The enzymes produced by psychrophilic organisms have adapted to temperatures close to the freezing point of water and typically display high catalytic rates and low stability as compared to their higher temperature adapted homologs i.e enzymes from mesophiles and thermophiles. Indeed many enzymes are incapable of carrying out their function under these low temperature conditions due to the reduced kinetic energy available at low temperatures, this effectively 'freezing' enzymatic motion. Currently it is hypothesised that psychrophilic enzymes have evolved an increased flexibility to overcome this (Collins, et al., 2003), thereby allowing for a high activity but also leading to the observed reduced stability. A reduced number or strength of intramolecular interactions are frequently reported for these enzymes as compared to their higher temperature adapted homologs (Gerday, et al., 1997) and it has ben hypothesized that these reduced interactions allow for the proposed flexibility of these enzymes and hence the enhanced activity at low temperatures. The actual molecular basis for the adaptation is enzyme specific however it still completely understood and direct evidence of the proposed increased flexibility is scant, with previous attempts to demonstrate this leading to conflicting results.

1. 1. 4. 1. The psychrophilic – mesophilic pair

This study is centred on a cold adapted thiol-disulphide oxidoreductase from a Gram negative psychrophilic bacterium (*Pseudoalteromonas haloplanktis* TAC125)

which has been isolated from an Antarctic coastal sea water sample collected in the vicinity of the French Antarctic station at Dumont d'Urville in Terre Adélie, Antarctica (66º 40' S; 140º 01' E) (Médigue, et al., 2005; Collins, et al., 2003). The gene encoding the cold-adapted enzyme has been cloned and the protein overexpressed using the pET22b(+)/*E. coli* BL21(DE3) expression system and purified from the periplasmic extracts. Production was carried out with Terrific Broth medium, using isopropyl β-D-1-thiogalactopyranoside (IPTG) for induction, and purification involved a combination of hydrophobic interaction chromatography and anion exchange chromatography. The production and purification procedures were not optimised and production levels were approximately 50 mg/L with large losses during purification being noted. Furthermore, precipitation of the protein, and in particular of the oxidised form, led to large losses of protein over time and no biochemical, dynamics or activity studies were carried out on this protein. Backbone and side-chain ^{1}H, ^{15}N and ^{13}C NMR assignments for the reduced form were however reported (Collins, et al., 2010a) and the NMR structure has been recently determined (Figure 4).

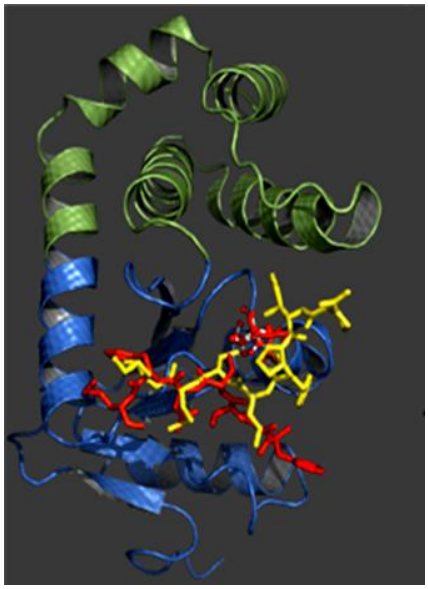


Figure 4: Structure of the psychrophilic PhDsbA revealing the thioredoxin domain in blue and in green the α-helical domain. Peptide substrate (yellow) and the oxidising loop of PhDsbB (red) were overlayed onto the structure of PshDsbAp by aligning it with the *E.coli* DsbA structural complexes DsbA-peptide and DsbA-DsbB. In: (Collins, et al., 2010a)

This cold adapted protein was found to be very similar to previously reported homologous mesophilic DsbAs with the 4 α-helices of the helical domain inserted into a thioredoxin like fold composed of a central 5 stranded β-sheet flanked by 3 α-helices.

In addition, this contains the consensus DsbA active site sequence (i.e. Cys-Pro-His-Cys) at a break in the first α-helix in the thioredoxin-like domain.

In contrast to the psychrophilic DsbA, a large number of mesophilic DsbAs have been studied in detail, both at the structural and biochemical levels (Heras, et al., 2009; Horne, et al., 2007; Schirra, et al., 1998). Examples include DsbAs from *Vibrio cholera* (Horne, et al., 2007), *Escherichia. coli* (Mössner, et al., 1998; Fabianek, et al., 2000), *Neisseria meningitidis* (Vivian, et al., 2009) *Salmonella enterica* serovar Typhimurium (Heras, et al., 2010) and *Staphylococcus aureus* (Williams, et al., 2010). Of these, that from *Vibrio cholera* is one of the best understood, with both NMR and crystallographic structures of both the oxidised and reduced states being reported as well as investigations of activity, stability and dynamics (by NMR) (Horne, et al., 2007). This mesophilic *Vibrio cholerae* DsbA is to be used for comparison in this project, it has already been cloned, successfully overexpressed in *E. coli* and purified, and protocols for these have already been optimised and reported (Horne, et al., 2007).

The availability of in-depth information for mesophilic enzymes homologous to the cold adapted protein of the present study and in particular for the *Vibrio cholera* DsbA should allow for a more comprehensive comparative analysis of the activity, stability, structure and dynamics of the enzymes. This should enable a better understanding of structure and function relationships in DsbAs as well as of cold adaptation in this enzyme.

1. 1. 4. THE pET22B(+)/*E. COLI* BL21(DE3) EXPRESSION SYSTEM

The first report of the Gram-negative, rod-shaped bacterium, *Escherichia coli*, was made in 1885 by Theodor Escherich. *Escherichia coli* is an abundant inhabitant of the mammalian colon and is one of the most thoroughly studied organisms known (Jeong, et al., 2009). It is well understood, easy to manipulate, grows rapidly on relatively cheap media (Khow & Suntrarachun, 2012) and is described as one of the most efficient vehicles for over-expression of both eukaryotic and prokaryotic proteins (Miroux & Walker, 1996). The current term ‘over-expression’ is here mentioned to define the capacity to produce target proteins at levels much higher than those of its own repertoire of proteins. Studies reveal that monomeric proteins that contain few cysteines and have an average size smaller than 60 kDa will give good production in an *E. coli* expression host (Bell, 2001). Indeed, in some cases up to 60 % of the total protein produced can be constituted by the recombinant protein.

The pET22b(+)/*E. coli* BL21(DE3) expression system based on the bacteriophage T7 promoter expression system is one of the most widely used laboratory systems for recombinant protein expression in *E. coli*. This is based on an inducible machinery that permits control of target gene expression. It consists of a *lac* operator sequence directly downstream of the T7 promoter, and the gene for the *lac* repressor (termed *lacI*) all encoded on the expression vector. The *E.coli* BL21 expression host used with this system contains a chromosomal copy of the T7 DE3 lysogen which comprises the T7 polymerase gene under control of the *E. coli lacUV5* promoter as well as a chromosomal copy of the *lacI* repressor gene. In DE3 lysogens the lac repressor acts not only at the *lacUV5* promoter in the host chromosome and thereby repressing T7 RNA polymerase gene transcription by the host polymerase, but also at the vector at the T7*lac* promoter, blocking the transcription of the target gene. The *lacUV5* and T7 *lac* promoters are inducible with IPTG or lactose, the addition of which to the growth medium 'inactivates' the lac I repressor and induces the production of the T7 RNA polymerase whereupon binding to the T7 *lac* promoter transcribes the target DNA (Figure 5).

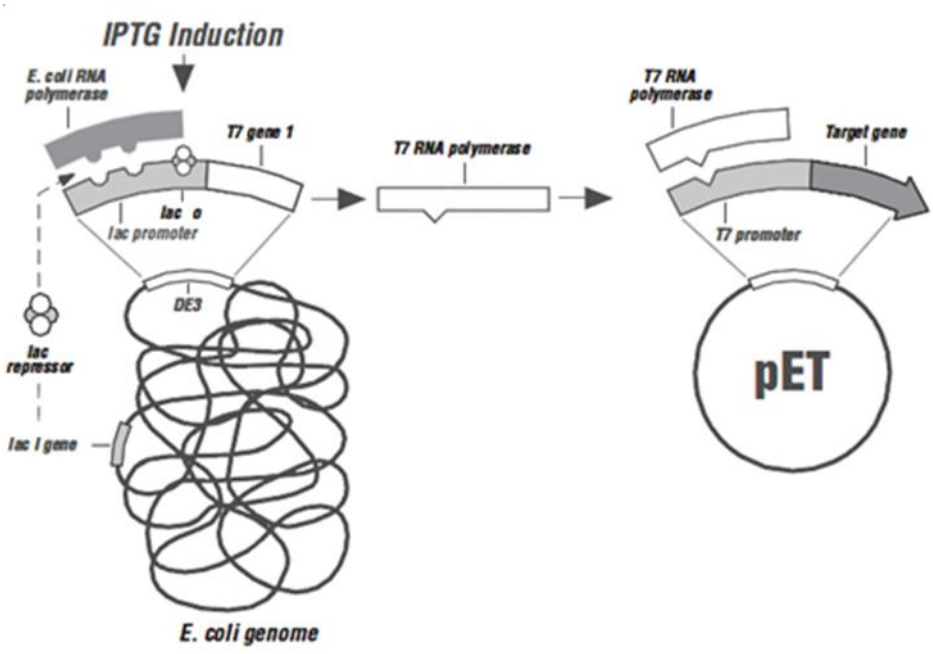


Figure 5: Representative scheme of IPTG induction in the pET/*E. coli* BL21(DE3) expression system. In: (Novagen, 2003)

Production with this system can make use of batch or fed-batch approaches, with batch production in shake flasks being the most common at a laboratory scale. Typically, the most frequently used shake flask production approach uses lysogeny

broth (LB) with IPTG induction at the mid-exponential phase of growth (Teulé, et al., 2009) but the use of richer media such as terrific broth (TB) or super broth (SB) has recently become common place. While being a highly used and efficient method for recombinant protein production in *E. coli*, this system does however sometimes suffer from low yields of protein product (mg/L) (Teulé, et al., 2009), with yields being dependent on the actual system used, the target protein, induction conditions and environmental factors. In fact, process optimisation to maximise productivity is an essential first step in the production of any recombinant protein.

In the case of the cold-adapted DsbA of the present study, the specific expression system used is the pET22b(+)/*E. coli* BL21(DE3) system already described above. Here, the use of the pET22b(+) expression vector allows for expression of unmodified and untagged DsbA in the host periplasm (Novagen, 2003). The wild-type signal sequence of DsbA which targets the produced protein to the periplasmic space and is removed during the translocation process is used.

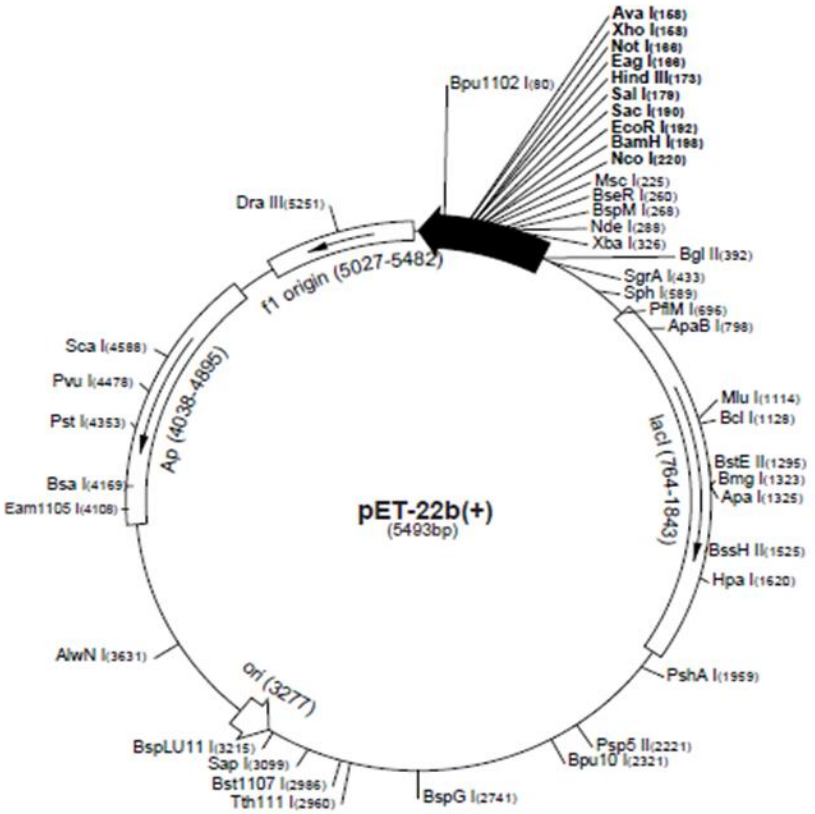


Figure 6: Schematic representation of pET22b(+) plasmid. In: (Novagen, 2003)

1. 1. 5. MEASUREMENT OF DsbA ACTIVITY

Several assays have previously been described for measuring DsbA activity. These assays are centred on the observation and/or quantification of the conformational and/or chemical differences between the two states of the protein, more precisely the reduced and oxidised states. The oxidised state displays a disulphide bond in the active site of DsbA and can oxidise any substrate that exhibits two free cysteines, itself becoming reduced in the process. Several strategies to measure DsbA activity have been reported, these include: an insulin activity assay, Ellmans assay, HPLC analysis, a fluorimetric assay, an SDS-PAGE based detection method and an assay using a synthetic fluorescent peptide. In this study the insulin assay will be used to monitor the reducing activity of the protein studied.

1. 1. 6. PSYCHROPHILIC DsbA PURIFICATION

Chromatography, which separates compounds on the basis of their differential partitioning between two phases (i.e. a mobile phase and a stationary phase), will be used to purify the cold adapted DsbA from the *E. coli* endogenous proteins. A variety of chromatographic approaches can be used for protein purification, including gel filtration chromatography, ion-exchange chromatography, hydrophobic interaction chromatography and affinity chromatography. Of these, hydrophobic interaction and ion-exchange chromatographies have been previously investigated for purification of the cold adapted homologs DsbA and will be encountered in this study.

Hydrophobic interaction chromatography (HIC): This technique makes use of subtle differences in protein surface hydrophobicity for separation. Here a reversible interaction occurs between exposed hydrophobic patches on the protein and hydrophobic ligands (e.g. phenyl, octyl, butyl, isopropyl etc.) on the column matrix. It is very similar to reverse phase chromatography but the ligands used are much less hydrophobic and hence less extreme elution conditions are required, thereby avoiding the denaturing conditions often used in reverse phase chromatography. Hydrophobic binding in HIC is often facilitated by use of neutral salts effective in ‘salting out’ (e.g. ammonium sulphate, NaCl), with these reducing protein solvation and leading to higher exposure of protein hydrophobic groups and thus improving binding. Reduction of the salt concentration is used in protein elution and these are eluted in order of increasing hydrophobicity (Amersham Pharmacia Biotech, 2000).

