
# Characterisation of the Doxorubicin pump of *Streptomyces peucetius* The DrrA component of the DrrAB ATP-binding cassette transporter

*By*

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***Summary***

The emergence of antibiotic resistant microorganisms is one of the most concerning problems in health welfare, and particularly, that of multi drug resistance. The active efflux of compounds from the cell is one of the key strategies used by microorganisms to avoid the noxious effects of toxic compounds and has become one of the most important mechanisms of multiple resistance, such transport processes being catalysed by an array of membrane associated proteins.

ATP-Binding Cassette Transporters, that carry out an energy dependent active transport process by which substances cross the cell membrane on the hydrolysis of ATP, also display the ability to translocate a number of unrelated molecules, including antibiotics. These transporters have been identified as one of the largest and most widely distributed families of such transmembrane transport systems. Virtually ubiquitous in nature, ABC transporters have been found in the genomes of every organism from the simplest archea through to man. The fact that they contribute the main pathway for resistance to anticancer drugs in humans highlights their importance and urgent need to study them.

In addition to the well known groups of antibiotics produced by members of the *Streptomyces* genus, that include aminoglycosides, tetracyclines, chloramphenicol, and some β-lactams, which inhibit the protein synthesis or the proper formation of bacterial cell walls, the species *Streptomyces peucetius* produces the antibiotics doxorubicin and daunorubicin. These latter compounds are classified into the anthracyclines group and possess antitumoral activity, expanding the arsenal of compounds with different activity produced by *Streptomyces*.

The manner, in which *S. peucetius* avoids the effect of the antitumorals it produces, is by pumping them out of the cells, and the system that it utilises is that of an ABC transporter. In this system two subunits are present, one of them, DrrA, a peripheral membrane protein that acts as the energy-transducing component, and the other, DrrB, the membrane carrier. This type of permease carries out export of antibiotics in an ATP-dependent manner.

The expression of DrrA in *E. coli* proved to be a challenging enterprise as only low yields were obtained for DrrA at 16 °C, that were only improved when DrrA was fused to Thioredoxin. The over-expression and purification of DrrA allowed a partial characterisation of the catalytic activity of DrrA fused to Thioredoxin, with traditional biochemical methods, complemented by additional characterisation assays in wild type DrrA. The characterisation was based on assays of the ATPase activity of Thio-DrrA and DrrA, which displayed a measurable catalytic activity compatible with a role in energising transmembrane transport. These proteins were shown to be cation dependent ATPases able of binding and hydrolysing ATP in a similar manner to the NBD proteins of well-characterised prokaryotic ABC transporters, also showing common features to other NBD's.

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**List of Abbreviations**

| | |
|---|---|
| **ABC** | ATP-Binding Cassette |
| **AC** | Acriflavin |
| **Art** | Artemisinin |
| **ATP** | Adenosin triphosphate |
| **AU** | Absortion Units |
| **AZ** | Azithromycin |
| **BCA** | Bicinchoninic acid |
| **Ber** | Berberine |
| **BLAST** | Basic Logical Alignment Search Tool |
| **Cc** | Chloroquine |
| **CCM** | Cytochrome C Maturation |
| **CCCP** | Carbonyl cyanide m-chlorophenylhydrazone |
| **CFTR** | Cystic Fibrosis Transmembrane Conductance Regulator |
| **CH** | Chlorhexidine |
| **CHH** | 2-chlorophenylhydrazine hydrochloride |
| **CHX** | Cycloheximide |
| **Cip** | Ciprofiaxacin |
| **CL** | Clarithromycin |
| **CP** | Chloramphenicol |
| **CPC** | Cetylpyridinium chloride |
| **CTX** | Cefotaxime |
| **CV** | Crystal Violet |
| **DAR** | Daunorubicin |
| **DHAP** | Dexamethasone |
| **DM** | Daunomycin |
| **DOC** | Deoxycholate |
| **DTT** | Dithiothreitol |
| **DXR** | Doxorubicin |
| **dNTP** | Deoxynucleotide triphosphate |
| **EB** | Ethidium bromide |
| **$EC_{50}$** | The Effective concentration that leads to 50% maximal response |
| **EDTA** | Ethylenediaminetetracetate |
| **Ery** | Erythromycin |
| **FQ** | Fluoroquinolones |
| **Fus** | Fusidic acid |
| **FZ** | Fluconazole |
| **GM** | Gentamicin |
| **GSH** | reduced glutathione |
| **GSSG** | oxidised glutathione |
| **H33342** | Hoechst 33342 |
| **HGNC** | Human Gene Nomenclature Committee |
| **ICD** | Intracellular domain |
| **IMAC** | Immobilised Metal Affinity Chromatography |
| **IPTG** | Isopropyl-_-D-thiogalactopyranoside |
| **IS** | Indolizine sulphones (e.g. SR33557) |
| **Kb** | Kilobase |
| **$K_m$** | Michaelis Menten constant |

| | |
|---|---|
| **KM** | Kanamycin |
| **KZ** | Ketoconazole |
| **LB** | Luria Bertani |
| **LTC4** | Cysteinyl leukotriene |
| **MANT-ATP** | 2'-(or 3')-O-(N-methylanthramiloyl) adenolsine 5' triphosphate |
| **MDR** | Multi-Drug resistance |
| **MESG** | 2-amino-6-mercapto-7-methylpurine riboside |
| **MF** | Major Facilitator |
| **MFQ** | Mefloquine |
| **MIC** | Minimal Inhibitory Concentration |
| **MMC** | Mitomycin |
| **MRP** | Multidrug Resistance Protein |
| **MTX** | Methotrexate |
| **MV** | Methyl viologen |
| **MZ** | Miconazole |
| **NA** | Nalidixic acid |
| **NBD** | Nucleotide-Binding Domain |
| **NBD-PC** | 7-nitrobenz-2-oxa-1,3-diazolphosphatidylcholine |
| **NBD-PE** | 1-myristoyl-2-(NBD)aminocaproyl]phosphatidylethanolamine |
| **NCBI** | National Centre for Biotechnology Information |
| **Neo** | Neomycin |
| **Nic** | Nicardipine |
| **Nor** | Norfloxacin |
| **Nov** | Novobiocin |
| **NQO** | 4-nitroquinoline N-oxide |
| **NTP** | Nucleotide triphosphate |
| **OL** | Oleandomycin |
| **OM** | Oligomycin |
| **PCR** | Polymerase Chain Reaction |
| **PF** | Proflavine |
| **P-gp** | P-glycoprotein |
| **PhMA** | Phenylmercuric acetate |
| **PI** | Pentamidine isothionate |
| **$P_i$** | Inorganic phosphate |
| **PM** | Puromycin |
| **PVDF** | Polyvinylidene fluoride |
| **PY** | Pyronine Y |
| **RND** | Resistance Nodulation Division |
| **RPM** | Revolutions Per Minute |
| **QA** | Quaternary amine compounds (including benzalkonium chloride and cetyltrimethylammonium bromide) |
| **QUN** | Quinine |
| **QUND** | Quinidine |
| **R6G** | Rhodamine 6G |
| **R123G** | Rhodamine 123G |
| **RB** | Rhodamine B |
| **RP** | Rifampicin |
| **SDS** | Sodium Dodecyl Sulphate |
| **SDS-PAGE** | Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis |
| **SO** | Safranin O |

| | |
|---|---|
| **SM** | Streptomycin |
| **SP** | Spiramycin |
| **SSF** | Stopped-Flow Fluorescence |
| **STDGEN** | Sexually Transmitted Disease Genome |
| **SU** | Sulphonamides |
| **TAM** | Tamoxifen |
| **TC** | Tetracycline |
| **TFPZ** | Trifluoroperazine |
| **TL** | Thiolactomycin |
| **TMD** | Trans-Membrane Domain |
| **$TPP^+$** | Tetraphenylphosphonium |
| **TX100** | Triton X-100 |
| **VB** | Vinblastin |
| **VC** | Vincristine |
| **VP** | Verapamil |
| **X-Gal** | 5-Bromo-4-Chloro-3-Indolyl-_-D-Galactoside |

# Chapter 1
# GENERAL INTRODUCTION

## 1. 1 The Resistance Problem

When antibiotics appeared, they were seen as a true miracle (although penicillin was discovered by Duchesne in 1896 (Duchesne, 1897; Ramon and Richou, 1946), and rediscovered by Fleming in 1929, its clinical use was introduced in 1942 and only widespread by 1946, after which a number of important antibiotics were being discovered; Levy, 1992). They were made by one microorganism and could stop growth and kill another one. Expectations were surpassed until it was realised that bacteria developed resistance against antibiotics (In 1946, 14% of all strains of *Staphylococcus* isolated in one hospital were found to be resistant to penicillin. By the end of that decade, the number had risen to 59%; Levy, 1992). Consequently, as more and more drugs were discovered and extensively used, bacteria were selected that were no longer killed by the antibiotic. These strains settled down in the environment and caused the re-emergence of infections such as tuberculosis, pneumonia, and some other nosocomial infections, that could not be treated by these compounds (Levy, 1998). Soon, it was observed that genes, which were responsible for conferring resistance to antibiotics, were copied and transferred by conjugation from resistant to sensitive organisms, spreading this resistance through bacterial populations (Franklin, 1992). In 1959, bacterial dysentery, linked to *Shigella dysenteriae*, was reported in Japan to be resistant to four different antibiotics. In 1965, a strain of *Salmonella typhimurium*, was shown to be simultaneously resistant to the antibiotics streptomycin, sulphonamides, tetracycline, ampicillin, neomycin, kanamycin and chloramphenicol (Smith, 1966), which has been an evident sign of concern since then.

The ability to transfer genes responsible for conferring resistance to different drugs by conjugation is due to the existence in the bacterial cell of transferable DNA elements of autonomous replication known as plasmids of resistance. This is one of the most important sources of resistance in diverse microorganisms. Other ways by which microorganisms acquire resistance are via transposons, and the events of transduction and transformation. Transposons are a very interesting resistance tool, as

they are mobile genetic elements, which can insert at random into plasmids or the bacterial chromosome independently of the host cell recombination system. In combination, these processes can enable a single bacterium to accumulate multiple antibiotic resistance genes harboured in a single plasmid that can in turn be passed between members of the same species or even across genera. In some transduction events, the host cell bears a special site for the attachment of bacterial viruses, also known as bacteriophages. If they integrate into the host chromosome, when the phage enters into a lytic phase it can carry pieces of that chromosome with its genetic information to a new target cell (Canchaya *et al.*, 2003). Apart from plasmids, during transformation some naked DNA is taken up by the receptor cell and can be incorporated in its genome (Lorencz and Wackernagel, 1994; Dubnau, 1999).

Nowadays, there is a perceived fear of not having useful drugs to combat multiresistant-pathogenical microorganisms. An example of this, was the emergence of vancomycin resistance in enterococcus as this could spread to staphylococcus and pneumococcus, making common infections such as otitis and pneumonia untreatable (Travis, 1994). That fear seemed to become a reality when the first clinical isolate of *Staphylococcus aureus* with decreased susceptibility to vancomycin (MIC = 8 μg/ml) was isolated in Japan in 1997 (Hiramatsu *et al.*, 1997). These forms designated as glycopeptide-intermediate (GISA) or vancomycin-intermediate *S. aureus* (VISA), are characterised by a thickening of their cell wall (Cui *et al.*, 2000) due to an increased number of D-ala-D-ala targets in their outer layers that may trap the vancomycin molecules, increasing their MIC for vancomycin (Hiramatsu, 2002). Although these isolates proved not to be the feared threat and the vancomycin MIC remained low enough (<16 μg/ml), in June 2002, a high-level vancomycin-resistant *S. aureus* (VRSA, MIC= 1024 μg/ml) was finally reported (Chang *et al.*, 2003).

### 1.1.1 Resistance mechanisms

Microorganisms defend themselves from the effects of different drugs by several resistance mechanisms:

1) Modification of the antibiotic or drug
2) Alteration of the drug target
3) Decreased permeability of the cell towards drug molecules

4) Active-efflux of the drug

### 1.1.1.1 Inactivation or enzymatic degradation of the drug

In 1940, eleven years after the crucial discovery of penicillin by Fleming, Abraham and Chain reported the existence of a bacterial enzyme that degraded the antibiotic by hydrolysis of its _-lactam ring, and discussed the possibility of its interference with future penicillin therapy (Abraham and Chain, 1940).

The β-lactamases, which inactivate the β-lactam antibiotics penicillin and cephalosporins, are the best-known example of this type of resistance mechanism. These antibiotics, containing a β-lactam ring, can act as pseudo substrates for the transpeptidases, enzymes that are responsible for the last stages of biosynthesis of the bacterial cell wall. Acylation of the active sites of the transpeptidases takes place. With a slow deacylation rate, these transpeptidases are slow to release the β-lactam ring from their active sites, preventing normal cross linking of peptide chains in the peptidoglycan layer, which remains mechanically weak and susceptible to lysis on changes in osmotic pressure (Walsh, 2000). The β-lactamases cleave the β-lactam ring, rendering these antibiotics inactive. Nowadays, more than two dozen of these enzymes, specified by different bacterial resistance genes, are known and are found in both Gram-positive and Gram-negative bacteria (Jacoby, 1994).

The therapeutic approach to counteract the action of these enzymes has consisted of producing many different semi-synthetic forms of the β-lactam antibiotic. However, bacteria have evolved with point mutations in the β-lactamases genes, resulting in enzymes with different amino-acid sequences and alteration of their substrate specifities (Jacoby and Medeiros, 1991). Additionally, the use of Augmentin (U.S. Trademark), a combination of amoxicillin and clavulanate, an inhibitor of β-lactamases, has also been an effective approach.

Another class of antibiotics that can be enzymatically inactivated are the amino-glycosides (e.g. streptomycin, kanamycin, and gentamicin). These molecules are covalently modified by enzymes such as phosphotransferases, nucleotidyltransferases and acetyltransferases, using the phosphoryl group of ATP, a

nucleotide triphosphate, and the acetyl group of acetyl-CoA, respectively (Shaw *et al.*, 1993; Azucena and Mobashery, 2001). As a result of modification, the antibiotics have reduced affinity for the 16S rRNA in the ribosomal 30S subunit (Fourmy *et al.*, 1996), and they can not exert their effect as inhibitors of protein synthesis. Whereas only one type of chemical modification in the β-lactam antibiotic (hydrolysis of its ring) produces bacterial resistance, a dozen different types of modification are found that leads to resistance to a broad range of aminoglycosides. Aminoglycoside-modifying enzymes that work by acetylation are distributed in several groups and are characterised by the AAC prefix, the best studied being AAC(6')-IV (also known as AAC(6')-Ib) (Okamoto and Suzuki, 1965) and AAC(3)-I (Williams and Northrop, 1978). Enzymes that modify aminoglycosides by adenylation also comprise several groups, best characterised being ANT(4') and ANT(2'')-I (Joshua *et al.*, 1993; Pedersen *et al.*, 1995). Fourteen groups of enzymes phosphorylate aminoglycosides, the best studied being APH(3')-IIIa (McKay *et al.*, 1994), APII(3')-IIa and APH(3')-Ia (Azucena and Mobashery, 2001). The enzymes involved in the chemical modification of amino-glycosides do not share more than 56% amino acid identity, and it is thought unlikely that these enzymes share a common ancestor.

In Gram-positive pathogens a bi-functional enzyme that modifies aminoglycosides has been found, being the only example of fused resistance genes. This enzyme encodes acetyl and phosphotransferase activities in separate protein domains, which have been acquired from different resistance genes (Davies, 1994; Ferretti *et al.*, 1986; Azucena *et al.*, 1997).

In the same way as the aminoglycosides, chloramphenicol is modified by acetylation, and this is carried-out by a dozen different chloramphenicol acetyl-transferases (CATs) (Bannam and Rood, 1991; Parent and Roy, 1992). The modified antibiotic is unable to bind to the ribosome and it cannot exert its effect. One CAT of note is the type I enzyme which acetylates chloramphenicol and also binds the antibiotic fusidic acid, thereby avoiding their action on protein synthesis. As with enzymes that inactivate aminoglycosides, the CATs seemed to have emerged through convergent evolution and do not seem to be related by point mutations to an ancestral gene (Bennett and Shaw, 1983).

The formation of glutathione adducts is a widely used mechanism to detoxify cell poisons in eukaryotes (e.g. herbicides in plants) and is another example of how antibiotics can be inactivated through modification. Although the formation of large quantities of glutathione is common in bacteria, the only example of this kind of modification is the inactivation of fosfomycin, another antibiotic that interferes with cell wall biosynthesis. A plasmid-encoded glutathione-*S*-transferase, which catalyses the formation of an inactive fosfomycin-glutathione adduct, is responsible for the resistance phenotype (Arca *et al.*, 1990). *Serratia marcensens* and *S. aureus* fosfomycin resistance genes were cloned and sequenced but were found not to be related at the sequence level (Suarez and Mendoza, 1991; Zilhao and Courvalin, 1990).

Other examples of resistance by modification of antibiotics are the *O*-phosphorylation of erythromycin and hydrolysis of its lactone ring (O'Hara *et al.*, 1989), and the *O*-nucleotidylation of lincosamides in Gram positive bacteria (Arthur *et al.*, 1987; Brisson-Noël *et al.*, 1988).

### 1.1.1.2 Alteration of the drug target

The main examples of this resistance mechanism relate to key macromolecules in some part of microbial metabolism, such as cell wall synthesis or DNA replication. An example of this mechanism is mutation of the transpeptidases involved in cell wall synthesis to create penicillin-binding proteins (PBPs) with lower affinities for the drug (Song *et al.*, 1987; Chu *et al.*, 1996). The ribosomes are other targets, and at least two different types of modification that render resistance to antibiotics are known.

To confer resistance, these mutations have to involve that part of the bacterial protein that interacts with the antibiotic (e.g. PBPs and ribosomes). When these regions are mutated they show less affinity for the drug and which cannot then interfere successfully with bacterial metabolic processes. *murMN* genes in *Streptococcus pneumoniae*, which are separate from PBPs genes, and which control the addition of the short dipeptide units seryl- or alanyl-alanine to the stem peptide lysine, appeared to build a chemically unusual cell wall enriched with branched

muropeptides (Filipe and Tomasz, 2000). These processes might also make a good target for the design of new antibacterial drugs that work synergistically to treat resistant pneumococcal infections. In ribosomes, methylation of the adenine residue A2058 in the 23S rRNA component of the 50S ribosomal subunit by the Erm methyl transferase (Bussiere *et al.*, 1998) does not impair protein biosynthesis but lowers the ability of erythromycin and pristamycin antibiotics to bind the rRNA.

One of the most concerning examples involving this type of resistance is found in *Mycobacterium tuberculosis*. To counteract the inhibiting effect of streptomycin on protein synthesis, mutations have appeared in the ribosome that prevents the antibiotic from binding. Mutations in the ribosomal gene *rspL*, which encodes the ribosomal protein S12, produce a high resistance to streptomycin when an original lysine residue is changed into arginine. Another mutation affects the *rrs* gene, which encodes 16S rRNA, and also produces resistant phenotypes (Finken *et al*, 1993).

Alteration of the drug target is an effective mechanism for conferring resistance to fluoroquinolones and rifampicins. Fluoroquinolones inhibit the metabolism of bacterial nucleic acids in Gram-positive and Gram-negative bacteria. They interact with the enzyme DNA gyrase, an $A_2B_2$ tetramer encoded by the *gyrA* and *gyrB* genes, which mediates DNA negative supercoiling. The drug forms a complex with the enzyme and doubly cleaved DNA covalently bound to GyrA subunits. In this way the enzyme cannot re-ligate the cleaved DNA, which is accumulated and cell death is induced (Hooper, 1995). Mutations in these genes are linked to resistance to the fluoroquinolones. Rifampicin acts on the bacterial RNA-polymerase β-subunit, encoded by the *rpoB* gene, preventing transcription. Point mutations, deletions and insertions in the 27 base pairs that encode the RNA-polymerase active site, confer resistance against rifampicin in *Escherichia coli* (Lisitsyn *et* al., 1984), *M. tuberculosis* (Mani *et al.*, 2001; Pozzi *et al.*, 1999) and *S. aureus* (Wichelhaus *et al.*, 1999).

We can also find this type of mechanism of resistance in eukaryotes. The resistance of pathogenic fungi to the synthetic nucleotide analogue 5-fluorocytosine (5FC) is the most studied. The nucleotide analogue is transported into the cell by a

cytosine permease and deaminated by a cytosine deaminase to 5-fluorouracil (5FU), then converted into 5-fluorouridylic acid, which is phosphorylated and incorporated into the fungus mRNA. This inhibits fungal growth as the transcript cannot be effectively translated. At the same time, 5FU is converted into 5-fluorodeoxyuridine mono-phosphate, which inhibits the enzyme thymidylate synthase, and by this mechanism prevents DNA synthesis and nuclear division. Resistance against this compound appears when some of the biosynthetic enzymes mutate so as not to utilise this compound, avoiding its incorporation into the RNA of the cell (Vanden Bossche *et al.*, 1994).

In the inhibition of ergosterol biosynthesis we find another example of resistance in eukaryotes. In this case, azole compounds act on the enzyme sterol 14_-demethylase (cytochrome $P_{450}$ 14DM or $P_{45051}$). Mutations in this enzyme reduce its affinity for azoles, thus conferring resistance (Vanden Bossche *et al*, 1994).

Finally, a less generally utilised mechanism is used to arrest the effect of tetracyclines, when some microorganisms (*Streptococcus spp; Staphylococcus spp; Listeria spp; Bacteroides spp*) produce a protein that binds to the ribosome (TC resistance classes M, O, S and Q, respectively), preventing the access of the antibiotic to the ribosomal target (Burdett, 1996).

### 1.1.1.3 Decreased permeability of the cell towards drug molecules

It is well known that the plasma membrane is a very selective barrier to the permeation of solutes between the cell and the extracellular environment. This property ensures the rapid entry to the cell of essential molecules like amino acids, glucose and lipids, the maintenance of metabolic intermediates and the ejection of waste compounds from the cell.

The decrease of cellular permeability seems to be an important mechanism of bacterial resistance, especially in Gram-negative bacteria, which possess an outer membrane. While porins in the outer membrane allow some small hydrophilic molecules to enter, antibiotics are excluded because they are bigger and charged (Nikaido, 1994). This was the first resistance mechanism reported for Penicillin (in

Levy, 1992). Nowadays, some synthetic β-lactam antibiotics can cross through porins and are effective in the absence of any other resistance mechanism. A mutation in the gene that encodes the porin can cause resistance against drugs that enter the cell through this pathway. The decrease of cellular permeability to the drug is effective when it is coupled synergistically with another mechanism of resistance (Nikaido, 2001; 2003).

Other bacteria like *Mycobacterium* possess an outer layer in their cell wall, characterised by the presence of mycolic acids. Resistance to isoniazid has been found in *M. tuberculosis*. This drug inhibits the synthesis of mycolic acids, which are incorporated into their cell wall and are essential for the permeability barrier function (Brennan and Nikaido, 1995).

#### 1.1.1.4 Active-efflux of antibiotics and other drugs

The phospholipid bilayer, the basic structural entity of biological membranes, is substantially impermeable to most water-soluble molecules, including essential molecules. There is a need to transport these molecules across cellular membranes and this is mediated by transport proteins inserted into the lipid bilayer.

The active efflux of compounds from the cell has become one of the most important mechanisms of multiple resistance. In 1980, McMurry reported that the plasmid-encoded resistance to tetracycline in *E. coli* was due to energy-dependent efflux (McMurry *et al*, 1980). This stimulated research into the mechanisms.

Transporters have usually been classified on the basis of three criteria, namely the energy source, the phylogenetic relationship, and the substrate specificity. The so-called primary active transporters mainly use chemical energy and constitute the bulk of drug efflux pumps in eukaryotic cells (Paulsen *et al.*, 1998). They are usually energised by ATP. Secondary active transporters predominate in bacteria (Paulsen *et al.*, 1998) (Table 1.1) and act as symports and antiports, coupling the movement of the solute to the transport of an ion along a concentration gradient (Poolman and Konings, 1993).

Within each of these two main classes of transporters, phylogenetic studies have led to the recognition of superfamilies, families and clusters, in correlation with their substrate specificity. According to their molecular assembly and the homology of their sequences, four families were known that presented this type of resistance mechanism in bacteria (Nikaido, 1994): the major facilitator (MF) superfamily, which shows homology to sequences of the glucose facilitators of mammalian cells (Saier *et al.*, 1999; Pao *et al.*, 1998; Ward *et al.*, 2001); the small multidrug resistance (SMR) family that consists of small transporters containing only four transmembrane helices (Paulsen *et al.*, 1996; Chung and Saier, 2001); and the resistance-nodulation-cell division (RND) that includes transporters for expelling cadmium, cobalt and nickel ions (Tseng *et al.*, 2003; Paulsen *et al.*, 1996; Saier *et al.*, 1994). These three families are classified as secondary active transporters (Fig. 1.1). The ATP-binding cassette (ABC) transporters comprise the fourth family. In prokaryotes, transport or extrusion of drugs is predominantly carried out by $H^+$-antiporters that belong to the first three families. The ABC transporters are less represented in prokaryotes, but they are much more prevalent in eukaryotic organisms. This family of transporters use ATP to drive the expulsion of antibiotics and other compounds from the cell. Two new families of transporters, named DME (drug metabolite efflux) and MATE (multidrug and toxic compound extrusion) (Putman *et al.*, 2000) have been added. A re-classification of SMR family as member of the DME has been suggested (Poole, 2004) and, a new superfamily named drug/metabolite transporter (DMT) has been proposed (Jack *et al.*, 2001), which includes the DME family.

Structurally, the MF family is characterised by having 12 membrane-spanning helices with a central loop between helices 6 and 7 that joins the two halves of the transporter, and as they are sequence-related, family members are thought to have arisen by gene duplication. There is another subfamily having 14 _-helices, which could have evolved from the insertion of an increasingly hydrophobic central loop into the 12 _-helices precursor (Saier *et al.*, 1999). The average size for the transporters of this family is 400 amino acid residues. Sugars and drugs are the main compounds transported by members of this family. TetA(B) and TetB(K) tetracycline transporters in *E. coli* and *S. aureus* respectively, are some of their best studied representatives. A particular case is shown where some MF (e.g. EmrB) and RND transporters can act in concert with an outer membrane protein such as TolC, which

acts as a protein channel that spans the outer membrane and the periplasm, for the translocation of drugs (Fig. 1.1).

The SMR transporters are the smallest of these four groups, composed approximately of 100 amino acids arranged into four helices (Paulsen *et al.*, 1996). Different studies of the *E. coli* multidrug transporter EmrE, that confers resistance to ethidium bromide and methyl viologen (Yerushalmi *et al.*, 1995, 1996), indicated that it could function as an oligomer (trimer) of similar tertiary structure to the MF transporters (Rotem *et al.*, 2001) or, as suggested by 2-D crystallisation trials, perhaps as a dimer (Borges-Walmsley *et al.*, 2003). Studies had indicated that EmrE was a tetramer, comprised by two "conformational heterodimers", whose polypeptides were chemically but not structurally identical (Ma and Chang, 2004). However, a homodimer conformation was confirmed for this protein in complex with its substrate tetraphenylphosphonium (TPP) (Pornillos *et al.*, 2005). In this conformation both subunits present opposite orientations in the membrane (they are antiparallel) and adopt slight different folds.

RND transporters also have a 12 _-helix topology but with a large periplasmic domain between helices 1 and 2, and 7 and 8, and are much bigger than those of the MF family, consisting of some 1000 amino acid residues. Again, the resemblance in the sequences of the two halves suggests an internal duplication of a 6 _-helix segment (Tseng *et al.*, 2003). All the members of this superfamily work as efflux transporters. AcrB, whose structure has been already determined (Murakami *et al.*, 2002), is one of the best-studied examples in this family of transporters, considered in Gram-negative bacteria, the most relevant multidrug efflux system in terms of resistance against clinically important agents (Poole, 2004). The DME family, recently described but poorly defined, seems to show similarities to the SMR family of proteins (Jack *et al.*, 2001).

MATE transporters are comparable in size to the MF transporters, with an average composition of 450 amino acids putatively arranged into 12 helices, but do not share similar sequences with members of the MF family. MATE transporters are a relatively new family among bacterial drug transporters, being less characterised than the former transporters. NorM and YdhE from *Vibrio*

*parahaemolyticus* and *E. coli* respectively, are examples of these transporters that have been characterised, with NorM revealed as being a multidrug $Na^+$-antiporter that confers resistance against dyes, fluoroquinolones and aminoglycosides (Morita *et al.*, 1998); and YdhE conferring resistance to cationic antimicrobials (Yang *et al.*, 2003) (Table 1.1; Borges-Walmsley *et al.*, 2003).

| Pump | Organism | Substrate/modulatory ligands | MFP/OMP | References |
|---|---|---|---|---|
| **MF** | | | | |
| TetA | *E.coli* | TC | | Yin *et al.*, 2000 |
| EmrB | *E.coli* | CCCP, NA, TL, CHH | EmrA/TolC | Lomovskaya, 1992 |
| EmrD | *E.coli* | CCCP | | Nishino *et al.*, 2001 |
| MdfA/Cmr/ CmlA | *E.coli* | EtBr, PM, TC, Ery, Neo, Nor, CP, IPTG, $TPP^+$, R6G, DAR, DXR, QA, RP | | Yang *et al.*, 2003 |
| FarB | *Neisseria gono-rrhoeae* | Fatty acids | FarA/MtrE | Lee and Shafer, 1999 |
| NorA | *Bacteroides fragilis* | Ery, Nor, PM | | Miyamae *et al.*, 1998 |
| VceB | *Vibrio cholera* | CCCP, NA, Cip, Ery, PhMA, DOC | VceA/TolC | Colmer *et al.*, 1998 |
| TetK | *S. aureus* | TC | | Guay *et al.*, 1993 |
| NorA | *S. aureus* | EtBr, AC, QA, FQ, R6G, $TPP^+$, PM | | Markham *et al.*, 1999 |
| QacA | *S. aureus* | EtBr, QA, CH, PI | | Mitchell *et al.*, 1999 |
| Bmr | *Bacillus subtilis* | EtBr, AC, QA, FQ, R6G, $TPP^+$, PM | | Neyfakh A., 1992 |
| LmrP | *Lactococcus lactis* | EtBr, DM, $TPP^+$, QUN, TX100 | | Van Veen *et al.*, 1999 |
| MdrL | *Listeria monocytogenes* | EtBr, Ery, CTX | | Mata *et al.*, 2000 |
| Tap | *M. tuberculosis* | TC | | Wu *et al.*, 1999 |
| Mmr | *M. tuberculosis* | EtBr, AC, Ery, TPP, SO, PY | | Wu *et al.*, 1999 |
| Tap | *Mycobacterium fortuitum* | TC, GM, 2-*N*'- and 2-N'-ethylnetilmicin, SM | | Ainsa *et al.*, 1998 |
| LfrA | *Mycobacterium smegmatis* | EtBr, AC, QA, FQ | | Sander *et al.*, 2000 |
| CaMDR1 | *Candida albicans* | FZ, NQO, benomyl, MTX | | Vanden Bossche *et al.*, 1998 |
| **SMR** | | | | |
| Smr/QacC | *S. aureus* | EtBr, CV, QA, MV, $TPP^+$ | | Littlejohn *et al.*, 1992 |
| EmrE | *E.coli* | EtBr, AC, MV, $TPP^+$ | | Yerushalmi *et al.*, 1995 |
| EbrAB | *B. subtilis* | EtBr, AC, $TPP^+$ | | Masaoka *et al.*, 2000 |
| YkkCD | *B. subtilis* | EtBr, PF, CV, PY, MV, CPC, CP, SM, TC, TPP | | Jack *et al.*, 2000 |
| Tbsmr | *M. tuberculosis* | AC, EtBr, MV | | Wu *et al.*, 1999 |
| Pasmr | *Pseudomonas aeruginosa* | AC, EtBr, MV | | Li *et al.*, 2003 |
| QacE | Gram-negative organisms | EtBr, QA, SU | | Kucken *et al.*, 2000 |
| QacEΔ1 | Gram-negative and positive organisms | Cip, EtBr, GM, QA, SU, TC | | Kucken *et al.*, 2000 |

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| | | | | |
|---|---|---|---|---|
| **RND** | | | | |
| AcrB | *E. coli* | EtBr, AC, CV, SDS, TX-100, bile salts, _-lactams, Nov, Ery, Fus, TC, CP, MMC, FQ, NA, organic solvents, NBD-PC | AcrA/TolC | Murakami *et al.*, 2002 |
| AcrB | *Haemophilus influenza* | EtBr, CV, Ery, RP, Nov | AcrA/ MexA/OprM | Sanchez *et al.*, 1997 |
| MexB | *P. aeruginosa* | AZ, Cip, CV, RP, TX-100 | | Li *et al.*, 2003 |
| SmeE | *Stenotrophomonas maltophila* | EtBr, Ery, TC | | Alonso and Martinez, 2000 |
| AcrB | *S. typhimurium* | AC, DOC, Cip, Ery, Fus, NA | | Lacroix *et al.*, 1996 |
| ArpB | *Pseudomonas putida* | Nor, Nov, RP, SDS, TC | | Kieboom and de Bont, 2001 |
| MtrD | *N. gonorrhoeae* | Cip, Ery, Nov, TC, TX-100, _-lactams, AZ, vertebrate peptide antimicrobials, spermicides | MtrC/E | Hagman *et al.*, 1995 |
| **MATE** | | | | |
| BexA | *Bacillus thetaiotamicron* | AC, EtBr, MV | | Miyamae *et al.*, 2001 |
| NorM | *Vibrio parahaemolyticus* | CP, EtBr, Nor | | Morita *et al.*, 1998 |
| YdhE | *E. coli* | AC, Cip, KM, Nor, SM, $TPP^+$ | | Morita *et al.*, 1998 |
| VcmA | *V. cholera* | Nor, Cip, DAR, DXR, SM, KM, EtBr, H33342 | | Huda *et al.*, 2001 |
| YdhE | *B. fragilis* | Nor, Cip, EtBr | | Miyamae *et al.*, 1998 |
| NorM | *N. gonorrhoeae* | AC, EtBr, Ber, Cip, Nor | | Rouquette-Loughlin *et al.*, 2003 |
| NorM | *Neisseria meningitidis* | AC, EtBr, Ber, Cip, Nor | | Rouquette-Loughlin *et al.*, 2003 |

**Table 1.1 Secondary transporters that act as drug pumps**

MFP and OMP stand for membrane fusion protein and outer membrane protein, respectively.

Substrate and ligand abbreviations: AC, acriflavin; AZ, azithromycin; Ber, berberine; CCCP, carbonyl cyanide m-chlorophenylhydrazone; CTX, Cefotaxime; CH, chlorhexidine; CHH, 2-chlorophenylhydrazine hydrochloride; Cip, ciprofloxacin; CL, clarithromycin; CP, chloramphenicol; CPC, cetylpyridinium chloride; CV, Crystal Violet; DAR, daunorubicin; DM, daunomycin; DOC, deoxycholate; DXR, doxorubicin; Ery, erythromycin; FQ, fluoroquinolones; Fus, fusidic acid; FZ, fluconazole; GM, gentamicin; H33342, Hoechst 33342; IPTG, isopropyl _-D-thiogalactoside; IS, indolizine sulphones (e.g. SR33557); KM, kanamycin; KZ, ketoconazole; LTC4, cysteinyl leukotriene; MMC, mitomycin; MTX, methotrexate; MV, methyl viologen; MZ, miconazole; NA, nalidixic acid; NBD-PC, 7-nitrobenz-2-oxa-1,3-diazolphosphatidylcholine; NBD-PE, 1-myristoyl-2-[6-(NBD)aminocaproyl]phosphatidylethanol-amine; Neo, neomycin; Nor, norfloxacin; Nov, novobiocin; NQO, 4-nitroquinoline N-oxide; OL, oleandomycin; OM, oligomycin; PF, proflavine; PhMA, phenylmercuric acetate; PI, pentamidine isothionate; PM, puromycin; PY, pyronine Y; QA, quaternary amine compounds (including benzalkonium chloride and cetyltrimethylammonium bromide); QUN, quinine; R6G, rhodamine 6G; R123G, rhodamine 123G; RB, rhodamine B, RP, rifampicin; SO, safranin O; SM, streptomycin; SP, spiramycin; SU, sulphonamides; TC, tetracycline; TL, thiolactomycin; $TPP^+$, tetraphenylphosphonium; TX100, Triton X-100; VB, vinblastin; VC, vincristine; VP, verapamil. (modified from Borges-Walmsley *et al.*, 2003).

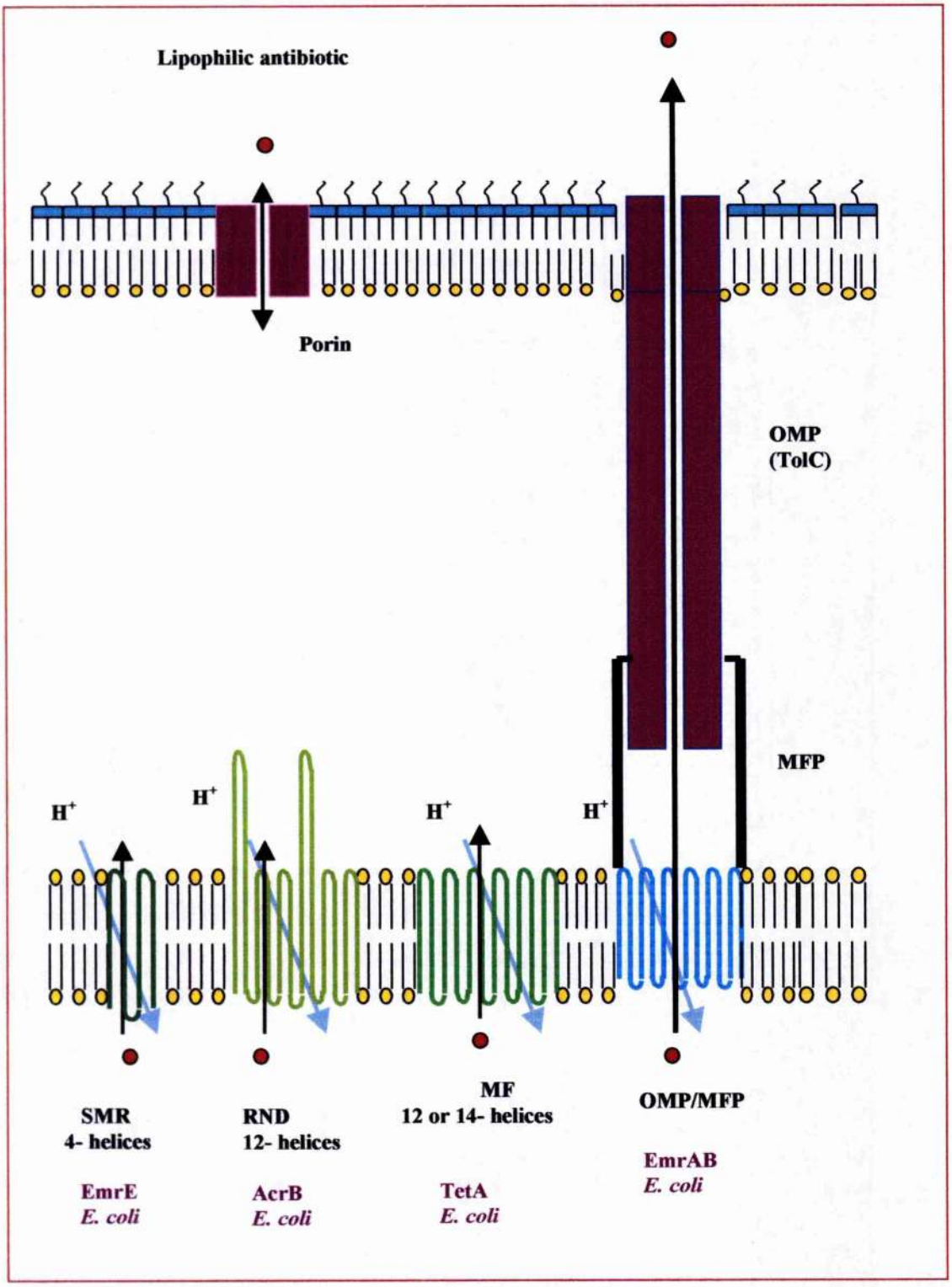

Lipophilic antibiotic
Porin
OMP
(TolC)
MFP
H+
H+
H+
H+
SMR
4- helices
EmrE
E. coli
RND
12- helices
AcrB
E. coli
MF
12 or 14- helices
TetA
E. coli
OMP/MFP
EmrAB
E. coli

**Figure 1.1 Schematic representation of the membrane topology of proton-driven drug pumps representing main Secondary transporters families found in Gram-negative bacteria.**

The helices of the pumps appear spanning the lipid bilayer, and lipophilic antibiotics (in red circles) freely permeate the lipid bilayer. The three classes of antiporters are shown: SMR, four-helix small multidrug transporters; RND, 12-helix resistance-nodulation-cell division transporters; MF, 12- and 14-helix major facilitator transporters. These transporters are shown using the proton motive force generated by respiration to eject antibiotics and other drugs into the periplasmic space between the inner and outer membranes. MF and RND transporters can act in concert with a membrane fusion protein (MFP) and an outer membrane protein (OMP), such as TolC, to translocate drugs across both the inner and outer membranes, expelling them from the cell. TolC acts as a protein channel that spans the outer membrane as a _-barrel, and also possibly spans the periplasm, as an -helical barrel (Koronakis *et al.*, 2000). Although the exact role of the MFP remains unknown, it may act as a protein channel translocating drugs between the inner-membrane transporter and the helical barrel of TolC. Alternatively, it is believed that MFP pulls the two membranes together, so that the inner-membrane transporter can pass drugs directly (based on diagram by Borges-Walmsley *et al.*, 2003, and McKeegan *et al.*, 2004).

ATP-Binding Cassette Transporters carry out an energy dependent active transport process by which substances cross the cell membrane driven by the hydrolysis of ATP (Higgins, 1992). They have also been proposed to have a 12 _-helix topology. The ability of these transporters to translocate a number of unrelated molecules, including antibiotics, and the fact that they contribute the main pathway for resistance to anticancer drugs in humans, highlights their importance and urgent need to study them.

The SMR transporter EmrE can exemplify a putative mechanism for multidrug transport involving the proton motive force in secondary transporters. Based upon site-directed mutagenesis experiments, the following steps have been proposed (Paulsen *et al.*, 1996; Mordoch *et al.*, 1999; Yerushalmi and Schuldiner, 2000; Schuldiner *et al.*, 2001) (Fig. 1.2):

a) Exchange between the drug and a pair of protons associated with a charged residue in the protein.
b) Translocation of the drug by a series of conformational changes driving it through a hydrophobic pathway (in the EmrE case), and
c) Replacement of the drug by a pair of protons in the external medium and return to the initial conformational state.

The overall result of this process is the exchange of the drug and the proton (antiport). Similar to proton antiporters, a conformational change of the ABC proteins involved in drug extrusion is possible and is probably triggered by drug binding and ATP hydrolysis (Sonveaux *et al*, 1996; Sharom, 1997).

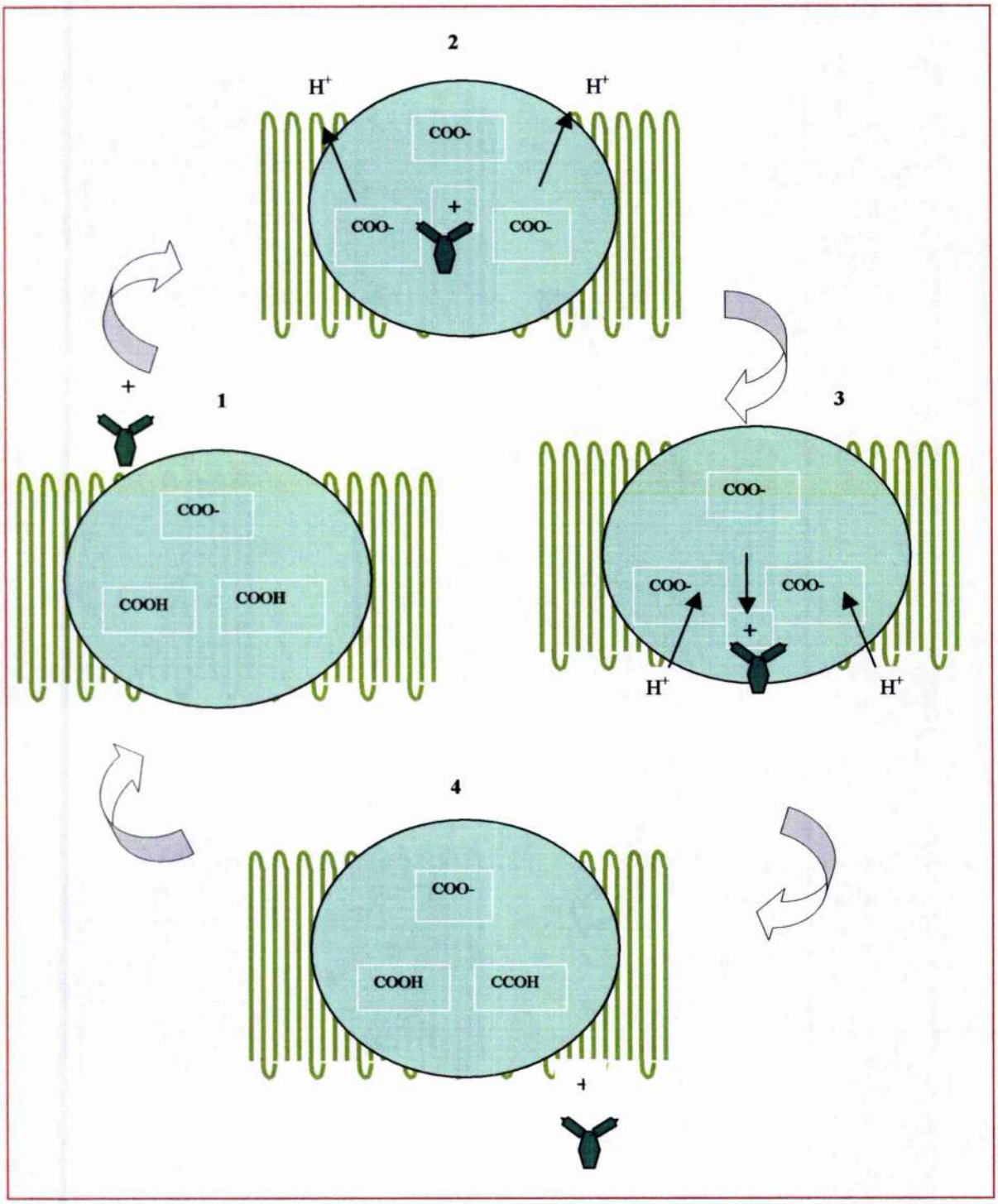

2
H+
H+
COO-
+
COO-
COO-
+
1
3
COO-
COOH
COOH
COO-
COO-
COO-
+
H+
H+
4
COO-
COOH
CCOH
+

**Figure 1.2. Possible multidrug resistance mechanism for *E. coli* SMR EmrE** (based on the Yerushalmy and Schuldiner model; Yerushalmy and Schuldiner, 2000). As the substrate approaches the hydrophobic binding pocket (left), two protons are released from the negatively charged glutamate cluster (top). When occupied by the substrate, the binding pocket becomes accessible to the other face of the membrane (right). The subsequent protonation of the carboxyl groups allows the release of the bound substrate (bottom). The protonated binding pocket relaxes back to the original membrane face so a new cycle can start.

## 1.2 The ATP-Binding Cassette Transporters

ABC proteins are the largest superfamily of proteins with more than 3500 members (Stieger and Biber, 2002). This family of transporters (also called traffic ATPases) includes many different transport proteins, found in organisms ranging from bacteria to humans (Lodish *et al*, 2000). In *E. coli*, about 5% of the entire genome encodes components of ABC transporters (Linton and Higgins, 1998).

While there are ABC groups involved only in the import or export of molecules, other members of this superfamily are not involved in transport (Dassa and Bouige, 2001). In prokaryotes, these transporters are localised exclusively in the cytoplasmic membrane of bacteria, while in eukaryotic organisms, they are found in the plasma membrane, endoplasmic reticulum membrane, mitochondrial inner membrane and intracellular vacuoles.

The histidine periplasmic permease system, HisJQMP, of *S. typhimurium* (Ames, 1972), and the maltose transport system, MalEFGK, of *E. coli* (Davidson and Nikaido, 1991) were pioneer systems in the gradual discovery of the family. The human multidrug resistance P-glycoprotein, which renders cells resistant to anti-cancer treatments, has become the paradigm for ABC transporters involved in the problem of resistance and has renewed interest in the study of these transporters (Sharom, 1997). The term ABC was coined in 1990 (Hyde *et al*., 1990) enhancing the recognition of the importance of this diverse family of proteins. Nowadays, many more ABC transporters have been recognised in resistance to diverse classes of drugs and other chemical compounds (Table 1.2).

| Pump | Organism | Substrate/modulatory ligands | References |
|---|---|---|---|
| MDR1/P-gp (ABCB1) | *Homo sapiens* | DNR, DOX, R6G, R123G, H3342, NBD-PE, NBD-PC, PM, VC, VB, CC, TAXOL, dexamethasone, steroids, VP, Nic, IS, antimalarials | Sauna *et al.*, 2001 |
| MRP1 (ABCC1) | *H. sapiens* | Similar specificity to P-glycoprotein + $LTC_4$ and other glutathione s-conjugates. | Leslie *et al.*, 2001 |
| $Pdr_5$ (ABCG subfamily) | *Saccharomyces cerevisiae* | R6G, R123G, RB, FZ, DNR, DOX, OM, CHX, CP, TAM, TFPZ, steroids, NBD-PE/VB, VC, TAXOL, VP, ionophoric peptides, QUN, CC. | Golin *et al.*, 2003 |
| $Cdr_1$ (ABCG subfamily) | *C. albicans* | FZ, KZ, MZ, steroids | Vanden Bossche *et al.*, 1998 |
| $Pfmdr_1$ | *Plasmodium falciparum* | MFQ, Art, Cc, a-factor (yeast pheromone). | Peel, 2001 |
| PGPA | *Leishmania spp.* | Pentostam, metal-thiol conjugates | Callahan *et al.*, 1994 |
| LmrA | *L. lactis* | EB, R6G, R123G, DNR, DOX, VB, VC, DM, CC, $TPP^+$, H33342, NBD-PE/VP, Nic, QUN, QUND, CA, IS. | van Veen *et al.*, 2000 |
| DrrAB | *Streptomyces peucetius* | DOX, DNR | Kaur, 1997 |
| MacB | *E. coli* | AZ, CL, Ery, OL | Kobayashi *et al.*, 2001 |
| Msr | *Streptomyces rochei* | DXR, Ery, OL, SP, TC | Fernandez-Moreno *et al.*, 1998 |
| YvcC | *B. subtilis* | P-gp homologue | Steinfels *et al.*, 2002 |
| MsbA | *E. coli* | Major transporters of lipids from cytoplasm to outer membrane | Chang and Roth, 2001 |
| Pst | *M. smegmatis* | FQ | Banerjee *et al.*, 2000 |

## Table 1.2 ABC drug pumps

Abbreviations in Table 1.2: ABC, ATP-binding cassette; Art, artemisinin; AZ, azithromycin; Cc, chloroquine; CC, colchicine; CHX, cycloheximide; CL, clarithromycin; CP, chloramphenicol; DM, daunomycin; DNR, daunorubicin; DOX, doxorubicin; EB, ethidium bromide; Ery, erythromycin; FQ, fluoroquinolones; FZ, fluconazole; H33342, Hoescht 33342; IS, indolizine; KZ, ketoconazole; $LTC_4$, cysteinil leukotriene; MFQ, Mefloquine; MZ, miconazole; NBD-PC, 7-nitrobenz-2-oxa-1,3-diazol-phosphatidylcholine; NBD-PE, 1-myrystoil-2-[6-(NBD) aminocaproyl]-phosphatidyl-ethanolamine; Nic, nicardipine; OL, oleandomycin; OM, oligomycin; PM, puromycin; QUN, quinine; QUND, quinidine; R6G, rhodamine 6G; R123G, rhodamine 123G; RB, rhodamine B, SP, spiramycin; TAM, tamoxifen; TC, tetracycline; TFPZ, trifluoroperazine; $TPP^+$, tetraphenylphosphonium; VB, vinblastin; VC, vincristine; VP, verapamil. (modified from original by Borges-Walmsley *et al.*, 2003).

### 1.2.1 Identification of ABC transporters

Nutrient uptake studies in *E. coli* gave the first indications of the existence of this remarkable group of proteins (Berger, 1973). Three systems were found responsible for nutrient transport: the phosphotransferase group, an osmotic shock-insensitive transporter group energised by the electrochemical gradient (secondary transporters), and a third group formed by primary, osmotic shock-sensitive systems energised by the hydrolysis of ATP and associated with periplasmic binding proteins. By using membrane vesicles was noticed that cells subjected to osmotic shock exhibited reduced transport rates for certain amino acids, whereas the uptake of others remained unaffected by the treatment. Subsequent studies showed that the transport in the shock-sensitive systems was abolished due to the loss of a vital substrate-binding protein (SBP) from the periplasm (Heppel, 1969).

Additional evidence for the presence of more of one type of transport was collected by studies of energy coupling mechanisms in *E. coli* (Berger, 1973; Berger and Heppel, 1974). SBP-dependant transporters were found to be insensitive to the effect of chemicals that uncoupled oxidative phosphorylation, whilst shock-insensitive transporters lost the capacity to uptake nutrients. Investigators concluded that the energy donors for the two classes of transporters were essentially different. The shock-sensitive transporters (SBP-dependant) required phosphate-bond energy to drive the transport process, whereas in the shock-resistant systems transport was driven by an energised membrane state, provided by the proton motive force generated via oxidative phosphorylation.

In 1982, the first complete sequence of one of these periplasmic transporters, the histidine transporter of *S. typhimurium*, was published (Higgins *et al.*, 1982). Apart from the periplasmic "substrate"-binding protein (HisJ), this transporter has three membrane-associated components (HisQMP). Shortly after this sequence was published, the sequence of a component of the *E. coli* maltose transporter (MalK) was also determined. A 32% identity between proteins MalK and HisP was reported (Gilson *et al.*, 1982). The similarity in both sequences suggested they evolved from a common ancestor. When the oligopeptide transporter of *S. typhimurium* OppD was also shown to share similarities with the aforementioned proteins (Higgins *et al.*,

1985), it was noticed that they included a consensus nucleotide-binding motif similar to those previously identified in ATP synthase, myosin and adenylate kinase (Walker *et al.*, 1982). From that moment, the involvement of these domains in coupling ATP hydrolysis with the transport process became a central feature.

Although the first eukaryotic example of this family of transporter, the human multidrug resistance P-glycoprotein, was identified in 1974 (Ling and Thompson, 1974; Juliano and Ling, 1976), the recognition that the ATP binding subunits from bacterial transporters described a large superfamily of proteins dates from 1986. Around this time, it was also proposed they were organised in a core of four domains. These domains were composed by two integral membrane subunits and by two nucleotide-binding subunits, which bind and hydrolyse ATP (Higgins *et al*, 1986). Actually, the general approach to the identification and analysis of ABC transporters consists in searching sequence conservation in the nucleotide-binding sites (NBDs) first. Thereafter, the search is directed to find genes that code for proteins with membrane spanning domains (MSDs) and proteins with solute-binding motifs.

As ATP-binding domains display a high degree of sequence similarity, conserved motifs can be deduced using the PROSITE web site (http://br.expasy.org/prosite/). The ABC signature sequence (LSGGQ) was used initially to characterise these domains and it is commonly present in members of the ABC family. Nowadays two additional internal domains are used for this purpose: the first domain is that of the Walker A motif, also known as the ATP/GTP-binding motif (PROSITE:PDOC00017). The second domain overlaps the ABC signature motif (also known as C motif) (PROSITE:PDOC00185) and the Walker B motif. Walker A and Walker B motifs can be present in ATPases that are not part of the ABC family.

Having identified NBDs, genes that can code for membrane-spanning domains (MSDs) are sought. Four or more transmembrane spanning sequences in proteins with NBDs or in gene products from NBD-adjacent genes become candidates for MSDs components. Some MSD-containing proteins (prokaryote permeases) also contain a conserved EAA---G---------I-LP motif located in a cytoplasmic loop

(PROSITE:PDOC00364), that can be used in the analysis of these transporters. This sequence is believed to interact with the NBD (Mourez *et al.*, 1997).

The current methodology underpinning these studies takes advantage of the STDGEN and ORALGEN databases as primary sources of protein and DNA sequences. Searches for similarity are based on the use of some BLAST tools from NCBI. The XDOM tool (Gouzy *et al.*, 1997) was also used to analyse the modular arrangement of protein domains. The PHDhtm program was used to predict the number and location of transmembrane regions and the pattern-matching program SIGNALP, to predict the signal peptide. The ClustalW (Thompson *et al.*, 1994) and MultAlign program (Corpet *et al.*, 1988) were used for multiple alignments.

### 1.2.2 The structural organisation of ABC transport proteins

Generally, ABC transporters have four domains arranged into two homologous halves. Each has a transmembrane domain arranged into six _-helices, and a nucleotide-binding domain (NBD) of about 200 amino acids (Ouellette *et al*, 1994) (Fig. 1.3). The transmembrane domains seem to be composed of very hydrophobic integral proteins, and their two six helix motifs are arranged so as to form a transmembrane pathway to transport the selected compounds. Additional membrane domains have been reported in some cases, but they do not seem to change the general behaviour of the system; for example, an extra five-helix membrane domain has been located at the N-terminus of the multidrug resistance protein (MRP) (Hou *et al.*, 2000). The other two domains that bind nucleotides are relatively hydrophilic and peripheral to the membrane. It is thought that they appear in the cytoplasm associated with the membrane. Their hydrolysis of ATP releases energy to drive the efflux of different compounds out of the cell (Van Bambeke, 2000; Lodish *et al.*, 2000).

In several cases, a single gene encodes the four domains (e.g. P-glycoprotein; Higgins, 1992). In other cases, each domain is encoded as a separate polypeptide (e.g. the OppBCDF transporter; Hiles *et al.*, 1987). Intermediate genetic organisation of ABC transporters can also exist, where separate genes encodes both membrane domains and both NBDs. A diagram of the topology of drug transporters belonging

to the ABC superfamily is presented (Fig. 1.4). Exceptions to the classical arrangement of four domains is exemplified by the multidrug transporter LmrA from *Lactococcus lactis*, where the *lmrA* gene encodes a protein with a fused single membrane and nucleotide binding domain (van Veen *et al.*, 1996), and *drrA* and *drrB* from *S. peucetius* (Kaur, 1997), which encode individually the NBD and the membrane domain respectively. Recent experiments have shown that DrrB forms at least eight helices instead of the six-helix model proposed for ABC transporters (Gandlur *et al.*, 2004). In cases in which one of the ABC domains appears to be absent, one of the remaining domains functions as a homodimer to maintain the proposed ABC complex (Higgins, 2001). As LmrA is thought to be assembled as a homodimer and DrrAB as a tetramer, it is believed that these transporters are topologically analogous to full size ABC transporters. In full size ABC transporters, the two halves of the protein resemble one another, suggesting that they have originated by gene duplication (Saier and Paulsen, 2001). Additional proteins are involved in some of these ABC systems. That is the case of importers (periplasmic binding-protein dependant (BPD)-ABC transporters), which employ an extra protein to bind the substrate to be transported (e.g. histidine and maltose transporters), but these proteins are associated in a reversible manner and do not form part of the four domain complex. Another example is the additional "R domain" present in the cystic fibrosis transmembrane conductance regulator (CFTR). The reversible phosphorylation of the R domain seems to regulate the function of the $Cl^-$ channel (Gadsby and Nairn, 1999).

