

Studies of the Histidine Permease of Salmonella typhimurium
in Escherichia coli and Methylophilus methylotrophus

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To my husband Tim

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AIMS OF THIS STUDY

The ultimate objective of this study was to introduce the histidine permease operon into methylophilus methylotrophus, a Gram negative organism with no known active amino acid uptake systems.

The initial objective was to identify the polypeptide components of the histidine permease; crude identification experiments had been attempted by G.F-L Ames and co-workers, however the proteins, which are synthesised in very small amounts, had not been overproduced sufficiently to clearly and unambiguously identify all the components.

The entire sequence of the histidine permease operon had recently been published prior to the commencement of this work. This sequence revealed a fourth open reading frame suggesting an additional membrane component HisM. The complete restriction map elucidated from the sequence would enable me to conveniently subclone the operon into expression vectors which ought to allow the overproduction of these protein components and therefore aid in both identification and localisation of each of the proteins within the cell.

From the onset of this work it was anticipated that overproduction of the membrane components of the periplasmic permeases might be lethal; therefore experiments were designed to attempt to overcome any such problems.

Ames and co-workers had formulated a model for the entire structure of the histidine permease prior to the onset of my work, however, most of the evidence available at that time stemmed from genetic mutation

analysis or more recently from examination of the protein structure from the translated nucleotide sequence; firm polypeptide analysis was not available. It was during the course of this work that additional biochemical analysis of the permease polypeptides emerged from Ames laboratory.

Having cloned these genes into an expression vector, it was hoped that they could then be transferred to a shuttle vector which is able to replicate in both E. coli and M. methylotrophus in order that the genes could be introduced into M. methylotrophus. Finally, once introduced, an assay system must be developed to determine whether the genes are expressed, the proteins assembled and the permease functional within this foreign organism.

CHAPTER 1

Introduction

1.1 Introduction

Biological membranes provide a highly selective permeability barrier which separates the cellular contents from the surrounding medium. Prokaryotic cells simply have their cytosol surrounded by one or two membrane layers together with a cell wall, whilst eukaryotic cells possess a number of membranes which form intracellular organelles such as mitochondria, nuclei, lysosomes and chloroplasts.

Membranes consist of lipid bilayers which prevent many substances from entering and leaving the cell. Although small non-polar molecules can cross this lipid bilayer by simple diffusion; bacteria rely mostly on specific active transport systems for both the accumulation of essential nutrients, such as sugars and amino acids, and the regulation of their intracellular pH by the extrusion of toxic end products of fermentation. These active transport processes are mediated by a multitude of specific protein molecules located within the membrane's lipid bilayer.

The transmembrane transport of molecules has proved difficult to study. Firstly, membrane proteins are generally highly hydrophobic and as such, are difficult to purify; secondly these proteins are generally present in very small amounts within the membrane. The advent of recombinant DNA techniques has circumvented these problems: The genes encoding the transport proteins can be cloned into plasmid vectors allowing increased

expression of these genes, and resulting in overproduction of the proteins, thus facilitating easier purification. Biochemical techniques employed to monitor the process of transport of molecules across biological membranes either in whole cells or membrane vesicles are unable to separate the multiple transport systems that may exist for any given substance, and also fail to determine the details of the molecular mechanisms for the individual transport systems. Genetic techniques have enabled transport deficient mutants to be isolated in bacteria proving invaluable in determining the number of proteins involved in each transport system and distinguishing the multiple transport systems for any single substrate molecule. DNA and protein sequence analysis can determine the sequence of amino acids constituting the membrane protein and, by comparison with other sequences of known function, can help identify the specific functional sites within this transport protein.

Having established the components of a transport system, the mechanism of action must then be elucidated. This could be achieved by having these components function in a much simpler system, such as membrane vesicles. These vesicles, which consist purely of cytoplasmic membrane and the proteins associated with such, enable reconstitution of a specific transport system. Such experiments have been performed for both histidine and glutamine transport (Hunt and Hong, 1981; Hunt and Hong, 1986). Both systems require the addition of the substrate binding protein and an energy source to achieve active transport into the vesicle. An alternative approach would be to use a Gram negative bacterium which lacks amino acid permeases. Methylophilus methylotrophus is a methylotroph with such properties and the experiments outlined in this thesis attempt to reconstruct the multicomponent histidine permease

within this organism. Success in this approach would provide an in vivo system for studying the assembly and functionality of the transport proteins expressed from a plasmid vector without the interference of any other amino acid permease.

1.2 Molecular architecture of the Gram-negative bacterial cell envelope

The bacterial cell is surrounded by a complex cell envelope; this usually consists of the cytoplasmic membrane and the cell wall. It is the difference in composition of the cell envelope which distinguishes Gram-positive and Gram-negative bacteria; the latter possessing an outer membrane located outside the layer of peptidoglycan. The structure of the Gram-negative cell envelope has been extensively reviewed (Nikaido and Nakae, 1979, Osborn and Wu, 1980, Hall and Silhavy, 1981, Lugtenberg and Van Alphen, 1983, Pugsley and Schwartz, 1985) and except where indicated, the following information has been taken from these reviews.

The Gram-negative bacterial cell envelope therefore consists of three layers, the cytoplasmic membrane, the peptidoglycan and the outer membrane (Fig. 1.1). Proteins are located in the inner cytoplasmic membrane, the outer membrane and the periplasmic space separating the two membrane layers. These envelope proteins under certain conditions (ie. slow growth) may amount to as much as 20% of total cellular protein.

Figure 1.1 Structure of the E. coli cell envelope

(Drawn by I.B. Holland

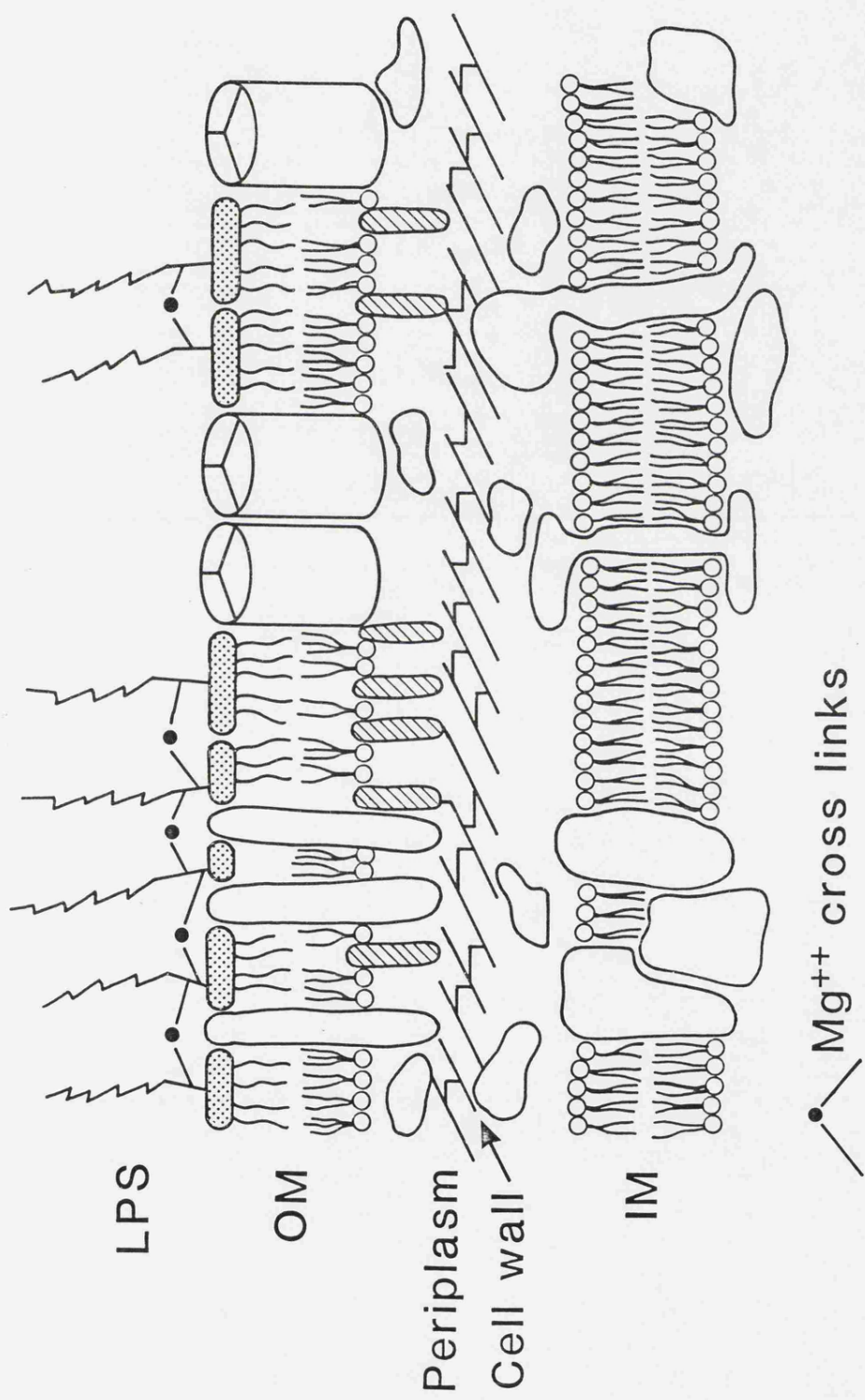
From Mackman et al., 1986)

LPS lipopolysaccharide

OM outer membrane

IM inner membrane

See text for details



1.2(i) The cytoplasmic membrane

The cytoplasmic membrane is a typical bilayer structure composed of phospholipids and proteins. There are numerous proteins within this membrane many of which are involved in the transport of nutrients, oxidative phosphorylation, electron transport and the synthesis of phospholipids, peptidoglycan and lipopolysaccharides. Some proteins may also be involved in cell division.

1.2(ii) The cell wall

The cytoplasmic membrane is surrounded by the cell wall. This is composed of a layer of peptidoglycan consisting of a network of linear amino sugar chains covalently linked to each other via cross links between the peptide side chains. This structure confers rigidity to the cell but at the same time is regarded as an entirely permeable layer forming an open network which readily allows the diffusion of solutes.

1.2(iii) The outer membrane

The outer membrane is composed of an asymmetrical bilayer of lipopolysaccharide to the outer surface and phospholipid to the inner surface.

In Gram-negative bacteria such as E. coli and S. typhimurium which live in the intestinal tract of animals, the outer membrane has evolved into an effective barrier against the detergent-like action of bile salts and degradation by proteolytic and lipolytic enzymes. The outer membrane

contains several different proteins which are involved in nutrient uptake, mediated by channel-forming proteins; lipid metabolism and maintenance of cell wall integrity. Many outer membrane proteins also serve as bacteriophage or colicin receptors. Another important function of the outer membrane is to provide the bacterial surface with strong hydrophilicity; this is important in evading phagocytosis, creates some complement resistance and enables the cell to avoid a specific immune attack by altering the surface antigen construction (Nikaido and Vaara, 1985).

1.2(iv) The periplasm

The periplasmic space is the gap between the outer and the cytoplasmic membrane. It is the location of peptidoglycan, and of the 'periplasmic proteins'. Three classes of periplasmic proteins can be distinguished with respect to their function.

- (1) Proteins with a catabolic function (e.g. 5' nucleotidase and alkaline phosphatase) convert solutes for which no transport system exists to a form that can be transported through the cytoplasmic membrane.
- (2) The binding proteins, which have affinity for nutrients like sugars, amino acids or ions (see Section 1.4.2(ii)).
- (3) Enzymes which are involved in the degradation or modification of harmful components such as antibiotics and heavy metals (e.g. β -lactamase).

1.3 Transport across the outer membrane

The selective permeability of the Gram-negative envelope is enforced by both the outer and inner membranes.

The outer membrane contains hydrophilic pores composed of pore forming proteins, porins, which provide extremely good permeability for nutrients and other solutes but at the same time allow the exclusion of bile salts and other harmful substances (Nakae and Nikaido, 1975, Decad and Nikaido, 1976).

The exclusion limits of these porins in E. coli and S. typhimurium was determined to be around 600 daltons (Nakae and Nikaido, 1975). Outer membrane vesicles reconstituted from phospholipids and outer membrane proteins were found to be permeable to low molecular weight oligosaccharides only when OmpF and OmpC outer membrane proteins were included for E. coli with the addition of OmpD outer membrane protein for S. typhimurium (Nakae, 1976b). Mutant strains deficient in one of these porin proteins display a greatly reduced ability to utilise low concentrations of several low molecular weight metabolites (Lutkenhaus, 1977). Thus, these porins function to form passive diffusion channels through the outer membrane and when extracted with SDS exist as undenatured trimers (Nakae et al., 1979). Electron microscopy shows the necessity of a tight association of these subunits for functionality (Dorset et al., 1983). The E. coli OmpF and OmpC proteins also act as receptors for bacteriophage Tu1a and Tu1b respectively (Datta et al., 1977).

Nutrients having a molecular weight greater than the exclusion limit of the porin proteins require their own more specific uptake system to pass through the outer membrane. For example, the uptake of iron as a complex with ferrichrome (740 daltons) or enterochelin (746 daltons) depends on the presence of specific receptors (Neilands, 1982).

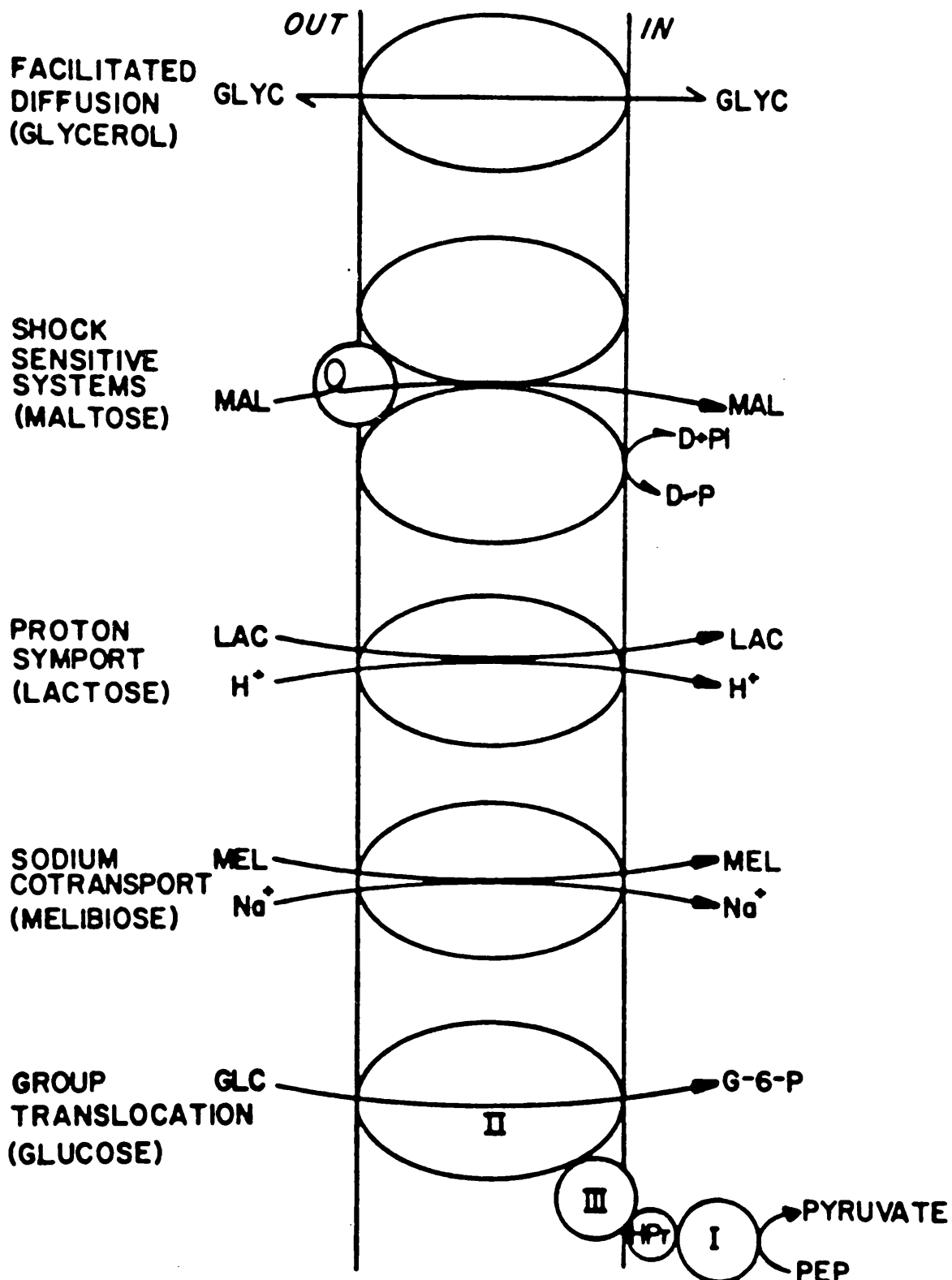
In E. coli the receptor of bacteriophage lambda is an outer membrane protein encoded by the lamB gene which also plays a role in the uptake of maltose and maltodextrins (Szmecman and Hofnung, 1975; Szmecman et al., 1976).

Von Meyenburg and Nikaido (1977) first indicated that the λ receptor also facilitates the diffusion of other nutrients; in vitro experiments reveal its ability to form a general transmembrane diffusion channel (Luckey and Nikaido, 1980, Boehler-Kohlem et al., 1979, Nakae, 1979a). Evidence for co-operation of the λ receptor with the periplasmic maltose binding protein in the uptake of maltose has been obtained (Wandersman et al., 1979), this interaction may also contribute to the preference of the pore for certain solutes. The protein has been purified and the active form found to be trimeric (Palva and Westerman, 1979). The protein has neither chemical (Endermann et al., 1978) nor immunological (Overbeek et al., 1980) similarities with other major outer membrane proteins; but its molecular structure evaluated from sequence data (Clement and Hofnung, 1981), protease accessibility, monoclonal antibody studies, phage resistant mutations and foreign epitope insertion suggest that the folding of other outer membrane proteins bears striking resemblance to the structure predicted for LamB (Gehring et al., 1987).

Figure 1.2 Carrier mediated transport mechanisms (Dills et al., 1980)

Examples are given in parenthesis of each defined system for the transport of molecules across the cytoplasmic membrane. At least one specific membrane protein is involved in every mechanism.

Abbreviations:	GLYC	glycerol
	MAL	maltose
	LAC	lactose
	MEL	melibiose
	GLC	glucose
	G-6-P	glucose-6-phosphate
	PEP	phosphoenolpyruvate
	I	enzyme I
	II	enzyme II
	III	enzyme III
	HPr	Heatstable protein



1.4 Transport across the cytoplasmic membrane

In contrast to the limited non specific permeability of the outer membrane, the inner, cytoplasmic membrane is impermeable to almost every solute unless a special transport system is present. These solute-specific transport systems fall into a number of different categories but all require at least one specific transport protein, the permease. These permeases exhibit marked selectivity for the transported molecule. Nutrient uptake usually constitutes the rate-limiting step in the metabolism of various carbon and energy sources. Many substrates e.g. histidine, can be transported by more than one system, indicating that the bacterium is able to adapt to changing environmental conditions. Low affinity transport systems may be constitutive whilst high affinity systems can be induced in times of need.

The different types of transport systems can be defined as follows; and are summarised in Fig. 1.2.

(1) Facilitated diffusion

There is only one system described for E. coli in which translocation of a hydrophilic compound through the cytoplasmic membrane is mediated by simple passive diffusion. The substrate is glycerol, and it appears that there is a specific glycerol facilitator within the membrane which exhibits specificity for glycerol and larger non-cyclic polyalcohols (Lin, 1976; Heller et al., 1980). This mechanism is therefore known as facilitated diffusion, the only driving force being the difference in concentration of the molecule across the membrane. The cells are not capable of accumulating substrate across a concentration gradient.

(2) Active transport

Active transport systems are energy coupled and accumulate substrates against a concentration gradient. There are three distinct classes of active transport in bacteria, as defined by the source of metabolic energy used.