Ion exchange chromatography (IEX): This technique is based on the reversible interaction between a charged protein and an oppositely charged chromatographic medium. Proteins carry many ionisable groups such as the basic groups on the side chains of lysine, arginine and histidine as well as the acidic groups on the side chains of aspartic acid and glutamic acid residues. The charge of these side chains is influenced by the dissociation constant (pK_a) of the side chains, the environment of these side chains (i.e. their neighbouring residues in space) as well as the pH of the solution. In turn, the charge, number and structural positioning of these ionisable groups determines the net charge of the protein in a particular condition. Indeed, knowledge of the pI of a protein, this latter being defined as the pH at which the protein carries a net zero charge i.e. equal number of positive and negative charges, and control of the pH of the solution can be used in deciding the type of ion exchange approach to use. Namely, at a pH above the pI proteins carry a net negative charge and will bind to an anion exchanger (positively charged matrix) whereas at a pH below the pI a net positive charge is displayed and a cation exchanger (negatively charged matrix) should be used (Amersham Biosciences, 2002).

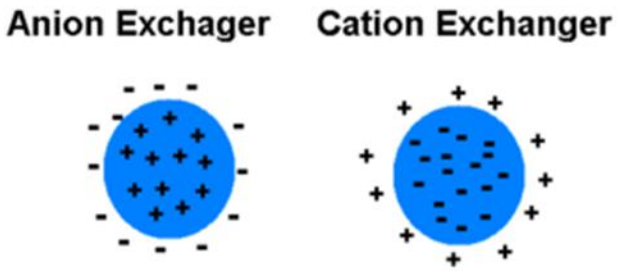


Figure 7: Anion and cation exchange chromatography. In anion exchange chromatography (shown at left) the matrix carries a positive charge and attracts negatively charged molecules. On the right cation exchange chromatography is illustred, this is based on the attraction of positively charged molecules by a negatively charged matrix. In: (Amersham Biosciences, 2002)

1. 1. 7. OBJECTIVES

The present study will be focused on the cold-adapted DsbA termed here as PhDsbA (Ph represents *Pseudoalteromonas haloplanktis*). Within the long term objectives of obtaining a better understanding of the structure-function relationship of DsbAs as well as of cold-adaptation in this enzyme, here we will focus on the following multiple objectives:

- **Optimisation of the batch production in shake flask of PhDsbA with the pET/*E. coli* BL21(DE3) expression system**

Current production levels are approximately 50 mg/L and here culture medium, culture aeration (via medium volume to flask volume ratio), induction time and induction period will be investigated in an attempt to improve production levels

- **Development of a simplified purification protocol for PhDsbA**

An initial attempt to purify this protein involved periplasmic extraction, HIC, dialysis and IEX, but this was found to result in large losses of PhDsbA. Here we will attempt to develop a simplified efficient purification protocol for this study.

- **Identify determinants of cold-adaptation.**

A comparative structural analysis of PhDsbA with its mesophilic homologs and in particular with that from *Vibrio cholera* will be carried out so as to identify mutations or alterations which may be important in adaptation to low temperatures. Primary, secondary and tertiary structures will be compared.

- **Construction of mutants**

Mutants identified in the structural comparison will be constructed so as to allow for identification of their role in the protein.

Chapter 2: Materials and methods

2. 1. BIOLOGICAL MATERIAL

2. 1. 1. Escherichia coli strains

In this study, the principal working strain used was *E. coli* BL21 (DE3), a descendent strain from the native *E. coli* strain B (Daegelen, et al., 2009). Transformants of this working strain that were constructed and used in this study to produce wild-type and mutant DsbA are described in Table 1.

Table 1: Strains used in this work

Stains	Genotype	Source/reference
BL21 (DE3)	F-, *omp*T, *hsd*S(r_B-, m_B-), *gal*, *dcm*, λDE3 (*lac*I, *lac*UV5-T7 gene 1, *ind*1, *sam*7, *nin*5)	Studier and Moffatt (1986)
BL21 (DE3)-pET22b(+)	BL21 (DE3) transformed with pET22b	This work
BL21 (DE3)-pET22b(+)-*DsbA*	BL21 (DE3) transformed with pET22b-*DsbA*	This work
BL21 (DE3)-pET22b(+)-*DsbA*-*Val64_Pro66del*	BL21 (DE3) transformed with pET22b-*DsbA*- *Val64_Pro66del*	This work
BL21 (DE3)-pET22b(+)-*DsbA*-*Val64_Ser65del*	BL21 (DE3) transformed with pET22b-*DsbA*- *Val64_Ser65del*	This work
BL21 (DE3)-pET22b(+)-*DsbA*-*Ser147_Leu149del*	BL21 (DE3) transformed with pET22b-*DsbA*- *Ser147_Leu149del*	This work
BL21 (DE3)-pET22b(+)-*DsbA*-*Ser147_Leu149+Ala151del*	BL21 (DE3) transformed with pET22b-*DsbA*- *Ser147_Leu149+Ala151del*	This work

All plasmids used in this work are listed and detailed in Table 2 with the respective characteristics and sources. The pET22b(+)-*dsbA* construct provided for this study contains the dsbA gene inserted in the pET22b(+) multiple cloning site between the *Nde*I and *Eco*RI restriction sites. The inserted sequence contains the wild–type N-terminal signal sequence for periplasmic expression.

Table 2: Plasmids used in this work

Stains	Characteristics	Source/reference
pET22b(+)	amp^R, T7*lac*, optional C-terminal His.Tag® sequence, signal sequence for potential periplasmic localization	Novagen
pET22b(+)-*dsbA*	pET 22b, *dsbA*	Tony Collins collection
pET22b(+)-*DsbA-Val64_Pro66del*	pET22b(+)-*DsbA-Val64_Pro66del*	This work
pET22b(+)-*DsbA-Val64_Ser65del*	pET22b(+)-*DsbA-Val64_Ser65del*	This work
pET22b(+)-*DsbA-Ser147_Leu149del*	pET22b(+)-*DsbA-Ser147_Leu149del*	This work
pET22b(+)-*DsbA-Ser147_Leu149+Ala151del*	pET22b(+)-*DsbA-Ser147_Leu149+Ala151del*	This work

2. 1. 2. DsbA Production: optimisation of medium, aeration and induction (time, period)

In an attempt to optimise production levels of the cold adapted DsbA we investigated various media (Table 3) and various production conditions for induced and non-induced cultures (Table 4).

Table 3: Composition of the media used in this work

Medium	Composition
LB	Bacto tryptone (1% w/v), yeast extract (0.5% w/v), and sodium chloride (0.5% w/v)
TB	Bacto tryptone (1.2% w/v), yeast extract (2.4% w/v), glycerol 99.5 % (0.4% w/v), 70 mM $K_2HPO_4.3H_2O$ and 20 mM KH_2PO_4
SB	Peptone (3.2% w/v), yeast extract (2% w/v) and sodium chloride (0.5% w/v)

The rich media TB, SB and LB were investigated for both induced and non-induced cultures. Aeration was investigated by varying the medium volume to flask volume ratio from 1:3 to 1:20, with the lower medium volume (i.e. higher ratio) allowing for better culture mixing and hence better aeration. Elapsed fermentation times (EFT) before induction with 1 mM IPTG of 12, 16, 24 and 28 hours, corresponding

respectively, to the early, mid, declining exponential and stationary phases of growth were also examined and compared to non-induced cultures. Induction periods of 2, 4, 6 and 12 hours after induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) were investigated. Cultivations in the absence of induction were carried out to determine growth curves. Biomass (OD_{600nm}) and DsbA production levels (SDS-PAGE) were compared for all production optimisation conditions.

PROTOCOL:

- Plate out glycerol cultures or fresh transformants of the producing strains on LB-agar plates containing 100 µg/mL of ampicillin as the selection marker and incubate overnight at 37 ºC.
- Inoculate 100 mL LB+ampicillin preculture in a 500 ml erlenmeyer with a cfu of the plate culture. Incubate for approximately 15 hours at 25 ºC and 200 rpm.
- Inoculate production cultures (in 500 mL erlenmeyers) to an initial OD_{600nm} of 0.1. Incubate at 20 ºC and 200 rpm (25 mm orbital).
- Induce when required with 1 mM IPTG. Collect 0.5 mL cell pellet samples at 2, 4, 6 and 12 hours after induction for determination of production levels by SDS-PAGE. Monitor biomass levels throughout the productions by measuring OD_{600nm}.

Table 4: Production conditions examined

Condition number	Medium	Ratio: flask volume to liquid volume	Induction with 1 mM IPTG	Elapsed Fermentation Time (EFT) of induction (hours of growth)
1	LB	1:3	No	
2	LB	1:5	No	
3	LB	1:10	No	
4	LB	1:20	No	
5	TB	1:3	No	
6	TB	1:5	No	
7	TB	1:10	No	
8	TB	1:20	No	
9	SB	1:3	No	
10	SB	1:5	No	
11	SB	1:10	No	
12	SB	1:20	No	
13	LB	1:5	Yes	0 hours
14	SB	1:10	Yes	12 hours
15	TB	1:10	Yes	12 hours
16	TB	1:10	Yes	16 hours
17	SB	1:10	Yes	16 hours
18	TB	1:5	Yes	24 hours
19	TB	1:10	Yes	24 hours
20	SB	1:5	Yes	24 hours
21	TB	1:5	Yes	28 hours
22	TB	1:10	Yes	28 hours
23	SB	1:5	Yes	28 hours

2. 1. 3. SDS-PAGE analysis

12 % SDS-PAGE was used for analysis of protein production levels as well as for monitoring the purification. Here proteins are linearized and imparted with a negative charge by SDS and DTT pre-treatment before being separated on the basis of differences in their size on an acrylamide-bis-acrylamide gel (see gel components in Table 5). Separated protein bands are then visualised by staining with Coomassie Blue which interacts ionically and hydrophobically with proteins.

Table 5: SDS-PAGE stacking and running gel composition.

Solution	Stacking gel	Running gel (12%)
Acrylamide 40%	216 µL	1.62 mL
Bis-acrylamide 2%	117 µL	960 µL
0.25 M Tris-HCl (pH 6.8), 2%SDS	1.1 mL	-
0.75 M Tris-HCl (pH 8.8), 2%SDS	-	2.8 mL
TEMED	3 µL	4.5 µL
APS (10%)	12.5 µL	30 µL
H_2O	750 µL	170 µL
Final volume	~ 2.2 mL	~ 5.6 mL

PROTOCOL (for preparation of production sample for SDS-PAGE):

- Collect cells from 500 µL production samples by centrifuging at maximum speed for 5 minutes and discard supernatant.
- Add 100 µL of a 50 mM Tris, 1 mM EDTA solution at pH 8 and mix well.
- Add 25 µL of SDS-PAGE loading solution (10 % SDS, 10 mM β-mercaptoethanol, 20 % glycerol, 0.2 M Tris at pH 6.8 and bromophenol blue) and vortex.
- Centrifuge at max speed for 25 minutes.
- Run 4 µL of supernatant on a 12 % SDS-PAGE gel at a constant current flow of 10 amps.

PROTOCOL (for preparation of purification samples for SDS-PAGE):

- Gently mix samples by inversion.
- To 20 µL of sample add 5 µL of SDS-PAGE loading solution.
- Mix to homogenize and run 20 µL in a 12 % SDS-PAGE gel at a constant current flow of 10 amps.

The Coomassie Blue staining solution is composed of 10 % acetic acid in deionised water with Coomassie Brilliant Blue R-250 addition until the solution attains a strong blue colour. The de-staining solution has the same composition with the exception of the Coomassie Brilliant Blue R-250 component.

ImageJ was employed for quantification of Coomassie Blue stained protein bands.

2. 1. 4. DsbA PURIFICATION

A previously used purification protocol was optimised in this project. This involved the following steps: DsbA periplasmic extraction, hydrophobic interaction chromatography, dialysis for buffer exchange and ion exchange chromatography.

A Pharmacia Biotech FPLC system composed of a LCC-501 Plus LKB controller, two P500 pumps, a UV-M II optical unit, a FRAC 100 fraction collector and a Rec 102 chart recorder was used for all chromatographic steps.

2. 1. 4. 1. DsbA periplasmic extraction

An osmotic shock when transferring cells from a high sucrose concentration to a dilute $MgSO_4$ solution in conjunction with a thermal shock by rapidly decreasing the temperature from approximately 25 ºC (room temperature) to 4 ºC allows for liberation of periplasmic proteins.

PROTOCOL:

- Collect cells by centrifugation at 7000 rpm for 5 minutes at 4 ºC.
- Add 1/40th the volume of the initial culture volume of 30 mM Tris-HCl at pH 8 and gently ressuspend pellet, on ice, with a Pasteur pipette.
- Add 1/40th the volume of initial culture volume of 2×PEB and mix by gentle inversion. (2×PEB is composed of 30 mM Tris-HCl at pH8, 40 % of sucrose and 2 mM EDTA)
- Transfer to 40 mL centrifuge tubes and leave for 20 minutes at room temperature.
- Centrifuge at 14000 rpm for 30 minutes at 20 ºC and discard supernatant.
- Immediately add 1/20th the volume of initial culture volume of cold 5 mM $MgSO_4$ and mix well.
- Leave tubes for 20 minutes on ice.
- Centrifuge tubes at 14000 rpm for 40 minutes at 4 ºC.
- Retain supernatant.

- Add 0.1 mM calcium and 5 -10 units of DNase (Fermentas®) and incubate for 10 minutes at room temperature.
- Store at 4 ºC.

2. 1. 4. 2. Hydrophobic interaction chromatography (HIC)

HIC is based on the reversible binding of proteins with exposed hydrophobic groups. A 1.6 cm x 20 cm, 40 mL Phenyl Sepharose High Performance (Pharmacia) column was used. This contains a hydrophobic phenyl group covalently coupled to a highly porous cross-linked 4 % agarose matrix. The sample solution is filtered through a 0.45 µm filter and 1 M ammonium sulphate added before loading to the column. The ammonium sulphate is added to enhance protein hydrophobicity and hence column binding and a gradient of decreasing ammonium sulphate (buffer B) is used for elution. See table 9 for details of the chromatographic conditions used.

Table 6: Details of gradient employed, buffer composition and loading speed used in HIC.