P-glycoprotein (ABCB1), which is a large plasma-membrane glycoprotein, has two similar halves, characteristic of many of the transporters of this superfamily, containing six putative transmembrane helices and one NBD (Loo and Clarke, 1995; Kast *et al.*, 1995). The two NBDs share 30-40 % amino acid sequence identity with each other and the equivalent domains of many other transporters included in the ABC family (Higgins *et al.*, 1997). Besides P-glycoprotein, which is considered the most important example of an ABC transporter because it is responsible for resistance in humans against antitumoral drugs, MRP (ABCC1) (multiple resistance protein), which acts in the lungs, is also an important resistance-related transporter (Cole *et al.*, 1992). Other ABC proteins of medical importance are the chloride-channel protein involved in cystic fibrosis (CFTR; ABCC7; Gadsby and Nairn, 1999), the human

adrenoleukodystrophy protein (ALDP; ABCD1), an ABC transporter associated with transport of very long chain fatty acids (VLCFAs) into the peroxisome (Mosser *et al*, 1993), and the endoplasmic reticulum (ER) peptide transporter involved in antigen presentation (TAP; ABCB2 and ABCB3) (Abele and Tampe, 1999).

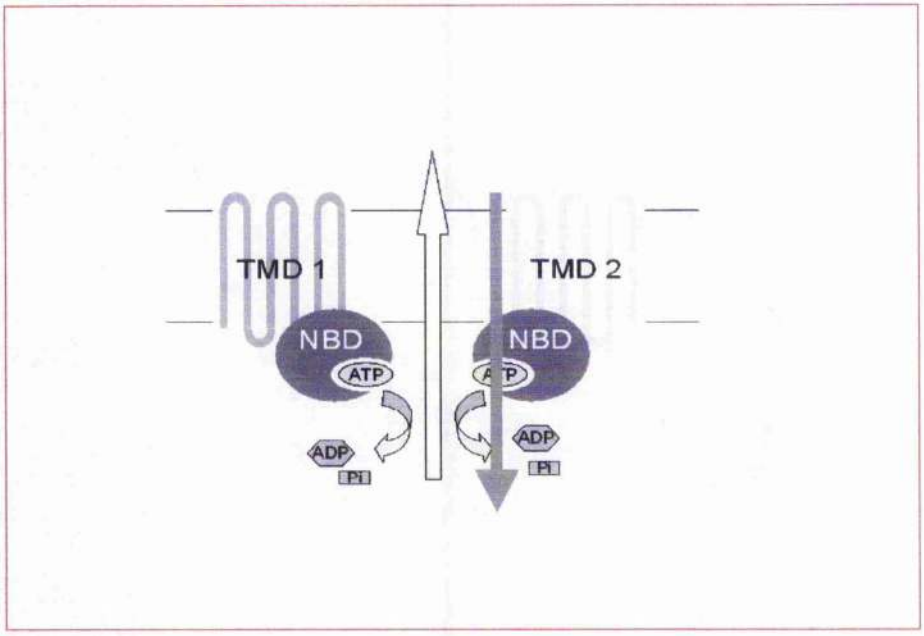



**Figure 1.3 Putative structural organisation of an ABC transporter**

TMD: Transmembrane domain

NBD: Nucleotide binding domain

ABC transporters have four domains arranged into two homologous halves. Each one possesses a transmembrane domain arranged into six α-helices, and a nucleotide-binding domain. Some transporters function as importers whilst other export molecules out of the cell.

This model depicts a structure with each half (TMD-NBD) of the transporter encoded by a single gene.

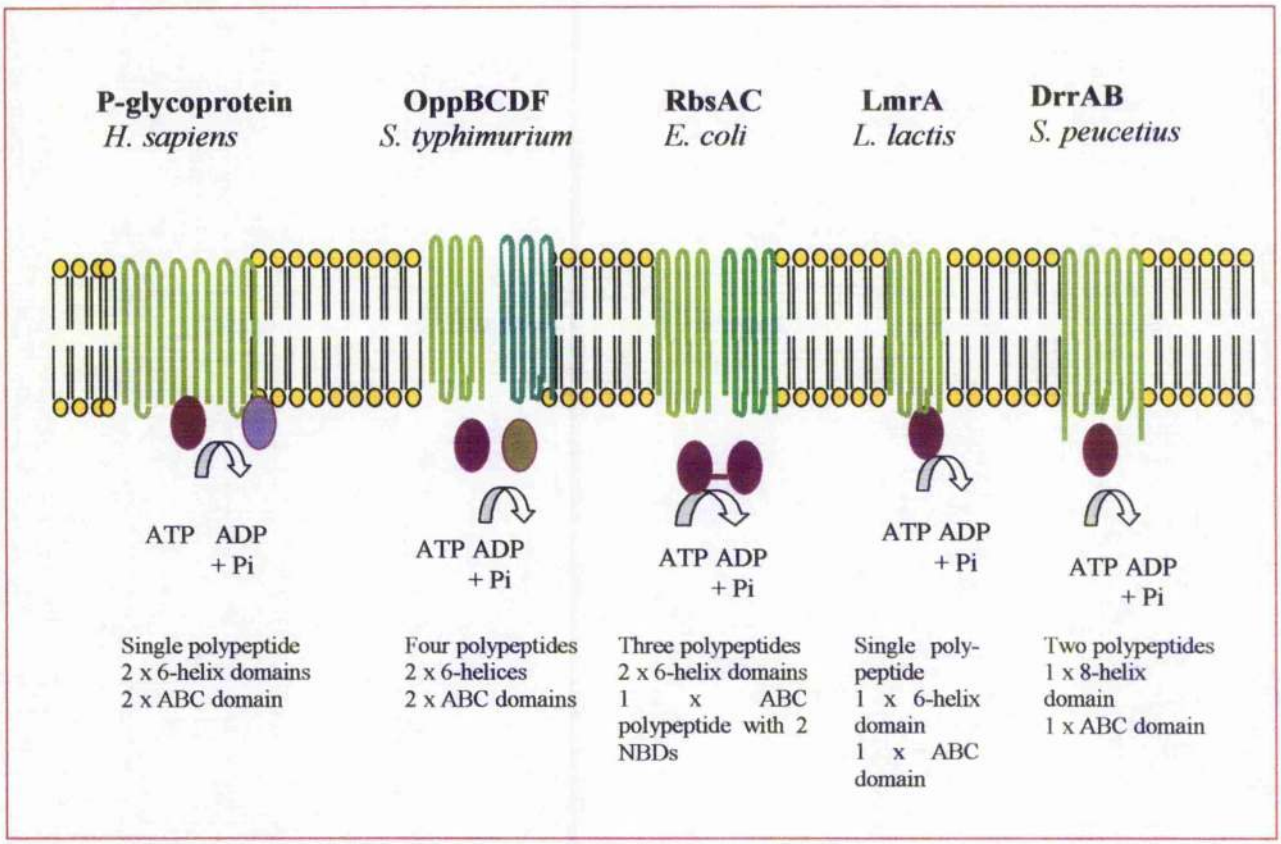



**Figure 1.4 Schematic representation of the membrane topology of diverse transporters belonging to the ABC superfamily**

The prototype of ABC transporter presents two NBDs and 12 membrane-spanning α-helices arranged in two groups of six. In P-gp, a single gene encodes all four domains (Higgins, 1992); in OppBCDF each of the four domains are encoded by different genes (Hiles *et al.*, 1987); in LmrA, a single gene encodes only one membrane and one NBD (van Veen *et al.*, 1996); for DrrAB, separate genes encode one membrane and one NB domain (Kaur, 1997)*. Intermediate genetic organisations are also found: a single gene encodes both NBDs of RbsAC whilst membrane domains are encoded separately (Lida *et al.*, 1984); or a single gene encodes both TMDs and NBDs but as heterodimers, as for TAP (Daumke and Knittler, 2001). MRP, C-class eukaryotic member (not shown) is also encoded by a single gene as P-gp, but it carries an additional five-helix TMD at the N-terminus (Hou *et al*, 2000).

*A new model has been proposed for the *S. peucetius* DrrB TMD arranged in a group of eight α-helices (Gandlur *et al.*, 2004).

### 1.2.3 The Nucleotide-Binding Domain (NBD)

The nucleotide-binding domains bind and hydrolyse ATP and couple this hydrolysis to solute translocation across the membrane. These domains consist of a core of about 200-230 amino acids, and contain the ABC signature sequence (LSGGQ), also known as the C-loop or linker-peptide that has been the key to identification of new members of this transporter family. Also included are the two highly conserved sequences, Walker A ($W_A$) and Walker B ($W_B$) motifs (Walker *et al.*, 1982), which are responsible for ATP hydrolysis (Fig. 1.5). It is the conservation of this entire domain which is important in defining the family (Higgins *et al.*, 1986). The Walker A motif is generally present with the sequence "G-X-X-G-X-G-K-S/T", X being any amino acid and a serine or threonine occupying the final position. The lysine in this motif interacts with the _- and _- phosphate groups of ATP, being essential for its hydrolysis. This region is also known as the phosphate (P-) loop. The Walker B motif is less conserved and is represented by the sequence "R/K-X-X-X-G-X-X-X-L-_-_-_-_-D", _ being any hydrophobic residue. An aspartate in the $W_B$ motif is responsible for co-ordinating the $Mg^{2+}$ ion of Mg-ATP, which is required for nucleotide binding. Mutations of the Walker sequences generally impair the ATP binding or hydrolysis, particularly when lysine in the Walker A motif or aspartate in the Walker B motif are involved, leading to a complete loss of the ATPase activity in the protein. The Walker A motif is located near the N-terminus of the NBD, followed by the ABC signature motif, that appears immediately upstream of the Walker B motif (Fig. 1.5).

Based on sequence similarities with the RecA protein in *E. coli*, the so-called "Switch region" has also been reported. This is located downstream of the Walker B motif and is characterised by the presence of a histidine in the last position that seems necessary to maintain the transport integrity (Fig. 1.5). Additionally, the Q loop, also called the _-phosphate switch, between the Walker A motif and signature sequence, and the D loop (Hopfner *et al.*, 2000), that contains an aspartic acid residue downstream of the Walker B motif, also forms part of the conserved sequences (Fig. 1.5). All of these sequences are involved in the interaction with ATP and its hydrolysis.

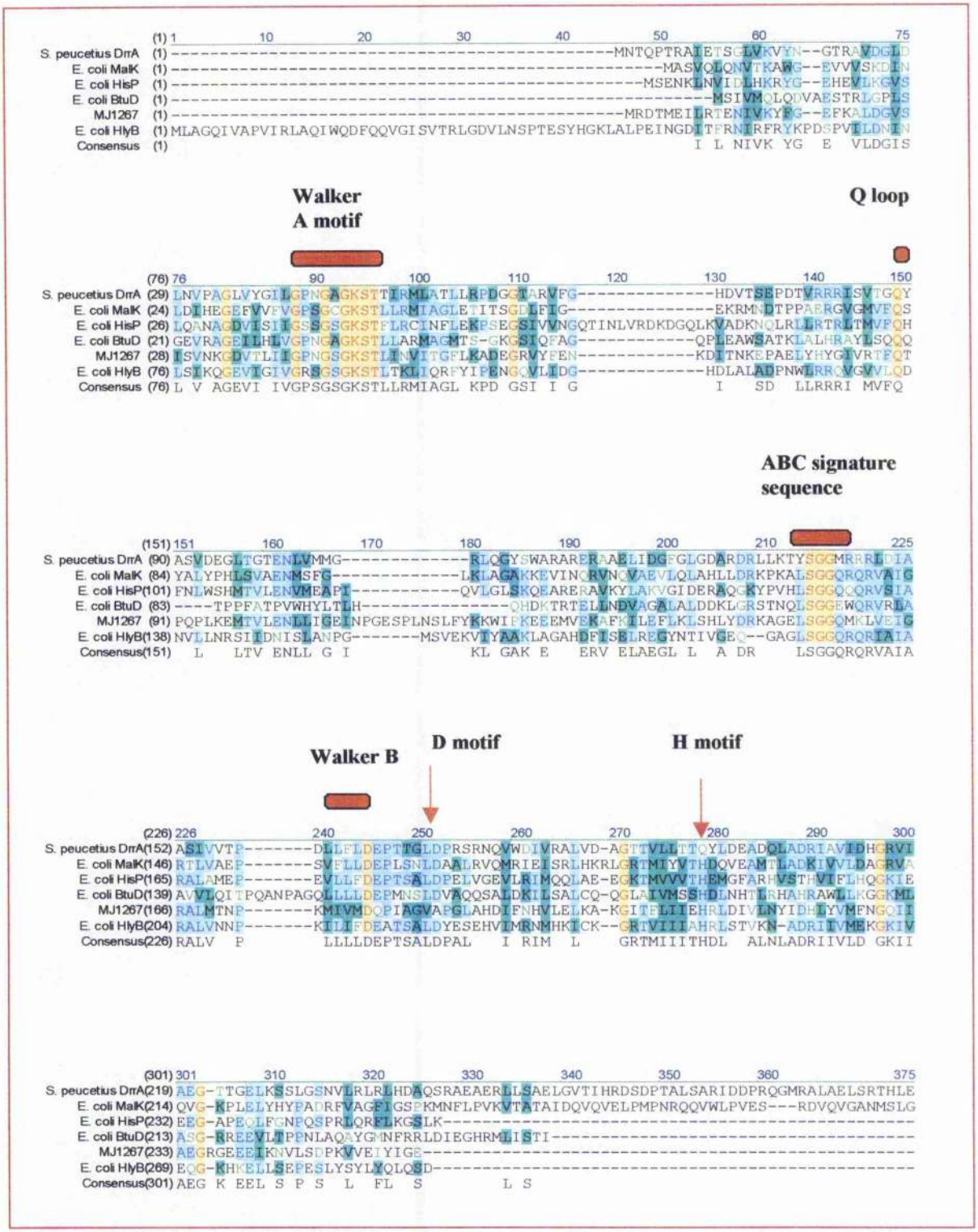



**Figure 1.5 Sequence alignment of *S. peucetius* DrrA and some well-known ABC ATPases**

Sequences are ordered to enable comparison with DrrA. Orange ovals or arrows indicate the main conserved amino acid motifs

### 1.2.3.1 Biochemical properties of ABC transporter Nucleotide-binding domains

The ability to bind and hydrolyse ATP and use the energy released to drive an active transport process, is the elemental feature that characterises these domains. Understanding how ABC transporters interact with the molecules or compounds that are transported is vital in elucidating their physiological role, and also in the design of strategies to circumvent the increasing problem of resistance to antimicrobials and cancer treatments.

The knowledge of the conformations that these NBDs acquire when they interact with the substrate (ATP) and when other components of the transporter system are present is necessary to clarify the whole nature of the process. This section presents information generated by studies of the biochemical factors involved in ATP hydrolysis and conformational changes gathered from work with isolated NBD's and intact ABC transporters alike.

The characterisation of ABC transporters has not proved an easy task. Whilst present in high numbers in eukaryotic cells, overexpression in the native host has not always been successful and has often resulted in low yields. Despite the predicted cytoplasmic localisation of the NBDs, many attempts to express them as soluble proteins or as fusion proteins have also met with limited success in the past. *E. coli* is the most common expression host for experiments of this sort and can overproduce recombinant protein from diverse organisms, retaining the proper folding of the native target protein. Some of the best-characterised ABC transporters that have been overexpressed in *E. coli* are proteins that occur naturally in that host (e.g. the maltose/maltodextrine transporter) or its close relatives (*S. typhimurium* maltose and histidine transporters). The characterisation of these transporter proteins requires the recovery and purification of sufficient amounts of soluble, stable and active protein for analysis by biophysical techniques such as X-ray crystallisation and nuclear magnetic resonance (NMR). Newer approaches such as the definition of domain boundaries within the protein to be overexpressed (Kerr *et al.*, 2003) may aid the effective expression of isolated NBDs.

Working from the four domain structure for an ABC active transporter, it has been suggested that both NBDs are involved in powering the translocation of the transported solute. In systems such as the maltose transporter of *S. typhimurium* and *E. coli*, the transporter is composed of the periplasmic maltose-binding protein MalE (MBP), the two integral cytoplasmic membrane protein subunits MalF and MalG, and two copies of the ATPase subunit MalK (Ehrmann *et al.*, 1998). Purified MalK was shown to have constitutive ATPase activity and to be insensitive to vanadate, a compound that causes strong inhibition to the assembled transport complex (Schneider, 2001). These results indicate that functional coupling of the NBD to those parts of the ABC transporter embedded in the membrane must exist in order to explain the action of vanadate. The favoured model proposes that the protein undergoes a conformational change upon binding of Mg-ATP, and then interacts with the membrane components (Mourez *et al.*, 1998). The proposed physical interaction between the NBDs and the transmembrane domains of the maltose transporter has been supported by site-directed chemical cross-linking experiments (Hunke *et al.*, 2000). Results obtained with a proteoliposome-reconstituted transport system suggested that MBP transmitted, through MalF and MalG, a signal to MalK inducing it to hydrolyse ATP (Davidson *et al.*, 1992). Co-operativity between the NBDs to drive the transport process has been reported for several systems, as in the case of P-glycoprotein (Buxbaum, 1999), MalK (Davidson and Sharma, 1997) and HisP (Nikaido and Ferro-Luzzi Ames, 1999). In isolated NBDs, *Methanococcus jannaschii* MJ0796 (Moody *et al.*, 2002) also showed co-operativity to hydrolyse ATP.

For the P-glycoprotein (MDR1; ABCB1), where both NBD subunits appear to be functionally equivalent (Loo and Clarke, 1994; Urbatsch *et al.*, 1995), Senior proposed a model for the NBD activity in which ATP hydrolysis occurs alternatively at each NBD (Senior *et al.*, 1995). In other transporters such as the multidrug resistance protein (MRP1), the NBDs are non-equivalent (Nagata *et al*, 2000). Photoaffinity labelling experiments revealed ATP binding exclusively at NBD1, whilst ADP trapping appeared predominantly at NBD2 of MRP1 (Hou *et al.*, 2000). The NBDs of TAP ($TAP_1$/$TAP_2$; ABCB2/ABCB3), the transporter associated with antigen processing; also show different nucleotide binding affinities. In photolabelling experiments with free 8-azido ATP[$\gamma$-$^{32}$P], the labelling efficiency for

$TAP_1$ was markedly higher than for $TAP_2$ (Alberts *et al.*, 2001), and identical mutations in the domains had different effects on peptide transport (Daumke and Knittler, 2001). On the other hand, it was reported that one intact ATP-binding subunit was sufficient to support ATP hydrolysis and translocation in the ABC transporter of histidine permease although at a rate half of that of the wild type transporter (Nikaido and Ames, 1999). These studies also indicated that only one NBD would be involved in the hydrolysis of ATP by isolated HisP.

The formation of a dimer as the active form of the NBD was proposed for HisP (Nikaido *et al.*, 1997). This conformation was also suggested for other NBDs of ABC transporters like the OpuAA of *Bacillus subtilis*, where equilibrium between monomeric and dimeric forms has been reported (Horn *et al.*, 2003) and GlcV from *Sulfolobus solfataricus* (Verdon *et al.*, 2003).

In addition to the site for nucleotide binding, these domains in P-glycoprotein also bear a site for ligands such as flavonoids, which modulate drug transport (Conseil *et al.*, 1998). When exposed to several drugs that P-glycoprotein ejects from the cell, the rate of ATP hydrolysis was increased several fold (Ambudkar *et al.*, 1992). This effect of stimulation by drugs however, is only noticed when all the subunits are part of the complex (Loo and Clarke, 1994). Although it is not part of the ABC family, the *E. coli* arsenical efflux pump ArsAB shares similar structural and functional features to ABC transporters like the human multidrug-resistance P-glycoprotein (Gottesman *et* al., 1996; Ambudkar *et al.*, 1999), and its ATPase subunit ArsA, has an allosteric site that exemplifies this behaviour. Although ArsA possess two nucleotide-binding sites (NBSs), its allosteric site is basically composed of three arsenite/antimonite binding sites formed by: His148 (A1) and Ser420 (A2); Cys113 (A1) and Cys422 (A2); and Cys172 (A1) and His453 (A2) (Zhou *et al.*, 2000). A1 and A2 refer to the two NBDs of the ArsA ATPase catalytic subunit of the ArsAB transporter. Each one of these sites can accommodate a molecule of arsenate or antimonite. It has been proposed that the binding of antimonite or arsenite makes the two halves of ArsA come together, and that this conformational change induces an increase in ATP activity (Rosen *et al*, 1999). In other transport NBDs like OleB (fused to maltose binding protein) and KpsT, involved in the export of oleandomycin and polysialic acid by *Streptomyces antibioticus* and *E. coli* (Buche *et al.*, 1997; Bliss and Silver,

1997) respectively, there are indications of an interaction with the substrate. Those results, however, do not rule out the possible participation of other components.

Although it is known the ABC transporter NBDs preference for ATP, other nucleotides can be accepted, usually with less affinity and reduced hydrolysis rate (e.g. in Buche *et al.*, 1997). A similar situation prevails for divalent cations different from $Mg^{2+}$ that are utilised to stimulate ATP hydrolysis, but in this case, the range and concentration of the cation can influence the catalysis. For example in the characterisation of HisP, the ATP-binding subunit of the histidine transporter, Nikaido and co-workers found that $Mn^{2+}$ was the best stimulator of HisP ATPase activity at high concentrations (above 1.5 mM) whilst $Co^{2+}$ was the best at low concentrations (<1 mM) (Nikaido *et al.*, 1997).

A number of inhibitors of ATP hydrolysis have been found to interact directly with NBDs. Vanadate is a specific inhibitor for P-type ATPases and its action is used to distinguish different types of pumps. Vanadate inhibits the enzymatic activity of P-glycoprotein by trapping ADP at the catalytic site, preventing the release of the nucleotide from the protein (Urbatsch *et al.*, 1995). Vanadate also catalyses the UV-dependent cleavage of the polypeptide backbone at both the ABC signature and the Walker A motifs when it is trapped in the nucleotide-binding site of the *E. coli* maltose transporter (Fetsch and Davidson, 2002). While reported for transport complexes, isolated NBDs remain insensitive to its effect (Table 1.3). Another inhibitor, N-ethylmaleimide (NEM), also affects intact transport proteins (Urbatsch *et al.*, 1994). A covalent modification of a cysteine present in the Walker A motif is pointed out as the target of this compound (Feng and Forgac, 1992). ADP and other non-hydrolysable analogues have also been shown to affect the ATPase activity of many ABC proteins (Schneider and Hunke, 1998).

Mutations in key amino acids in any of the conserved sequences in the NBD can be used to abolish ATPase activity but not the binding of ATP. The affinity of ABC transporters for ATP shows a very broad range of variability (Table 1.3). It is probable that in some cases, the purification conditions and the status of its association with the full complex have affected the reported $K_m$ and $V_{max}$.

**Table 1.3 Enzymatic properties of some purified ABC transport systems and their isolated components**

| Protein | $K_m$ (ATP) (μM) | $V_{max}$ (nmol/min/mg prot.) | Inhibitor | Reference |
|---|---|---|---|---|
| $MalFGK_2$ (*E. coli*) | 74 | 860 | Vanadate | Davidson *et al.*, 1992, 1996 |
| MalK (*S. typhimurium*) | 23.9 | 322 | Insensitive to vanadate | Morbach *et al.*, 1993 |
| MalK (*E. coli*) | 120 | 275 | Insensitive to vanadate | Mourez *et al.*,1998 |
| $HisQMP_2$ (*S. typhimurium*) | 8000 | 350 | ---------- | Liu and Ames, 1997 |
| HisP (*S. typhimurium*) | 205 | 500 | Insensitive to vanadate | Nikaido *et al.*, 1997 |
| MglECA (*S. typhimurium*) | nd | 20 | Vanadate | Richarme *et al.*, 1992 |
| MglA (*S. typhimurium*) | 60 | 140 | Vanadate | Richarme *et al.*, 1993 |
| HlyB-ABC (*E. coli*) | 200 | 1000 | Vanadate | Koronakis *et al.*, 1993 |
| OleB (N-term.) | 1 | 19400 | Azide | Aparicio *et al.*, 1996 |
| P-glycoprotein (chinese hamster) | 940 | 300 | Vanadate, NEM | Shapiro and Ling, 1994 |
| P-glycoprotein (human) (ABCB1) | 400 | 1650 | Vanadate, bafilomycin | Sharom *et al.*, 1995 |
| P-gly-NBD2 (chinese hamster) | 24 | 20000 | Nd | Sharma and Rose, 1995 |
| P-gly-NBD1 (human) | nd | 180 | Vanadate | Shimabuku *et al.*, 1992 |
| PstB (*M. tuberculosis*) | 71.5 | 122 | -------- | Sarin *et al.*, 2001 |
| Trx-DrrA (*M. tuberculosis*) | 84.8 | 52 | --------- | Nash, 2003 |
| CFTR NBD1 (human) | 110 | 30 | Azide | Ko and Pedersen, 1995 |
| CFTR NBD2 (human) | 86 | 6 | Ap5A | Randak *et al.*, 1997 |

Ap5A, $p^1$, $p^5$ –di (adenosine-5') pentaphosphate; NEM, N-ethylmaleimide; NBD, nucleotide binding domain. (This table is an adaptation from Schneider and Hunke, 1998).

## 1.3 Structure of ABC transporters

### 1.3.1 Nucleotide-binding domains

The periplasmic histidine permease transporter ($HisQMP_2$), present in *E. coli* and *S. typhimurium* is one of the best characterised ABC transporters and may be regarded as a good example of this superfamily. It consists of a membrane-bound complex comprising the integral membrane subunits HisQ and HisM, and two copies of the ATP-binding subunit HisP, which has properties intermediate between those of integral and peripheral membrane proteins (Kerppola *et al.*, 1991), being accessible from both sides of the membrane (Baichwal *et al.*, 1993). These two HisP subunits form a dimer as suggested by their cooperativity in ATP hydrolysis (Liu *et al.*, 1997). The X-ray crystal structure of HisP from *S. typhimurium* was elucidated in 1998 (Hung *et al.*, 1998) (Fig. 1.6).

The overall fold shape derived from the crystal structure of the HisP monomer (Fig. 1.6) is considered to be different from any other known protein, resembling an "L" with two thick arms (described as arm I and arm II). The ATP-binding pocket, containing the "phosphate-binding loop", is localised near the end of arm I, which also includes the Walker B motif. The ABC signature motif forms part of arm II, characterised by a higher _-helices composition. Limited similarities can be found between the structures of HisP and RecA (Story and Steitz, 1992) and the _- and _-subunits of bovine $F_1$-ATPase (Abrahams *et al.*, 1994).

Thereafter, when structures were resolved for other bacterial ABC transporters (e.g. MalK, MJ1276, TAP1), a consensus fold for the NBD was revealed (Diederichs *et al.*, 2000; Karpowich *et al.*, 2001; Yuan *et al.*, 2001; Gaudet and Wiley, 2001; Chang and Roth, 2001; Locher *et al.*, 2002; and Smith *et al.*, 2002). This fold shows the two armed L shape and localisation of the P- and C- loops that was first reported for HisP by Hung and collaborators (1998).

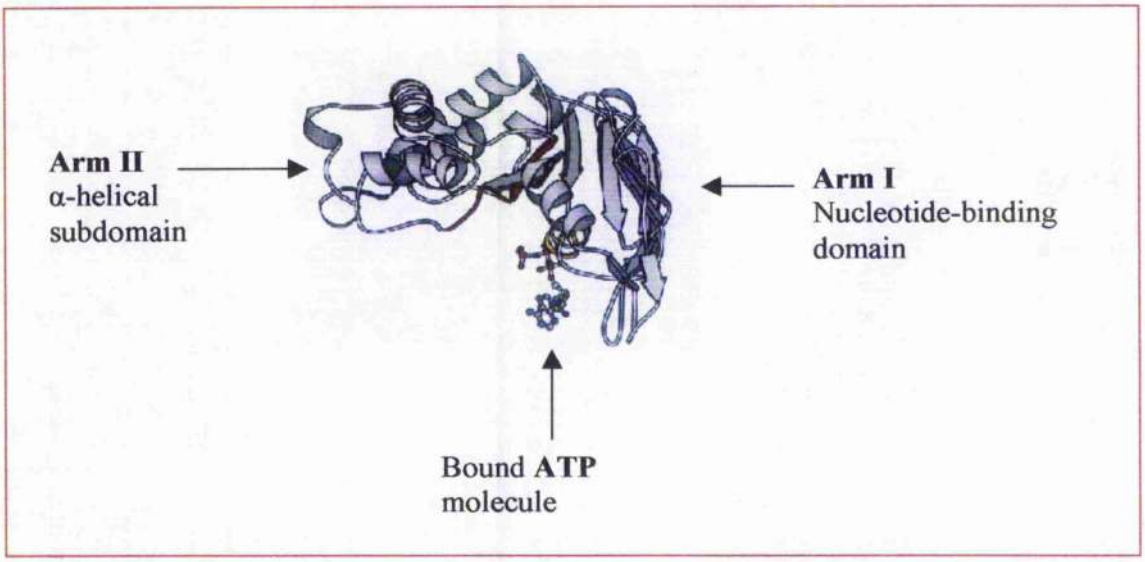



**Figure 1.6 X-ray crystal structure of *S. typhimurium* HisP monomer (Hung *et al.*, 1998)**

A dimer conformation has been suggested as the active form of several NBDs, and although the monomer conformation was similar, the manner of how monomers were associated to form the dimer was significantly different (Janas *et al.*, 2003). Dimer conformations have been indicated in some crystal structures like those of MalK from the archeon *Thermococcus litoralis* (Diederichs *et al.*, 2000), and the structures of wild type MJ0796 (Yuan *et al.*, 2001) and mutant MJ0796-E171Q of *Methanococcus janaschii* (Smith *et al.*, 2002). However, the best dimer model for NBD:NBD interaction is based upon ABC domains not from a transporter, but from the DNA repairing enzyme Rad50. In this dimer structure (Fig. 1.7), two nucleotides are clamped at the interface between two monomers, inducing them to dimerise (Hopfner *et al.*, 2000). In this model, the ATP molecules are sandwiched between the Walker A and B motifs of one monomer and the C-loop of the other monomer. The NBDs form a dimer with ATPase activity, they share homology with ABC-type ATPases and they are structurally similar to the monomeric MalK and HisP proteins. This NBD dimer from Rad50 is stable in solution in the presence of $Mg^{2+}$ and the non-hydrolisable ATP analogue adenyl imido diphosphate (AMP-PNP) (Hopfner *et al.*, 2000).

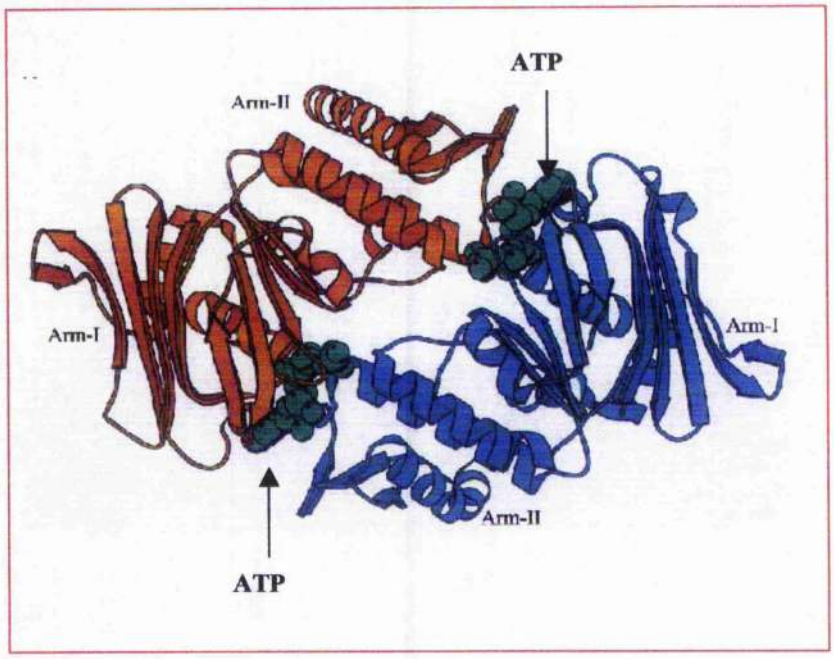



**Figure 1.7** ***Pyrococcus furiosus*** **ATP-bound Rad50 dimer crystal structure** (Hopfner *et al.*, 2000)

To support this dimer model, a MalK structure, which resembles Rad50, was obtained from *E. coli* (Chen *et al.*, 2003) (Fig. 1.8). In the structure, it can clearly be seen that the ATP molecules interact with the Walker A motif (in red) of one monomer and the signature (in purple) of the other monomer in a sandwich type manner. MalK was crystallised in the absence and presence of ATP and showed a stable dimer conformation in the three different crystal conformations obtained. The stability of these dimer structures in comparison to those of other NBDs can be attributed to an additional C-terminal sequence called the regulatory domain (not shown in Fig. 1.8), that contributes substantially to the dimer interface.

The architecture of this ATP-bound MalK dimer is consistent with biochemical data obtained with the intact maltose transporter. Maltose-binding protein (MBP) stimulates ATP hydrolysis by $MalFGK_2$, and vanadate traps a complex of MgADP, MBP, and $MalFGK_2$ in the catalytic transition state (Chen *et al.*, 2001). The highly specific photocleavage of the MalK subunits in the presence of UV light at the Walker A and LSGGQ motifs mediated by vanadate, demonstrates that both motifs lie very close to the position of the ATP $\gamma$ phosphate in the catalytic transition

state, and since the ABC signature motif is well separated from the Walker A motif in one subunit, specific cleavage at both sides provides strong evidence that the LSGGQ motifs complete the nucleotide binding sites of the opposing subunits (Chen *et al.*, 2003).

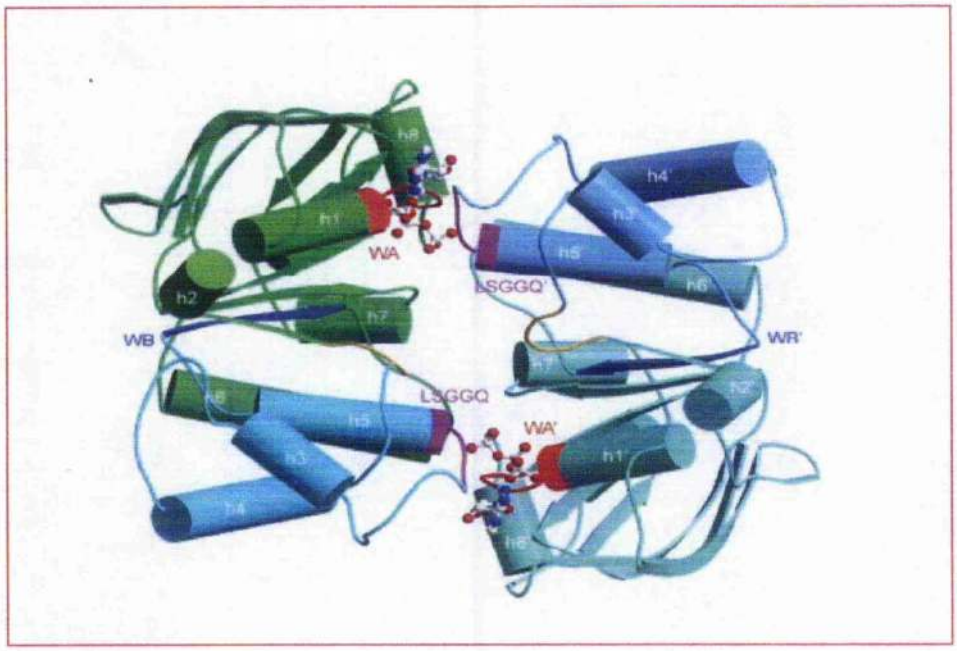



**Figure 1.8 *E. coli* MalK dimer crystal structure** (Chen *et al.*, 2003)

Dimer structures similar to the configuration shown for Rad50 were also discovered for NBDs of the vitamin B12 transporter BtuCD (Locher *et al.*, 2002) and the *E. coli* maltose transporter (Chen *et al.*, 2003).

### 1.3.2 Transmembrane Domains

Detailed structural studies of membrane proteins have been particularly difficult in the past. A prerequisite for these studies is to overexpress such proteins in large quantities. *E. coli* is widely adopted as the system of choice for this purpose due to its high growth rate, well-characterised genetics and the wide range of available vectors. However, the production of properly folded transmembrane proteins and their

purification has not been easily achieved. One obstacle in working with proteins that carry hydrophobic domains is the requirement for detergents. Whilst detergents generally aid solubility, they can also result in denaturation of proteins and can be detrimental to their enzymatic activity (Swiderek *et al.*, 1997). Although advances in the crystallisation of single NBDs have occurred, the transmembrane domains show less conservation of sequence than NBDs and the involvement of the membrane components in the global interaction with the NBDs still needs to be clarified.

The existence of mutations allowing maltose transport in the absence of the MBP led to the identification of sites in MalF and MalG thought to be important for the interaction with the MBP (Covitz *et al.*, 1994). A highly conserved motif of 20 amino acids, EAA-X(3)-G-X(9)-I-X-L-P (Saurin and Dassa, 1994), called the EAA loop, has been suggested to interact with the NBD in the ABC importer-type proteins (Hunke *et al.*, 2000; Mourez *et al.*, 1997). This region is hydrophilic and lies in a loop facing the cytoplasm in all of these proteins of recognised topology (Saurin *et al.*, 1994). This EAA motif seems to be of functional importance since several mutations have been characterised that have reduced or abolished transport in different uptake systems (e.g. maltose, Dassa 1990; iron (III) hydroxamate, Koster and Bohm, 1992; and phosphate, Webb *et al.*, 1992).

Site-directed mutagenesis experiments by Mourez and collaborators (1997) showed that single substitutions in the EAA region of MalG abolished transport completely, but the same mutation in MalF had no effect, suggesting that these EAA motifs and perhaps the MalG and MalF proteins do not function symmetrically. In some cases, double mutants possessed properties that were not equivalent to the combined characteristics of their corresponding single mutants. This result suggested some kind of cooperativity between the EAA regions of MalG and MalF in the formation of a functionally important site. Two classes of mutants were also found that indicated two probable roles for the EAA motifs. Firstly, they may play a fundamental role in the association of MalK with the membrane and the formation of a functional transporter. This has been inferred from the observation that some EAA mutations result in localisation of MalK to the cytoplasm (in the normal transporter, 80 % of MalK appears in a particulate fraction extracted by Triton X-100, which also contains MalF and MalG). Suppressor mutations in *malK* that reconstitute the

transporter function indicate that mutations in the EAA loop can be corrected by mutations in the ATPase ("helical domain"). The second role inferred for the EAA regions from mutagenesis is that they might form part of a substrate binding site or a site that transduces a signal to MalK (Mourez *et al.*, 1997).

Mourez and co-workers presented the following conclusions from their analysis: mutations in EAA loops might affect their direct participation in the membrane–NBD association, or indirectly, inducing a conformational change in other region of the proteins more directly involved in the interaction with MalK. EAA mutations might also disrupt the formation of the MalF-MalG heterodimer, resulting in the release of MalK in the cytoplasm. Another possibility is that EAA mutations alter a functional site that involves the three proteins MalF, MalG and MalK (Mourez *et al.*, 1997). An EAA-like motif has been identified in some eukaryotic ABC transporters that may indicate a more generalised role for this sequence (Schneider and Hunke, 1998).

Although the EAA---- sequence might be distinctive in membranes involved in influx processes, an equivalent sequence has not been reported for transporters involved in efflux. Interactions between substrates and TM domains are not completely understood but they seem to be dependent on the nature and architecture of the drug-binding sites in the membrane, rather than the presence of particular conserved sequence motifs. This indeed might explain the broad substrate specificity found in multidrug transporters such as P-glycoprotein. The existence of many separate binding sites as well as the presence of a single flexible site that can accommodate different chemical structures within different sub-regions, have been proposed (Neyfakh, 2002). Several separate binding sites have been reported for P-glycoprotein: a lipid-exposed substrate binding site (Qu and Sharom, 2002), two non-identical drug interaction sites in the human protein (Dey *et al.*, 1997), and a minimum of four distinct drug binding sites for substrates and modulators of P-glycoprotein transport (Martin *et al.*, 2000). Nevertheless, the idea of a common drug-binding pocket prevails for other investigators (Loo *et al.*, 2003; Shapiro *et al.*, 1999). The existence of many separate binding sites to accommodate all the known different chemical classes of drugs known to be expelled by P-glycoprotein seems improbable. A single large flexible drug binding pocket might explain better the

translocation of multiple different substrates. Structural analysis of a number of soluble multidrug-recognising proteins have shown the presence of large hydrophobic binding sites and that the binding of substrates is due to a combination of hydrophobic effects and electrostatic attraction rather than specific interactions (Neyfakh, 2002).

The concept of a single flexible binding site was based on research into the *Bacillus subtilis* transcriptional regulator BmrR (Neyfakh *et al.*, 1991; Vazques-Laslop *et al.*, 2000; Zhelesnova *et al.*, 2000; Zhelesnova and Brennan, 2001). The X-ray crystal structure of this soluble multidrug-binding protein in complex with one of its substrates has been determined (Zhelesnova and Brennan, 2001). The drug is held in place within the protein core by extensive van der Waals and stacked hydrophobic interactions, and with positively charged drugs, electrostatic interactions with a buried glutamate residue are important. In this system, each compound forms a different set of atomic contacts within the protein, in a flexible binding site. There is no special mechanism for multiple drug recognition by the BmrR protein, an idea that has been suggested for other proteins involved in multidrug transport (Sharom *et al.*, 2001).

The staphylococcal protein QacR activates expression of the multidrug pump QacA in *S. aureus* (Schumacher *et al.*, 2001), Although QacR is not an ABC transporter, it is the best example of how multidrug resistance proteins interact with their substrates. Six QacR structures bound to different substrates have been obtained, establishing that one large drug-binding pocket accommodates the different ligands. Aromatic and polar residues are present within the large pocket that also comprises several multiple drugs-binding mini pockets. Four glutamate residues, E57, E58, E90 and E120 are involved in the charge neutralisation for different cationic drugs (Schumacher and Brennan, 2002). Conformational changes were observed when drugs were bound, allowing the hydrophobic core of the pocket, buried in the absence of drugs, to be exposed. On binding a drug, two aromatic residues Tyr92 and Tyr93, present in the drug-free state pocket, are expelled resulting in the displacement of glutamate 90 into the pocket and creation of the drug-binding pocket (Kaur, 2002). Two independent binding-sites have been identified for R6G and for ethidium, and a third site that overlaps part of the other sites (Schumacher and Brennan, 2003).

Many of the features of the QacR structure are also found in BmrR (Kaur, 2002). Although QacR and BmrR are not transporters, the architecture of the pocket of these structures appears to be well suited as a general characteristic of the MDR transporters.

### 1.3.3 Structure of complete ABC transporters

The recent determination of the crystal structure of various bacterial ABC transporters, such as the transporters for Lipid A and Vitamin B12 (e.g. *E. coli* MsbA; Chang and Roth, 2001 and BtuCD; Locher *et al.*, 2002), is helping to elucidate the structure and mode of operation of ABC pumps. Although impeded for many years by the difficulty of expression and purification of the target proteins, the first high-resolution X-ray crystal structures of these membrane proteins are now available. Until recently, the only three-dimensional structure of a complete ABC transporter was a 25 Å resolution structure determined by electron diffraction (Rosenberg *et al.*, 1997). In 2001, Geoffrey Chang and Christopher Roth determined the first crystal structure of a complete ABC transporter at a resolution of 4.5 Å, that of the *E. coli* MsbA transporter (Chang and Roth, 2001). This particular ABC transporter is a lipid flippase that translocates lipids from the inner layer of the cell membrane to the outer layer, and is more closely related to the mammalian P-glycoproteins than any other bacterial ABC transporter (Chang and Roth, 2001).

The overall organisation is consistent with most bacterial MDR-ABC transporters. The *msbA* gene encodes a half transporter that contains a single membrane-spanning region fused to a NBD, which is assembled into a homodimer. Hydropathy analyses confirm a six membrane spanning region and the localisation of the NBD on the cytoplasmic side of the membrane. Chang and Roth's approach was to explore the cloning, overexpression and purification of more of than 20 full-length bacterial transporters and their homologues derived from several MDR-ABC transporter families and 12 bacterial species. From these, they sought the best subject for crystallisation trials, attempting not less than 96,000 crystallisation conditions with about 20 detergents to finally obtain crystals of 35 different membrane proteins.

The crystal structure obtained is compatible with that expected for this transporter; a homodimer, with each subunit composed of two domains. The structure also shows an additional intracellular domain (ICD) connecting the NB and TM domains. All the transmembrane _-helices are tilted between 30° and 40° with respect to the plane of the membrane and give a cone shape to the structure (Fig. 1.9). The outer leaflet is the region where both TM halves contact at the top of the chamber cone holding the two monomers of the transporter together. The base of the structure facing the cytoplasm is ~ 45 Å in its widest dimension, being sufficient volume to accommodate the Lipid A molecule. There is no contact between NBDs, which are separated by ~ 50 Å at the closest point. Although in the structure of the NBDs the Walker A motif section was not well resolved, the ATP-binding sites are predicted to face away from the centre of the dimer. The inner leaflet is characterised by a cluster of positively charged amino acids contrasting with the hydrophobic environment in the outer membrane side. TM2 and TM5 (shown circled in Fig. 1.9) lead the contact between TM halves in the formation of the cone, and with TM6, have been proposed to play a role in the substrate recognition. The ICD region occupies an important part in the structure and shows considerably contact with both the TM and NB domains, including a direct contact with the NBD and TM 2, 5 and 6.

Chang and Roth (2001) suggested a possible mechanism to flip hydrophobic substrates in the *E. coli* MsbA transporter: the substrate (Lipid A as model) moves towards the base of the chamber presumably *via* the inner leaflet of the membrane. ATP binding and hydrolysis is triggered in the NBDs. Recruitment of the substrate and closure of the chamber is caused by the rearrangement of the TMs 2, 5 and 6 and the two NBDs. The polar environment in that part of the membrane presents an unfavourable environment for hydrophobic substrates and the substrate is flipped to a more favourable position in the outer leaflet bilayer of the chamber where it can establish hydrophobic interactions. The flipping of the substrate induces the chamber to undergo additional re-arrangements that include the separation of the NBDs and the repositioning of the main TM helices, enabling the complete expulsion of the substrate to the exterior and re-setting of the system. This particular mechanism explains the ability of multidrug transporters to drive the efflux of a number of different hydrophobic substrates. Additionally, the size and shape of the chamber allows the accommodation of a wide diversity of amphipathic molecules. This

structure provides a foundation for the understanding of the bioenergetics of lipid/drug "flip flop" for the entire MDR-ABC transporter family.

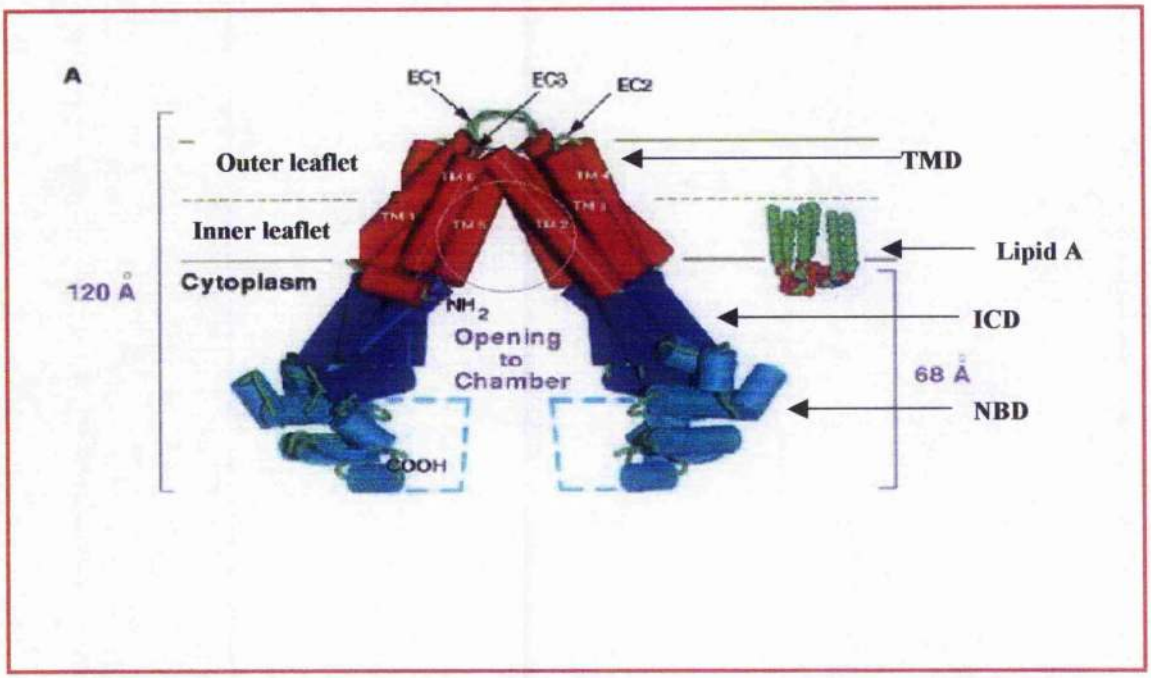



**Figure 1.9 Structure of the *E. coli* MsbA homodimer (Chang and Roth, 2001)**
TMD: transmembrane domain; ICD: intracellular domain; NBD: nucleotide binding domain

A second complete ABC transporter structure has been obtained, that of the *E. coli* vitamin $B_{12}$ importer BtuCD, at 3.2 Å resolution. All critical parts have been ordered and resolved (Locher *et al.*, 2002). With a similar approach to the MsbA crystallisation, a number of different transporters were obtained from cloning and overexpression in *E. coli* and assayed in crystallisation trials. Crystals of high quality were obtained for the BtuCD transporter and its structure is shown in Figure 1.10. This transporter consists of two copies of the TMD BtuC, and two copies of the NBD BtuD, with subunits showing that the two ABC cassettes are in close contact with each other. The assembled complex is ~ 90 Å tall, 60 Å wide and ~ 30 Å thick, and viewed from the front face, shows an overall shape resembling an inverted gate (Locher *et al.*, 2002). Its structure contrasts with that of MsbA; in this case the TMD shows an asymmetric ordering and in a number higher than the 12 predicted transmembrane helices, there are not indications of the presence of the ICD, and both NBDs are in close contact with each other.

Each of the two BtuC subunits crosses the membrane 10 times creating a total of 20 helices in the transporter. Although very different from canonical transporters like MsbA that possess 12 helices, it is similar to the predicted transmembrane helices for other transporters such as the human antigen transporter TAP (19 helices; Lankat-Buttgereit and Tampe, 2002), and the ferrichrome-transporting FhuB protein (20 helices; Groeger and Koster, 1998). The 10 transmembrane helices found per subunit are packed together in a rather entangled way with no similarities to the MsbA monomer. The interface between two membrane-embedded subunits allows a cavity to be formed (top section in the Figure) that is believed to correspond to the translocation pathway, being of sufficient size to accommodate the Vitamin $B_{12}$ molecule.

The BtuCD structure shows the NBDs to be associated in a manner very similar to that reported for Rad50, where the P-loop of one monomer closely contacts the ABC signature region in the other monomer. This dimer interface consists mainly of amino acid residues from the highly conserved Walker A (P-loop) and D-loop, and from the moderately conserved Switch region, indicating that this dimer arrangement may be common to all members of the ABC family. Nevertheless, this dimer interface between both cassettes is rather small for a specific dimer contact surface and smaller than the interfaces between each pair of BtuC-BtuD subunits or between the two BtuC subunits. The structure suggests that the TM domains are required to stabilise the dimeric conformation of NBDs (Locher *et al.*, 2002); in their absence the weak forces holding the dimers together may be insufficient to maintain their association. This would explain the divergence in dimer conformations for HisP and MalK.

Although there is not an ICD that links the TM and NB domains as occurs in the MsbA dimer, a long cytoplasmic loop between TM 6 and 7 from BtuC that folds into two short helices (L1 and L2; shown in yellow in the Figure), provides the largest contribution to the TM-NB interface. This L loop has resemblance to the EAA motif of bacterial importers (Mourez *et al.*, 1997), to the fourth intracellular loop of CFTR (ICL4) and to the first cytoplasmic loop in drug exporters, and it is believed that it may represent a general interface between ABC cassettes and membrane-spanning domains (Locher *et al.*, 2002). The mechanism proposed for BtuCD is again based on

a series of binding and rearrangement events coupled to ATP hydrolysis by the NBD. In this case, it involves a permanent contact between the TM and NB domains. It is proposed that the binding of the periplasmic binding protein-vitamin $B_{12}$ complex to the periplasmic domain of BtuC induces ATP hydrolysis by BtuD. Consequently, this energy powers further re-arrangement of the NB and TM domains to allow substrate translocation to a large water-filled space in the cytoplasmic side of the membrane. Release of ADP re-sets the transporter to its original state.

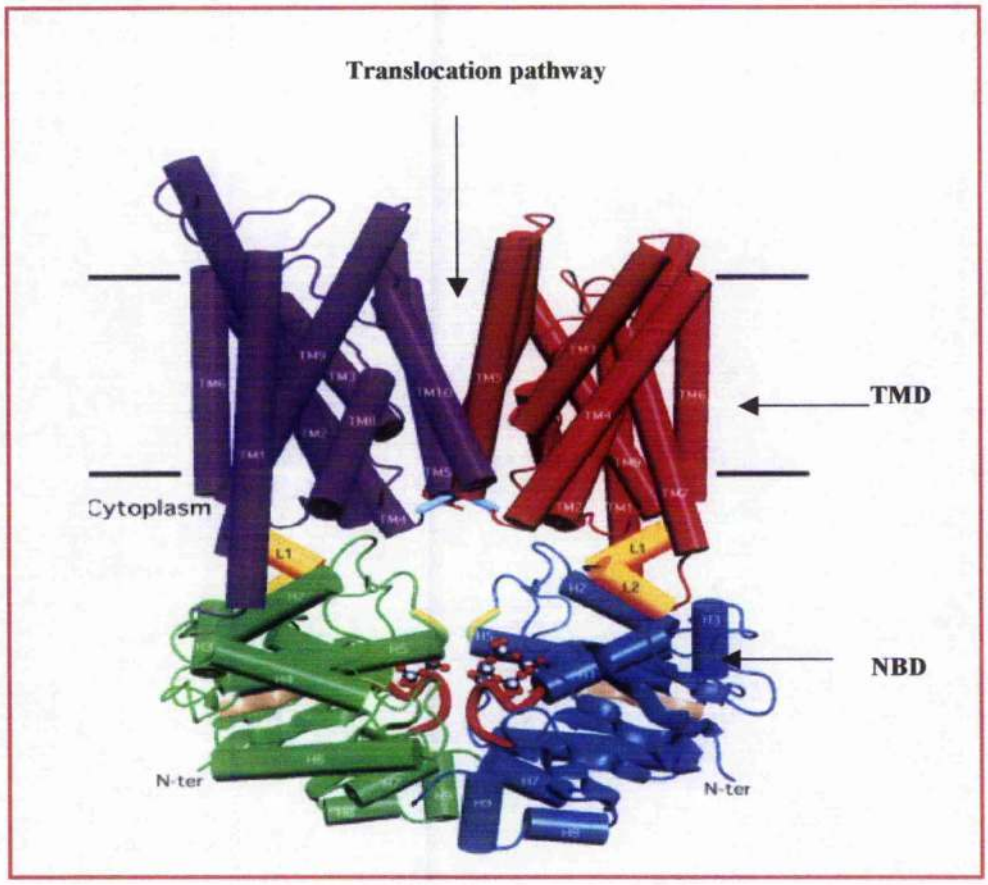



**Figure 1.10 Structure of *E. coli* BtuCD (Locher *et al.*, 2002)**

The differences noted between crystal structures may be also influenced by the crystallographic techniques, leading to non-physiological interactions. Although a useful tool to determine the architecture of ABC transporters, it is still necessary to verify as far as possible proposed modes of action. The crystal structure of *Vibrio*

*cholera* MsbA (Chang, 2003) has been informative in this regard since it shows the same features of its predecessor *E. coli* MsbA, but with a notable difference. In this structure (Fig. 1.11) the NBDs do not present an interface separation as in *E. coli* MsbA but they are in very close contact. This finding, again, supported the suggested close interaction between both nucleotide-binding subdomains and indicated that the *E. coli* and *V. cholera* transporters crystal structures might differ because they represent different stages in the transport process.

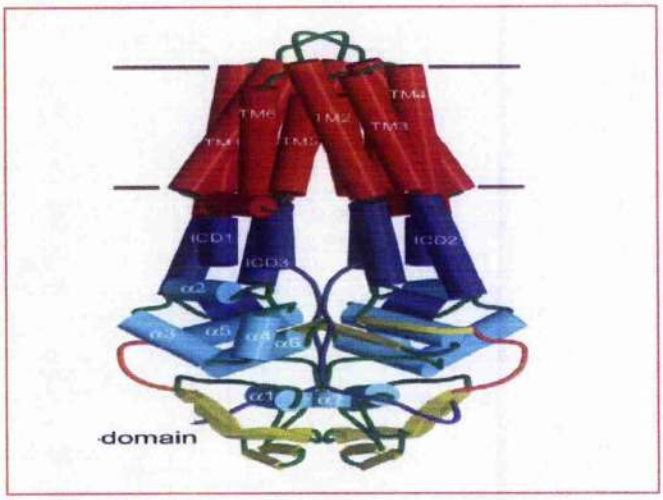



**Figure 1.11 Structure of *V. cholera* MsbA** (Chang, 2003)

More recently, the structure of *S. typhimurium* MsbA has been added to the understanding of this transporter, providing further knowledge on its architecture and that of ABC transporters in general (Reyes and Chang, 2005). This new structure, obtained in complex with Mg•ADP•Vanadate and Ra Lipopolysaccharide to a resolution of 4.2 Å, seems to show a post-hydrolysis intermediate (Fig. 1.12). In this structure, the NBDs appear folded as expected, and face each other in close proximity to perform ATP hydrolysis and possibly drive substrate translocation. The resolution of this new X-ray structure by Chang's group further supports the "flip flop" mechanism described earlier for the translocation of lipids from the inner layer of the cell membrane to the outer layer (see p. 39, and Chang and Roth 2001).