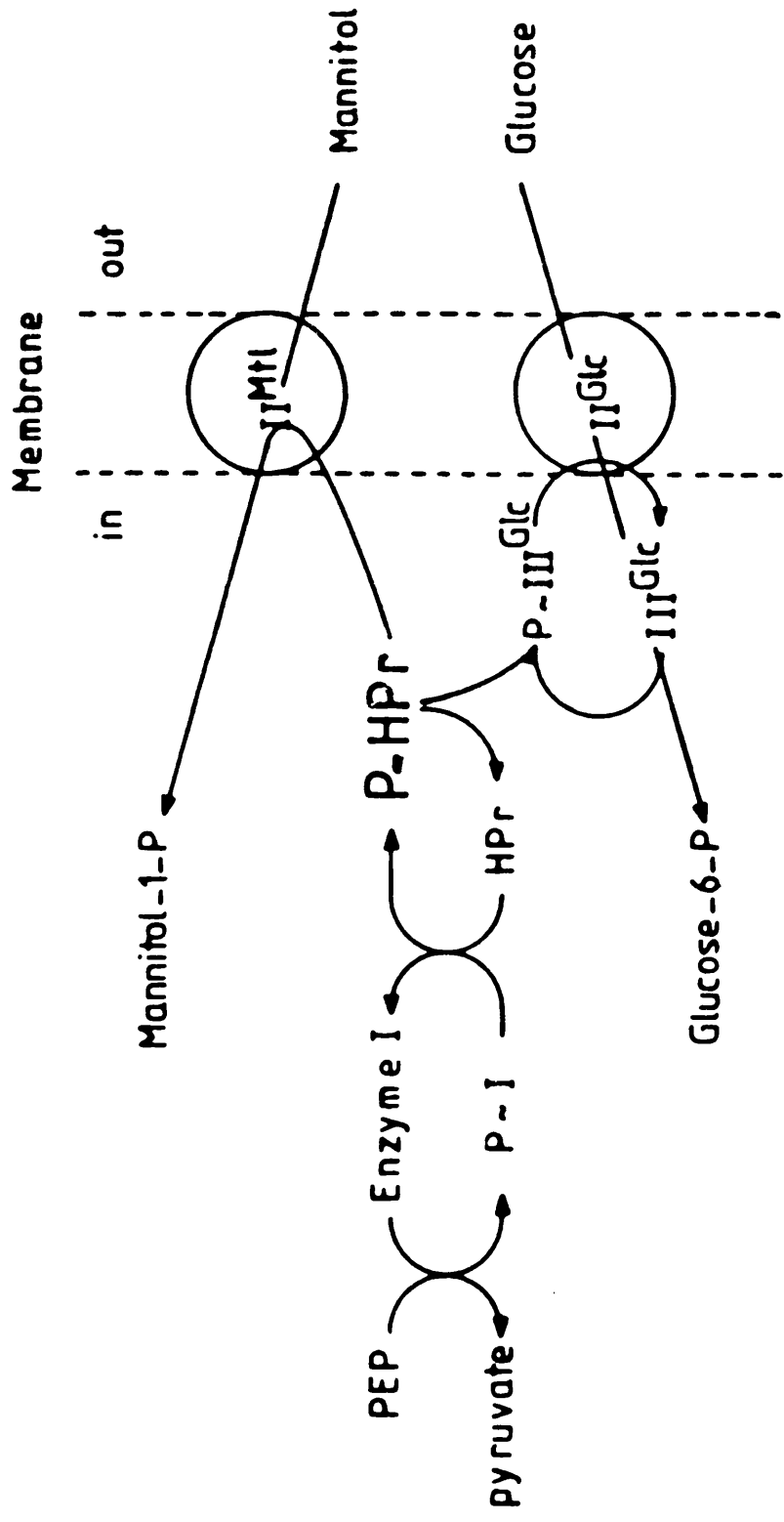
(i) Group translocation

During this process the transport of molecules across the membrane is coupled with a chemical modification of the substrate. The finding that some sugars were accumulated by bacteria as phosphorylated derivatives led to the discovery of this transport process which involves the substrate molecule undergoing phosphorylation as it is transported across the membrane against a concentration gradient (Saier, 1977; Postma and Lengeler, 1985). This phosphotransferase system (PTS) consists of a number of soluble and membrane bound proteins each of which can exist in the phosphorylated and non-phosphorylated state (Fig. 1.3). The soluble proteins, Enzymes I (I) and the low-molecular weight heat-stable protein (HPr), initiate phosphoryl transfer from phosphoenol pyruvate (PEP) resulting in the phosphorylation of HPr. During translocation of a substrate through the membrane, the phosphoryl group from phospho-HPr is transferred to the substrate probably by means of a phospho-enzyme II or III intermediate. Enzymes II and III are both associated with the cytoplasmic membrane; enzyme III being located on the periplasmic face, whereas enzyme II complexes are integral components of the membrane.

Figure 1.3 The phosphotransferase system (PTS)

Different enzyme IIs are specific for different carbohydrates, II^{Glc} from glucose II^{M+L} for mannitol.

Enzyme I and Heat-stable protein (HPr) are required for the first step in transport and phosphorylation of all PTS carbohydrates, resulting in the phosphorylation of HPr at the expense of phosphoenol pyruvate (PEP) the phosphoryl donor. See text for details.



It is enzyme II which appears to serve as the sugar recognition component such that different enzyme IIs recognise a series of structurally related carbohydrates e.g. glucose. This system is able to provide the cell with carbohydrates in the first intermediate form of their catabolism - the carbohydrate phosphate, thus providing a tight linkage between uptake and subsequent metabolism. No free carbohydrate is found inside the cell; however the cell is provided with intracellular carbohydrate phosphates as a source of carbon and energy.

(ii) The permeases

Over 80% of known transport systems in E. coli and S. typhimurium fall into the final category which consists of two distinct groups. These groups can be distinguished according to their response to a physical treatment, osmotic shock (Neu and Heppel, 1965), into shock sensitive and shock resistant permeases. Osmotic shock involves plasmolysing the cells in sucrose in the presence of EDTA and Tris buffer to 'weaken' the outer membrane, followed by a rapid dilution in cold distilled water which causes the outer membrane to rupture. During this process the contents of the periplasm are released.

(a) Shock resistant permeases

Members of this class of transport system are often referred to as the membrane bound systems since all the proteins required for transport are firmly associated with the cell membrane and are retained in isolated membrane vesicles. Molecules are transported across a concentration gradient without any change to their composition. The classic

representative of this class of active transport system is the transport of lactose by the β -galactoside permease (Overath and Wright, 1983). This protein is the product of the lacY gene and was the first transport protein to be isolated and purified from the E. coli cytoplasmic membrane.

An additional distinction between the shock resistant and shock sensitive permeases lies in the nature of the mechanism of energy coupling (Berger and Heppel, 1976). It has been postulated that shock sensitive permeases depend on substrate level phosphorylation energy (section 1.8) whilst the membrane bound systems utilise the proton motive force across the membrane to drive active transport (West and Mitchell, 1973). Lactose enters the cell via the β -galactoside permease together with a proton, a process known as symport. Both the single component nature of the lactose permease and the process of energy coupling have been verified in vitro using membrane vesicles (Newman and Wilson, 1980).

Protons are the usual coupling ions in E. coli, however a gradient of Na^+ ions has been shown to drive the transport of melibiose (Stock and Roseman, 1971; Tsuchiya et al., 1977), glutamate (Halpern et al., 1973) and proline (Cairney et al., 1984). Of the shock resistant transport systems analysed all appear to be unicomponent with only one protein required to mediate transport of the substrate molecule across the membrane. However, since only the lactose permease system has been characterised in depth it is premature to generalise that every shock resistant permease consists of a single protein.

(b) The shock sensitive permeases

Finally the class of transport process, which will be the topic of this work, is the periplasmic permeases. These are so called because transport is inactivated during osmotic shock, due to the loss of an essential periplasmic protein component. This component is responsible for high affinity binding of the substrate being transported. Together with this periplasmic protein, several membrane proteins are involved in transporting substrate into the cell.

The high affinity histidine transport system of E. coli and S. typhimurium is the system under study here and it was this periplasmic permease of S. typhimurium which was the first transport operon sequenced (Higgins et al., 1982). This sequence data provided evidence for the involvement of more than one membrane protein. The maltose uptake system of E. coli is another well characterised system and I shall discuss these two in detail with respect to this complex transport system (section 1.7). The complexity of these periplasmic permeases may well correlate to the high efficiency displayed by such, since all the systems characterised are capable of concentrating substrate inside the cell against a very large concentration gradient (10^5 fold in the case of maltose, Szmecman et al., 1976). They are also able to scavenge solutes at very low concentrations. The apparent K_m for uptake is $0.01 \mu\text{M}$ for the histidine permease (Ames and Lever, 1970) and $1 \mu\text{M}$ for the maltose permease (Szmecman et al., 1976); by comparison, the apparent K_m for the transport of lactose through the β -galactoside permease is $190 \mu\text{M}$ (Winkler and Wilson, 1966).

A number of the periplasmic permeases have been analysed in great detail including those for transport of histidine, maltose, branched-chain amino acids, oligopeptides, β -methyl galactose, ribose, arabinose and phosphate; the advent of DNA technology in recent years has accelerated this analysis. Since it is the histidine permease which is the subject of this thesis, a detailed analysis of the system will be presented in section 1.6 together with a comparison between the histidine permease system and the other characterised periplasmic permeases (section 1.7).

1.5 Assembly of the protein components of the Gram-negative envelope

This thesis is mainly concerned with the introduction of the complex histidine permease system into a Gram-negative organism Methylophilus methylotrophus and attempts to demonstrate that the system is then functional. General permease assembly into the cytoplasmic membrane and the periplasm must therefore be considered in order to assess the possibility of obtaining a functional permease in M. methylotrophus. Obviously the substrate must also be able to cross the outer membrane. However, since this barrier is not totally impermeable one must assume that histidine is able to pass this membrane by means of a possible 'porin' like protein. Periplasmic proteins are thought to cross the cytoplasmic membrane from their site of synthesis by a mechanism proposed in the signal sequence hypothesis. This subject has been extensively reviewed (Michaelis and Beckwith, 1982; Randall and Hardy, 1984), and extends beyond the scope of this discussion. In general it is thought that periplasmic proteins are synthesised on membrane bound ribosomes (Smith et al., 1977). The 5' end of the gene transcript codes for 15-30 amino acids of a mainly hydrophobic nature, the signal sequence. As the polypeptide being

synthesised emerges from the ribosome this signal sequence is thought to engage a soluble secretory complex which in turn causes polysomes to bind to the membrane. This binding is suggested to recruit membrane proteins which, together with ribosomal components, constitute a membrane pore through which the growing polypeptide chain is co-translationally extruded to the periplasmic side of the membrane whereby the signal peptide is cleaved off (Blobel and Dobberstein, 1975).

The cytoplasmic membrane proteins, however, if synthesised by the same mechanism as described above obviously have to find a mechanism for their retention within the membrane. Several E. coli inner membrane proteins including the penicillin binding proteins PBP5 and PBP6, have also been shown to be synthesised in precursor form (Pratt et al., 1981) but to localise exclusively in the inner membrane. PBP5 is a D-alanine carboxypeptidase which removes the terminal D-alanine from the pentapeptide side chain of pre-existing peptidoglycan (De Pedro and Schwarz, 1981). Since the substrate to this enzyme is located in the periplasm it is predicted that a substantial portion of PBP5 is translocated across the inner membrane. Moreover, it has now been shown that the protein is anchored to this membrane by a specific sequence at the C-terminus (Pratt et al., 1986). The presence of a signal sequence is therefore not surprising. Furthermore, this signal sequence has been shown to contain all the essential information necessary to initiate translocation of OmpF to the outer membrane (Jackson et al., 1985).

The majority of E. coli and S. typhimurium inner membrane proteins are synthesised without a cleavable N-terminal signal sequence. This indicates that these proteins may be synthesised via a different

mechanism than that proposed for the periplasm and outer membrane proteins. These proteins include lactose permease (Ehring et al., 1980), the F_o component of ATPase (Nielsen et al., 1981) and His Q and M of the histidine permease (Higgins et al., 1982).

The N-terminal amino acid sequence of lactose permease synthesised in vitro is identical to the enzyme isolated from the cytoplasmic membrane (Ehring et al., 1980); DNA sequencing confirms this finding (Buchel et al., 1980). The overall amino acid sequence and previous biochemical evidence suggests a highly hydrophobic protein, with several long stretches of hydrophobic amino acids suggesting that the protein may simply partition into the membrane.

The F_o ATPase complex provides a well characterised example of the assembly of a multicomponent inner membrane protein complex (Fig. 1.4). The entire ATPase complex is composed of two structurally distinct entities, the F_1 and F_o complexes. The F_1 complex consists of five different subunits, α to ϵ , all of which are peripheral to the membrane protruding into the cytoplasm; these catalyse the hydrolysis of ATP. The F_o complex is buried within the cytoplasmic membrane and is composed of three kinds of subunit a, b and c, which are thought to provide a transmembrane channel for protons (Senior, 1985).

F_o subunits a and b bind F_1 to the membrane via subunits δ and ϵ (Smith and Sternweis, 1977; Sternweis and Smith, 1977; Walker et al., 1982; Downn and Heppel, 1981), however, all three subunits are necessary for proton conduction (Friedl et al., 1983). By studying the F_o components in F_1 depleted inverted membrane vesicles their arrangement within the membrane was established (Hoppe et al., 1983). A large portion of the b

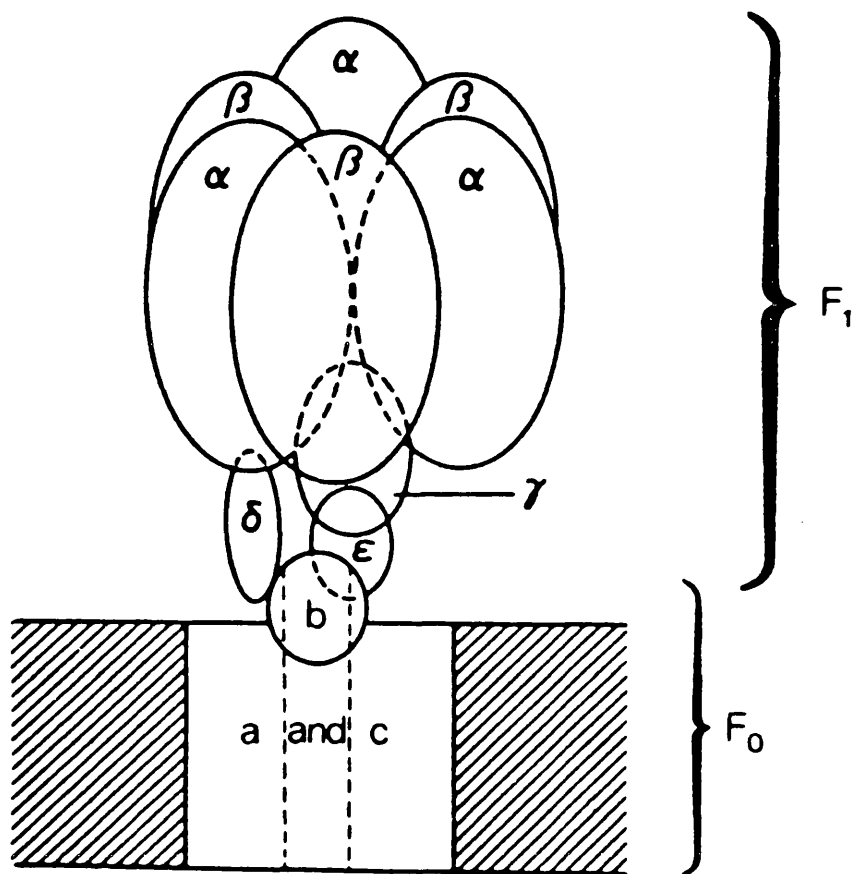
Figure 1.4 Organisation of polypeptides in the ATP-synthase complex.

Redrawn from Walker et al., 1982.

The hydrophobic F_0 subunits, a and c, are buried in the membrane. a and c possibly interact with the hydrophobic amino terminal region of the third F_0 subunit, b. The rest of b is very polar and lies outside the lipid bilayer, making contact with the γ and ϵ subunits of F_1 .

F_1 subunits protrude into the cytoplasm and are responsible for ATP synthesis.

The three F_0 subunits are all required to form a transmembrane proton channel (dotted line).



subunit, an amphiphilic protein, extends from the membrane into the cytoplasm since the protein is highly susceptible to protease digestion in inverted vesicles; the F_1 sector is able to protect against proteolysis confirming that b is responsible for binding F_1 . The a and c polypeptides are very hydrophobic and are less susceptible to attack by proteases, the c subunit being totally protected. This suggests that it is embedded in the membrane, together with the a subunit whose amino acid sequence is typical of an integral membrane protein. The integration into the membrane of the a or b subunits is independent of any other F_o or F_1 subunits (Friedl et al., 1983). Although subunit c was not studied, its hydrophobicity suggests it too is independent of its counterparts. In contrast the F_1 subunits are intimately dependent on each other for binding to the membrane (Sternweis and Smith, 1977) indicating that the F_1 complex forms prior to its binding to the membrane (Futai, 1977). Furthermore, absence of any of the three F_o subunits does not abolish the F_1 complex binding (Friedl et al., 1983). Functionality of this system however is dependent on the presence of the entire complement of the ATPase complex.

The HisQ and M proteins, like ATPase F_o subunits a and c, are highly hydrophobic (Section 1.6) suggesting these proteins too may be able to integrate into the membrane by virtue of their hydrophobicity.

1.6 The components of the histidine permease of *S. typhimurium*

Although the majority of research has been undertaken using the histidine permease of *S. typhimurium*, the high affinity histidine transport of *E. coli* has also been characterised. It is thought that the two systems are analogous in physiological, biochemical and genetical properties (Ardeshir and Ames, 1980).

The histidine permease of *S. typhimurium* as discussed in section 1.4 belongs to the shock sensitive family of permeases. Sequence data (Higgins *et al.*, 1982) revealed four reading frames corresponding to the four proteins involved in high affinity histidine uptake. Early evidence for a multicomponent system arose from data indicating that transport deficient mutants could be obtained which had an intact periplasmic histidine binding protein (Ames and Lever, 1970). Furthermore, the temperature sensitivity of a mutant histidine binding protein could be correlated to temperature sensitivity of transport (Ames and Lever, 1972).

The histidine permease is composed of one periplasmic substrate-binding protein and three inner-membrane-associated proteins. The genes encoding this permease together with the regulatory locus *dhuA* comprise an operon located at 48.5 minutes on the *S. typhimurium* chromosomal map (Ames *et al.*, 1977) (Fig. 1.5).

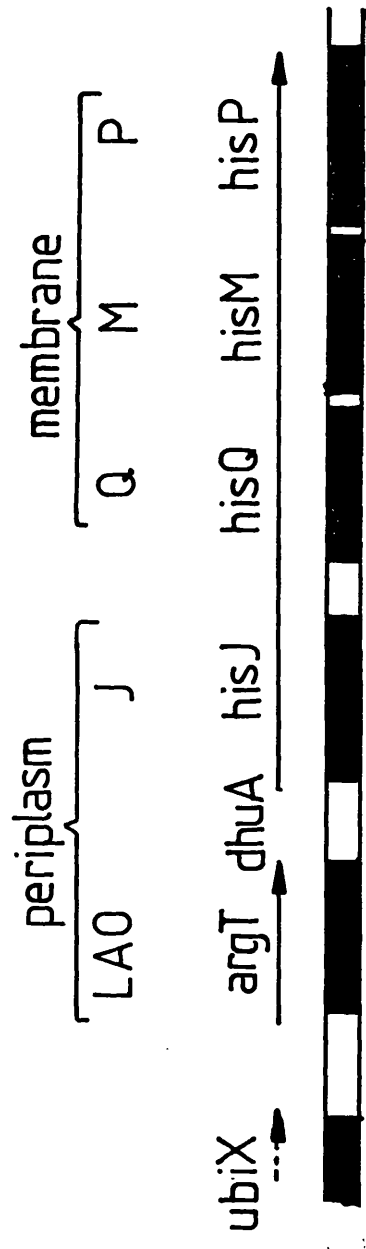
The *dhuA* promoter is able to regulate expression of the histidine permease via the availability of nitrogen (Kustu *et al.*, 1979; Higgins and Ames, 1982; Stern *et al.*, 1984). Transcription is increased under

Figure 1.5

Schematic representation of the histidine permease operon and of the argT gene coding for the LAO protein.

The black areas represent structural genes whereas the white areas represent regulatory regions or non-coding regions.

The horizontal arrows indicate the direction of transcription and presumed size of mRNAs. The presumed location of the proteins is also indicated.



nitrogen limiting conditions, however, the histidine permease operon is still expressed in nitrogen rich conditions and experiments using lac fusions introduced throughout the operon, reveal an induction increase merely of between 1.4 and 2.3 in nitrogen starved cells (Stern et al., 1984).

The hisJ gene encodes the periplasmic histidine binding protein, HisJ, and sequence data revealed only one reading frame encoding a protein with a molecular weight of 25,000 (Higgins and Ames, 1981). This protein is synthesised in precursor form and is processed during translocation to the periplasm.

The hisQ and hisM genes encode two typical hydrophobic membrane proteins (Fig. 1.6) with predicted molecular weights 24,500 and 26,400 respectively (Higgins et al., 1982). The two proteins are produced in very small amounts with respect to HisJ. The hisQ and hisM genes bear strong homology to each other (Ames, 1985) indicating that they originated by a gene duplication. This homology together with their similar hydropathicity plots (see Fig. 1.6) has suggested that the two proteins form a pseudo dimer in the membrane (Ames, 1985). Genetic evidence also suggests that HisQ or HisM might carry a substrate binding site since mutations within these two genes show altered specificity of transport (Higgins et al., 1982).

The final component of the histidine permease is the hisP gene product. This gene encodes a protein of 28,700 molecular weight which is not recognisably hydrophobic despite the fact that it is apparently membrane bound (Ames and Nikaido, 1978) (see section 1.7) in fact Fig 1.6 demonstrates that the hydropathicity of HisP shows closer similarity to

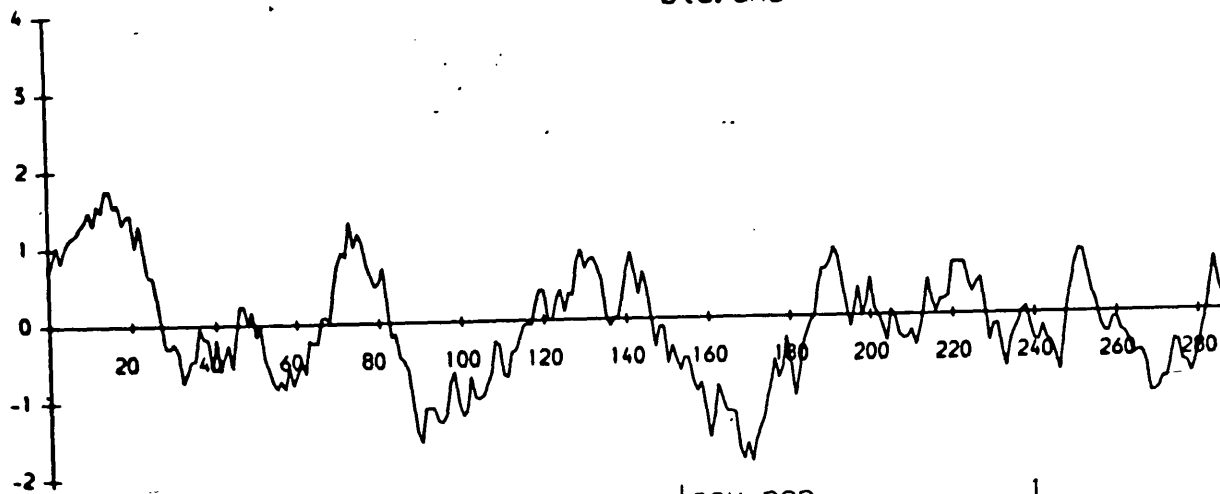
Figure 1.6 Hydrophobicity plots for HisJ, HisQ, HisM and HisP together with another example of cytoplasmic (CAT) periplasmic (β -lactamase), inner membrane (lactose permease) and outer membrane (OmpF) protein.