Gradient (percentage of buffer B)	**Elapsed time (minutes)**
0 to 100%	53
100%	80
100 to 0%	82
0%	115
Buffer A	20 mM Tris-HCl, 1 mM EDTA, 1 M $(NH_4)_2SO_4$ at pH 8
Buffer B	20 mM Tris-HCl, 1 mM EDTA at pH 8
Buffer load speed	3 mL/min
Fraction size	5 mL (i.e. 1.67 minutes)

2. 1. 4. 3. Dialysis

This is based on the diffusion of solutes across a semi-permeable membrane from a region of high concentration to a region of low concentration. It is used for the exchange of buffers.

PROTOCOL:

Load sample into a pre-wetted 12000-14000 kDa MWCO dialysis tubing and dialyse, with constant mixing, overnight in 4 to 5 litres of the appropriate buffer (i.e. buffer A for the IEX).

2. 1. 4. 4. Ion exchange chromatography (IEX)

Ion-exchange chromatography separates proteins based on their charge. A 1.6 cm x 20 cm, 40 mL DEAE Fast Flow Sepharose (Pharmacia) column was used. This anion exchanger contains the positively charged reactive group diethylaminoethanol (DEAE) covalently linked to a sepharose (a polysaccharide polymer) matrix. A gradient of increasing NaCl concentration (buffer B) is used for protein elution.

In the present study the pH of the equilibration buffer used was optimised to maximise DsbA binding: pH 7.2, 7.5 and 8.0 were investigated.

Table 7: Details of the gradient employed, buffer composition and running speed used in IEX.

Gradient (percentage of buffer B)	**Elapsed time (minutes)**
0 to 50%	56
50 to 100%	59
100%	79
100 to 0%	81
0%	105
Buffer A	10 mM MOPS, 1 mM EDTA tested at pH 7.2; 7.5 and 8.
Buffer B	10 mM MOPS, 1 mM EDTA, 1 M NaCl tested at pH 7.2; 7.5 and 8.
Buffer load speed	5 mL/min
Fraction size	5 mL (i.e. 1 minute)

2. 1. 5. DsbA reducing activity assay

DsbA reducing activity was confirmed according to a procedure described by Arne Holmgren in 1979. This assay is based on the reduction of insulin disulphide bonds by DsbA under the conditions used and measuring the resultant insulin precipitation by monitoring the increase in absorbance at 650 nm. DTT is used to recycle oxidised DsbA following catalysis.

The assay was used to monitor the purification process as well as to determine the activity of wild-type and mutant DsbAs.

PROTOCOL (for insulin solution preparation at 10 mg/mL):

- Add 50 mg of insulin to 4 mL of 0.05 M Tris-HCl at pH 8.
- Add 1M HCl to adjust pH between a range of 2 or 3.
- Immediately add 1M NaOH to adjust pH to 8.
- Bring the volume to 5 mL with deionized water.

PROTOCOL:

- Add a known concentration of DsbA to a 1 mL cuvette and add 2 mM EDTA, 0.75 mg/mL insulin and 20 mM MOPS at pH 7 to bring the final volume to 1 mL.
- Mix gently by inversion and blank the spectrophotometer at 650 nm with this solution
- Add 0.33 mM DTT, mix by inversion.
- Immediately monitor the change in A_{650nm} over time. A Genesys 20 (R) spectrophotometer from Thermo Spectronic (R) was used.
- A negative control with all components except DsbA should also be monitored.

2. 1. 6. Sugar detection assay

Sugar detection was performed according to a protocol first described in 1956 by Dubois and collaborators (Dubois, et al., 1956). This is a quantitative and sensitive colorimetric test where sulphuric acid in the presence of phenol is used for sugar detection. The sugars are converted to hydroxymethylfufurals in the hot acidic conditions and form a green product on interaction with phenol. Even small quantities of sugars can be detected and quantified based on the direct relationship between the enhancement of colour (A_{490nm}) and the sugar quantity.

PROTOCOL:

- To 1 mL of sample solution (in water) add 1 mL of a 5 % phenol solution.
- Add 5 mL of 96 % sulphuric acid to each tube and mix well.
- Leave 10 minutes at room temperature, mix and place in a water bath at 25 to 30 ºC for 20 minutes.
- Blank spectrophotometer with water.
- Read absorbance at 490 nm.

- Calculate the amount of total carbohydrate using a standard curve prepared using 0.02 – 0.1 mg/mL glucose.

All reactions were carried out in glass tubes in a laminar flow hood.

2. 1. 7. Bradford assay for protein quantification

The Bradford assay was used to calculate protein concentration spectrophotometrically at 595 nm. This method is based on the reaction between the NH_3^+ and possibly also the aromatic groups of amino-acids with the coomassie blue reagent (Kruger, 2002). For this purpose a standard curve with known concentrations of BSA was used.

Bradford Reagent: Dissolve 100 mg Coomassie Blue G-250 in 50 ml 95% ethanol, add 100 ml 85% (w/v) phosphoric acid to this solution and dilute the mixture with 850 mL of water. Leave to agitate overnight and filter twice.

PROTOCOL:

- Place 5 to 20 µL of sample solution in a 1 mL cuvette and bring to a final volume of 100 µL with distilled water.
- Add 1 mL of Bradford solution and mix immediately.
- Leave at room temperature to allow reaction to develop for 10 minutes.
- Blank spectrophotometer with the mixture without enzyme sample solution.
- Read absorbance at 595 nm and calculate concentration from a standard curve prepared with BSA concentrations of 5 to 500 µg/ml.

2. 1. 8. MUTANT CONSTRUCTION

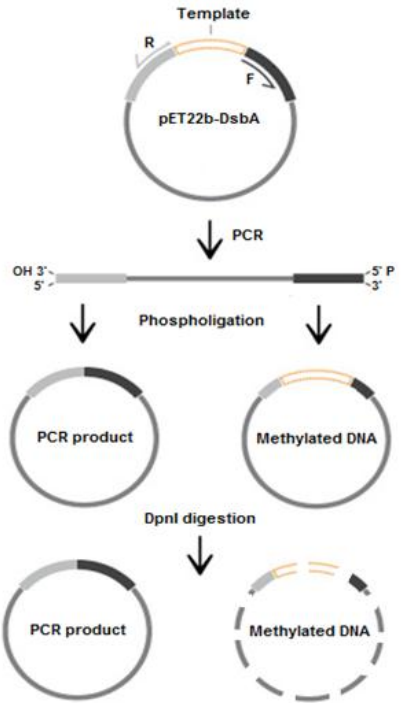
In an attempt to better understand adaptation to temperature, structural differences between DsbAs adapted to various temperatures were identified and a number of mutants designed and prepared so as to investigate these differences. This involved a number of steps.

- Mutant selection: sequence and structure comparisons so as to identify structural differences in DsbAs adapted to various temperatures.

- Design of PCR primers for introduction of desired mutations.

– Introduction of selected mutations into cold-adapted DsbA gene sequence by inverse PCR, recircularisation of vector by ligation, and transformation to an expression host (Scheme 1).

– Plasmid isolation, confirmation of mutation by restriction digestion analysis and sequencing.



Scheme 1: Representation of process used for deletion of amino acids in the PhDsbA sequence.

2. 1. 9. 1. Mutant selection: structure comparisons

Here, the primary, secondary and tertiary structures of the cold-adapted enzyme were compared to homologous DsbAs. Major differences in the cold-adapted DsbA primary sequence (and in any other cold-adapted DsbA sequence available in the UniProtKB databank) were first identified by sequence comparisons of DsbAs available at the UniProtKB databank. Mutations identified in the primary sequences were then investigated in more detail by comparisons of the tertiary structures of the cold-adapted (reduced state) DsbA with the *E. coli* and *V. cholera* DsbAs.

– DsbA sequences for primary structure analysis were identified and retrieved from the uniprotkb database by a similarity search with the PhDsbA amino acid sequence (e-value) and by manually retrieving sequences based on a bibliography

search. Cold-adapted and mesophilic sequences were identified among these by a bibliography search.

– Amino acid sequence comparisons were carried out with the basic local alignment search tool (blast) available at the NCBI (http://blast.ncbi.nlm.nih.gov/blast.cgi?page=proteins). The blastp 2.2.26 algorithm with the Blosum62 (blocks of amino acid substitution matrix number 62) matrix was used with default parameters.

2. 1. 9. 2. Design of PCR primers

Primers were designed so as to allow for introduction of the desired mutations in the cold-adapted DNA sequence in pET22b(+) by inverse PCR. Fast PCR and Primer3 were used for design and analysis of primers and the Finnzymes *Tm* calculator (http://www.diagnostics.finnzymes.fi/tm_determination_old.html) was used for annealing temperature calculation. This latter allows for calculation of the annealing temperature when using polymerases such as the Phusion polymerase as the DNA binding domain fused to this leads to a tighter binding and hence higher melting and annealing temperatures. Complementarity of primers as calculated with the Fast PCR program was taken into account in minimising dimer formation, this has a scoring range from 0 (no propensity for formation) to 7 (high propensity). Primers of 20 to 30 bp with a GC content of 40 to 60 % were chosen where possible, primer pairs with similar melting temperatures were designed where possible.

2. 1. 9. 3. Site directed mutagenesis

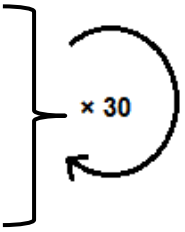
Mutations were introduced on the circular DsbA-pET22b(+) template by inverse PCR using the appropriate primers resulting in linearised mutated product (see Scheme I). Phusion High-Fidelity DNA Polymerase (kindly provided by Professor Björn Johansson) was used as this allows for high fidelity, highly processive replication of large fragments. It consists of a novel *Pyrococcus furiosus* DNA polymerase fused to a DNA binding domain. PCR was carried out with a 96 well PCR thermal cycler (MyCycler from Bio-Rad®) and standard conditions were used as recommended by the polymerase supplier and as described below (Table 8 and 9).

Table 8: Details of PCR mix.

Compound	Concentration	Volume added	Final concentration in total solution
dNTP mix	10 mM	1 µL	0.2 mM
Ultra-pure H_2O		35 µL	
Phusion HF Buffer	5×	10 µL	1×
Template	33 ng/µL	0.5 µL	16.5 ng
Reverse Primer	20 µM	1.25 µL	0.5 µM
Forward Primer	20 µM	1.25 µL	0.5 µM
Phusion HF DNA polymerase (Finnzymes)	2000 units/mL	1 µL	2 units
Total volume		50 µL	

Table 9: PCR cycle details

PCR step	Temperature	Time
Pre-denaturation	98 ºC	2 minutes
Denaturation	98 ºC	30 seconds
Annealing	Calculated using Finnzymes T_m calculator	20 seconds
Extension	72 ºC	2 minutes and 30 seconds
Final extension	72 ºC	10 minutes
	4 ºC	∞



As already mentioned, the annealing temperature was calculated according to the Finnzymes online calculator. To ensure best results the Phusion HF DNA polymerase supplier recommends using the lower T_m given by the calculator for annealing when primers have less than 20 nucleotides. However, for primers greater than 20 nucleotides it is recommended to use an annealing temperature 3 ºC higher than the lower T_m given by the calculator program.

2. 1. 9. 4. Re-circularization of PCR product

Re-circularisation of DNA is carried out by use of a polynucleotide kinase (PNK) that phosphorylates the 5' phosphate and a ligase (T4 ligase) that links this to the 3' hydroxyl group of the linear PCR product.

PROTOCOL:

- To 18.5 µL of PCR product in an eppendorf tube add 2.5 µL of 10× ligase buffer and 2 µL of T4 polynucleotide kinase (PNK) at 10 units/µL (both from Fermentas®).
- Leave 30 minutes at 37 ºC.
- Add 2 µL of PNK (10 units/µL) and leave for 30 minutes at 37 ºC.
- Add 2 µL of T4 ligase (Fermentas®) and leave at 37 ºC for 2 hours.

Following phospholigation the restriction digestion enzyme *Dpn*I was added to digest the methylated DNA of the template used in the PCR reaction. Buffer Tango (final concentration 1×) and 10 units of *Dpn*I per 10 µL of reaction mix were added and incubated for 1 hour at 37 ºC (both the digestion enzyme and respective buffer are from Fermentas®).

2. 1. 9. 5. Preparation of *E. coli* competent cells and transformation

E. coli BL21 (DE3) competent cells were prepared using a rapid procedure first described in 1987 (Hanahan, 1983). In this procedure the transformation efficiency of the cells is enhanced by using rubidium chloride. Some modifications were made to the original protocol and the complete procedure utilized is described below.

PROTOCOL:

- Inoculate 5 mL LB with a cfu of *E. coli* BL21 (DE3).
- Grow over night at 37 ºC in an orbital incubator at 200 rpm.
- Transfer 300 µL to a 2 L Erlenmeyer flask containing 400 mL LB and incubate in an orbital incubator at 37 ºC, 200 rpm until an OD_{600} of 0.4 – 0.6 is reached.
- Divide culture in 8 frozen 50 mL falcons.
- Centrifuge for 5 minutes at 4500 rpm at 4 ºC
- Discard supernatant
- Ressuspend pellet in TEB1 solution (Table 10) with mixing.

- Incubate tubes for 5 minutes on ice.
- Centrifuge 5 minutes at 4500 rpm and 4 ºC.
- Discard supernatant.
- Add 20 mL of TEB2 solution (Table 10) to the first falcon, ressuspend, transfer to the second falcon, ressuspend and so on until all pellets are ressuspended.
- Incubate during 45 to 60 minutes on ice.
- Distribute into eppendorfs, 100-200µL to each.
- Store frozen at -80 ºC.

Table 10: Detailed composition of TEB1 and TEB2 solutions used in competent cells preparation..

TEB1		**TEB2**	
Reagent	Concentration	Reagent	Concentration
CH_3COOK	3 M	MOPS	1 M
$CaCl_2$	1 M	$CaCl_2$	1 M
$MnCl_2$	2 M	$RbCl_2$	0.12% (w/v)
$RbCl_2$	1.2% (w/v)	Glycerol (99,5%)	15%
Glycerol (99,5%)	15%		

The transformation of competent *E. coli* cells was made with an adaptation of a commonly utilized protocol from Inoue and co-workers (1990). The entire procedure is described below and it consists of making the competent cells susceptible to uptake of DNA by a thermal shock treatment (Inoue, et al., 1990).

- Defreeze 200 µL of competent cells on ice.
- Add approximately 100 ng of circular DNA to each tube.
- Leave tubes on ice for 30 minutes.
- Proceed to a thermal shock effectuated for 45 seconds at 42 ºC with gentle agitation of tubes.
- Leave tubes on ice for 10 minutes and add 800 µL of preheated LB.
- Incubate for 1 hour at 37 ºC with 200 rpm agitation.
- Centrifuge the mixture at 14500 rpm for 1 minute.
- Reject 800 µL of the supernatant and ressuspend the remaining 200 µL of culture.
- Plate cells on solid LB medium supplemented with ampicillin (100 µg/mL) and incubate over night at 37 ºC.