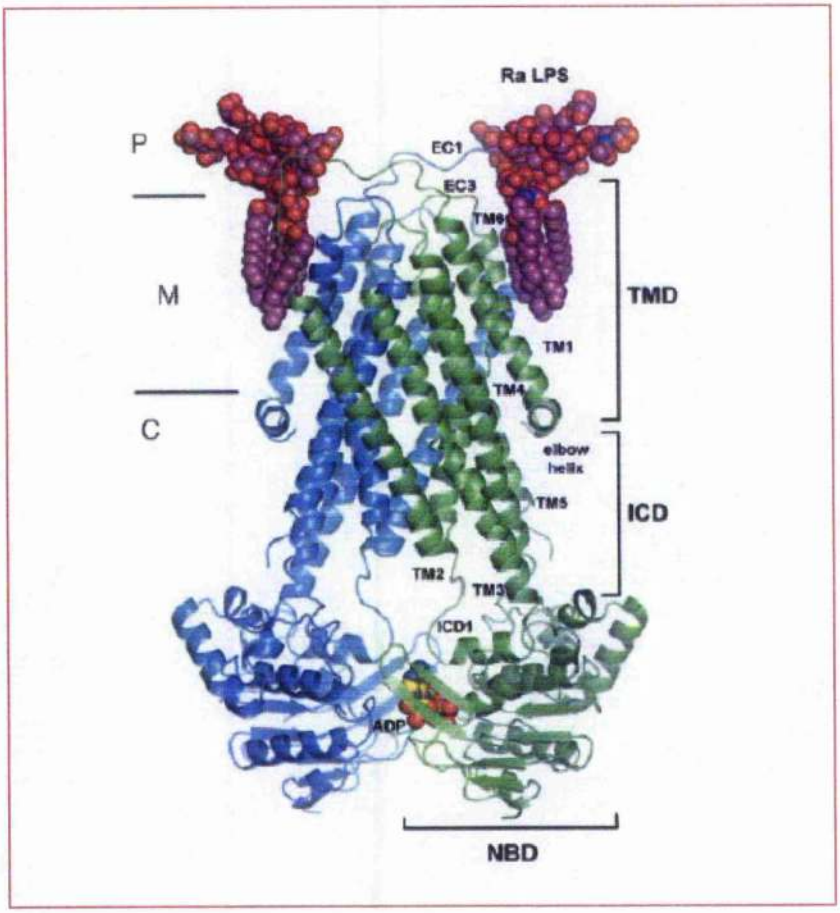



**Figure 1.12 Structure of *S. typhimurium* MsbA in complex with Mg•ADP•Vanadate and Ra Lipopolysaccharide** (Reyes and Chang, 2005)

P: periplasm; M: lipid bilayer matrix; C: cytoplasm; Ra LPS: Ra lipopolysaccharide; TMD: transmembrane domain; ICD: intracellular domain; NBD: nucleotide binding domain

## 1.4 Distribution and classification of the ABC transporter superfamily

ABC systems are involved in transport as importers or exporters, and this feature is the primary approach to their classification, although they are also implicated in many cellular processes and in their regulation. Importers comprise mostly the prokaryotic "transport substrate" binding protein-dependent (BPD) transporters and are involved in the acquisition of essential nutrients by bacteria. Exporters are extensive in both prokaryotes and eukaryotes and are implicated in the expulsion of harmful substances, the secretion of extracellular toxins and the targeting of membrane components (Fath and Kolter, 1993). A third class of ABC systems is

not apparently involved in transport but in cellular processes such as DNA repair (Thiagalingam and Grossman, 1993), translation and regulation of gene expression (Belfield *et al.*, 1995).

A global phylogenetic tree of ATP-binding modules of ABC systems based on the MalK protein sequence was prepared in order to classify ABC transporters (Saurin *et al*, 1999). Two main branches were constructed, with one branch corresponding to proteins involved in export, while the other branch grouped proteins involved in import processes. Prokaryotes and eukaryotes were present in the first branch but the second one included only prokaryote proteins and mainly components of BPD transporters. ABC transporters were then divided in the families ABC-A and ABC-B. ABC genes in eukaryotes had already been classified in different families from ABCA to ABCG, based on gene organisation and primary sequence homology (Anjard *et al*, 2002; Klein *et al.*, 1999). Due to the broad distribution of ABC systems, more detailed use was made of the functions associated with these systems and three classes were primarily defined on the basis of cluster sequences analysis (Fig. 1.13) (Dassa and Bouige, 2001). Class 1 contains the majority of known exporters with fused nucleotide-binding domains (NBDs) and transmembrane domains (TMDs). Class 2 includes all systems with no known TMDs associated with ABC modules and consists of systems involved in antibiotic resistance and cellular processes other than transport. Class 3 contains all BPD importers and other poorly characterised systems which seem not to be involved in import. The general properties of the systems to which they belong appear summarised in Table 1.4.

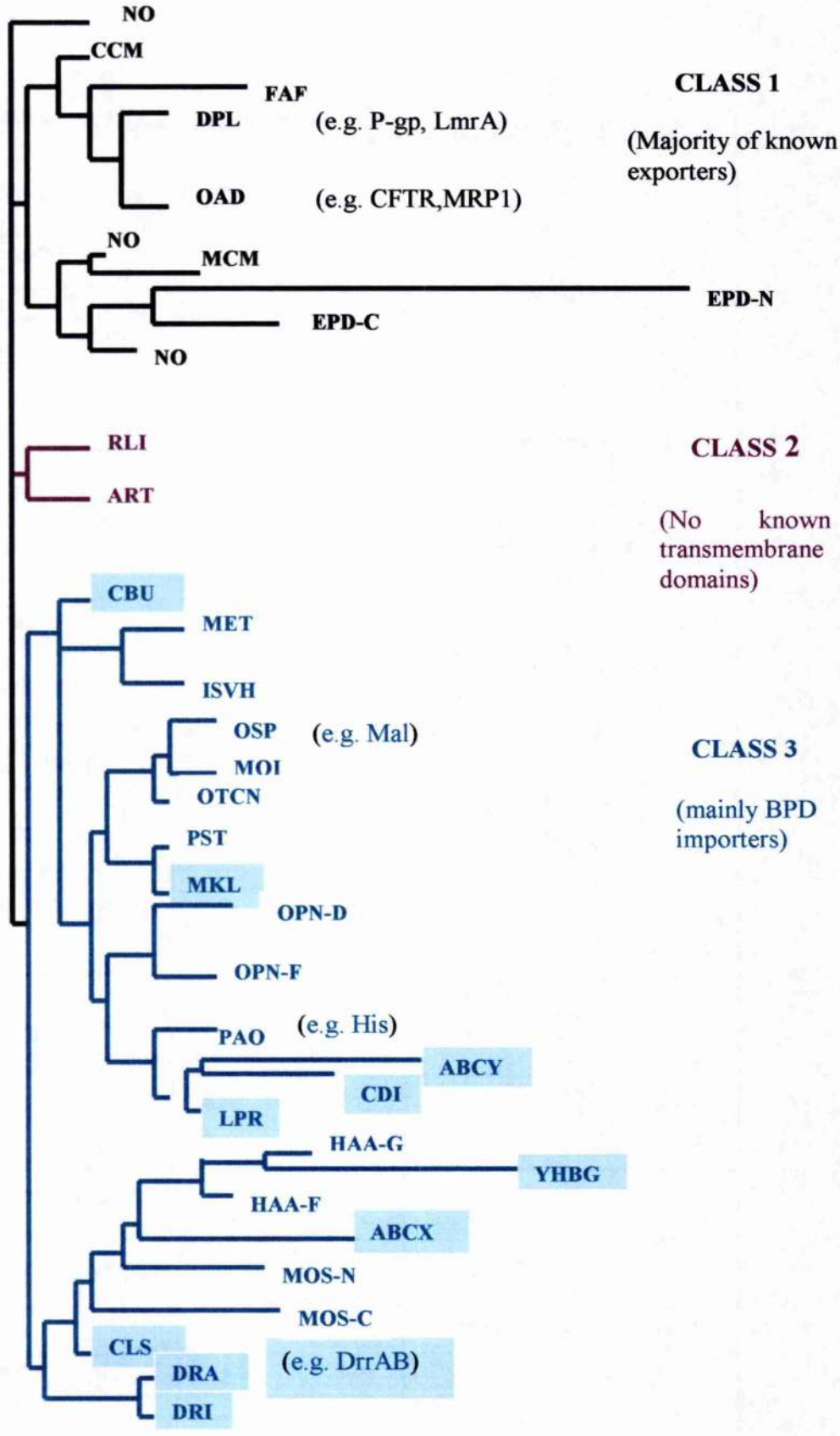

NO
CCM
FAF
DPL
(e.g. P-gp, LmrA)
OAD
(e.g. CFTR,MRP1)
NO
MCM
EPD-N
EPD-C
NO
CLASS 1
(Majority of known exporters)
RLI
ART
CLASS 2
(No known transmembrane domains)
CBU
MET
ISVH
OSP
(e.g. Mal)
MOI
OTCN
PST
MKL
OPN-D
OPN-F
PAO
(e.g. His)
ABCY
CDI
LPR
HAA-G
YHBG
HAA-F
ABCX
MOS-N
MOS-C
CLS
DRA
(e.g. DrrAB)
DRI
CLASS 3
(mainly BPD importers)

**Figure 1.13 Unrooted phylogenetic tree of ABC proteins and domains presented by Dassa and Bouige**

Only the branches pointing to families were drawn. The three classes and their main branches are indicated by different colours; black for class 1, violet for class 2 and teal blue for class 3. In class 3 systems that are not conclusively related to import are highlighted by blue light squares. NO represent a few sequences with unknown function and apparently unrelated to neighbouring families. OPN-D and OPN-F, and HAA-F and HAA-G, correspond to the two different ABC subunits of OPN and HAA systems respectively. –C and –N added to a family name correspond to the C- and N- terminal ABC domains of systems with $(TM\text{-}ABC)_2$, $(ABC\text{-}TM)_2$ and $ABC_2$ organisations; ABC refers to the nucleotide-binding domain, and TM, to the transmembrane domain. The ABC families are mainly named according to their functions. CCM: Cytochrome C maturation or Heme biogenesis; FAE: Long chain fatty acids or enzymes; DPL: Drugs, peptides, lipids; OAD: Organic anions, ions, drugs; MCM: Methanogenesis; EPD: Eye pigment precursors, drugs; RLI: RNAse L inhibition, RNAse stability; ART: Antibiotic resistance, translation, regulation; CBU: Cobalt uptake; MET: Metals; ISVH: Iron-siderophores, vitamin B12, hemin; OSP: Oligosaccharides and polyols; MOI: Mineral and organic ions; OTCN: Osmoprotectants, taurine, cyanate, nitrate; PST: Not mentioned; MKL: Unknown; OPN: Oligopeptides and nickel; PAO: Polar amino acids, opines; ABCY: Unknown; CDI: Cell division; LPR: Release of lipoproteins from membrane; HAA: Hydrophobic amino acids and amines; YHBG: Unknown; ABCX: Unknown; MOS: Monosaccharides ; CLS: Capsular, Lipopolysaccharides, teichoic acids; DRA: Drug and antibiotic resistance; DRI: Drug resistance, bacteriocin immunity (based on diagram by Dassa and Bouige, 2001).

| Family | Saier (a) | HGNC (b) | Function | Substrate type or process | ABC protein prototype (c) | Organisation (d) |
|---|---|---|---|---|---|---|
| FAE | FAT | ABCD | EXPORT | Long chain fatty acids or enzymes | ALD_HUMAN | TM-ABC |
| DPL | (5) | ABCB | EXPORT | Drugs, peptides, lipids | HLYD_ECOLI<br>MDR1_MOUSE | TM-ABC (TM-ABC)2 |
| OAD | (9) | ABCC | EXPORT | Organic anions, ions, drugs | CFTR_HUMAN<br>MRP1_HUMAN | (TM-ABC)2 |
| EPD | (8) | ABCG | EXPORT | Eye pigment precursors, drugs | WHIT_DROME<br>PDR5_YEAST | ABC-TM (ABC-TM)2 |
| CCM | HemeE | | EXPORT | Heme biogenesis | CCMA_ECOLI | 2TM, ABC |
| MCM | | | Unknown | Methanogenesis | Methyl coenzyme M reductase A | ABC2 |
| ART | (7) | ABCF | Cellular processes | Antibiotic resistance, translation, regulation | MSRA_STAEP<br>EF3_YEAST | ABC2 |
| RLI | | ABCE | " | RNase L inhibition, RNA stability | RNA SELI | ABC2 |
| UVR | | | " | DNA repair, antibiotic resistance | UVRA_ECOLI | ABC2 |
| MET | MZT | | IMPORT | Metals (Fe, Zn, Mn) | ZNUC_ECOLI | BP, 2TM, ABC |
| ISVH | (3) | | IMPORT | Iron-siderophores, vitamin B12, hemin | FHUC_ECOLI | OMR, BP, 2TM, ABC |
| OSP | CUTI | | IMPORT | Di- or oligosaccharides and polyols | MALK_ECOLI | BP, 2TM, ABC |
| MOI | (1) | | IMPORT | Minerals and organic ions | CYSA_ECOLI<br>POTD_ECOLI | BP, 2TM, ABC |
| OTCN | (2) | | IMPORT | Osmoprotectants, taurine, cyanate, nitrate | TAUB_ECOLI | BP, TM, ABC |
| OPN | PepT | | IMPORT | Oligopeptides and nickel | OPPD_SALTY<br>NIKE_ECOLI | BP, 2TM,2ABC |
| PAO | PAAT | | IMPORT | Polar amino acids, opines | HISP_SALTY | BP, 2TM, ABC |
| HAA | HAAT | | IMPORT | Hydrophobic amino acids and amides | LIVG_ECOLI | BP, 2TM, 2ABC |
| MOS | CUT2 | | IMPORT | Monosaccharides | RBSA_ECOLI | BP, TM, ABC2 |
| CBU | CoT | | Unknown | Cobalt uptake? | CBIO_SALTY | TM, ABC |
| MKL | | | Unknown | Unknown | MKL_MYCLE | SS,TM,ABC |
| ABCY | | | Unknown | " | ABC_ECOLI | LPP, TM,ABC |
| YHBG | | | " | " | YHBG_ECOLI | SS, TM, ABC |
| CDI | | | " | Cell division | FISE_ECOLI | TM, ABC |
| LPR | DevE | | " | Release of lipoproteins from membranes | LOLD_ECOLI | MFP or SS, (2)TM, ABC |
| ABCX | | ABCA | EXPORT | Unknown | ABCX_CYAPA | ABC, 2 CYT |
| DRA | (10) | | " | Drug and antibiotic resistance | DRRA_STRPE | 2TM, ABC (TM-ABC)2 |
| DRI | (6) | | " | Drug resistance, bacteriocin immunity | BCRA_BACLI | 2TM, ABC |
| CLS | (4) | | | Capsular, lipopolysaccharides, teichoic acids | KST1_ECOLI | OMA, MPA2, TM, ABC |

**Table 1.4. Families of ABC systems identified in living organisms (Dassa and Bouige, 2001)**

The three classes of ABC systems appear with colour codes similar to presented in Fig. 1.13. Family names are abbreviations of the substrate or the biological process handled by the systems. For families comprised of systems with unknown function, an arbitrary name was assigned. (a) Names adopted by M. Saier for the classification of transporters www.biology.ucsd.edu/~msaier/transport/titlepage2.html. Some phylogenetic families described in this table are separated by his author in subfamilies according to substrate type. (1) = SulT, + PhoT + MolT + FeT + POPT + ThiT; (2) = QAT + NitT + TauT; (3) = VB12T + FeCT; (4) = CPSE + LPSE + TAE; (5) = LipidE + GlucanE + Prot1E + Prot2E + Pep1E + Pep2E +Pep3E + DrugE2 + DrugE3 + MDR + CFTR + Ste + TAP + HMT + MPE; (6) = NatE; (7) = DrugRA1 + DrugRA2; (8) = EPP + PDR; (9) = CT1 + CT2; (10) = DrugE1 + CPR. (b) HGNC: these are the names adopted by the Human Gene Nomenclature Committee www.gene.ucl.ac.uk/users/hester/abc.html. (c) When available, the Swissprot ID of the prototype protein is given. (d) Schematic representation of the organisation of ABC systems. BP = extracellular substrate binding protein; LPP = extracytoplasmic protein with a lipoprotein type signal sequence; SS = uncharacterised secreted protein with a signal sequence; TM = integral cytoplasmic membrane domain or protein; ABC = ATP binding cassette module; CYT = cytosolic protein; OMR = high affinity outer membrane receptor; OMP = outer membrane protein; OMA = outer membrane auxiliary protein; MFP = membrane fusion protein; MPA2 = type 2 cytoplasmic membrane periplasmic auxiliary protein

## 1.5 Physiological role of ABC transporters

Different physiological roles have been listed for this superfamily of transporters, which is not too surprising if we take into account the many representatives from diverse lineages that form part of this group of transporters. Transport substrates comprise from very simple (e.g. ions) to complex molecules (e.g. proteins, complex polysaccharides and lipids). In some cases, these transporters are very specific, but in others, they show broad compound specificity. This is the case for transporters involved in multidrug resistance, which not only transport molecules with different physico-chemical properties but also compounds that vary significantly in size.

Apart from humans, fungi and bacteria, ABC transporters are also found in several parasitic protozoa, responsible for important illness around the world. For instance, the P-glycoprotein homologous gene, *pfmdr1* has been reported for the chloroquine resistant *Plasmodium*, the causative agent of malaria (Foote *et al.*, 1989; Wilson *et al.*, 1989). Arsenate and antimony resistant *Leishmania*, where the gene *ltpgpA* has been isolated and its product shown to be more similar to the multidrug-resistance-associated protein (MRP) and to the cystic fibrosis transmembrane regulator (CFTR) than to P-glycoproteins (Cole *et al.*, 1992); emetine-resistant *Entamoeba histolytica*, which causes the universal amoebiasis (Descoteaux *et al.*, 1992), and metronidazole resistant *Trichomonas vaginalis* (Johnson, 1993) are other important examples of parasitic protozoa producing severe diseases in developing countries. In yeast, the *STE6* gene of *Saccharomyces cerevisiae* encoded the first non-mammalian P-glycoprotein described so far (McGrath and Varshavsky, 1989). This protein is not involved in the transport of drugs but of a pheromone, which is necessary for mating. Other ABC transporters are involved in drug resistance, examples of those transporting drugs are the products of the *SNQ2* gene, that confers resistance to 4-nitroquinolone-N-oxide and others chemicals (Servos *et al.*, 1993); and *STS1* and *PDR5* that confer a multidrug resistance phenotype when they are overproduced (Bissinger and Kuchler, 1994). A more extended view of some of the different roles displayed by ABC transporters, with their respective examples, is presented in Table 1.5 to illustrate the importance of this family.

**Table 1.5. Physiological role of ABC transporters**

| Physiological role | ABC transporter representative | Substrate | Organism | Reference |
|---|---|---|---|---|
| Nutrient uptake | HisJQMP | Histidine | *S. typhimurium* | Ames, 1972 |
| | MalEFGK | Maltose | *E. coli* | Davidson and Nikaido, 1991 |
| | BtuCD | Vitamin B12 | *E. coli* | Reynolds *et al.*, 1980 |
| | ModBC | Molybdenum | *E. coli* | Maupin-Furlow *et al.*, 1995 |
| Polysaccharide export | KpsTM | K5 capsullar polysaccharide | *E. coli K5* | Reizer *et al.*, 1992 |
| Peptide and protein export | HlyBD | Haemolysin | *E. coli* | Fath and Kolter, 1993 |
| | BcrABCD | Bacitracin | *Bacillus licheniformis* | Podlesek *et al.*, 1995 |
| | PrtED | Proteases | *Erwinia chrysantemii* | Létoffé *et al.*, 1990 |
| | NisT | Nisin precursor | *L. lactis* | Engelke *et al.*, 1992 |
| | LcnC | Lactococcins | *L. lactis* | Franke *et al.*, 1999 |
| | CvaB | Colicin V | *E. coli* | Wu and Tai, 2004 |
| Cellular signalling and differentiation | NodIJ | Nod factors | *Rhizobium leguminosarum* | Spaink *et al.*, 1995 |
| | YbdAB[a] | Unknown[a] | *B. subtilis* | Isezaki *et al.*, 2001 |
| Multidrug resistance | P-gp (ABCB1) | Multidrugs | *H. sapiens* | Gottesman *et al.*, 1996 |
| | MRP (ABCC1) | Multidrugs | *H. sapiens* | Cole and Deeley, 1998 |
| | LmrA | Multidrugs | *L. lactis* | Van Veen *et al.*, 1998 |
| | Pdr5 | Multidrugs | *S. cerevisiae* | Bissinger and Kuchler, 1994 |
| | MacB | Macrolides | *E. coli* | Kobayashi *et al.*, 2001 |
| | Pgh1[b] | Multidrugs[b] | *P. falciparum* | Ruetz *et al.*, 1996 |
| Intracellular export | TAP[c] | Peptides[c] | *H. sapiens* | Beck *et al.*, 1992 |
| | Atm1p | Iron/sulphur clusters | *S. cerevisiae* | Leighton and Schatz, 1995 |
| | ADLP (ABCD1) | Long-chain fatty acids | *H. sapiens* | Mosser *et al.*, 1993 |
| Ion channels | CFTR (ABCC7) | Chloride anions | *H. sapiens* | Luckie *et al.*, 2003 |
| | SUR[d] (ABCC8/ABCC9) | Potassium cations[d] | *H. sapiens* | Aguilar-Bryan and Bryan, 1999 |

[a] A regulatory role in the sporulation of *B. subtilis* has been suggested (Isezaki *et al.*, 2001)

[b] The multidrug transport Pgh1and its resistance role in *P. falciparum* has remained controversial due to contradictory results obtained by different authors (Bray *et al.*, 1992; Ginsburg and Krugliak, 1992, Krogstad *et al.*, 1987, 1992)

[c] Antigen processing

[d] Potassium channels ATP dependent

## 1.6 The drug-efflux system of *Streptomyces peucetius*

### 1.6.1 Actinomycetes

The Actinomycetes are a group of microorganisms that show characteristics of both bacteria and fungi. The growth habit of Actinomycetes is hyphal and may form a mycelium characteristic of fungi, although hyphae are always of smaller diameter and usually do not exceed one micrometre (Alexander, 1961). Additionally, Actinomycetes are able to produce an aerial mycelium and conidia characteristic of fungal species. When they are grown in pure liquid culture, the turbidity usually associated with bacterial growth is absent, and in many species the growth rate is not exponential as it is in bacteria (Alexander, 1961). However, unlike fungi, actinomycetes are prokaryotes. They have been placed in the bacterial group of $Gram^{+}$, and in the order Actinomycetales. Actinomycetes play both detrimental and beneficial roles in nature. Among their negative attributes is their ability to cause opportunistic diseases in animals, humans, forestry and plants, and spoilage of hay, straw, cereal grains, seeds, bagasse, plant fibres, wood, pulp, paper, wool, hydrocarbons, rubber and plastic. Biodegradation by these organisms, on the other hand, plays an extremely useful role in waste removal and as an integral part of the recycling of materials in nature. Actinomycetes are numerous in soil, where they have three important environmental roles: decomposition of the organic matter; binding clay particles to their filaments that is conducive to crop production; and responsible in part, for the earthy odour of soil. Many Actinomycetes are also good chitin degraders because Actinomycetes are "late colonisers" and play vital roles in degradation processes and the formation of humus (Schrempf, 1995). Actinomycetes decompose lignocellulose from plant residues and have potential for bioconverting waste materials into chemicals. Mesophilic and thermophilic species produce cellulases, xylanases, amylases, proteases and ligninases. Thermotolerant and thermophilic species are active in composts degrading plant polymers and generating heat. The ability of certain Actinomycetes to degrade lignin and/or cellulose is potentially important in the production of liquid fuel and chemicals from lignocellulose (Piret and Demain, 1988).

Aerobic Actinomycetes that are frequently found in environmental microbiology include *Streptomyces, Rhodococcus, Nocardia, Mycobacterium, Actinomadura, Nocardiopsis*, and *Dermatophilus* (Grant and Long, 1981). Most attention has been focused on the ability of Actinomycetes to synthesise antibiotic compounds. Thus, the search for novel species and the mass screening of species for secondary metabolites has been a major focus of pharmaceutical companies worldwide.

Natural antibiotics, particularly those from the genus *Streptomyces*, are as important as the _-lactam antibiotics, which are derived from chemical modification of existing antibiotics.

Actinomycetes are rather difficult to identify at the species level but cell wall type, extracted sugars, morphology and the colour of mycelia and sporangia, G + C content, membrane phospholipid composition, and the heat resistance of spores are all useful in this regard. Comparison of 16S rRNA sequences and pulse-field electrophoresis of large DNA fragments produced by restriction endonuclease digestion are also used for classification purposes.

### 1.6.2 *Streptomyces*

The order *Actinomycetales* is divided into 10 suborders. The *Streptomycineae*, is the most relevant suborder as it contains the *Streptomyces* genus, many species of which are ecologically and medically important. Research on *Streptomyces* indicates that they, along with other fungi, may play an important role in the degradation of lignin. They play important roles in degradation in compost piles. *Streptomyces* are also important agents in the degradation of organic matter in soil and contribute to the formation of stable humus (Metting, 1992). A few species are pathogenic for animals and humans (McNeil and Brown, 1994); *Streptomyces somaliensis* is known to be pathogenic in humans, causing actinomycetoma (Nasher and Hay, 1998), which is an infection of subcutaneous tissues that produces swelling, abscesses and even bone destruction. Other species are phytopathogens.

The *Streptomyces* genome is about twice the size of *E. coli*, and the organism is characterised by the presence of L,L-diaminopimelic acid (L,L-DAP) in its cell wall peptidoglycan. It also possesses complex polar lipids that typically contain diphosphatidylglycerol. Members of the genus are aerobes and chemoorganotrophic, having an oxidative type of metabolism. They are catalase positive and generally reduce nitrates to nitrites, degrade adenine, esculin, casein, gelatin, hypoxanthine, starch and L-tyrosine, and use a wide range of organic compounds as sole sources of carbon for energy and growth (Goodfellow and Cross, 1974). *Streptomyces albus* is the species prototype and the differentiation of species in this genus is difficult. There are over 50 named species, and their differentiation is based on a combination of pigmentation, morphological and physiological traits.

On isolation, colonies are discrete and lichenoid, leathery or butyrous; initially relatively smooth surfaced but later they develop a wide variety of aerial mycelia that may appear granular, powdery, velvety or floccose (Alexander, 1991). *Streptomyces* produce a wide variety of pigments responsible for colours of the vegetative mycelium, aerial mycelium and substrate, being the colour of mature sporulated aerial mycelia, white, grey, yellow and some other colours in between. *Streptomyces* are slow growing organisms and isolation plates are often incubated for 1 to 2 weeks to allow differentiation and adequate growth of the colonies (Fig. 1.14).

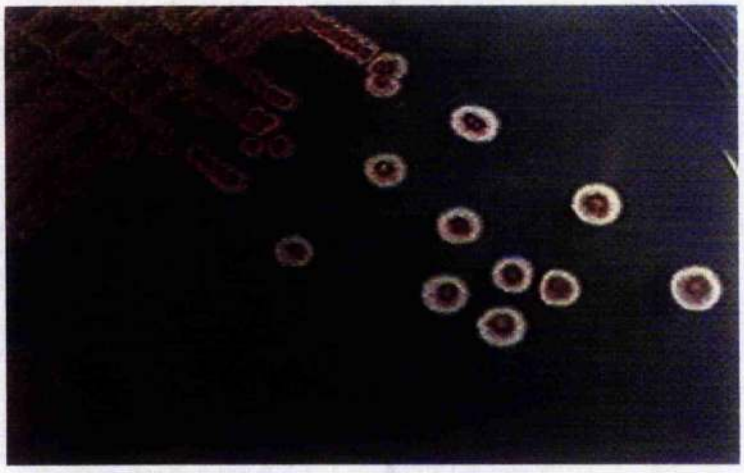


**Figure 1.14 *Streptomyces* colonies on agar** (reproduced from http://filebox.vt.edu/users/chagedor/biol_4684/Microbes/strep.html)

Besides producing many secondary metabolites, extracellular enzymes, enzymatic inhibitors and pigments (Schrempf, 1991), the bacterial genus *Streptomyces* is noted for the production of antibiotics, as much in volume as in diversity. Practically all the different chemical families of antibiotics active against bacteria, fungi, algae, viruses or tumours are produced by some representative from *Streptomyces*.

### 1.6.3 Drug export as self-protection mechanism in antibiotic producing organisms

Diverse mechanisms to protect antibiotic-producing organisms have been described (Ohnuki *et al.*, 1985; Levy, 1992). In tetracycline producers, cytoplasmic resistance determinants (e.g. TetM, OtrA) are associated with the ribosome and prevent its interaction with the antibiotic (Roberts, 1994; B_hal and Hunter, 1995). The microorganism may lack the target site for the antibiotic it produces (Vining, 1979). Modification of the antibiotic or its target site in the producing organism and active transport out of the cell seem to be the most common mechanisms to avoid the effect of these autotoxic compounds. A resistance mechanism implied for producers of antibiotic peptides (e.g. subtilin, nisin, lacticin) seems to require the presence of a couple of ABC transporters, one of them being necessary for the re-translocation of exported peptides that have penetrated the membrane from the external media (Abee, 1995). Another ABC peptide exporter also involved in producer self-protection is the bacitracin transporter (Bcr) of *B. licheniformis* (Podlesek *et al.*, 1995). Both proton-dependent transmembrane electrochemical gradients and ABC pumps are represented in *Streptomyces* self-resistance mechanisms. The self resistance of *S. peucetius* to the anti-tumour anthracycline compounds daunorubicin and doxorubicin, is one of the known examples that involves active transport of antibiotic compounds out of the producing cell to avoid its toxicity (Guilfoile and Hutchinson, 1991; Kaur, 1997). Other *Streptomyces* species possess self-resistance mechanisms that involve ABC transporters; some examples include the tylosin-resistance protein TlrC from *Streptomyces fradiae* (Rostek *et al.*, 1991), which is similar to the DrrA protein; the oleandomycin transporter OleC from *Streptomyces antibioticus* (Olano *et* al., 1996); the *Streptomyces argillaceus* mithramycin resistant-determinant (Fernandez *et al.*, 1996); the Cb carbomycin-resistance determinant in *Streptomyces thermotolerans*

(Epp *et al.*, 1987); and *Streptomyces rochei msr* that is another gene that would encode a multidrug ABC transporter (Fernandez-Moreno *et al.*, 1998).

A summary of some examples of ABC transporters responsible for antibiotic resistance mechanisms in antibiotic producing microbes can be seen in Table 1.6. As many ABC transporters have been located in antibiotic biosynthetic gene clusters, it is logical to link their presence to some natural function for the export of the product across the cellular membrane to avoid their autotoxic effects (Mendez and Salas, 2001).

**Table 1.6. ABC transporters in antibiotic-producing actinomycetes**

| Synthesised antibiotic | Producer organism | ATP binding protein | Membrane protein | References |
|---|---|---|---|---|
| **Type I** | | | | |
| Daunorubicin | *S. peucetius* | DrrA | DrrB | Guilfoile and Hutchinson, 1991 |
| Mithramycin | *S. argillaceus* | MtrA | MtrB | Fernandez *et al.*, 1996 |
| Oleandomycin | *S. antibioticus* | OleC | OleC5 | Rodriguez *et al.*, 1993 |
| Tetronasin | *S. longisporoflavus* | TmrB2 | TmrB3 | Linton *et al.*, 1994 |
| Macrotetrolide | *S. griseus* | Non-orf5 | Non-orf6 | Smith *et al.*, 2000 |
| Kasugamycin | *S. kasugaensis* | KasK | KasI., KasM | Ikeno *et al.*, 2000 |
| Rapamycin | *S. hygroscopicus* | OrfX | ---------- | Schwecke *et al.*, 1995 |
| **Type II** | | | | |
| Carbomycin | *S. thermotolerans* | CarA | - | Schöner *et al.*, 1992 |
| Spiramycin | *S. ambofaciens.* | SrmB | - | Schöner *et al.*, 1992 |
| Tylosin | *S. fradiae* | TlrC | - | Rosteck *et al.*, 1991 |
| Oleandomycin | *S. antibioticus* | OleB | - | Olano *et al.*, 1996 |
| A201A | *S. capreolus* | Ard1 | - | Barrasa *et al.*, 1995 |
| Lincomycin | *S. lincolnensis* | LmrC | - | Peschke *et al.*, 1995 |
| Frenolicin | *S. roseofulvus* | FnrD | - | AF058302 |
| Virginiamycin | *S. virginiae* | VarM | - | Kawachi *et al.*, 2000 |
| **Type III** | | | | |
| Bleomycin | *Stv. Verticillum* | Ble-Orf7 | In type III transport both domains are fused | Calcutt and Schmidt, 1994 |
| Streptomycin | *S. glaucescens* | StrV, StrW | | Beyer *et al.*, 1996 |
| Chloroeremomycin | *A. Orientalis* | Orf2 | | Wageningem *et al.*, 1998 |
| Novobiocin | *S. sphaeroides* | NovA | | Steffensky *et al.*, 2000 |
| Nystatin | *S. noursei* | NysG and NysII | | Brautaset *et al.*, 2000 |

In producer organism column: A is *Amycolatopatopsis*; S is *Streptomyces* and; Stv is *Streptoverticillum*. In type I transporters, ATP-binding domains and membrane domains are produced in separate proteins. In type II transporters, membrane domains have not been reported. In type III transporters, ATP-binding domains are fused to membrane domains in a single protein. This table is an adaptation of the table presented by Mendez and Salas, 2001.

### 1.6.4 The DrrAB pump system

In addition to antibiotics like aminoglycosides, tetracyclines, chloramphenicol, and some β-lactams that are produced by members of the genus *Streptomyces*, *S. peucetius* produces the antibiotics doxorubicin and daunorubicin (Guilfoile and Hutchinson, 1991). These compounds are classified into the anthracyclines and possess antitumoral activity (Arcamone, 1981), expanding the arsenal of compounds with different activities produced by *Streptomyces*.

The way in which *S. peucetius* avoids the effects of the anthracyclines they produce, is by pumping them out of the cell (Guilfoile and Hutchinson, 1991) using an ABC transporter (Gottesman and Pastan, 1993). Two subunits are present, one of them, DrrA, is a peripheral membrane protein that acts as the energy-transducing component, and the other, DrrB, is the membrane carrier. This type of permease carries out export of antibiotics in an ATP-dependent manner.

In 1991, Guilfoile and Hutchinson cloned, sequenced, and transcribed the genes that confer resistance to the daunorubicin and doxorubicin compounds. The transformation of *Streptomyces lividans* allowed the analysis and recognition of the *drrAB* locus by resistance selection to the anthracyclines (Guilfoile and Hutchinson, 1991). Transcription of both genes could not be detected early in growth, suggesting that *drrAB* transcription might be co-regulated with the genes for antibiotic production.

Having identified start and stop codons for each reading frame of the operon, they could predict that *drrA* would encode a predominantly hydrophilic protein containing 330 amino acids with a molecular mass of 35,668 Da and a pI of 5. Similarly, *drrB* would encode a very hydrophobic protein, composed of 283 amino acids, a molecular mass of 30,614 Da and a pI of 7.5. They concluded that the sequence of the predicted product of the *drrA* gene was similar to the products of other transport and resistance genes, notably with P-glycoprotein from mammalian tumour cells and that it was probably involved in the transport of the same substrates. DrrB, on the other hand, showed no significant similarity to other known proteins. Later investigations reported that DrrB exhibited statistically significant sequence

homology to integral membrane components of four bacterial ABC transporters, such as NodJ from NodIJ, involved in oligosaccharide export in *R. leguminosarum* (Evans and Downie, 1986), KpsM from KpsTM, involved in capsular polysaccharide export in *E. coli* (Smith *et al.*, 1990; Pavelka *et al.*, 1991), and its capsular polysaccharide export homologues BexB from *H. influenzae* BexABC (Kroll and Moxon, 1990) and CtrC from *N. meningitidis* CtrDCB (Frosch *et al.*, 1991). In conjunction with the homology that DrrA presents with the ATP binding constituents of these four transporters, suggested a novel subclass of transporters to be named the ABC-2 family (Reizer *et al.*, 1992).

The open-reading frames of the *drrrA* and *drrB* genes were sub-cloned and expressed in *E. coli* expression vectors as single or coupled gene constructs (Kaur, 1997). Both gene products showed a molecular mass that corresponded to that predicted from their nucleotide sequences (36 kDa for DrrA and 31 kDa for DrrB). The DrrA protein was present in the cytosolic fraction and was also located in the membrane. The DrrB protein was scarcely expressed in the absence of the *drrA* gene, and was shown to be toxic for *E. coli* growth. DrrA was shown to bind ATP and GTP in the presence of magnesium ions in a UV cross-linking assay. The binding of nucleotide to DrrA was enhanced by the presence of doxorubicin, indicating that the activity of the pump was regulated by its substrate (Kaur, 1997; Kaur and Russell, 1998).

It has been suggested that a very close relationship between both subunits is required for the activity of this system, and subsequently, for the resistance of *S. peucetius* towards its own antibiotic. DrrA was expressed as an active form that could bind ATP only when it was in complex with DrrB. UV cross-linking studies with [$\alpha$-$^{32}$P] ATP showed that only the membrane-bound form of DrrA in cells containing both DrrA and DrrB, was in a conformation competent to bind and/or hydrolyse ATP (Kaur and Russell, 1998). Kaur and Russell's work emphasised that ATP interacted with membrane-bound DrrA and not with DrrA present in the cytoplasmic fraction, irrespective of whether or not it was over-expressed in the presence of DrrB.

Although DrrB seems to be translated in the absence of DrrA, it was only detected after translation of both the *drrA* and *drrB* genes (Kaur, 1997; Kaur, and

Russell, 1998) indicating a requirement upon the *drrA* gene product for stabilisation of the DrrB protein. Chemical cross-linking studies also suggested a direct interaction between DrrA and DrrB with a probable stoichiometry of the complex as $DrrA_2DrrB_2$. The authors proposed that DrrA is an allosteric protein that acquires different conformations depending on the presence or absence of DrrB and the ligand doxorubicin.

Another gene, *drrC*, is also involved in *S. peucetius* resistance to anthracycline antibiotics, specifically daunorubicin (Dnr). However, DrrC is not part of the DrrAB pump; it is proposed to protect DNA from Dnr as soon as its production starts, and that DrrA and DrrB work to export the antibiotic after its concentration rises in the cell (Furaya and Hutchinson, 1998). A regulatory role for the *dnrI* gene product in the production of DrrC, DrrA, and DrrB proteins has been proposed by these authors.

An equivalent DrrAB(C) system was also reported and characterised for *M. tuberculosis* (Choudhuri *et al.*, 2002; Nash, 2003), although its role in this microorganism is not yet clear. DrrA, particularly, shares some features with other NBDs, notably its preference for ATP as substrate and its dependence upon divalent cations like magnesium. When purified in isolation and assayed, ATPase activity is not affected by the presence of the transport substrate and interaction with membrane components does not take place unless the protein is reconstituted into proteoliposomes.

Most ABC transporters consist of two nucleotide-binding domains (NBDs) and two transmembrane spanning domains (TMDs) of six-helices each. DrrAB consists of one NBD and one TMD, equivalent to a half transporter, and it is thought further association is required to form a full transporter structure. A seven-helix topology had been suggested for DrrB, different from the six-helix reported in the transmembrane domains of many other transporters. Recent experiments (Gandlur *et al.*, 2004), support an eight-helix topology (Fig. 1.15). Fusion of DrrB to several reporter genes indicated the presence of the N- and C-termini in the cytoplasm.

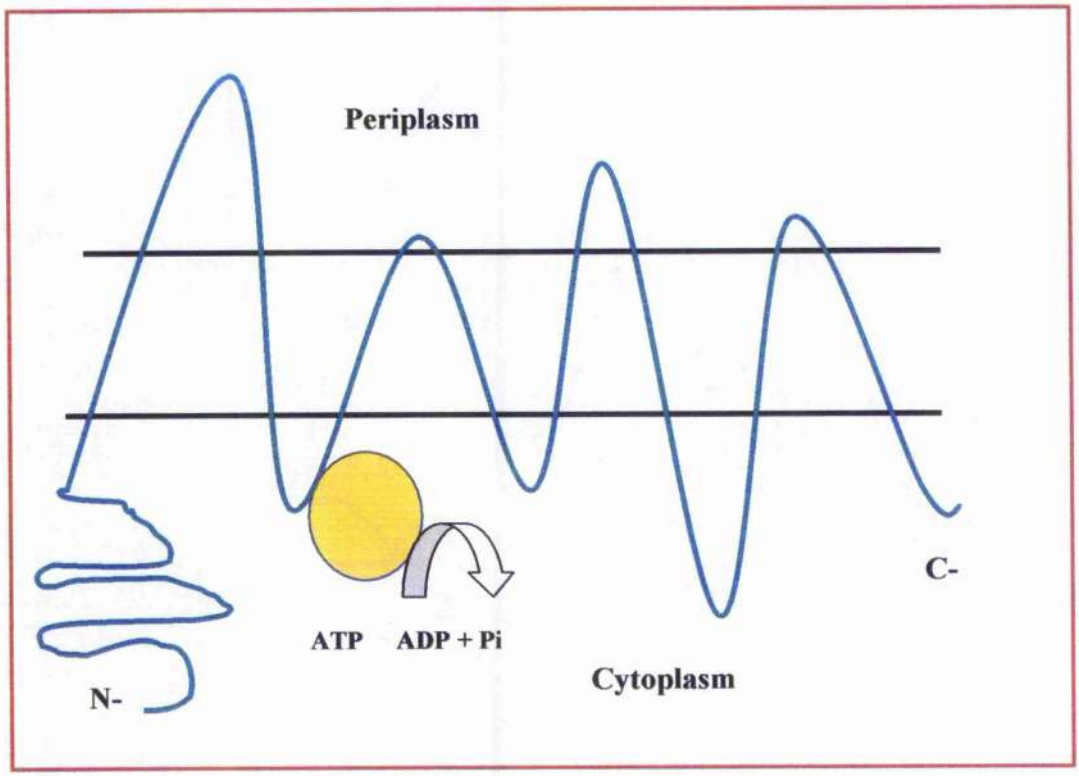



**Figure 1.15 Diagrammatic presentation of the membrane topology of the two transport domains from *S. peucetius DrrAB* transporter.**

Two polypeptides are encoded by different genes; the membrane domain (eight α-helices) by *drrB*, and the nucleotide-binding domain, by *drrA*. The figure above represents a half transporter. Two TM and two NB domains would form the canonical structure for the ABC transporters.

New experiments from Kaur's group (Kaur et al., 2005), have used single-cysteine DrrB mutants in chemical cross-linking experiments. Single cysteine residues were created in the transmembrane and cytosolic loops by site-directed mutagenesis, and subsequently attempts were made to link the DrrB proteins to a primary amine in DrrA. This was used to map possible sites of DrrA DrrB interaction. Interestingly, a motif that might be equivalent to the EAA sequences reported in ABC importers was identified in the N-terminal cytoplasmic tail of DrrB, and shown by sequence analysis to be also present in exporters.

## 1.7 Aims of the project

The goal of this project is to determine the molecular mechanisms of antibiotic binding and translocation for the doxorubicin pump, DrrAB, from *S. peucetius*.

Principally, the approach will be to prepare proteins for transient kinetic experiments to provide mechanistic information. If sufficient protein can be obtained, it might also be used for crystallisation trials to provide structural information. An additional potential goal would be to use the DrrA protein as a component of a biochip to screen for inhibitors of the antibiotic pump.

If the ATP binding domain can be functionally separated from the membrane domain of the transporter it may then be possible to overproduce these domains separately for detailed structure-function studies. Knowledge of the structure of DrrA will be important in elucidating the architecture of the antibiotic binding-site. As such, Drr is an important model system for the study of the molecular mechanism of resistance pumps. Understanding the structure and function of one of these pumps will be vitally important in combating the escalating threat posed by multidrug resistant pathogens and in cancer chemotherapy.

# Chapter 2

# MATERIALS AND METHODS

## 2.1 Reagents and materials

### 2.1.1 Source of reagents and materials

Chemicals were mainly acquired from Sigma and BDH, but as well from Aldrich, Riedel-de Häen, and Fischer. Difco and Oxoid supplied microbial growth media. Enzymes used in molecular biology were obtained from Promega, Roche, or Gibco. Additional reagents or materials for special applications are noted in the respective section in this chapter.

### 2.1.2 Preparation of chemical and media solutions

General chemicals were weighed on an AND digital balance and dissolved in distilled/de-ionised water. Small amounts were weighed on a Metler Toledo digital fine balance.

### 2.1.3 Sterilisation of reagents and materials

Reagents and media to be sterilised were either autoclaved or filter sterilised, depending on their stability to heat. Sterilisation by autoclaving was performed at 121°C for 15 minutes at 4 atmospheres pressure. Filter sterilisation was used for heat labile liquid reagents by filtration through a 0.2 μm Millipore syringe filter.

### 2.1.4 Centrifugation

Routine room temperature centrifugation of 1.5 ml microcentrifuge tubes was performed in a MSE MicroCentaur benchtop microcentrifuge, fitted with a 24 place fixed angle rotor. When cooling was required, a Jouan CRi benchtop refrigerated centrifuge was used. Larger volumes of liquids were processed in a Beckman-Coulter Avanti J-E refrigerated centrifuge accommodating several different fixed angle rotors. A Beckman JA-10 rotor was used to process volumes up to 3 litres in 500 ml plastic containers, whilst a Beckman JA-21 rotor was used to process up to 400 ml in 50 ml

tubes. High-speed ultracentrifugation was performed in a Beckman L8M ultracentrifuge using a Beckman Ti50 fixed angle rotor.

### 2.2 Microorganisms; growth and storage

A list of all bacterial strains used in this thesis is presented in the Table 2.1.

#### 2.2.1 Growth media

Basic liquid cultures for propagation of *E. coli* strains used two main types of growth media:

Luria-Bertani (LB) medium:

| | |
|---|---|
| NaCl | 10 g/L |
| Tryptone | 10 g/L |
| Yeast extract | 5 g/L |

2X YT medium:

| | |
|---|---|
| NaCl | 5 g/L |
| Tryptone | 16 g/L |
| Yeast extract | 10 g/L |

**Table 2.1 Bacterial strains used in this study**

| Microorganism | Genotype | Origin | Application |
|---|---|---|---|
| *Escherichia coli* Novablue | *endA1 hsdR17($r_{k12}^-$ $m_{k12}^+$) supE44 thi –1 recA1 gyrA96 relA1 lac [F' proA$^+$B$^+$lac$^q$Z_M15::Tn10(tet$^R$)]* | Novagen | General cloning |
| *Escherichia coli* XL1-Blue | *recA1 endA1 gyrA96 thi –1 hsdR17supE44 relA1 lac [F' proA$^+$B$^+$lac$^q$Z_M15Tn10(tet$^R$)]* | Stratagene | General cloning |
| *Escherichia coli* XL10 Gold | *Tet$^r$ _(mcrA)183 _(mcrCB-hsdSMR-mrr)173 end A1 suoE44 thi-1 recA1 gyrA96 relA1 lac The[F' proAB lac$^q$Z _M15 Tn10 (Tet$^r$) Amy Cam$^r$* | Stratagene | General cloning |
| *Escherichia coli* TOP10 | *F$^-$ mcrA _(mrr-hsdRMS-mcrBC) _80lacZ_M15 _lacX74 recA1 deoR araD139 _(ara-leu)7697 galU galK rpsL (Str$^R$) endA1nupG* | Invitrogen | pBADTOPO cloning and mediated recombinant expression |
| *Epicurian coli* BL21 (DE3) | *E. coli B F$^-$ omp had S ($r_B^-$ $m_B^-$) dcm$^+$ Tet$^r$ gal EndA The[argU ileY leuW Cam$^r$]* | Stratagene | pET21-a mediated recombinant expression |
| *Epicurian coli* BL21 AI | *F$^-$ ompT$^-$ hsdS$_B$ ($r_B^-m_B^-$) gal dcm araB::T7RNAP-tetA* | Invitrogen | pET21-a mediated recombinant expression |
| *Escherichia coli* BMH mutS | *Thi, sup*E, *_(lac-pro*AB, [*mut*S:Tn10] [F', *pro*AB, *lac*_qZ_M15 | Promega | Site-directed mutagenesis |
| *Escherichia coli* DH5 pro | *_80lacZ_M15 recA1 endA1 gyrA96 thi-1 hsdR17($r_k^-$ $m_k^+$) supE44 relA1 deoR _(lacZYA-argF)U169* | Promega | General cloning |
| *Escherichia coli* LMG194 | *F$^-$ mcrA _lacX74 gal E thi rpsL _phoA (Pvu II) _ara714 leu::Tn10* | Invitrogen | pBADTOPO cloning and mediated recombinant expression |
| *Escherichia coli* M15[pREP4] | *Nal$^s$ Str$^s$ Rif$^{ss}$ Thi$^-$ Lac$^-$ Ara$^-$ Gal$^+$ Mtl$^-$ F$^-$ RecA$^+$ Uvr$^+$ Lon$^-$* | Qiagen | Protein expression |

### 2.2.2 Growth of bacteria

*Escherichia coli* strains were routinely cultured in Luria-Bertani (LB) broth, supplemented with the appropriate antibiotic where necessary. When solid media were prepared, agar was added at 1.5% w/v prior to autoclaving. To provide higher cell densities when large volumes of cells were required for expression cultures, 2X YT broth was used. Other media such as SOC and $NZY^+$ were used in some specific cases according to the requirements of the particular protocol.

Inoculation in liquid broth was achieved by transferring a single colony from a plate to an appropriate volume of medium. The culture was incubated, with rotary shaking at 200 rpm and 37 °C, in a 25-ml conical bottomed universal container.

Culture on solid media was achieved by streaking or spreading organisms onto the agar surface, using a sterile microbiological loop or sterile spreader. Plates were placed in a 37 °C incubator, in an inverted position, for 18 hours.

### 2.2.3 Storage of bacteria

Stock cultures of each bacterial strain used were prepared in order to maintain their long term integrity and viability. Stocks were typically prepared by mixing 800 µl of an overnight liquid culture with 200 µl of sterile 80% glycerol, and stored at -80 °C. These strains were retrieved by scraping a small amount of the frozen stock using a sterile loop, and then streaking this material onto solid growth medium for overnight incubation at 37 °C.

### 2.2.4 Antibiotics

The use of antibiotic markers in a culture is a useful tool to ensure the presence of plasmids and the selection of strains that carry specific genetic markers such as antibiotic resistance genes, avoiding contamination by other microorganisms. Antibiotics were made up at the following final concentrations unless otherwise stated and filter sterilised before use: ampicillin 100 mg/ml; carbenicillin 100 mg/ml; kanamycin 50 mg/ml; doxorubicin 2 mg/ml in DMSO.

## 2.3 Isolation and analysis of nucleic acids

### 2.3.1 Agarose gel electrophoresis

Electrophoresis in horizontal gel slabs was used for analysis of DNA. Agarose was dissolved at 0.9-1% (w/v) in TAE buffer by boiling the mixture in a microwave. The solution was allowed to cool slightly before the addition of the DNA staining reagent ethidium bromide at a final concentration of 0.1 μg/ml. The mixture was poured into a horizontal gel-tray and allowed to set solid by cooling. Wells for sample loading were created in to the agarose slabs by fitting a comb into one end of the molten agarose solution. The agarose slabs were then placed into electrophoresis tanks and submerged in TAE buffer prior to sample loading.

The DNA samples were mixed with 6X loading buffer and applied to the gel wells, whilst a 1 kb DNA ladder (Roche Molecular Diagnostics; 5 μg) was used for reference. Gels were typically run at 110 V.

The presence of the dyes bromophenol blue and xylene cyanol in the loading buffer, allowed the progression of the samples through the gel to be observed so that we could determine when the DNA had migrated a sufficient distance. Then the gel was examined on an ultraviolet light transilluminator to visualise the DNA. If necessary, the gel was photographed using a Polaroid MP4 instant camera system or a SynGene Bio Imaging system.

Buffer compositions:
1X TAE:
4.84 g Tris base
1.142 ml glacial acetic acid
2 ml 0.5 M EDTA
Made up to 1 L in distilled water

6X DNA loading buffer:
100 mM EDTA (pH 8.0)
1% w/v SDS
0.1% w/v bromophenol blue

0.1% w/v xylene cyanol
50% glycerol

### 2.3.2 Small scale preparation of plasmid DNA

Bacterial strains harbouring plasmids were cultured in 5 ml LB broth, containing appropriate antibiotics, and harvested by centrifugation. Plasmid DNA was then isolated using the QIAprep Spin Miniprep Kit (Qiagen), according to the manufacturer's instructions. The procedure uses the modified alkaline lysis method of Birnboim and Doly (Birnboim and Doly, 1979), and the lysate is subsequently neutralised and adjusted to high-salt conditions in one step, before centrifugation. The next step comprises the absorption of plasmid DNA onto a silica-gel membrane housed in the QIAprep spin column. The final steps comprise the washing and elution of purified plasmid DNA from the column.

### 2.3.3 Extraction of DNA from agarose gels

DNA was extracted from agarose gels for cloning. This was accomplished using the QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's instructions. The sample containing the DNA target was separated on an agarose gel containing ethidium bromide. After visualisation of the DNA on a UV transilluminator, the bands required were excised from the gel for extraction. The gel slices were mixed with solubilisation buffer (solution QG) at a ratio of 300 µl per 100 mg of agarose and incubated in a water bath at 50 °C, until complete dissolution of the agarose had been achieved. The sample was then applied to a spin column, allowing DNA adsorption to its silica-membrane. Other components of the solution were cleared by centrifugation through the spin column. Subsequent washing removed traces of agarose and residual buffers. The DNA was eluted in a last step.

### 2.3.4 Alternative Procedure for gel extraction/purification/concentration of DNA

Alternatively, the Qiaex II resin (Qiagen) was also used for DNA gel extraction following the manufacturer's instructions.

## 2.4 DNA amplification, cloning and sequencing

### 2.4.1 Use of the Polymerase chain reaction (PCR)

PCR was mainly used to amplify the *drrA* gene for insertion into different vectors. Alternatively, PCR was also used for site-directed mutagenesis and assessment of the orientation of genes inserted in the pBADTOPO thio vector.

#### 2.4.1.1 Template

*Streptomyces peucetius drrA*, or *drrA* and *drrB*, were prepared by PCR using the cosmid pWHM612 (the kind gift of Guilfoile and Hutchinson) as template.

#### 2.4.1.2 Oligonucleotide primers for PCR

Oligonucleotide primers for PCR (Table 2.2) were synthesised at a 50 nmol scale by Life Technologies and supplied desalted and lyophilised. Primers were re-suspended in the appropriate volume of 10 mM Tris-HCl pH 8.0 to achieve a stock concentration of 50 pmol per microlitre.

**Table 2.2 Oligonucleotide primers for PCR**

| Primer name | Sequence (5' to 3') |
|---|---|
| pET21a-*drrA* forward | CAT ATG AAC ACG CAG CCG ACA CGG GCC ATC GAA |
| pET21a-*drrA* reverse | CTC GAG TGC CAC CTT CTC CTC TTC CGC CGC TTC TTC |
| pET21a-*drrAB* forward | GCT AGC CAT CAT CAT CAT CAT CAT AAC ACG CAG CCG ACA CGG GCC ATC |
| pET21a-*drrAB* reverse | AAG CTT TCA GTG GGC GTT CTT GTT GCG GTA CAG ACG CAT GGT CAG CGG |
| pET33b-*drrAB* forward | GCT AGC AAC ACG CAG CCG ACA CGG GCC ATC |
| pET33b-*drrAB* reverse | AAG CTT TCA GTG GGC GTT CTT GTT GCG GTA |
| pBADTOPO-*thiodrrA* forward | GTG AAC ACG CAG CCG ACA CGG GCC ATC GAA |
| pBADTOPO- *thiodrrA* reverse | TGC CAC CTT CTC CTC TTC CGC CGC TTC TTC |
| PUC18-*drrAB forward* | GAA TTC GCA CCA CCA CCA CCA CAA CAC GCA GCC GAC ACG GGC CAT CGA |
| PUC18-*drrAB* reverse | AAG CTT TCA GTG GGC GTT CTT GTT GCG GTA CAG ACG CAT GGT CAG CGG |
| pQE-*drrA* forward | GAG CTC GTG AAC ACG CAG CCG ACA CGG GCC ATC |
| pQE-*drrA* reverse | AAG CTT TCA TGC CAC CTT CTC CTC TTC CGC CGC |

### 2.4.1.3 Standard PCR

PCR was carried out using HotStarTaq™ thermostable DNA polymerase (a derivative of *Thermus aquaticus* DNA polymerase) (Qiagen) and its respective buffers. This enzyme requires a period of incubation of 15 minutes at 95 °C to be active. This characteristic of the polymerase increases the probability of specific priming and extension. Typical reaction composition is presented below with cycling parameters (Table 2.3). Particular PCR conditions will be presented in the respective Results section.

A one-tube format reaction was performed using the following reaction components:

| | |
|---|---|
| Final volume | 100 µl |
| 10X PCR buffer | 10 |
| dNTPs (10 mM) | 2 |
| Primer 1 (50 pmol/µl) | 2 |
| Primer 2 (50 pmol/µl) | 2 |
| DNA polymerase | 1 |
| Millipore water | 82 |
| Template DNA (40 ng/µl) | 1 |

Deoxyribonucleotides dATP, dCTP, dGTP and dTTP were obtained as 100 mM stock solutions. A mixture of the four dNTP's was prepared with each one at a concentration of 10 mM.

On some occasions the use of the proprietary reagent Q-solution (supplied with HotStar Taq DNA polymerase) was essential to the success of the amplification of the DNA target sequence. This solution alters the melting properties of duplex DNA.

**Table 2.3 Cycling parameters for standard PCR reaction**

| Segment | Cycles | Temperature | Time |
|---|---|---|---|
| Hot Star Taq activation | 1 | 95 °C | 15 minutes |
| Denaturation | 30 | 94 °C | 45 seconds |
| Annealing | | Gradient 50-60 °C | 45 seconds |
| Extension | | 72 °C | 1 min. 30 sec. |
| Final extension | 1 | 72 °C | 10 minutes |

PCR was performed on either an Eppendorf Gradient Thermocycler or a Techne Cyclogene Thermocycler. Both machines have heated lids which prevent evaporation of the reaction mixes during the cycling process. These lids were set to 105 °C, removing the need for a layer of mineral oil over each reaction.

Reactions were set up on ice and transferred to the thermocycler for PCR. Thin-walled PCR reaction tubes and sterile tips were used for all reactions.

## 2.4.2 Vectors

### 2.4.2.1 pGEMT-Easy (Promega)

This vector was used for the cloning of all the PCR products except for the product to be inserted into the pBADTOPO thiofusion vector. These vectors are convenient systems for cloning of PCR products as they present single terminal T overhangs, which not only impede the recircularisation of the vector, but also provide compatible overhangs for PCR products that have been generated by thermostable polymerases with terminal transferase activity. These enzymes often add a single deoxyadenosine to the 3' ends of the amplified fragments.

#### 2.4.2.2 pET21a (Novagen)

*Nde*I and *Xho*I restriction sites flanking the *drrA* gene were incorporated into the primers used for amplification by PCR, and these sites in the pET21a plasmid were used for cloning. In this vector (Fig. 2.1), the sub-cloned gene is placed under control of the strong bacteriophage T7 promoter. Sub-cloning of both the *drrA* and *drrB* genes into pET21a was also performed. For this reaction, *Nhe*I and *Hind*III restriction sites were used.

#### 2.4.2.3 pET33b (Novagen)

The vector pET33b was also used to sub-clone *drrA* and *drrB* together. *Nhe*I and *Hind*III restriction sites flanking the *drrAB* sequence were designed into the 5' termini of the primers.

#### 2.4.2.4 pBADTOPO thiofusion (Invitrogen)

For cloning into this vector, *drrA* primers did not carry any restriction sites as the vector carries the enzymatic machinery for ligation of the PCR product. This plasmid (Fig. 2.2) contains the *araBAD* promoter that can be tightly regulated to produce recombinant protein in a more controlled fashion.

#### 2.4.2.5 pQE100 (Qiagen)

*Sac*I and *Hind*III restriction sites were designed into *drrA* primers to enable ligation between these sites in the pQE100 plasmid. In pQE vectors, expression of the sub-cloned gene is achieved from the bacteriophage T5 promoter.