Graphs were plotted according to the method of Kyte and Doolittle (1982), scanning the protein 19 amino acids at a time. Thus the first point represents the hydropathic index averaged for amino acids 1-19, the second point the average hydropathic index for amino acids 2-20 and so on. Positive values are hydrophobic, negative values are hydrophilic. In all cases the sequence used is that of the initial translation product.

HisJ	periplasmic HisJ (precursor)
HisQ	inner membrane HisQ
HisM	inner membrane HisM
HisP	apparent inner membrane HisP
β la	periplasmic β -lactamase (precursor)
LacY	inner membrane lactose permease
CAT	cytoplasmic chloramphenicol acetyl transferase
OmpF	outer membrane OmpF (precursor)

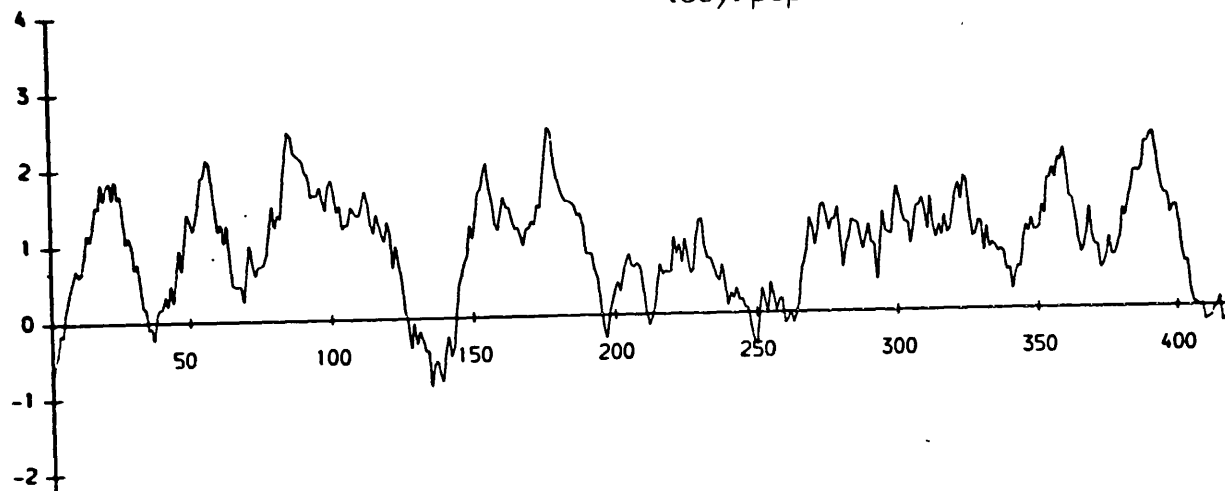
bla. dna

1



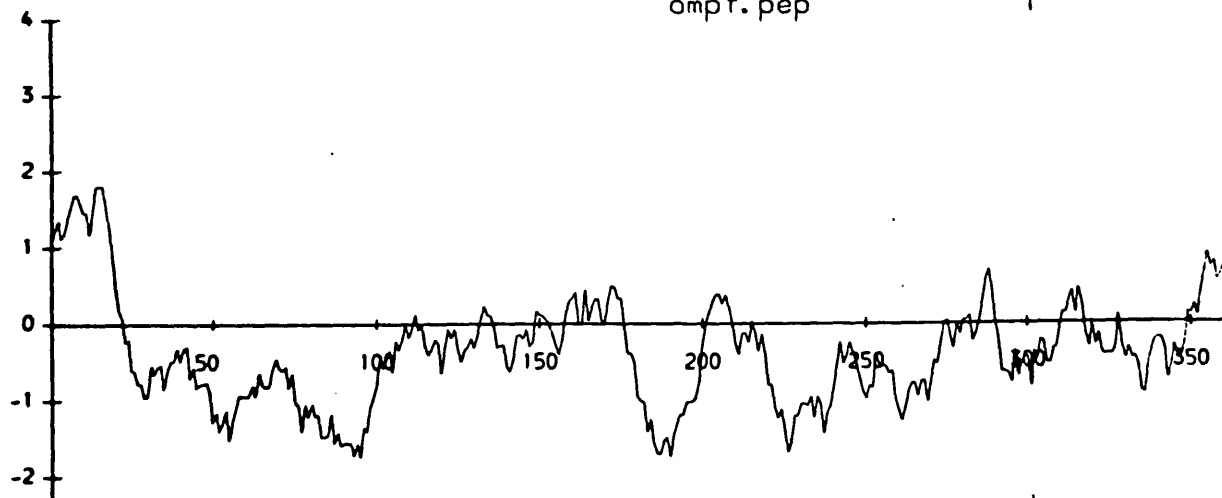
lacy. pep

1



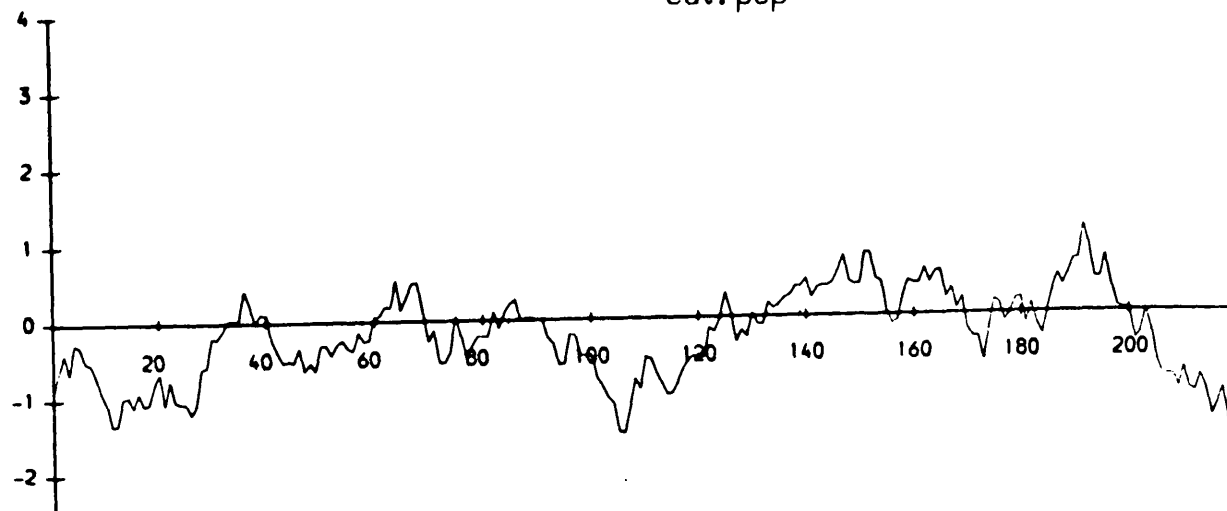
omp f. pep

1



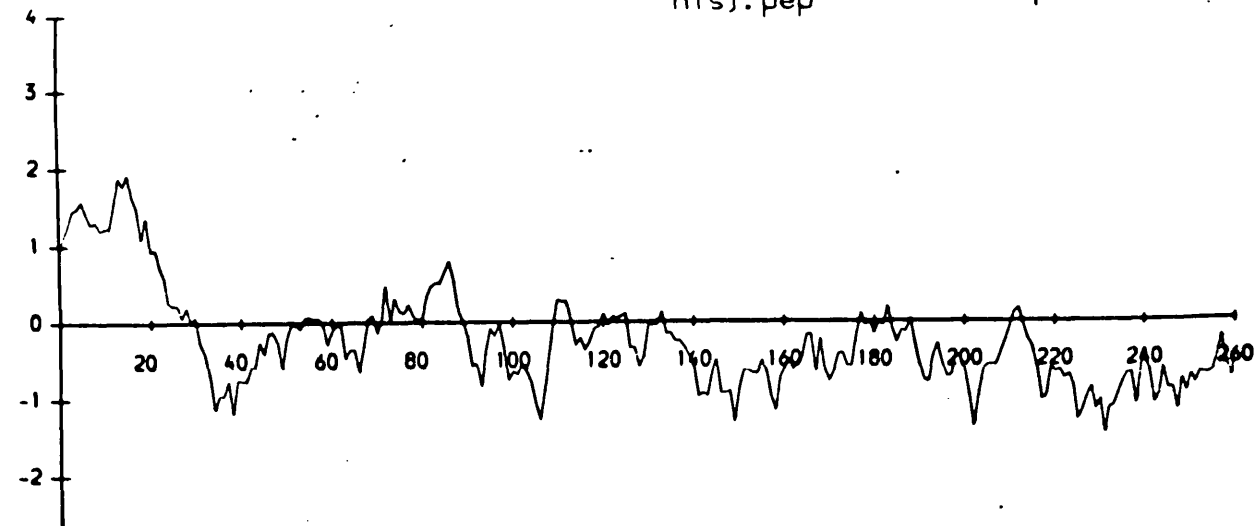
cat. pep

1



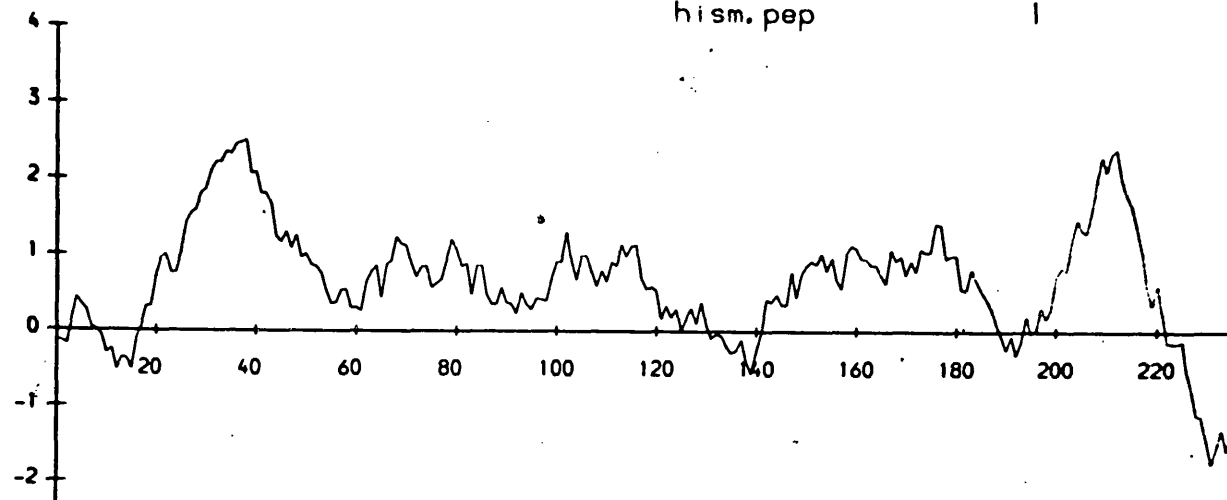
hisj. pep

1



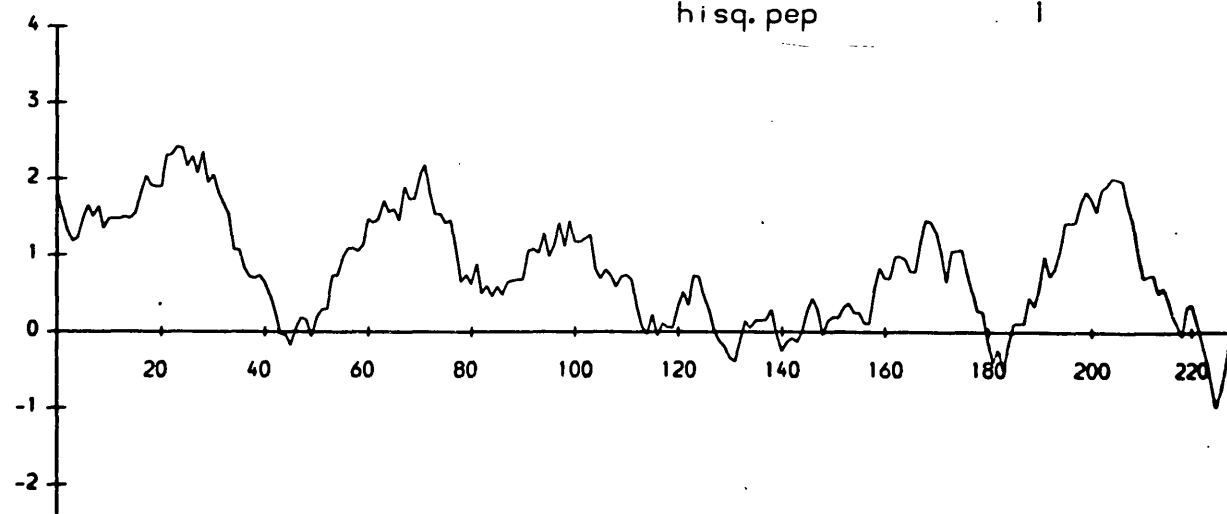
hism. pep

1



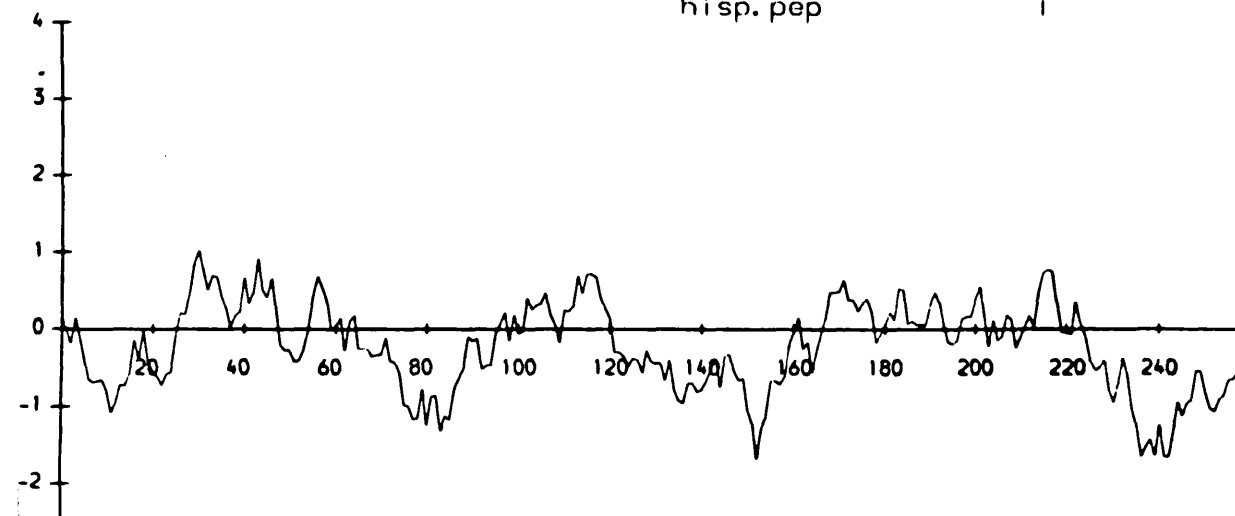
hisq. pep

1



hisp. pep

1



the cytoplasmic chloramphenicol acetyl transferase (CAT) protein than membrane proteins. Recent evidence suggests that HisP carries a nucleotide binding site since the protein is able to bind 8-azido-ATP (Hobson et al., 1984). Competition of 8-azido-ATP with a variety of nucleotide-containing compounds suggested that ATP and/or GTP are the natural substrates of the HisP protein. As discussed in a following section (section 1.8) this protein is believed to belong to family of proteins, each having a similar role within these so-called periplasmic permeases.

1.7 A comparison of all the characterised periplasmic permeases

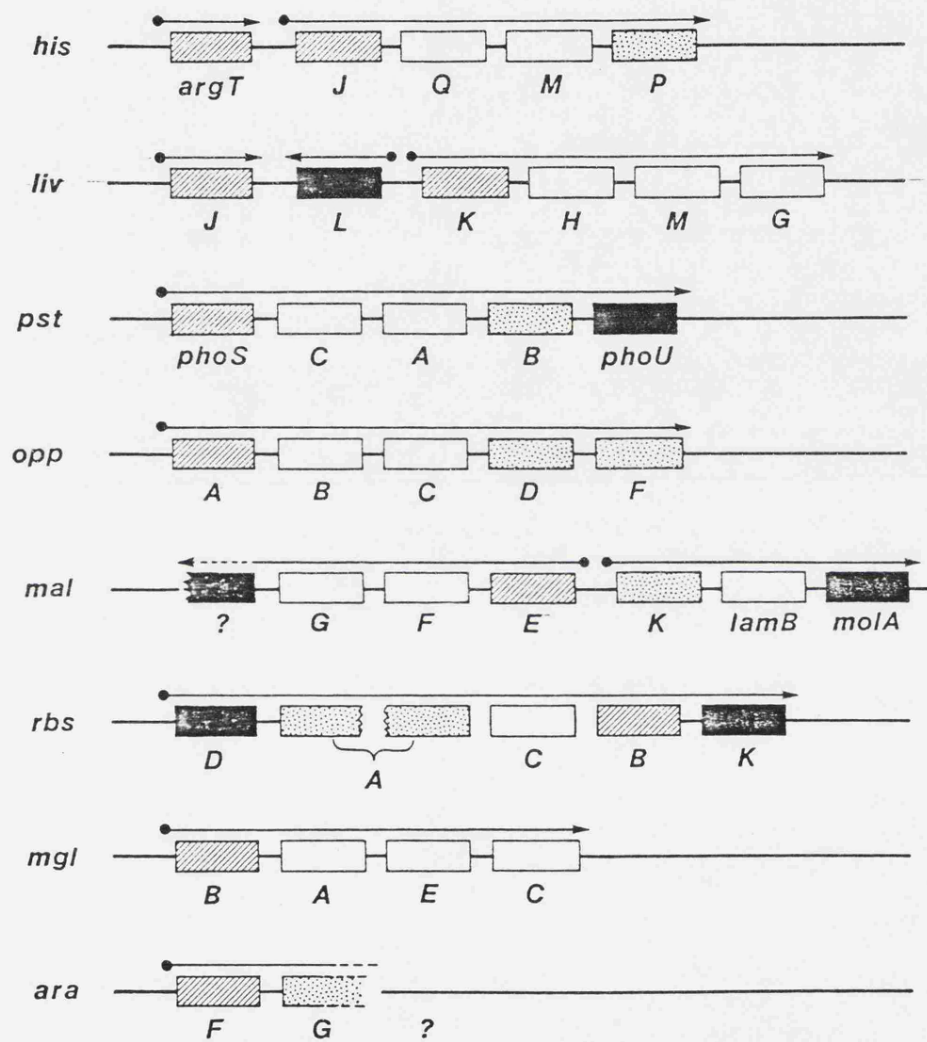
Accumulating evidence shows that the periplasmic permeases are composed of one periplasmic substrate binding protein, two typical inner membrane proteins and a membrane associated protein. This has been found to be true for seven permeases specific for : histidine (Higgins et al., 1982), maltose (Hengge and Boos, 1983), branched chain amino acids (Landick et al., 1985), oligopeptides (Hiles and Higgins, in press), ribose (Bell et al., in press), β -methyl galactoside (Harayama et al., 1983) and phosphate (Amemura et al., 1985). The genetic structure of all these permeases is shown in Fig. 1.7. In all cases a single operon contains all known transport components. In addition, the histidine and branched-chain amino acid permeases contain a separately regulated gene encoding a second periplasmic binding protein of different specificity (argT and livJ). The argT gene encodes the binding protein (LAO) involved in the transport of lysine, arginine and ornithine via the Q, M and P proteins of the histidine permease.

Figure 1.7

Genetic structure of periplasmic transport operons. Each operon codes for at least one substrate binding protein (hatched boxes). Black boxes represent genes that are not known to be involved in transport. Stippled boxes represent genes encoding the family of homologous membrane bound components. The open boxes represent genes encoding the integral membrane proteins. The sequence of liv, mgl and ara is not yet complete and their structure has been established by genetic means.

The arrows indicate the direction of transcription, however, the start and termination sites have not been fully established in many cases.

Taken from Ames, 1986.



Of these, it is the systems for maltose transport and histidine transport which have been extensively studied, revealing strong similarities between the two systems. As evidence is accumulating for the other transport systems mentioned; strong homology is being observed to the histidine and maltose permeases. I shall discuss the histidine and maltose transport systems in detail and provide data for the other transport systems where applicable.

One fundamental difference between maltose and histidine transport is that the transport of maltose requires a specific pore within the outer membrane, the LamB protein, whereas no specific pore appears to be necessary for histidine. From the periplasm inwards the components of the two systems appear to be very similar.

Both systems require a periplasmic substrate binding protein, HisJ or MalE. Indirect evidence suggests an interaction between the HisJ protein and the membrane associated HisP protein, and between MalE and both MalG and MalF, the integral inner membrane components in the maltose pathway. It is the peripheral membrane protein MalK protein which has shown homology to the HisP protein (Gilson et al., 1982) suggesting the two proteins may be serving slightly different roles. However, interaction has also been reported between the MalG membrane protein and MalK indicating that each component is able to interact sequentially. There has been no direct evidence to confirm these observations. However, genetic evidence from the histidine permease suggests that a mutant HisJ protein which is able to bind histidine cannot function in transport (Kustu and Ames, 1974). This suggests that the HisJ protein has separate binding sites for both histidine and interaction with the membrane

complex. Furthermore, a mutation within the hisP gene region can suppress this hisJ mutation suggesting that HisJ interacts with HisP (Ames and Spudich, 1976). Further evidence for an interaction with the membrane comes from the finding that the amino acid sequence of the HisJ protein is homologous to that of the closely related LAO binding protein. Two portions of these molecules show greater than 90% homology (Higgins and Ames, 1981) and the mutation in hisJ referred to above is located within one of these regions. Since both HisJ and the LAO protein require HisQ, M and P for function this suggests that the two proteins are involved in identical function; the interaction with a common membrane component. Finally, hisJ mutants have been characterised which interfere with the proper functioning of the membrane components; presumably by binding irreversibly to one of them (Ames, unpublished data cited in Ames, 1986a).