2. 1. 9. 6. Plasmid isolation

Plasmid isolation from *Escherichia coli* was carried out with the GenElute™ Plasmid miniprep kit (Sigma®). This kit provides a simple method based on the affinity to silica of the smaller sized plasmids (as compared to the chromosomal DNA) of a cell lysate for isolating plasmid DNA from recombinant *E. coli* cultures. Bacterial cells are collected through centrifugation, exposed to an alkaline-SDS lysis solution and the DNA adsorbed to silica in the presence of high salt concentrations. Thereafter, contaminants are removed through a simple wash step and bound plasmid is eluted using a solution of low salt concentration.

PROTOCOL:

- Grow 6 mL of inoculated LB supplemented with 100 µg/mL of ampicillin over night at 37 ºC in an orbital incubator agitating at 200 rpm.
- Pass culture to 2 mL collection tubes centrifuge at 12000 × g for 5 minutes and discard supernatant.
- Ressuspend cells with 200 µL of the ressuspension solution (supplied with RNase A) and pipette up and down or vortex the mixture.
- Add 200 µL of the lysis solution and invert gently to mix. Allow to clear for less than 5 minutes.
- Add 350 µL of neutralization solution and invert tubes 4 to 6 times to mix.
- Centrifuge tubes for 10 minutes at maximum speed and discard supernatant.
- Prepare binding columns by adding 500 µL of the column preparation solution supplied and centrifuge at 12000 × g for 1 minute.
- Discard flow-through, transfer cleared lysate into binding columns and spin for 30 seconds to 1 minute. Discard flow-through.
- Add 750 µL of wash solution supplemented with ethanol to columns and centrifuge 30 seconds to 1 minute then discard flow-through.
- Spin for 1 minute to remove residual ethanol.
- Transfer column to new collection tube.
- Add 50 µL of elution solution (ultrapure water) and spin for 1 minute.

DNA concentration was quantified using a NanoDrop ND-1000 spectrophotometer. Absorbances at 260 nm (for DNA quantification) and 280 nm (for analysis of protein contamination) were determined.

2. 1. 9. 7. Mutation confirmation

So as to confirm a correct introduction of mutations the isolated plasmids were subjected to restriction digestion analysis where possible. The software programs Geneious and SerialCloner were employed for identification of restriction enzymes for use in the mutation analysis.

PROTOCOL:

- In an eppendorf tube join 10 µL of the DNA sample (plasmid isolate) with 2.5 µL of ultra-pure water, 10 units of the appropriate enzyme and 1.5 µL of the appropriate buffer (10×) for the enzyme used (buffer A from Roche® with *Eae*I and buffer R from Fermentas® with *Bsu*RI).
- Leave for 3 hours at 37 ºC.

Digested DNA was run on a 2 % agarose gel using Loading Dye 6× Orange (final concentration 1×) from Thermo Scientific to load samples and GeneRuler 1kb DNA ladder Plus as a molecular weight marker. Results from gels were observed with a UV transluminator.

Sequencing of the DsbA gene sequence of the selected clones was carried out using the T7 forward primer by Eurofins MWG Operon so as to ensure correct introduction of the desired mutations and absence of other mutations.

Chapter 3: results and discussion

3.1. PRODUCTION OPTIMIZATION

The objectives of this task were to maximise batch production of PhDsbA in shake flasks at 20 ºC by optimising various process variables for non-induced and induced cultures. In particular we examined 1) various rich media; 2) various liquid volume to flask volume ratios so as to optimise aeration; 3) cultivation time (for non-induced cultures); 4) elapsed fermentation time at induction (i.e. stage of growth when induced); and 5) induction period (hours after induction). Both the biomass levels (OD_{600nm}) and target protein production levels (SDS-PAGE, Coomassie Blue staining) were monitored.

3. 1. 1. Non-Induced PhDsbA Production Optimisation

Here both the medium and aeration conditions allowing for maximum biomass and PhDsbA production by non-induced *E. coli* BL21(DE3)/pET22b(+)-PhDsbA cultures in shake flasks were investigated and compared.

The 20 ºC growth curves for non-induced cultures in the media tested (i.e. LB, TB and SB) at various levels of aeration (i.e. liquid volume to flask volume ratios of 1:3, 1:5, 1:10 and 1:20) are shown in Figure 9. It can be seen that highest biomass levels were achieved with TB and SB, with growth rates increasing with higher levels of aeration. Growth curves were generally similar for TB and SB but, interestingly, at the highest liquid volume to flask volume ratio tested (1:20) TB was found to allow for a significantly higher biomass production than TB and LB.

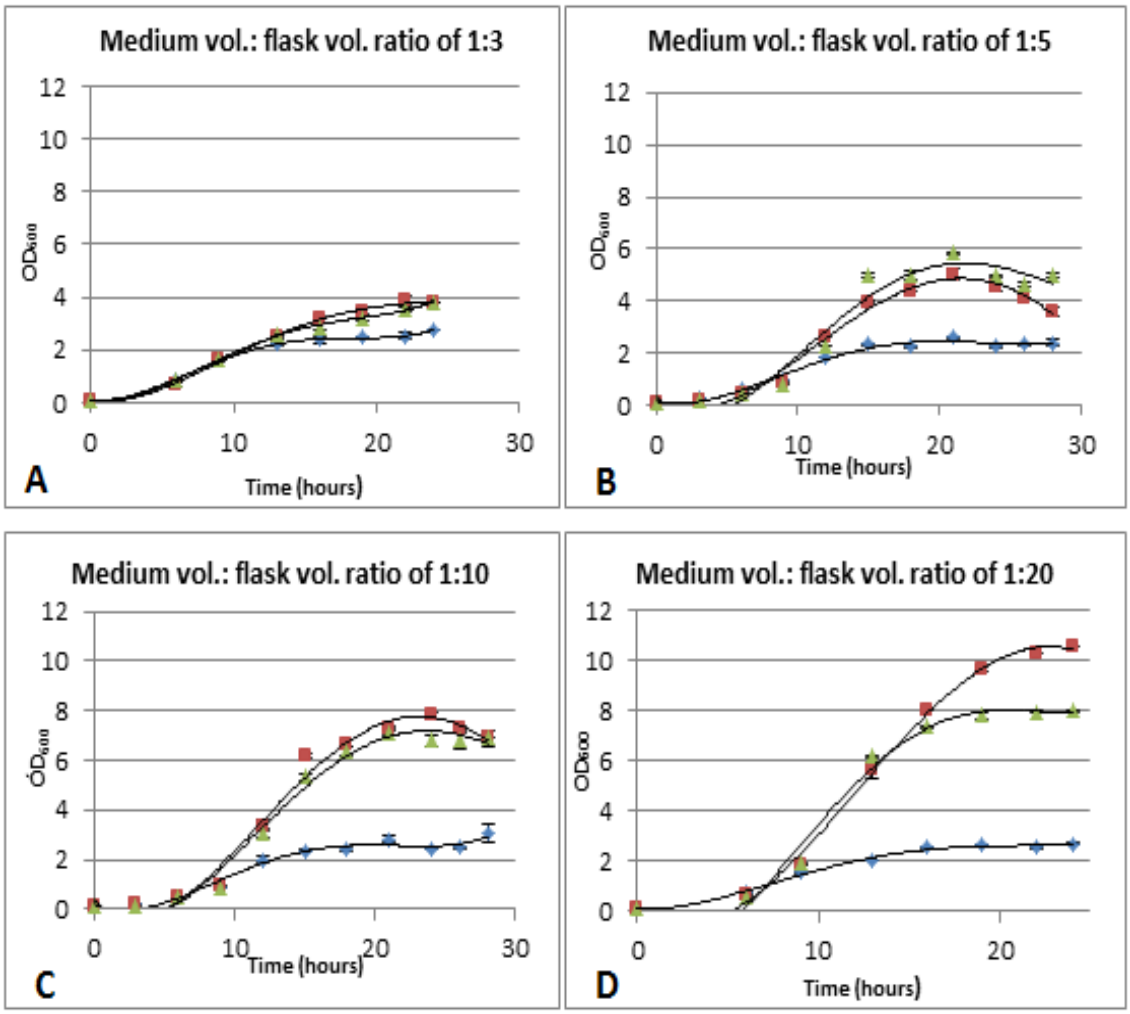


Figure 8: Comparisons of growth curves at 20 ºC for non-induced cultures in three different rich media (LB, SB and TB) at four ratios of medium volume to flask volume i.e. 1:3 (A), 1:5 (B), 1:10 (C) and 1:20 (D). The green triangles represent growth in SB, the red squares characterize growth in TB and the blue diamonds denote the growth in LB.

SDS-PAGE was used to analyse PhDsbA production levels at various time points throughout the non-induced cultures cultivations (every 2 hours until 28 hours of growth) and Figure 10 shows the results for the time points of highest production ('best producers') at each of the conditions tested. Interestingly, PhDsbA was found to start accumulating during the mid-log phase even in the absence of induction, with maximum production being observed in the stationary phase before a reduction in the late stationary phase (not shown). Highest PhDsbAp production was obtained with TB with a medium volume to flask volume ratio of 1:5.

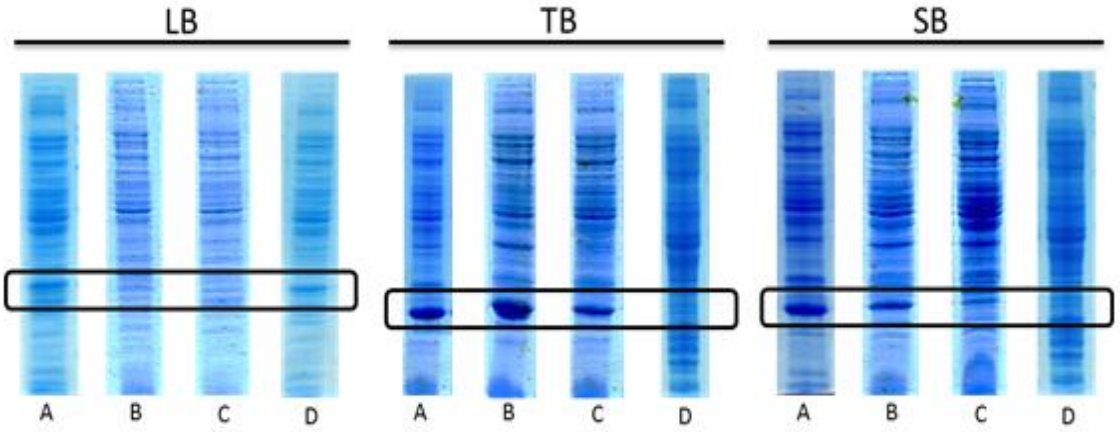


Figure 9: 12% SDS- PAGE of cellular extracts from non-induced cultures under the various production conditions tested. The time point of highest production is shown in each case i.e. samples taken during the stationary phase. A represents a medium to flask volume ratio of 1:3; B, a ratio of 1:5; C, a ratio of 1:10 and D a ratio of 1:20. The black boxes indicate the position of the bands corresponding to PhDsbA.

Figure 10 gives an overview of the quantitative comparison of biomass and PhDsbA production levels for the 'best PhDsbA producers' under the various conditions examined and Figure 11 and 12 show the variation of these when using TB, the medium allowing for highest production.

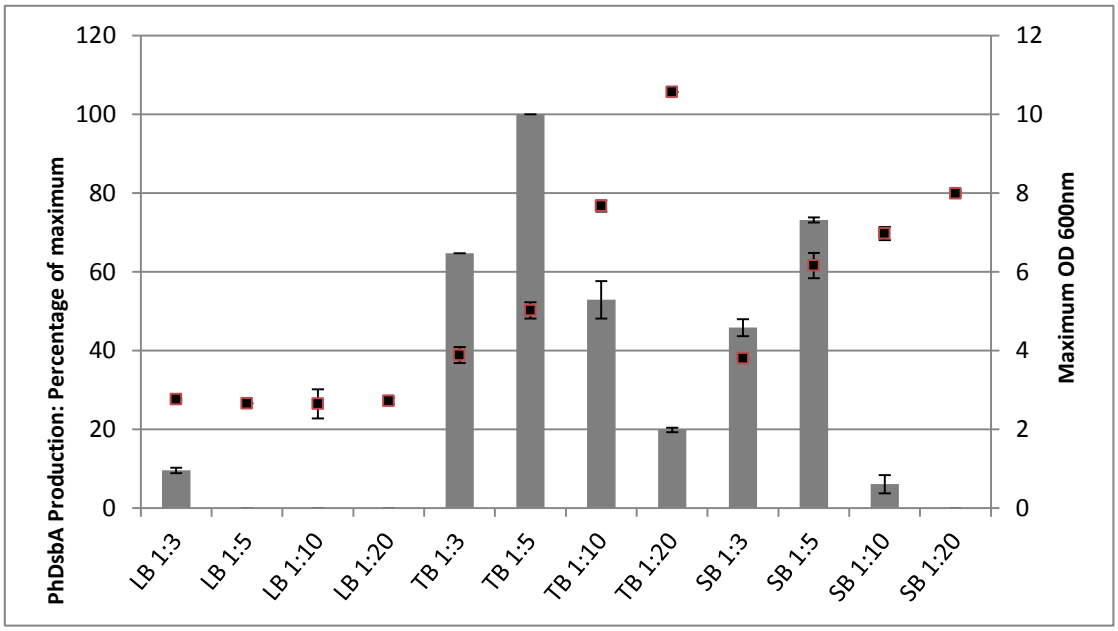


Figure 10: Comparison of maximum biomass (black points) and PhDsbA (grey bars) production levels obtained with the various media (LB, TB, SB) and aeration conditions (1:3, 1:5, 1:10, 1:20) examined. The PhDsbA production levels (grey bars) were compared using SDS-PAGE and ImageJ for quantification and are expressed as a percentage of the hignest production level observed. Maximum biomass levels attained (black points) are reported as the highest OD_{600nm} observed during culturing.