### 2.4.3 PCR based DNA sequencing

Sequencing of DNA was carried out by BaseClear (Leiden, The Netherlands), and by the University of Durham. Purified DNA template and, were necessary, gene specific primers, were sent to the service providers. Sequence data were returned in the form of text and chromatogram files for manipulation and analysis. Data were analysed using Vector NTI 6.0 bioinformatics software (InforMax Inc.).

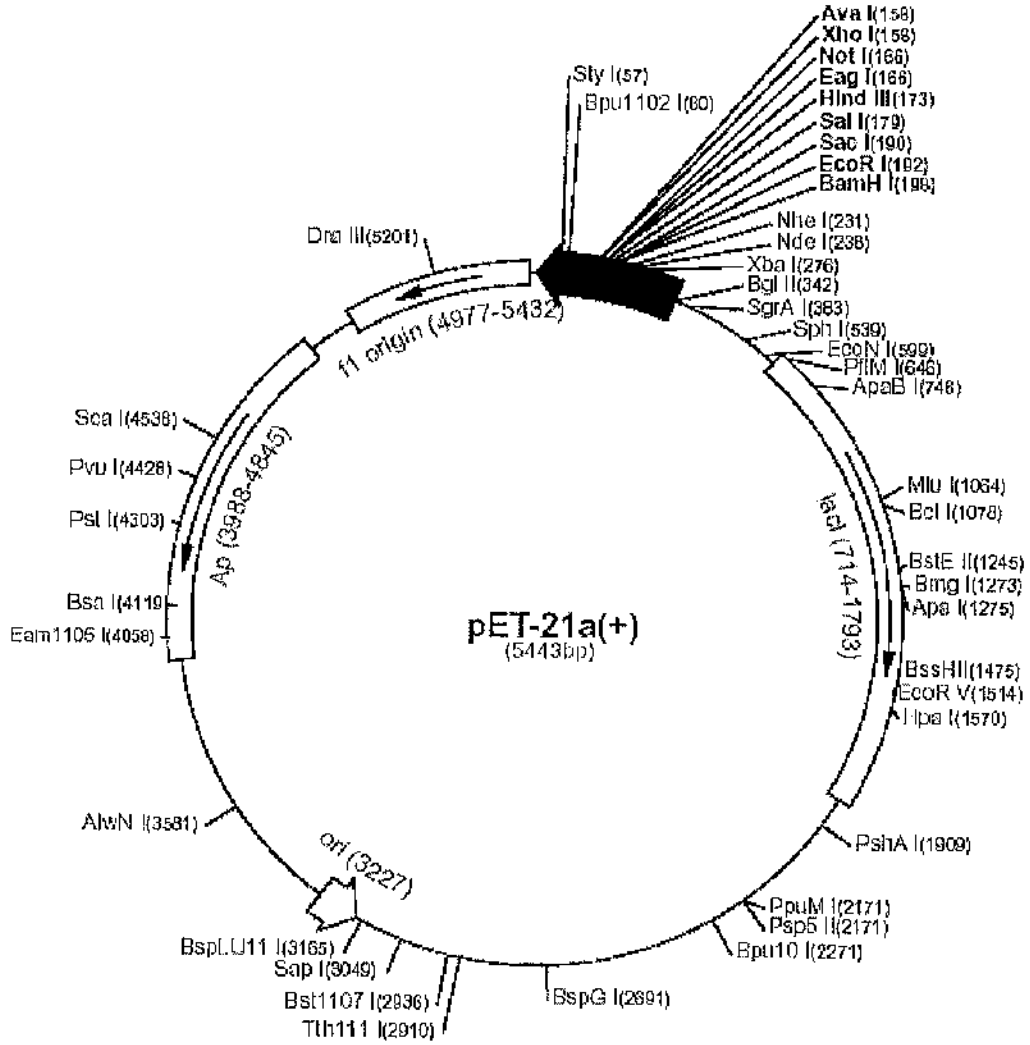



**Figure 2.1 Map of the pET-21a vector used to clone the *drrA* and *drrAB* genes** (from Novagen)

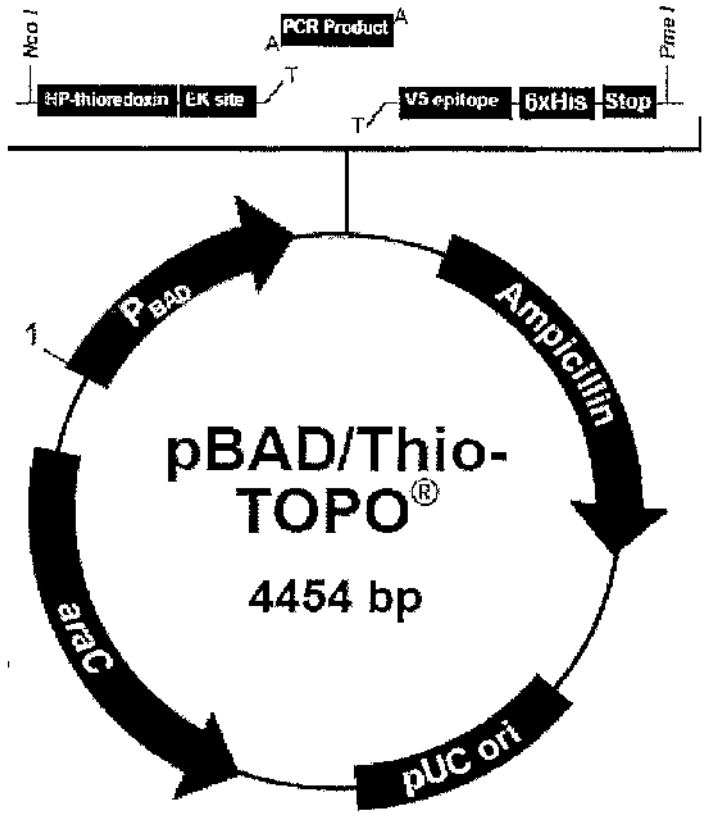



**Figure 2.2 Map of the pBADTOPO thiofusion vector used to clone the *drrA* gene** (from Invitrogen)

## 2.5 Enzyme mediated manipulations of DNA

### 2.5.1 Restriction enzyme digestion

Digestion of DNA with restriction endonucleases was performed in sterile 0.5 ml microcentrifuge tubes at 37 °C in an incubator. Reaction mixes contained the appropriate enzyme buffer, restriction endonuclease and an appropriate amount of DNA. Sterile distilled water was added to a final volume of 20 µl. A standard reaction mix contained:

| | |
|---|---|
| 10X restriction enzyme buffer | 2 µl |
| Experimental DNA sample | 2-5 µl (up to 1.5 µg) |
| Restriction enzyme | 1 µl (2-10 units) |
| MilliQ water | Up to 20 µl |

Digestion was allowed to proceed for 1-3 hours, depending on template concentration and the intended application. After the incubation period, samples were analysed by agarose gel electrophoresis.

### 2.5.2 Ligation of DNA to plasmid vectors

Vector systems used for cloning were linearised in preparation for ligation with the DNA of interest. For the cloning of PCR products, T-A cloning was used. For cloning of restriction fragments cohesive ligations were performed by cutting the vector to generate ends compatible with those of the DNA to be cloned.

#### 2.5.2.1 T-A cloning:

For general propagation of PCR amplicons the pGEMT-Easy vector system was used (Promega). Reaction components were as follows:

| | |
|---|---|
| pGEMT-Easy vector DNA | 1 µl (50 ng) |
| 2X rapid ligation buffer | 5 µl |
| Gel purified PCR amplicon | 3 µl (2:1 insert to vector ratio) |
| T4 DNA ligase | 1 µl (5 Units) |

Ligation was allowed to proceed for 1 hour at room temperature or overnight at 4 °C prior to transformation.

### 2.5.2.2 pET cloning

PCR products amplified from *S. peucetius* were cut out of the pGEMT-Easy vector and ligated into different *E. coli* expression vectors (particularly pET21a). DNA excised from pGEMT-Easy by restriction enzyme digestion was ligated into linearised expression vectors that had been digested with the same enzymes, such that the two DNA fragments possessed complimentary ends. Components were:

| | |
|---|---|
| Digested pET vector DNA | 1 µl (50 ng) |
| Digested DNA to be cloned | 3 µl (3:1 insert to vector ratio) |
| 5X ligase buffer | 4 µl |
| T4 DNA ligase | 1 µl (5 Units) |
| Sterile distilled water | to 20 µl |

Ligation was allowed to proceed for 1 hour at room temperature prior to transformation, or incubated at 16 °C overnight to guarantee a high number of recombinant DNA molecules.

### 2.5.2.3 pBADTOPO thio cloning

For the construction of a recombinant protein expression system using the pBAD fusion vector, the *drrA* PCR product was ligated using an activation factor (Topoisomerase I) and the single overhanging 3' deoxythymidine (T) residues contained in the vector. Reaction components were:

| | |
|---|---|
| Fresh PCR product | 3 µl |
| Salt solution | 1 µl |
| Sterile water | to a total volume of 5 µl |
| TOPO$^{R}$ vector | 1 µl |
| Final volume | 6 µl |

The reaction mix was incubated for 5 minutes at room temperature for ligation. Subsequently, 2 µl of this reaction mixture was used to transform chemically competent LMG 194 *E. coli* cells according to standard protocols. The transformed bacteria were plated onto solid LB agar media supplemented with 100 µg/ml carbenicillin, and recombinant cells were isolated after incubation overnight at 37 °C.

## 2.6 Transformation of *E. coli* with DNA plasmid

### 2.6.1 Transformation of chemically competent *E. coli* cells

Chemically competent *E. coli* strains were acquired from various commercial sources, or produced in the laboratory. Transformation protocols were followed according to the manufacturer's instructions. A typical protocol is described below.

Ligation reactions or plasmid preparations (2.5 µl) were added to 50 µl of thawed competent cells on ice and gently mixed. The mixture was then incubated on ice for 30 minutes. Heat shock was applied by immersion of the tube in a 42 °C water bath for 45 seconds. Tubes were rapidly restored to an ice bath for two minutes, 250 µl of SOC medium (or equivalent) was added to each tube, and the suspension was shaken at 220 rpm and 37 °C for 1 hour. Cells were then plated onto LB agar containing the appropriate antibiotic and incubated at 37 °C overnight.

When fresh competent cells were prepared, the $CaCl_2$ method was used. In this procedure, cells from an overnight culture are diluted 50 fold into a volume of 10 ml and grown until an $OD_{600nm}$ of 0.6 is reached. An aliquot of 1 ml is centrifuged (30-60 secs), the supernatant is discarded, and the pellet, kept on ice, is re-suspended in 0.5 ml of cold 50 mM $CaCl_2$ for 10 minutes, or overnight at 4 °C. After a second short spin, the pellet is again re-suspended in 0.3 ml of $CaCl_2$ and kept on ice for 30 minutes, or again overnight at 4 °C. At that stage, cells are ready for transformation.

### 2.6.2 Selective screening of recombinant bacterial clones

The presence of one or several antibiotic resistance determinants was a feature of all the plasmid vectors used in this work. This ensured that only successfully transformed bacteria could grow on media supplemented with antibiotics. DNA from

these clones was then screened for the presence of insert using restriction digestion and/or sequence analysis. Ampicillin resistance was a feature of almost all the vectors used in this study.

**Blue/white screening of recombinant pGEMT-Easy clones**

Identification of pGEMT-Easy is aided by insertional inactivation of a _-galactosidase reporter gene and the loss of hydrolysis of the substrate analogue X-gal (5-bromo-4-chloro-3-indolyl-_-D-galactoside). Expression of the _-galactosidase gene is under the control of the *lac* promoter and successful transcription requires the presence of the inducer IPTG (isopropyl-_-thiogalactopyranoside). Colonies with non-recombinant plasmids appear blue on the indicator plates because the _-galactosidase gene has not been disrupted and the X-gal substrate can be digested. In contrast, cells that carry recombinant plasmid remain white under these conditions.

To make indicator plates, LB agar plates containing 100 µg per ml ampicillin were spread with 100 µl of sterile 100 mM IPTG. Prior to inoculation, 100 µl of 2% (w/v) X-gal in dimethyl formamide, was spread onto the agar surface. These components were allowed to diffuse and dry on the plates for at least 1 hour before use. Plates were incubated in an inverted position, at 37 °C for 18 h, and white colonies picked and propagated for further analysis.

## 2.7 Heterologous expression of recombinant proteins

### 2.7.1 Expression of recombinant proteins from pET21a in *Epicurian coli* BL21 (DE3)

*Epicurian coli* BL21 (DE3) cells were used for the production of recombinant proteins derived from pET21a carrying the *S. peucetius* DrrAB system. This bacterial strain is lysogenic for the lambda bacteriophage and carries an IPTG-inducible bacteriophage T7 RNA polymerase that is required for transcription from the T7 promoter. Bacterial strains harbouring expression constructs were streaked onto an LB agar plate containing 100 µg per ml carbenicillin. Following overnight incubation and growth, a single colony was used to inoculate a starter culture. This culture was grown at 37 °C and 200 rpm overnight and used to inoculate a larger expression

culture at a 1/1000 dilution. All expression cultures were grown in baffled flasks with rotary shaking at 200 rpm to promote aeration, and supplemented with the appropriate antibiotic to maintain the retention of the pET plasmid. Growth continued until the culture reached an approximate $OD_{600}$ of 0.6. Expression was then induced by addition of IPTG to a final concentration of 1 mM, and was allowed to proceed for 3-4 hours. Through interaction with the *lac* repressor, this triggered de-repression of the chromosomal *lacUV5* promoter and hence the production of the T7 RNA polymerase, required for transcription of the target gene from the T7 promoter.

To assess the extent of heterologous protein expression, 1 ml aliquots were removed from the expression culture prior to induction and at 1h intervals thereafter. The cells were harvested by centrifugation at 16,600 x *g* for 1 minute, and the cell pellet was prepared for analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), as described later.

Alternatively, the expression culture was grown until the cells reached an $OD_{600}$ of approximately 0.25. The temperature was then gradually reduced about 5 degrees each 15 minutes until it reached 19°C. When an $OD_{600}$ of 0.6 was reached, cultures were induced by addition of IPTG. The reduction in temperature was continued to 17 °C, and the culture was then grown overnight.

### 2.7.2 Expression of recombinant proteins from pBADTOPO thiofusion in *E. coli* LMG 194 and Top 10 cells.

Single colonies of transformed *E. coli* LMG 194 or *E. coli* TOP 10 cells were picked to liquid medium and grown at 200 rpm and 37 °C overnight. Expression cultures were prepared by inoculation with a 1/1000 volume of overnight culture. Cultures were grown until the cells reached an approximate $OD_{600}$ of 0.6-0.7 before induction with arabinose.

To find the appropriate concentration of inducer, a pilot experiment was carried out using different concentrations of arabinose to induce protein expression at 37 °C in 10 ml cultures that had reached mid-log phase. Arabinose concentrations were varied from 0.0002% to 2% (w/v) to identify the optimal concentration required

for expression of soluble protein. Protein expression was induced for 4 hours and monitored at intervals by withdrawing 1 ml samples for centrifugation and re-suspension of cell pellets in 100 µl of 4X SDS-PAGE buffer. Samples (10 µl) were then analysed by SDS-PAGE.

Having explored the influence of arabinose concentration on protein expression, larger scale expression cultures were induced by addition of arabinose to a final concentration of 0.002%. Induction was allowed to proceed for 3-4 hours.

Similar to work with pET vectors, low temperature expression was used. Cultures were grown to an $OD_{600}$ of approximately 0.25, and the temperature was then dropped about 5 degrees each 15 minutes. When the temperature reached 19 °C and the cultures were at an $OD_{600}$ of 0.7, protein expression was induced by addition of 0.002% arabinose. The temperature continued to be reduced to 17 °C, and the culture was induced overnight under these conditions.

### 2.7.3 Electrophoresis under denaturing conditions by Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis (PAGE)

Analysis of protein derived from expression experiments was achieved by separation of the proteins by electrophoresis. Pre-cast polyacrylamide mini-gel systems NuPAGE® Bis-Tris (Invitrogen) were employed under denaturing conditions. These gels were buffered at pH 6.4 and contained 12% acrylamide. Although they do not contain SDS, they are intended for denaturing gel electrophoresis. NuPAGE MOPS SDS was used as running buffer.

### 2.7.4 Electrophoresis of protein by Polyacrylamide Gel Electrophoresis (PAGE) under native conditions

Pre-cast polyacrylamide mini-gel systems NuPAGE® Bis-Tris-Acetate (Invitrogen) were employed. These gels were buffered at pH 8.0 and had 3-8 % of acrylamide. NuPAGE Tris Glycine was used as running buffer, whilst NuPAGE SDS sample buffer from which SDS had been omitted, was used as sample buffer.

### 2.7.5 Preparation of samples for SDS-PAGE

Samples for SDS-PAGE were prepared from bacterial pellets or from fractions collected during the purification of protein from disrupted bacterial cells. In the first case, for direct analysis, cell pellets were re-suspended in an appropriate volume of concentrated SDS-PAGE loading buffer, heated at 70 °C for 5 minutes and placed on ice until required.

In the second case, fractions from affinity chromatography were mixed at a 3:1 ratio with SDS-PAGE loading buffer, and then heated at 70 °C for 5 minutes.

### 2.7.6 Preparation of samples without SDS for PAGE

In this case, samples purified by affinity chromatography were mixed 3:1 with PAGE loading buffer, and then applied directly to the gel. Running buffer and the pre-cast gel did not contain SDS.

### 2.7.7 Electrophoresis protocol

To each sample well of the gel, 10-20 µl of protein sample was added using a 20 µl capacity Gilco micropipette or equivalent. Each gel included a lane of molecular weight markers (pre-stained SecBlue standards from Invitrogen). A voltage of 200 V was applied and electrophoresis was allowed to run for 45 minutes.

### 2.7.8 Analysis of SDS-PAGE gels

Polyacrylamide gels were usually stained using Gelcode® Blue Protein Stain (Pierce). The gel was removed from its container and transfered into a 10 cm x 10 cm petri dish filled with distilled water. It was washed twice (10 minutes each wash) with gentle agitation to remove SDS, which could inhibit the staining process. After the second wash, 20 ml of protein stain was added and the staining was allowed to proceed for 1 hour. After this, the stain was discarded and replaced with water. Protein bands could be visualised after 15 minutes. The longer the gel remained in water, the greater the resolution of the bands.

### 2.7.9 Analysis of non SDS-PAGE gels

Analysis of these gels was performed in a similar way but the initial washing was omitted.

## 2.8 Analysis of recombinant expression by Western blotting

Western immunoblotting was used to confirm the presence of recombinant proteins carrying a histidine tag ($His_6$).

### 2.8.1 Western transfer of proteins onto PVDF membranes

Western blotting, also called immunoblotting, is one of the best methods for detecting a particular protein in a complex mixture. Its three-steps comprise the separation of proteins through an SDS gel, the reaction of the target protein with specific antibodies, and detection by enzyme conjugates.

Electrophoresis was carried out as previously described for SDS-PAGE. Gels were run in duplicate, one for transfer and the other for direct analysis by staining. Proteins were transferred electrophoretically to a polyvinylidene fluoride (PVDF) membrane using a constant voltage of 30 V for 1 hour 30 minutes. The membrane was subsequently blocked by washing with a solution of non-fat milk powder and then probed with primary antibody (1:300 dilution of mouse anti-$His_6$ monoclonal antibody (Bio-Rad)), secondary antibody (1:1000 dilution of goat anti-mouse alkaline phosphatase conjugated antibody), and substrate (BioRad CDP-Star® chemiluminescent substrate). The membrane was thoroughly washed after each antibody incubation to remove unbound protein. The enzyme (AP) conjugated to the secondary antibody catalyses a light-emitting reaction when substrate is added, resulting in the exposure of X ray film.

## 2.9 Purification of DrrA

Recombinant DNA techniques usually permit the construction of fusion proteins in which specific affinity tags are added to the protein sequence of interest. These tags simplify the purification of recombinant fusion proteins by employing

affinity chromatography methods. All heterologous expression constructs were designed in such a way as to encode six consecutive histidine residues at either the N- or C- terminus of the recombinant protein. This sequence enables the expression of the recombinant protein to be monitored by immunochemical methods, and purification by affinity chromatography. The residues bind strongly to certain divalent cations, particularly $Ni^{2+}$, allowing an efficient purification of the recombinant protein using "Immobilised Metal Affinity Chromatography" (IMAC). Nickel-nitrilotriacetic ($Ni^{2+}$-NTA) affinity chromatography was used.

Usually a 10 L culture in 2X YT medium containing carbenicillin (100 μg/ml) was induced to overexpress the target protein. The induction was accomplished with IPTG for the pET constructs carrying DrrA, or DrrA and DrrB. Arabinose was used to induce pBAD constructs to overexpress the Thio-DrrA fusion. In all the constructs DrrA was produced with a histidine tag, to aid further purification.

Cells were collected by centrifugation at 4,000 x *g* for 8 min at 4 °C, the supernatant discarded, and the pellet placed on ice. The cells were re-suspended in an appropriate volume of re-suspension buffer (usually 30 ml Tris buffer pH 8.0 per L of culture) to allow for a concentration factor of 34 with respect to the original culture volume. The cell suspension was sometimes treated with lysozyme (1 mg/ml final concentration) and incubated for 1 hour before disruption in a homogeniser at 20 PSI. Cells were forced under high pressure through a small aperture in the base of a cell disrupter cup and passed twice through the chamber to assure they were successfully broken. The disrupted material was recovered from the disrupter cup and debris and insoluble proteins were harvested by centrifugation at 22,000 x *g* for 20 minutes.

In an alternative protocol, cell disruption was performed using an Ultrasonic disintegrator (Sanyo Soniprep 150). Batches of 30-40 ml of cells were processed individually with 6 sonication pulses of 15 seconds and an interval of resting of at least 45 seconds between each pulse. The amplitude was maximised using the power control and the process was performed manually for the indicated times. The sample

vessel (usually plastic Universal tubes were employed) was immersed on ice to avoid damage to the protein due to the heat generated.

### 2.9.1 DrrA purification under native conditions

#### 2.9.1.1 DrrA purification from the soluble fraction

**Affinity chromatography**

The supernatant from the 22,000 x *g* centrifugation step was decanted into 20 ml ultracentrifuge tubes (Sorvall or Beckman) and ultra-centrifuged at 149,000 x *g* for 1.5 hours to remove cell membranes. The supernatant thus obtained (representing the soluble fraction of the cell extracts), was used in Nickel affinity chromatography. This fraction was shaken gently at 4 °C for 1 hour or overnight, with $Ni^{2+}$-NTA agarose affinity resin (Qiagen;1 ml per 10 L of culture), and the slurry was loaded into a 0.8 cm diameter gravity fed chromatography glass column (Bio-Rad). The nickel cation in the $Ni^{2+}$-NTA complex interacts with the histidine tag present in the DrrA protein derivative. The pellet collected during ultracentrifugation comprised the membranes and insoluble proteins. This was dissolved in a buffer containing an appropriate detergent before Nickel affinity chromatography (see next section).

After allowing the slurry to accumulate in the column (a batch usually corresponding to 2 or 2.5 L of culture), it was washed with increasing imidazole concentrations up to 50 mM prepared in the same buffer as the sample. Then, 10 ml of elution buffer (300 mM imidazole) was loaded onto the column in 1 ml aliquots, to elute the protein. The recovery of protein at stages through the purification process was assessed by SDS-PAGE.

Solubilisation buffers:

Phosphate buffer: 50 mM $Na_2HPO_4$, 300 mM NaCl, 10% (v/v) glycerol, 10 mM imidazole, pH 8.0

Tris buffer: 50 mM Tris-HCl, 150 mM NaCl, 10% (v/v) glycerol, 10-20 mM imidazole, pH 8.0. Specific adjustments were made for particular assays, such as

NaCl concentration adjusted to 400 mM, higher and lower glycerol concentrations, the addition of detergent and reducing agent. These are presented in the respective Results chapter.

MOPS buffer and a different Phosphate buffer formulation (pBADTOPO thiofusion manual) were also tested and are presented in the respective Results chapter.

### 2.9.1.2 DrrA purification from the membrane fraction

The purification of recombinant proteins from the membrane fraction followed the general procedure stated above. Cell membranes were separated from the soluble fraction after the 149,000 x *g* centrifugation step. To solubilise these membranes (membrane pellet obtained from a 10 L culture), 50 mM Tris-buffer (200 ml) with 2% of dodecyl maltoside (DDM), was used. The membrane solubilisation proceeded for 14 hours at 4 °C with gentle shaking. After that period, the mixture was ultra-centrifuged at 149,000 x *g* for 30 minutes at 4 °C and non-solubilised material was discarded. The supernatant was then ready for affinity chromatography following the standard procedure already described, with the addition of 0.2% DDM in the wash buffers.

## 2.9.2 DrrA purification under denaturing conditions. Protein refolding

When heterologous proteins expressed in *E. coli* are present only as insoluble protein in inclusion bodies, solubilisation and refolding of the protein under denaturing conditions has been successful for some proteins.

In this procedure, the disrupted cell pellet was collected and solubilised in Tris-HCl lysis buffer with 8 M urea. Phosphate buffer was also tested. The suspension was shaken for 2 h at 4 °C. A further round of centrifugation at 6,000 *g* removed material that remained insoluble. Tris-urea buffer was 50 mM Tris-HCl, 150 mM NaCl, 10% (v/v) glycerol, 10 mM imidazole, 8 M urea, pH 8.0.

### 2.9.2.1 Refolding with Urea

Refolding of DrrA solubilised from inclusion bodies was done using a decreasing urea gradient from 8 to 0 M. This was carried out on a column. To accomplish this, 200 µl of disrupted cell pellet was mixed in 10 ml of binding buffer for 1 hour, on ice. Five hundred µl of $Ni^{2+}$-NTA agarose was added to the column, and washed with 10 ml binding buffer to pre-equilibrate. The sample was then added, and the flow-through collected slowly over 15 min. The column was washed with 10 ml of binding buffer containing 8 M urea, and 5 mM imidazole, followed by 10 ml of washing buffer containing 8 M urea, 20 mM imidazole. Having immobilised the protein onto the column, re-naturation was attempted by running a 20 ml gradient comprising 8 to 0 M urea through the chromatography medium. After a final wash with 20 ml binding buffer without urea, the re-natured protein was eluted with 3 ml elution buffer (0 M urea, 0.3 M imidazole), and collected in 500 µl aliquots, followed by a single pulse of 5 ml of elution buffer.

All the buffers were 20 mM Tris, 0.5 M NaCl and pH 8.0. Binding buffer was 5 mM imidazole and 8 M urea. Washing buffer was the same as binding buffer except imidazole was 20 mM. Elution buffer was 300 mM imidazole. This protocol was also employed using 50 mM Tris and 0.15 M NaCl in the buffer recipe.

### 2.9.2.2 The FoldIt Screen Refolding

The FoldIt Screen Refolding kit (Hampton Research) was also used in an attempt to find conditions for refolding of the DrrA protein under denaturing conditions. FoldIt is a fractional factorial-folding screen designed for use with proteins expressed from *E. coli*. In this protocol, inclusion bodies were solubilised in 4 M Guanidine-HCl, 0.1 M Tris and 0.1 M NaCl, and a set of 16 different experimental conditions were assayed. Parameters included the type and concentration of buffer, salt, presence and absence of denaturing agents, type of cation chelator, polar and non-polar additives, presence and type of detergent, reducing agents, ligand addition, and protein concentration (Tables 2.4 and 2.5). Treated protein preparations were dialysed, checked on gels, and their activity was evaluated in ATPase activity assays and fluorescence spectroscopy.

### 2.9.3 Dialysis of purified protein

Those eluted fractions which had the highest concentrations of protein as visualised on polyacrylamide gels, were collected and pooled to be dialysed against solubilisation buffer without imidazole.

Purified DrrA was dialysed before quantification and further experiments, as imidazole interferes with the BCA protein determination. Dialysis cassettes (Pierce Slide-A-Lyzer®) of different sizes were employed according to the volume of the sample. Occasionally dialysis tubing was used as well. For this, membrane (12-14 kDa cut off) was cut into 8 cm sections and boiled for 10 minutes in water before use.

Dialysis of the protein was performed in 3 L Tris buffer pH 8 (50 mM Tris-HCl, 150 mM NaCl, and 10% glycerol) or equivalent, usually over 4 hours if the sample volume was less than 6 ml. The process was run at 4 °C with gentle stirring in a volume of 800 ml of buffer, being the buffer replaced each hour. If necessary, fractions were concentrated using a centrifugal concentrator (Amicon Centricon A10) or a Stirred Ultrafiltration Cells device (Millipore) according to the protein volume.

### 2.9.4 Size exclusion chromatography

Purification by size exclusion was tested as a method for separation of Thio-DrrA from contaminating proteins. The ÄKTA purification system of Amersham Pharmacia Biotech was used for this purpose, and 16/60 Superdex 75 and 200 columns were employed to run the chromatography. Each of these columns was equilibrated with 50 mM Tris-HCl buffer pH 8, 150 mM NaCl, and loaded with 1 ml of sample. A flow of 0.5 ml/min was set for the run and 1.5 X the volume of the column was used to elute the samples.

**Table 2.4 FoldIt Screen Formulations**

| Buffer | Salt (NaCl, KCl) | PEG 3350 (%) | Guanidine HCl | Cation/Che–lator | Polar and Nonpolar Additives |
|---|---|---|---|---|---|
| 1) Tris pH 8.2 | 264 mM, 11 mM | 0.055 | 0 mM | EDTA | None |
| 2) MES pH 6.5 | 10.56 mM, 0.44 mM | None | 550 mM | $MgCl_2$, $CaCl_2$ | None |
| 3) MES pH 6.5 | 10.56 mM, 0.44 mM | 0.055 | 550 mM | EDTA | Sucrose, L-Arginine |
| 4) Tris pH 8.2 | 264 mM, 11 mM | None | 0 mM | $MgCl_2$, $CaCl_2$ | Sucrose, L-Arginine |
| 5) MES pH 6.5 | 264 mM, 11 mM | None | 0 mM | $MgCl_2$, $CaCl_2$ | Sucrose |
| 6) Tris pH 8.2 | 10.56 mM, 0.44 mM | 0.055 | 550 mM | EDTA | Sucrose |
| 7) Tris pH 8.2 | 10.56 mM, 0.44 mM | None | 550 mM | $MgCl_2$, $CaCl_2$ | L-Arginine |
| 8) MES pH 6.5 | 264 mM, 11 mM | 0.055 | 0 mM | EDTA | L-Arginine |
| 9) MES pH 6.5 | 264 mM, 11 mM | 0.055 | 550 mM | $MgCl_2$, $CaCl_2$ | Sucrose |
| 10) Tris pH 8.2 | 10.56 mM, 0.44 mM | None | 0 mM | EDTA | Sucrose |
| 11) Tris pH 8.2 | 10.56 mM, 0.44 mM | 0.055 | 0 mM | $MgCl_2$, $CaCl_2$ | L-Arginine |
| 12) MES pH 6.5 | 264 mM, 11 mM | None | 550 mM | EDTA | L-Arginine |
| 13) Tris pH 8.2 | 264 mM, 11 mM | None | 550 mM | EDTA | None |
| 14) MES pH 6.5 | 0.56 mM, 0.44 mM | 0.055 | 0 mM | $MgCl_2$, $CaCl_2$ | None |
| 15) MES pH 6.5 | 0.56 mM, 0.44 mM | None | 0 mM | EDTA | Sucrose, L-Arginine |
| 16) Tris pH 8.2 | 264 mM, 11 mM | 0.055 | 550 mM | $MgCl_2$, $CaCl_2$ | Sucrose, L-Arginine |

All buffers were 55 mM.

EDTA was 1.1 mM; $MgCl_2$ and $CaCl_2$ were 2.2 mM.

Sucrose was 440 mM, and L-arginine was 550 mM

**Table 2.5 FoldIt Additions – Pipetting and dilution table for FoldtIt**

| Reagent # | Reagent volume (µL) | 0.1 M DTT (µL) | 30 mM Lauryl Maltoside (µL) | 100 mM GSH (µL) | 10 mM GSSG (µL) | Ligand (mM) | Protein (µL) | Final [Prot.] (mg/ml) |
|---|---|---|---|---|---|---|---|---|
| 1 | 950 | 10 | - | - | - | Up to 10 | 50 | 0.1 |
| 2 | 950 | - | 10 | 10 | 10 | - | 50 | 0.1 |
| 3 | 950 | - | - | 10 | 10 | Up to 10 | 50 | 0.1 |
| 4 | 950 | 10 | 10 | - | - | - | 50 | 0.1 |
| 5 | 950 | - | - | 10 | 10 | Up to 10 | 50 | 1.0 |
| 6 | 950 | 10 | 10 | - | - | - | 50 | 1.0 |
| 7 | 950 | 10 | - | - | - | Up to 10 | 50 | 1.0 |
| 8 | 950 | - | 10 | 10 | 10 | - | 50 | 1.0 |
| 9 | 950 | 10 | - | - | - | - | 50 | 0.1 |
| 10 | 950 | - | 10 | 10 | 10 | Up to 10 | 50 | 0.1 |
| 11 | 950 | - | - | 10 | 10 | - | 50 | 0.1 |
| 12 | 950 | 10 | 10 | - | - | Up to 10 | 50 | 0.1 |
| 13 | 950 | - | - | 10 | 10 | - | 50 | 1.0 |
| 14 | 950 | 10 | 10 | - | - | Up to 10 | 50 | 1.0 |
| 15 | 950 | 10 | - | - | - | - | 50 | 1.0 |
| 16 | 950 | - | 10 | 10 | 10 | Up to 10 | 50 | 1.0 |

Ligand was doxorubicin 30 µg/ml.

## 2.10 Protein determination

Protein concentrations were determined using the BCA Protein Assay Kit (Pierce Chemical Co.) with bovine serum albumin as standard. In this colorimetric assay, the protein reacts with the BCA reagent to give a violet colour of intensity equivalent to its concentration in a range between 20-2000 μg/ml, and that is measured at an absorbance of 562 nm. The Pierce BCA assay is a formulation based on bicinchoninic acid and combines the well-known reduction of $Cu^{2+}$ to $Cu^{1+}$ by protein in an alkaline medium (Biuret reaction) with the colorimetric detection of the cuprous cation ($Cu^{+1}$) using a special reagent containing bicinchoninic acid. The colour is the product of the chelation of two molecules of BCA with one cuprous ion.

Protein concentration was determined with reference to standards of bovine serum albumin (BSA), and although the test tube procedure was the most accurate method to determine protein concentration, the microplate procedure was chosen for routine use since only 25 μl of sample was required. In this procedure, 25 μl of the protein sample was added to a well into the microplate followed by 200 μl of the BCA reagent. The solutions were mixed, and incubated at 37 °C for 30 minutes. After the incubation period, sample absorbance values were measured in a microplate reader at 610 nm. The absorbance values were compared with those of a protein standard curve, and related back to protein concentration.

## 2.11 Characterisation of Thio-DrrA

### 2.11.1 DrrA protein sequence analysis

Protein analyses based on sequence were performed using NTI Vector and Wabim programs.

### 2.11.2 ATPase Activity

#### 2.11.2.1 EnzCheck Phosphate Assay (Molecular Probes)

This method is based on a method described by Webb (Webb, 1992). The EnzChek Phosphate Assay System was used to run a number of ATPase activity assays to evaluate the success of the refolding procedures for DrrA prepared from

inclusion bodies (see sections 2.9.2 and 2.9.2.2). The system relies upon the spectrophotometric detection of inorganic phosphate ($P_i$) released into the assay medium by the activity of ATPase enzymes. Phosphate release is enzymatically coupled to phosphorolysis of the artificial substrate 2-amino-6-mercapto-7-methylpurine riboside (MESG). The phosphorolysis reaction produces a molecule with a strong absorption at 360 nm that can be monitored in real-time. The reaction was initiated by the addition of $MgCl_2$ and the ATPase activity was monitored as an increase in absorbance of the reaction mixture at 360 nm over time.

To follow the kinetics of $P_i$ release, an enzyme reaction was set up with the following components in a total volume of 1 ml of reaction mixture:

| | |
|---|---|
| 20X reaction buffer | 50 µl |
| MESG substrate solution | 200 µl |
| Purine nucleotide phosphorylase | 10 µl (1 Unit) |
| ATP | x µl |
| $MgCl_2$ | y µl |
| Experimental enzyme | z µl |
| Tris-HCl buffer (50mM) | 740 – x – y – z µl |

It is the purine nucleotide phosphorylase component of the reaction mixture which couples release of $P_i$ by the ATPase enzyme to phosphorolysis of the MESG substrate.

### 2.11.2.2 Malachite green Assay

A colorimetric assay to determine free phosphate, based on the use of the ammonium molybdate/malachite green reagent (Nash, 2003; Maehatma, 2001; Harder *et al.*, 1994), was used in microplates. Malachite green in the presence of molybdate binds inorganic phosphate, which gives rise to a complex that can be measured

between 620-660 nm (Ekman and Jäger, 1993). A total reaction volume of 500 µl was used for each sample. This included the DrrA protein (DrrA or Thio-DrrA), ATP, and assay buffer. The mixture was pre-incubated for 5 minutes at 37 °C. The reaction was then initiated by addition of $MgCl_2$. Immediately after the induction of ATPase activity, and at various time points thereafter, 45 µl of sample were withdrawn and pipetted into each well of the microplates, where 5 µl of 0.5 M EDTA had been previously added to sequester $Mg^{2+}$ ions and stop the reaction. At the end of the reaction time course, 100 µl of Malachite green assay solution was added to each sample in the well, and the microplate was immediately read at an absorbance of 610 nm. Two experiments were run simultaneously, and protein of 1 µM concentration was usually sufficient to follow the development of colour in the reaction with malachite green in standard conditions. Routine experiments and characterisation were performed with this method and using 450 µM $Mg^{2+}$ and 250 µM of ATP, final concentrations.

Preparation of Malachite green Assay solution

1. - Solution A: 4.2% (w/v) ammonium molybdate in 4 N HCl.
2. - Solution B: 0.045% (w/v) Malachite green solution in $H_2O$.
3. - 1 part of A is mixed with 3 parts of B.
4. - The resultant solution is filtered, giving a yellow colour.

Assay buffer was usually 50 mM Tris-HCl, 150 mM NaCl, glycerol 10%, pH 8.0.

### 2.11.3 Dependence on ATP concentration

ATP concentrations between 0.1 to 2 mM (prepared in assay buffer), were assayed as described in section 2.11.2.2 to find the concentration that induced the highest ATP-ase activity in recombinant DrrA proteins, enabling calculation of $K_m$.

### 2.11.4 Dependence on divalent cation concentrations: $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Zn^{2+}$ and $Ca^{2+}$

Concentrations of divalent cations between 0.1 and 2 mM (prepared in assay buffer), were assayed as described in section 2.11.2.2 substituting for $Mg^{2+}$, to determine the efficiency of their stimulation upon the DrrA ATPase activity.

### 2.11.5 Dependence on pH

A range of pH between 6.0 and 9.0 was tested to assess the optimum for DrrA proteins ATPase activity. MES/Na buffer was used for pH values between 6.0-6.5. MOPS/Na was used for pH range 6.6-7.5. Tris/Cl for pH range 8.0-9.0. All buffers were 50 mM.

### 2.11.6 Substrate specificity and inhibitors

The nucleotides triphosphate CTP, GTP, and TTP (prepared in assay buffer), were evaluated as substrates for Thio-DrrA. Forty µl of 3.125 mM stocks of each of the NTPs, were added to the reaction mix to obtain a final concentration of 250 µM. Reactions were initiated by addition of $Mg^{2+}$ as described above. The effect of ADP on the ATPase activity of Thio-DrrA was also assayed. A final concentration of 250 µM was used.

Ortho-vanadate and NaCl were assayed as inhibitors of the DrrA ATPase activity. Vanadate concentrations between 10-1000 µM were added to a standard DrrA assay for ATPase activity and its kinetics were followed. NaCl concentrations between 150-2000 µM were evaluated to determine if NaCl inhibited the Thio-DrrA ATPase activity. The conditions in these experiments were standardised with 250 µM ATP and 450 µM $MgCl_2$.

### 2.11.7 Dependence on DrrB presence

DrrA and Thio-DrrA were assayed for ATPase activity in presence of the DrrB protein (the kind gift of Dr. McKeegan, Wolfson Institute, University of Durham). Protein concentration was 1 µM, and ATPase kinetics between 25 and 2000 µM ATP was estimated. $MgCl_2$ concentration was 450 µM. The assay buffer contained 0.2% DDM detergent.

### 2.11.8 Dependence on the presence of Doxorubicin or Daunorubicin

The effect of the presence of doxorubicin was assayed for both DrrA and Thio-DrrA. Concentrations of doxorubicin of 2, 10 and 20 µg/ml (~3, 17 and 34 µM, respectively) were used in reaction mixtures with DrrA 37 µg/ml (~1 µM), to

follow ATPase kinetics. The effect of the presence of daunorubicin (3 μM) was also assayed on Thio-DrrA ATPase activity.

### 2.11.9 Dependence on the presence of DrrB and Doxorubicin

The effect of both DrrB and doxorubicin on the ATPase activity of DrrA was evaluated. These two components were added to a reaction mixture containing DrrA protein and the kinetics of ATP hydrolysis was followed. DrrA and DrrB were 1 μM, whilst doxorubicin was 2 μM.

## 2.12 DrrA Site-Directed Mutagenesis

Site-directed mutagenesis is a valuable tool for the study of DNA function as well as for the analysis of protein structure and function. It is accomplished by hybridisation and extension of a synthetic oligonucleotide that is complementary to the target template except for a region of mismatch near the centre, and also by PCR methods. This mismatched region contains the desired mutation. In both cases, double-stranded DNA is obtained and used to transform an *E. coli* host.

### 2.12.1 GeneEditor protocol (Promega)

This protocol was used to obtain a single amino acid mutation in the protein Thio-DrrA for stopped-flow experiments based on the fluorescence of the amino acid tryptophan. This system uses antibiotic selection to obtain high frequency mutants. Oligonucleotides provided with the system encode mutations that alter the ampicillin resistance gene, creating a new additional resistance to the GeneEditor antibiotic selection mix. The selection oligonucleotide is annealed to a single or double-stranded DNA template at the same time as a mutagenic oligonucleotide. Subsequent synthesis and ligation of the mutant strand links the two oligonucleotides. The resistance to the GeneEditor antibiotic selection mix encoded by this mutant DNA facilitates selection of the desired mutation. A summary of the procedure is presented here.

As single stranded DNA (ssDNA) was required as template for mutagenesis reactions, alkaline denaturation of double stranded DNA (dsDNA) was achieved in a reaction that included plasmid DNA (dsDNA; 0.5 pmol ~2μg), 2 M NaOH, 2 mM EDTA (2μl), and $dH_2O$. The DNA was precipitated with 2 M ammonium acetate (2 μl) and 100% ethanol at -70°C (75 μl) for 30 minutes, and pelleted by centrifugation. The ssDNA pellet was washed in 70% ethanol (200 μl), re-collected by centrifugation, air-dried, dissolved in TE buffer (100μl), and a 10 μl aliquot was analysed by agarose gel electrophoresis in order to estimate the reaction success and yields.

ssDNA template (10 μl) was mixed with the phosphorylated selection oligonucleotide (1 μl; 0.25 pmol), the phosphorylated mutagenic oligonucleotide, (1.25 pmol), 10X annealing buffer (2 μl), and $dH_2O$ up to 20 μl. The mixture was heated at 75 °C for 5 minutes, and slowly cooled to 37 °C (1.5 °C per minute). During this period the oligonucleotides anneal to the template DNA.

Synthesis of the mutant strand and ligation was accomplished in a reaction containing $dH_2O$ (5 μl), 10X synthesis buffer (3 μl), T4 DNA polymerase (5-10 U; 1 μl), and T4 DNA ligase (1-3 U; 1 μl). The reaction mixture was incubated at 37 °C for 90 minutes to allow mutant strand synthesis.

The DNA was then transformed into competent *E. coli BMH 71-18 mutS* cells. This strain has a mutation in the *mut*S DNA repair gene which allows the cells to tolerate plasmids with small double strand mismatches. The cells were transformed with 3 μl of the mutant plasmid mixture prepared in the previous step. Transformation was performed by a standard heat-shock protocol. The entire mixture was incubated in 4 ml of LB medium containing the proprietary selection antibiotic overnight (37 °C), allowing time for plasmid replication and segregation in such a way as to enhance the number of cells carrying the mutated plasmid.

Mutant plasmids obtained from the previous step were then transformed into competent *E. coli* JM109 cells, and selected on solid LB-agar medium containing the selection antibiotic. JM109 clones resistant to the antibiotic selection mixture were

used to prepare plasmid DNA. The successful introduction of specific mutations was determined by DNA sequencing.

### 2.12.2 QuickChange Site-Directed Mutagenesis (Stratagene)

The QuikChange site-directed mutagenesis protocol (Stratagene), was also used for the same purpose. This method uses a supercoiled double-stranded DNA vector with an insert of interest as template, and two synthetic oligonucleotides primers containing the desired mutation. It is performed using *Pfu Turbo*® DNA polymerase and a temperature cycler. This enzyme replicates both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers, each complementary to opposite strands of the vector. Incorporation of oligonucleotide primers generates a mutated plasmid containing staggered nicks. After temperature cycling, the product is treated with *Dpn* I endonuclease, which is specific for methylated and hemimethylated DNA, to digest the parental DNA template. The only DNA left is the newly synthesized DNA containing the mutation. This nicked vector containing the desired mutation is then transformed into XL1-Blue supercompetent cells. A summary of the procedure is presented here. Two complementary oligonucleotides containing the desired mutation, flanked by unmodified nucleotide sequence, were synthesised for the mutagenesis experiment (Table 2.6).

**Table 2.6 Primers used for Site-directed mutagenesis in pBADTOPO *thio-drrA***

| Primer name | Mutation in Thio-DrrA | Sequence (5' to 3') |
|---|---|---|
| *drrA* Y140 forward (Y140W) | Original Y was replaced by W in the ABC signature region | CGG CTC CTG AAG ACC **TGG** TCC GGT GGC ATG CGG |
| *drrA* Y140 reverse (Y140W) | Same | CCG CAT GCC ACC GGA **CCA** GGT CTT CAG GAG CCG |
| *drrA* Y37 forward (Y37W) | Original Y position 37 in the DrrA sequence, was mutated to W | CCG GCC GGT CTC GTC **TGG** GGG ATC CTG GGG CCG |
| *drrA* Y37reverse (Y37W) | Same | CGG CCC CAG GAT CCC **CCA** GAC GAG ACC GGC CGG |
| *drrA* A45 forward (A45W) | A in position 45 in the Walker A motif, was mutated to W | CTG GGG CCG AAC GGC **TGG** GGC AAG TCC ACC ACC |
| *drrA* A45 reverse (A45W0 | Same | GGT GGT GGA CTT GCC **CCA** GCC GTT CGG CCC CAG |
| *drrA*-T50 forward (T50W) | T position 50 beside the Walker A motif, was mutated to W | GCC GGC AAG TCC ACC **TGG** ATC CGC ATG CTG GCG |
| *drrA* T50 reverse (T50W) | Same | CGC CAG CAT GCG GAT **CCA** GGT GGA CTT GCC GGC |
| Thio forward | The 2 original W present in Thioredoxin, were mutated to G | ATC CTG GTT GAT TTC **GGG** GCA CAC **GGG** TGC GGT CCG TGC AAA |
| Thio reverse | Same | TTT GCA CGG ACC GCA **CCC** GTG TGC **CCC** GAA ATC AAC CAG GAT |
| PBAD forward priming site | To check mutations in thioredoxin gene | ATG CCA TAG CAT TTT TAT CCA |
| *drrA*-T50 –forward (W50T) | T50 mutation was cancelled, and the original T replaced the mutation W | GCC GGC AAG TCC ACC **ACC** ATC CGC ATG CTG GCG |
| *drrA*-T50 –reverse (W50T) | Same | CGC CAG CAT GCG GAT **GGT** GGT GGA CTT GCC GGC |

The sample reaction was prepared according to the following indications:

5 µl of 10X reaction buffer

X µl (5-50 ng) of dsDNA template

X µl (125 ng) of oligonucleotide primer #1

X µl (125 ng) of oligonucleotide primer #2

1 µl of dNTP mix

dd$H_2O$ to a final volume of 50 µl

One µl of *PfuTurbo* DNA polymerase (2.5 U/µl) was added. A control reaction was performed as well. The reaction was cycled using the parameters outlined in the Table 2.7.

The reaction mixture was placed on ice for 2 minutes and allowed to cool to 37 °C. One µl of the *Dpn*I restriction enzyme (10U/µl) was added to the amplification reaction, and this was thoroughly mixed by pipetting, briefly centrifuged, and incubated for 1 hour at 37 °C to digest the parental supercoiled dsDNA.

One µl of the *Dpn*I-treated DNA was added to 50 µl of XL1-Blue supercompetent cells, previously thawed on ice, and the mix was incubated on ice for 30 minutes. The transformation reaction was heat-shocked for 45 seconds at 42 °C, and then placed on ice for 2 minutes. $NZY^+$ broth (0.5 ml) preheated to 42 °C was added to the transformation reaction, and this was incubated at 37 °C for 1 hour with rotary shaking at 225 rpm. The transformation reaction (250 µl) was subsequently plated on agar plates containing the appropriate antibiotic for the plasmid vector.

**Table 2.7 Program for DNA amplification designed for QuickChange site-directed mutagenesis**

| Segment | Cycles | Temperature | Time |
|---|---|---|---|
| 1 | 1 | 95 °C | 30 seconds |
| 2 | 12-18 | 95 °C | 30 seconds |
| | | 55 °C | 1 minute |
| | | 68 °C | 1 minute/kb of plasmid length |

For single amino acid changes segment 2 was adjusted to 16 cycles, as suggested per manufacturers.

## 2.13 Ligand Binding Analysis

### 2.13.1 Steady-state protein fluorescence measurements

Protein fluorescence measurements were assayed at room temperature using a Jasco FP-750 fluorescence spectrophotometer. The excitation light was set to 285 nm via a series of chromating mirrors and the protein fluorescence was measured at right angles to the direction of the incident light using the amplified signal provided by a photomultiplier tube.

Volumes of 200 µl of sample were prepared to load a Helma Suprasil quartz fluorescence cuvette and protein fluorescence spectra were obtained at wavelengths between 295 and 400 nm. Excitation and emission bandwidths were selected to be 5 nm, and usually, the detection sensitivity of the photomultiplier tube was set to medium.

Changes in the fluorescence protein spectra would be measured when small volumes of ligand binding components were added at a high concentration to the protein solution. DrrA proteins, ATP, MANT-ATP and Mg were the basic components involved in the ATP system that was assayed.

### 2.13.2 Stopped-flow analysis of nucleotide protein interactions

Nucleotide interactions can produce transient changes in the fluorescence of proteins. Interactions involving the nucleotide-binding domain (NBD) of the pump DrrAB were assessed in a stopped-flow device from Applied Photophysics SMV 18. The stopped-flow system allows the rapid mixing of the protein with the ligand and enables one to time-resolve changes in the fluorescence on a millisecond time scale. Protein and ligand were injected in different channels that were directed in equal volumes to a mixing chamber at room temperature, by nitrogen-driven hydraulic plungers. Light excitation at 285 nm was provided by a xenon lamp and two serial monochromators, whilst a photomultiplier tube (PMT) was used to detect the emitted light.

Tryptophan excitation light was fixed at 280 nm and emission light was filtered to select for the appropriate wavelength with 335 or 420 nm cut-off filters placed in front of the detection photomultiplier tube, where fluorescence data were collected. Alterations in the level of fluorescence were visualised as alterations in the voltage output of the tube. Ligand-binding experiments were set up to be performed under pseudo first-order conditions, having the ligand concentration in excess over the protein concentration. In that way, an exponential change in the fluorescence of the protein could be fitted by non-linear regression to an exponential equation defining a first order binding process.

The protein samples corresponding to the *S. peucetius* DrrAB NBD were assayed by this technique. For the assays, protein samples, $Mg^{2+}$, and ATP or MANT-ATP in 50 mM Tris-HCl buffer with 150 mM NaCl and 10% glycerol, were used.

# Chapter 3

# Cloning and sequence analysis of genes in the *Streptomyces peucetius drrAB* operon

The *drrAB* operon of *Streptomyces peucetius* was originally recognised by Guilfoile and Hutchinson (1991). Sequence analysis showed the presence of two genes, *drrA* and *drrB*, which could encode the ATP-binding and transmembrane domains of an ABC transporter, respectively, and mediate self-protection against the anthracyclines compounds daunorubicin and doxorubicin. These compounds are important in cancer treatment as they show anti-tumour activity. The *drrA* gene was predicted to express a product with similar sequence to the products of other transport and resistance genes, such as P-glycoprotein from mammalian tumour cells.

## 3.1 PCR amplification of the *drrA* and *drrB* genes.

Oligonucleotide PCR primers were designed for the specific amplification of the *S. peucetius drrA* and *drrB* genes from the cosmid pWHM612 (Guilfoile and Hutchinson, 1991). The published *drrA* and *drrB* genes sequences (NCBI site) present in the GenBank database under the accession number M73758 were used in primer design. The *drrAB* operon is 1,841 bp long. The *drrA* ORF is 993 bp long, and extends from position 471 to 1,463. The *drrB* ORF is 852 bp long and its beginning overlaps the *drrA* stop codon; it extends from position 1,460, to 2,311.

Additional nucleotides at the 5' and 3' ends of the gene(s) were included in all the primers except in those used to amplify the gene for ligation into the pBADTOPO thiofusion vector (section 2.4.1.2. in Methods). This step was necessary to allow excision of the sequences from the initial cloning vector. A two-step strategy was planned to simplify the process of sub-cloning from the initial cloning vector into the expression vectors chosen for over-expression studies. To sub-clone *drrA* into pET21a, the 5' primer contained an ATG start codon as part of an *Nde*I site that was substituted for the original GTG *drrA* start codon. In this construct, the original *drrA* stop codon was omitted from the 3' end to allow the incorporation of a six-histidine tag in the C-terminus of the DrrA protein. A *Xho*I site was also incorporated at the 3' terminus to enable subcloning.

The amplification of *drrA* was successful using QIAGEN Q solution and a gradient between 50-60 °C (Table 3.1; Fig. 3.1). When the temperature gradient was not used, the temperature was set to 55 °C to amplify the gene. The amplification of the *drrAB* operon for other vectors was also successful with similar programs (Fig. 3.2).

**Table 3.1 Standard PCR program (Cycling parameters)**

| Segment | Cycles | Temperature | Time |
|---|---|---|---|
| Hot Star Taq activation | 1 | 95 °C | 15 minutes |
| Denaturation | 30 | 94 °C | 15-60 seconds |
| Annealing | | Gradient 50-60 °C | 30-60 seconds |
| Extension | | 72 °C | 1 min. 30 seconds |
| Final extension | 1 | 72 °C | 10 minutes |

For the amplification of the *drrA* gene, 15 and 30 seconds were set for denaturation and annealing, respectively. A time of 60 seconds was used in the same stages for amplification of *drrAB*.

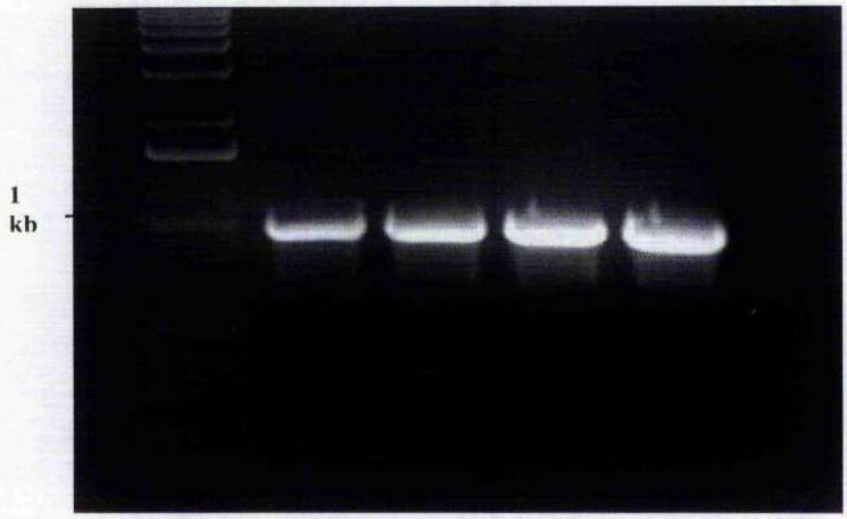



**Figure 3.1 PCR amplification of the *drrA* gene**

The *drrA* gene was amplified from pWHM612 using 30 cycles under the following reaction conditions: 94 °C denaturation for 15 sec, annealing for 30 sec at temperatures ranging between 50 and 60 °C, 72 °C extension for 90 sec. Lane M: 1 kb DNA molecular markers. Lanes 1-4: *drrA* PCR product from replicated reactions with identical conditions.

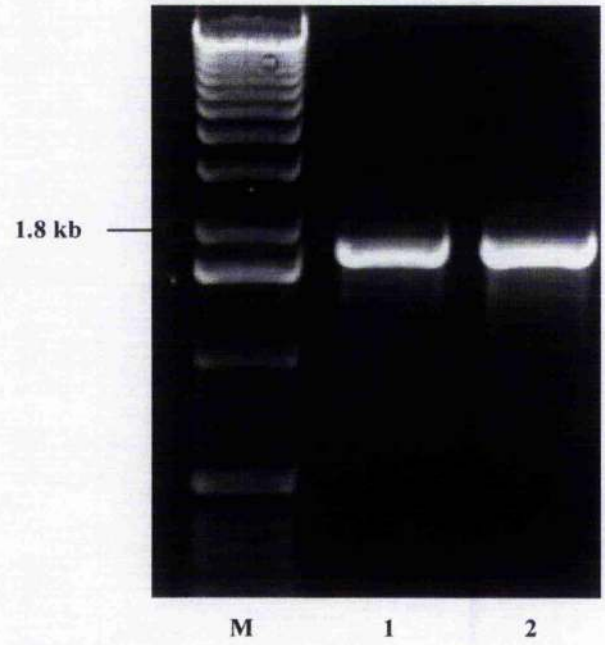



**Figure 3.2 PCR amplification of the *drrAB* operon**

The genes *drrA* and *drrB* were amplified from pWHM612 using 30 cycles under the following reaction conditions: 94 °C denaturation for 1 min, annealing for 1 min at temperatures ranging between 50 and 60 °C, 72 °C extension for 1 min. Lane M: 1 kb DNA molecular markers. Lanes 1-2: *drrAB* PCR product from replicated reactions with identical conditions.

### 3.2 Cloning of the *drrA* gene

The amplified *drrA* gene was purified from an agarose gel and cloned into the pGEMT-Easy vector (Fig. 3.3) (Section 2.3 in Methods). *Nde*I and *Xho*I endonuclease restriction sites had been designed to form part of the *drrA* gene at its ends, for sub-cloning in the pET21a vector. *Sac*I and *Hind*III endonuclease restriction sites were present instead when the sub-cloning was intended for the pQE100 vector. No endonuclease restriction sites were designed into the ends of *drrA* for cloning into the pBADTOPO thiofusion vector (Table 2.2 in Methods). For cloning into pBAD, PCR products were isolated from several bands cut from analytical gels, and pooled into a single, large well, to get a high concentration of the PCR product for further TOPO ligation (Fig. 3.4).

### 3.3 Cloning of the *drrAB* operon

Several PCR products bearing the sequence of the *drrA* and *drrB* genes were also obtained (Fig. 3.2) to allow future co-expression with different vectors (e.g. pET21a, pET33b). In this case, *Nhe*I and *Hind*III restriction sites were incorporated into the primers, an *Nhe*I site in the 5' terminus of the *drrA* gene, and a *Hind*III site in the 3' terminus of the *drrB* gene.