With regard to the maltose transport system again genetic evidence was obtained for interaction between the maltose binding protein and MalF and MalG. Thus, mutants of MalF and MalG allow transport of maltose in the absence of binding protein; however the introduction of wild-type maltose binding protein MalE completely inhibits maltose uptake (Treptow and Shuman, 1985), suggesting that these membrane components possess binding sites for both the substrate binding protein and the substrate itself. The maltose binding protein has also been shown to interact with the outer membrane protein LamB (Bavoil and Nikaido, 1981; Wandersman et al., 1979).

The two integral inner membrane components of the periplasmic permeases characterised so far are all produced in very small amounts and none have been purified. Sequence data emerging for several of these systems is

revealing strong homology between these two components. Homology has been shown between HisQ and HisM (Ames, 1985 and section 1.6), between MalF and MalG (Dassa and Hofnung, 1985) and between PstC and PstA of the phosphate transport system (Ames and Doolittle, unpublished data, cited in Ames, 1986).

MalK, like HisP is thought to be membrane bound but to not exhibit the typical hydrophobicity of an inner membrane protein (Gilson et al., 1982). Initial evidence suggests that attachment to the membrane by this component might vary from one system to another. MalK is thought to be a peripheral membrane protein since it is released upon sonication (Shuman and Treptow, 1985) and can be recovered in the soluble fraction in mutants defective in MalG, indicating that it may be attached to the membrane via the MalG protein (Shuman and Silhavy, 1981). Ames has recently attempted similar experiments with HisP but has shown that HisP always remains tightly bound to the cytoplasmic membrane (Ames and Niakaido, unpublished data, cited in Ames, 1986), however, work carried out in this thesis does not draw such a definite conclusion.

1.8 Sequence homology between a family of nucleotide binding proteins

Amino acid sequence data deduced from the nucleotide sequences has revealed strong homology between several nucleotide binding components of the periplasmic permeases. Homology was shown between HisP and MalK (Gilson et al., 1982) and between HisP, MalK and OppD (Higgins et al., 1985). Furthermore, a consensus nucleotide-binding sequence (Walker et al., 1982) has been identified in each of these three proteins, within those regions where greatest sequence homology is shown (Fig. 1.8).

Figure 1.8

Alignment of various prokaryotic and eukaryotic sequences to illustrate the conservation of amino acids in the nucleotide-binding sites of these proteins. The consensus sequence (Walker et al., 1982) is shown at the base of the column.

Adapted from Higgins et al. (1986).

Figure 1.8

Protein	Species	Sequence
OppD	<i>S. typhimurium</i>	GETLGI VESCCKSQSLR
OppF	<i>S. typhimurium</i>	GESLG VVESCCKKSTFAR
HisP	<i>S. typhimurium</i>	GDVOSOCSSCSCLSTFLR
MalK	<i>E. coli</i>	GEFVVFVGPSCCKKSTLLR
PstB	<i>E. coli</i>	NOVTAFI GPSCCKKSTLLR
RbsA(N)	<i>E. coli</i>	GRVMALVGENCACKSTM MK
HlyB	<i>E. coli</i>	GEVIGIVGRSCSKSTLTK
NodI	<i>R. leguminosarum</i>	GECFGLLGPNCACKSTITR
FtsE	<i>E. coli</i>	GEMAFLTGHSGACKSTLLK
ATPase	<i>E. coli</i>	GQRELIIGDRQTGKTALAI
ATPase	<i>E. coli</i>	GGKVGLFGGAGVCKTVNMM
RecA	<i>E. coli</i>	GRIVEIYGPESGKTTLTL
UvrD	<i>E. coli</i>	RSNLLVLAGAGSKTRVLV
ATPase	Bovine	GGKIGLFGGAGVCKTVGIM
Adenylate Kinase	Rabbit	SKIIFVVGPGSGKGTQCE
Myosin	Rabbit	NQSILITGESGACKTVNTK
EF-Tu	<i>E. coli</i>	HVNVTIGIHDHGKTTLTA
Consensus sequence		-----GAGGVCKS-----
		T

Recently, it has become apparent, that as more sequence data for periplasmic permeases accumulates; a family of membrane associated proteins is emerging each possessing a nucleotide binding site (Higgins et al., 1986).

Some of these conserved regions also share homology with proteins known to have ATP binding sites such as α and β subunits of the proton-translocating ATPase and adenylate kinase (Higgins et al., 1985). The MalK protein has recently been purified and found to bind ATP with an affinity constant of 10^{-4} M (Nikaido, unpublished data, cited in Ames, 1986). A fusion protein between OppD and β -galactosidase was also shown to react with the ATP analogue 5'-p-fluorosulfonyl-benzoyladenosine, and furthermore OppD protein has been shown to bind a Cibacron blue column and be eluted by ATP (Higgins et al., 1985). This apparent nucleotide binding site suggests that HisP, MalK and OppD may be involved in an energy coupling mechanism (Higgins et al., 1985; Higgins et al., 1986; Hobson et al., 1984), however, attempts to demonstrate ATP hydrolysis by any of these proteins have failed, (Ames and Speiser, unpublished data, Niakaido and Nikaido, unpublished data, cited in Ames, 1986), and thus, the possibility that the ATP-binding serves a purely regulatory role in transport cannot be eliminated.

All the proteins so far related to the group appear to be involved in energy requiring processes such as amino acid uptake (HisP), cell division (FtsE) DNA repair (UvrD) or export functions (HlyB) within the cell and most of these systems involve membrane-associated functions. Very recent publications ignite even more interest in the role of these proteins; the same homology is found in mammalian multidrug resistance (Mdr) (Gerlach et al., 1986; Gros et al., 1986b; Chen et al., 1986).

Thus, a class of mammalian membrane glycoproteins are thought to be associated with the development of resistance to cytotoxic agents in tumour cells causing failure of chemotherapy treatment in cancer. Obviously the elucidation of this mechanism is very important in order to develop improved methods for treating tumour cells. Drug resistant cell lines have been isolated and a genomic DNA domain, amplified in multidrug-resistant cell lines has been cloned (Gros et al., 1986a; Gros et al., 1986c). Furthermore, cDNA has been prepared from an overexpressed mRNA from these cell lines and when inserted into an appropriate expression vector, is capable of conferring multidrug resistance to otherwise drug sensitive cells (Gros et al., 1986d). This gene mdr has been sequenced and hence the homology determined with the nucleotide binding proteins (Gros et al., 1986b).

The mechanism by which drug-resistant cells achieve a lower intracellular drug level is not well understood. Two principal mechanisms have been proposed, reduced membrane permeability leading to a decreased rate of drug entry (Ling and Thompson, 1974; Riordan et al., 1985) or increased rate of removal of drug from the cell via an energy-dependent efflux mechanism (Dana, 1973; Skovsgaard, 1978).

The deduced amino acid sequences from the mdr cDNAs correspond to proteins of around 140,000 daltons. The protein structure suggests a hydrophobic region and also two duplicated hydrophilic regions each containing a nucleotide binding site, these regions show strong homology to the family of bacterial membrane transport proteins. Furthermore, these structures show marked similarity to the membrane glycoproteins gp170 thought to be associated with multiple drug resistance; work is

currently in progress to determine whether the mdr genes are infact encoding gp170 proteins. The regions of strong homology shown between these Mdr proteins and the bacterial membrane transport proteins suggest that multiple drug resistance involves an energy dependent transport mechanism. A model has been put forward (Ames, 1986b) whereby the Mdr protein, which appears to be a large protein in comparison to the bacterial membrane transport proteins, has incorporated into a single molecule the features of the entire membrane-bound complex of the bacterial counterpart - two homologous hydrophobic domains and two homologous hydrophilic domains involved in ATP hydrolysis. Substrates (ie. drugs) can bind to this molecule and the ATP-binding domain is somehow involved in energizing its transport function. Although not yet isolated, substrate binding proteins may be involved in a concentration process, 'trapping' the drug and delivering it to the Mdr complex. These substrate binding proteins may be substrate-specific but use a common Mdr protein, thus increasing the specificity of transport.

These findings have therefore created further interest in the bacterial periplasmic permeases, since prokaryotic cells are generally easier to work with than the complex eukaryotic cells. The complete elucidation of the mechanism for histidine transport in E. coli or S. typhimurium could assist in explaining the mechanism of multiple drug resistance and therefore help in devising a mechanism to overcome this problem. Furthermore, introducing a functional periplasmic permease into a foreign organism such as M. methylotrophus would encourage the idea that such complex transport systems can be transferred successfully from one organism to another. This in turn would raise the possibility of the mdr genes could also be introduced into E. coli whereby these cells would

become resistant to drugs. Genetic manipulations would be simpler in this organism and providing that the system was functional the mechanism of multiple drug resistance could readily be analysed.

1.9 Possible models for transport by the periplasmic permeases

The accumulating evidence with respect to the identity and location of the components making up the histidine transport system has led to the derivation of several models.

Two molecular models for histidine transport were first proposed by Higgins et al. (1982), the pore model and the binding site model. Both were based on the evidence that the periplasmic protein HisJ binds histidine (Ames and Lever, 1972), inducing a conformational change. Extensive spectral changes in the HisJ protein have been identified by proton nuclear magnetic resonance spectroscopy, upon the binding of histidine (Ho et al., 1980). This conformational change then allows the HisJ-histidine complex to interact with a membrane-bound protein, possibly HisP (Ames and Spudich, 1976).

The two basic models proposed by the Ames and Higgins group both assume that proteins HisP, HisQ and HisM form a cytoplasmic membrane-bound complex since complementation studies suggest interaction between HisQ and HisP (Ames and Nikaido, 1978). The HisP protein was first shown exposed to the periplasmic side of the inner membrane. Genetic evidence suggested interaction between HisJ and HisP since mutants in HisP could suppress defects in HisJ (Ames and Spudich, 1976). This organisation was updated by Hobson et al. (1984) with the discovery of a nucleotide

Figure 1.9 Schematic representation of Histidine transport

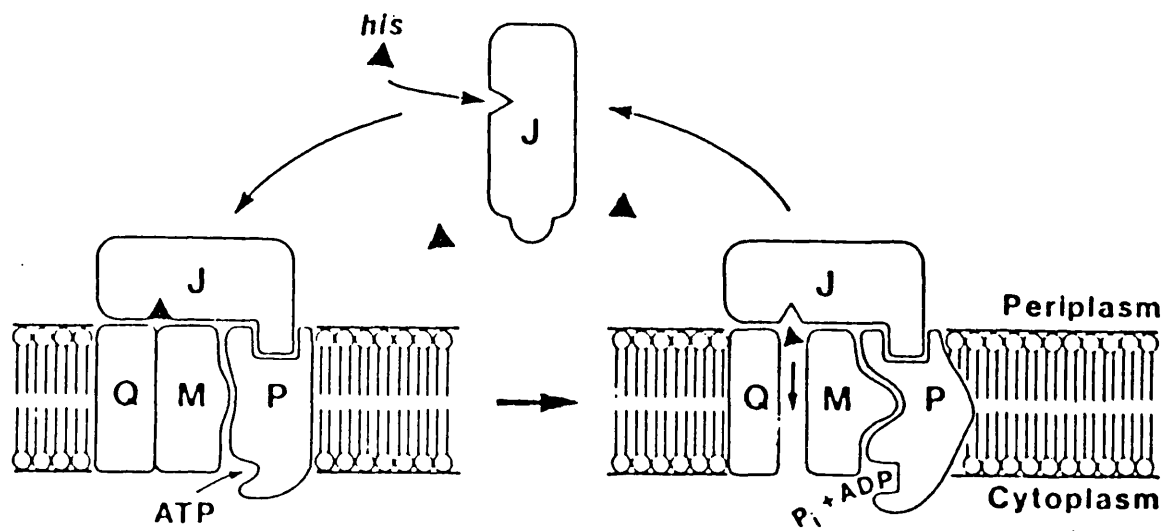
A. The pore model

The interaction between J (with histidine bound to it) and P allows the binding of ATP to the cytoplasmic face of P. Hydrolysis of ATP allows a conformational change to occur in P which is transmitted to the other proteins allowing the opening of a pore through which histidine can diffuse. The release of J and loss of ADP + Pi from P would complete the cycle, ending in the closure of the pore (Hobson et al., 1984).

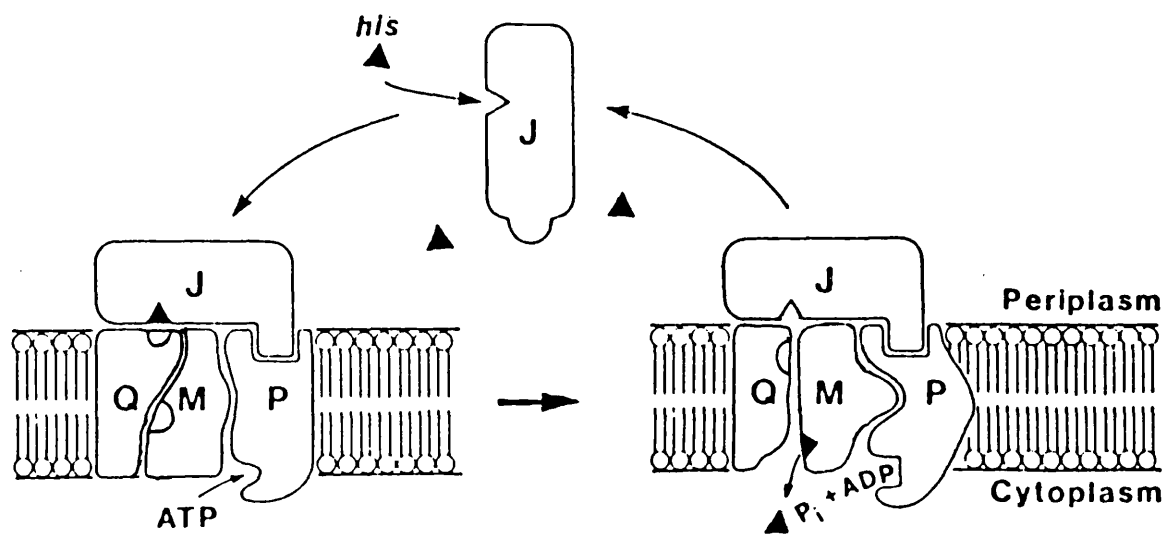
B. Binding site model

Substrate binding sites (indicated by hemispheres) are sequentially activated in a cascade like manner, with histidine passing from one protein to the next, thus being carried across the membrane (Higgins et al., 1982). The mechanism, involving conformational changes again requires energy, provided by ATP hydrolysis.

A)



B)



binding site in HisP; since no evidence is available suggesting ATP can reach the periplasm the portion of HisP protein which interacts with ATP must be accessible to the cytoplasmic side of the membrane. This assumption was confirmed by the use of impermeant membrane-labelling reagents (Hobson et al. unpublished results).

The two models, outlined in Fig. 1.9 suggest that the membrane bound complex made up of HisQ, HisM and HisP can either form a simple diffusion pore to allow the passage of histidine into the cytoplasm; (model A-pore model) or that the substrate binding sites suggested by Ames and Nikaido (1978) and Ames and Spudich (1976) create a cascade-like mechanism whereby each is activated sequentially and histidine is passed through the membrane from one protein component to the next (model B-binding site model). Genetic evidence favours the binding site model since wild-type histidine permease transports L-histidine efficiently and also transports D-histidine and the histidine analogue HIPA with lower affinity. Two types of mutants have been found; mutants within the hisQ gene which transports L-histidine normally but does not transport D-histidine or HIPA (Higgins et al., 1982), and mutants originally isolated on the basis of resistance to inhibitory analogues transported by the histidine permease (Ames et al., 1977) and later mapped in the hisM region. These have been shown by sequence analysis to have deleted a 12 base pair sequence, leading to an inframe deletion of a tetrapeptide in the translated product (Payne et al., 1985), resulting in an inability to transport L-histidine and several of its analogues; however, the mutant gains the ability to transport L-histidinol. These results suggest that the mutations have caused an alteration in the substrate binding site. In the maltose system, mutations have also been isolated in malF and malG

and in a strain deleted for malE (i.e. lacking the periplasmic binding protein) which are able to transport maltose (Shuman, 1982; Treptow and Shuman, 1985). Spheroplasts prepared from such mutants are able to transport maltose, indicating that the mutation has altered the membrane component thus enabling the system to function without the aid of a periplasmic binding protein. In mutants lacking HisJ protein, or in spheroplasts or membrane vesicles, no transport has been detected. These results provide further evidence for a conformational change to occur in the membrane components in order to expose the substrate binding site within the membrane component.

Both the substrate binding model and the pore model require energy to power these proposed conformational changes; Berger and Heppel (1974) suggested that hydrolysis of a phosphate bond was responsible for powering the periplasmic permeases, as opposed to shock-resistant systems which were driven by a proton-motive force. Unfortunately, the experiments undertaken involved treating the cells with metabolic poisons which could have affected multiple metabolic pathways and so the results were taken with caution.

It is now clear that there is a nucleotide binding protein component in each of the periplasmic permeases studied so far (Higgins et al., 1986). The ability of these proteins to bind ATP suggest that these periplasmic permeases are driven by the hydrolysis of ATP or a closely related nucleotide, perhaps in a manner analogous to the ATP-driven ion pumps (Kyte, 1981; Amzel and Pedersen, 1983), although as mentioned in section 1.8 attempts to demonstrate ATP hydrolysis have been unsuccessful.

The models put forward suggest that not only must HisP bind the periplasmic protein HisJ during histidine transport, but it must also be accessible to the cytoplasm in order to hydrolyse ATP. These functions suggest a protein which spans the membrane, however, as seen in section 1.7 the hydropathicity of this protein does not suggest a protein with membrane spanning characteristics. Experiments carried out in this thesis have attempted to determine the location of HisP and several of the events experienced have prompted a further model to be postulated.

CHAPTER 2

Materials and Methods

2.1 Bacterial strains and bacteriophage

The bacterial strains used in this study are listed with their genotypes in Table 2.1. E. coli cultures were maintained on nutrient agar (NA) plates at 4°C when in current use and frozen at -20°C in nutrient broth (NB) containing 20% v/v glycerol for long term storage.

M. methylotrophus cultures can be stored on minimal methanol agar (MMA) plates for very short periods and frozen stocks in minimal methanol medium (MMM) containing 20% v/v glycerol stored at -80°C for long term storage.

2.2 Media and buffers

All media and buffers used are described in Table 2.2. Plasmid bearing strains were grown in, and maintained on freshly prepared media containing the appropriate antibiotic(s) as shown in Table 2.2.

2.3 Growth of bacteria in liquid culture

Liquid cultures were grown in a New Brunswick Gyrotary shaker at 37°C and mass increase followed by measuring absorbance at 450 nm or 600 nm using a Gilford Microsample spectrophotometer 300N.

Table 2.1

Bacterial strains

E. coli K-12

<u>Strain</u>	<u>Genotype</u>	<u>Source</u>
BW162 [R64d _{rd} 11]	F ⁻ , <u>glnV</u> , <u>lacY1</u> , <u>leuB6</u> , <u>thi1</u> , <u>thr1</u> , <u>tonA21</u> , <u>supE44</u> , ColI ^r Plasmid : R64d _{rd} 11	B.M. Wilkins
CSH26ΔF6	<u>ara</u> , <u>thi</u> , Δ(<u>lac</u> , <u>pro</u>), Δ(<u>recA</u> , <u>srl</u>) <u>rpsL</u>	J. Pratt
C600	F ⁻ , <u>glnV</u> , <u>lacY</u> , <u>leuB6</u> , <u>thi1</u> , <u>thr1</u> , <u>tonA21</u> <u>supE44</u>	B.M. Wilkins
DS410	<u>sup</u> ⁰ , <u>lacY</u> , <u>minA</u> , <u>minB</u> , <u>rpsL</u> , <u>ara</u> , <u>malA</u> <u>azi</u>	J. Pratt
MC4100	<u>araD139</u> , Δ(<u>lacIPOZYA</u>) U169, <u>thiA</u> , <u>relA1</u> , <u>rpsL</u>	P. Bassford
NM522	Δ(<u>lac proAB</u>) <u>thi</u> , <u>supE</u> r ⁻ m ⁺ [F' <u>proAB lacI</u> ^q Z Δ M15]	A. Boyd
TA3476	hisΔ461 (OGDCBHAFIE) hisΔ700 (J, Q, <u>PacK</u> <u>pta</u>)	G. F-L. Ames
TA3457	As above with plasmid pFA6	G. F-L. Ames
TA3598	As above with plasmid pFA13	G. F-L. Ames
JA200[pTD101]	Host F ⁺ , <u>trpE5</u> , <u>thr</u> , <u>leu</u> , <u>lacY</u> , <u>recA</u> with plasmid : pTD101	W. Wickner
159	<u>gal</u> <u>uvrA</u> <u>rpsL</u>	G.S. Plastow

Methylophilus methylotrophus

AS1	WT NC1B No 10515	D. Byrom
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Cont....

Table 2.1 Continued 2

Bacteriophage lambda strains

λ ::Tn5 cI⁸⁵⁷O_{am} W.J. Brammar

Genotype symbols are defined in Bachmann (1983). ColI^r is the phenotype symbol for resistance to colicin Ib-P9.