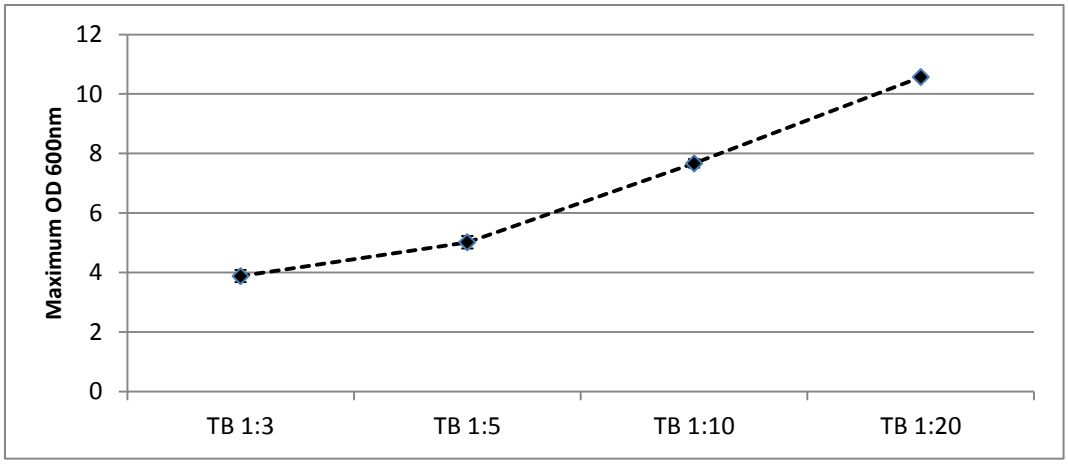


Figure 11: Comparison of maximum OD_{600nm} observed with non-induced TB cultures under the various medium volume to flask volume ratios tested.

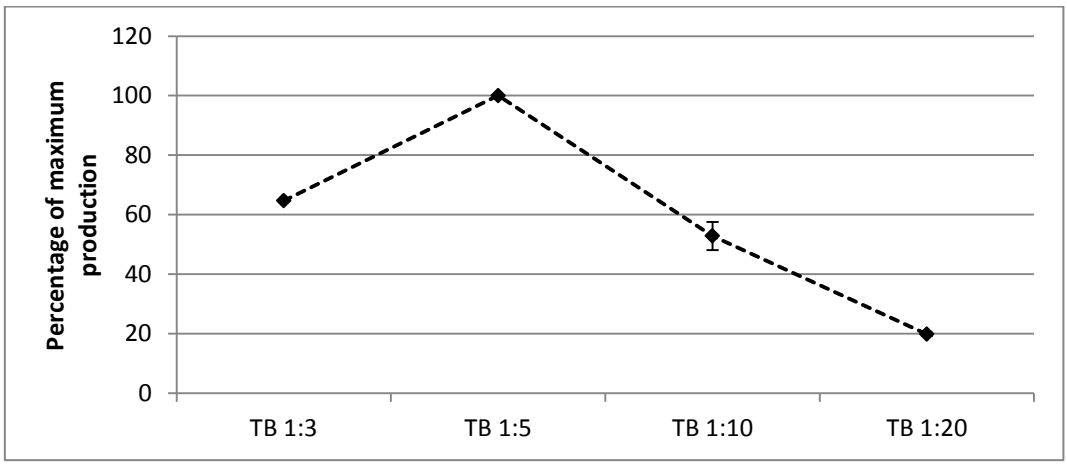


Figure 12: Comparison of maximum PhDsbA production levels observed with various medium volume to flask volume ratios for non-induced TB cultures.

It can be seen that while increased aeration allows for improved biomass levels, highest PhDsbA production with the non-induced TB culture is attained at a reduced medium volume to flask volume ratio.

3. 1. 2. Induced PhDsbA Production Optimisation

Having examined production under non-induced conditions we then investigated production upon induction with 1 mM IPTG. Here various media, levels of aeration and the induction time and period were investigated. Inductions times were at 0 hours, 12 hours (early log phase), 16 hours (late log), 24 hours (stationary) and 28 hours (late stationary phase) of elapsed fermentation time and samples were collected after 2, 4, 6 and 12 hours of an induction period.

Figure 13 gives examples of some of the results for the SDS-PAGE analyses while Figure 14 gives a comparative overview of the principal results obtained. In all cases the 'best producing' conditions are shown, corresponding to 4 to 6 hours after induction.

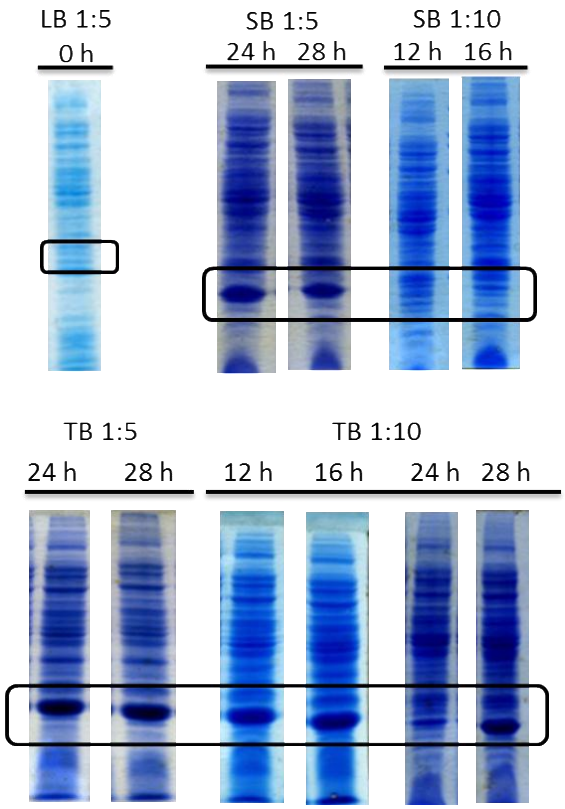


Figure 13: 12% SDS-PAGE analysis of induced cultures. The band corresponding to PhDsbA is indicated within the black boxes.

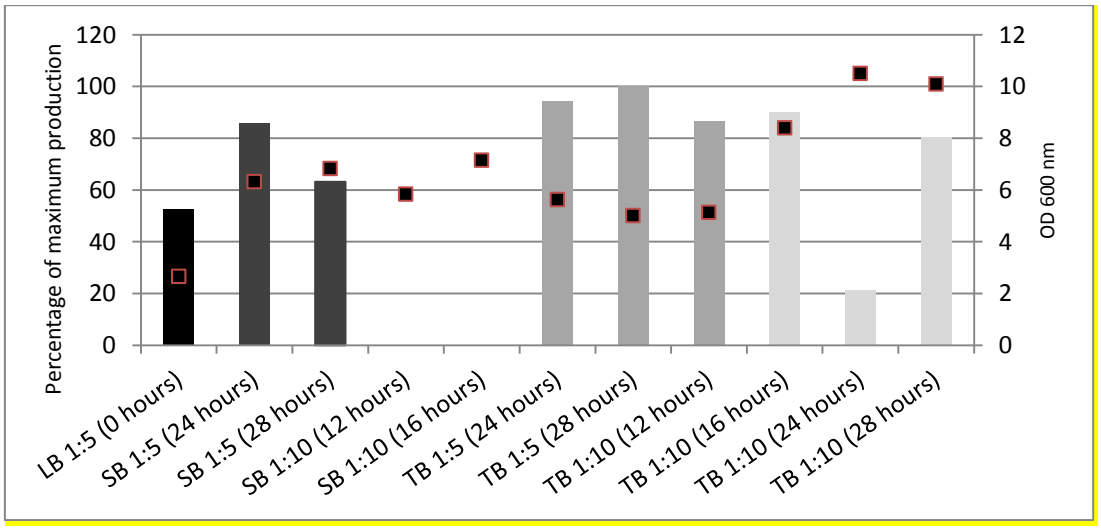


Figure 14: Comparison of maximum PhDsbA production (bars) and maximum OD_{600nm} (black squares) upon induction at distinct cultivation times with the various cultivation conditions shown in Figure 14.

It can be seen that optimum PhDsbA production levels were obtained with TB medium induced during the stationary phase of growth.

3. 1. 3. Induced versus Non-induced PhDsbA production

The optimised production conditions for induced and non-induced cultures were compared and as can be seen from Figure 15 and 16 a slightly improved production was obtained with the induced culture.

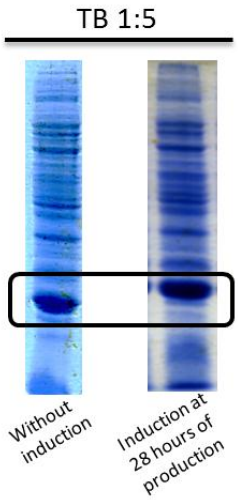


Figure 15: 12% SDS-PAGE comparison of optimised induced and non-induced PhDsbA productions. The band corresponding to PhDsbA is indicated within the black box. Gels were stained with Coomassie Blue.

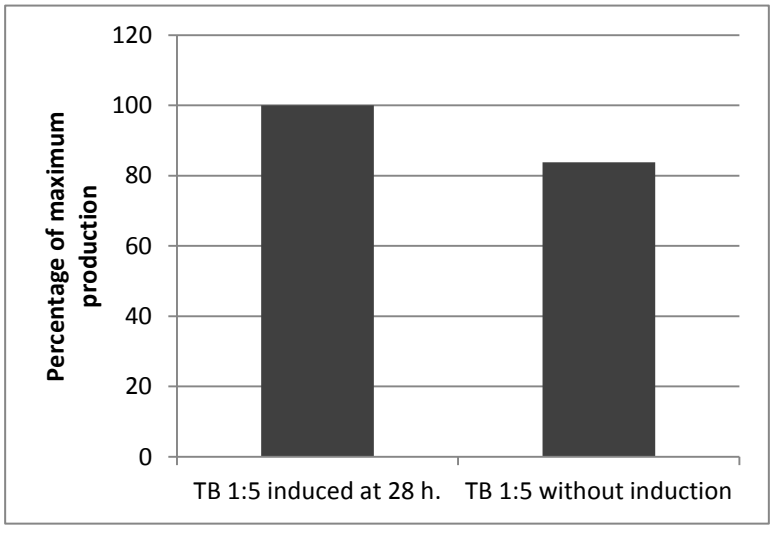


Figure 16: Comparison of relative levels of PhDsbA production with the optimised induced and non-induced production conditions.

3.2. PHDSBA PURIFICATION OPTIMIZATION

A purification protocol for PhDsbA was developed here. This involved periplasmic extraction, HIC at pH 8.0 in the presence of ammonium sulphate and IEX at pH 8.0.

3. 2. 1. Hydrophobic Interaction Chromatography (HIC)

HIC was carried out in the presence of 1 M ammonium sulphate at pH 8.0 with elution being achieved with a gradient of decreasing ammonium sulphate concentration. Figure 18 shows the chromatogram and SDS-PAGE analysis of fractions indicated PhDsbA to elute in 0 % ammonium sulphate in a large peak corresponding to fractions 32 to 50.

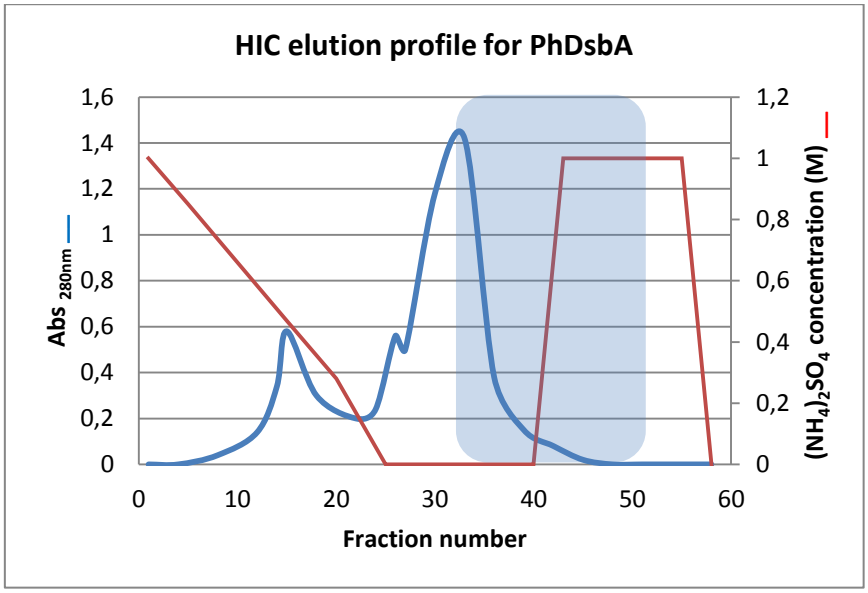


Figure 17: HIC chromatogram for purification of PhDsbA from the periplasmic extract. HIC was carried with 10 mM MOPS buffer at pH 8.0 with a decreasing ammonium sulphate gradient for elution. The transparent blue square represents the fractions containing PhDsbA as determined by SDS-PAGE..

3. 2. 2. Ion Exchange Chromatography (IEX)

Previously, purification of PhDsbA involved IEX at pH 7.2 but we observed large losses of the protein (approx. 40 %) in the void at this pH due to poor matrix binding. Therefore we investigated various pHs in an attempt to improve protein recovery during IEX.

Table 11 shows the amount of protein recovered after IEX at each of the pHs investigated and it can be seen that pH 8.0 allowed for minimum losses of the protein during IEX. Furthermore, use of this pH for IEX allowed for direct loading of the pool following HIC, hence avoiding the necessity for buffer pH exchange by dialysis.

Table 11: Comparison of pH used for IEX and PhDsbA recoveries.

Buffer pH (10 mM MOPS)	% protein recovered following IEX
7.2	~ 50 %
7.5	~ 70 %
8.0	~ 95 %

Figure 18 shows the chromatogram for IEX at pH 8.0 and it can be seen that PhDsbA elutes at low NaCl concentrations under the condition used.

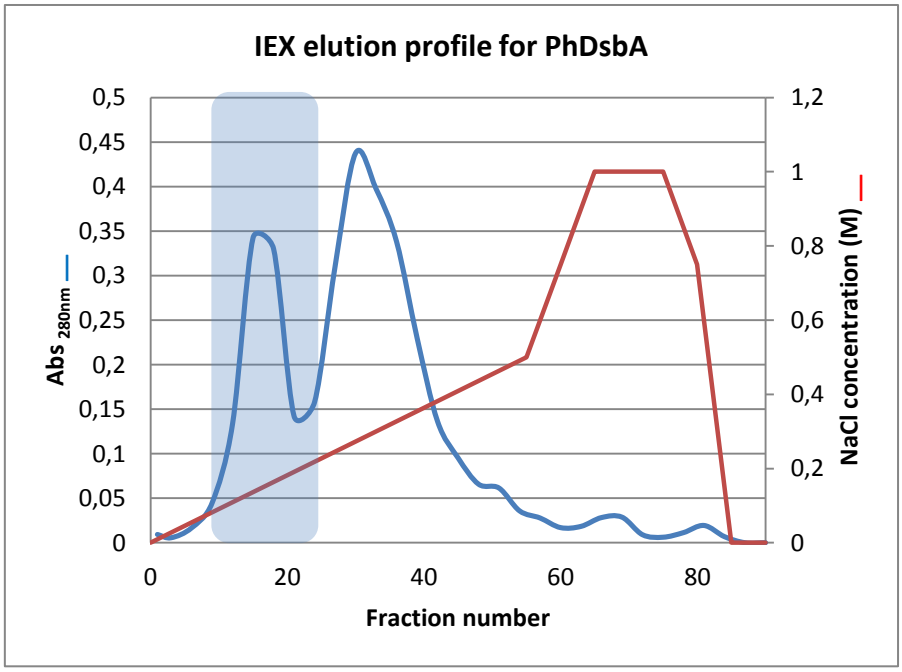


Figure 18: IEX chromatogram for purification of PhDsbA at pH 8.0 in 10 mM MOPS. Elution was carried out with an increasing NaCl concentration. The transparent blue square indicates the fractions containing PhDsbA as determined by SDS-PAGE.