The product was cloned into the pGEMT-Easy vector and insertion confirmed by digestion with *Nhe*I and *Hind*III (Figure 3.5). The release of bands of around 3 kb (pGEMT vector) and 1.8 kb (*drrAB* operon) confirmed the success of this cloning.

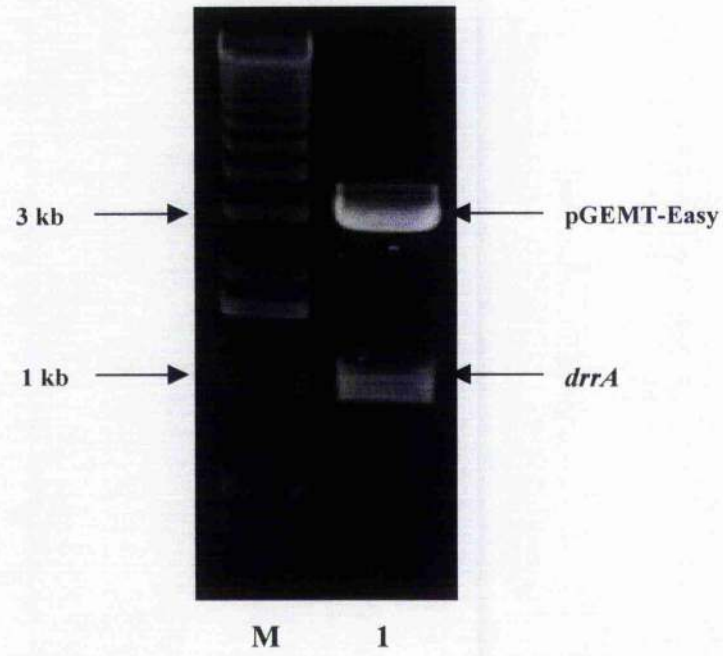



**Figure 3.3 Restriction analysis of the pGEMT Easy-*drrA* plasmid**

Plasmid DNA from transformed bacteria was isolated and digested with *Nde*I and *Xho*I before analysis on an agarose gel. M: 1 kb DNA molecular markers. 1: DNA fragments after digestion with *Nde*I and *Xho*I showing the vector and excised *drrA* insert (indicated).

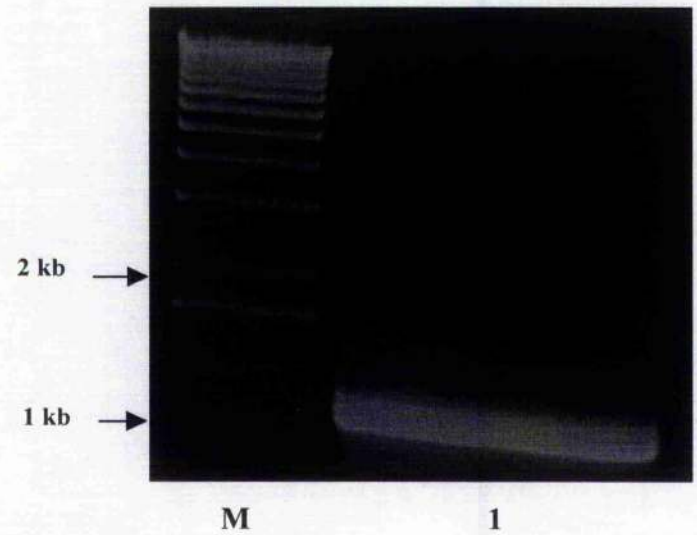



**Fig. 3.4 Electrophoresis of DNA in agarose gel showing concentrated *drrA* PCR product**

DNA from PCR *drrA* product was isolated from several bands cut from analytical gels, and pooled into a single, large well, to obtain a high concentration of the PCR product for cloning into pBADTOPO thiofusion. M: 1 kb DNA molecular markers. 1: concentrated *drrA* PCR product with no restriction sites at its ends

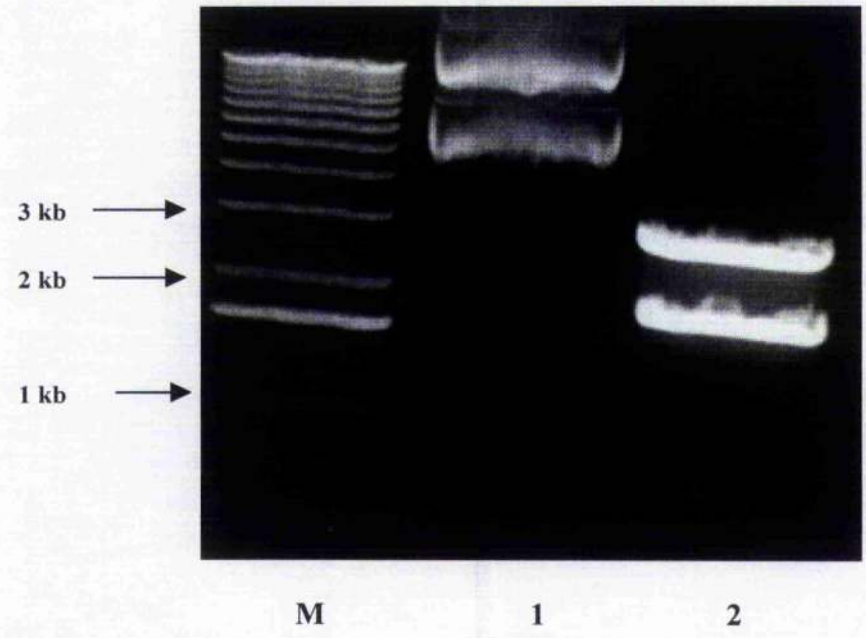



**Figure 3.5 Restriction analysis of the pGEMTEasy-*drrAB* plasmid**

Plasmid DNA from transformed bacteria was isolated and digested with *Nhe*I and *Hind*III before analysis on an agarose gel. M: 1 kb DNA molecular markers. 1: DNA fragments before digestion with *Nhe*I and *Hind*III showing the undigested pGEMT-easy *drrAB* construct. 2: DNA fragments after digestion with *Nhe*I and *Hind*III showing the vector and excised *drrAB* insert

## 3.4 Initial construction of pET *drrA* plasmids for over-expression

pET vectors were considered to be a good option to sub-clone the *drrA* gene for production of soluble protein for further assays. In particular, pET21a offered several immediate advantages to accomplish this objective. Apart from possessing a convenient resistance determinant (ampicillin), a varied multi-cloning site and a poly histidine-coding region suitable for affinity chromatography purification, it carries a powerful promoter (T7) to overexpress heterologous proteins. The *E. coli* RNA polymerase does not interact with this promoter, and hence "leaky" expression of the target protein is minimised in the uninduced state. The transcription of the heterologous gene is accomplished by transforming the expression construct into a host cell with a chromosomal copy of the T7 RNA polymerase, itself under control of the *lacUV5* promoter. The expression of the T7 RNA polymerase is induced by the addition of IPTG to a log phase culture. This in turn results in a very high transcription of the target gene sequence.

### 3.4.1 Sub-cloning of the *drrA* gene into a suitable expression vector for *Escherichia coli*.

The DNA segment of the *drrA* gene was obtained by digesting the initial pGEMT-Easy construct with *Nde*I and *Xho*I and ligation of the small released fragment into pET21a (Fig. 2.1 in Methods, and Fig. 3.6). The *drrA* gene had been designed with the particular restriction sites *Nde*I and *Xho*I, to minimise any additional DNA sequence between the end of the gene and the encoded his-tag sequence, which could disturb the conformation of the expressed protein. As the start codon ATG forms part of the *Nde*I site, it substituted the original *S. peucetius drrA* gene start codon GTG, which was not necessary to include.

Transformants carrying the pET21a-*drrA* construct were selected on the basis of vector encoded antibiotic resistance. Recombinant expression plasmids were isolated by mini-prep and checked for the presence of the inserted *drrA* gene by restriction digest. A diagram showing the general cloning process for *drrA* gene (Fig. 3.6) and a restriction analysis of recombinant pET expression plasmids (Fig. 3.7) are presented on the following pages.

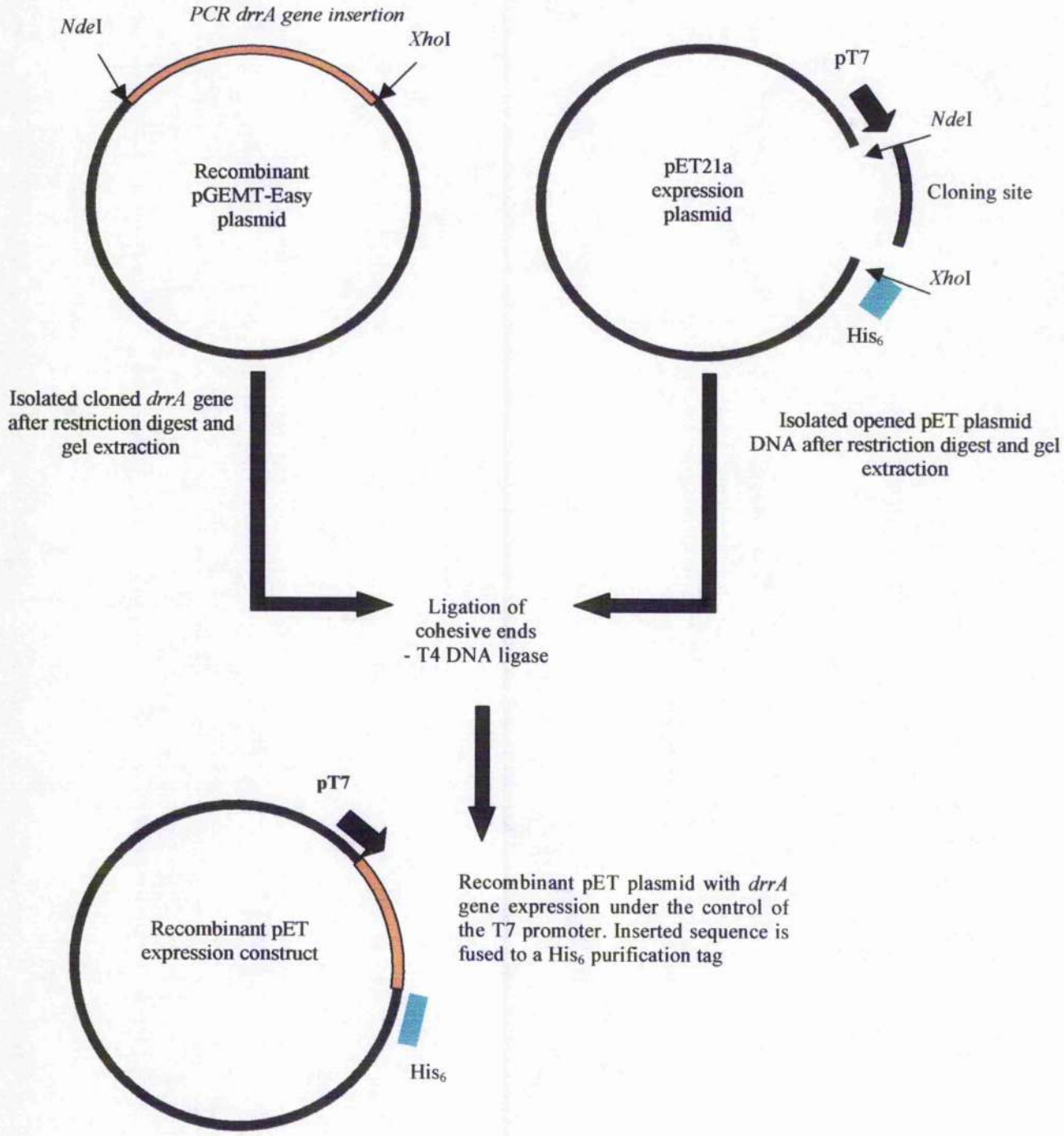



**Fig. 3.6 Diagram showing the cloning of the *drrA* gene into the pET21a vector**

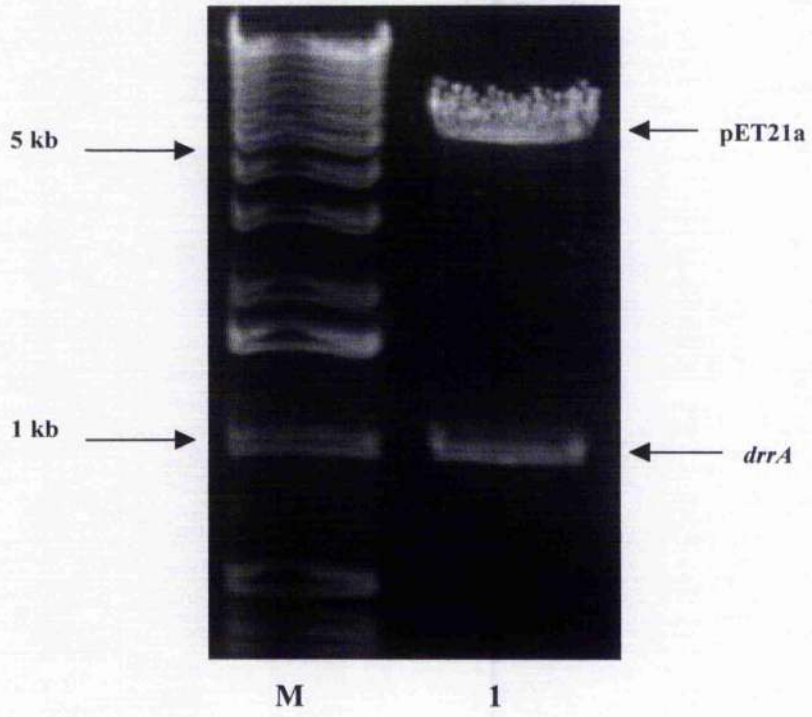



**Fig. 3.7 Restriction analysis of the pET21a-*drrA* plasmid**

Plasmid DNA from transformed bacteria was isolated and digested with *Nde*I and *Xho*I before analysis on an agarose gel. M: 1 kb DNA molecular markers. 1: DNA fragments after digestion with *Nde*I and *Xho*I showing the vector and excised *drrA* insert (indicated).

### 3.4.2 Sub-cloning of the *drrAB* operon into a suitable expression vector for *Escherichia coli*

To sub-clone the operon sequence corresponding to *drrA* and *drrB* for over-expression, a sequence coding for a histidine tag had to be added to the 5' terminus of *drrA* to make DrrA suitable for immobilised-metal affinity chromatography purification. When the sub-cloning was performed into pET33b, no histidine tag coding sequence had been included in the *drrA* gene, as the vector presented these sequences at both ends of the operon.

Additionally, some primers to clone this sequence in the vector pUC 18 were also prepared (Table 2.2 in Methods). Although the expression of genes in this vector is not high, former research with the *drrAB* system employed this vector (Kaur and Russell, 1998).

The successful sub-cloning of *drrAB* in pET21a can be seen in the restriction analysis of this plasmid (Fig. 3.8).

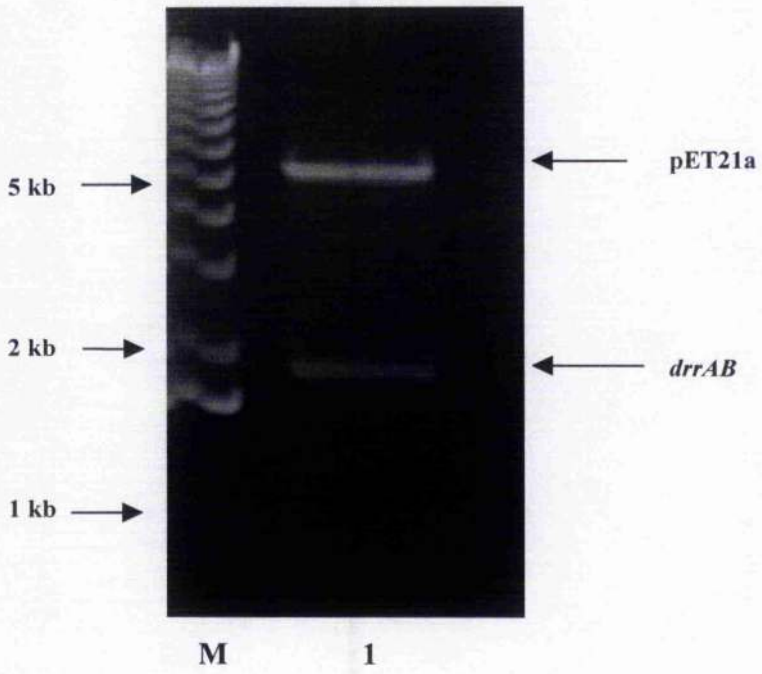


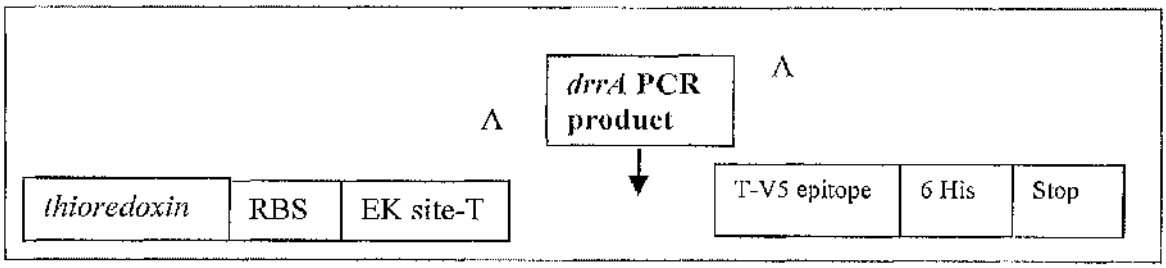

**Fig. 3.8 Restriction analysis of the pET21a-*drrAB* plasmid**

Plasmid DNA from transformed bacteria was isolated and digested with *Nhe*I and *Hind*III before analysis on an agarose gel. M: 1 kb DNA molecular markers. 1: DNA fragments after digestion with *Nhe*I and *Hind*III showing the vector and excised *drrAB* insert

### 3.5 Initial construction of pBADTOPO *thio-drrA*

The pBADTOPO thiofusion vector (Fig. 2.2 in Methods and below) was also used to clone the *drrA* gene for expression of DrrA. In this system, a fusion to Thioredoxin was created. No restriction sites were designed into the *drrA* primers as the pBADTOPO vector contains the enzymatic machinery to ligate PCR products. This vector also includes a sequence encoding a poly-histidine tag to ease protein purification by Immobilised metal affinity chromatography (IMAC). It was hoped that as Thioredoxin is a small and very soluble protein, it could help in the solubilisation of DrrA and minimise the production of inclusion bodies.

Insertion of *drrA* into the pBADTOPO thiofusion vector would create a single protein of 51.7 kDa. This protein would correspond to the DrrA protein (35.7 kDa) fused to an N-terminal HP-Thioredoxin (13 kDa) and a 6 histidine tag at the C-terminus (3 kDa). Some problems were experienced encountered in obtaining the construct, as it was difficult to discern if the constructs had the *drrA* gene inserted with the proper orientation. Uncommon single restriction sites were present in both the plasmid and the *drrA* gene, and it was difficult to assess the orientation of the insert on gels. Employing a PCR methodology for the same purpose produced contradictory results; the first four constructs to be tested were correctly inserted according to PCR amplification, but the analysis of their sequences showed that all of the four inserts were in the wrong orientation. Finally, after exhaustive analysis, a positive clone was obtained with the insert correctly orientated according to a restriction analysis with *Eco*RV and *Nar*I. This was confirmed by sequence analysis. Expression from this construct was induced with arabinose and could be tightly regulated according to the used inducer concentration. A diagram showing the localisation of the *drrA* gene PCR product inserted into the vector pBADTOPO thiofusion is shown below:



### 3.6 Transformation of *E. coli* cells

The pET21a vector, carrying the *drrA* gene alone, or with *drrB*, was used to transform *E. coli* XL-10 for propagation of the plasmids. *E. coli* BL21 DE3 was transformed for protein expression. Similarly, *E. coli* DH5_ pro cells were used for the pET33b plasmid. Standard transformation procedures were followed incorporating a 90 seconds heat shock at 42 °C that produced sufficient transformants for screening.

When transformation was carried out with the pBADTOPO *thio-drrA* construct, *E. coli* LMG194 or TOP-10 cells were employed. Heat shock at 42 °C for 45 seconds, as suggested in the manual, produced sufficient transformants.

### 3. 7 Sequence analysis of the cloned *drrAB* genes

The nucleotide sequences of the cloned PCR amplicons were consistent with those of the published *drrA* and *drrB* genes, except where restriction sites had been added to the primers, and where poly histidine tags were included in the construct. For example, a *Xho*I site was introduced at the 3' end of the *drrA* gene sequence for cloning of *drrA* alone into when the pET21a vector, and no restriction site was included at the *drrA* 3' terminus when cloning included *drrB*. The sequence data confirmed the success of the initial PCR reactions with genomic DNA and hence the transformed *E. coli* strains were laid down as glycerol stock cultures. An *in silico* analysis of the cloned sequences was also performed to establish that each encoded the expected DrrA and DrrB proteins.

#### 3.7.1 Sequence analysis of the *drrA-thio* fusion

The *drrA* gene fused to *thioredoxin* bore some additional sequence from the pBADTOPO thiofusion vector comprising nucleotides for the Enterokinase (EK) site upstream from the 5'terminus of *drrA*, and the V5 epitope downstream from the 3' terminus of *drrA*.

The whole coding sequence in the pBADTOPO *thio-drrA* construct was 1,446 bp, encoding a protein of 481 amino acids. 990 bp were derived from *drrA* and of the

remaining 456 bp, 18 coded for the histidine tag, 42 for the V5 epitope, and almost all the remainder, for the *thioredoxin* gene sequence.

## 3.8 Discussion

Vectors of pET series, and specially pET21a, were considered to be the best option for cloning and expressing the *drrA* gene. These vectors are popular because they offer several options for selection with antibiotics, tags for purification, such as the histidine repeat can be positioned at the N- or C-termini of the translation product and multiple cloning sites are available. In addition, they carry a strong promoter (T7), suitable for the transcription and expression of soluble proteins. Previous publications (Guilfoile and Hutchinson, 1991; and Kaur, 1997; Kaur and Russell, 1998) reported that DrrA was a hydrophilic soluble protein, peripheral to the membrane. This suggested that vectors such as the pET series would be good vehicles to overexpress appropriate quantities of soluble DrrA for functional analysis. The pET21a vector seemed to be the most suitable of these pET vectors to achieve this aim.

In designing primers, care was taken to ensure the resulting DrrA protein would carry as few non-native residues as possible. By using *Nde*I and *Xho*I restriction sites, the gene could be cloned into pET in frame with tags and additional bases within the multiple-cloning site could be excluded. In addition, the presence of the histidine tag at the C-terminus of DrrA would avoid the co-purification by nickel-agarose affinity chromatography of products degraded from this terminus of the protein, ensuring the purification of a full length product.

The choice of the pBADTOPO thiofusion vector formed an alternative option for production of soluble DrrA protein. TOPO ligation is a very effective way for insertion of PCR products but it suffered the disadvantage that products could ligate into the vector in either orientation. As it was not easy to find appropriate single restriction sites, it proved difficult to identify clones with the intended orientation.

A further option of the co-expression of *drrA* and *drrB* genes was established as previous work suggested that there was some degree of interdependence in the DrrAB system (Kaur, 1997; Kaur and Russell, 1998). These studies concluded that DrrB was not overexpressed at all in the absence of DrrA, and that active DrrA protein could only be obtained when co-expressed with DrrB.

This chapter reports the successful cloning of the *S. peucetius drrA* gene, and its sub-cloning into vectors for overexpression of the proteins. This was an important target in order to accomplish the project aims of purification of large quantities of these proteins in order to further study the biochemical, structural and physiological properties of ABC transporters in general and the function of the *S. peucetius* DrrA protein in particular.

# Chapter 4

## Heterologous expression of recombinant proteins

On the assumption that the cloned *drrA* gene would encode the nucleotide-binding domain (NBD) of the *S. peucetius* DrrAB pump, the next stage of the investigation involved its over-expression to obtain adequate quantities of protein for characterisation. After purification, it was planned that these studies would include the analysis of the ATPase activity of DrrA. This enzyme would utilise energy from ATP to drive the transport of the anthracycline compounds daunorubicin and doxorubicin out of the cell, avoiding their toxic effects.

*Streptomyces* is a soil microorganism, and its growth and handling are not as straightforward as for laboratory organisms. To overcome this handicap and avoid the requirement for special culturing facilities, it was decided that a biochemical study of DrrA would be founded upon its expression in a heterologous host. *E. coli* was selected since it has been used to overexpress successfully an enormous number of different proteins from biologically diverse species. Another very important reason for the use of *E. coli* is that genetic systems for the expression of heterologous proteins in this bacterium are well developed and characterised.

### 4.1 Heterologous over-expression of the *S. peucetius* DrrA protein

It was initially thought that *drrA* would be more easily cloned without *drrB*, and that the mainly hydrophilic properties of DrrA would favour the production of soluble protein for further characterisation.

#### 4.1.1 Over-expression of the DrrA protein using the pET21a plasmid

The *drrA* gene was cloned into the pGEMT-Easy vector and sub-cloned into the pET21a vector. The start codon of *drrA* was thereby placed downstream of a bacteriophage T7 promoter, a *lac* operator sequence and an *E. coli* ribosome-binding site. These features should have ensured the repression of protein expression in the uninduced state, and high level transcription when the plasmid was transformed into a suitable host strain and induced with IPTG. The *E. coli* strain chosen for the purpose

of expressing this gene was BL21 (DE3). This strain lacks two major cellular proteases, Lon and OmpT, which may interfere with the expression and purification of intact, full-length, recombinant protein; it also has a DE3 lysogen, which is stably incorporated into the *E. coli* chromosome and encodes an IPTG inducible bacteriophage T7 RNA polymerase. It is the induction of this polymerase that allows transcription of the recombinant gene sequence. The vector also provided a six-histidine tag at the C-terminus of DrrA (see Ch. 3, sections 3.1 and 3.4.1).

#### 4.1.1.1 Initial analysis of cell fractions

Cells of *E. coli* BL21 carrying pET21a-*drrA* were grown at 37 °C to exponential phase ($OD_{600nm}$ = 0.6) in complex media (LB broth), and IPTG was added to 1 mM to induce expression. Growth continued for 3 hours and the cells were recovered by centrifugation. After disruption, a supernatant fraction and a pellet were obtained. The insoluble material in the disrupted cell pellet was prepared for analysis by incubation in 8 M urea Phosphate lysis buffer for 2 hours (see Ch. 2, section 2.9.2). The crude fractions were analysed for the presence of DrrA by addition of SDS-PAGE sample buffer and subsequent electrophoresis. A band on an SDS-PAGE gel with a molecular mass similar to that of the recombinant protein (37 kDa) was present in the insoluble fraction from the disrupted cells (designated the inclusion body fraction). The appearance of this band seemed to depend upon induction with IPTG (Fig. 4.1). The size of the recombinant protein and its expression in the presence of IPTG supported the notion that it was likely to be DrrA.

#### 4.1.1.2 Optimisation of conditions for production of soluble protein

Attempts to shift the proportion of DrrA present in the insoluble fraction towards the cytosolic fraction, led us to test induction at lower temperatures, and induction with lower IPTG concentrations. Assays to obtain soluble DrrA protein using lower temperatures (30 and 25 °C) (Fig. 4.2) and lower IPTG concentrations (0.2 mM) did not increase the yield of soluble protein in early experiments using low volumes of media (1 L). Afterwards, induction at 18 °C provided some protein in the soluble fraction for purification by affinity chromatography (IMAC; see Ch. 2, section 2.9), but the amounts were too low to proceed with characterisation assays (Fig. 4.3).

Initially the yield of soluble DrrA was no higher than 30 μg protein/L of culture. In further experiments, a concentration of 60±20 μg protein/L of culture was obtained by induction at 17 °C with 1 mM IPTG. The first two fractions eluted from five IMAC purifications (each purification corresponding to approximately 2 L of culture) were collected. About 1 mg of DrrA in approximately 9 ml of pooled elution buffer was collected and dialysed.

#### 4.1.1.3 Over-expression of DrrA from the pET21a plasmid in strain BL21 AI

Expression of the pET21a construct was tested in the *E. coli* strain BL21 AI to establish if it was possible to improve the yields of DrrA by using a different bacterial strain. In this system, IPTG and arabinose inducers provide stringent regulation over the expression of DrrA. In spite of expectations, the expression of DrrA at 37 °C and 17 °C was not improved relative to the BL21 (DE3) strain, although multiple combinations of inducers were used. Induction with 0.01 mM IPTG had the weakest effect on the expression of DrrA in this system (Fig. 4.4), while 0.1 mM was the best, rendering the highest yield of soluble protein.

#### 4.1.1.4 Analysis of expression of DrrA from the pET21a vector by Western blot

Western blotting was used to demonstrate the presence and identity of DrrA protein expressed from different constructs, exploiting the presence of a $His_6$ epitope tag. Antibodies raised against DrrA were not available.

The recombinant 37 kDa protein was produced by induction with 0.1-1 mM IPTG (and with Arabinose and IPTG in the BL 21 AI strain). After transfer to PVDF, blots were probed with antibody against the histidine tag. At 37 °C, almost all the protein appeared in the insoluble cytoplasmic fraction, presumably in inclusion bodies. However, at 18 °C a small amount of DrrA could be detected in the soluble cytosolic fraction. A proportion of DrrA also appeared in the membrane fraction (Fig. 4.5).

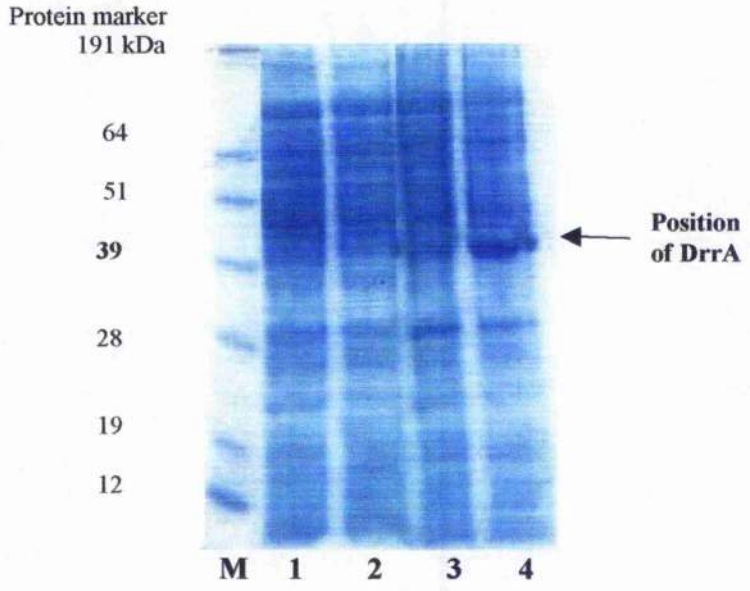



**Figure 4.1 Preliminary fractionation of *E. coli* BL21(DE3) grown at 37 °C to determine sub-cellular localisation of DrrA**

Bacteria carrying the pET21a expression construct were grown at 37 °C to exponential phase and induced with 1 mM IPTG for 3 hours. In a control experiment, cells were treated identically but for the omission of IPTG. Soluble cytosolic proteins were obtained by cell disruption and centrifugation. Proteins recovered from the disrupted pellet following addition of urea were termed the inclusion body fraction. Samples were analysed by SDS PAGE and GelCode blue staining.

M: Protein molecular weight markers

Lane 1: Crude soluble fraction - IPTG

Lane 2: Crude soluble fraction + IPTG

Lane 3: Inclusion body fraction - IPTG

Lane 4: Inclusion body fraction + IPTG

Approximately 20 µg of protein was loaded per well. The 37 kDA protein presumed to be DrrA is indicated.

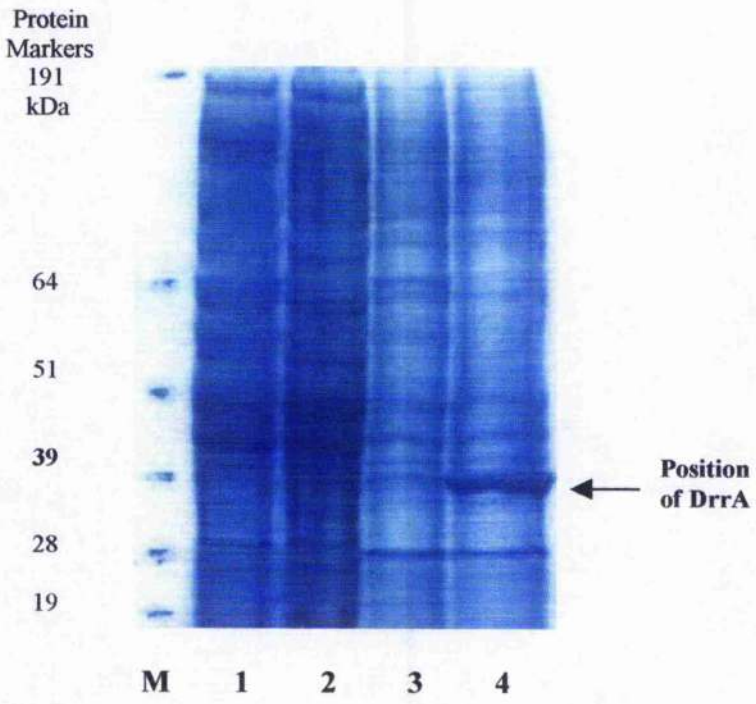



**Figure 4.2 Preliminary fractionation of *E. coli* BL21(DE3) grown at 25 °C to determine the sub-cellular localisation of DrrA**

Bacteria carrying the pET21a expression construct were grown at 37 °C to exponential phase, shifted to 25 °C and induced with 1 mM IPTG overnight. In a control experiment, cells were treated identically but for the omission of IPTG. Cell fractionation and analysis was carried out as described in Figure 4.1

M: Protein molecular weight markers

Lane 1: Crude soluble fraction - IPTG

Lane 2: Crude soluble fraction + IPTG

Lane 3: Inclusion body fraction - IPTG

Lane 4: Inclusion body fraction + IPTG

Approximately 30 µg of protein from the soluble fractions and 20 µg from the inclusion bodies fractions were loaded to the gel.

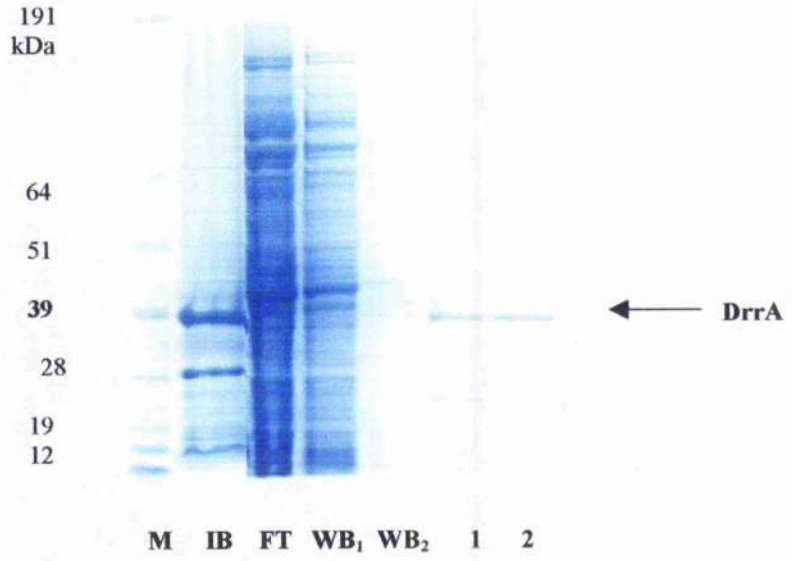



**Figure 4.3 Expression and purification of DrrA from *E. coli* BL21 (DE3) cultures induced at 18 °C**

Bacteria carrying the pET21a expression construct were grown at 37 °C to exponential phase, shifted to 18 °C and induced with 1 mM IPTG overnight. Proteins from the soluble cytoplasmic fraction were purified by IMAC. Samples were analysed by SDS PAGE and GelCode blue staining.

M: Protein molecular weight markers

IB: Inclusion body fraction

FT: Flow-through from the IMAC column

$WB_1$: Wash fraction from the column (25 mM imidazole)

$WB_2$: Wash fraction from the column (50 mM imidazole)

Lanes 1 and 2: Fractions eluted from the IMAC column with 300 mM imidazole

20 µg of protein from the inclusion body fraction was loaded to the gel. IMAC was carried out with material prepared from about 2 L of induced culture.

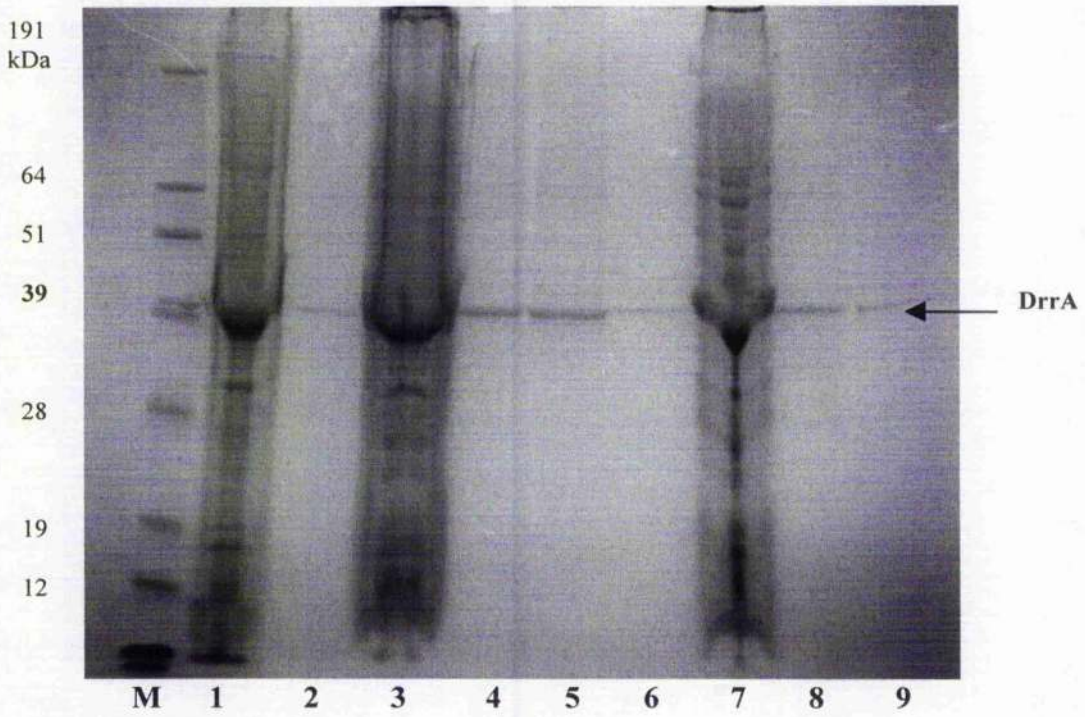



**Figure 4.4 Expression and purification of DrrA from *E. coli* BL21 AI cultures induced at 17 °C**

Bacteria carrying the pET21a expression construct were grown at 37 °C to exponential phase, shifted to 17 °C and induced with 0.002% arabinose and the IPTG concentrations indicated, overnight. Proteins from the soluble cytoplasmic fraction were purified by IMAC. Samples were analysed by SDS PAGE and Coomasie blue staining.

M: Protein molecular weight markers

1: Inclusion body fraction (induction with 0.01 mM IPTG)

2: Protein eluted from IMAC column after loading with soluble cytosolic fraction (induction with 0.01 mM IPTG)

3: Inclusion body fraction (induction with 0.1 mM IPTG)

4-6: Fractions eluted from IMAC column after loading with soluble cytosolic fraction (induction with 0.1 mM IPTG)

7: Inclusion body fraction (induction with 1 mM IPTG)

8-9: Fractions eluted from IMAC column after loading with soluble cytosolic fraction (induction with 1 mM IPTG)

In each case, IMAC was carried out with material prepared from about 2 L of induced culture.

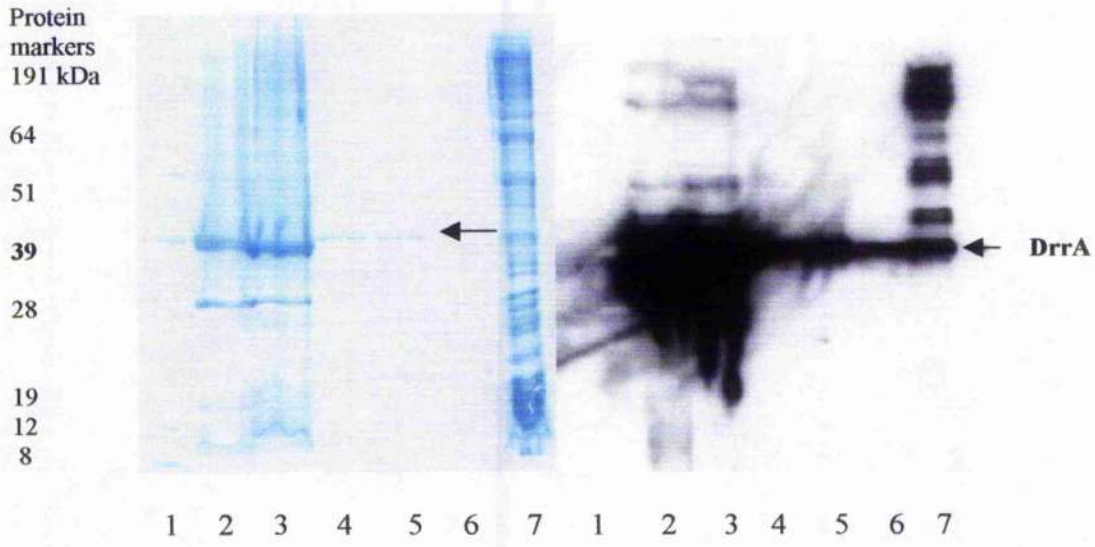



**Figure 4.5 Expression, purification and blotting analysis of DrrA from *E. coli* BL21 (DE3) cultures induced at 18 °C**

Bacteria carrying the pET21a expression construct were grown at 37 °C to exponential phase, shifted to 18 °C and induced with 1 mM IPTG overnight. Proteins from the soluble cytoplasmic fraction were purified by IMAC and analysed by Samples were analysed by SDS PAGE and GelCode blue staining (left panel) and Western blotting with an antibody against the histidine purification tag (right panel).

1: Protein molecular weight markers

2: Inclusion body fraction from induction at 18 °C

3: Inclusion body fraction from induction at 30 °C

4-6: First 3 fractions eluted from IMAC column after loading with soluble cytosolic fraction (induction at 18 °C)

7: Membrane fraction (induction at 18 °C)

IMAC was carried out with material prepared from about 1 L of induced culture.

## 4.2 Heterologous over-expression of DrrA and DrrB using pET vectors

It has been reported that the expression of DrrA is dependent upon the presence of DrrB, indicating a tight interaction between both proteins (Kaur, 1997, 2005; Kaur and Russell, 1998). Therefore, the expression of DrrA was investigated as a component of the whole DrrAB complex. Plasmids pET21a and 33b were chosen as vectors for the *drrAB* operon to test whether more soluble DrrA could be obtained when expression was translationally coupled to DrrB.

### 4.2.1 Heterologous over-expression of DrrA and DrrB proteins using the pET21a plasmid

When both *drr* genes were cloned into the pET21a vector for expression studies DrrA could not be detected on gels after induction with 1 mM IPTG. Yields could not be improved by induction at 16 °C. Sequencing confirmed that part of the DNA sequence encoding the histidine-tag was missing from the construct, but a new vector preparation in which this feature was corrected also failed to express DrrA.

### 4.2.2 Heterologous over-expression of DrrA and DrrB proteins using the pET33b plasmid

The same approach was attempted using the pET33b vector. The *drrAB* PCR product was cloned into the expression vector and transformed into *E. coli* BL21(DE3). Transformants were grown and induced with 1 mM IPTG at 37 °C. No signs of DrrA expression could be detected when samples were analysed on gels. The temperature at induction was dropped to 17 °C and IPTG added to 1 or 0.1 mM, Soluble cytosolic proteins were purified by IMAC for gel analysis. This revealed several bands on the gel including one that matched the molecular weight expected for DrrA (~37 kDa). Two additional bands of similar concentration were present around 39 kDa (Fig. 4.6) but the yield and purity of the material was insufficient to allow further progress.

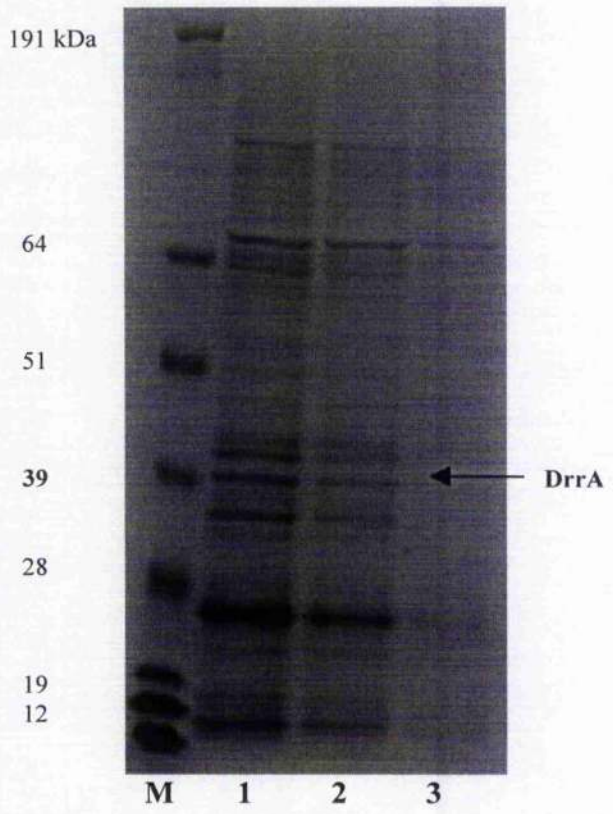



**Figure 4.6 Expression and purification of DrrA from *drrAB* construct induced at 17 °C**

Bacteria carrying the pET33b expression construct were grown at 37 °C to exponential phase, shifted to 17 °C and induced with 0.1 mM IPTG overnight. Proteins from the soluble cytoplasmic fraction were purified by IMAC and analysed by SDS-PAGE and Coomasie blue staining..

M: Protein molecular weight markers

Lanes 1-3: Fractions eluted from the IMAC column

Soluble protein from a 2 L culture was loaded to the column

## 4.3 Heterologous over-expression of Thio-DrrA from the pBADTOPO thiofusion plasmid

Since only minimal quantities of soluble DrrA were obtained by expression of the pET constructs, a different approach was tested. The pBADTOPO thiofusion plasmid encodes for Thioredoxin, a protein of low molecular weight and high solubility, and the vector enables convenient fusion with the product of a cloned gene. It was considered that the properties of Thioredoxin might increase the proportion of DrrA protein in the soluble cytosolic fraction, although forming part of a fusion protein. As this type of vector can be tightly regulated by an appropriate concentration of arabinose, a small-scale culture at 37 °C was used to determine the arabinose concentration that induced the highest expression of the Thio-DrrA fused protein. When the *E. coli* transformant were grown to an $OD_{600}$ of about 0.5, they were treated with different arabinose concentrations to induce expression of the DrrA protein (0.00002-0.2%; see section 2.7.2). Following a four-hour incubation period, the cells were recovered by centrifugation and samples from each culture were mixed and lysed with SDS NuPAGE buffer, and run on a gel. A Thioredoxin-DrrA fusion protein, of approximately 52 kDa molecular weight, was clearly expressed when cultures were induced with arabinose at concentrations between 0.002 and 0.2% (Fig. 4.7).

Having chosen an arabinose concentration of 0.002% to induce the expression of the fused DrrA, experiments were carried out on a much larger scale (10 L). Although some protein appeared in the soluble cytosolic fraction when cultures were grown and induced at 37 °C (Fig. 4.8), most of the protein was present in the insoluble fraction (assumed to be inclusion bodies). Dropping the temperature of induction to 25 °C only produced a slight improvement in solubility (Fig. 4.9). Only when cultures were induced at 17 °C, did better yields of soluble Thio-DrrA emerge (Fig. 4.10). The presence of the histidine purification tag was confirmed by Western blotting using specific antibody (Fig. 4.11).

It was found that standardised experimental conditions of overnight culture at 17 °C could produce Thio-DrrA at a concentration of 250 µg/ml in the eluate (12 ml). Using IMAC for purification, the first three fractions eluted from four purification

runs (corresponding to 7 L of culture) yielded Thio-DrrA at 430 μg/L of culture. As DrrA comprises a 72.5% of the fused Thio-DrrA protein, it is possible to deduce that _ of this protein correspond to the DrrA fraction. This result shows that the inherent production of DrrA from the pBAD construct was about 3.6 fold higher than when the pET21a construct was used.

### 4.4 Comparative analysis of DrrA from expression vector systems

A comparative Western blot of DrrA proteins expressed from pET21a and pBADTOPO thiofusion vectors after induction at 17 °C and 37 °C was performed. When *E. coli* cultures carrying these plasmids were induced at 37 °C, a very faint band of Thio-DrrA appeared on SDS-PAGE gels, whilst no DrrA could be detected from the pET21a expression system. The yield of DrrA from the pET21a construct when induced at 17 °C was scarcely more prominent than that obtained for Thio-DrrA produced at 37 °C, according to data from SDS-PAGE and Western blotting (Fig. 4.12 and 4.13). Thus, it was concluded that the pET21a expression system worked best (if not ideally) when induced at 17 °C and grown on overnight.

In contrast, the pBADTOPO *thio-drrA* construct showed more promise as a vehicle for expression of recombinant DrrA. When induced with arabinose at 17 °C, this system yielded at least five times more Thio-DrrA than was obtained from other preparations as determined by SDS-PAGE (Fig. 4.12) and the determination of protein concentration. An additional band of similar size to DrrA appeared on the gel (Fig 4.12) and was reactive with anti-tag antibody in Western blots (Fig. 4.13). This was probably being a degradation product of the fused protein. Yields of soluble protein from the pET21a expression system were approximately 60 μg per litre of culture, whilst yields for Thio-DrrA were 375 μg/L (Table 4.1).

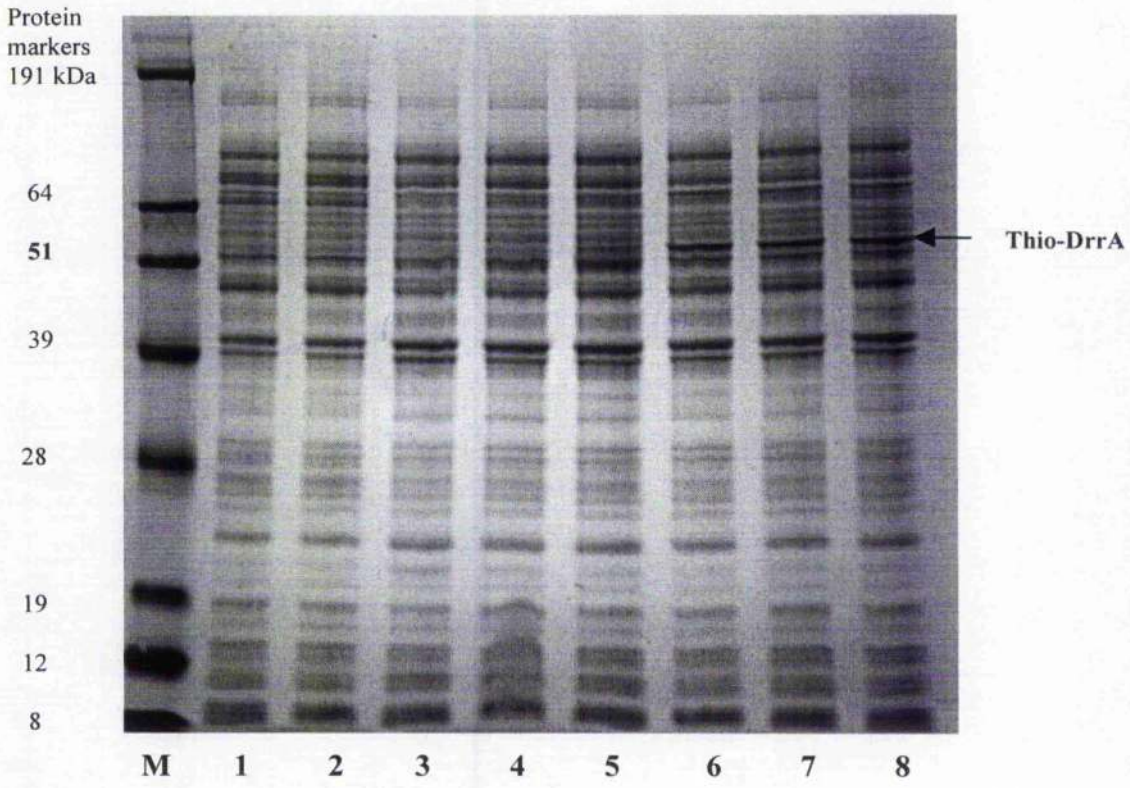



**Fig. 4.7 Expression of Thio-DrrA in *E. coli* LMG 194 after induction with different concentrations of arabinose**

Cultures of *E. coli* were grown at 37 °C to exponential phase, treated with varying concentrations of arabinose, and incubated for an additional 4 hours. Total cellular protein was analysed by SDS-PAGE and GelCode blue staining.

M: Protein molecular weight markers

1: Control taken at time of induction, no arabinose added

2: Control at the end of the induction period, no arabinose added

3: Culture induced with 0.000002% arabinose

4: Culture induced with 0.00002% arabinose

5: Culture induced with 0.0002% arabinose

6: Culture induced with 0.002% arabinose

7: Culture induced with 0.02% arabinose

8: Culture induced with 0.2% arabinose

A protein of 52 kDa consistent with the size of the Thioredoxin-DrrA fusion protein is indicated.

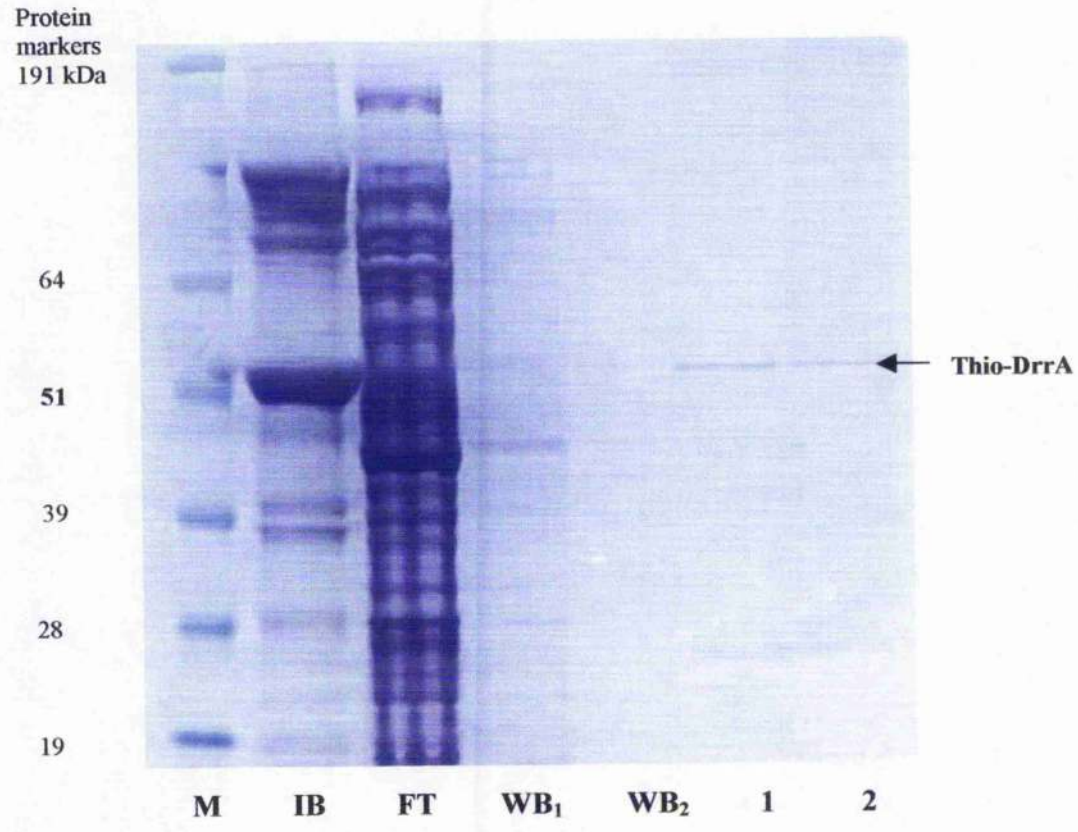



**Figure 4.8 Expression and purification of Thio-DrrA from *E. coli* LMG 194 cultures induced at 37 °C**

Cultures of *E. coli* were grown at 37 °C to exponential phase, treated with 0.002% arabinose and incubated for an additional 4 hours. Cells were fractionated and recombinant protein purified from soluble cytoplasmic extracts by IMAC. Samples were analysed by SDS PAGE and Coomasie blue staining.

M: Protein molecular weight markers

IB: Inclusion body fraction

FT: Flow-through from the IMAC column

WB1: Washings from the column (20 mM imidazole)

WB2: Washings from the column (50 mM imidazole)

1 and 2: Fractions eluted from the column (300 mM imidazole)

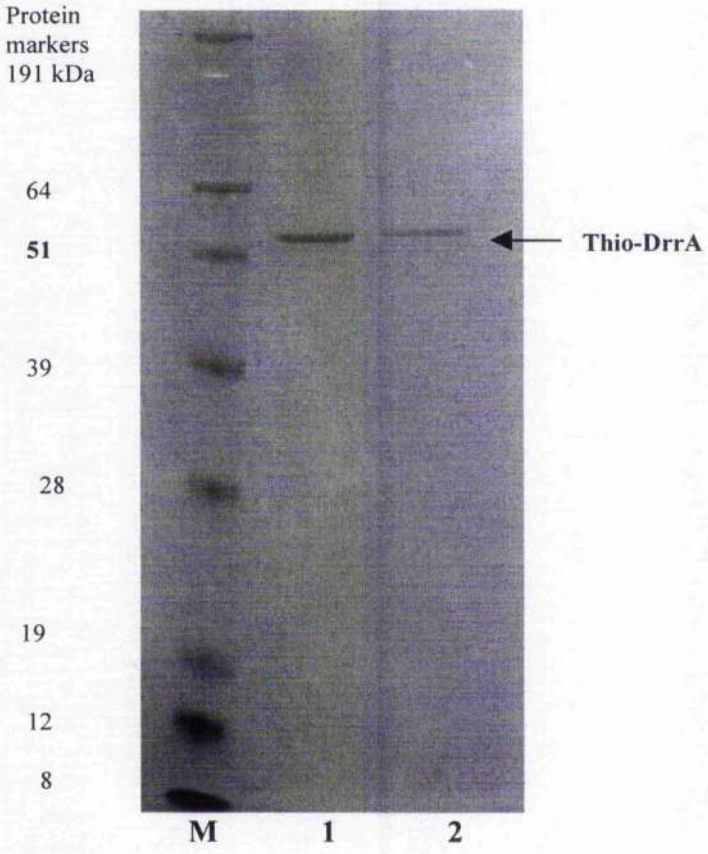



**Figure 4.9 Expression and purification of Thio-DrrA from *E. coli* LMG 194 cultures induced at 25°C**

Cultures of *E. coli* were grown at 37 °C to exponential phase, shifted to 25 °C and treated with 0.002% arabinose overnight. Cells were fractionated and recombinant protein purified from soluble cytoplasmic extracts by IMAC. Samples were analysed by SDS PAGE and GelCode blue staining.