Plasmids

Plasmid	Phenotype	Source
pFA6	Ap	G.F-L. Ames
pFA13	Ap Tc	G.F-L. Ames
pUC12	Ap	J. Messing
pBR325	Ap Tc Cm	Lab stock
pGSS33	Ap Tc Cm Sm	G.S. Sharpe
pTTQ18	Ap	M. Stark
pTD101	Ap	W. Wickner

All plasmids with the prefix "pYCP" are described in this thesis.

Table 2.2 Media and Buffer

Media

Nutrient broth (NB)	Oxoid No.2 nutrient broth	2.5% w/v
Luria broth (LuB)	Tryptone	1.0% w/v
	Yeast extract	0.5% w/v
	NaCl	0.5% w/v
M9 minimal medium	Na ₂ HPO ₄	0.6% w/v
	KH ₂ PO ₄	0.3% w/v
	NH ₄ Cl	0.1% w/v
	NaCl	0.05% w/v
	Glucose (autoclaved separately)	0.4% w/v
	MgSO ₄ " "	10 mM
	CaCl ₂ " "	1 mM
	Plus, as required :	
	Thiamine (filter sterilised)	10 µg ml ⁻¹
	Amino acids	40 µg/ml ⁻¹
Trypticase agar	Oxoid Trypticase peptone	1% w/v
	NaCl	0.5% w/v
	Agar	1.5% w/v
	Soft agar only contains 0.7% agar	

Cont....

Table 2.2 Continued 2

Methanol minimal medium
(MMM)

1L of MMM contained	(NH ₄) ₂ SO ₄	0.18% w/v
	MgSO ₄ .7H ₂ O	0.02% w/v
	NaH ₂ PO ₄ . 2H ₂ O	0.14% w/v
	K ₂ HPO ₄ .3H ₂ O	0.25% w/v

(These constituted a x 1 stock salts solution)

- 5 ml methanol
- 1 ml Trace element solution

1 litre of trace element contained

FeCl ₃	0.098%w/v
CuSO ₄ .5H ₂ O	0.002%w/v
MnSO ₄ .4H ₂ O	0.01% w/v
ZnSO ₄ .7H ₂ O	0.01% w/v
CaCO ₃	0.18% w/v
1 M HCl	36.6 ml

Agar plates were prepared by solidifying media with 1.5% Difco Agar.

Cont...

Table 2.2 Continued 3

Antibiotics

		<u>E. coli</u>	<u>M.methylotrophus</u>
Ampicillin	(Ap)	25 µg ml ⁻¹	25 µgml ⁻¹
Streptomycin	(Sm)	100 µg ml ⁻¹	-
Tetracycline	(Tc)	10 µg ml ⁻¹	2 µgml ⁻¹
Chloramphenicol	(Cm)	25 µg ml ⁻¹	25 µgml ⁻¹
Kanamycin	(Km)	50 µg ml ⁻¹	- -

Ap and Sm filter sterilised, stocks of Tc and Cm made in 50% ethanol.

Buffers

Bacterial buffer	Na ₂ HPO ₄	0.7% w/v
	NaCl	0.4% w/v
	KH ₂ PO ₄	0.1% w/v
	MgSO ₄ .7H ₂ O	0.01% w/v
Lambda buffer	Tris-Cl (pH7.2)	6 mM
	MgSO ₄	10 mM
	Gelatin	0.5% w/v

2.4 Growth of bacteriophage λ ::Tn5 CI⁸⁵⁷Oam

(a) Titration of phage stocks: An overnight culture of E. coli C600 was supplemented with 10 mM MgCl₂. 0.1 ml was added to 0.1 ml of a serial dilution of phage in λ buffer, and left at room temperature for 10 min to allow adsorption. 3 ml molten Trypticase soft agar (0.7% w/v) were added, briefly mixed and overlaid on Trypticase agar plates. The plates were incubated at 37°C overnight.

(b) Preparation of phage stocks: Dilutions of phage were plated as described above. The trypticase agar plates were freshly poured. The plate on which the plaques were just confluent was taken and the soft agar scraped into a McCartney bottle. A few drops of CHCl₃ were added, and the bottle vortexed. The debris was pelleted by centrifugation (3,000 rpm 5 min in a MSE bench centrifuge) and the supernatant transferred to another McCartney bottle. A few drops of CHCl₃ were added, and the lysate stored at 4°C. Titres obtained were usually greater than 10¹⁰ pfu ml⁻¹.

2.5 Mutagenesis by Tn5 insertion

Strain E. coli 159 (pYCP2), grown in Luria broth + 0.2% maltose was infected with λ ::Tn5 CI⁸⁵⁷Oam at an infectivity of 1 A₅₅₀ unit cells to 2 x 10⁹ pfu phage. After 15 min incubation at room temperature, to allow adsorption of the phage, the cells were grown in 0.8 ml LuB at 42°C for 5 min to inactivate the cI gene product followed by 30 min at 37°C. The culture was plated onto LuA containing 50 μ g ml⁻¹ kanamycin, to select Tn5 and 25 μ g ml⁻¹ ampicillin, to select for the plasmid.

Colonies were pooled from the plates and plasmid DNA prepared using a scaled up Mini Birnboim preparation. This DNA was transformed into a suitable E. coli strain and selected for on 50 $\mu\text{g ml}^{-1}$ Kan. Individual colonies were then screened and Tn5 insertions mapped.

2.6 Mobilization of IncQ-derivative plasmids

M. methylotrophus does not have a transformation system, neither have any phage been isolated; therefore the only method for introducing foreign DNA is by transferring it from an E. coli donor strain by plate matings on solid media.

A colony of the recipient strain was suspended in 50 μl of sterile bacterial buffer and spread over half the surface of a selective minimal methanol agar plate (MMA). Plate colonies of the donor strain were then streaked in one direction across the plate into the recipient. The selective MMA plates were designed so that only recipient cells containing plasmid from the donor were able to grow.

Plates were incubated at 37°C for 48 hours and single colonies were then purified on selective medium plates by restreaking several times.

Contamination by E. coli donor strains was checked by streaking a colony onto an LuB plate, if the colony is pure recipient growth is inhibited.

2.7 Preparation of plasmid DNA

When handling DNA, disposable gloves were worn, all buffers, reagents, glass and plastic ware used were sterilised by autoclaving, and all glassware coming into contact with the DNA was siliconised.

(a) Rapid plasmid preparation for screening. (Adapted from the method by Birnboim and Doly, 1979) 1.5 ml of an overnight culture of bacteria in LuB plus the appropriate antibiotic(s) was transferred to an Eppendorf tube and centrifuged 3 min in an Eppendorf microfuge. The cells were resuspended by vortexing in 100 μ l of TEG buffer (Table 2.3). The tube was incubated on ice for 10 min. 200 μ l of alkaline SDS (Table 2.3) solution was added and the contents of the tube mixed by several sharp inversions followed by a further 5 min incubation on ice. 150 μ l of potassium acetate (Table 2.3) was added, the contents gently mixed and the tube incubated for a further 10 min on ice. The chromosomal clot was pelleted by centrifugation for 3 min and the supernatant removed into a fresh Eppendorf tube using a Gilson P200 pipette. An equal volume of phenol mix (Table 2.3) was added to the supernatant, the tube was vortexed, and then centrifuged for 2 min.

The aqueous layer was carefully removed, taking care not to disturb the interface. An equal volume of chloroform : isoamyl alcohol mix (Table 2.3) was added to the aqueous layer to remove any phenol, the tube was vortexed and then centrifuged for 2 min. The aqueous layer was removed and nucleic acids were then precipitated by the addition of 2.5 volumes of ethanol, vortexing briefly and leaving on ice for 5 min. The precipitate was collected by centrifuging for 5 min, the pellet was then drained. 1 ml of 70% ethanol was added to the pellet, the tube vortexed briefly and recentrifuged. The supernatant was removed, the pellet dried briefly under vacuum and resuspended in 50 μ l TE containing ribonuclease A (20 μ g ml⁻¹) to degrade contaminating RNA (Table 2.3). For restriction digests 10 μ l of DNA was used.

(b) Large scale plasmid preparation. A 400 ml culture was grown overnight. The cells were then chilled, harvested and washed with bacterial buffer (Table 2.2). After harvesting, the cells were resuspended in 3 ml Tris-sucrose (Table 2.3) and 0.5 ml lysozyme (10 mg ml^{-1})/Ribonuclease A ($300 \text{ } \mu\text{g ml}^{-1}$) solution was added. The mixture was incubated at room temperature for 5 min. 1 ml 0.25M EDTA was then added and the incubation continued for 5 min. 4 ml Triton X-100 lysis mix (Table 2.3) were added and the tube inverted several times until lysis was complete. The lysate was cleared by centrifugation (Sorvall SS34 rotor, 18,000 rpm, 20 min, 4°C) and the supernatant decanted into a sterile Sorvall SS34 tube. Two thirds volume of PEG/NaCl solution (Table 2.3) was added and left on ice for more than two hours. The precipitate was collected by centrifugation (Sorvall HB4 rotor, 7K, 10 min), drained and resuspended in exactly 1.1 ml TES buffer (Table 2.3). The lysate was transferred to a Beckman VTi65 self-sealing tube by overlaying onto 4 ml CsCl-EtBr solution (Table 2.3). The refractive index of the CsCl-EtBr solution had been adjusted to 1.393 by the addition of further CsCl or TE buffer. The tube was centrifuged to equilibrium (Beckman Vti65 rotor, 55,000 rpm, 3 h, 15°C with slow acceleration). Chromosomal and plasmid bands were clearly visible with long wave UV. The plasmid band was removed with a syringe inserted through the side of the tube, transferred into a siliconized glass tube and extracted with an equal volume of CsCl/saturated propan-2-ol (Table 2.3) to remove ethidium bromide. The CsCl was removed by dialysis against TE buffer, and the DNA then phenol extracted to remove nucleases. The phenol was removed by extraction with chloroform : isoamyl alcohol mix. The plasmid solution was finally concentrated by ethanol precipitation, adding 1/10 vol of sodium acetate and 2.5 vol of absolute

Table 2.3 Plasmid DNA preparation

TEG buffer	Tris-Cl (pH8.0)	25 mM
	EDTA	10 mM
	Glucose	50 mM
Alkaline SDS	NaOH	200 mM
	SDS	1% w/v
Potassium acetate	5 M KAc	60 ml
	Glacial acetic acid	11.5 ml
	H ₂ O	28.5 ml
Phenol mix	Phenol	500 g
	Chloroform	500 ml
	Leave to dissolve then add	
	8 hydroxyquinoline	0.5 g
	Isoamylalcohol	20 ml
	Store under 1 cm layer of TE buffer at	4°C
Chloroform mix	Chloroform	24 ml
	Isoamylalcohol	1 ml
	Store at 4°C	
TE buffer	Tris-Cl (pH7.5)	10 mM
	EDTA	1 mM

Cont...

Table 2.3 Continued 2

Tris-Sucrose	Tris-Cl (pH8.0)	50 mM
	Sucrose	25% w/v
TES buffer	Tris-Cl (pH8.0)	50 mM
	EDTA	5 mM
	NaCl	50 mM
Triton lysis mix	Triton X-100	2% v/v
	Tris-Cl (pH8.0)	50 mM
PEG-NaCl solution	Polyethylene glycol 6000	25% w/v
	NaCl	1.25 M
CsCl-EtBr solution	CsCl	80 g
	TES buffer	52 ml
	EtBr solution (5 mg/ml)	8 ml
Caesium chloride- propan-2-ol	Saturated CsCl solution	10 ml
	Propan-2-ol	250 ml

Add 10 ml amounts of H₂O and mix well with
all aqueous phase persists, and all CsCl is in
solution

ethanol. The solution was left at -20°C overnight, the pellet containing nucleic acids collected by centrifugation and resuspended in TE buffer.

2.8 Preparation of chromosomal DNA

A 5 ml overnight E. coli culture or a 10 ml thick M. methylotrophus culture was pelleted in the MSE Chilspin at 4,000 rpm for 10 min. After a wash in TE, cells were resuspended in 5 ml TE and incubated at 37°C for 10 min. 0.4 ml 20% SDS was added and the incubation continued for 30 min to allow the cells to lyse. 0.54 ml 3 M Na acetate and 3.2 ml isopropanol were added to precipitate the DNA. These DNA strands can be easily removed by spooling onto a micropipette and transferred into a 15 ml siliconised glass test tube. The DNA was redissolved in 2 ml TE and RNA removed by incubating $100\text{ }\mu\text{g ml}^{-1}$ with RNase at 37°C for 30 min. Protein was removed by phenol extraction followed by chloroform extraction to remove the phenol. The DNA was precipitated with 1/10 volume 3M Na acetate and 2.8 ml isopropanol and pelleted at 9,000 rpm for 10 min in the Sorvall HB4 rotor. The pellet was resuspended in 0.3M Na acetate and transferred to an Eppendorf tube. 1 ml ethanol was added and the DNA precipitated at -80°C for 10 min followed by a 10 min centrifugation. The pellet was washed in 70% ethanol and then dried under vacuum and resuspended in 400 μl TE.

2.9 Agarose gel electrophoresis

Horizontal agarose slab gels (0.5-1.0% w/v) were prepared by boiling agarose in electrophoresis buffer (Table 2.4) to dissolve and adding

Table 2.4 Agarose gel electrophoresis

Electrophoresis buffer	Tris-acetate (pH8.0)	267 mM
	EDTA	1 mM
Sample buffer	Tris-Cl (pH6.8)	125 mM
	Glycerol	20% w/v
	EDTA	5 mM
	Bromophenol blue	0.05%

ethidium bromide to $0.5 \mu\text{g ml}^{-1}$ before pouring. All samples were mixed with 1/3 volume of agarose gel sample buffer (Table 2.4) before loading. Electrophoresis was carried out with the gel completely submerged in electrophoresis buffer at 100 volts, until the dye front had migrated through 3/4 of the gel. DNA was visualised by transillumination with short wave (260 nm) ultraviolet light. λ DNA cut with HindIII restriction endonuclease was used to give a set of molecular weight markers.

2.10 Restriction endonuclease digestion

0.5 μg plasmid DNA was used per restriction digest; if the DNA was heavily contaminated with RNA, 0.5 μl of 6 mg ml^{-1} ribonuclease A (previously boiled for 5 min to destroy DNase activity) was added to the restriction mixture. Final reaction volume was usually 20 μl , thus 2 μl X10 restriction buffer (Table 2.5) was added, but this volume was increased if more DNA was to be restricted (e.g. for a ligation). In all cases total reaction volume was at least 10 times the volume of enzyme added. Digestions were carried out for 1 hr at 37°C and if necessary (for ligation) enzymes were then inactivated by a further 10 min incubation at 65°C .

2.11 Ligation of DNA fragments

DNA to be ligated was first phenol extracted several times and ethanol precipitated (see Section 2.7). After resuspension of the precipitate in TE buffer to a concentration of approximately $50 \mu\text{g ml}^{-1}$, the DNA fragments to be ligated were mixed in a vector to insert mass ratio of

Table 2.5 Restriction and ligation of DNA

Restriction buffers (x 10)

	Reagents (Mm Concentrations)			
	Tris-Cl (pH7.5)	MgCl ₂	NaCl	DTT
Low salt	100	100	-	10
Med salt	100	100	500	10
High salt	500	100	1,000	10

Ligation buffer (x 10)

1M Tris-Cl (pH7.4)	660 μ l
1M MgCl ₂	100 μ l
10mM ATP	100 μ l
DTT	15 mg
BRL nuclease free BSA (10 mgml ⁻¹)	10 μ l
H ₂ O	90 μ l

DTT = dithiothreitol

4:1 for 'sticky end' ligations or 2:1 for blunt end. Sticky ends were melted by heating at 65°C and then left on ice for 60 min to allow reannealing. 1/10 volume of X10 ligation buffer (Table 2.5) and 1 µl T4 DNA ligase were then added and the mixture incubated overnight at 16°C. 2-10 µg DNA was usually used per ligation and 0.5 µg samples taken before addition of ligase, and after overnight incubation to be run on a gel to check the efficiency of the ligation reaction.

2.12 Filling recessed 3' ends of DNA

DNA to be filled in was first phenol extracted, ethanol precipitated and resuspended to a concentration of no less than 100 µg ml⁻¹ in TE. For 1-2 µg DNA a 50 µl reaction was prepared with 5 µl X10 medium salt buffer and 5 µl each of dNTPs at a concentration of 0.5 mM. 3 units of Klenow fragment of DNA polymerase were added and the reaction incubated at room temperature for 30 min.

2.13 Transformation of bacteria with plasmid DNA

(a) Preparation of competent cells

A culture of the bacteria to be transformed was grown in LuB from A₄₅₀ 0.05 to A₄₅₀ 0.4; 10 ml of these cells were centrifuged in a sterile universal (5 min 5,000 rpm) before being resuspended in 5 ml of sterile 100 mM MgCl₂. Immediately the cells were pelleted and resuspended in 5 ml of sterile 100 mM CaCl₂. After incubation on ice for 20 min, the bacteria were harvested and resuspended in 0.5 ml of 100 mM CaCl₂. Storage of cells at 4°C overnight increases their competence.

(b) Transformation of cells with DNA

200 μ l of competent cells was added to an Eppendorf tube containing the DNA and the mixture incubated on ice for 60 min. After 3 min heat shock at 42°C the bacteria were diluted into 2 ml prewarmed nutrient broth and grown for 2 h at 37°C. 0.1 ml aliquots of undiluted culture, 10^{-1} and 10^{-2} dilutions were then spread over nutrient agar plates containing antibiotics to select for plasmid transformed cells. Dilutions of 10^{-5} and 10^{-6} were also plated onto nutrient agar to assay viable cells. Plates were incubated overnight at 37°C. Transformants were restreaked and purified on nutrient agar containing the appropriate antibiotics. With untreated, supercoiled plasmid DNA 1 μ g was used per transformation; half of a ligation mix was used in a transformation mix.

2.14 Recovery of DNA from agarose gels by electrophoresis onto DEAE-cellulose paper (Dretzen *et al.*, 1981)

It is important that only the highest quality of agarose can be used in this method if the DNA is required for further manipulations involving enzymes. Most grades of agarose contain sulphated polysaccharides which are extracted from the gel together with the DNA, these substrates are potent inhibitors of most of the enzymes commonly used in subsequent cloning steps.

A 0.5% agarose gel was prepared using 2 cm wide slots such that a large amount of DNA could be loaded. The gel was run in the usual manner and then visualised over UV light. Using a sharp scalpel, the piece of

agarose containing the required band was removed and wrapped in a small strip of Whatman DE81 DEAE-cellulose paper. The piece was then returned to the gel and squeezed firmly against the gel to close the incision. The gel was then returned to the tank and electrophoresis resumed at 200 V until the DNA had entered the paper strips; this can be monitored with an overhead UV lamp. The method allows for recovery of bands that are close together. Paper above the bands of interest prevents contamination by larger fragments.

The DE81 paper containing the DNA was removed from the gel and excess paper discarded. The paper was then placed in a 750 μ l Eppendorf tube containing a small plug of glasswool and 400 μ l elution buffer added (1M NaCl, 50 mM Tris-Cl pH8.0, 1 mM EDTA). The paper was shredded by vortexing and the tube incubated at 37°C for 3 hours followed by 5 min at 65°C. A small hole was made in the bottom of the tube with a fine needle and the tube then placed inside a topless 1.5 ml Eppendorf tube and the tubes were centrifuged for 5 min in a microfuge.

The eluate was extracted with phenol, followed by chloroform and then precipitated with ethanol. The DNA was resuspended in 1/20 volume TE buffer.

2.15 SDS polyacrylamide gel electrophoresis

(a) Preparation and running of gels. The procedure was based on that of Laemmli (1970) using vertical slab gels.

The buffers, solutions and gel recipes used are given in Table 2.6. Gels were usually 1 mm thick, and composed of a 7% acrylamide stacking

Table 2.6 SDS PAGE solutions, buffers and gel recipes

Gel composition

		13%	15%	
Plug gel	Buffer A	13.5	13.5	ml
Separating gel	Acrylamide	8.0	9.2	ml
	H ₂ O	4.8	3.6	ml
	Ammonium persulphate (APS)	0.95	0.95	ml
	freshly made, 10 mg/ml			
	N,N,N',N'-tetramethyl	75	75	μl
	ethylene diamine (TEMED)			
		7%		
Stacking gel	Buffer B	10.0		ml
	Acrylamide	3.3		ml
	H ₂ O	6.7		ml
	APS	0.5		ml
	TEMED	40		μl

TEMED was always added immediately before the gel was poured.

<u>Buffer A</u>	Tris-Cl (pH8.8)	0.75 M
	SDS	0.2% w/v
<u>Buffer B</u>	Tris-Cl (pH6.8)	0.25 M
	SDS	0.2% w/v

Cont.....

Table 2.6 Continued 2

<u>Acrylamide</u>	Acrylamide	4.4% w/v
	N,N'-methylene-bis-acrylamide (bis)	0.8% w/v
<u>Electrophoresis buffer</u>	Trizma base	0.125 M
	Glycine	0.192 M
	SDS	0.1% w/v
<u>Sample buffer</u>	Tris-Cl pH6.8	0.125 M
	Glycerol	20% v/v
	β -mercaptoethanol	10% v/v
	SDS	4% w/v
	Bromophenol blue	0.05% w/v
1/5 volume of this sample buffer was added to each sample before boiling and electrophoresis.		
<u>Destain</u>	Propan-2-ol	25% v/v
	Acetic acid	10% v/v
<u>Stain</u>	Coomassie brilliant blue	0.1% w/v in destain

gel with a 13% or 15% separating gel, 1 cm of effective stacking distance was allowed between the sample wells and the surface of the separating gel. An 11% plug gel was used to prevent leakage. All samples were boiled for 5 min before loading and electrophoresis carried out at 25 mA, until the dye front was within 5 mm of the bottom of the gel. Gels were then either fixed by shaking in 200-300 ml of destain (Table 2.6) for at least 30 min, or stained by shaking overnight in 200-300 ml stain solution (Table 2.6), followed by a destain by diffusion in several changes of destain solution, shaking throughout.