Figure 19 shows the SDS-PAGE analysis of the pools for each of the purification steps. It can be seen that PhDsbAp was successfully purified using the optimised protocol (HIC at pH 8, IEX at pH 8 with simplified two step production process).

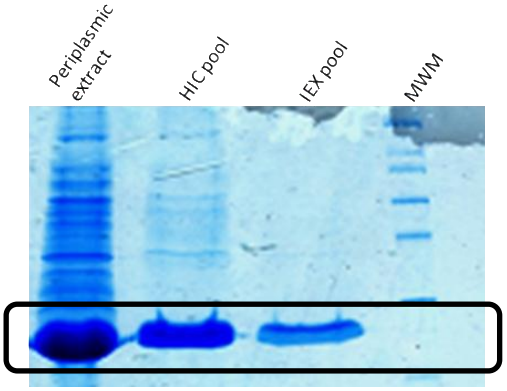


Figure 19: 12% SDS-PAGE of sample pools after each step of the optimised PhDsbA purification protocol. The band corresponding to PhDsbA is indicated by black boxes. The molecular marker is the Broad Range SDS-PAGE Molecular Weight Standards Marker (Bio-Rad).

Analysis of the purification process indicated that the protein was purified with a final yield of approximately 90 %, allowing for 250 mg of purified PhDsbA per liter of production culture. This is approximately five times that previously obtained.

Following purification samples were precipitated with approximately 80 % ammonium sulphate, ressuspended in storage buffer and stored at 4 ºC.

Previous studies had indicated the presence of sugars in the final purified PhDsbA solution (possibly fixed to the protein) but analysis of the purified solution obtained using the optimised protocol of the present study indicated an absence of sugar contaminants with the sugar detection assay used.

The optimised purification protocol developed here for the wild-type PhDsbA was also successfully used for purification of the four mutants prepared in this study (see below for description of mutants).

3.3. MUTANT CONSTRUCTION

3. 3. 1. Comparative structural analysis

A comparison of the PhDsbA sequence with homologs available at the UniProtKB/SwissProt database indicated this to be distinguished by two short insertions, a three residue and four residue insertion, as compared to most of its homologous sequences. Indeed, closer examination and a literature study indicated

that the observed insertions were only found in DsbA sequences isolated from organisms inhabiting low temperature environments (i.e. marine psychrophiles/psychrotrophs).

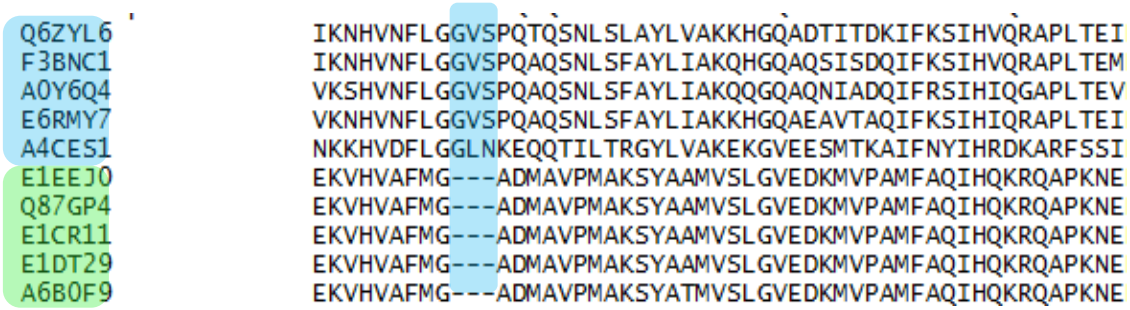


Figure 20: Partial sequence alignment of various DsbAs, represented here by their UniProt identifier codes, showing a possible 3 amino acid insertion in 'cold' DsbAs. Codes shown with a blue background represent DsbAs identified in organism inhabiting low temperature environments and those shown with a green background are the mesophilic DsbAs. PhDsbA is represented here as Q6ZYL6. The blue stripe seen in the middle of the sequences represents a possible insertion site as observed with this alignment.

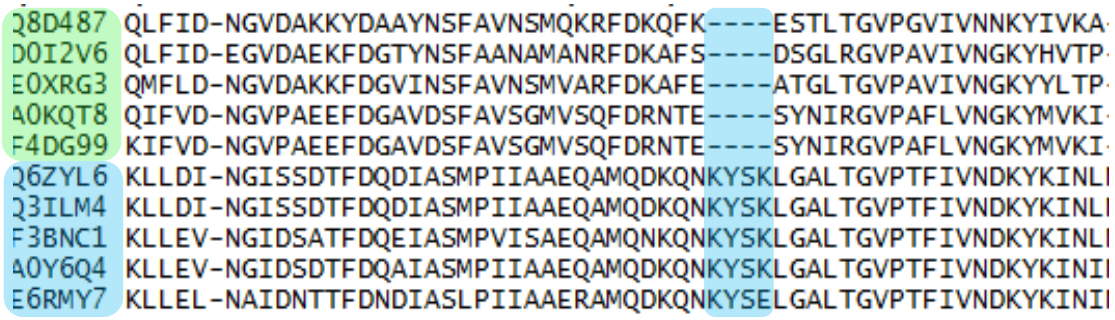


Figure 21: Partial sequence alignment of various DsbAs, represented here by their UniProt identifier codes, showing a possible 4 amino acid insertion site in 'cold' DsbAs. Codes shown with a blue background represent DsbAs identified in organism inhabiting low temperature environments and those shown with a green background are the mesophilic DsbAs. PhDsbA is represented here as Q6ZYL6. The blue stripe seen in the middle of the sequences represents a possible insertion site as observed with this alignment.

Tertiary structure comparisons of PhDsbA with its mesophilic homologs (Figure 22 - 24 indicated these insertions to be located in an inter-domain loop (the 3 residue insertion) and at the end of the long backbone α-helix (the 4 residue insertion).

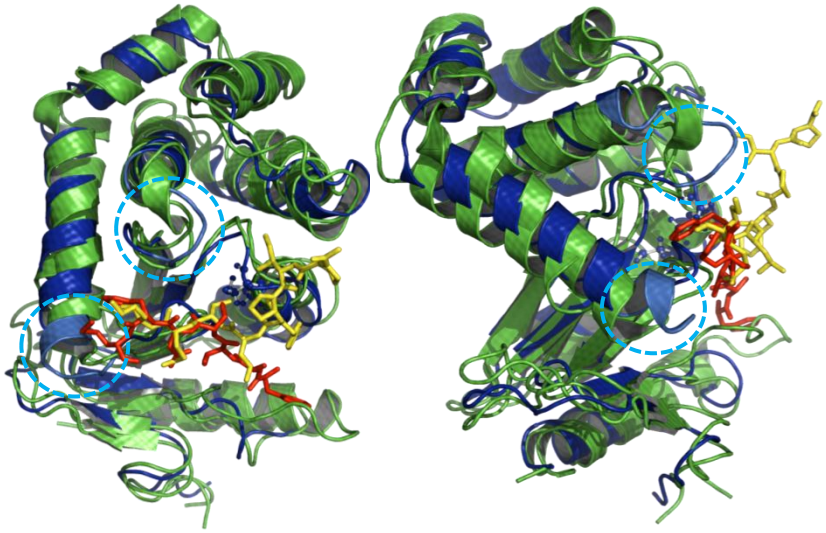


Figure 22: Overlay of PshDsbAp (blue) with its mesophilic homologs from *Vibrio cholerae* (pdb: 2IJY) and *E. coli* (pdb: 1A23). Both mesophiles are shown in green. The two insertions in the cold-adapted DsbA are displayed in light blue and are circled.

More detailed tertiary structure comparisons with Pymol and Dali are illustrated in Figures 23 and 24.

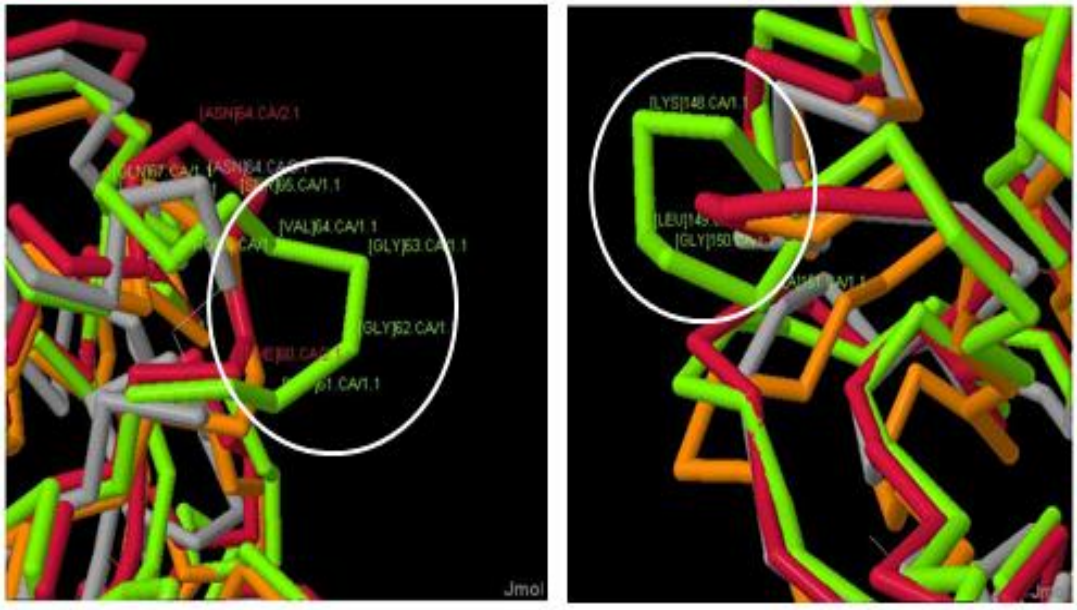


Figure 23: Structural alignments using DALI of PhDsbA in green with with its mesophilic homologs from *Vibrio cholerae* (pdb: 2IJY) and *E. coli* (pdb: 1A23). The left image represents the region containing the 3 residue loop insertion and the right the 4 residue insertion, The structural differences induced by the insertions are highlighted by the white circles.

It can be seen that the insertions results in elongated loops with directional changes in PhDsbA as compared to its mesophilic homologs. The insertions result in

residues Gly[62], Gly[63] and Val[64] in the three residue insertion and Ser[147], Lys[148], Leu[149], Gly[150] and Ala[151] in the four residue insertions protruding out from the structure.

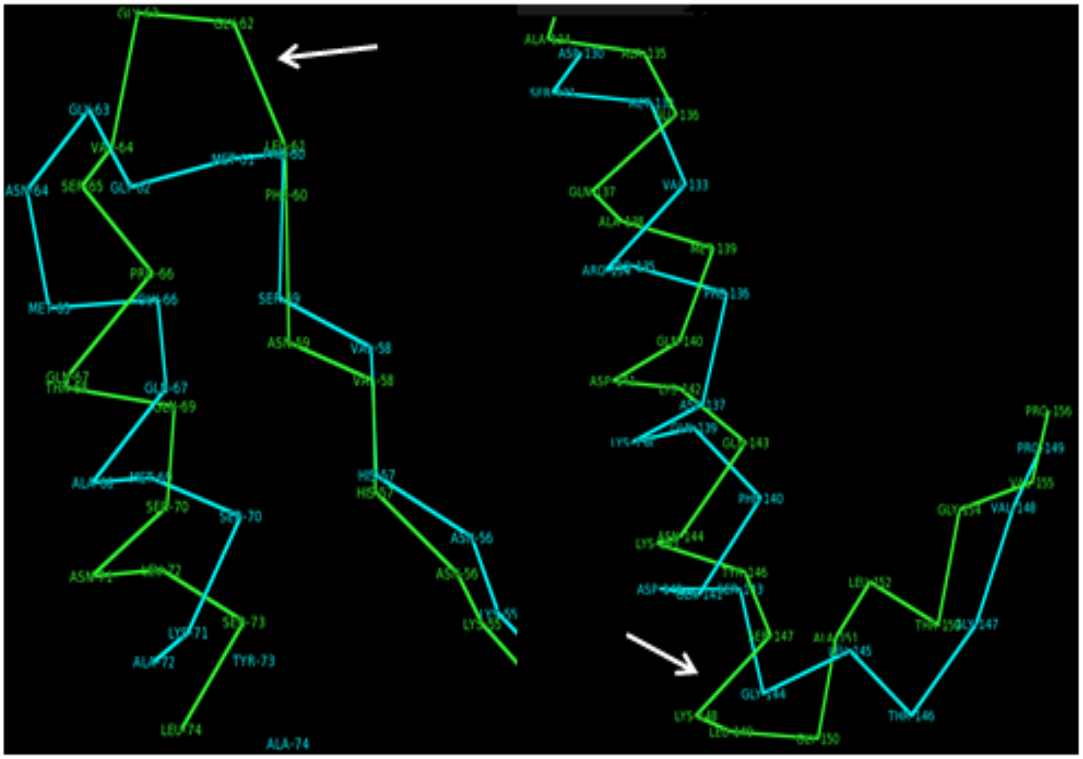


Figure 24: Structural alignments using Pymol to compare PhDsbA, represented in green, with the mesophilic homologous protein, DsbA from *Vibrio cholera*, in blue. The left image represents the region containing the 3 residue loop insertion and the right mage conatians the 4 residue insertion. The structural differences induced by the insertions are highlighted by arrows.

A closer look at the three residue insertion indicates that it is Pro[66], a conserved residue in all cold-adapted DsbA sequences, that appears to induce a change in direction in the PhDsbA loop with the residues Val[64], Ser[65] increasing the length of this deviated loop and resulting in the protrusion of residues 62 to 64 from the structure.

In the case of the four residue insertion the insertion of residues Ser[147], Lys[148], Leu[149], Gly[150] and/or Ala[151] appear to be determinant for the loop protrusion. Importantly, the conservation of a Gly corresponding to Gly[150] in many mesophilic homologs indicates the importance of this residue in DsbAs.