M: Protein molecular weight markers

Lane 1-2: First two fractions eluted from IMAC column (300 mM imidazole)

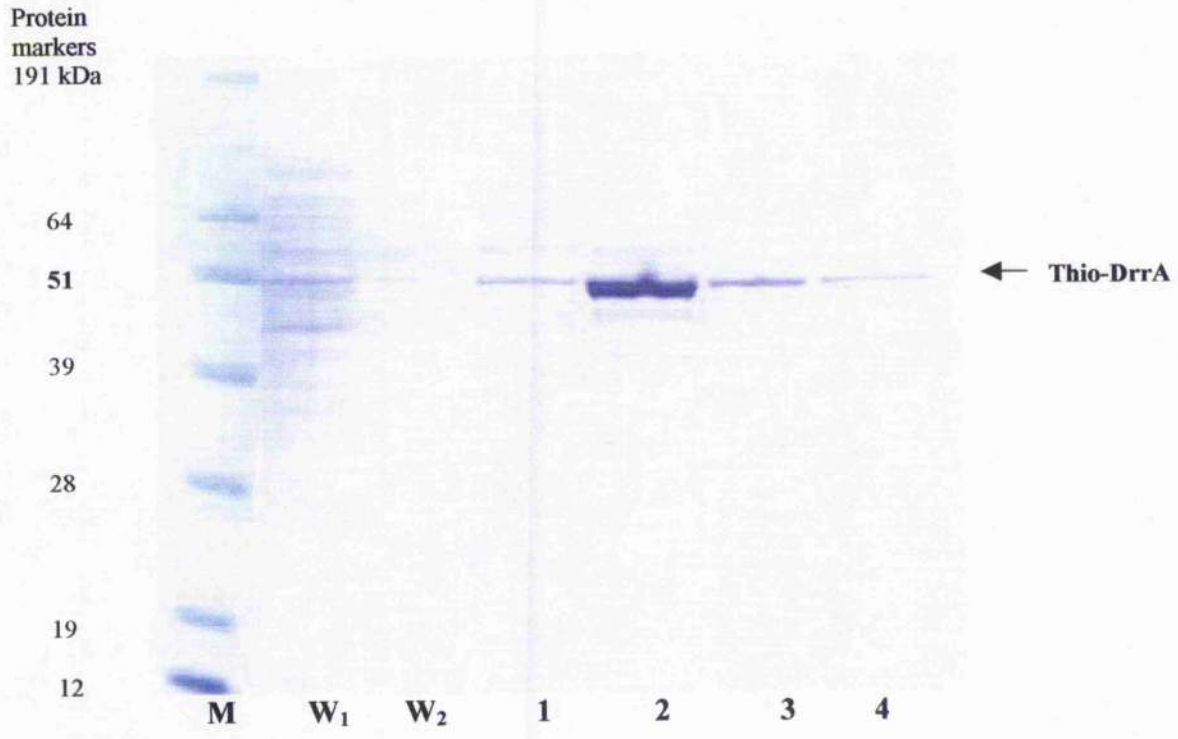



**Figure 4.10 Expression and purification of Thio-DrrA from *E. coli* LMG 194 cultures induced at 17 °C**

Cultures of *E. coli* were grown at 37 °C to exponential phase, shifted to 17 °C and treated with 0.002% arabinose overnight. Cells were fractionated and recombinant protein purified from soluble cytoplasmic extracts by IMAC. Samples were analysed by SDS PAGE and Coomasie blue staining.

M: Protein molecular weight markers

$W_{1\text{-}2}$: Washings from the IMAC column (25 and 50 mM imidazole, respectively)

1-4: First four fractions eluted from the IMAC column (300 mM imidazole)

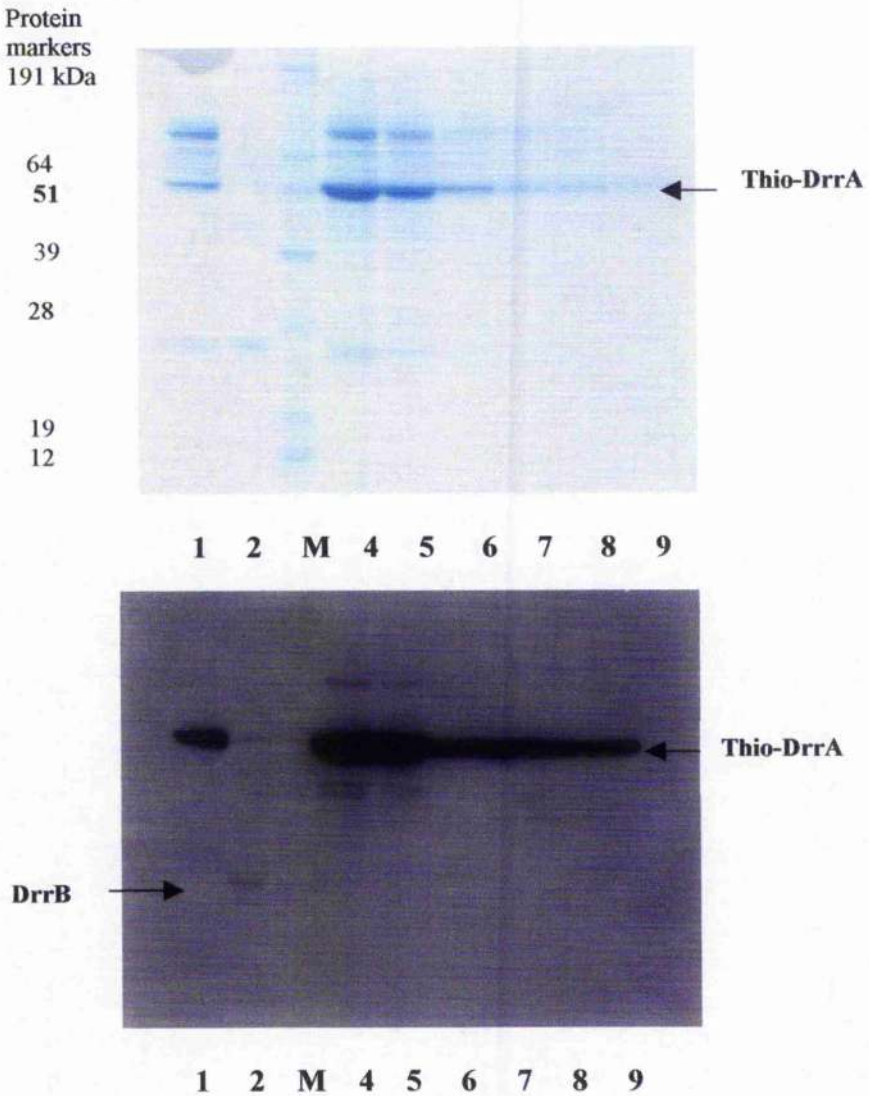



**Figure 4.11 Expression, purification and blotting analysis of Thio-DrrA from *E. coli* LMG 194 cultures induced at 17 °C**

Cultures of *E. coli* were grown at 37 °C to exponential phase, shifted to 17 °C and treated with 0.002% arabinose overnight. Cells were fractionated and recombinant protein purified from soluble cytoplasmic extracts by IMAC. Samples were analysed by SDS-PAGE and GelCode blue staining (upper panel) and Western blotting (lower panel) using antibodies against the histidine tag.

Lanes 1: First eluted fraction from IMAC column (300 mM imidazole)

Lane 2: DrrB prepared from a pET21 construct in a different experiment

M (lane 3): Protein molecular weight markers

Lanes 4-9: Fractions 2-7 eluted from IMAC column (30 mM imidazole)

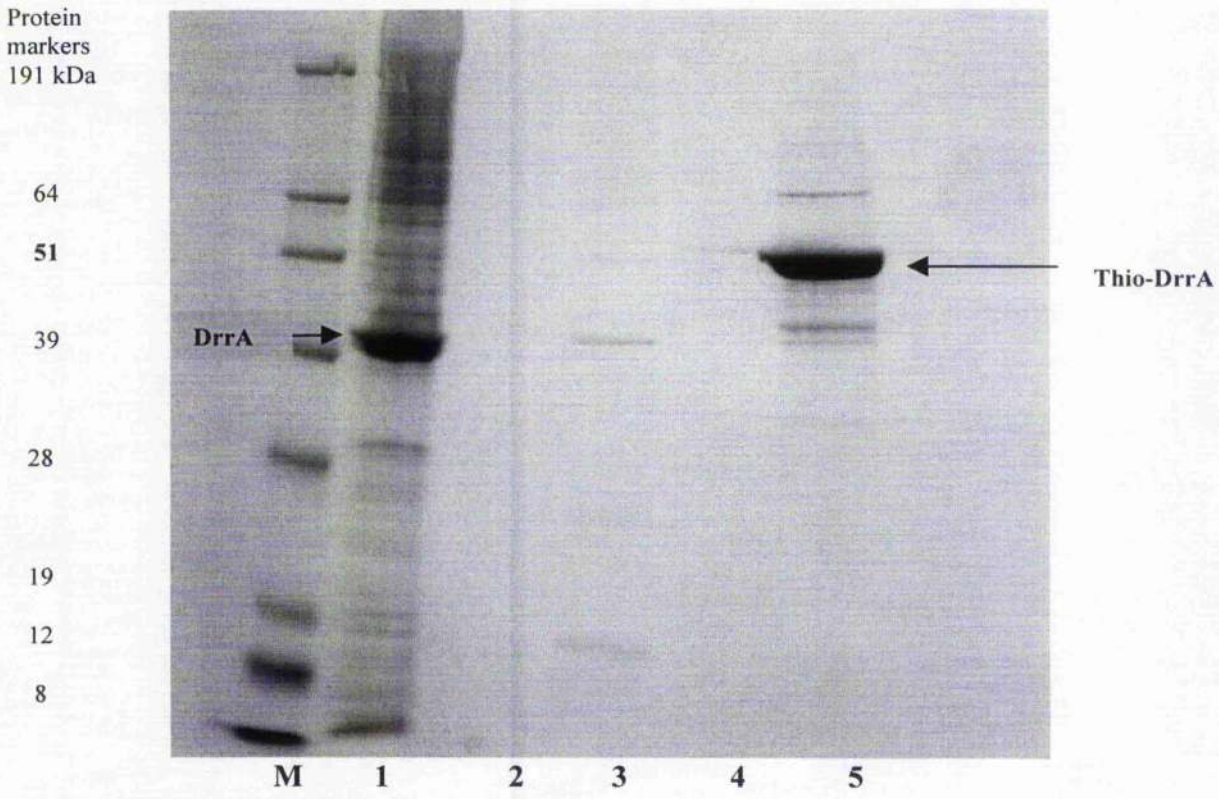



**Figure 4.12 Analysis of recombinant DrrA expressed as a single protein from pET21a and as a Thio-DrrA fusion protein**

*E. coli* cultures were induced with 1 mM IPTG for expression of DrrA and with arabinose for expression of the Thio-DrrA fusion protein. Samples were analysed by SDS PAGE and GelCode blue staining.

M: Protein molecular weight markers

1: DrrA present in inclusion body fraction prepared from cultures induced at 37 °C

2: DrrA present in the soluble fraction prepared from cultures induced at 37 °C

3: DrrA present in the soluble fraction prepared from cultures induced at 17 °C

4: Thio-DrrA present in the soluble fraction prepared from cultures induced at 37 °C

5: Thio-DrrA present in the soluble fraction prepared from cultures induced at 17 °C

Sample loadings represent 0.1 % of the disrupted cell pellet from 1 L of culture. DrrA in the inclusion body fraction (lane 1) was loaded as a reference marker for the migration of DrrA

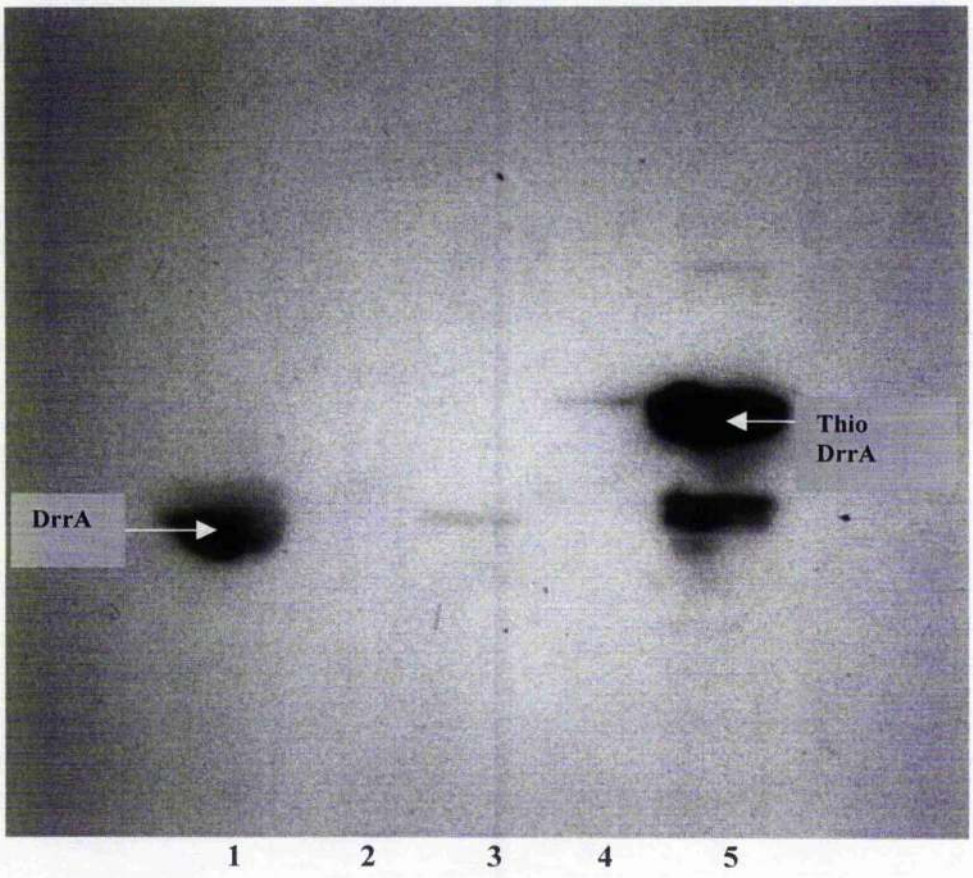



**Figure 4.13 Western blot analysis of DrrA expressed as a single protein from pET21a and as a Thio-DrrA fusion**

Samples were prepared as in Figure 4.12

1: DrrA present in inclusion body fraction prepared from cultures induced at 37 °C

2: DrrA present in the soluble fraction prepared from cultures induced at 37 °C

3: DrrA present in the soluble fraction prepared from cultures induced at 17 °C

4: Thio-DrrA present in the soluble fraction prepared from cultures induced at 37 °C

5: Thio-DrrA present in the soluble fraction prepared from cultures induced at 17 °C

**Table 4.1 Comparison of protein yields**

| | Thio-DrrA | DrrA |
|---|---|---|
| Volume of culture (L) | 8 | 8 |
| Volume eluted from IMAC columns (ml) | 12 | 8 |
| Protein concentration (μg/ml) in the eluted material | 250 | 60 |
| Total protein obtained (mg) | 3 | 0.48 |
| Protein yield (μg/L) | 375 | 60 |

Data was gathered from four independent purifications each using 2 L of culture, whose material was pooled for analysis. Protein yields represent the average from three determinations. In each case, *E. coli* strains were grown at 37 °C to exponential phase, shifted to 17 °C, treated with inducer (0.002% arabinose for Thio-DrrA, 1 mM IPTG for DrrA) and incubated overnight to induce expression. Proteins were isolated from soluble cytoplasmic fractions using IMAC.

## 4.5 Discussion

Expression of transporter proteins has been a difficult task in the past. Membrane proteins need an appropriate environment for expression and purification besides intrinsic requirements for functionality. NBDs, on the other hand, have been classified as cytosolic domains, peripheral to the membrane, but they also are usually expressed with difficulty in hydrophilic environments. To overcome this drawback, researchers have usually had to search for particular approaches linked to the system under study. The following examples illustrate the different approaches investigators have used to overexpress transporters, particularly those of the ABC family:

a) Overexpression of eukaryotic genes using eukaryotic systems.

To overexpress eukaryote genes, eukaryotic vectors have been preferred to those of *E. coli*. Some examples include: mouse *mdr1*, where the biological activity of cDNAs were tested after cloning into the expression vector p91023b (Azzaria *et al.*, 1989). This vector system had been used previously to express *mdr1* (Gros *et al.*, 1986) and carries the adenovirus major late promoter and simian virus 40 enhancer to direct high levels of expression of cloned cDNAs. Wild-type human P-glycoprotein (ABCB1, *MDR1* gene product), has been expressed with a C-terminal histidine tag in *S. cerevisiae* plasma membranes using the yeast expression plasmid YEpMDR1HIS (Al Shawi *et al.*, 2003). Similarly, studies of human MRP1used baby hamster kidney (BHK-21) cells transfected with pNUT-MRP/His (Chang *et al.*, 1997). The original vector (pNUT) also contains a mutant dihydrofolate reductase (DHFR) gene under the control of the SV40 early promoter, allowing for selection of transfectants with methotrexate

b) Overexpression of eukaryotic genes using *E. coli* cells

Examples include that of the carboxyl-terminal NBD from Chinese hamster P-gp. This was cloned and overexpressed in the vectors pT7-7 and pET22b, and purified to allow structure-function studies of the NBDs. The overall yield of purified protein was of 25-50 μg/L of cell culture (Sharma and Rose, 1995). Mdl1 from *S. cerevisiae* has also been studied in this way to investigate its ATP hydrolysis cycle and properties of its NBD (Janas *et al.*, 2003).

c) Overexpression of prokaryotic genes using related prokaryotic systems

Overexpression of genes from *E. coli* or related bacteria has usually been achieved with success. Examples include *E. coli* MsbA expressed in *E. coli* Novablue DE3 cells harboring a pET28b construct containing *msbA* (Reuter *et al.*, 2003); *V. cholera* MsbA, that was cloned into pET19b with a 23-residue fusion leader containing an N-terminal deca-histidine tag, and expressed in *E. coli* BL21(DE3) (Chang, 2003). In studies of the Mal transporter from *E. coli*, the components MalF, MalG, and MalK were overproduced simultaneously from two plasmids, pFG23, that carried *malF* and *malG* under control of the *trc* promoter, and pMRll, that carried *malK* under the same promoter (Davidson and Nikaido, 1991). These plasmids were transformed into a strain HN597 carrying a deletion of the $F_oF_1$-ATPase. Mal proteins were recovered from membrane preparations and transport activity was reconstituted in proteoliposome vesicles. The maltose transport complex $MalFGK_2$, modified with a polyhistidine tag at the N terminus of MalK, was also overexpressed in *E. coli* by Sharma and Davidson (Sharma and Davidson, 2000). The isolated MalK protein was purified from strain BL21(DE3) carrying the plasmid pMF8 with *malK* under control of a T7 promoter. Another example of a prokaryotic transporter gene expressed in a prokaryotic system is that of LmrA, which was overexpressed in *L. lactis* using nisin A-inducible vectors pNHLmrA, pNHLmrA-E314A, and pNHLmrA-E512Q (Balakrishnan *et al.*, 2004).

d) Overexpression of divergent prokaryotic genes in *E. coli*

Recombinant proteins have been expressed in *E. coli* with varying degrees of success, depending in many cases of the approach of the research. MJ1267 from *M. janaschii* was cloned into pET28a and expressed in *E. coli* BL21(DE3) for further purification and crystallisation (Karpowich *et al.*, 2001). For another extremophile, *glcV* from *Sulfolobus solfataricus* was inserted into the expression plasmid pET15b and its product shown to undergo dimer formation in the presence of Mg-ATP (Verdon *et al.*, 2003). Of direct relevance to the present study, DrrA and DrrB from *S. peucetius* were subcloned and expressed either individually or together (Kaur and Russell, 1998). In a previous study (Kaur, 1997), the *drrA* and *drrB* genes were modified at to introduce restriction sites at their start codons. This aided subcloning to place expression under the control of the *lac* promoter in pSU2718, a vector

previously used for expression of *arsA*. The *drrA* and *drrB* genes were also subcloned separately into pSU2718 at the *Nde*I-*Hin*dIII sites after PCR amplification of the genes.

Despite the attraction of exceptionally high transcription and translation, the literature has plenty of examples where overexpression of recombinant proteins from the T7 promoter has given rise to the formation of inclusion bodies. Given that some transporters genes placed under the control of the T7 promoter have been overexpressed with success (e.g. the SMR of EmrE (Ma and Chang, 2003), the NBDs of HisP (Nikaido *et al.*, 1997) MalK (Schmees *et al.*, 1999) and others), cloning of *drrA* under the control of this promoter in pET21a was an option worthy of exploration. It enabled very high levels of expression but almost all the recombinant protein was present in a fraction of insoluble protein, presumed to be inclusion bodies. No soluble DrrA was expressed at 37 °C whilst at 17 °C, some soluble protein was present and could be purified in low yield by IMAC. It is also worth noting that some DrrA appeared in the membrane fraction, but it was neither quantified nor was its ability to hydrolyse ATP assayed at this stage. One interpretation of these results is that the protein is less hydrophilic in character than indicated from SOSUI hydropathic analysis (not shown) and reported by other investigators (e.g. Guilfoile and Hutchinson, 1991; and Kaur, 1997). Utilisation of protein produced from pET21a would thus depend upon purification of DrrA under denaturing conditions to solubilise the protein from inclusion bodies (Chapter 5). In this phase of the project, the other strategy explored was to seek greater yields of soluble protein by expression in different plasmid vectors.

We wished to assay co-expression of DrrA with DrrB as an option to improve the production of DrrA in the cytosol, but co-expression only seemed to be successful using the pET33b vector, and it did not show any improvement in DrrA yield over that from independent expression in pET21a. Moreover, IMAC isolated many other proteins from the soluble fraction, making this option even less satisfactory. The other strategy employed was to fuse DrrA to another protein of high solubility.

Fusion to soluble proteins has allowed the overexpression of many proteins that previously formed inclusion bodies. NBDs such as mouse Pgp C-terminal NBD2 (Baubichon-Cortay *et al.*, 1994) and MRP1 (Cool *et al.*, 2002), both fused to Glutathione-*S*-transferase (GST), were recovered as soluble proteins. Similarly, NBDs such as those of Pgp (Sharma and Rose, 1995; Wang *et al.*, 1999; Berridge *et al.*, 2003), MRP1 (Cool *et al.*, 2002), and OpuAC (Horn *et al.*, 2005), have been fused to maltose–binding protein (MBP) and overexpressed as soluble proteins.

On the past decade, LaVallie *et al.* (1993) developed a promising *E. coli* expression system based on the use of *E. coli* thioredoxin as a gene fusion partner. Previously expressed as inclusion bodies, a variety of mammalian cytokines and growth factors could be expressed as soluble proteins to high levels when they were fused to Thioredoxin. Equally promising, fusion of Thioredoxin to the D-lactate dehydrogenase VanH, produced soluble, functional protein when other expression systems had completely failed. The intact fusion protein was used for kinetic studies and crystallization trials. Michaelis constants for NADPH, NADH, and pyruvate were derived for VanH and found to be comparable to those reported for the native dehydrogenase (Stoll *et al.*, 1998). The effect of Thioredoxin fusion on the production and folding of single chain Fv (scFv) antibodies in the cytoplasm of *E. coli* is another interesting example of protein fusion that resulted in high level expression and the retention of the correct folding pattern even in the absence of disulfide-bond isomerase Dsbc, an enzyme which has been shown to act as a chaperone for scFvs in the cytoplasm. When compared to MBP, Thioredoxin fusion was more effective in this regard. Another important feature of this Thio fusion was that antigen-binding assays showed that the scFv moiety maintained its affinity for the antigen, despite the presence of its fusion partner. It was concluded that Thio-scFv fusions could be used without removal of the Thioredoxin fusion partner and that the Thioredoxin acted largely as an intramolecular protein chaperone (Jurado *et al.*, 2006). An additional example where a Thioredoxin fusion has been important to overcome the formation of inclusion bodies is that of *S. aureus* gyrase A (Strahilevitz *et al.*, 2006). For this protein, the expression vector pBADTOPO thiofusion, was modified by the addition of DNA sequences encoding a hexahistidine tag upstream and a cleavage site for tobacco etch virus protease downstream of the gene for Thioredoxin. A high level

expression of soluble GyrA was achieved using this expression system in *E. coli* TOP 10 strain and GyrA was purified to over 95% homogeneity.

In this project, improvement in expression of soluble DrrA was achieved through fusion to Thioredoxin. What advantages prompted the use of Thioredoxin over other potential fusion partners? Thioredoxin is a low molecular weight protein (11.7 kDa) that is characterised by high solubility: It was hoped that this feature might help to maintain DrrA in solution under those conditions where the pET21a construct failed. If so, it was thought that the low size of Thioredoxin might not affect other properties of the fused protein under study. In addition, the pBADTOPO thioredoxin fusion vector provided tight regulation over expression through incorporation of the arabinose promoter. Although many studies have shown MBP to be effective in maintaining fusion partners in soluble form, its size (44 kDa) is significant and can be higher than the fused moiety. This can affect the intrinsic properties of the target protein, and makes necessary its elimination from the fusion complex. This can be done by cleave with proteases such as Factor Xa but this requires the introduction of a thrombin cleavage site to release MBP and assumes that the protease will not alter the protein of interest. At 29 kDa, Glutathione-*S*-transferase suffers similar limitations.

Studies using the pBADTOPO thiofusion system confirmed that higher yields of DrrA protein could indeed be obtained by fusion to Thioredoxin. Although yields of the DrrA fusion protein at 37 °C remained very poor, reduction below 21 °C provided more protein that was soluble, and suggested that characterisation studies with recombinant DrrA would be possible.

# Chapter 5

## Purification of DrrA

The purification of proteins over-expressed from the different vectors was a necessary step before proceeding to studies aimed at their characterisation. The production of these proteins with a tag such as the histidine repeat enabled the use of immobilised metal affinity chromatography (IMAC) for purification. Whilst these tags were present in several of the vectors used for the study, in other instances they were added to the PCR products to appear at the N- or C- termini of the protein. This feature provides a useful means to rapid and efficient purification.

### 5.1 Purification of DrrA under denaturing conditions

A common obstacle to the expression of recombinant proteins in foreign host cells is the accumulation of insoluble, aggregated protein in morphological structures called inclusion or refractile bodies. Several reasons can explain the origin of these elements: the target proteins may require specialised post-translational modification for full biological activity utilising enzyme systems that are lacking in the host organism used for expression. The natural conformation of the recombinant protein may not be able to form in the particular conditions prevailing in the host cell cytosol (e.g. when extracellular secreted proteins are expressed as intracellular recombinant proteins). In the natural producing organism, a variety of additional protein factors may be involved in assisting the protein to fold, and their level and specificity may differ in the expression host thereby affecting the refolding and solubility of the recombinant protein. Another important reason that may be responsible for this phenomenon is that the recombinant proteins may be produced at a rate and to a level that exceeds the limits of physiological solubility (Thatcher *et al.*, 1996).

Although the formation of inclusion bodies may not be desirable, typically the target protein comprises 70-90% of the structure and this represents a great advantage in the purification process, as the enclosed protein is concentrated. Given this, solubilisation and refolding need not yield large amounts of functional, recombinant protein to make possible other studies. Recovery of functionally active protein

commonly requires the solubilisation of inclusion bodies under strong denaturing conditions to effectively separate and unfold the aggregated protein, followed by refolding. This can be accomplished by dialysis or dilution of the denaturing agent.

### 5.1.1 Refolding of DrrA from inclusion bodies using an 8 M urea gradient

Solubilisation of inclusion bodies is usually carried out in 8 M urea or 6 M guanidine hydrochloride at alkaline pH and in the presence of a reducing agent. In the case of a-chymotrypsin, Hibbard and Tulinsky (Hibbard and Tulinsky, 1978) showed the effective penetration of the interior of the enzyme by urea, in contrast to guanidine hydrochloride which showed no significant changes in the protein interior. Urea bound within the interior of a protein is presumed to stabilise the partly folded protein chains (Guo and Clark, 2001). Thus, for the purpose of unfolding/refolding proteins in inclusion bodies, urea appears to be a suitable choice as it is expected to stabilize the unfolded intermediates and simultaneously help unfolding the insoluble protein bodies sufficiently to correct any misfolded structures. Moreover, urea is one of the denaturing agents most commonly employed in protein refolding processes, due to the fact that it is cheap, easily available, and can be easy removed without irreparable modification of the protein under investigation.

Work to this point established that the over-expression of DrrA always rendered the protein insoluble, and reducing the temperature and inducer (IPTG) concentrations below 37 °C and 1 mM, respectively, failed to raise the proportion of soluble recombinant protein. Since inclusion bodies containing DrrA were readily available, it was decided to attempt purification from this starting material. Conventional methods for refolding insoluble recombinant proteins include slow dialysis or dilution into a large volume of refolding buffer or chromatographic refolding using packed columns. Chromatographic methods can include solvent-exchange, size exclusion chromatography and immobilization of the denatured protein onto a column or gel matrix, with subsequent dilution of denaturant to promote refolding.

Examining reports in the literature, the advantages of on-column chemical refolding included: lack of dependence upon protein concentration, high yields of

soluble protein, simultaneous purification and refolding and amenability to high-throughput refolding. Furthermore, physical separation of the molecules, for example by immobilising them on an IMAC resin, is thought to reduce the possibility of unfavourable protein-protein interactions occurring during the refolding process that can lead to aggregation and eventual precipitation of the target protein. Therefore, refolding of DrrA from the inclusion bodies was attempted, using as starting material *E. coli* cells carrying the pET21a-*drrA* construct, grown at 37 °C and induced with 1 mM IPTG. Refolding was attempted on-column using a decreasing urea gradient (8-0 M).

The DrrA inclusion bodies were solubilised in Tris-HCl buffer with 8 M urea (see Methods sections 2.9.2 and 2.9.2.1), mixed with $Ni^{2+}$-NTA agarose resin to bind the histidine tag carried by the protein, and loaded into a glass chromatography column. The refolding process consisted of washing the protein-NiNTA resin complex in the column with decreasing concentrations of urea over an 8-0 M range. Finally, the protein was eluted from the column using a high concentration of imidazole (250-300 mM). The DrrA protein appeared in all eluted fractions and showed good purity on gel analysis (Fig. 5.1). After dialysis of the eluted purified material, DrrA fluorescence changes were initially monitored in the absence and presence of ATP-$Mg^{2+}$ as a means to evaluate the success of the refolding process. This analysis failed to show that the changes detected on the protein fluorescence were genuinely caused by the interaction between DrrA and the ATP-$Mg^{21}$ complex (see Chapter 7 section 7.1). Therefore, this methodology was replaced by an ATPase activity assay using the Molecular Probes EnzCheck phosphate assay.

As the EnzCheck phosphate assay (up to 150 μM of Pi detected) did not reveal any ATPase activity from the putatively refolded DrrA, further refolding experiments were devised to assess the ATPase activity of DrrA with a method that was more sensitive (1 to 30 _M; 50 to 1500 picomoles of $P_i$) and easier to set up, the Malachite green assay (Harder *et al.*, 1994).

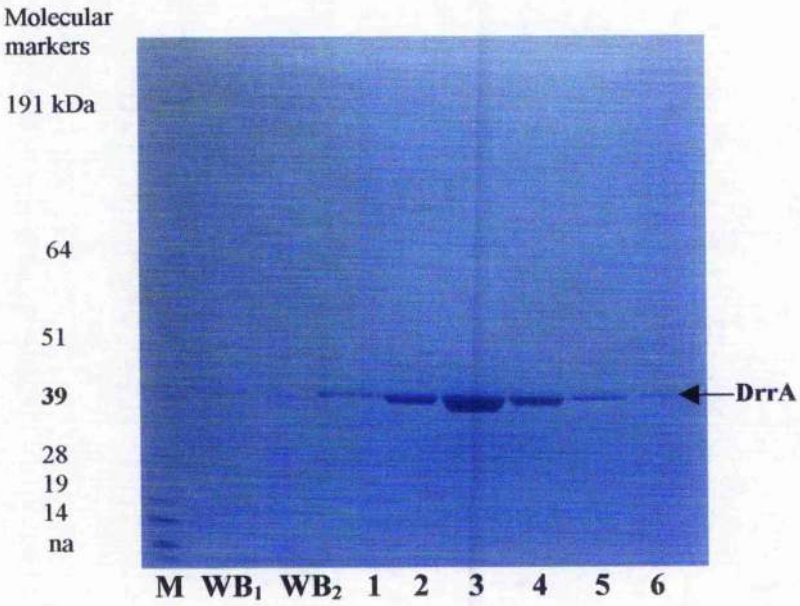



**Figure 5.1 Purification of DrrA after urea gradient treatment on an IMAC column**

Bacteria carrying pET21a-*drrA* were grown at 37 °C to exponential phase and induced with 1mM IPTG for 4 hours. 200 µg of insoluble protein ("inclusion bodies") was solubilised in 8 M urea and loaded to the IMAC column. After progressive reduction in the urea concentration and washing, protein was eluted with imidazole. Samples were analysed by SDS PAGE and Coomasie blue staining.

M: Protein molecular markers

WB (1-2): Wash fractions using 25 and 50 mM imidazole respectively

1-6: Eluted fractions (300 mM)

A protein consistent with the predicted size of DrrA is indicated

Again, inclusion bodies from the disrupted cell pellet were solubilised in 8 M urea and the process of binding to NiNTA and reduction in the concentration of urea was repeated (section 2.9.2). The six fractions eluted from the IMAC column (0.5 ml each) contained DrrA protein, and fractions 3, 4 and 5, which had the highest protein concentrations, were pooled, dialysed and tested for ATP-ase activity using Malachite green. The kinetics of ATPase is typically followed for 15 minutes, but in this instance, the reaction was monitored for 1 hour to allow complete ATP hydrolysis. Again, the release of inorganic phosphate by the "refolded" DrrA could not be detected.

This experiment was repeated with 2-6 fold more starting material (up to 3 ml of disrupted cell pellet paste) solubilised in 8 M Urea, to increase the chances of detection of even small amounts of "refolded" protein in the ATP-ase assay. Although it was possible to visualise intensely-staining bands corresponding to DrrA on polyacrylamide gels, there were no signs of ATP-ase activity using the Malachite green assay. These results confirmed that the solubilisation of DrrA from inclusion bodies using urea and on-column removal of the denaturant did not yield appropriately folded DrrA protein.

### 5.1.2 Assay with a refolding kit

The Hampton Research FoldIt Screen allows researchers to determine with a reasonable degree of confidence if a protein of interest can be folded from inclusion bodies. Sixteen different refolding formulations are arranged, and assayed to find the best conditions for proper refolding of proteins; these were assayed for the DrrA protein (Fig. 5.2 and 5.3) in another attempt to try to obtain active protein for characterisation. This system allows convenient variation of multiple variables (Tables 2.2 and 2.3 in Chapter 2) that include the type and concentration of buffer, salt, presence and absence of denaturing agent, type of cation chelator, polar and non-polar additives, presence and type of detergent, reducing agents, ligand addition, and protein concentration. Reagents to enable protein refolding should favour the formation of the native fold and minimise the aggregation of folding intermediates. Reagents such as polar additives (arginine), osmolytes (polyethylene glycol), detergents (lauryl maltoside), and chaotropes (guanidine hydrochloride) can minimise

aggregation and increase the yield of properly folded protein (FoldIt Screen Hampton Research manual).

Inclusion bodies were solubilised with 6 M guanidine hydrochloride then diluted into the test conditions in order to reduce the denaturant concentration. Refolding was assayed with protein alone, and in the presence of doxorubicin (Fig. 5.2 and 5.3). A summary of the refolding parameters tested is presented in Table 5.1.

To measure the success of refolding, ATPase activity was assayed using the Molecular Probes EnzCheck phosphate method. Although DrrA protein samples did not show signs of precipitation in the dialysis tubes under several sets of conditions (Fig. 5.3), none showed any ATPase activity. Binding of the ATP analogue MANT-ATP in the presence and absence of doxorubicin was also tested, but there were no fluorescence changes that would suggest some interaction between the protein and the ligands.

As attempts to refold DrrA from inclusion bodies were unsuccessful, alternative procedures were considered to express the protein in an active form and in sufficient quantities to proceed with further characterisation.

**Table 5.1 Hampton Research Foldlt Screen refolding parameters tested for DrrA**

| | |
|---|---|
| Protein concentration | 0.1 mg/ml vs. 1.0 mg/ml |
| Presence of polar additive | +/- 550 mM L-Arginine |
| Presence of detergent | +/- 30 mM Lauryl Maltoside |
| pH | pH 6.5 vs. pH 8.2 |
| RedOx potential | 100 mM DTT vs. 100 mM GSH |
| Presence of chaotropic salt | +/- 550 mM Guanidine Hydrochloride |
| Ionic strength | 264 mM NaCl +11 mM KCl vs. 10.56 mM NaCl + 0.44 mM KCl |
| Presence of cations or chelator | 2.2 mM CaCl + 2.2 mM MgCl vs. 1.1 mM EDTA |
| Presence of osmolyte | +/- 0.055% (w/v) PEG 3350 |
| Presence of non-polar additive | +/- 440 mM Sucrose |
| Presence of ligand | +/- 30 µg/ml doxorubicin |

The detailed formulation is presented in the Methods section 2.9.2 in Chapter 2

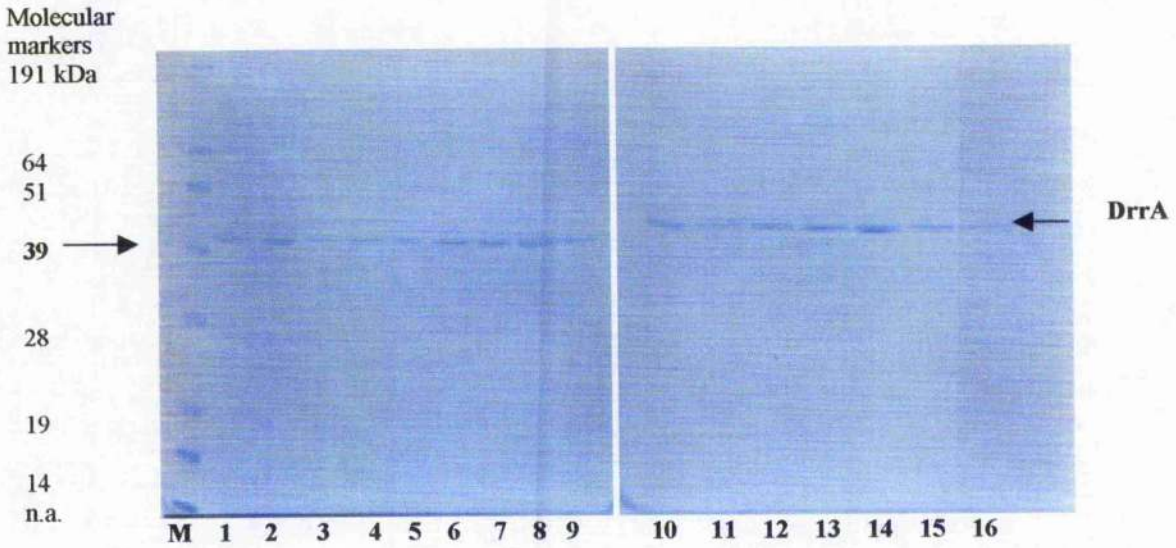



**Figure 5.2 DrrA after treatment with 16 FoldtIt Screen refolding formulations (FOLDLT SCREEN from Hampton Research)**

Bacteria carrying pET21a-*drrA* were grown at 37 °C to exponential phase and induced with 1 mM IPTG for 4 hours. Insoluble protein ("inclusion bodies") was solubilised in 6 M Guanidine-HCl at 0.1 mg/ml protein concentration according to the FoldtIt Screen refolding formulations from Tables 2.5 and 2.6 presented in the Methods section 2.9.2.2 and Table 5.1. After incubation for 7 hours, samples were dialysed in 20 mM Tris buffer. Samples were analysed by SDS PAGE and Coomasie blue staining.

M: Protein molecular markers

1-16: DrrA from FoldtIt Screen refolding formulations 1-16

A protein consistent with the predicted size of DrrA is indicated

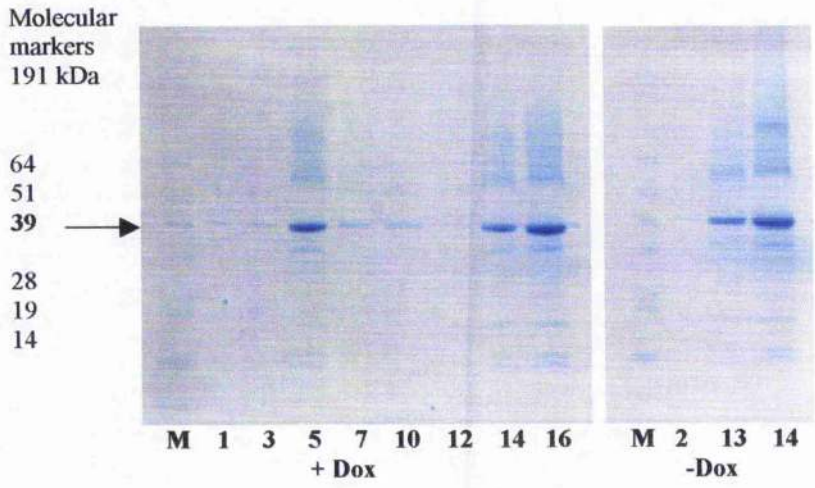



**Figure 5.3 DrrA after treatment with different FoldtIt Screen refolding formulations (FOLDLT SCREEN from Hampton Research) in presence and absence of doxorubicin**

Bacteria carrying pET21a-*drrA* were grown at 37 °C to exponential phase and induced with 1 mM IPTG for 4 hours. Insoluble protein ("inclusion bodies") was solubilised in 6 M Guanidine-HCl at 0.1 mg/ml protein concentration except in samples number 5, 13, 14 and 16, where 1 mg/ml of protein was used. After incubation for 7 hours, samples were dialysed in 20 mM Tris buffer. Samples were analysed by SDS PAGE and GelCode blue staining.

M: Protein molecular markers

1, 3, 5, 7, 10, 12, 14, and 16: DrrA from the same numbered FoldtIt Screen refolding formulations in presence of ligand doxorubicin

2, 3, and 14: DrrA from the same numbered FoldtIt Screen refolding formulations in absence of ligand doxorubicin

A protein consistent with the predicted size of DrrA is indicated

### 5.2 Native purification of DrrA and Thio-DrrA

Initially phosphate buffer had been used to solubilise DrrA from extracts of *E. coli* cells, but Tris-HCl buffer pH 8.0 was preferred in the experiments that followed. By using Tris buffers, phosphate was excluded and the sample was better suited for assays of ATPase activity. A buffer containing 50 mM Tris-HCl, 150 mM NaCl, and 10% glycerol, adjusted to pH 8.0, seemed to be adequate for primary DrrA or Thio-DrrA protein purification.

Soluble extracts were prepared by ultracentrifugation to remove inclusion bodies and membrane components. The soluble fraction was divided into batches (1/2-3 L culture), mixed with $Ni^{2+}$ agarose resin, incubated for 1 h and loaded onto an Econo glass column. After washing, the protein was eluted with 300 mM imidazole, and purified DrrA or Thio-DrrA was obtained under these conditions.

The eluted fractions that had the highest concentrations of protein, as determined from gel analysis, were pooled. This represented the total yield of soluble DrrA or Thio-DrrA obtained from the original culture (usually 10 L). Generally, these were the first two fractions eluted off the column. Some other bands with different sizes also appeared on the gel. Dialysis was necessary to remove the high concentration of imidazole used for elution, as it interferes with protein determination by the BCA method. Usually, four hours of dialysis in a volume of 3 L, was enough for pooled sample of less than 10 ml volume. If necessary, fractions were concentrated using an Amicon Centricon A10 centrifugal concentrator, or Stirred Ultrafiltration Cell that seemed to reduce low molecular weight contaminants as well. In the last case Millipore filter membranes with a cut off separation range of 30 and 10 kDa were used for Thio-DrrA (approximate size of 52 kDa) and DrrA (approximate size of 39 kDa), respectively.

By including imidazole at concentrations of up to 50 mM in the wash buffers, the purification protocol yielded preparations of DrrA and Thio-DrrA of sufficient purity, as judged from gels by direct visualisation. However, some additional bands were present and further trials were attempted to achieve 95% purity, and also increase protein yields by about 20%.

### 5.3 Trials for improved purification of Thio-DrrA using IMAC

Some changes in the concentration and/or the composition of the purification buffer were tested. The Tris buffer composition was varied to try and reduce the presence of contaminant bands in the protein preparation, and if possible, to also improve the yield of the Thio-DrrA protein. The addition of a reducing agent (2-mercaptoethanol) and detergent (dodecyl maltoside) was tested along with increases in glycerol and salt concentrations in the buffer. None of these changes produced an improvement in the purification of Thio-DrrA without detriment to the protein yield (Fig. 5.4 and 5.5). A buffer containing 50 mM Tris-HCl, 150 mM NaCl, 10% glycerol and 10 mM imidazole, pH 8.0, was retained as the basic composition of purification buffers.

The other approach to increase purity was to employ a lower concentration of inducer for the Thio-DrrA expression. The arabinose concentration was reduced 10-fold, from 0.002 % to 0.0002 % (Fig. 5.6). The protein produced and purified under these new conditions showed fewer contaminating proteins in the higher molecular weight range, but it was also much less concentrated than protein induced with 0.002% arabinose, reducing the protein yield. It is probable that the partial disappearance of upper bands on the gel was caused by the low protein concentration that was found in that preparation, when compared to induction with 0.002% arabinose.

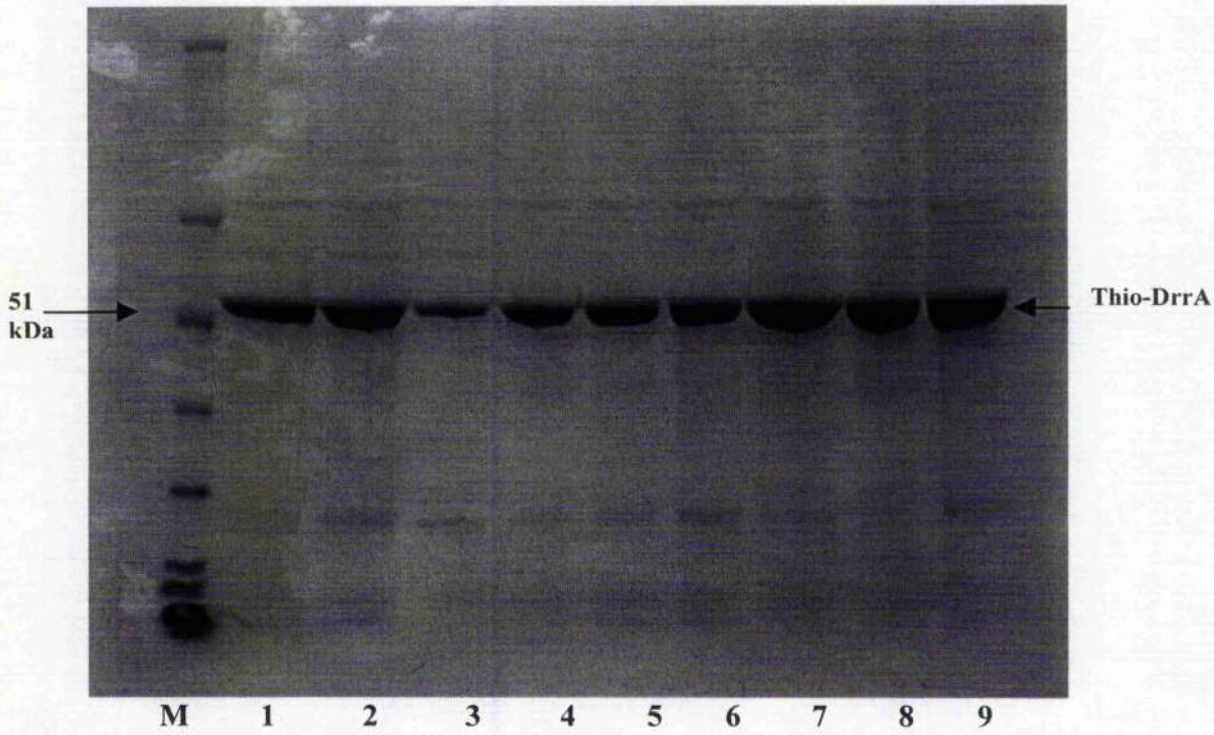



**Figure 5.4 IMAC purification of Thio-DrrA under different Tris-HCl buffer conditions: presence of DDM and 2-Mercaptoethanol**

Cultures of *E. coli* LMG 194 carrying pBADTOPO *thio-drrA* were grown at 37 °C to exponential phase, shifted to 17 °C and treated with 0.002% arabinose overnight. Cells were fractionated and recombinant protein purified from soluble cytoplasmic extracts by IMAC. Buffer composition was 50 mM Tris-HCl pH 8.0, 150 mM NaCl and 10% glycerol. Additionally, elution buffer was 300 mM imidazole. Samples were analysed by SDS PAGE and Coomasie blue staining.

M: Protein molecular weight markers

1-3: First three fractions from sample solubilised in buffer containing 10 mM 2-Mercaptoethanol (ME) eluted from the IMAC column

4-6: First three fractions from sample solubilised in buffer containing 0.05% Dodecyl-maltoside (DDM) eluted from the IMAC column

7-9: First three fractions from samples solubilised in control 50 mM Tris-HCl buffer 150 mM NaCl and 10% glycerol

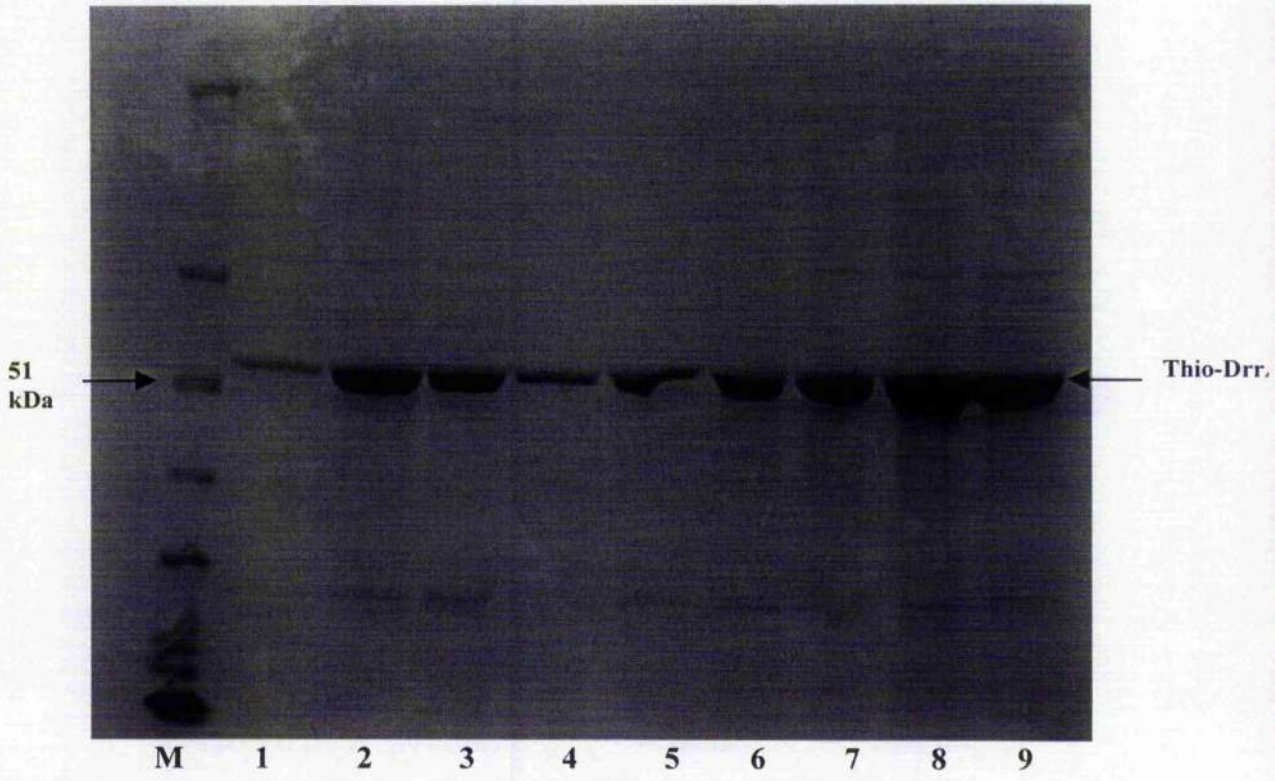



**Figure 5.5 IMAC purification of Thio-DrrA under different Tris-HCl buffer conditions: 20% of glycerol and 400 mM NaCl**

Cultures of *E. coli* LMG 194 carrying pBADTOPO *thio-drrA* were grown at 37 °C to exponential phase, shifted to 17 °C and treated with 0.002% arabinose overnight. Cells were fractionated and recombinant protein purified from soluble cytoplasmic extracts by IMAC. Buffer composition was 50 mM Tris-HCl pH 8.0, 150 mM NaCl and 10% glycerol. Additionally, elution buffer was 300 mM imidazole. Samples were analysed by SDS PAGE and Coomasie blue staining.

M: Protein molecular weight markers

1-3: First three fractions from sample solubilised in buffer containing 20% glycerol eluted from the IMAC column

4-6: First three fractions from sample solubilised in buffer containing 400 mM NaCl eluted from the IMAC column

7-9: First three fractions from samples solubilised in control 50 mM Tris-HCl buffer 150 mM NaCl and 10% glycerol

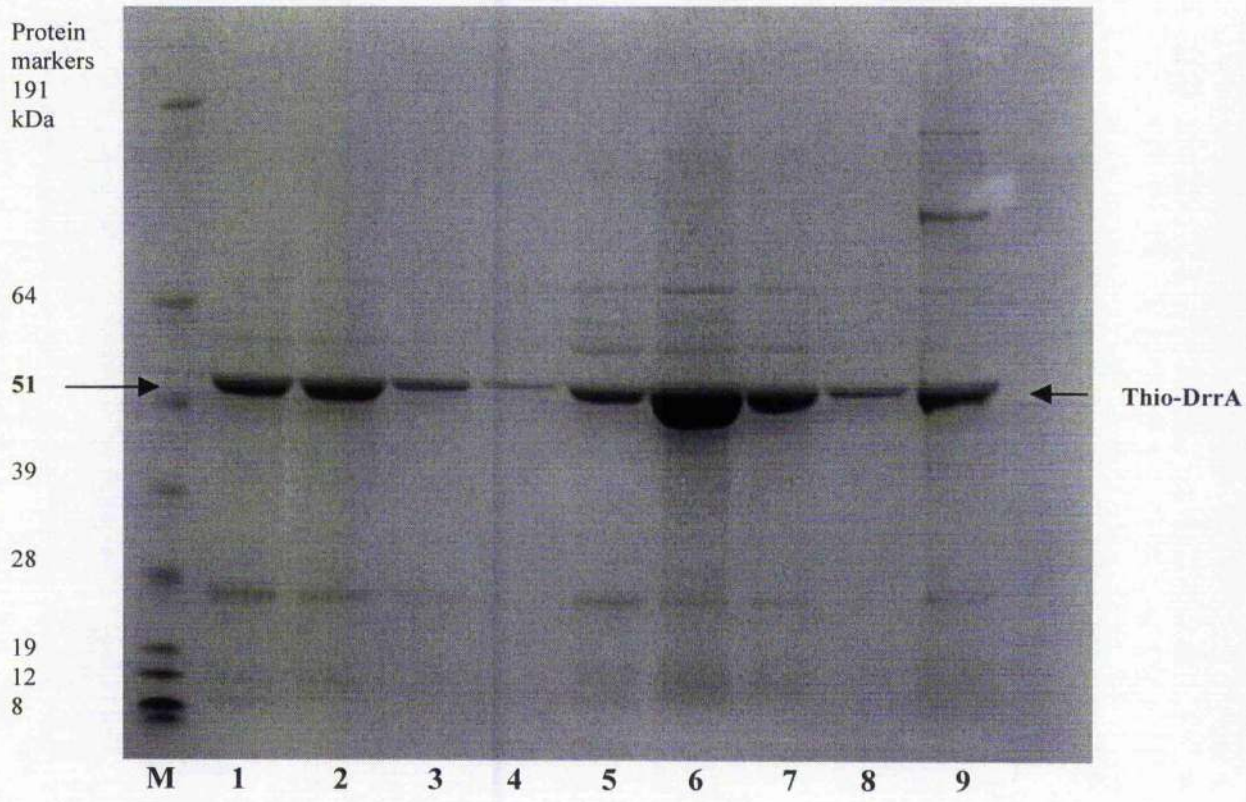



**Figure 5.6 Expression of Thio-DrrA after induction with 0.0002 and 0.002% arabinose**

Cultures of *E. coli* LMG 194 carrying pBADTOPO *thio-drrA* were grown at 37 °C to exponential phase, shifted to 17 °C and treated with 0.002% or 0.0002% arabinose overnight. Cells were fractionated and recombinant protein purified from soluble cytoplasmic extracts by IMAC. Buffer composition was 50 mM Tris-HCl pH 8.0, 150 mM NaCl and 10% glycerol. Additionally, elution buffer was 300 mM imidazole. Samples were analysed by SDS PAGE and Coomasie blue staining.

M: Protein molecular weight markers

1-4: First four fractions containing Thio-DrrA from cultures induced with 0.0002% arabinose, eluted from the IMAC column

5-8: First four fractions containing Thio-DrrA from cultures induced with 0.002% arabinose, eluted from the IMAC column

9: Control Thio-DrrA dialised

## 5.4 Purification of DrrA from the membrane fraction

In a 10 L trial with pET21a-*drrA*, the soluble and the membrane fractions were purified and checked for the presence of DrrA. Although in the past some DrrA could be detected by Western blot in the membrane fraction, its yield was low. When the induction temperature was reduced to 16 °C, an appreciable quantity of DrrA protein partitioned to the membrane fraction suggesting that under these conditions, higher amounts of DrrA might be obtained from membrane fractions. The level of over-expression can be estimated when samples from soluble and membrane fractions from the same culture are compared (Fig. 5.7 and 5.8). A yield of 65 μg/L was found for DrrA from the membrane fraction, against 40 μg/L found for DrrA from the soluble fraction in the same experiment.

When DrrA isolated from membrane fractions failed to show any ATPase activity, it was thought that the high amount of detergent used in the solubilisation of the membrane and in the purification protocol might have affected the integrity of the protein. Reducing the concentration of the detergent DDM from 2 to 1 % for solubilisation, and to 0.05 % for purification, failed to recover active DrrA.