(b) Molecular weight markers

For radioactive molecular weight markers a [^{14}C]-methylated protein mixture was used supplied by Amersham International. This contained proteins of the following molecular weights myosin (200 000) phosphorylase B (100 000, 92 500), bovine serum albumin (69 000), ovalbumin (46 000), carbonic anhydrase (30 000) and lysozyme (14 000). For stainable markers a mixture of proteins supplied by BRL was used which contained the above proteins with the substitution of α chymotrypsinogen for carbonic anhydrase and the addition of β -lactoglobulin (18 400). A 10 μl solution contained 0.5 μg of each protein.

(c) Autoradiography and fluorography. For autoradiography fixed gels were dried onto a sheet of Whatman No.17 chromatography paper using a Biorad slab gel drier model 1125. The dried gels were placed in a cassette with a sheet of Kodak XR P5 X-ray film for exposure. The films were developed using Kodak DX-80 developer, a 1% acetic acid wash, and Kodak FX-40 fixer.

Fluorography was carried out using the method of Bonner and Laskey (1974)
: fixed gels were dehydrated by two 30 min washes in 300 ml
dimethylsulphoxide (DMSO), after which the gels were soaked for 90 min in
200 ml 22% PPO w/v dissolved in DMSO and then rehydrated by soaking in
water for 60 min. The gels were then dried and autoradiographed as
above, except the cassettes were stored at -80°C during exposure.

2.16 Preparation of bacterial cell lysates

3 ml of bacterial culture was centrifuged in the Sorvall SS34 rotor at
7,000 rpm for 5 min. The pellet was resuspended in 2.5 ml 20 mM Tris-Cl
(pH7.5), 50 mM NaCl and the A_{450} read. 5 A_{450} units of cells were added
to an Eppendorf tube and pelleted in a microfuge. The cells were then
resuspended in 150 μl bacterial sample buffer lacking bromophenol blue
and incubated at 60°C for 5 min followed by 100°C for 5-10 minutes until
the suspension was clear. The suspension was transferred to a 50 Ti tube
and centrifuged in the Beckman 50 Ti rotor at 30,000 rpm for 30 min. The
supernatant was transferred to an Eppendorf tube and 1 μl bromophenol
blue added before analysis by SDS PAGE.

2.17 Preparation of bacterial cell envelopes from sonicated lysates

A 150 ml culture of bacteria was set up and grown to an A_{450} 0.5 before
being induced with $50\text{ }\mu\text{g ml}^{-1}$ IPTG for 2 hours; for non-inducible strains
a culture grown to an A_{450} 1.5-2.0 was used. Cells were harvested by
centrifugation (Sorvall GSA rotor 8,000 rpm 10 min 4°C) and resuspended
in 7 ml ice cold 10 mM sodium phosphate buffer (pH7.2). The cells were
transferred to a 25 ml beaker and sonicated for three 30s intervals,

with 30s cooling periods, using the 3/4 inch diameter probe in a 150 watt MSE ultrasonic disintegrator.

The sonicated sample was transferred to a centrifuge tube and centrifuged (Sorvall SM24 rotor, 7,000 rpm, 5 min 4°C) to remove unlysed cells. The supernatant was transferred to a Beckman 50 Ti centrifuge tube and the envelope pelleted (Beckman 50 Ti rotor, 35,000 rpm, 40 min, 4°C). The supernatant was transferred to an SM24 centrifuge tube on ice and 1/10 volume 50% TCA added to precipitate the cytoplasmic and periplasmic proteins. The pellet was resuspended in 10 mM sodium phosphate buffer pH7.2 and the envelopes repelleted (Beckman 50 Ti rotor, 35,000 rpm, 40 min, 4°C). The washed envelope pellet was resuspended in 150 µl sodium phosphate buffer and 150 µl sample buffer added. To separate the outer and inner membrane the washed pellet was resuspended in 150 µl 0.5% w/v sarkosyl NL97. This was incubated at room temperature for 30 min to solublise the membranes and centrifuged (Beckman 50 Ti, 35,000 rpm, 90 min, 4°C) to pellet the outer membrane. The supernatant was transferred to an Eppendorf tube. This fraction contained the inner membrane. The outer membrane pellet was gently rinsed with sodium phosphate buffer, drained and the tube walls carefully dried.

The pellet was resuspended in 150 µl sodium phosphate and transferred to an Eppendorf tube. Both membrane fractions were prepared for SDS-PAGE by addition of 150 µl SDS lysis buffer. The TCA precipitate was centrifuged (Sorvall SM24, 7,000 rpm 10 min 4°C) to pellet the proteins and the pellet resuspended in 150 µl SDS lysis buffer, the pH was adjusted with concentrated Tris buffer and the volume made up to 300 µl with water. All samples were boiled for 5 min. Samples were stored at -20°C and reboiled before loading onto a gel.

For radioactively labelled cells the same procedure was followed, however, unlabelled carrier cells of the same strain are added prior to sonication to give 10 A₄₅₀ units.

2.18 Preparation of periplasmic fractions by osmotic shock

(a) E. coli

A 25 ml culture grown in LuB to an A₄₅₀ of 1 was harvested (SS34 8K 10 min) and the cells washed twice with 10 mM Tris-Cl (pH7.1), 30 mM NaCl. The cells were then resuspended in 2.5 ml 20% Sucrose, 30 mM Tris-Cl (pH7.1), 2.5 ml 40% sucrose/Tris was added followed by 10 µl 50 mM EDTA, all solutions were at room temperature. The cells were then incubated shaking at room temperature for 8 min and then harvested in the MSE bench centrifuge at room temperature.

The pellet was resuspended in 5 ml ice cold 5mM MgCl₂ and the cells shaken on ice for 8 min before being harvested in the MSE Chilspin. The supernatant containing the periplasmic fraction was concentrated 10 times by TCA precipitation and an equal volume of sample buffer was added. 40 µl of sample was loaded on a gel.

(b) M. methylotrophus

A 50 ml culture was grown to late exponential phase and the cells harvested and washed in 25 mM Tris-Cl (pH7.5). The pellet was then resuspended in 5 ml 25 mM Tris-Cl (pH7.5), 10% sucrose, 1 mM EDTA and the cells shaken at room temperature for 10 min. The cells were centrifuged

and the supernatant fraction collected, this contained the periplasmic proteins. An equal volume of sample buffer was added and 40 μ l samples loaded on a gel.

2.19 The maxicell system

The strain used for this in vivo expression system was CSH26 Δ F6, which carries a recA deletion and is therefore highly sensitive to UV-irradiation. The method used here is based on that of Sancar et al. (1979), who found that low UV doses cause extensive degradation of chromosomal DNA in recA strains, whilst most plasmid DNA copies remain intact due to a lower probability of receiving a UV hit. Plasmid encoded proteins can therefore be identified by labelling the proteins synthesised after chromosomal degradation has occurred.

Plasmid bearing strains of CSH26 Δ F6 were grown in M9 minimal medium containing the appropriate antibiotics, from A_{450} 0.05 to A_{450} 0.5. 2.5 ml of each strain was transferred to a 2" diameter sterile petri dish, and irradiated, while shaking, with a dose of 4 Joules, from a Hanovia bactericidal lamp measured with a Latarget dosimeter. A control culture of plasmid free CSH26 Δ F6 was also irradiated. 2 ml of each culture was then transferred to a foil covered 6" x 5/8" test tube, and the tubes shaken at 37°C for 60 min. Cycloserine was then added to a final concentration of 200 μ g ml⁻¹ in order to kill surviving cells, before shaking the tubes overnight at 37°C.

1 ml of irradiated cells of each strain were collected by centrifugation in sterile Eppendorf tubes, and bacteria resuspended in 0.5 ml fresh, prewarmed minimal medium containing 200 µg cycloserine. The tubes were incubated at 37°C for 60 min, then 2 µl of ^{35}S -methionine ($10\text{ }\mu\text{Ci ml}^{-1}$) was added to each, to label the plasmid encoded proteins. After 60 min incubation at 37°C, 10 µl of 8 mg ml^{-1} methionine was added to each tube. The bacteria were then pelleted in an Eppendorf centrifuge (5 min) and resuspended in 60 µl of bacterial buffer. 60 µl of SDS-PAGE sample buffer was added to each sample before boiling and the proteins analysed on an acrylamide gel.

2.20 Minicells

The minicell producing strain DS410 was used; the minicells were purified from a 400 ml overnight culture grown in Luria broth containing appropriate antibiotics. The cells were harvested for 15 min at 8,000 rpm, resuspended gently in 3 ml of M9 minimal medium, and layered onto a sucrose gradient in a clear SS34 tube (20% sucrose in M9 minimal medium, previously frozen in the tubes and allowed to thaw overnight at 4°C) which were centrifuged at 5,000 rpm for 20 min in the HB4 rotor. The minicells banding in the central region of the gradient, were removed with a pasteur pipette and pelleted in the SS34 rotor for 10 min at 13,000 rpm. The pellet was resuspended in 1 ml M9 minimal medium and layered onto another sucrose gradient and centrifuged as before. The top half of the minicell band was removed and pelleted as before. The pellet was resuspended in 200 µl of M9 minimal medium, 10 µl of minicells were plated out for a viable count, and the cells were also checked under a phase contrast microscope.

To label plasmid encoded proteins, 50 μ l of minicells were transferred to an Eppendorf tube and incubated at 37°C for 60 min, then 30 μ Ci of [³⁵S]-methionine was added and incubation continued at 37°C for 30 min. 10 μ l 8 mg ml⁻¹ methionine was then added and the cells incubated for a further 5 min. The minicells were pelleted in the microfuge and resuspended in 60 μ l bacterial buffer. 60 μ l of SDS PAGE sample buffer was added before boiling and analysing by SDS PAGE.

2.21 Fractionation of minicells

Cytoplasmic/periplasmic and envelope proteins were prepared using a procedure modified from section 2.17. Minicells were labelled using the method above, harvested and resuspended in 7 ml of envelope buffer (10 mM phosphate buffer, (pH7.2)) before being lysed by sonication; sonication was carried out for 40s with 20s cooling since minicells require longer sonication. Remaining whole cells were removed by centrifugation (13,000 rpm; 10 min) and cell membranes were sedimented by centrifugation (35,000 rpm; 30 min). Since only a small number of cells are present in minicells 'cold carrier' membrane was added in order to enhance pelleting of membrane. The soluble and membrane fractions were treated in the same way as section 2.17.

2.22 Western blotting

Method as described by Towbin et al. (1979) was followed. The antibody reaction involved a 1:200 dilution of the primary antibody in PBS tween, and a 1:1000 dilution of secondary anti-rabbit antibody and of PAP. A 0.02% solution of chloronaphthol in 20% methanol was used for staining

combined with 0.1% hydrogen peroxide. This reaction is stopped by water and the filter immediately dried in the dark.

2.23 Colony hybridisation

(a) Preparation of colonies for hybridisation

Colonies were patch plated onto selective plates covered with a gridded nitrocellulose disc and allowed to grow at 37°C overnight.

Petri dish lids were prepared by lining with 2 sheets of 3 mM paper and soaking each pad thoroughly in the solutions described in the Table 2.7. The nitrocellulose disc was removed from the plate and placed colony side up on each pad for the given time indicated in Table 2.7. Residual colony debris was blotted from the filter prior to baking at 80°C for 2 hr. The filters were then ready for hybridisation in conditions of 6 x SSC.

(b) Hybridisation

Filters were prehybridised and hybridised in sealed perspex boxes at 65°C. Pre and hybridisation mixes contain 5 x Denhardts solution 3 x SSC, 6% PEG 6000 and 100 µgml⁻¹ sheared, denatured salmon sperm DNA to reduce background. Prehybridisation took place for 2-4 hr after which the solution was removed and replaced by 20 ml prewarmed solution containing 5 x 10⁵ - 5 x 10⁶ cpm radiolabelled DNA probe which had been boiled for 2 min. Hybridisation generally took place overnight at 65°C. Filters were washed in 4, 30 min washes in 3 x SSC with 0.1% SDS at 65°C. Filters were then dried, mounted on card, covered in

Table 2.7 Preparation of colonies for hybridisation

<u>Solution</u>		<u>Time</u>
0.5 M	NaOH	7 min
1.0 M	Tris-Cl (pH7.4)	2 min
1.0 M	Tris-Cl (pH7.4)	2 min
0.5 M	Tris-Cl (pH7.4), 1.5 M NaCl	4 min

Table 2.8 Hybridisation solutions

5 x Denhardt's Solution	0.1%	Bovine Serum Albumin
	0.1%	Polyvinyl pyrrolidone
	0.1%	Ficoll 400
1 x SSC Solution	0.15M	NaCl
	0.15M	Sodium Citrate (pH7.0)

Table 2.9

Labelling buffer

10 μ l OLB
2 μ l BSA (10 mg/ml)
DNA in agarose (upto 32.5 μ l)
5 μ l [32 P]dCTP (10 μ Ci/ μ l)
2 μ l Klenow (1 U/ μ l)
x μ l H₂O
50 μ l Total

OLB

Solution 0

1.25 M Tris-Cl (pH8.0)
0.125 M MgCl₂

Solution A

1 ml solution 0
18 μ l 2-mercaptoethanol
5 μ l dATP (100 mM)
5 μ l dTTP (100 mM)
5 μ l dGTP (100 mM)

Solution B

2 M Hepes titrated to pH6.6 with 4 M NaOH

Table 2.9 continued

Solution C

Hexadeoxyribonucleotides (pd(N)₆)

evenly suspended in TE at 90 OD.units/ml

Mix solutions A:B:C in a ratio of 100:250:150 to make OLB

Stop solution

20 mM NaCl

20 mM Tris-Cl (pH7.5)

2 mM EDTA

0.25% SDS

1 μ M dCTP

clingfilm and exposed to X-ray film in the presence of an intensifying screen at -80°C for 5-24 hr depending on the activity of the probe.

2.24 Southern blotting

DNA to be hybridised was electrophoresed through a thin 0.7% agarose gel in the normal manner. The gel was then soaked for 7 min in 0.25 M HCl followed, after a rinse, by a 40 min soak in 0.5 M NaOH, 1.5 M NaCl to denature the double strands. Neutralisation then took place in 1 M Tris-Cl (pH5.5), 3 M NaCl for 1 hr. The blot was set up as described by Southern (1975) and blotting usually occurred overnight at room temperature.

The nitrocellulose containing the blotted DNA was washed in 3 x SSC prior to baking at 80°C for 2 hr.

Hybridisation was the same as that described for colony hybridisation using conditions of 3 x SSC.

2.25 Preparation of probe for hybridisation

(a) Nick translation

Method followed using Nick translation kit, supplied by Amersham International.

(b) Oligolabelling

The DNA to be used as a probe was isolated from a 0.6% low gelling temperature agarose gel. All the excess agarose was removed and the band transferred into a preweighed 1.5 ml microcentrifuge tube; the tube was then reweighed.

Water was added at a ratio of 3 ml H₂O per gram of gel. The tube was then placed in a boiling water bath for 7 min to dissolve the gel and denature the DNA; this DNA can then be stored at -20°C.

Prior to labelling a 20-30 ng of DNA were taken and reboiled for 3 min and then incubated at 37°C for 30 min. The labelling reaction was carried out at room temperature; labelling reagents shown in table 2.9 were added and incubated for at least 5 hr. The reaction was then stopped using 200 µl of the stop solution (Table 2.9).

CHAPTER 3

Identification of the hisJ gene product

3.1 Introduction

Plasmid pFA6 and phage λ gt4-Sa1 (Ardeshir and Ames, 1980) both contain the entire histidine permease operon of Salmonella typhimurium within a 12.4 kb EcoR1 fragment. Plasmid pFA6 is a pBR322 derivative containing this 12.4 kb fragment cloned into the EcoR1 site. A deletion around the EcoR1 site (ΔE , Fig. 3.1) had been created during the construction of this plasmid and the plasmid bearing strain TA3457 was grown only with great difficulty. Further manipulation of this cloned DNA was necessary. The histidine permease operon is 3.5 kb in size and is therefore flanked by a large amount of partially uncharacterised DNA within this 12.4 kb fragment in pFA6. Since the protein products of the histidine permease operon are produced in very small amounts, it was necessary to attempt to overproduce the gene products in order to identify them in order to subsequently study their expression in M. methylotrophus. It had been suggested (C. Higgins, personal communication) that overproduction of the histidine permease may be lethal, I therefore decided to clone the structural genes of histidine permease into pUC12 under the control of the lac promoter. Gene expression was expected to be controllable, remaining in a repressed state due to the presence of the lac repressor. The addition of IPTG for short time periods, however, induces expression of the cloned genes.

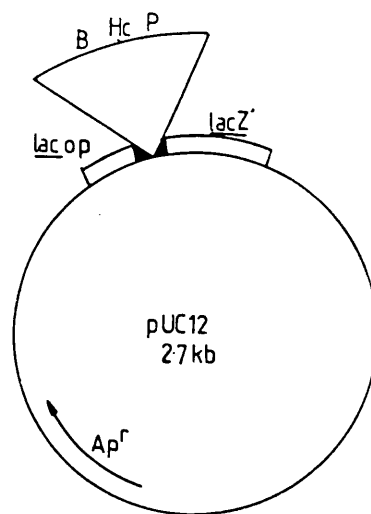
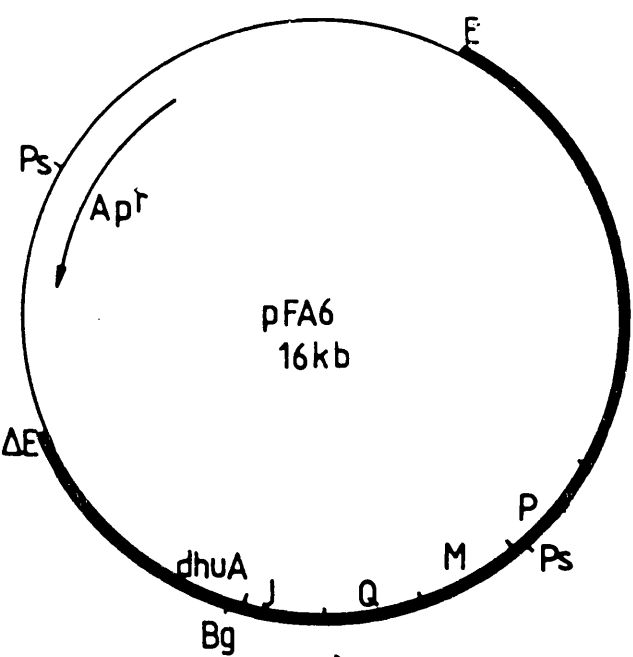
Figure 3.1

The construction of plasmid pYCP2.

Plasmid pFA6 contains the entire histidine permease operon within a 12.4 kb EcoRI fragment. During construction of this vector (Ardeshir et al., 1981) one EcoRI site spontaneously deleted as indicated by ΔE .

Restriction sites: Ps = PstI
 E = EcoRI
 Bg = BglII
 Hc = HincII
 H = HindIII

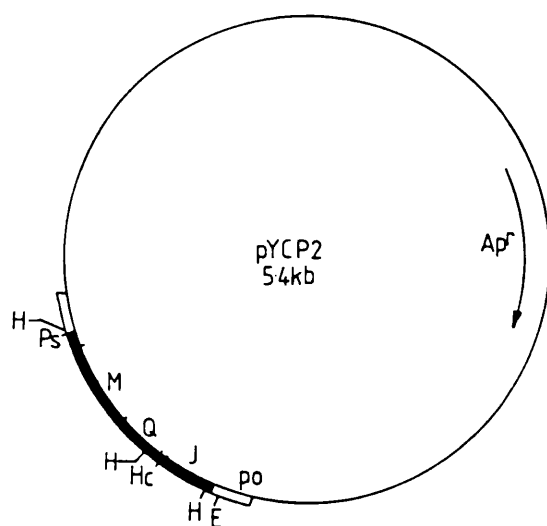
These plasmids are not drawn to scale.



1) BglII
2) PstI

1) BamHI
2) PstI

ligase



3.2 Construction of plasmid pYCP2

Fig. 3.1 shows the strategy used for cloning a 2.5 kb BglIII-PstI fragment from pFA6 into BamH1-PstI cut pUC12. This fragment contains hisJ, hisQ and hisM genes together with the promoter-proximal portion of the hisP gene. The resulting plasmid - pYCP2 - carries the his permease genes lacking their own promoter and now under control of the lac promoter.

3.3 Analysis of the gene products of pYCP2

The ligation mixture was used to transform E. coli NM522 and the plasmid-bearing cells selected on LuB containing ampicillin. X-gal and IPTG were also included in the plates to allow easy selection of plasmids containing an insert. Plasmid DNA prepared from these white colonies was screened by the restriction enzymes shown in Fig. 3.2 to confirm the identity of plasmid pYCP2.