3. 3. 2. Mutant Construction

Based on the comparative structural analysis a number of PhDsbA deletion mutants were prepared, namely deletions of: (Val[64], Ser[65]); (Val[64], Ser[65] Pro[66]); (Ser[147], Lys[148], Leu[149]) and (Ser[147], Lys[148], Leu[149], Ala[151]).

Primers were designed for deletion of these residues as described in Table 12 and mutagenesis carried out by the inverse PCR procedure described in the materials and methods.

Table 12: Primers designed for mutation of DsbA sequence.

Deleted amino-acids	Type	Amino-acids sequence (5' to 3')	Length (bp)	% of GC	Melting temperature	Self-complementarity
VSP	Reverse	GCCGCCTAAAAAGT TAACGTG	21	47,6	64,7 ºC	4
VSP	Forward	CAAACACAAAGTAAC TTGAGCCTAGC	26	42,3	65,1 ºC	2
VS	Forward	CCACAAACACAAAGT AACTTGAGC	24	41,7	63,9 ºC	3
SKL	Reverse	GTATTTATTTTGTTTA TCTTGCATTGCTT	29	24,1	63,3 ºC	1
SKL	Forward	GGTGCGTTAACAGG CGTTC	19	57,9	65,9 ºC	1
SKLA	Reverse	ACCGTATTTATTTTG TTTATCTTGC	25	28	60,5 ºC	2
SKLA	Forward	TTAACAGGCGTTCCT ACTTTTATTG	25	36	63,3 ºC	0

Restriction digestion analysis of the various constructs obtained indicated successful deletions and gene sequencing confirmed these deletions as well as the absence of other mutations. All four mutant constructs were then transformed to *E. coli* BL21(DE3) and produced and purified using the procedures optimised for the wild type enzyme.

3.4. ACTIVITY ASSAY

The insulin assay was used to determine whether the purified wild type and mutant PhDsbAs produced maintained a reducing activity. The results of this assay with similar concentrations of DsbA are shown in Figure 26 where it was found that all mutants displayed activity. It can be seen that the VS deletion appears to have a higher reducing activity under the conditions used while the SKL+A mutant retained high activity as compared with WT. In contrast, the SKL has poor reducing activity. SKL_A mutant has not been studied at this level.

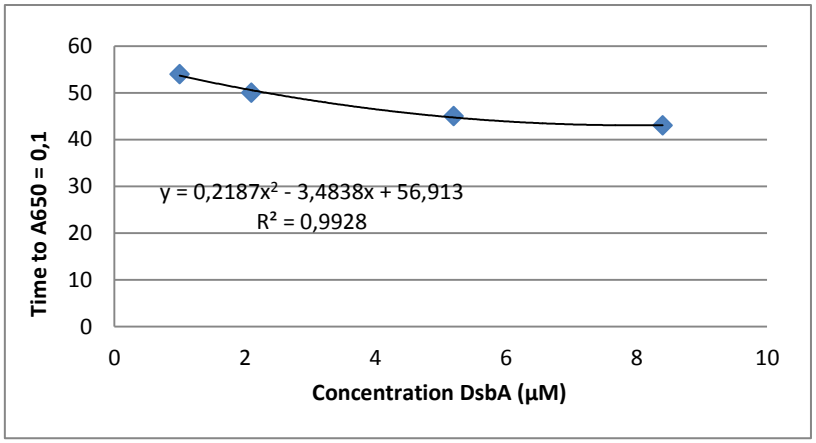


Figure 25: PhDsbA concentration dependence of precipitation.

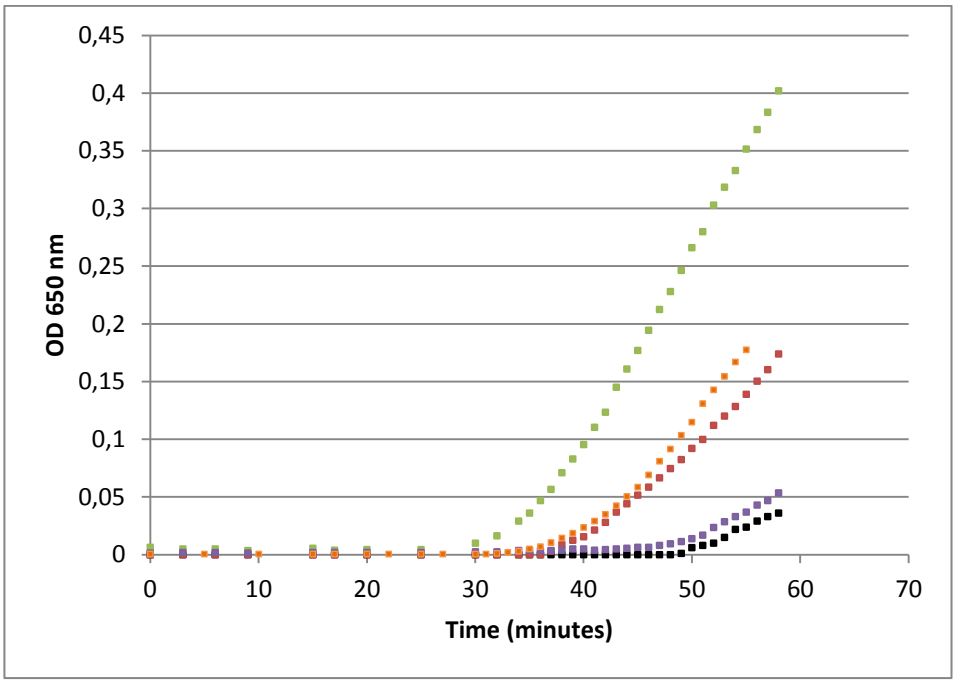


Figure 26: Insulin activity assay demonstrating activity of the various mutants produced. Green squares represent the *DsbA-Val64_Ser65del* mutant (VS deletion); red squares the *DsbA-Val64_Pro66* mutant (VSP deletion); purple squares the *DsbA-Ser147_Leu149del* mutant (SKL deletion), black squares the negative control and orange squares the wild type. The rate of insulin precipitation, as indicated by an increase in the OD_{650nm} value, is indicative of the reducing activity of the DsbA.

Chapter 4: Final remarks and future perspectives

DsbAs catalyse disulphide bond formation in newly synthesised proteins and due to the importance of these covalent bonds in the structure, stability and activity of many proteins they are of much fundamental and applied interest. Approximately 30% of currently produced pharmaceutical proteins contain disulphide bonds and hence the use of DsbAs as an aid in recombinant protein production has a strong potential. More recently, their crucial role in the correct folding and functioning of virulence factors produced by pathogenic bacteria has opened up a potential role in medicine and in particular in the development of antimicrobial agents. In an attempt to better understand DsbAs and their structure-function relationship and hence to develop their potential in the above mentioned fields we have initiated a comparative study of DsbAs adapted to various temperatures. We are using homologous cold-adapted and mesophilic DsbAs as model enzymes as we believe that such a comparative study would reveal much more information and better identify determinants of activity and stability in DsbAs as compared to studies of individual enzymes.

As an initial part in our overall study of DsbAs, the present study is focused on two main areas:

1) developing and optimising the production and purification protocols for a recombinantly produced cold-adapted DsbA from *Pseudoalteromonas haloplanktis* (PhDsbA).
2) identifying structural determinants of cold-adaptation and construction of a number of mutants so as to allow for future studies investigating these.

In the first part of the study the production of the cold-adapted DsbA with the *E. coli* BL21(DE3)/pET22b(+) expression system was investigated in shake flasks. Even though this expression system is based on a controlled induction we nevertheless obtained strong production even in the absence of added inducer. In fact this has already been noted in the past by other groups (Collins, et al., 2013; Guda, et al., 1995; Nair, et al., 2009) and has been attributed to low levels of lactose contamination in the media used. While this was not examined here, it is possibly also the cause of the ‘uninduced’ production observed as complex unrefined media ingredients such as yeast extract and tryptone peptone were used. As might be expected, maximum cell growth was observed with the richer media TB and SB as compared to LB, with TB showing the highest levels at the highest medium volume to flask volume ratios (i.e. highest aeration rates) tested. Even though SB is a richer medium (higher concentrations of yeast extract and tryptone peptone) the higher biomass levels

achieved with TB is probably a result of the buffered (phosphate buffer) and glycerol supplemented nature of this. These reduce the negative effects of the co-products produced (namely acetate) at the higher growth rates achieved when higher medium volume to flask volume ratios are used (Collins et al, 2013). Finally, DsbA periplasmic expression was found to increase with increasing aeration up to a medium volume to flask volume ratio of 1:5 only, with a decreased production being noted thereafter. This reduced yield of periplasmic protein is attributed to losses to the extracellular environment at the higher medium volume to flask volume ratios tested and is probably a result of a greater physical force on the cells under these conditions.

Similar results and conclusions to those observed with the 'non-induced' productions were obtained for the induced production optimisation study i.e. optimal growth and production was obtained with TB with intermediate (1:5) medium volume to flask volume ratios. Highest production was observed with induction during the stationary phase of growth with at least 4 to 6 hours of induction. Typically recombinant protein production is induced during the exponential phase of growth as it is believed that this is when the transcription and translational machinery are most active (Babaeipour, et al., 2007). Nevertheless, previous studies have demonstrated that expression systems with ampicillin as the selection marker display a rapid loss of the production plasmid immediately following induction and thereby drastically decreased product formation following induction (Collins et al, 2013). This obviously diminishes the effectiveness of early induction and long post induction times and indicates that protein production levels with these systems are strongly dependent on cell density before induction. The attainment of a high cell density before induction thus maximises production levels as was seen in the present study.

A combination of hydrophobic interaction chromatography (HIC) and ion-exchange chromatography (IEX) have been previously described for the purification of mesophilic DsbAs from *E. coli* (Wunderlich & Glockshuber, 1993) and *Vibrio cholera* (Horne et al, 2007) and hence this approach was investigated for the purification of the homologous cold-adapted DsbA of this study. Following osmotic shock for extraction of the periplasmic protein and addition of 1 M ammonium sulphate for improved column binding, HIC with a decreasing ammonium sulphate concentration allowed for removal of the majority of contaminating proteins. Initial attempts involving dialysis and anion exchange chromatography at pH 7.2 resulted in losses of up to 50% of the protein of interest in the void and hence higher pHs were investigated for improved column

binding. Anion exchange at a pH of 8.0 not only allowed for almost complete DsbA recovery but also allowed for the direct loading of the HIC pool to the IEX column, thereby avoiding the requirement for dialysis and hence simplifying the purification procedure. Following this IEX step, sufficiently pure DsbA, as demonstrated by SDS-PAGE analysis, was recovered with a yield of approximately 90%. Approximately 250 mg of purified DsbA per litre of production culture was obtained using the optimised production and purification protocols developed in this study, this equating to about 5 times that obtained during initial tests.

In the second part of the study an investigation into the structural determinants of cold-adaptation in DsbAs was initiated. Sequence and structure alignments clearly show the cold-adapted enzyme to be distinguished by two short insertions in regions believed to be important in DsbA activity. An insertion of 3 residues occurs in an inter-domain loop which is believed to be important in substrate binding and inter-domain movement while the second insertion (4 residues) occurs at the interface of the C-terminal end of a long backbone α-helix and at the start of a long loop believed to be important in catalytic activity. It is possible to hypothesise that these insertions allow for improved inter-domain movement, improved substrate binding and improved movement of the loop important in enzyme activity and hence allow for the improved flexibility required for activity at low temperatures. Interestingly, these insertions were found to be conserved in all DsbAs isolated to date from low temperature environments and hence points to a central role of these Insertions in temperature adaptation of DsbAs. These loop regions are obviously important for DsbA activity and hence we have designed and prepared a number of deletion mutants in which these insertions were targeted. Four mutants were prepared, produced and purified and shown to be active. Interestingly one of the mutants was found to have a higher reducing activity than the wild-type enzyme under the conditions used while all others displayed a lower reducing activity. Further, more in-depth studies are required to characterise these mutants and compare them to their mesophilic and psychrophilic homologs in an attempt to better understand their role. In particular, studies comparing both the reducing and oxidising activities at various temperatures, most probably by HPLC (Zapun et al, 1993), as well as comparative stability studies (DSC, CD, irreversible inactivation) are required to obtain a better understanding of the effects of the mutations and to better characterise structure-function relationships in DsbAs. The information gained could enable a better design of DsbA inhibitors in the future, possibly even targeting the loop regions identified in this study. Finally, it is suggested

that in the future the cold-adapted enzyme be investigated for use in cell free protein production systems as the expected high activity of this enzyme should offer advantages over currently used mesophilic DsbAs.

Bibliography

Alexeeva, S., Hellingwerf, K. & Teixeira de Mattos, M., 2002. Quantitative Assessment of Oxygen Availability: Perceived Aerobiosis and Its Effect on Flux Distribution in the Respiratory Chain of Escherichia coli. *Journal of bacteriology,* Volume 184, pp. 1402-1406.

Amersham Biosciences, 2002. *Ion exchange chromatography.* s.l.:s.n.

Amersham Pharmacia Biotech, 2000. *Hydrophobic interaction chromatography.* s.l.:s.n.

Antonio-Pérez, A., Ramón-Luing, L. A. & Ortega-López, J., 2012. Chromatographic refolding of rhodanese and lysozyme assisted by the GroEL apical domain, DsbA and DsbC immobilized on cellulose.. *Journal of chromatography A,* Volume 1248, pp. 122-129.

Babaeipour, V. et al., 2007. A proposed feeding strategy for the overproduction of recombinant proteins in Escherichia coli. *Biotechnology and Applied Biochemistry.*

Bardwell, J., McGovern, K. & Beckwith, J., 1991. Identification of a protein required for disulfide bond formation in vivo. *Cell,* Volume 67, pp. 581-589.

Bell, P., 2001. E. coli expression systems. In: *Molecular Biology Problem Solver: A Laboratory Guide.* s.l.:Wiley-Liss, Inc., pp. 461-490.

Bentahir, M. et al., 2000. Structural, Kinetic, and Calorimetric Characterization of the Cold-active Phosphoglycerate Kinase from the Antarctic Pseudomonas sp. TACII18. *The journal of biological chemistry,* Volume 275, p. 11147–11153.

Boehr, D., Dyson, J. & Wright, P., 2006. An NMR Perspective on Enzyme Dynamics. *Chemical review,* Volume 106, pp. 3055-3079.

Collet, J.-F. & Bardwell, J., 2002. Oxidative protein folding in bacteria. *Molecular microbiology,* Volume 44, pp. 1-8.