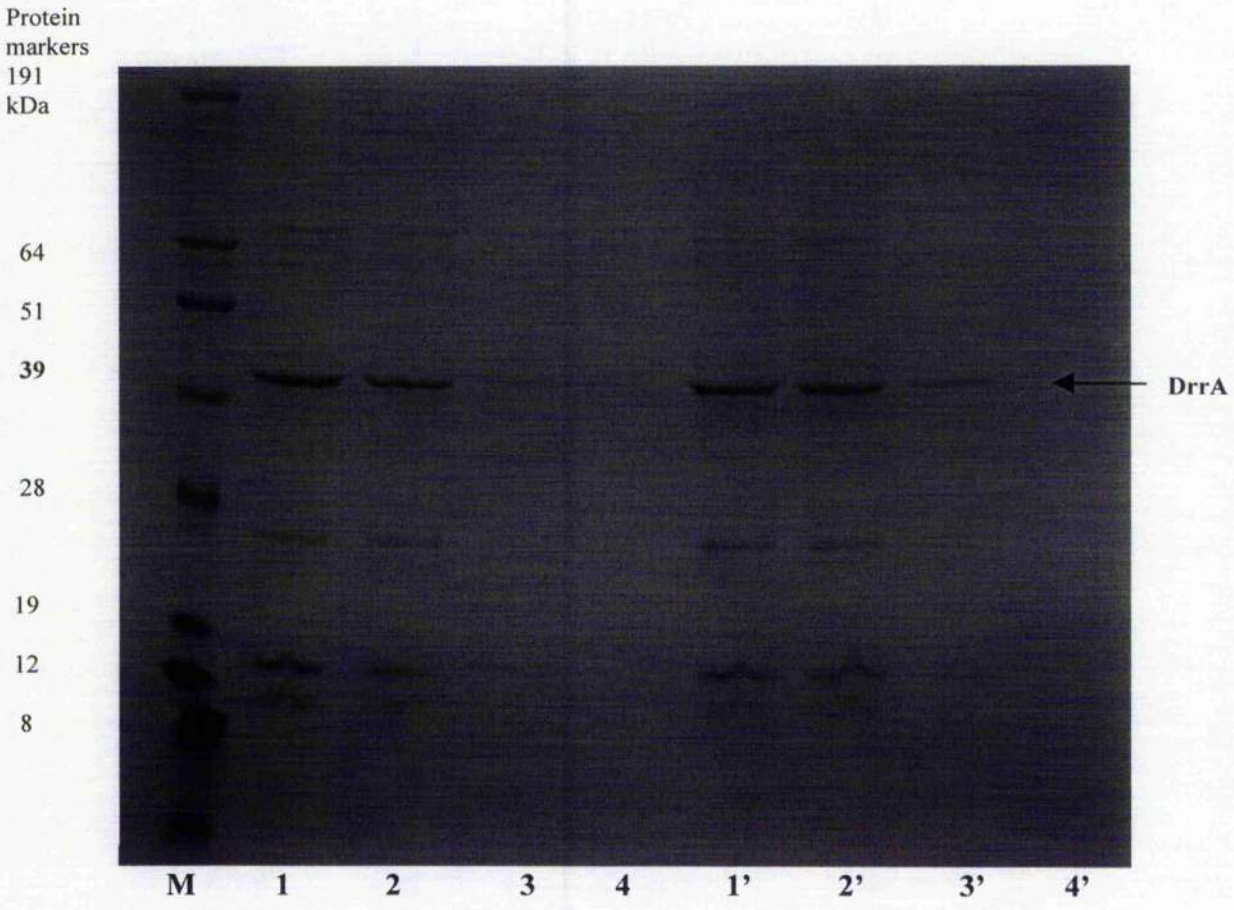



**Figure 5.7 Overexpression of soluble DrrA from 10 L of media in four batches of 2.5 L**

Bacteria carrying the pET21a expression construct were grown at 37 °C to exponential phase, shifted to 17 °C and induced with 0.2 mM IPTG overnight. Cells were fractionated and recombinant protein purified from soluble cytoplasmic extracts by IMAC. Samples were analysed by SDS PAGE and Coomasie blue staining.

M: Protein molecular markers

1-4: Fractions of the first purification batch (corresponding to 2.5 L of culture) eluted from the IMAC column

1'-4': Fractions of the second purification batch (corresponding to 2.5 L of culture) eluted from the IMAC column

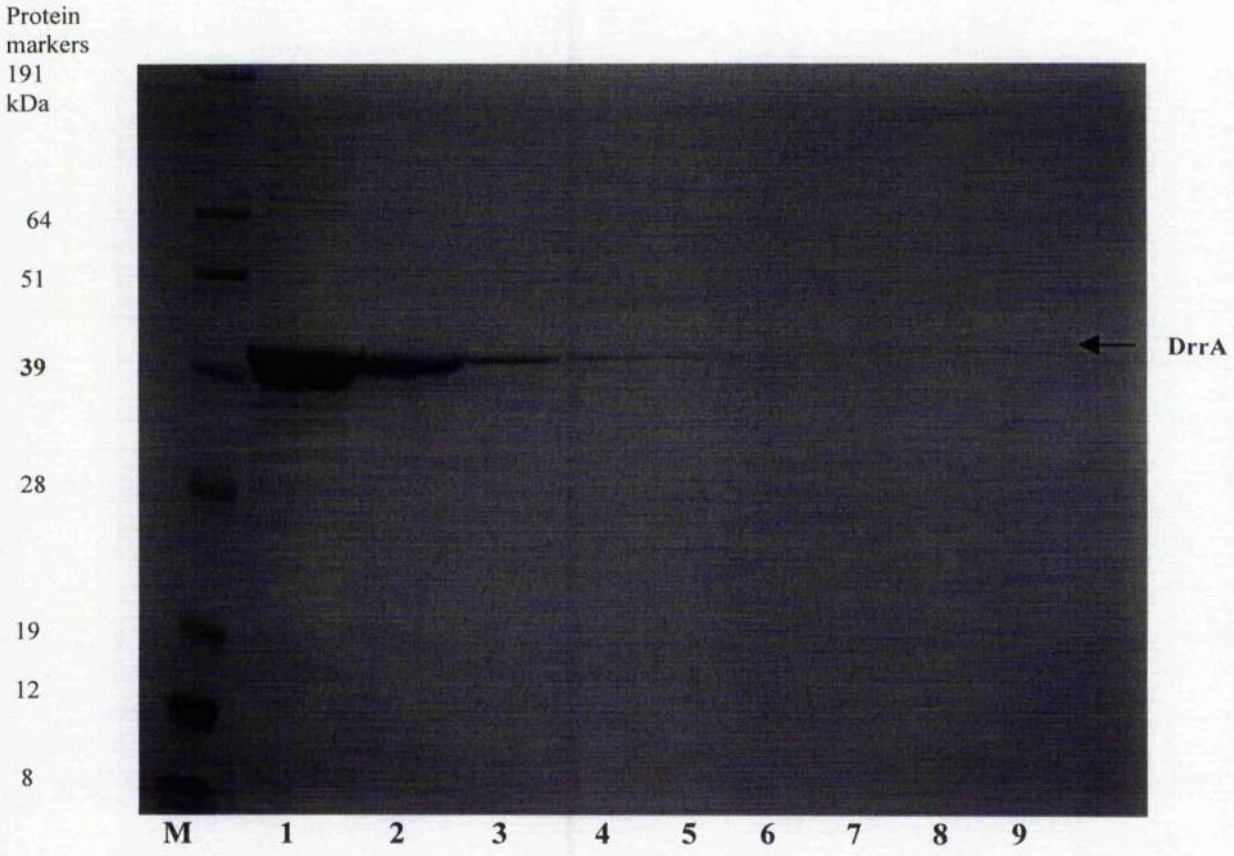



**Figure 5.8 Overexpression of DrrA from 10 L of media and its purification from the membrane fraction**

Bacteria carrying the pET21a expression construct were grown at 37 °C to exponential phase, shifted to 17 °C and induced with 0.2 mM IPTG overnight. Cells were fractionated and recombinant protein purified from membrane extract by IMAC. Solubilisation buffer for the DrrA membrane fraction was 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% glycerol and 10 mM Imidazole plus 2% DDM. Washing buffers were the same Tris buffer with 0.2% DDM. Elution buffer was 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% glycerol and 300 mM Imidazole plus 0.2% DDM. Samples were analysed by SDS PAGE and Coomasie blue staining.

M: Protein molecular markers

1-9: Fractions of the membrane purification (corresponding to 10 L of culture) eluted from the IMAC column

## 5.5 Discussion

The use of a histidine tag in the different constructs allowed the purification of the different forms of DrrA in a few stages. Immobilised metal affinity chromatography (IMAC) was effective even when material from inclusion bodies was used. The insoluble cytoplasmic fraction formed the richest source of DrrA from all the vectors when cells were cultivated at 37 °C.

Solubilisation of inclusion bodies that contain recombinant proteins and refolding appeared an option worth investigating, although the efficiency of refolding was uncertain at best. Strong denaturing conditions such as the use of high concentrations of chaothropic agents like urea or guanidine hydrochloride in alkaline conditions are necessary to effectively separate and unfold the aggregated protein present in inclusion bodies. The use of 8 M urea for denaturation and refolding of recombinant proteins has been reported by a number of authors. Zhang (Zhang *et al.*, 2005) reported studies in which pro-carboxypeptidase was successively refolded using urea gradient gel filtration. Further chromatography resulted in an enzyme preparation that was active and 90% pure. Zhang *et al.* (Zhang *et al.*, 1998) worked with urea-denatured adenylate kinase and monitored the formation of secondary structure, changes in surface hydrophobicity, and the recovery of catalytic activity during a refolding protocol. The outer membrane protein and ferric enterobactin receptor FepA from *E. coli* has also been successfully refolded from solubilised inclusion bodies using a combination of sulfobetaine 3-14 and sodium dodecylsulfate, prior to purification by using anion exchange and gel filtration chromatography (Buchanan, 1999). Finally, Liu *et al.* recently described the refolding of urea-denatured transglutaminase from *S. fradiae* expressed from a pET21a vector using an on-column refolding procedure (Liu *et al.*, 2006). Using a similar approach, the solubilisation of DrrA in 8 M urea, and its gradual refolding was investigated to try and make best use of the target protein from inclusion bodies. However, these attempts to refold DrrA as an active protein were not successful.

Denaturing conditions generated with guanidine hydrochloride have also been reported for the refolding of diverse recombinant proteins with varying degrees of success. Rehm *et al.* have described the purification of class II polyhydroxyalkanoate

(PHA) synthase from *P. aeruginosa* (Rehm *et* al., 2001). The enzyme was overexpressed from the strong _10 promoter in *E. coli* BL21(DE3), leading to the formation of inactive inclusion bodies, comprising approximately 30% of total cellular protein. Inclusion bodies were dissolved and denatured with 6 M guanidine hydrochloride, and after the protein was immobilized on a $Ni^{2+}$-nitrilotriacetate–agarose matrix, it was refolded by gradual removal of the denaturing reagent. Refolded PHA synthase showed a specific enzyme activity corresponding to 27% of the maximum specific activity of the native enzyme; Lee *et* al., 2002, expressed a single-chain antibody derived from a murine monoclonal specific for human apolipoprotein B 100 in *E. coli* from a T7 promoter. Refolding was achieved through slow dilution into refolding buffer, and the soluble scFv was purified by affinity chromatography to obtain an active fraction with antigen-binding activity comparable with that of native Fab.

When generic refolding procedures are being developed, several arrays are available that aid the identification of appropriate refolding conditions. Among them, the FoldtIt screen from Hampton Research has been developed from reports by Armstrong *et al.*, (1999), Chen and Gouaux, (1997), and Rudolph and Lilly, (1996). This resource allowed convenient testing of 16 different folding conditions, and additional trials were assayed to seek properly folded DrrA from inclusion bodies. In spite of the diverse the conditions tested, none enabled the recovery of active DrrA, leaving the perception that refolding conditions would prove elusive, limiting the value of this purification strategy.

The small quantities of DrrA that were expressed finally as soluble protein from the pET21a construct, were active in hydrolysing ATP (see next chapter). These results did not support Kaur's thesis that the catalytic component DrrA takes on an active conformation able to bind and/or hydrolyse ATP only when it is in complex with DrrB. Kaur and Russell (1998) showed in UV cross-linking studies with [_-$^{32}$P]ATP that only the membrane-bound form of DrrA in cells containing both DrrA and DrrB was in a conformation competent to bind ATP (Kaur, 1997; Kaur and Russell, 1998). Nonetheless, it is worth noting that Kaur's work was done in cell extracts, whilst in the experiments reported here, DrrA came from IMAC purification. As there are several examples in the literature of the binding and/or hydrolysis of ATP

by isolated NBDs (HisP, Nikaido and Ames (1999); MalK, Walter *et al.*, (1992); MJ0796, from *M. jannaschii*, Moody *et al.*, 2002), the observation that purified DrrA shared these properties is not implausible.

The partitioning of DrrA into the membrane fraction at low induction temperatures might be responsible in part for the reduced DrrA concentration in the soluble fraction. This effect suggests the existence of a greater degree of hydrophobic characteristics in the protein than was expected. However, DrrA obtained from this source did not show any ATPase activity, even when milder detergent conditions were employed in purification. This result is not in contradiction with Kaur's proposal with respect to the functional conformation of membrane-bound DrrA. However, isolated bound-membrane MalK could be overexpressed without its cognate Mal transport membrane proteins and recovered from inclusion bodies displaying ATPase activity (Walter *et al.*, 1992).

The Thio-DrrA fusion protein became the main source of DrrA protein for the project, as protein could be expressed and purified to an acceptable degree and with sufficient yield for further characterisation studies. The yield of protein obtained from the pBAD construct could be improved to a modest extent and it was employed for the different characterisation trials that are presented in the following chapter. Efforts to improve the degree of purification of DrrA and obtain more rigorously purified Thio-DrrA protein were not successful and essentially, any slight improvements in purity were at the expense of the protein yield.

# Chapter 6

## Characterisation of DrrA proteins

The characterisation of DrrA was one of the main goals of this work. Given its classification as a member of the ABC family, it was intended to test if DrrA could drive the efflux of the anthracyclines compounds daunorubicin and doxorubicin, via the DrrAB system from *S. peucetius*. Having established conditions for the expression and purification of soluble Thio-DrrA, the fusion protein was chosen for the analysis of biochemical properties. The low levels of soluble DrrA expressed from the pET21a vector created an obstacle to routine analysis of this form of the protein.

The characterisation of other ABC transporter NBDs has given rise to a wealth of experimental data on their biochemical properties. The study of P-glycoprotein, and other medically important ABC transporters like MRP and CFTR, has enhanced the research on other ABC transporters to reveal the essential structural and biochemical features of the transport process. This is of particular interest with regard to the phenomenon of drug resistance, since an understanding of transport could provide better approaches to the design of drugs or treatments to combat it.

## 6.1 Characterisation of the ATPase activity of DrrA proteins.

All ABC transporter NBDs identified to date have the ability to bind and hydrolyse ATP and, using the energy released by the phosphate bond, to drive transport processes. The role of DrrA as an ATPase to direct the extrusion of the self-produced anti-tumour compounds daunorubicin and doxorubicin out of the cells of *S. peucetius* was indicated in the works of Guilfoile and Hutchinson (1991), as also its similarity to P-glycoprotein, involved in resistance to anti-cancer drugs. One aim was therefore to characterise the ATPase activity of DrrA employing the Thio-DrrA fusion protein as representative of NBD.

### 6.1.1 ATPase activity

To assess the ATPase activity of Thio-DrrA the protocol of Harder and collaborators (1994), based on the malachite green assay, was employed with some minor modifications (Nash, 2003; Maehatma, 2000). The assays, performed in 96 well microtitre plates, displayed linearity over a range of $P_i$ concentrations between 50 and 4000 pmoles. Standard phosphate curves were prepared in order to quantify the amount of $P_i$ released for each batch of reagent. The reagent was stable when stored at 4 °C; at room temperature, with ageing, some precipitation and colour differences were found. Data generated for a typical standard curve can be seen in Figure 6.1.

The ATPase activity is a process dependent on the presence of divalent cations, in particular magnesium although other divalent cations have been shown to stimulate this activity to varying degrees. Total volumes of 500 µl were used in the reaction, in which the test protein was pre-incubated in the presence of ATP for five minutes at 37 °C; additional components were added after this pre-incubation stage. The addition of $Mg^{2+}$ was used to initiate the reaction and 45 µl of sample were removed from the mix at 5 minutes intervals over a fifteen-minute time course reaction, and transferred to the wells of a microtitre plate well containing 5 µl of EDTA. The role of EDTA is to sequester $Mg^{2+}$ and in consequence, stop the reaction. Having collected the required samples, 100 µl of malachite detection reagent was added to each sample and the results were collected immediately by measuring the absorbance at 610 nm. Although different authors (e.g. Mahatma *et al.*, 2000) recommend waiting some time to allow colour development (15-30 minutes), the strong acidic conditions of the reagent causes the ATP in the samples to be gradually hydrolysed in the absence of enzymatic activity. This can mean that samples taken at zero time or controls that lack magnesium can also show development of green colour.

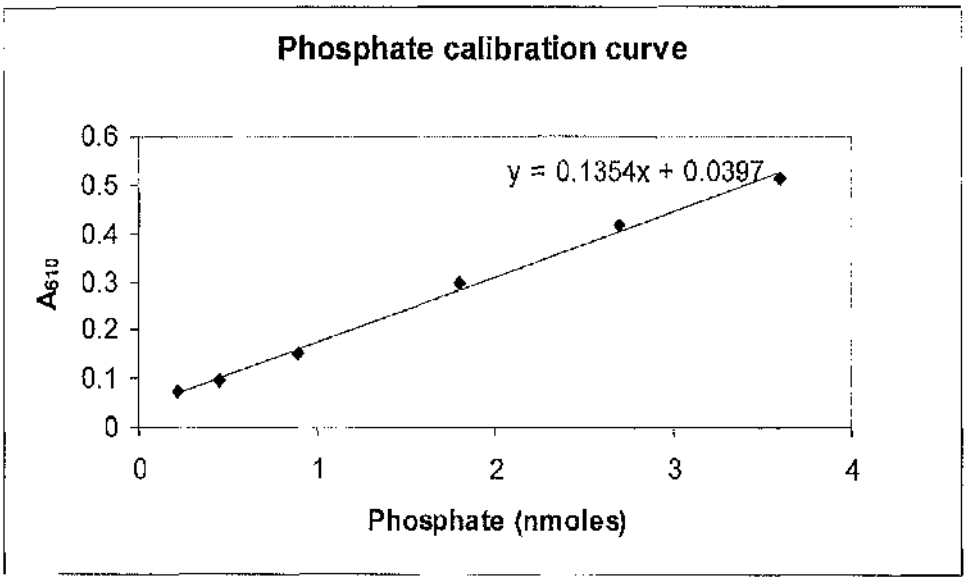



**Figure 6.1 Standard curve to determine inorganic phosphate concentration by the Malachite green assay.**

The standard curve above shows the response of the malachite green $P_i$ assay over a range of 50 to 3500 picomoles of $P_i$.

Samples containing different amounts of phosphate were prepared by dilution of a 1 mM $Na_2HPO_4$ stock into Tris assay buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8.0). An aliquot of 45 µl of each standard was mixed with 5 µl of 500 mM EDTA in separate wells of a 96-well microtitre plate. The assay was performed by adding 100 µl of Malachite green detection reagent to each well and measuring the absorbance of samples at 610 nm in a plate reader.

The curve showed linearity up to 3.5 nanomoles of $P_i$

In initial experiments to measure ATPase activity, Thio-DrrA was used at a concentration of 50 μg/ml following assays set up with *M. tuberculosis* Trx-DrrA (Nash, 2003). This concentration corresponds to approximately 1 μM fusion protein. It was found that a 15-minute reaction only produced an incipient light green colour. The reaction time was extended to 60 minutes in order to allow enough time for enzymatic hydrolysis of ATP (monitored as green colour development). DrrA fused to Thioredoxin, at a protein concentration of 50μg/ml, showed a 15-minute lag in activity after initiation of the reaction by the addition of $Mg^{2+}$ and incubation for 60 minutes. This delay could not be completely reversed by doubling the Thio-DrrA concentration to 2 μM and therefore it was necessary to use a higher concentration of protein. This resulted in an $A_{610nm}$ of 0.34 in a reaction time of 15 minutes, without the aforementioned delay. Thus, in the following experiments, reactions were run for 15 minutes but with higher protein concentrations that in the first experiments.

A Thio-DrrA concentration of 120 μg/ml (~2.5 μM) was usually sufficient to follow the development of colour in the reaction with Malachite green over a 15 minute reaction time. Routine experiments were carried out using final concentrations of 450 μM $Mg^{2+}$ and 250 μM ATP. Figure 6.2 shows a standard ATPase activity assay for Thio-DrrA, stimulated by $Mg^{2+}$, and results gathered in the absence of $Mg^{2+}$. As clearly seen in the Figure, ATPase activity could be demonstrated for Thio-DrrA by the release of $P_i$.

ATPase activity could also be detected for the DrrA protein, expressed from the pET21a-*drrA* construct. Here, 1 μM DrrA, corresponding to a concentration of 37 μg/ml, was used, giving an $A_{610nm}$ of 0.35 after 15 minutes of reaction.

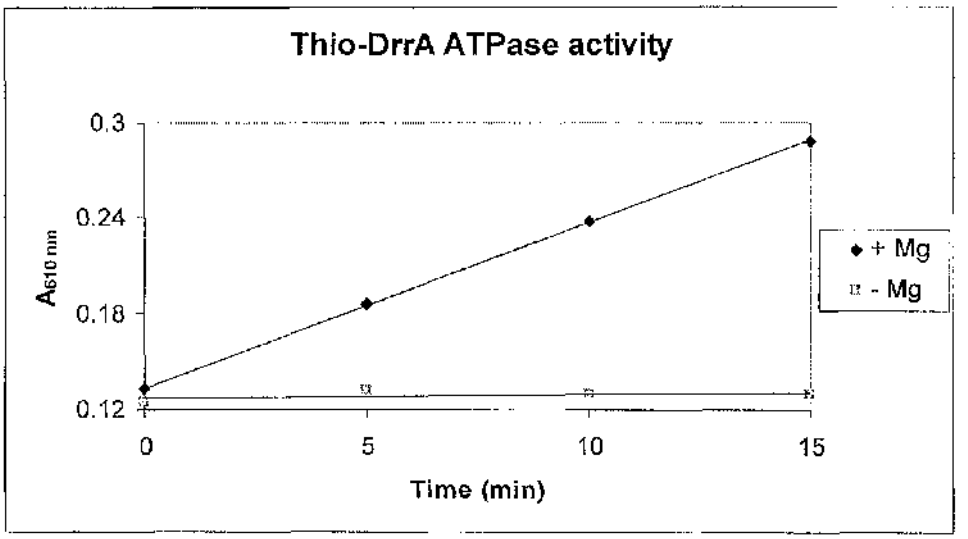



**Figure 6.2 Standard ATPase activity assay for Thio-DrrA**

Two identical reaction mixtures (see above) were incubated at 37 °C for five minutes prior to the addition of 450 μM $MgCl_2$ (diamonds), or $H_2O$ (squares). Forty-five μl samples were withdrawn from the reaction mixtures at five minute intervals, deposited in separate wells of a microtitre plate, and mixed with 5 μl of 500 μM EDTA, to halt the reaction. Samples were analysed for $P_i$ content by reading their absorbance at 610 nm after the addition of 100 μl of the Malachite green detection reagent. Released $P_i$ values were obtained by comparison with a standard curve.

### 6.1.2 Dependence on divalent cations: $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$, and $Ca^{2+}$

The ATPase activity of all known ABC transporter NBD's has been shown to depend on the presence of divalent cations. These cations are potential stimulators of ATPase activity, $Mg^{2+}$ being the most common stimulator of these systems. As expected, magnesium ions were necessary for the ATPase activity of Thio-DrrA and DrrA (Figure 6.2). In order to determine if other divalent cations could substitute for $Mg^{2+}$, ATPase assays were performed with different divalent cations. Manganese, cobalt, calcium and zinc, used as dichloride salts, were tested for their ability to modulate the ATPase activity of Thio-DrrA. Other authors (e.g. Nikaido *et al.*, 1997; Aparicio *et al.*, 1996) have shown that some cations other than $Mg^{2+}$ can be strong activators of the enzymatic hydrolysis of ATP, depending on the concentration of the divalent cation in the reaction mix. In ABC transporter NBD's the Walker B sequence is characterised by a main set of hydrophobic amino acids and by the presence of a highly conserved aspartate residue that is involved in the co-ordination of the divalent cation.

The stimulating effect of different divalent cations was tested over a concentration range between 0.1 and 2 mM. All assays to determine the inorganic phosphate released from the reactions employed the Malachite green method, and were performed at 37 °C, for 15 minutes and with a Thio-DrrA concentration of 100 μg/ml (~2 μM). The Thio-DrrA ATPase activity reached a maximum (specific activity of 44 nanomoles/min/mg of protein) with about 500 μM $MgCl_2$ (Fig. 6.3). This maximal activity was maintained up to a concentration of $Mg^{2+}$of 600 μM. These data indicated an $EC_{50}$ of about 500 μM. $Mg^{2+}$ concentrations above 1 mM seemed to impair stimulation of the ATPase activity of the protein (data not shown).

Manganese ions also activated the ATP hydrolysis by Thio-DrrA but to about one third the level of that obtained for $Mg^{2+}$ (Fig. 6.4). Cobalt ions appeared rather better (Fig. 6.5), stimulating the ATPase activity of Thio-DrrA to a maximum of 9 nanomoles $P_i$/min/mg of protein, which was roughly a half that reached with $MgCl_2$. This was obtained with concentrations between 400-600 μM of $CoCl_2$.

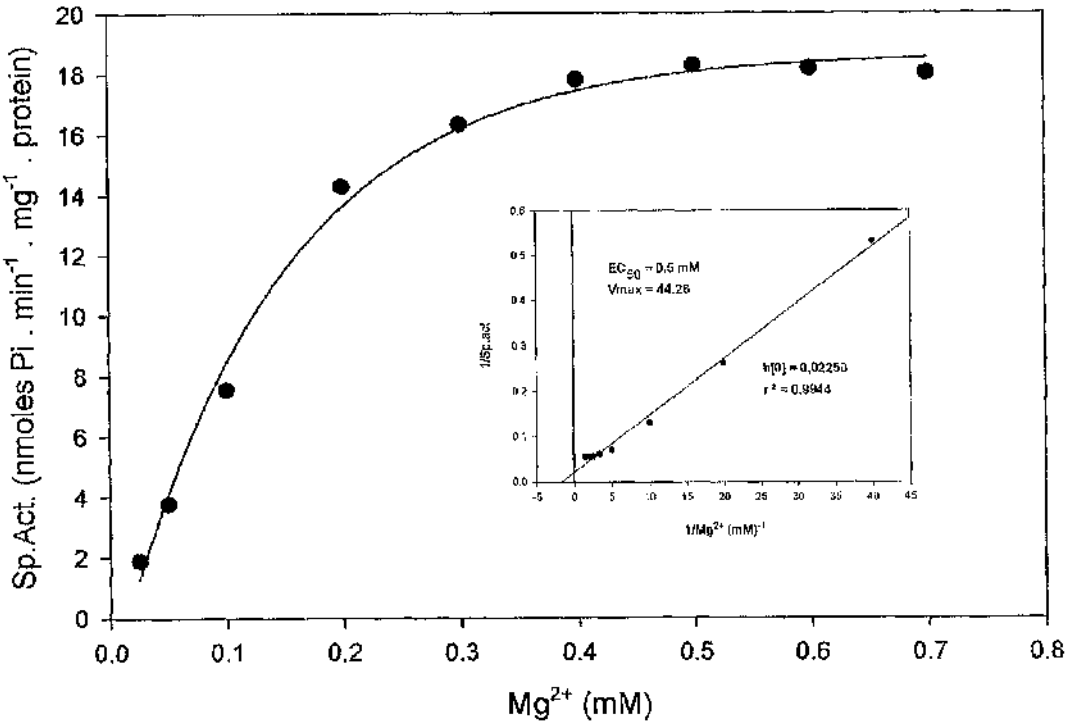



**Figure 6.3 Effect of $Mg^{2+}$ ions on the ATPase activity of Thio-DrrA**

ATPase activity was initiated by the addition of $Mg^{2+}$ at the concentrations indicated. The Malachite green $P_i$ release assay was used to calculate the rate of ATP hydrolysis from 15-minute ATP hydrolysis kinetics at each $Mg^{2+}$ concentration. Data are presented as protein specific activity (nanomoles of $P_i$ liberated/min/mg of protein).

All assays were performed at 37 °C in a total volume of 500 µl.

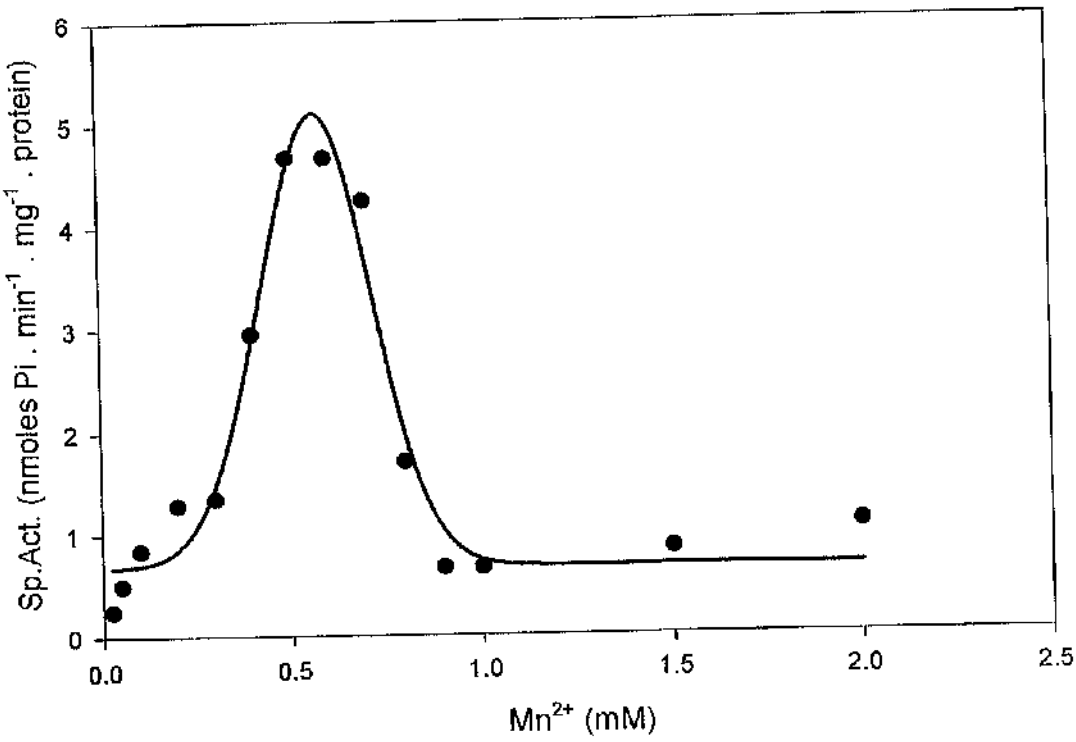



**Figure 6.4 Effect of $Mn^{2+}$ ions on the ATPase activity of Thio-DrrA**

ATPase activity was initiated by the addition of $Mn^{2+}$ at the concentrations indicated. The Malachite green $P_i$ release assay was used to calculate the rate of ATP hydrolysis from 15-minute ATP hydrolysis kinetics at each $Mn^{2+}$ concentration. Data are presented as protein specific activity (nanomoles of $P_i$ liberated/min/mg of protein).

All assays were performed at 37 °C in a total volume of 500 µl.

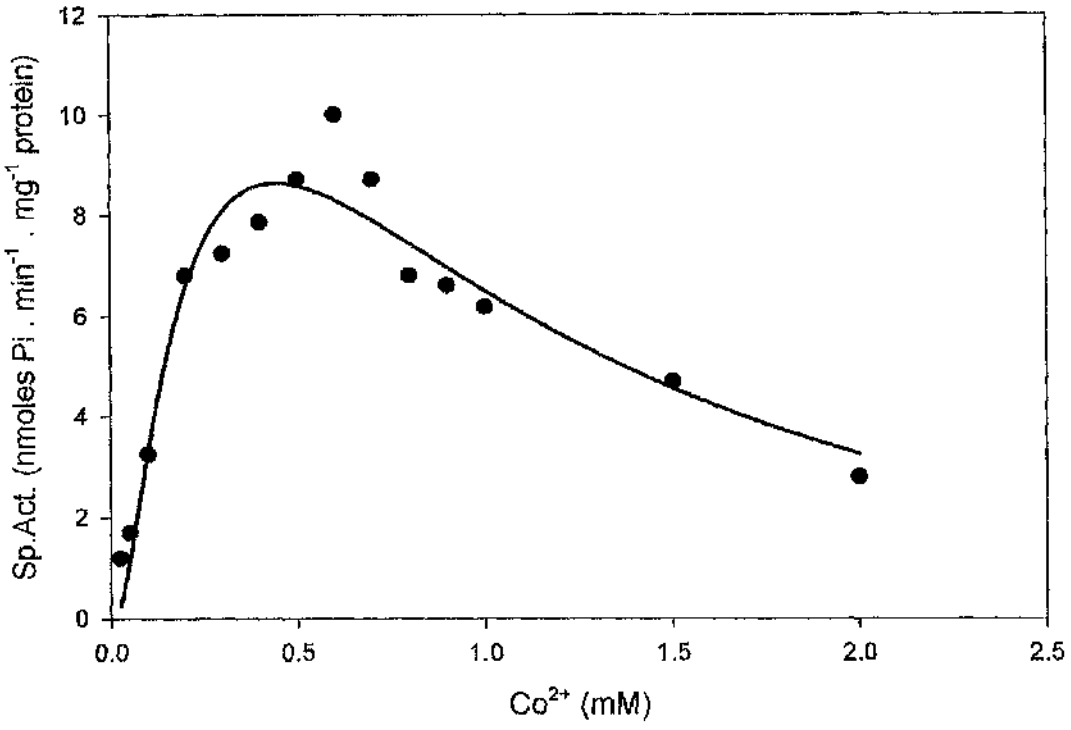



**Figure 6.5 Effect of $Co^{2+}$ ions on the ATPase activity of Thio-DrrA**

ATPase activity was initiated by the addition of $Co^{2+}$ at the concentrations indicated. The Malachite green $P_i$ release assay was used to calculate the rate of ATP hydrolysis from 15-minute ATP hydrolysis kinetics at each $Co^{2+}$ concentration. Data are presented as protein specific activity (nanomoles of $P_i$ liberated/min/mg of protein).

All assays were performed at 37 °C in a total volume of 500 µl.

Calcium ions were the least efficient modulators of the activity of Thio-DrrA, being ineffective between 0.1-1.0 mM (data not shown). Similarly, $Zn^{2+}$ failed to stimulate ATPase activity at any concentration (data not shown).

The behaviour of Thio-DrrA was not dissimilar to that of other NBDs, such as HisP (Nikaido *et al.*, 1997) and *M. tuberculosis* Trx-DrrA (Nash, 2003). For these proteins, optimal cation concentrations were found to be in a range of 1-2 mM.

### 6.1.3 Dependence on pH

Thio-DrrA was active between pH 7.0-9.0, with maximum activity at pH 8.0-8.5 (Fig. 6.6). At pH 9.0 only a slight decrease in the Thio-DrrA ATP hydrolysis was observed. Values below 7 were detrimental to the ATPase activity of this protein; when a pH of 6.5 was used, the enzymatic activity was completely abolished.

### 6.1.4 Dependence on protein concentration

The plot of ATPase activity against protein concentration was non-linear (Fig. 6.7), suggesting that Thio-DrrA molecules do not hydrolyse ATP independently of one another. This result might suggest that ATP hydrolysis for this protein is a cooperative process, where more than one Thio-DrrA molecule is necessary for ATPase activity.

### 6.1.5 Dependence on temperature

Temperatures of 24, 37 and 44 °C were assayed to evaluate the ATPase activity kinetics of Thio-DrrA (Fig. 6.8 and Table 6.1). A temperature of 37 °C was well-suited to the assay, as the rate of ATP hydrolysis at 24 °C was 50% lower than at 37 °C, and the velocity of the reaction at 44 °C was not significantly superior (Fig. 6.8). The temperature range at which inactivation of Thio-DrrA took place was not investigated.

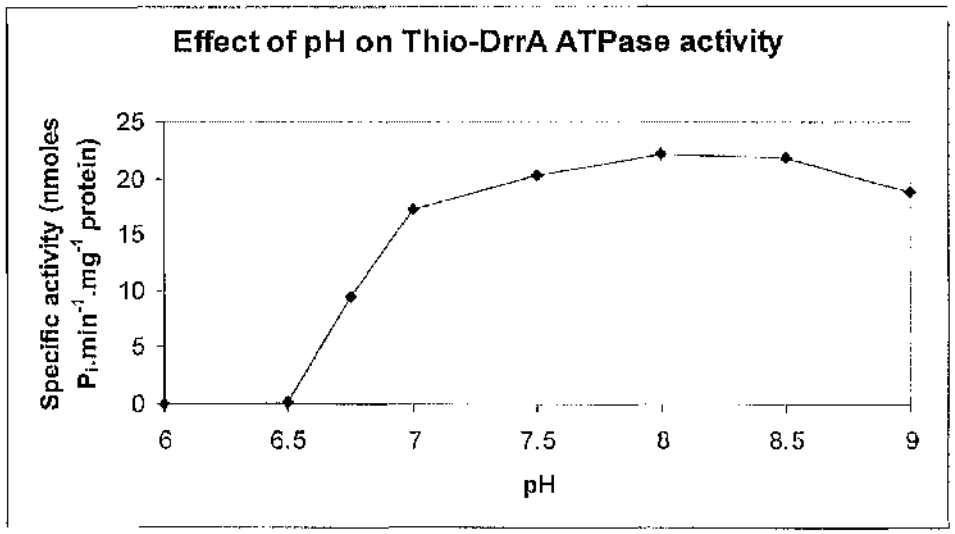



**Figure 6.6 Effect of the pH on the ATPase activity of Thio-DrrA**

The Malachite green $P_i$ release assay was used to calculate the rate of ATP hydrolysis from 15-minute ATP hydrolysis kinetics in each pH experiment. Data are presented as protein specific activity (nanomoles of Pi liberated/min/mg of protein).

Different buffers were required to achieve the pH values for the assay. MES was used for the range pH 6.0 – 6.5, MOPS was used at neutral pH and Tris was used for pH 8 – 9. Other reaction conditions were as standardised

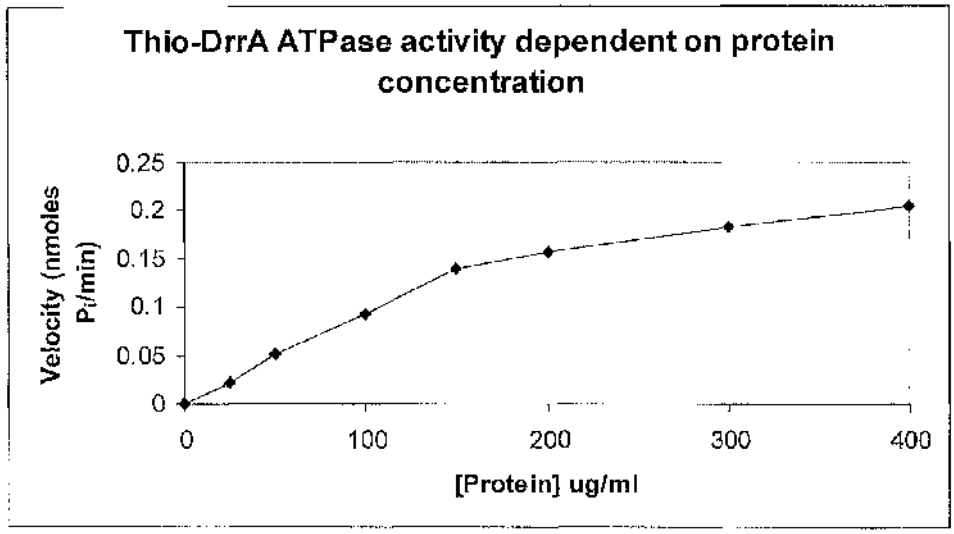



**Figure 6.7 Effect of the protein concentration on the ATPase activity of Thio-DrrA**

The Malachite green $P_i$ release assay was used to calculate the rate of ATP hydrolysis from 15-minute ATP hydrolysis kinetics with each protein concentration. The rate of ATP hydrolysis by Thio-DrrA was shown to vary linearly with increasing protein concentration (data not shown). From those data, the ATP hydrolysis rates (nanomoles of $P_i$ liberated/min) obtained, were plotted against Thio-DrrA concentration.

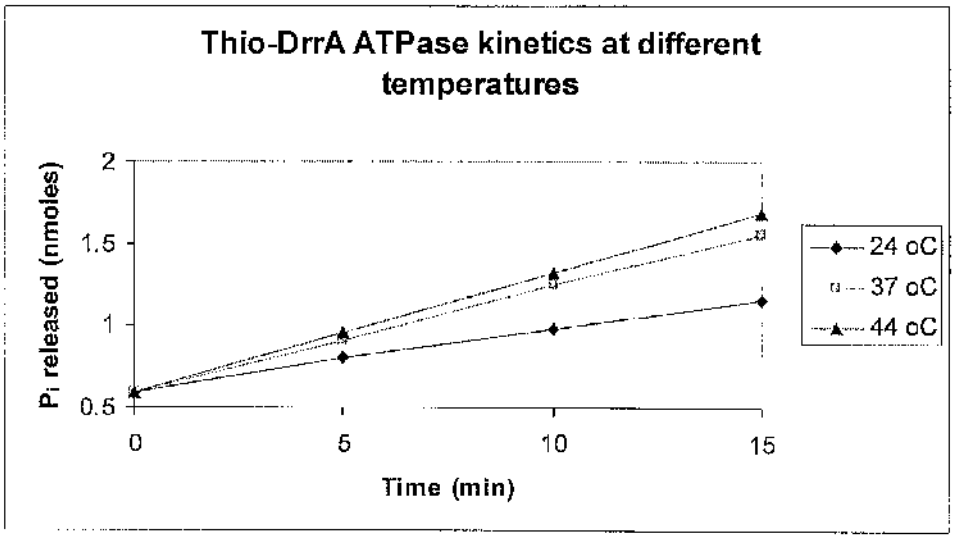



**Figure 6.8 Effect of the temperature on the ATPase activity of Thio-DrrA**

The Malachite green $P_i$ release assay was used to calculate the rate of ATP hydrolysis in each experiment. The ATPase activity of Thio-DrrA is displayed in 15-minute ATP hydrolysis kinetics, when the experiments were incubated at three different temperatures.

**Table 6.1 Effect of the temperature on the Thio-DrrA ATPase activity**

| Temperature | Specific activity (nanomoles Pi released /min/mg protein) |
|---|---|
| 24 °C | 11.2 |
| 37 °C | 19.2 |
| 44 °C | 20.1 |

Specific activities were calculated on the basis of triplicated measurements of the velocities calculated as in Fig. 6.8

## 6.2 Substrate Specificity

### 6.2.1 Dependence of Thio-DrrA and DrrA activity on the concentration of ATP. Determination of $V_{max}$ and $K_m$

The determination of the steady-state kinetics parameters $V_{max}$ and $K_m$, gives useful information about the catalytic activity of the protein under study, allowing these parameters to be compared with similar proteins. $K_m$ is the parameter that measures the affinity of the enzyme for its substrate, whilst $V_{max}$ measures the maximal velocity of the reaction at saturating substrate concentrations. ATPase activity data were obtained over a 200-fold range of ATP concentrations from 50 μM to 2 mM. The concentration of Thio-DrrA in these assays was chosen in order to obtain measurements of absorbance at 610 nm of approximately 0.34 at the end of the 15-minute ATP hydrolysis reaction. This makes possible informative comparison between the different experiments. Thus, 3.7 μM protein was used for the determination of $V_{max}$ and $K_m$. The rate of ATP hydrolysis varied in a hyperbolic manner with increasing ATP concentrations, indicative of simple Michaelis-Menten type kinetics. Calculation of the affinity of Thio-DrrA for ATP gave a $K_m$ value of 1.25 mM (Fig. 6.9), while for DrrA on its own, this value was in the order of 143 μM (Fig. 6.10), indicating different affinities for the substrate. Calculation of $V_{max}$ showed a substantial difference between the forms of DrrA, with values of 30 and 111 nanomoles Pi/min/mg protein for Thio-DrrA and DrrA, respectively. The low $V_{max}$ derived here for Thio-DrrA might be explained by the presence of additional amino acid sequences from Thioredoxin or a conformation that might diminish the original catalytic potential of DrrA, assuming that independently expressed DrrA retains its full ATPase activity.

A comparison of $K_m$ and $V_{max}$ values for Thio-DrrA and DrrA with NBDs reported by other authors is shown in Table 6.2. The $K_m$ for the DrrA proteins does not suggest a high affinity for substrate, and although the $K_m$ for DrrA lies between values for MalK (*S. typhimurium*) and HisP (*S. typhimurium*), the $K_m$ for Thio-DrrA is 10-fold higher and the highest in the Table. With reference to the $V_{max}$ parameter, DrrA showed again a value in the range reported for other systems, but Thio-DrrA displayed the lowest, suggesting an inappropriate state of that preparation.

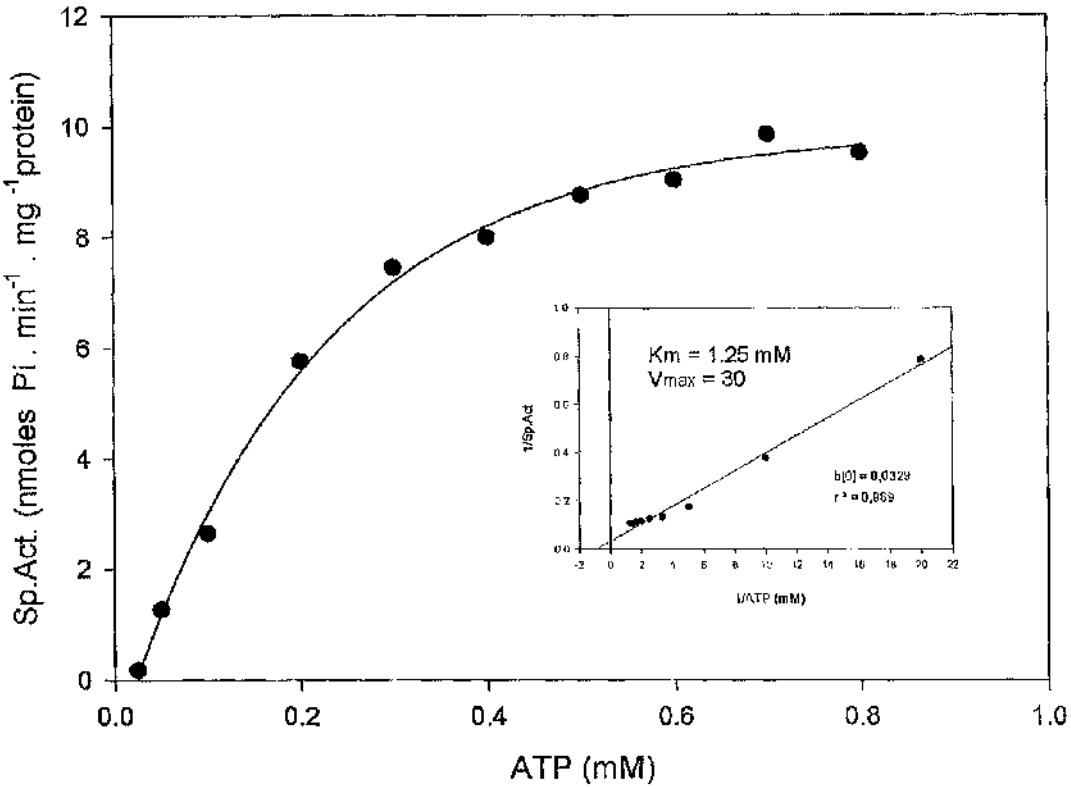



**Figure 6.9 Effect of ATP concentration on the ATPase activity of Thio-DrrA**

The Malachite green $P_i$ release assay was used to calculate the rate of ATP hydrolysis from 15-minute ATP hydrolysis kinetics at each ATP concentration experiment. ATP assays were performed at 37 °C and initiated by the addition of 45 µl of $MgCl_2$. The total volume for each assay was 500 µl with 45 µl of sample analysed for $P_i$ content at 5-minute time intervals over a time course of 15 minutes. A standard curve was used to convert absorbance values at 610 nm to nanomoles of $P_i$. Final values were expressed as specific activity in nanomoles of $P_i$ released/minute/mg of protein.

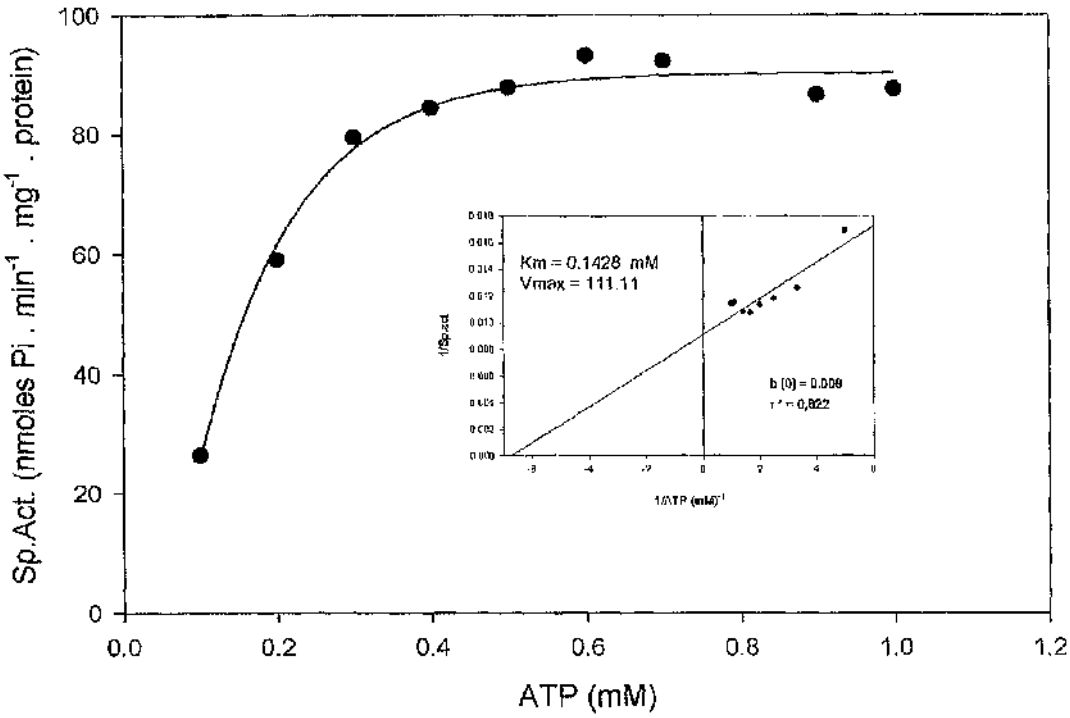



**Figure 6.10 Effect of ATP concentration on the ATPase activity of DrrA**

The Malachite green $P_i$ release assay was used to calculate the rate of ATP hydrolysis from 15-minute ATP hydrolysis kinetics at each ATP concentration experiment. ATP assays were performed at 37 °C and initiated by the addition of 45 µl of $MgCl_2$. The total volume for each assay was 500 µl with 45 µl of sample analysed for $P_i$ content at 5-minute time intervals over a time course of 15 minutes. A standard curve was used to convert absorbance values at 610 nm to nanomoles of $P_i$. Final values were expressed as specific activity in nanomoles of $P_i$ released/minute/mg of protein.

**Table 6.2 Comparative analysis of $K_m$ and $V_{max}$ values for different nucleotide-binding domains.**

| Protein | $K_m$ (μM) | $V_{max}$ (nanomoles $P_i$/min/mg protein) | Reference |
|---|---|---|---|
| MalK (*E. coli*) | 23.9 | 322 | Morbach *et al.*, 1993 |
| MalK (*S. typhimurium*) | 80 | 700 | Schneider *et al.*, 1995 |
| HisP (*S. typhimurium*) | 205 | 500 | Nikaido *et al.*, 1997 |
| PstB (*M. tuberculosis*) | 71.5 | 122 | Sarin *et al.*, 2001 |
| Trx-DrrA | 84.8 | 52 | Nash, 2003 |
| Thio-DrrA | 1250 | 30 | This study |
| DrrA | 143 | 111 | This study |

As low specific activities were obtained for a number of different preparations of the protein, we thought this could be linked to the instability of the Thio-DrrA preparations.

### 6.2.2 Other Nucleotide Triphosphate as substrates for Thio-DrrA

Cytosine triphosphate (CTP), thymine triphosphate (TTP) and guanidine triphosphate (GTP) nucleotides were tested as alternative substrates for Thio-DrrA in a standard ATP reaction (Fig. 6.11). CTP did not act as substrate for Thio-DrrA at all and the TTP behaviour was not very different, although a minimal activity signal could be detected at the end of the reaction. GTP was the only NTP apart from ATP that acted as a substrate for this protein, suggesting that Thio-DrrA has a preference for purine substrates.

### 6.2.3 Inhibitors of the ATPase activity of DrrA proteins

The effect of ADP on the ATPase activity of Thio-DrrA was tested. When used at the same concentration as ATP, it inhibited the Thio-DrrA ATPase activity by 60% (Fig. 6.12). In the absence of ATP, no hydrolysis of ADP by Thio-DrrA could be detected.

Vanadate possessed the dual properties of a phosphate transition state ATPase analogue, and a photoactive chemical (Fetsch and Davidson, 2002). Therefore vanadate was tested as a potential inhibitor of the ATPase activity of DrrA and Thio-DrrA. Vanadate concentrations ranging from 10 μM to 4 mM were assayed. At a concentration of 500 μM, 50 % inhibition of the ATPase activity of DrrA was detected; higher concentrations up to 4 mM did not enhance the level of inhibition (Fig. 6.13). The $IC_{50}$ value of 0.5 mM was not dissimilar to the value reported by Nikaido and collaborators for the Histidine permease NBD, assayed in isolation from the other components of the histidine transporter. These authors reported a much more potent effect for vanadate when tested with the complete His transporter, reporting an $IC_{50}$ of 6.5 μM (Nikaido *et al.*, 1997). Similar results were reported for MalK, the ATPase subunit of the maltose transporter (Schneider, 2001).

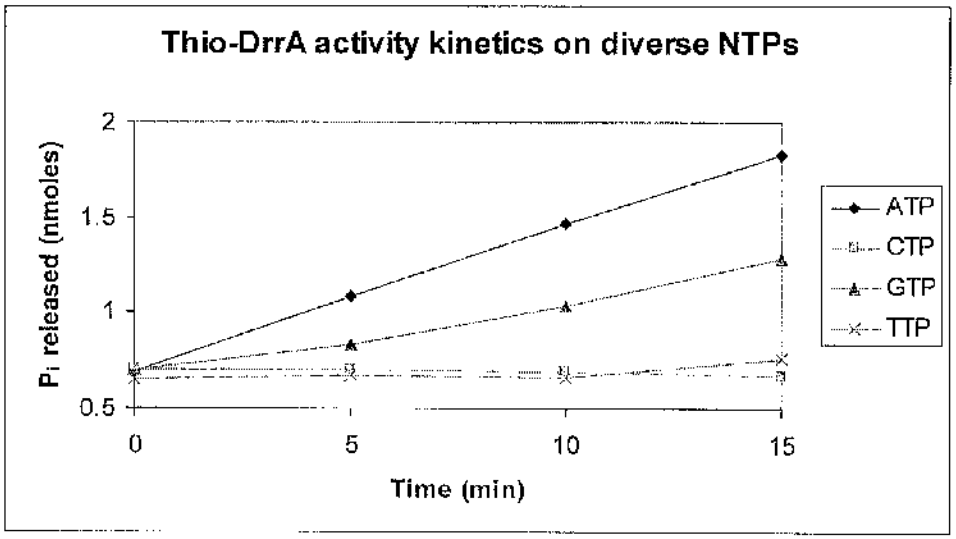



**Figure 6.11 Nucleotide substrate specificity for Thio-DrrA**

The Malachite green $P_i$ release assay was used to calculate the rate of nucleotide triphosphate (NTP) hydrolysis in each experiment. NTP assays were performed at 37 °C and initiated by the addition of 45 µl of $MgCl_2$. The NTPase activity of Thio-DrrA is displayed in 15-minute NTP hydrolysis kinetics, when the experiments were incubated with the different NTPs.

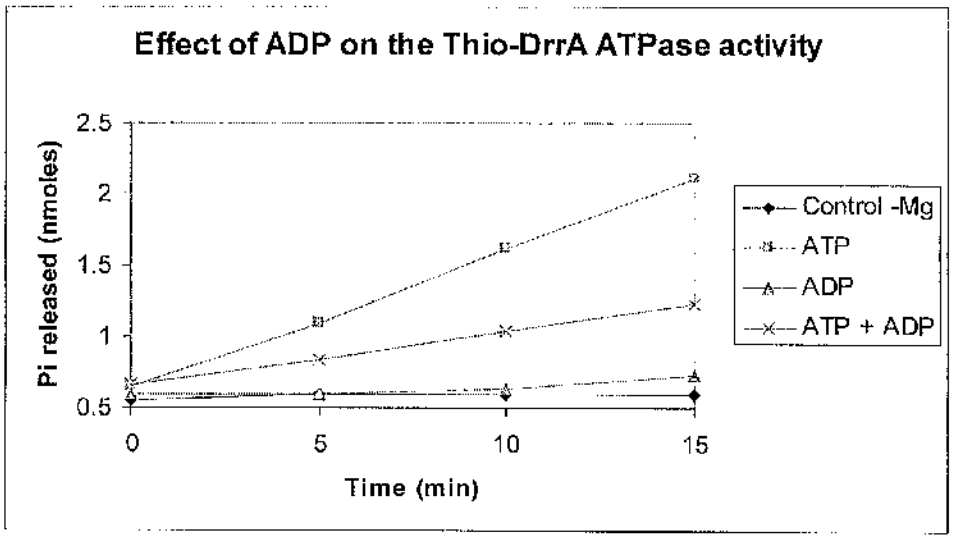



**Figure 6.12 Effect of ADP on the ATPase activity of Thio-DrrA**

The Malachite green $P_i$ release assay was used to calculate the rate of ATP hydrolysis in each experiment. ATP assays were performed at 37 °C and initiated by the addition of 45 µl of $MgCl_2$. The ATPase activity of Thio-DrrA is displayed in 15-minute ATP hydrolysis kinetics. ADP was added at a concentration of 250 µM.