Plasmids pUC12, pYCP2, pFA6 and pFA13 were used as templates in an in vitro transcription-translation system (Zubay, 1973) and the protein products analysed by SDS PAGE. Plasmid pFA13 is a derivative of pFA6 containing the entire histidine permease operon now within a smaller 5.5 kb fragment (see section 4.1), therefore providing cleaner results when analysing the gene products. These plasmids were also used to transform E. coli strain CSH26ΔF6, a strain normally used for the preparation of maxicells (see section 2.19). Maxicells could not however be prepared from CSH26ΔF6 (pFA6) since the strain was unable to grow in minimal medium; as there is no problem incurred when using CSH26ΔF6 (pFA13) it

Figure 3.2

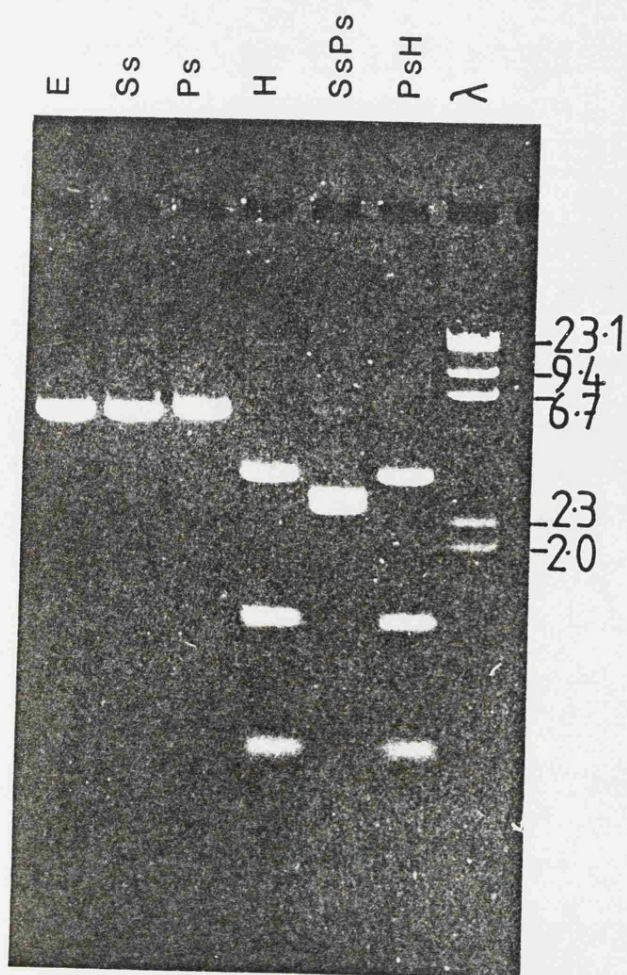
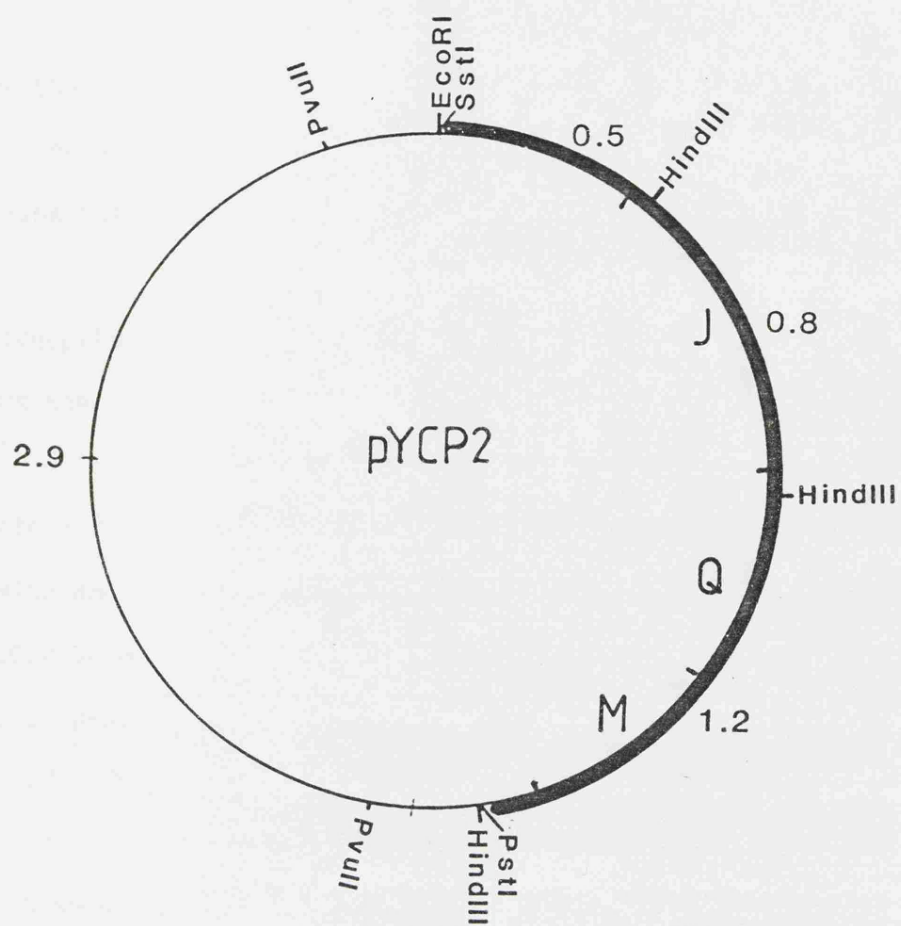
Plasmid pYCP2 and restriction digests of the plasmid.

Diagram showing a scaled map of plasmid pYCP2 together with restriction digests confirming it to be the expected recombinant. Plasmid pYCP2 is 5.4 kb in size and confers resistance to ampicillin.

The heavy line represents the area of the histidine permease operon.

Restriction sites: E = EcoR1
 Ss = Sst1
 Ps = Pst1
 H = HindIII

This plasmid is drawn to scale.



appears that a region within the 12.4 kb fragment of pFA6 which is outside the his permease operon and therefore not present in pFA13 must be causing these effects.

The polypeptide products from all these experiments were analysed by SDS PAGE and the results shown in Fig. 3.3.

It is clear from Fig. 3.3a that plasmid pYCP2 encodes a polypeptide in vitro with an apparent molecular weight of 31,000 (as indicated), above the 30,000 band of pre β -lactamase. A similar polypeptide is also encoded by pFA6 and pFA13 suggesting that this protein is a component of the histidine permease.

Fig. 3.3b shows a similar result, however, since maxicells express plasmid genes in vivo the gene products are mature and the band representing β -lactamase now shows an apparent molecular weight of 29,000. The unique polypeptide from pYCP2 and pFA13 is again clear as indicated and it is also apparent that the amount of this protein produced is much greater for pYCP2 than pFA13. Although it is not clear from Fig. 3.3, the new polypeptide of about 31,000 in vivo runs slightly faster than the presumed precursor form in vitro; this is evident when samples from an in vitro and in vivo (maxi-cell) experiment are analysed on the same gel (data not shown). From these experiments it is evident that this 30,000 polypeptide is expressed from the his permease operon.

Osmotic shock of the maxicell samples shows that this protein, together with β -lactamase, is isolated from the shocked supernatant; this indicates that the protein is located in the periplasmic space of

Figure 3.3

Products of in vitro and in vivo expression of pYCP2 and the parent vectors.

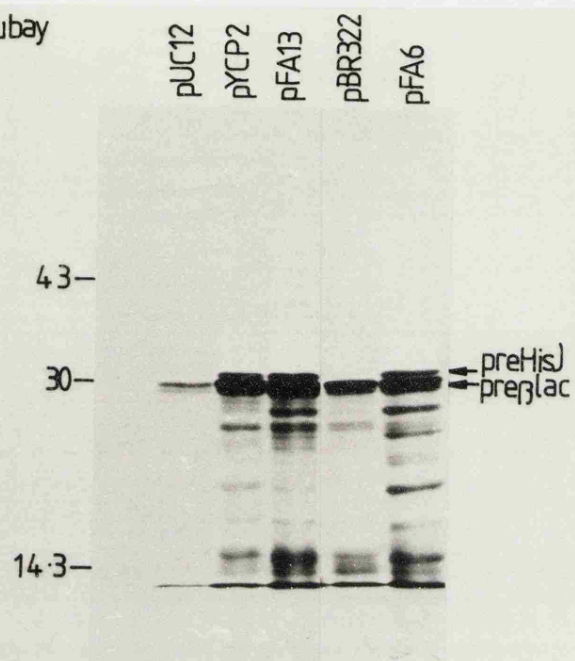
- (a) Polypeptide products resulting from in vitro transcription-translation of the plasmid DNA, as described by Zubay (1978). Polypeptides are in precursor form.
- (b) Polypeptide products from plasmids expressed in vivo by maxicells. Polypeptides are primarily in mature form.

In both experiments, proteins were labelled with [³⁵S] methionine and analysed by 13% PAGE followed by fluorography.

The position of precursor and mature β lactamase are shown together with the proposed HisJ protein.

The identity of β lactamase was confirmed by a western blot of these samples, blotting with antibody raised against β lactamase protein. (Data not shown).

a) zubay



b) maxicell



E. coli. (Data not shown but stained profile shown in Section 3.7). I therefore propose that this polypeptide corresponds to the hisJ gene product.

3.4 Identification of the hisJ gene product by Tn5 mutagenesis

In order to determine the identity of the 31,000 polypeptide produced from plasmid pYCP2, insertion mutagenesis by transposon Tn5 was carried out. Tn5 insertion mutagenesis represents a relatively rapid method for defining regions of plasmid DNA encoding particular genes. A useful vector for mutagenesis consists of transposon Tn5 carried on a λ phage λ CI857 O_{am} . The phage can enter neither the lytic cycle (due to the O_{am}), nor the lysogenic cycle if maintained in an E. coli sup⁰ host at 37°C. The infection was carried out as described in section 2.5 and DNA prepared from plasmids containing the Tn5 insertions.

3.5 Mapping the position of the Tn5 insertions

The positions of the Tn5 insertions were mapped using the restriction enzymes EcoRI and HindIII, followed by EcoRI PvuII using the following criteria.

Plasmid pYCP2 contains a unique EcoRI site and three HindIII sites whilst transposon Tn5 contains no EcoRI site and two HindIII sites, Fig. 3.4(b). Digestion of pYCP2 with EcoRI-HindIII produces four fragments A,B,C and D : insertion of Tn5 into one of these will cause the disappearance of the fragment from the gel, Fig. 3.4(c). Two new fragments will be produced whose total size will equal the size of the deleted fragment

Figure 3.4

- (a) Restriction map of Transposon Tn5. Heavy lines show the 1.5 kb terminal inverted repeat, whilst the central portion is a unique sequence of 2.7 kb.
- (b) Restriction map of plasmid pYCP2 showing the four fragments resulting from an EcoR1-HindIII restriction digest.

Fragment A represents the pUC12 portion of the plasmid.

Fragment B, includes the whole of the hisM gene and all but the first 37 codons of the hisQ gene.

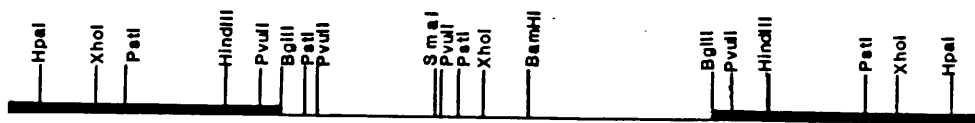
Fragment C includes the hisJ gene.

Fragment D includes the region between the lac promoter and the proximal portion of hisJ.

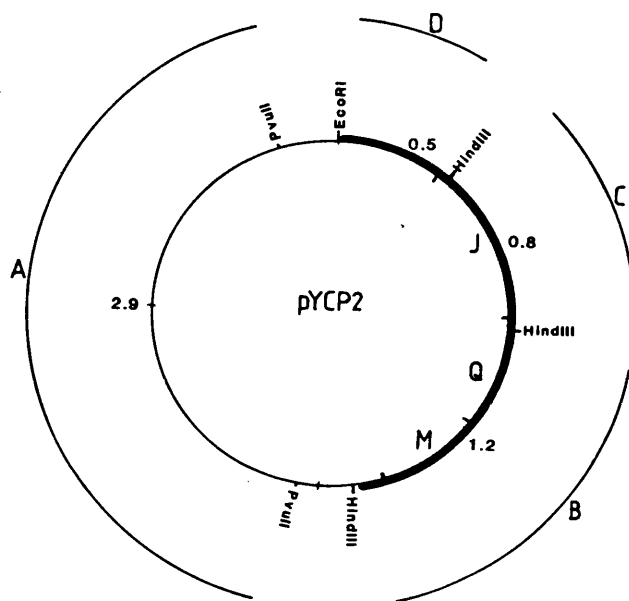
The heavy region represents the fragment of the histidine permease operon.

- (c) The expected restriction pattern on an agarose gel of pYCP2 DNA digested with EcoR1 and HindIII, together with the altered pattern after insertion of Tn5. (New restriction bands expected after insertion, not shown).

A



B



		pYCP2				Tn5 INSERTION				kb
		A	B	C	D	A	B	C	D	
Tn5	—	—	—	—	—	—	—	—	—	3.1
A	—	—	—	—	—	—	—	—	—	2.9

C

B	—	—	—	—	1.2
C	—	—	—	—	0.8
D	—	—	—	—	0.5

plus the fused flanking regions of Tn5. The sizes of these two new fragments will depend on the exact position of the Tn5 insertion within the particular fragment of pYCP2.

This exact position can be determined by restriction with EcoR1-PvuII. Plasmid pYCP2 contains two PvuII sites (Fig. 3.4b), the large 4 kb fragment containing the cloned his permease. Tn5 contains four PvuII sites giving fragments of 0.35, 0.7 and 1.8 kb flanked at either end by 1.4 kb of DNA (Fig. 3.4a). It is the size of these flanking regions which will differ depending on the position of the transposon insertion and since the approximate position has already been determined, the exact position of the insertion within B, C or D can be elucidated.

Fig. 3.5 shows the restriction digest patterns of all the inserts mapping within the cloned fragment and the positions deduced for these Tn5 insertions within the histidine permease genes. Sample 63 is included as an example of an insertion mapping outside the cloned fragment within fragment A (Fig. 3.4b) since the 2.9 kb band is missing in this track.

From Fig. 3.5 three insertions within the hisJ gene are evident (samples 47, 61 and 78). These all appear to map in the same position towards the distal end of the gene and most likely represent the same insertion event in the original mutagenesis. Tn5 insertions are also present in hisQ and hisM and maxicells prepared from these samples may enable these less abundant gene products to be recognised when analysed by SDS PAGE.

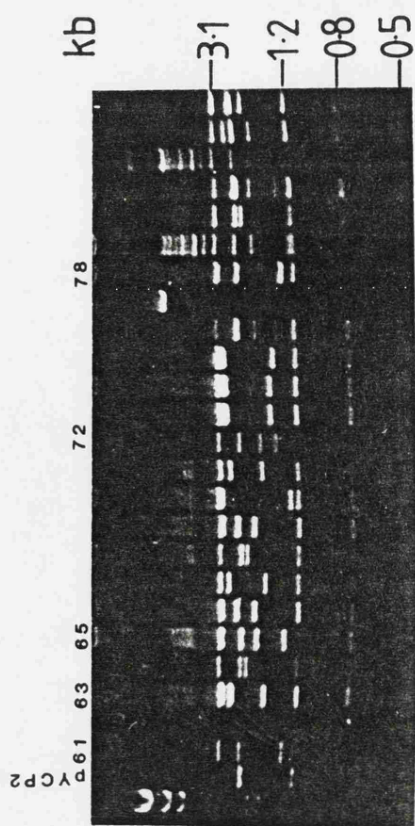
Figure 3.5

Position of Tn5 within the his permease genes of pYCP2.

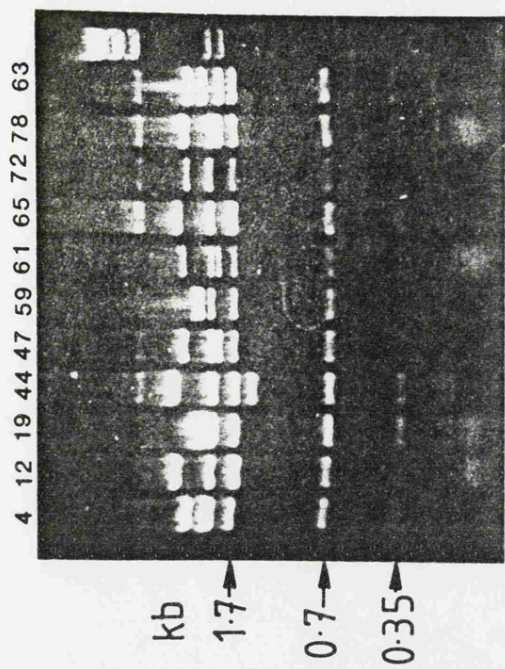
- (a) Results of restriction digests using EcoR1-HindIII. Only DNA samples with Tn5 within the his permease genes are numbered.
- (b) Restriction digests, using EcoR1-PvuII, of all the samples mapping Tn5 within the his permease region, after the first screening (a). Sample 63 represents a sample with Tn5 mapping outside the his permease.
- (c) Positions of Tn5 within plasmid pYCP2 linearised with EcoR1.

Restriction sites : E = EcoR1
H = HindIII
Pv = PvuII

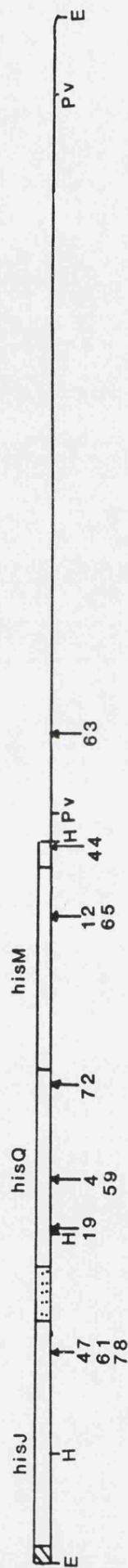
A



B



C



3.6 Analysis of the polypeptides encoded by pYCP2::Tn5

: Identification of HisJ

Maxicells were prepared from a selection of samples containing pYCP2::Tn5 plasmids, where Tn5 maps within the his permease genes. Fig. 3.6 shows the position of the Tn5 insertions within his permease and also the SDS PAGE polypeptide profile of the corresponding samples.

The gel in Fig. 3.6 shows the gene products of plasmid pYCP2 clearly showing the β -lactamase band and the 30,000 polypeptide above this. Samples 78 and 47 both contain a Tn5 insertion within the hisJ gene and the gel clearly shows the absence of the pYCP2 specific 30,000 polypeptide. The other tracks on the gel correspond to insertions downstream of the hisJ gene and clearly in all tracks the 30,000 band is again evident. This 30,000 polypeptide is again localised within the periplasmic fraction when the cells are subject to osmotic shock (section 3.7). This experiment clearly shows that the protein with an apparent molecular weight of 30,000 corresponds to the HisJ polypeptide.

Since the Tn5 insertion maps towards the distal end of the hisJ gene, we might expect to see truncated HisJ polypeptides within these samples. These are not evident perhaps being obscured by the neomycin phosphotransferase polypeptide whose molecular weight of 23,000 corresponds to the estimated size of the truncated polypeptides.

This experiment was designed with the view to also identifying the hisQ and/or hisM gene products. Careful comparison of the polypeptides from sample 65 with those from samples 19, 4 and 72, however, shows no sample

Figure 3.6

Polypeptide products from maxicells of pYCP2 and pYCP2::Tn5 clones.

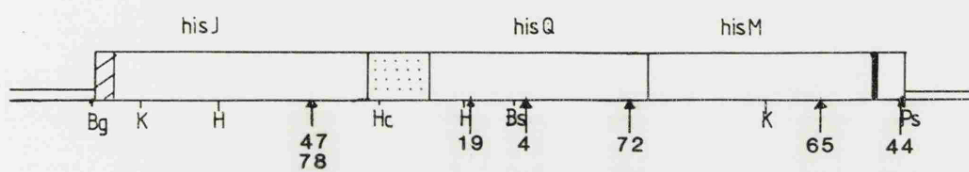
(a) Position of Tn5 inserts within the portion of the his permease operon of pYCP2.

(b) Polypeptide products from maxicells prepared from pYCP2::Tn5.

β la = β lactamase.

Neo = Neomycin phosphotransferase.

Proteins were electrophoresed on a 13% SDS polyacrylamide gel followed by autoradiography.



specific bands with an apparent molecular weight between 20-30,000. No candidates for HisQ or HisM were therefore identified.

3.7 Expression of hisJ in pYCP2 is not tightly regulated by IPTG

The in vivo and in vitro experiments described in the previous sections were carried out in the absence of IPTG. Since LacI may be present in low levels in expression systems (ie. maxicells, minicells), relative to highly expressed plasmid encoded genes, the lac promoter may not be fully repressed and induction with IPTG therefore minimal. Indeed, since the HisJ polypeptide is clearly synthesised in abundance the lac promoter must be promoting relatively constitutive expression of the his permease operon.