Collins, T. et al., 2013. Batch production of a silk-elastin-like protein in E. coli BL21(DE3): key parameters for optimisation. *Microbial Cell Factories .*

Collins, T. et al., 2010a. *NMR Solution Structure of a Cold-Adapted Thiol-Disulphide Oxidoreductase.* s.l.:s.n.

Collins, T., Matzapetakis, M. & Santos, H., 2010b. Backbone and side chain 1H, 15N and 13C assignments for a thiol-disulphide oxidoreductase from the Antarctic bacterium Pseudoalteromonas haloplanktis TAC125. *Biomolecular NMR assignments,* Volume 4, pp. 151-154.

Collins, T., Meuwis, M.-A., Gerday, C. & Feller, G., 2003. Activity, Stability and Flexibility in Glycosidases Adapted to Extreme Thermal Environments. *Journal of molecular biology,* Volume 328, p. 419–428.

Daegelen, P. et al., 2009. Tracing Ancestors and Relatives of Escherichia coli B, and the Derivation of B Strains REL606 and BL21(DE3). *Journal of Molecular Biology,* pp. 634-643.

Dailey, F. E. & Berg, H. C., 1993. Mutants in disulphide bond formation that disrupt flagellar assembly in Escherichia coli. *Proceedings of the National Academy of Sciences,* Volume 90, pp. 1043-1047.

D'Amico, S. et al., 2002. Molecular basis of cold adaptation. *Philosophical transactions of the royal society,* Volume 357, pp. 917-925.

D'Amico, S. et al., 2006. Psychrophilic microorganisms: challenges for life. *Embo reports,* Volume 7, p. 385–389.

Daniell, H. et al., 1997. Hyperexpression of a synthetic protein-based polymer gene. *Methods in molecular biology ,* Volume 63, pp. 359-371.

Davail, S., Feller, G., Narinx, E. & Gerday, C., 1994. Cold Adaptation of Proteins: purification, characterization, and sequence of the heat-labile subtilisin from the antarctic psychrophile Bacillus TA41. *The journal of biological chemistry,* Volume 269, pp. 17448-17453.

Dubois, M. et al., 1956. Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry,* Volume 28, pp. 350-356.

Durand, E. et al., 2009. Structural biology of bacterial secretion systems in gram-negative pathogens– potential for new drug targets. *Infectious Disorders - Drug Targets,* Volume 9, pp. 518-547.

Dutton, R. et al., 2010. Inhibition of bacterial disulfide bond formation by the anticoagulant warfarin. *Proceedings of the National Academy of Sciences,* Volume 107, pp. 297-301.

Fabianek, R., Hennecke, H. & Thöny-Meyer, L., 2000. Periplasmic protein thiol:disulphide oxidoreductases of Escherichia coli. *FEMS Microbiology reviews,* Volume 24, pp. 303-316.

Fabianek, R. H. H. & Thöny-Meyer, L., 2000. Periplasmic protein thiol:disul¢de oxidoreductases of Escherichia coli. *FEMS Microbiology reviews,* Volume 24, pp. 303-316.

Feller, G., 2007. Life at low temperatures: is disorder the driving force?. *Extremophiles,* Volume 11, p. 211–216.

Feller, G., Arpigny, J.-L., Narinx, E. & Gerday, C., 1997. Molecular adaptations of enzymes from psychrophilic organisms. *Comparative Biochemistry and Physiology . A,* Volume 118, pp. 495-499.

Fields, P., 2001. Review: Protein function at thermal extremes: balancing stability and flexibility. *Comparative biochemistry and physiology,* Volume 129, pp. 417-431.

Fields, P. & Somero, G., 1998. Hot spots in cold adaptation: Localized increases in conformational flexibility in lactate dehydrogenase A4 orthologs of Antarctic notothenioid fishes. *Proceedings of the National Academy of Sciences of U.S.A.,* Volume 95, p. 11476–11481.

Früh, V. et al., 2010. Application of fragment-based drug discovery to membrane proteins: identification of ligands of the integral membrane enzyme DsbB. *Chemistry and biology,* Volume 17, pp. 881-891.

García-Arribas, O. et al., 2007. Thermodynamic stability of a cold-adapted protein, type III antifreeze protein, and energetic contribution of salt bridges. *Protein science,* Volume 16, pp. 227-238.

Georlette, D. et al., 2004. Some like it cold: biocatalysis at low temperatures. *Microbiological reviews,* Volume 28, pp. 25-42.

Gerday, C. et al., 1997. Psychrophilic enzymes: a thermodynamic challenge. *Biochimica et biophysica acta,* Volume 1342, p. 119–131.

Gerday, C. et al., 2000. Cold-adapted enzymes: from fundamentals to biotechnology. *Trens in Biotechnology,* Volume 18, pp. 103-107.

Glazyrina, J. et al., 2010. High cell density cultivation and recombinant protein production with Escherichia coli in a rocking-motion-type bioreactor. *Microbial Cell Factories.*

Gruber, C. et al., 2006. Protein disulfide isomerase: the structure of oxidative folding. *Trends in biochemical sciences,* Volume 31, pp. 455-464.

Guda, C. et al., 1995. Hyper expression of an environmentally friendly synthetic polymer gene.. *Biotechnol Lett .*

Guddat, L. et al., 1997. Structural analysis of three His32 mutants of DsbA: Support for an electrostatic role of His32 in DsbA stability. *Protein Science,* Volume 6, pp. 1893-1900.

Hanahan, D., 1983. Studies on transformation of Escherichia coli with plasmids.. *Journal of molecular biology,* pp. 557-580.

Hanahan, D., 1983. Studies on transformation of Escherichia coli with plasmids.. *Jornal of molecular biology,* pp. 557-580.

Harrison, D. E. & Pirt, S. J., 1967. The influence of dissolved oxygen concentration on the respiration and glucose metabolism of Klebsiella aerogenes during growth. *Journal of general microbiology,* Volume 46, pp. 193-211.

Henikoff, S. & Henikoff, J., 1992. Amino acid substitution matrices from protein blocks. *Proceedings of the National Academy of Sciences,* Volume 89, pp. 10915-10919.

Heras, B. et al., 2009. DSB proteins and bacterial pathogenicity. *Microbiology,* Volume 7, pp. 215-225.

Heras, B. et al., 2010. Structural and Functional Characterization of Three DsbA Paralogues from Salmonella enterica Serovar Typhimurium. *Journal of Biological Chemistry,* Volume 285, p. 18423–18432.

Holmgren, A., 1979. Thioredoxin Catalyzes the Reduction of Insulin Disulfides by Dithiothreitol and Dihydrolipoamide. *The journal of biological chemistry,* Volume 254, pp. 9627-9632.

Horne, J. et al., 2007. Probing the Flexibility of the DsbA Oxidoreductase from Vibrio cholerae—a 15N - 1H Heteronuclear NMR Relaxation Analysis of Oxidized and Reduced Forms of DsbA. *Journal of molecular biology,* Volume 371, pp. 703-716.

Inaba, K. & Ito, K., 2002. Paradoxical redox properties of DsbB and DsbA in the protein disulfide-introducing reaction cascade. *European Molecular Biology Organization Journal,* Volume 21, pp. 2646-2654.

Inaba, K. & Ito, K., 2008. Structure and mechanisms of the DsbB-DsbA disulfide bond generation machine. *Biochimica et Biophysica acta,* Volume 1783, pp. 520-529.

Inoue, H., Nojima, H. & Okamaya, H., 1990. High efficiency transformation of Escherichia coli with plasmids. *Gene,* pp. 8-23.

Ito, K. & Inaba, K., 2008. The disulfide bond formation (Dsb) system. *Current Opinion in Structural Biology,* Volume 18, p. 450–458.

Jeong, H. et al., 2009. Genome Sequences of Escherichia coli B strains REL606 and BL21(DE3). *Journal of molecular biology,* Volume 394, pp. 644-652.

Khow, O. & Suntrarachun, S., 2012. Strategies for production of active eukaryotic proteins in bacterial expression system. *Asian Pacific Journal of Tropical Biomedicine,* pp. 159-162.

Kruger, N., 2002. The bradford method for protein quantification. In: *The protein protocols handbook.* s.l.:Humana Press Inc., pp. 15-21.

Kuroita, T. et al., 2007. Functional similarities of a thermostable protein-disulfide oxidoreductase identified in the archaeon Pyrococcus horikoshii to bacterial DsbA enzymes. *Extremophiles.*

Lasica, A. & Jagusztyn-Krynicka, E., 2007. The role ofDsb proteins of Gram-negative bacteria in the process of pathogenesis. *FEMS Microbology review,* Volume 31, pp. 626-636.

Lin, D., Rao, C. & Slauch, J., 2008. The Salmonella SPI1 type three secretion system responds to periplasmic disulfide bond status via the flagellar apparatus and the RcsCDB aystem. *Journal of bacteriology,* Volume 190, pp. 87-97.

Lonhienne, T., Gerday, C. & Feller, G., 2000. Psychrophilic enzymes: revisiting the thermodynamic parameters of activation may explain local flexibility. *Biochimica et biophysica acta,* Volume 1543, pp. 1-10.

Madonna, S. et al., 2006. The thiol-disulfide oxidoreductase system in the cold-adapted bacterium Pseudoalteromonas haloplanktis TAC 125: discovery of a novel disulfide oxidoreductase enzyme. *Extremophiles,* Volume 10, pp. 41-51.

Médigue, C. et al., 2005. Coping with cold: The genome of the versatile marine Antarctica bacterium Pseudoalteromonas haloplanktis TAC125. *Genome Research,* Volume 15, p. 1325–1335.

Miroux, B. & Walker, J., 1996. Over-production of Proteins in Escherichia coli: Mutant Hosts that Allow Synthesis of some Membrane Proteins and Globular Proteins at High Levels. *Journal of Molecular Biology,* Volume 260, pp. 289-298.

Mössner, E., Huber-Wunderlich, M. & Glockshuber, R., 1998. Characterization of Escherichia coli thioredoxin variants mimicking the active-sites of other thiol/disulfide oxidoreductases. *Protein Science,* Volume 7, pp. 1233-1244.

Nair, R. et al., 2009. Yeast extract mediated autoinduction of lacUV5 promoter: an insight.. *N Biotechnol .*

Noguchi, A., Nishino, T. & Nakayama, T., 2009. Kinetic and thermodynamic characterization of the cold activity acquired upon single amino-acid substitution near the active site of a thermostable α-glucosidase. *Journal of Molecular Catalysis B,* Volume 56, p. 300–306.

Novagen, 2003. *pET system manual.* s.l.:s.n.

Parrilli, E., Duilio, A. & Tutino, M., 2008. *Heterologous protein expression in psychrophilic hosts.* s.l.:Springer.

Paxman, J. et al., 2009. The Structure of the Bacterial Oxidoreductase Enzyme DsbA in Complex with a Peptide Reveals a Basis for Substrate Specificity in the Catalytic Cycle of DsbA Enzymes. *The journal of biological chemistry,* Volume 284, p. 17835–17845.

Poleksic, A., 2009. Island method for estimating the statistical significance of profile-profile alignment scores. *BMC bioinformatics,* pp. 100-112.

Reckenfelderbäumer, N. et al., 2000. Identification and Functional Characterization of Thioredoxin from Trypanosoma brucei brucei. *The journal of biological chemistry,* Volume 275, p. 7547–7552.

Ruddock, L., Hirst, T. & Freedman, R., 1996. pH-dependence of the dithiol-oxidizing activity of DsbA (a periplasmic protein thiol : disulphide oxidoreductase) and protein disulphide-isomerase : studies with a novel simple peptide substrate. *Biochemical journal,* Volume 315, pp. 1001-1005.

Schirra, H. et al., 1998. Structure of Reduced DsbA from Escherichia coli in Solution. *Biochemistry,* Volume 37, p. 6263–6276.

Shouldice, S. et al., 2011. Structure and function of DsbA, a key bacterial oxidative folding catalyst. *Antioxidants & redox signaling,* Volume 14, pp. 1730-1752.

Siddiqui, K., Bokhari, S., Afzal, A. & Singh, S., 2004. A Novel Thermodynamic Relationship Based on Kramers Theory for Studying Enzyme Kinetics under High Viscosity. *IUBMB Life,* Volume 56, pp. 403-407.

Soini, J., Ukkonen, K. & Neubaeur, P., 2008. High cell density media for Escherichia coli are generally designed for aerobic cultivations – consequences for large-scale bioprocesses and shake flask cultures. *Microbial Cell Factories.*

Teulé, F. et al., 2009. A protocol for the production of recombinant spider silk-like proteins for artificial fiber spinning. *Nature protocols,* Volume 4, pp. 341-355.

Tomatis, P. E. et al., 2008. Adaptive protein evolution grants organismal fitness by improving catalysis and flexibility.. *Chemical Biology,* Volume 20605, pp. 105-152.

Uribe, S. & Sampedro, J., 2003. Measuring Solution Viscosity and its Effect on Enzyme Activity. *Biological Procedures Online,* pp. 108-115.

Vivian, J. et al., 2009. Structure and Function of the Oxidoreductase DsbA1 from Neisseria meningitidis. *Journal of Molecular Biology,* Volume 394, p. 931–943.

Williams, M., Chalmers, D., Martin, J. & Scanlon, M., 2010. Backbone and side chain 1H, 15N and 13C assignments for the oxidised and reduced forms of the oxidoreductase protein DsbA from Staphylococcus aureus. *Biomolecular NMR Assignments ,* Volume 4, pp. 25-28.

Wunderlich, M. & Glockshuber, R., 1993. Redox properties of protein disulfide isomerase (DsbA) from Escherichia coli.. *Protein Sci.*

Wu, X., Liu, N., He, Y. & Chen, Y., 2009. Cloning, expression, and characterization of a novel diketoreductase from Acinetobacter baylyi. *Acta Biochimica et Biophysica Sinica,* Volume 41, pp. 163-170.

Xu, Y., Feller, G., Gerday, C. & Glansdorff, N., 2003. Moritella cold-active dihydrofolate reductase: are there natural limits of catalytic efficiency at low temperature?. *Journal of bacteriology,* Volume 185, pp. 5519-5526.

Zapun, A., Bardwell, J. & Creighton, T., 1993. The reactive and destabilizing disulphide bond of DsbA, a protein required for rotein disulphide bond formation in vivo. *Biochemistry,* Volume 32, pp. 5083-5092.

Zapun, A., Cooper, L. & Creighton, T., 1994. Replacement of the Active-Site Cysteine Residues of DsbA, a Protein Required for Disulfide Bond Formation in Vivo. *Biochemistry jornal,* Volume 33, pp. 1907-1914.

Zecchinon, L. et al., 2001. Did psychrophilic enzymes really win the challenge?. *Extremophiles,* Volume 5, p. 313–321.