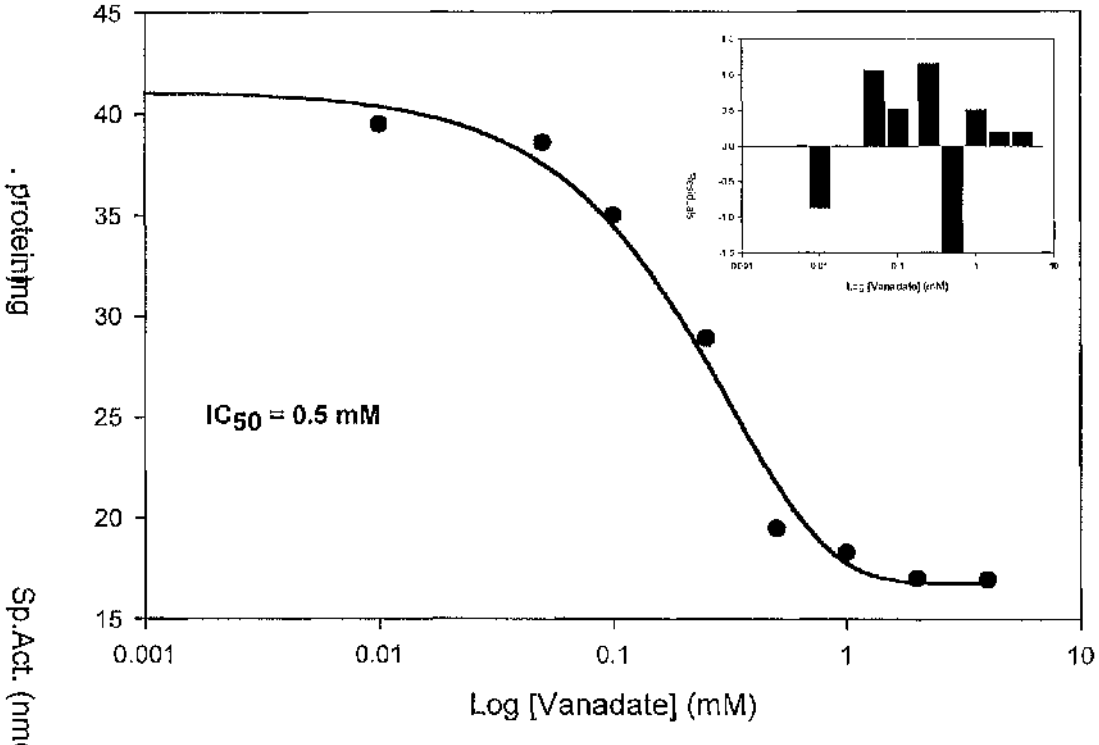



**Figure 6.13 Effect of Vanadate on the ATPase activity of DrrA**

The Malachite green $P_i$ release assay was used to calculate the rate of ATP hydrolysis from 15-minute ATP hydrolysis kinetics at each vanadate concentration. ATP assays were performed at 37 °C in presence of different vanadate concentrations, and initiated by the addition of 45 µl of $MgCl_2$. The total volume for each assay was 500 µl with 45 µl of sample analysed for $P_i$ content at 5-minute time intervals over a time course of 15 minutes. A standard curve was used to convert absorbance values at 610 nm to nanomoles of $P_i$. Final values were expressed as specific activity in nanomoles of $P_i$ released/minute/mg protein.

Thio-DrrA was used to test if increasing NaCl concentrations would inhibit ATPase activity, as reported for other NBDs (Nikaido *et al.*, 1997). NaCl concentrations above 300 μM were found to inhibit the ATPase activity of Thio-DrrA substantially, as can be seen in Figures 6.14. For purification and assay of the protein, retaining a concentration of NaCl of 150 mM was thus validated.

### 6.2.4 Effect of the presence of DrrB on the ATPase activity of Thio-DrrA and DrrA

The presence of DrrB did not appear to influence the ATPase activity of Thio-DrrA, but the addition of the protein did appear to elevate background signals in the assay. A similar experiment was run using DrrA with a different preparation of DrrB that did not seem to generate background signal. According to these data (Fig. 6.15), DrrB did not elevate the ATPase activity in the reaction. It thus seemed unlikely that the proteins in their free state could interact in the same manner as is probably in the proposed complex $DrrA_2B_2$.

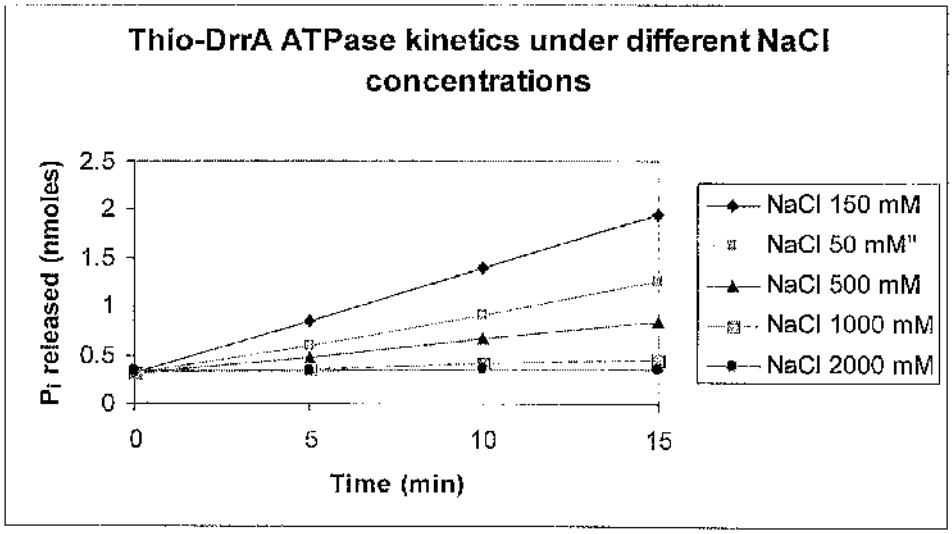



**Figure 6.14 ATPase activity of Thio-DrrA under different NaCl concentrations**

The Malachite green $P_i$ release assay was used to calculate the rate of ATP hydrolysis from 15-minute kinetics in presence of different NaCl concentrations. ATP assays were performed at 37 °C and initiated by the addition of 45 µl of $MgCl_2$. The total volume for each assay was 500 µl with 45 µl of sample analysed for $P_i$ content at 5-minute time intervals over a time course of 15 minutes. A standard curve was used to convert absorbance values at 610 nm to nanomoles of $P_i$.

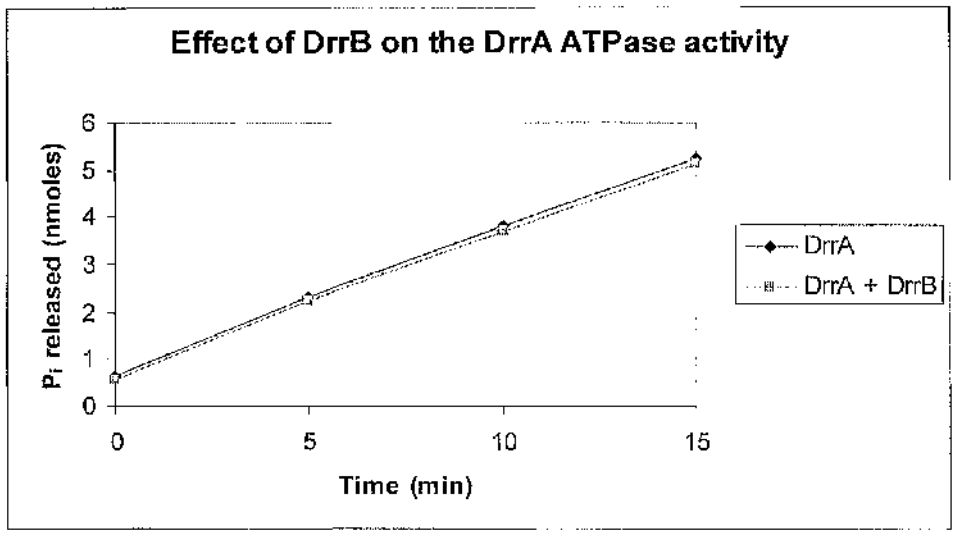



**Figure 6.15 Effect of DrrB on the ATPase activity of DrrA**

The Malachite green $P_i$ release assay was used to calculate the rate of ATP hydrolysis in each experiment. Assays were performed at 37 °C in the presence of DrrA (1 μM ) in the absence or presence of DrrB (1 μM; the kindly gift of Dr. McKeegan, Wolfson Institute, University ofn Durham). Reactions were initiated by the addition of 45 μl of $MgCl_2$. The total volume for each assay was 500 μl with 45 μl of sample analysed for $P_i$ content at 5-minute time intervals over a time course of 15 minutes. A standard curve was used to convert absorbance values at 610 nm to nanomoles of $P_i$. Final values were expressed in nanomoles of $P_i$ released.

### 6.2.5 Effect of Doxorubicin on the ATPase activity of Thio-DrrA and DrrA

When the compound doxorubicin was added to a reaction mix containing Thio-DrrA or DrrA, no change in ATPase activity could be detected. Doxorubicin concentrations from 1-20 μg/ml (1.5-35 μM) were assayed to evaluate the effect on the ATPase activity of DrrA (Fig. 6.16). A link with other elements involved in the DrrAB system has not been established when DrrA has been expressed without the remainder of the DrrAB system. Some expectations had been raised about the possibility that anthracyclines compounds could act as modulators of the ATPase activity of DrrA, as occurs in the ATPase subunit ArsA of the *E. coli* non ABC transporter ArsAB, with its substrates arsenate and antimonite (Rosen *et al.*, 1999). Although that pump is not an ABC transporter, it shows some similarities in conformation and function with the ABC transporters.

The combined presence of the DrrB protein and doxorubicin was also assayed to monitor any change in the catalytic activity of DrrA. Once more, the DrrA activity was unaffected (Fig. 6.17).

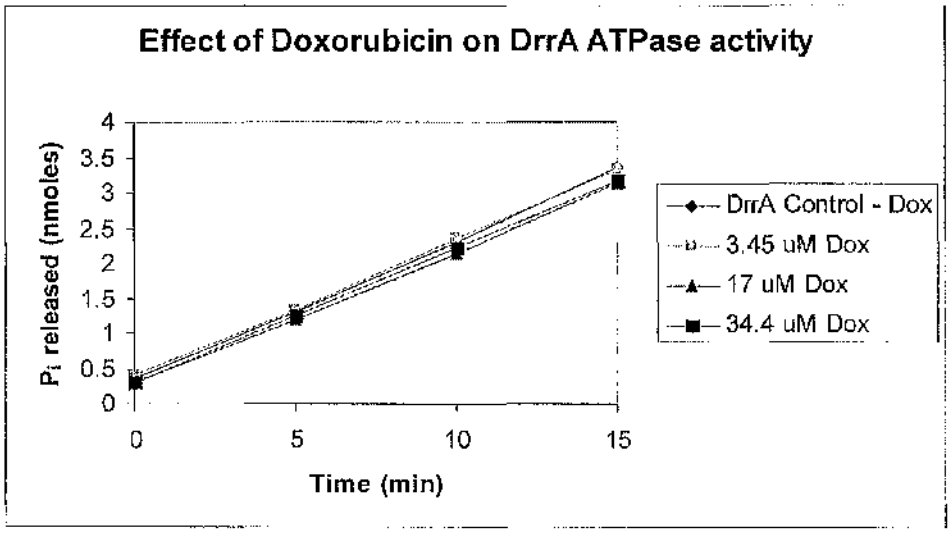



**Figure 6.16 Effect of Doxorubicin on the ATPase activity of DrrA**

The Malachite green $P_i$ release assay was used to calculate the rate of ATP hydrolysis in each experiment. ATP assays were performed at 37 °C in the presence of doxorubicin at several concentrations. Reactions were initiated by the addition of 45 µl of $MgCl_2$. The total volume for each assay was 500 µl with 45 µl of sample analysed for $P_i$ content at 5-minute time intervals over a time course of 15 minutes. A standard curve was used to convert absorbance values at 610 nm to nanomoles of $P_i$. Final values were expressed as nanomoles of $P_i$ released.

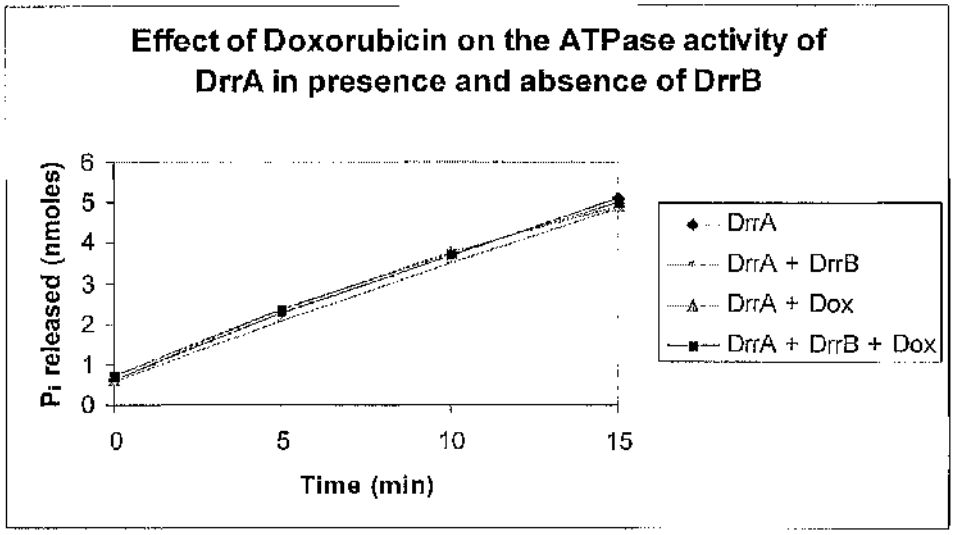



**Figure 6.17 Effect of Doxorubicin on the ATPase activity of DrrA in the presence of DrrB**

The Malachite green $P_i$ release assay was used to calculate the rate of ATP hydrolysis in each experiment. ATP assays were performed at 37 °C with DrrA (1 μM) in the presence of doxorubicin (2 μM), and / or DrrB (1 μM). Reactions were initiated by the addition of 45 μl of $MgCl_2$. The total volume for each assay was 500 μl with 45 μl of sample analysed for $P_i$ content at 5-minute time intervals over a time course of 15 minutes. A standard curve was used to convert absorbance values at 610 nm to nanomoles of $P_i$. Final values were expressed as nanomoles of $P_i$ released.

## 6.3 Comparative analysis between the proteins DrrA and Thio-DrrA

Overexpression of DrrA at 16 °C from the pET21a vector and of Thio-DrrA from the pBADTOPO thiofusionvector, provided the best sources of DrrA proteins in terms of purity and quantity. The properties of the DrrA preparations are compared in Table 6.3. It is interesting to notice the higher stability of the DrrA protein when it is stored at -20 °C, as it maintains almost 100% of activity over a period of two weeks, whilst Thio-DrrA appeared to be more sensitive to loss of activity during storage.

**Table 6.3 Comparative analysis between experimental DrrA proteins**

| | Thio-DrrA | DrrA |
|---|---|---|
| ATPase activity ($K_m$) | $K_m$=1.25 mM | $K_m$=143 μM |
| Velocity of the reaction ($V_{max}$) | 30 nanomoles $P_i$/min/mg protein | 111 nanomoles $P_i$/min/mg protein |
| Biophysical properties such as induction by divalent cations, pH, inhibitors | Similar | |
| Activity stability after ageing | Loss of 50 % activity in 2 weeks time at –20 °C | Loss of 5 % activity in 2 weeks time at –20 °C |
| Post-purification stability | Variable and low | High |
| Protein yield | 250 μg/L | 40 μg/L |

Thio-DrrA was overexpressed from the pBADTOPO *thio-drrA* fusion vector, induced with 0.002 % arabinose, whilst DrrA was over-expressed from the pET21a-*drrA* vector and induced with 1.0 mM IPTG.

Data on protein yields were taken from single experiments.

## 6.4 Discussion

A partial characterisation of Thio-DrrA was made using established biochemical methods, complemented by the characterisation of DrrA. The main property assayed was the ATPase activity of the proteins. This was of primary importance as there are reports that DrrA expressed in the absence of DrrB does not possess ATPase activity (Kaur, 1997; Kaur and Russell, 1998).

Essentially, the characterisation of the Thio-DrrA fusion protein showed features shared with other NBD's, such as HisP and MalK from *S. typhimurium*, MalK from *E. coli* and DrrA from *M. tuberculosis* (Liu *et al.*, 1997; Nikaido *et al*, 1997; Morbach *et al.*, 1993; Nash, 2003). Although the *S. peucetius drrAB* operon has been characterised by others (Guilfoile and Hutchinson, 1991), and the ability of DrrA to bind ATP has been reported (Kaur, 1997; Kaur and Russell, 1998), the data presented here are the first to show the hydrolysis of ATP by DrrA.

Thio-DrrA and DrrA possessed detectable catalytic activity consistent with a role in energising transmembrane transport. The protein was shown to be a cation dependent ATPase able of binding and hydrolysing ATP in a similar manner to the NBDs of well-characterised prokaryotic ABC transporters.

The $V_{max}$ value obtained for the Thio-DrrA fusion was markedly lower than that for DrrA and other NBDs, suggesting that the fusion protein at the time of assay had a low ATPase activity. This might reflect the inappropriate folding or reduced integrity of the protein under the conditions of expression or purification that were employed rather than its intrinsic activity. This fact seems to be supported by the results with DrrA expressed in isolation; the average velocity for this species was comparable with other NBDs. $K_m$ for DrrA was also in the range of values reported for other NBDs, but for Thio-DrrA the same parameter, was 9-fold higher, indicating a reduced affinity for ATP. Although higher Km values for ABC transporters have been reported (3.0 mM for MRP; Chang *et al.*, 1997) it appears possible that the fusion affected the character and catalytic activity of DrrA. Characterisation of Thio-DrrA therefore allow qualitative assessment of the features of DrrA. Whilst data from analysis of DrrA might be considered more informative with regard to the kinetics of the DrrA system ($K_m$ and $V_{max}$), very limited amounts of the protein were available for

experimentation due to its insolubility. Also, early reports make mention that DrrA is only functionally active when forming part of the membrane fraction. Hence, the potential necessity of membrane interactions needs to be considered before definitive evaluation of the ATPase activity of DrrA can be made.

Inhibition of ATPase activity by high NaCl concentrations has been reported for some NBDs (Nikaido *et al.*, 1997), however, it was surprising to find inhibition of Thio-DrrA at concentrations below those (300 mM) recommended for IMAC purification. This might explain the low activities detected when NaCl was used at 400 mM for the extraction and purification of some preparations of the protein.

Although an interaction of DrrB and doxorubicin with DrrA might be expected, if any interaction took place between these species it failed to enhance the ATPase activity of the reaction. It is clear that these experiments do not emulate the natural conditions of the assembled transporter in the cell. It has been reported that isolated NBDs do not show the same pattern of behaviour as the complete transporter in the presence of inhibitors like vanadate and *N*-ethylmaleimide. Vanadate strongly inhibits the ATPase activity in assembled ABC transporters (e.g. $HisQMP_2$; Nikaido *et al.*, 1997) but scarcely affects their NBDs when tested in isolation. Several of these single NBDs have shown low sensitivity to these agents. It is not clear whether the inhibition of DrrA ATPase activity by vanadate is of biological significance. The pattern observed (Fig. 6.13) shows an inhibitory effect that appears real but it is only evident over vanadate concentrations (in excess of 0.1 mM) that are high by comparison to those that inhibit intact ABC transporter systems (e.g. $HisQMP_2$ is inhibited by vanadate with an $IC_{50}$ of 6.5 μM whereas the ATPAse activity of HisP is unaffected by concentrations of 0.5 mM; Nikaido *et al.*, 1997).

In common with the finding that doxorubicin does not enhance the ATPase activity of DrrA or Thio-DrrA, the basal ATPase activity of P-glycoprotein Mdr1 is not enhanced by drugs when its NBDs or N- or C-terminal half-molecules have been separately expressed (Loo and Clarke, 1994; Buxbaum, 1999). It is now known that the drug-binding sites in P-glycoprotein are localised in the transmembrane domain (Ambudkar *et al.*, 1999). If we assume that the drug-binding sites in DrrAB are also

located in its TMD in an equivalent manner to P-glycoprotein, this might explain why the addition of doxorubicin to DrrA did not enhance ATPase activity. However, studies on the mechanism of the *E. coli* ArsA ATPase have shown that ArsA possesses allosteric sites for As(III) and Sb(III), that are transport substrates for the ArsAB pump (Rosen *et al.*, 1999). Antimonite stimulated ATP hydrolysis by ArsA by 10- to 20-fold whilst with arsenite, the stimulation was lower. Similar allosteric sites might occur in ABC NBDs, and the existence of sites for ligands in DrrA was a possibility worth investigating.

It has been suggested that cooperativity between NBDs is a probable feature for all the NBDs in ABC transporters. A dimeric form of the NBD in the mitochondrial ABC transporter Mdl1p (Janas *et al.*, 2003) was suggested when its ATPase activity was dependent in a non-linear fashion on protein concentration and confirmed by the formation of intermediate dimeric transition states trapped by vanadate or beryllium fluoride. In addition, an active NBD dimer has been reported for the OpuA system of *B. subtilis*, evidenced by size exclusion chromatography (Horn *et al.*, 2003). There was little evidence that the ATPase activity of Thio-DrrA departed from a linear relationship with protein concentration, but the result is far from conclusive. One feature of the Thio-DrrA protein was its high aggregation state, a property that impeded the successful use of size exclusion chromatography to assess whether a dimer was favoured as the active form for DrrA. Clearly, this might have bearing on the potential formation of dimers with ATPase activity different from that of monomeric forms. This issue could not be addressed with independently expressed DrrA, as insufficient soluble protein could be recovered for this type of assay.

# Chapter 7

## Fluorescence spectroscopy of DrrA proteins

Spectroscopic methods can be used to monitor changes in the conformation of transporter proteins and time-resolve these changes. Fluorescence spectroscopy is preferred for monitoring ligand-membrane protein interactions whilst stopped-flow fluorescence (SFF) spectroscopy is favoured for determination of the mechanisms of ligand binding and translocation. The latter method is more sophisticated and uses rapid techniques to measure the rate constants governing the translocation of the substrate across the membrane (Walmsley, 2000).

## 7.1 Intrinsic tryptophan fluorescence of DrrA refolded from inclusion bodies in a urea gradient

Based on the fluorescence properties of the aromatic amino acids tryptophan and tyrosine, fluorescence spectroscopy has proved to be a powerful tool for investigating the properties of ABC transporter complexes (e.g. P-glycoprotein; Sharom *et al.*, 1998, 1999, and 2001). The technique relies upon the unique responses that small changes in the environment generate in the fluorescence properties of individual tryptophan residues within a protein. Therefore, conformational changes in a protein, induced by events such as ligand binding, can often be correlated with a change in the fluorescence of tryptophan residues.

Refolding of DrrA from inclusion bodies produced with the pET21a vector did not appear to generate protein with ATPase activity (see Chapter 5, section 5.1). Fluorescence spectroscopy techniques were used to seek evidence that the DrrA was able to interact with its substrate but unable to hydrolyse any bound ATP. Fluorescence changes in DrrA were monitored in the presence of the ligand doxorubicin and the nucleotide analogue MANT-ATP (Fig. 7.1). The peak in the spectrum in the absence of ligand was maximumal at 340 nm. This was consistent with a folded rather than a denatured structure as tryptophans in proteins that have been denatured by urea or guanidine hydrochloride exhibit a $_{max}$ of 350 nm (Reshetnyak and Burstein, 2001). The emission intensity fell by 29% in the presence of doxorubicin or MANT-ATP, and was reduced slightly more in the presence of

both. When magnesium was added, emission intensity rose to match that of the doxorubicin-DrrA peak. The data thus suggested that MANT-ATP was binding the DrrA protein. The MANT-ATP, on the other hand, displayed a peak at 440 nm that remained the same even in the presence of the protein.

Although the protein displayed these spectroscopic properties, no ATPase activity was detectable for DrrA. Although this could be interpreted as evidence for the interaction of DrrA with the ligands, the absence of ATPase activity did not justify further investigation.

## 7.2 Intrinsic tryptophan fluorescence of the fused protein Thio-DrrA

Protein fluorescence data, collected under steady-state conditions, can be used to calculate biochemical parameters such as the equilibrium dissociation constant and/or the stoichiometry of a particular protein-substrate interaction. These can also be time resolved to give mechanistic information. The detailed kinetic mechanism describing the activity of the *E. coli* ArsA ATPase was determined by Walmsley and collaborators using a combination of these techniques (Walmsley *et al.*, 2001). Conformational changes associated with ATP binding, metalloid binding, ATP hydrolysis and product release were all detected through changes in the tryptophan fluorescence of the protein.

In order to evaluate whether similar conformational changes might be detected during the binding of ATP to Thio-DrrA and its further hydrolysis, steady-state tryptophan emission spectra were collected for protein both in the presence and absence of nucleotide. A typical spectrum is shown in Figure 7.2. In absence of nucleotide, Thio-DrrA exhibited an emission maximum at a wavelength of 334 nm. The addition of 1 mM ATP to the protein resulted in a quench in the fluorescence (64 % decrease), and the subsequent addition of 1 mM $MgCl_2$ to the mix caused a further quench of 4%. Neither addition had any effect on the $\lambda_{max}$ value that remained constant at 334 nm.

The reduction in emission intensity after addition of ATP initially indicated that some of the four tryptophan residues present in Thio-DrrA was responsible for a

nucleotide induced conformational change in the protein structure. Nevertheless, the adenine ring of ATP interferes with fluorescence measurements by absorbing photons, thereby reducing the intensity of both the excitation beam and of the observed emission spectrum. This phenomenon is known as "inner filter effect", and has to be considered when measuring the steady-state fluorescence of ATP-binding proteins (Nash, 2003). The extent of the inner filter effect was evaluated for different concentrations of ATP (1.0-0.01 mM), and it was determined that the decrease in emission intensity for Thio-DrrA could be attributed to an inner filter phenomenon. The magnitude of the effect was substantial and it was judged that any further changes in tryptophan fluorescence of Thio-DrrA that might arise from specific interaction with ATP, were unlikely to be discernible by this technique. Further doubt was cast upon the value of this approach in control experiments with bovine albumin. BSA showed the same quench in protein fluorescence as DrrA protein when 1 mM ATP was added.

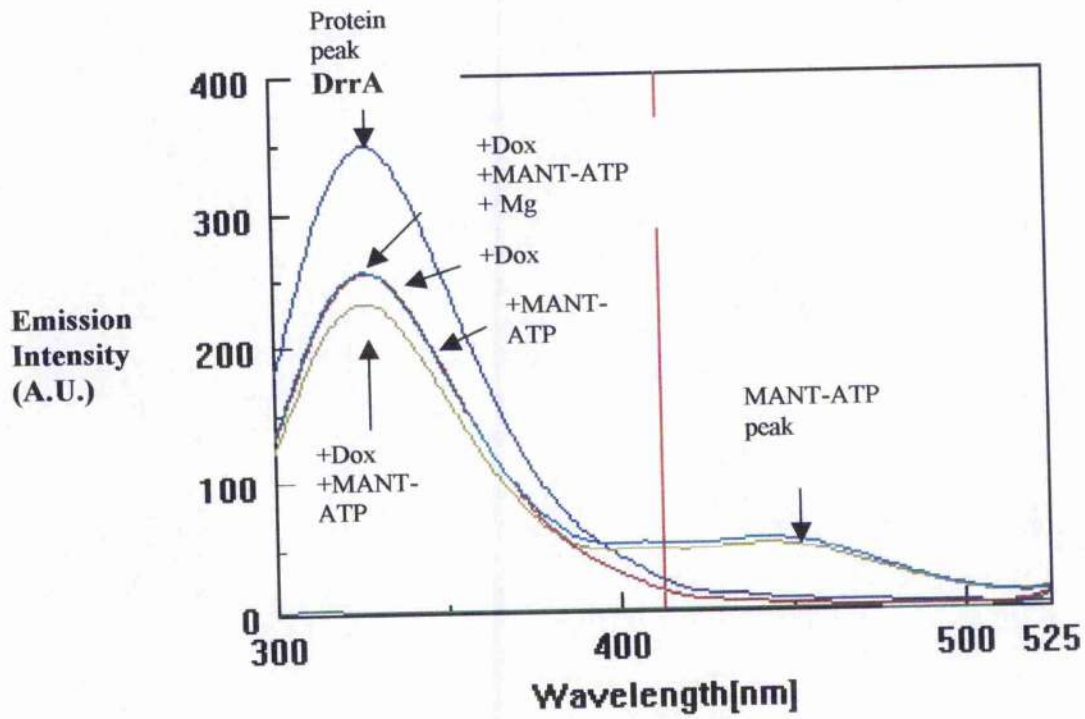



**Figure 7.1 Tryptophan fluorescence emission spectra of refolded DrrA in the presence of substrate and ligand**

Protein was excited at 285 nm and emission scanned between 300 and 525 nm. Protein fluorescence peaks appeared at 340 nm. The composition of the test samples is indicated. The ATP analogue MANT-ATP has a fluorescence maximum peak at 440 nm (indicated). Fluorescence data are shown in arbitrary units (A.U.).

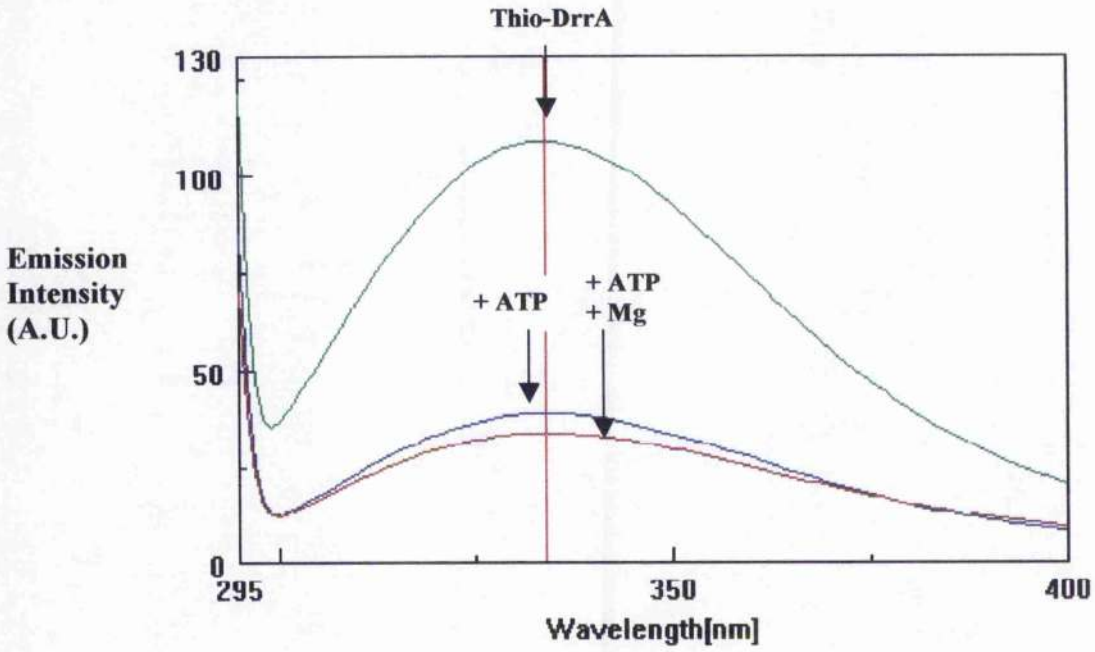



**Figure 7.2 Tryptophan fluorescence emission spectra of Thio-DrrA**

The fluorescence emission spectrum of 2.5 μM Thio-DrrA was measured at room temperature in a buffer containing 50 mM Tris-HCl and 150 mM NaCl, pH 8.0. Tryptophan fluorescence was excited at a wavelength of 285 nm and emission data gathered from samples (composition as indicated) between 295 and 400 nm. Fluorescence data are shown in arbitrary units.

## 7.3 Stopped-flow fluorescence studies of Thio-DrrA

Nucleotide interactions can produce transient changes in the fluorescence of proteins. Stopped-flow fluorescence spectroscopy allows the very rapid detection of changes in protein fluorescence in response to ligand binding. The method allows the rapid mixing of the protein with the ligand, and enables one to time-resolve changes in the fluorescence on a millisecond time scale (see Chapter 2, section 2.13.2).

Contrary to measurement of changes under steady-state conditions, the use of stopped-flow techniques negates inner filter effects because any reduction in signal due to the presence of ATP occurs prior to the onset of data collection. A second advantage of the technique is that even very small and rapid changes in fluorescence that may be missed under steady-state conditions can be detected and time-resolved to reveal mechanistic details of the interaction (Walmsley *et al.*, 2001). Stopped-flow experiments involve mixing two (or more) solutions together as rapidly as possible and getting them immediately to the optical observation point. The faster this happens, the earlier into the reaction one may observe (i.e. the smaller the dead time). Some stopped-flow instruments are capable of achieving dead times of less than 1.0 millisecond.

### 7.3.1 Production of tryptophan mutants in Thio-DrrA for fluorescence experiments

Due to intrinsic fluorescence properties of the aromatic amino acids tryptophan and tyrosine, it is possible to measure changes in their signals when a ligand binds a protein with these residues adequately exposed. When this type of interaction was assessed for the nucleotide-binding domain of the pump DrrAB (Thio DrrA) in a stopped-flow device, no changes were detected in the presence of the natural substrate ATP, or its fluorescent-analogue MANT-ATP.

Site-directed mutagenesis is a valuable tool for studies of gene and protein structure and function that allows one to specifically change any given base in a cloned DNA sequence. In an attempt to obtain detectable signals, single mutations were constructed with the aim of introducing additional tryptophan residues in

sections of the protein sequence that could be involved in the catalytic active site, or close to it. Tryptophan is more responsive to any change than tyrosine. Thio-DrrA contains four tryptophan residues, with two of them forming part of the Thioredoxin protein. The initial aim was to obtain a mutation in the protein ABC signature sequence to enhance its fluorescence signal without affecting its intrinsic ATPase activity. If this signal was not satisfactory, it was argued that different residues involved in the Walker A motif could be targeted later (Fig. 7.3).

**Mutagenesis in Thio-DrrA**

Fluorescence quenching following the addition of ATP had been previously reported for NBD's (Schneider *et al.*, 1994, working with MalK). The alleged ABC signature-sequence motif (also known as linker peptide) is one of the most highly conserved regions in ABC transporter NBD's and it is generally represented by the consensus "LSGGQ". It is located in the ABC transporter-specific α-helical sub-domain of the NBD. A number of crystallographic NBD dimers suggest this motif is part of a complex ATP binding site formed by juxtaposition of one NBD's Walker A motif with the signature-sequence motif of a second molecule (Hopfner *et al.*, 2000; Smith *et al.*, 2002; Chen *et al.*, 2003) . In *S. peucetius* DrrA the signature-sequence motif is represented by the sequence 'YSGGM', and it was selected as target for site-directed mutagenesis. Although not common, the same YSGGM signature sequence was reported for *Streptococcus pyogenes* OppD and OppF (Podbielski *et al*, 1996), and *M. tuberculosis* DrrA (Nash, 2003). Mutation of the motif's initial tyrosine residue (Y140) to a potentially fluorescent tryptophan residue (W) was devised. Being part of the most conserved sequence in these transporters, this region might well be intimately involved in both dimerisation of the NBD and the mechanism of ATP hydrolysis.

To produce this mutation in Thio-DrrA, primers were designed according to the protocols for the Promega Gene Editor system, and later, the QuickChange site directed mutagenesis system from Stratagene. In both cases, TAC codon was mutated to TGG, to replace tyrosine with tryptophan.

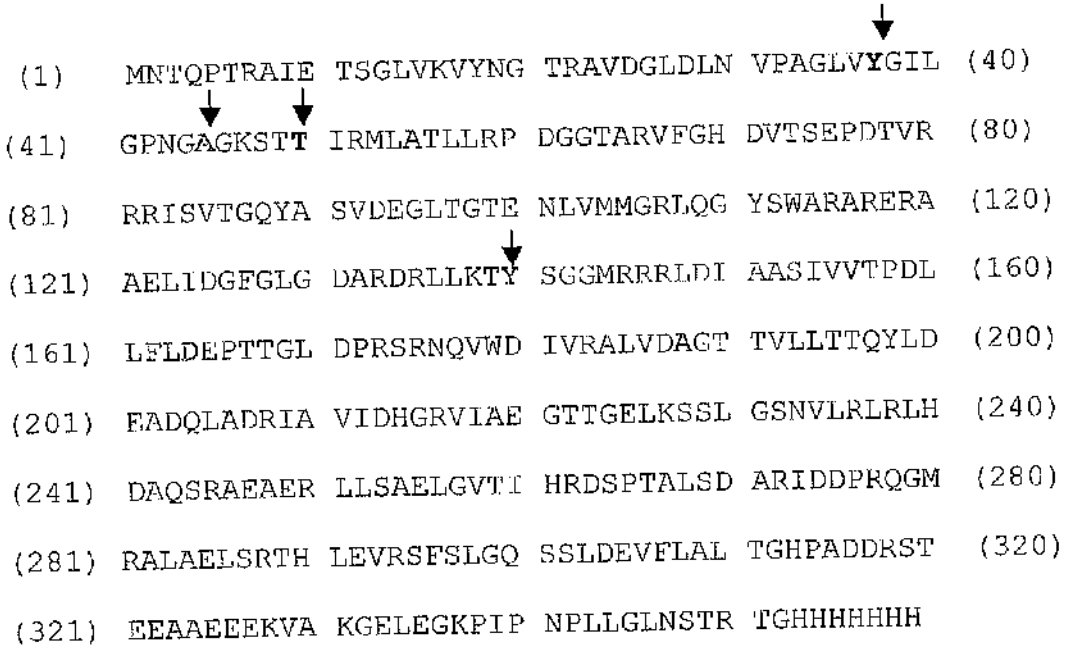



**Figure 7.3 Positions of tryptophan mutations in the DrrA component of Thio-DrrA**

Residues selected for mutation by site directed mutagenesis are indicated in bold and shown by the arrow, corresponding to the mutations Y37, A45 and T50 around the Walker A motif, and the mutation Y140 that corresponds to the former ABC signature sequence mutation. In all these mutations, the original aminoacid was replaced by a tryptophan. Red text indicates the DrrA Walker A motif, blue shows Walker B and green highlights the ABC transporter signature-sequence.

### 7.3.1.1 GeneEditor System (Promega):

To use the GeneEditor system, only a single primer was needed to produce the mutation and synthesise the mutated DNA. Its sequence was:

Primer SP DrrA
Sequence (5' to 3'): CTC CTG AAG ACC TGG TCC GGT GGC

The original TAC codon was converted into TGG (fifth base triplet in the primer)

The first step in this protocol was to denature by alkali the plasmid DNA containing the gene of interest, and then use single stranded DNA as template for synthesis. This step was accomplished (Fig. 7.4), but no bacteria could be recovered that were transformed with the newly synthesised DNA plasmid. Numerous attempts to repeat the alkali denaturation step did not show the expected pattern on gels with the single stranded DNA migrating ahead of the double stranded (Fig. 7.5). Although modifications were introduced such as the use of higher temperatures (70-80 °C) or longer incubation times (10-30 minutes) instead of room temperature and the five minutes suggested by the protocol, the electrophoresis pattern expected for denatured DNA could not be detected and transformation of competent cells with newly synthesised mutant DNA proved elusive.

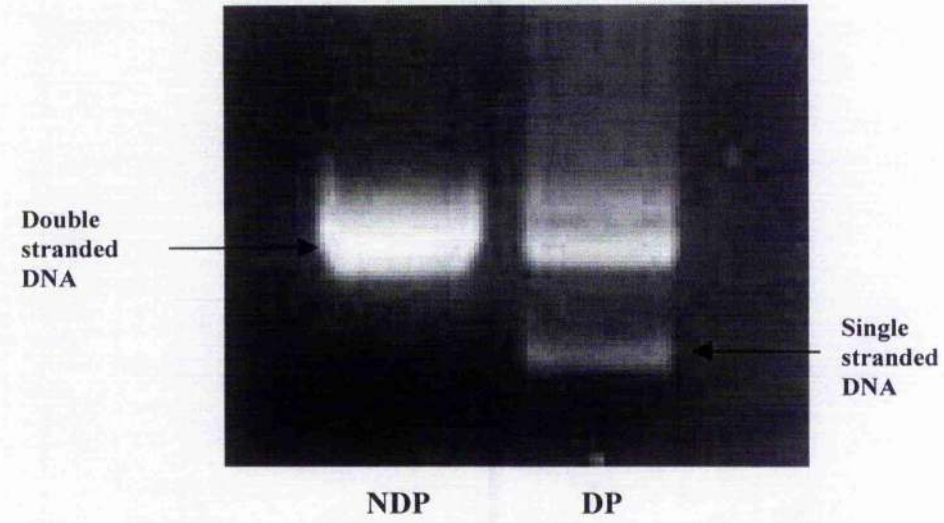



**Figure 7.4 Alkali-mediated DNA denaturation of pBADTOPO *thio-drrA***

NDP: Non-denatured plasmid DNA

DP: Denatured pBAD *thio-drrA* plasmid

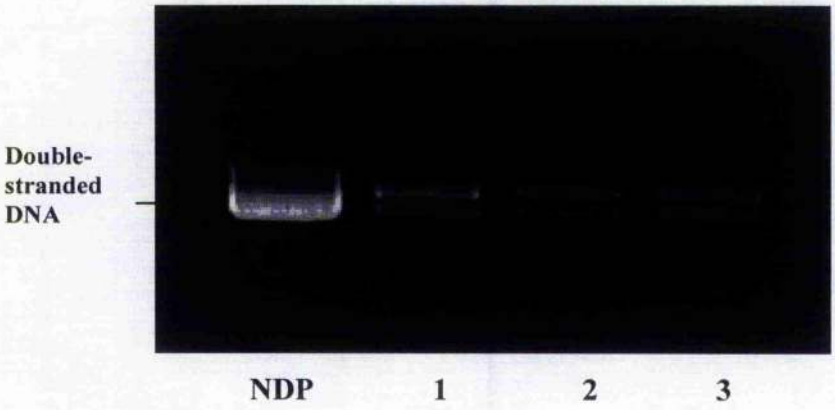



**Figure 7.5 Unsuccessful attempts to generate alkali denatured DNA from pBADTOPO *thio-drrA***

NDP: Non-denatured plasmid DNA

1-3: Plasmid preparations treated with alkali

### 7.3.1.2 QuickChange Site-Directed Mutagenesis (Stratagene)

Site-directed mutagenesis was also attempted with this methodology from Stratagene. In this protocol, it was necessary to construct a pair of complementary primers each carrying the mutation towards the middle of the primer, with five codons of native sequence at either side.

#### 7.3.1.2.1 Mutagenesis in the Thio-DrrA ABC signature

The complementary primers bearing the mutant sequence are shown below:

Primer DrrA Y140 forward
Sequence (5' to 3'): CGG CTC CTG AAG ACC TGG TCC GGT GGC ATG CGG

Primer DrrA Y140 reverse
Sequence (5' to 3'): CCG CAT GCC ACC GGA CCA GGT CTT CAG GAG CCG

Twenty-five and 50 ng of double-stranded pBADTOPO *thio-drrA* DNA were used as template, and with both reaction mixtures, transformants were obtained that carried the mutation. Transformants were selected for resistance to ampicillin, the antibiotic marker present in the plasmid.

The mutant DNA carried the original pBADTOPO *thio-drrA* plasmid sequence with the original tyrosine codon (YSGGM) mutated to tryptophan (WSGGM). The mutation was confirmed by DNA sequencing. Plasmid DNA was purified and transformed into *E. coli* LMG194 for expression of the mutated Thio-DrrA. The ATPase activity of this modified protein was similar to that of wild-type Thio-DrrA (Table 7.1). The protein was used in stopped-flow spectroscopy experiments, but no improvement could be detected in fluorescence signals in the presence of ATPase reaction components.

#### 7.3.1.2.2 Mutations inside and around the Thio-DrrA Walker A motif

In ABC transporters, two other regions have highly conserved motifs and are implicated in nucleotide binding and hydrolysis, the Walker A and Walker B motifs.

The Walker A site is implicated in the binding of phosphate and consequently is known as the phosphate loop. If a tryptophan, generated by a single mutation in this region of the protein was fluorescence-responsive, an interaction with the substrate should change the fluorescence signal.

Single amino-acid mutations were designed for Thio-DrrA to either sides of the Walker A motif, and within the motif. In all mutants, a tryptophan residue replaced the original amino acid. In the first mutation, a tyrosine at position 37 was altered to tryptophan (Fig. 7.3). In the second, the alanine inside the Walker A motif was changed as illustrated below:

Native DrrA Walker A motif 41- **G** P N G A **G K** S -48

↓

Mutant (A45) 41- **G** P N G W **G K** S -48

The highly conserved residues in the motif are in bold. A third mutation involving substitution of a threonine at position 50 was devised to the other flank of the Walker A motif (Fig. 7.3). All these plasmids were then purified from *E. coli* XL1 Blue and transformed into *E. coli* LMG194 for expression of the Thio-DrrA mutants.

Although the Thio-DrrA mutants showed ATPase activity (Table 7.1), they did not give appropriate fluorescence signals in the stopped flow assays. Mutant Thio-DrrA Y37 showed 40% of the ATPase activity of wild-type Thio-DrrA, but its fluorescence was not responsive to the binding of ATP (Fig 7.6 A and B). For Thio-DrrA A45W, the protein lost almost all ATPase activity, indicating that the integrity of the Walker A motif is essential for activity (Fig. 7.6 C and D). For Thio-DrrA T50W, the protein retained full ATPase activity but did not give a fluorescence signal in stopped flow, although some signal amplification was suggested by the data (Fig. 7.6 E and F).

Considering the SFF spectra obtained for the Thio-DrrA mutants, there are no net changes in fluorescence that appear to be linked to ATP binding or hydrolysis. Variation in the ATP concentration did not alter the strength of the fluorescent signal. The ATP analogue MANT-ATP possessed a fluorescence signal several times higher

than that of ATP, but no improvement was noticed using this reagent, the main problem appearing to be a low signal-to-noise ratio. Additional mutations were generated to convert the tryptophan residues present in the Thioredoxin moiety into glycine to reduce the background signal. For example, a tryptophan-free Thioredoxin moiety was exchanged into the Thio-DrrA T50 mutant. Assays with different ATP concentrations, ADP and MANT-ATP were performed in the stopped-flow instrument, but no improvement in the tryptophan fluorescence signal could be detected.

**Table 7.1 ATPase activity of mutant Thio-DrrA proteins constructed for stopped-flow assays**

| Thio-DrrA Protein | Specific activity (nmoles Pi/min/mg protein) |
|---|---|
| Wild-type | 40±2 |
| Y140W | 34±4 |
| Y37W | 15±3 |
| A45W | 3±0.5 |
| T50W | 37±3 |

The malachite green $P_i$ release assay was used to calculate the rate of ATP hydrolysis. ATP assays were performed at 37 °C and initiated by the addition of 45 µl of $MgCl_2$. The total volume for each assay was 500 µl with 45 µl of sample analysed for $P_i$ content at 5-minute time intervals over a time course of 15 minutes. A standard curve was used to convert absorbance values at 610 nm to nanomoles of $P_i$. Final values were expressed as specific activity in nanomoles of $P_i$ released/minute/mg of protein.

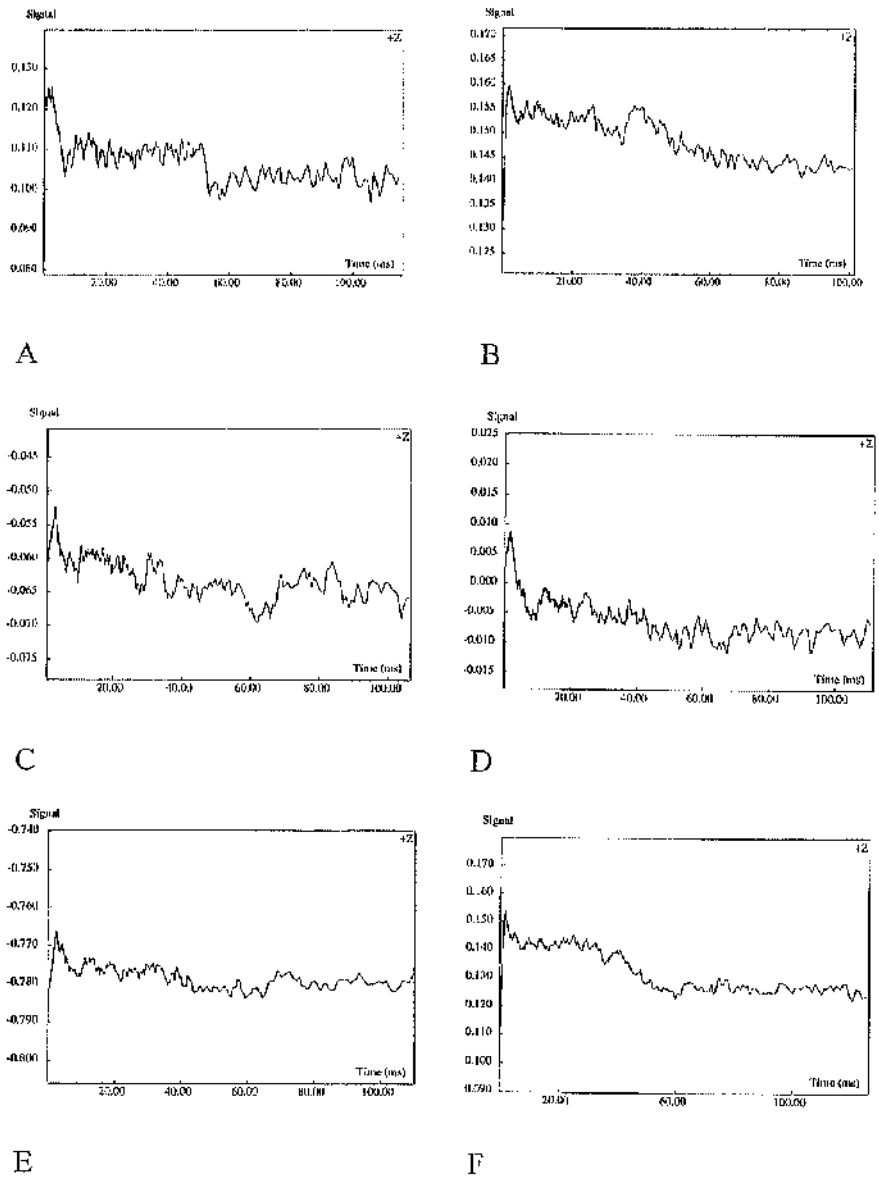



**Figure 7.6 Stopped flow fluorescence spectra of Thio-DrrA mutant proteins in presence and absence of ATP and $Mg^{2+}$**

Stopped-flow fluorescence traces generated by mixing 5 _M Thio-DrrA proteins with 1 mM ATP and 2 mM $MgCl_2$ (final concentrations in the mixing chamber). ATP fluorescence was excited at 285 nm and emission data collected above 320 nm.

A and B represent **Y37 Thio-DrrA**; C and D, **A45 Thio-DrrA**; E and F, **T50 Thio-DrrA**, all in absence and presence of ATP and $Mg^{2+}$ respectively.

## 7.4 Summary

Analysis of the DrrA proteins was carried out by fluorescence spectroscopy to monitor changes in tryptophan fluorescence in presence of ligands. Intrinsic tryptophan fluorescence was assayed for DrrA purified from inclusion bodies, denatured and subsequently refolded using a urea gradient, to evaluate the success of the procedure. The data were consistent with the protein possessing a folded conformation but one that lacked ATPase activity.

Intrinsic tryptophan fluorescence was also assayed for Thio-DrrA proteins. With these proteins, a high inner filter effect was noted from ATP. This obscured any changes in tryptophan fluorescence that might be caused by ligand interaction with the protein.

Stopped flow-fluorescence was employed in an effort to detect and evaluate changes in Thio-DrrA fluorescence in response to ligand binding. Substantial and consistent changes in tryptophan fluorescence could not be detected. To overcome this, mutations were introduced at locations close to or within the motifs implicated in ATPase activity of the protein, such as the Walker A (Y37, A45, and T50) and the ABC signature (Y140) motifs. Although the mutants retained ATPase activity, no improvement in tryptophan fluorescence was obtained. Finally, a pair of tryptophan residues present in the Thioredoxin moiety was converted to glycine residues in an attempt to eliminate the background fluorescence of the fusion proteins. Analysis of the resulting Thio-DrrA constructs did not show any improvement.

# Chapter 8

## Final Discussion

*"DrrA is a component of the DrrAB pump of S. peucetius and its role is to drive the efflux of the anthracycline compounds doxorubicin and daunorubicin out of the cell. DrrA is a protein located peripheral to the membrane and has the characteristics of a cytoplasmic protein".*

ABC transporters are formed by several functional units and organised into modules consisting of a minimum of four functional domains - two ATP-binding/hydrolysing domains and two membrane-spanning domains (Higgins *et al.*, 1986). In contrast, the model for the *S. peucetius* ABC transporter DrrAB proposes that it is composed of two functional domains: a nucleotide binding domain (NBD), and a transmembrane domain (TMD). It has been suggested that the NBD was duplicated and that this event also happened with the original TMD, resulting in a $DrrA_2B_2$ stoichiometry for the complex (Kaur and Russell, 1998).

That was the state of knowledge about the experimental system chosen for this project at its start. If the ATP binding domain could be functionally separated from the membrane domain of the transporter, it was argued that it might be possible to overproduce the domains separately for detailed structure-function studies. As the doxorubicin energy-dependent pump was thought to comprise distinct membrane and ATPase domains, this goal seemed achievable. The wider importance of the study remains that if the molecular mechanism for pumps linked with drug resistance can be better understood, this will provide a rational basis for combating the threat posed by microorganisms resistant to multiple antibiotics, and improving cancer chemotherapy.

Many other soluble proteins from bacteria have been successfully overexpressed as heterologous proteins in *E. coli*. There was no *a priori* reason to think that this would not be the case for DrrA and the expectation was that DrrA would be expressed in sufficient quantities to perform a complete study of its properties. However, this did not prove to be the case. Although substantial quantities

of recombinant protein were overexpressed using a pET-based construct, the vast majority of the product was present as inactive, insoluble inclusion bodies. The lack of biological function associated with this form of the protein impeded further biochemical analysis of native DrrA. Diverse trials were conducted to refold the aggregates from inclusion body into an active conformation but this proved to be futile. It has been suggested that certain structural features of the NBD promote non-physiological interactions between individual molecules, resulting in aggregation and insolubility (Yuan *et al.*, 2001), and it is now known that inclusion body formation is a rather common event when overexpressing NBDs of ABC transporters.

Since DrrA could not be expressed as a native protein and refolding proved unsuccessful, an alternative mode of expression was sought. By expressing DrrA as a translational fusion to *E. coli* Thioredoxin, a stable soluble Thio-DrrA was eventually produced in quantities just sufficient for analysis.

Similar approaches had been used extensively for the heterologous overexpression of both prokaryotic and eukaryotic ABC transporter NBDs. Even so, expression of a DrrA fusion protein from a pBADTOPO *thio* vector was not easy. Low concentrations of inducers did not really improve the yield of soluble protein at 37 °C. A change of temperature to 25 °C at induction was not sufficient to provide soluble Thio-DrrA. This goal was only reached by dropping the temperature close to 17 °C, when finally a small quantity of soluble, active DrrA and rather more Thio-DrrA were obtained. These results led to the characterisation of Thio-DrrA, a form of DrrA that showed signs of ATPase activity. The presence of additional sequence, not least the Thioredoxin moiety, could impact upon the behaviour of DrrA compared to its native form.

Given these problems, might the overexpression of DrrA have been easier in a host closer to *S. peucetius*? This strategy was seen as inconvenient from the beginning of the project. There was no previous experience in the team to support research along these lines, and the longer culture times for *Streptomyces* was seen as an obstacle. In contrast, *E. coli* was extensively used by others in the research team for the expression of heterologous proteins and its well characterised genetics,

biochemistry and physiology and the extensive availability of vectors for overexpression were seen as major advantages.

The characterisation of DrrA was mainly based on its ATPase activity and this was assessed by release of phosphate, as determined by a malachite green/ammonium molybdate assay. To evaluate this activity in the different trials assayed and to observe clearly their differences with control experiments, concentrations of Thio-DrrA were used that would generate $A_{610nm}$ values close to 0.34 at the end of the reaction. A protein concentration of 1 μM (about 52 μg/ml) was found to carry activity too low to meet this criterion for some preparations. This variation suggested that the protein was not being expressed with complete ATPase activity and that some variation in this property was evident, batch to batch. In consequence, protein concentrations between 1 and 3 μM were required to meet the assay criteria. This approach was adequate for qualitative comparisons. The ATPase activity of the Thio-DrrA fusion protein was shown to exhibit cation dependence, a feature consistent with its proposed role as the energy transducing sub-unit of an ABC transporter. The structural integrity of the Walker A nucleotide-binding motif, a feature shared by other ATPase families, was also important and a mutant protein in which this motif was disrupted displayed much reduced levels of ATP hydrolysis. Thio-DrrA was shown to exhibit a broad range of substrate preferences with regard to nucleotide triphosphates and divalent cations. This feature is not uncommon amongst isolated bacterial NBDs and may be seen as a further indication that the behaviour of Thio-DrrA is typical for an NBD. These data are the first to show that the *S. peucetius* DrrA protein is a functional ATPase.

However, the low specific activity of Thio-DrrA compared to other ATPases was notable and disappointing. Although the $V_{max}$ data showed variations between experiments, it was reasonable to assume that the $K_m$ of the DrrA proteins would be constant. However $K_m$ values derived for Thio-DrrA were a ten times higher than the same parameter for the isolated DrrA protein, indicating that something was affecting the properties of the fused protein. Heterogeneous ATPase activities might be linked to the presence of mixtures of soluble but inactive DrrA fusions with active forms of the protein. This important phenomenon has been encountered by other investigators and purification strategies have been proposed based upon monodispersity (see

Nomine *et al.*, 2001). Expression of isolated DrrA from the pET vector system always produced protein with a high activity, although yields were consistently low and could not be improved.

A number of biochemical and structural data suggest that the ATPase active form of ABC transporter NBDs is a dimer, as shown for the Rad50 and MalK (Hopfner *et al.*, 2000; Chen *et al.*, 2003). A dimer formation for Thio-DrrA could not be further corroborated because of aggregation problems with Thio-DrrA, and insufficient quantities of DrrA protein for size exclusion chromatography.

The ATPase activity of Thio-DrrA and DrrA did not appear to be modulated by the presence of the putative DrrAB substrates doxorubicin and daunorubicin. This argues against the presence of an antibiotic binding site or sites on DrrA. DrrA thus does not seem to be an allosteric protein like ArsA as has been suggested by Kaur (Kaur, 1997). Interaction between DrrA and DrrB could not be detected and overall these experiments failed to take forward an understanding of the manner in which DrrA interacts with the substrates and the membrane components of this pump. This phenomenon has been reported in other systems (Nash, 2003; Nikaido *et al.*, 1997; Morbach *et al.*, 1993), where isolated components of ABC systems behave differently when assayed in dilute solution rather than as part of the entire transporter complex. It seems clear that isolated DrrA retains it ATPase activity but how ATP hydrolysis mechanism is coupled to multidrug extrusion remains unclear.

Since the ATP binding domain is strongly conserved among ABC transporters with various specificities, it seems unlikely that this domain is involved in initial substrate recognition. As a consequence, identification of an ABC-type nucleotide binding domain alone cannot be taken as evidence for a putative MDR transporter (Allikmets *et al.*, 1993; Karow and Georgopolous, 1993). It should be stressed that alterations in the drug resistance profile have been observed as result of mutations in the nucleotide binding domain, which indicate an intimate relationship with the hydrophobic domain(s) (Beaudet and Gross, 1995). Both biochemical and structural data are beginning to suggest that the ABC transporter function is highly dependent upon interactions between the various domains of the complex. As such, a complete

understanding of its function may require researchers to adopt a "system-approach" to its analysis, rather than more traditional techniques.

To achieve a more detailed characterisation of the NBD of the *S. peucetius* DrrAB pump, in particular the mechanism by which DrrA interacts with its substrate, efforts should be directed to finding a better vehicle for overexpression and purification. Fusion with maltose binding protein and use of a Nus tag has been promoted for improving yields of soluble protein, but it is clear that no fusion system can guarantee overcoming aggregation problems. Vectors containing promoters weaker than T7, should be assayed but it should also be clarified if insertion of DrrA into the bacterial membrane independently or as a component of the DrrAB system influences its activity.

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