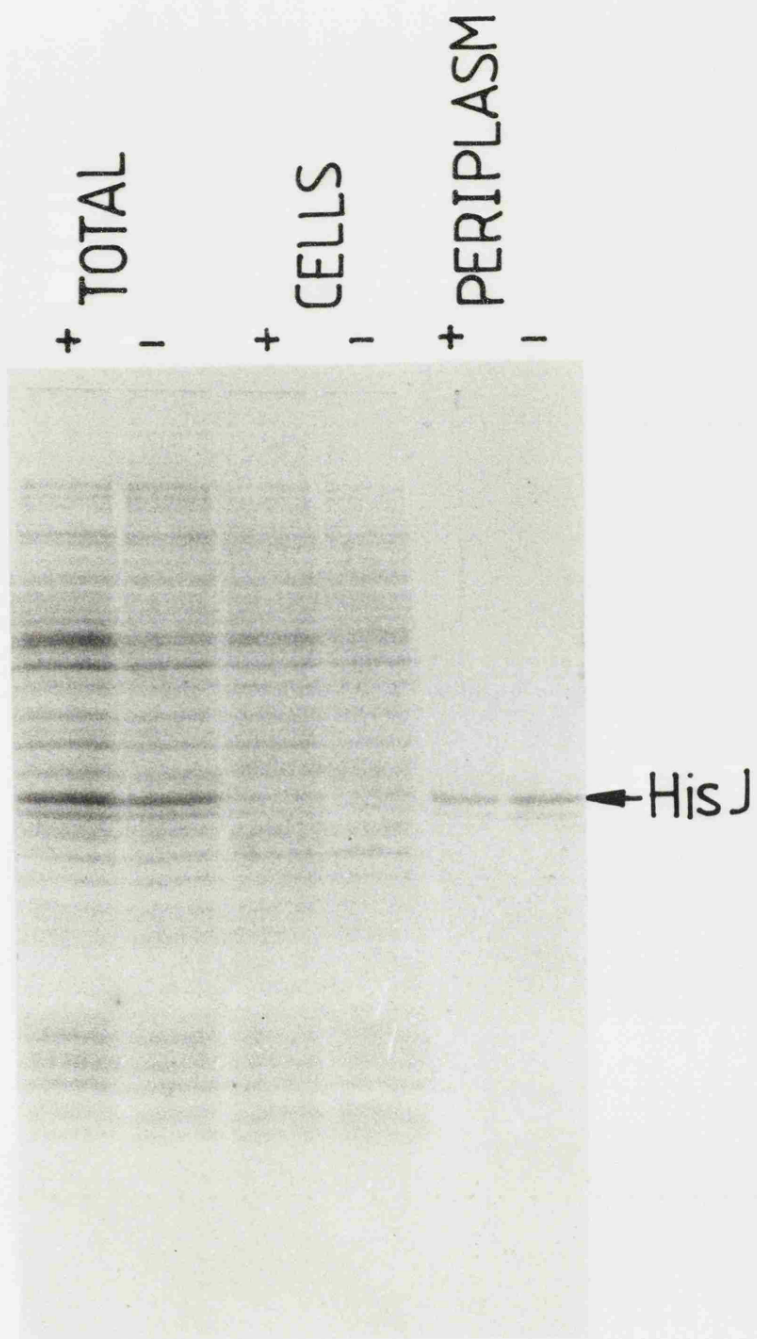
Cell lysates, and osmotic shockates were then prepared from NM522 (pYCP2) grown with and without IPTG and are shown in Fig. 3.7. Firstly, it is clear that the 30,000 HisJ polypeptide, is localised in the periplasm banding above the less abundant β -lactamase polypeptide on the gel. Secondly, this polypeptide is, with and without IPTG, synthesised in equal amounts indicating that lac-mediated expression is occurring also under these conditions in the absence of the inducer IPTG and therefore the hisJ gene is again constitutively expressed.

Strain NM522 in fact contains a lacI^Q gene carried upon an F' plasmid; this synthesises a LacI repressor protein constitutively, but only one copy per cell of the gene is present. Plasmid pUC12 and its derivatives are present in 50-100 copies per cell (Messing, 1983) and therefore the number of copies of the operator greatly exceeds the amount of repressor.

Figure 3.7

Total cell lysates and osmotic shock fractions of NM522(pYCP2).

NM522(pYCP2) were grown with and without a two hour induction by IPTG. A portion of the cells were osmotically shocked as described in section 2.18 to separate the periplasmic proteins from the rest of the cell. The cell fractions were analysed on a 13% SDS PAGE and stained with Coomassie blue.



For this reason genes expressed from the lac promoter within pUC12 cannot be totally repressed by LacI.

3.8 Discussion

These experiments have enabled me to identify HisJ by SDS PAGE and this protein is clearly present in the periplasmic fraction as expected (Ames and Lever, 1972). DNA sequence analysis of hisJ (Higgins and Ames, 1981) revealed a reading frame which could encode a protein with a molecular weight of 25,000 and this matched the amino acid sequence also with a 25,000 molecular weight (R.N. Hogg, 1981). G.F-L. Ames (1974) has previously identified the HisJ polypeptide from whole cell samples of S. typhimurium by SDS PAGE and has reported a polypeptide which migrates to the same position in the gel as the 25,000 molecular weight marker. However, close examination of these results shows that the '25,000' molecular weight marker is in fact the HisJ protein and therefore the apparent molecular weight may not be accurate. Also the gel conditions were different to those I have used.

Plasmid pYCP2 also contains the 5' end of the hisP gene, therefore one might expect to see a truncated HisP protein. However, the 84 base pairs of the hisP gene would encode a protein of approximately 3,500 daltons - too small to be detected by the gel conditions used in these experiments. HisQ and HisM polypeptides could not be identified from these experiments. These proteins are presumably synthesised in much less abundance than HisJ thus making identification difficult. Since the three His polypeptides are predicted to be of similar size, the HisQ and M polypeptides may be obscured by another polypeptide, β -lactamase and neomycin phosphotransferase have molecular weights in this 23 to 29,000 region.

CHAPTER 4

Cloning of the entire histidine permease operon into pUC12

4.1 Introduction

After several unsuccessful attempts at obtaining a plasmid containing the hisJ, Q, M and P genes under lac control in pUC12 (data not shown), I decided that the observed constitutive expression from the lac promoter (Chapter 3) must be having deleterious effects on the cell. The facts that the hisJ, Q and M genes can be expressed from the lac promoter and, that the hisJ gene product appears to assemble correctly in the cell (Chapter 3) suggest that it is the hisP gene product which causes the adverse effects. This may be due to its nucleotide binding capacity, an increase in which could starve the cells of essential energy to support growth. Alternatively, it may be due to the increase in the amount of protein in the membrane disrupting this important barrier. The entire operon had already been successfully cloned into pBR322 to produce pFA13 (G.F-L. Ames, personal communication) and this plasmid appeared stable. The failure to place the structural histidine permease genes under the control of the strong lac promoter therefore suggests that this increased expression must exceed the threshold whereby the amount of protein produced is deleterious to the cells. In an attempt to circumvent this problem I decided to clone the whole permease operon including the dhuA promoter/operator region into pUC12. The experiment was designed so that the operon could insert in either orientation into pUC12. The presence of the dhuA promoter would allow expression of the histidine permease genes. Success in obtaining a recombinant in the opposite orientation to

Figure 4.1

Strategy for inserting the entire his permease operon into pUC12.

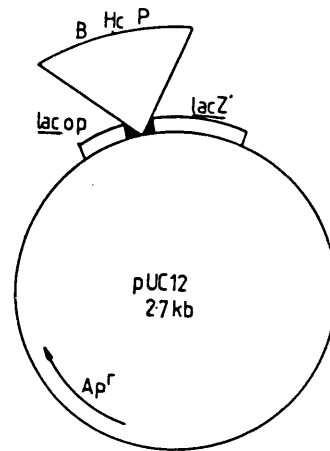
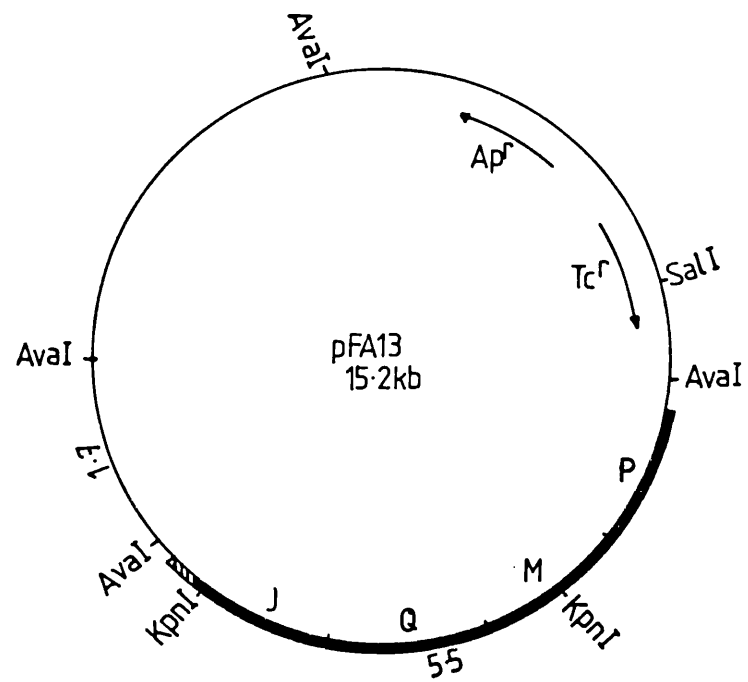
The 5.5 Kb AvaI fragment of pFA13 contains the entire his permease operon. This fragment can be purified and the recessed 3' ends filled in using the Klenow fragment of DNA polymerase I. The blunt ended fragment can be inserted into the HincII site of the pUC12 polylinker. This fragment may ligate in either of two orientations A and B.

Orientation A:- The his permease operon will be in the same orientation as the lac promoter.

Orientation B:- The his permease operon will be in the opposite orientation to the lac promoter, however, the genes can be expressed from the dhuA promoter.

Restriction sites :	H	=	<u>Hind</u> III
	Pv	=	<u>Pvu</u> II
	P/Ps	=	<u>Pst</u> I
	Bs	=	<u>Bst</u> EII
	Bg	=	<u>Bgl</u> II
	Ss	=	<u>Sst</u> I
	Hc	=	<u>Hinc</u> II
	B	=	<u>Bam</u> HI

The plasmids are drawn to scale.



1)AvaI
2)purify
his fragment
3)klenow

HincII

ligase

OR

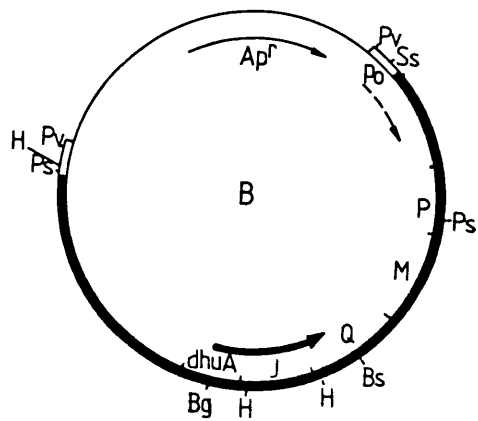
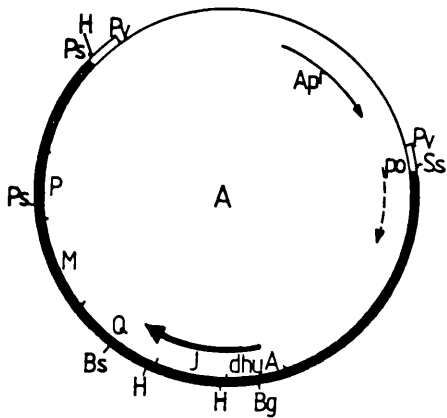


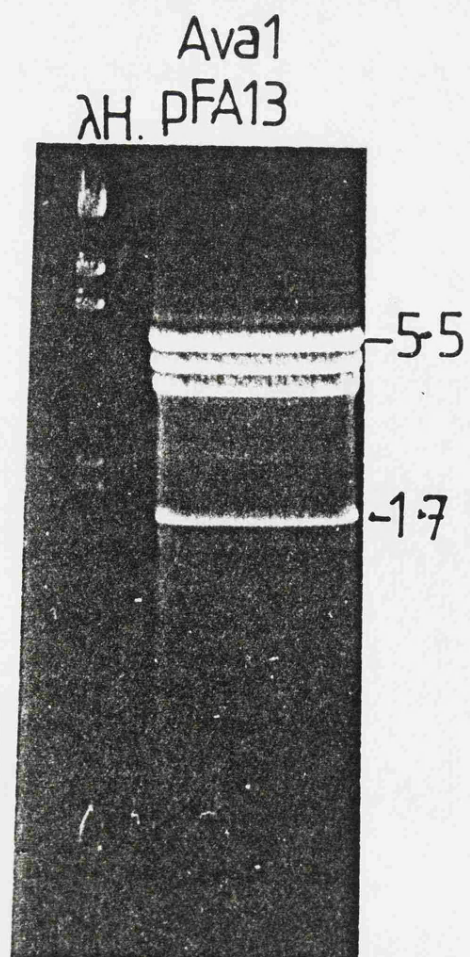
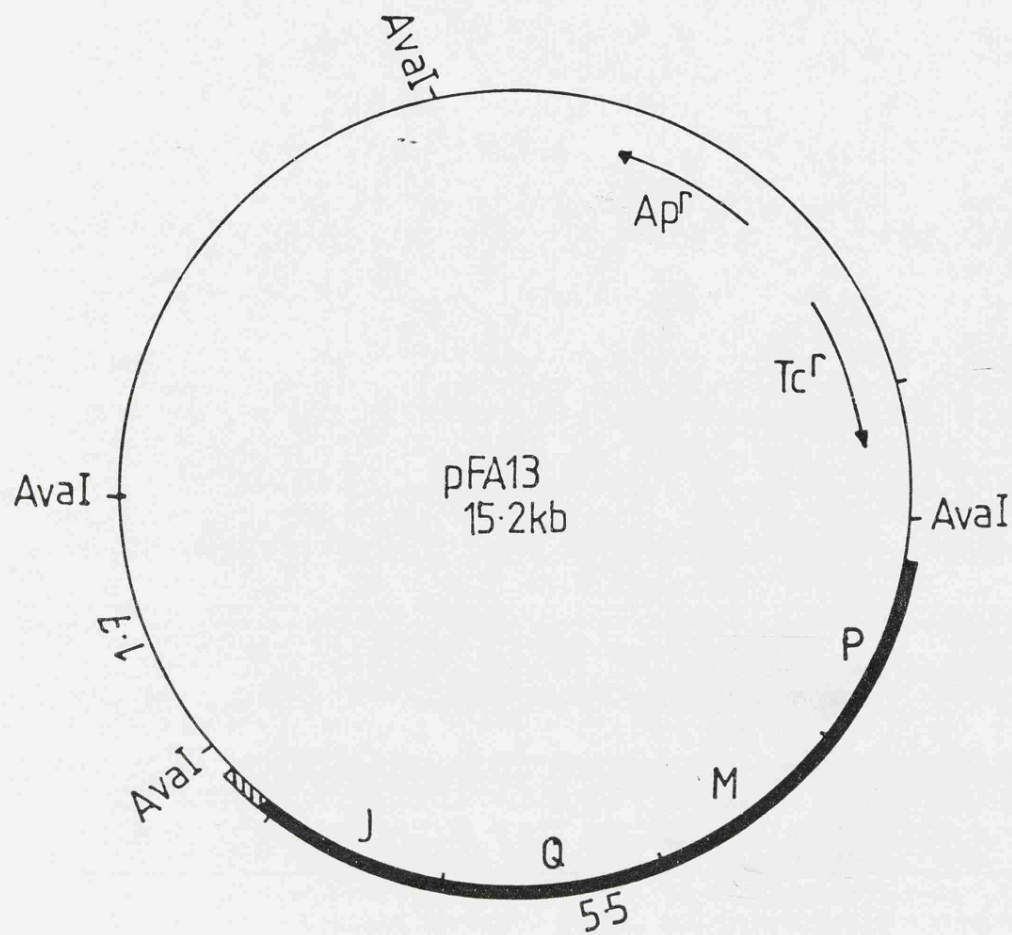
Figure 4.2

Ava1 restriction digest of plasmid pFA13.

The plasmid map shows the restriction sites of Ava1, the his permease operon is located within the 5.5 Kb fragment.

The restriction digest shows Ava1 digested pFA13 run on a 0.5% preparative agarose gel. The 5.5 Kb fragment is shown together with the 1.7 Kb fragment which was used as a control during this cloning experiment.

This plasmid is drawn to scale.



lac would show that the copy number of the permease genes can be increased to the level of the high copy number vector pUC12, and these genes expressed at this level. However, failure to place this operon in the same orientation as the lac promoter would suggest that increased expression (due to the strong lac promoter), together with increased gene copy renders the cells inviable.

Construction of this vector would also provide a convenient intermediate for the subsequent cloning of the whole permease operon into a broad host range vector for introduction into M. methylotrophus. Finally, should any vector contain the operon in the lactose orientation (orientation A in Fig. 4.1) straightforward DNA manipulation would enable the removal of the fragment containing the dhuA promoter thus enabling a further attempt to place the permease genes under inducible, lac, control.

4.2 Cloning the entire his permease operon in both orientations in pUC12

Fig. 4.1 shows the strategy for construction of these plasmids. The 5.5 kb AvaI fragment from plasmid pFA13 contains the whole of the histidine permease operon (GF-L Ames, personal communication). This fragment was purified from an agarose gel (Fig. 4.2) and the single-stranded ends filled in using the Klenow fragment of E. coli DNA polymerase 1 prior to being ligated with HincII digested pUC12. The ligation mix was used to transform E. coli. Due to the indications above that cloning histidine permease into multicopy vectors might be lethal the following precautions were taken:

Figure 4.3

Restriction map and digest pattern of plasmid pYCP5.

Plasmid pYCP5 contains the entire his permease operon in the opposite orientation to the lac promoter (orientation B).

The heavy line represents the 5.5 Kb AvaI fragment whilst the open circle represents the polylinker and flanking regions of pUC12.

The single line represents pUC12.

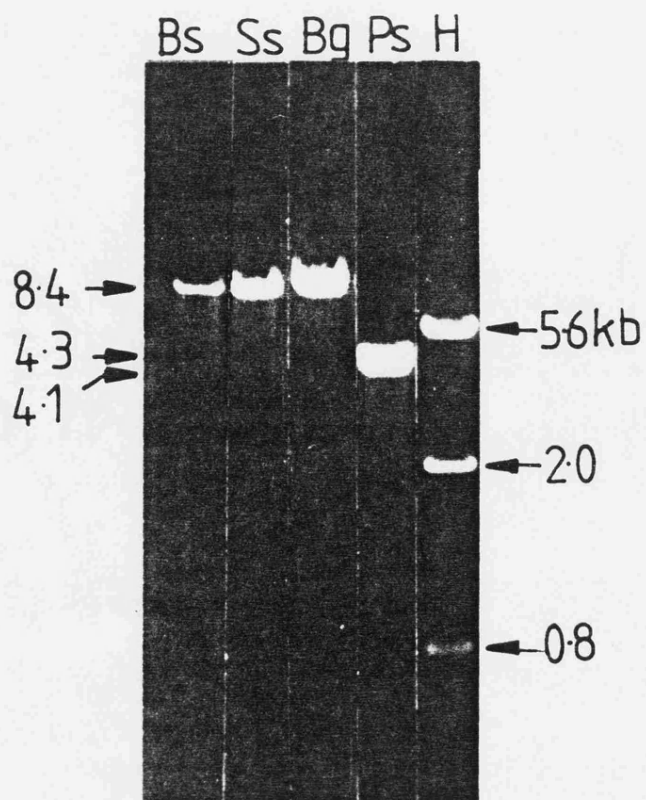
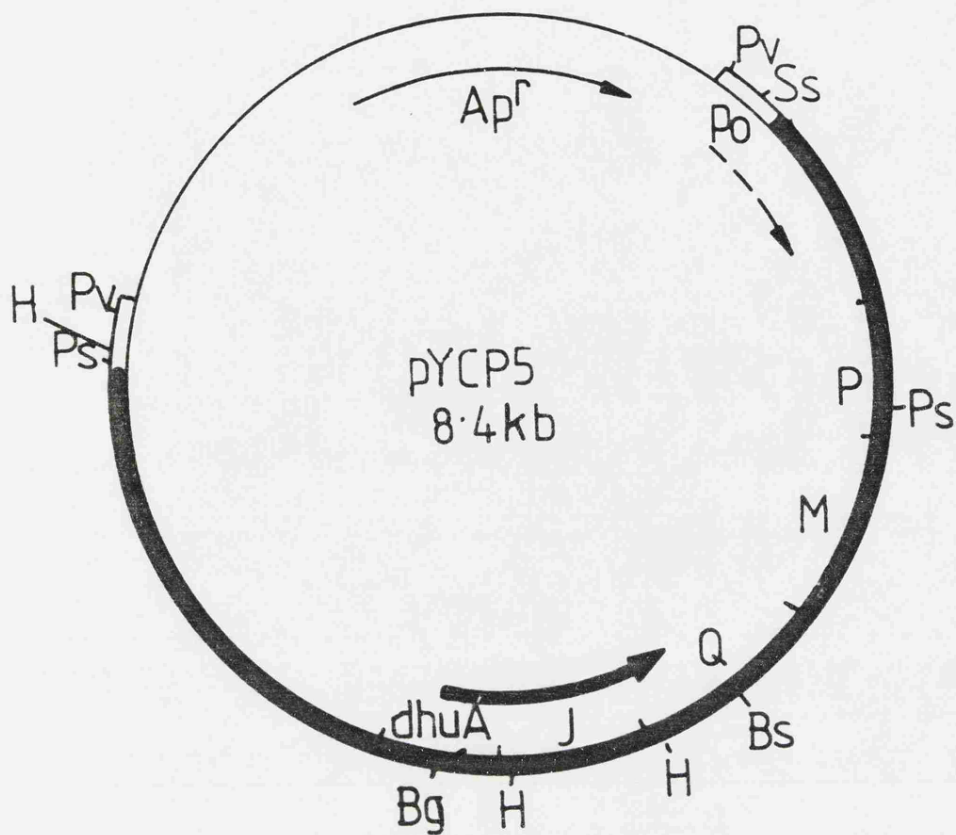
The restriction digest confirms orientation B of the AvaI fragment.

The PstI restriction pattern differs considerably depending on the orientation of the fragment.

Orientation	Expected <u>Pst</u> I fragments (Kb)
A	7.2
	1.2
B	4.3
	4.1

Restriction sites : Bs = BstEII
 Ss = SstI
 Bg = BglII
 Ps = PstI
 H = HindIII
 Pv = PvuII

This plasmid is drawn to scale.



- (1) E. coli strain NM522 which carries the complementary lac genes together with the lacI^Q gene was used, and the initial transformation was plated onto Luria agar (LuA) plates containing ampicillin.
- (2) The resulting transformants were then plated onto LuA plates containing X-gal and IPTG and screened for the occurrence of white colonies.
- (3) The material propagated was never from colonies growing on IPTG, but from the master plate (LuA + Ap) to avoid problems associated with high level expression.

Several white colonies were obtained but restriction analysis of plasmid DNA prepared from these revealed only two to contain the his permease operon and in both these the operon was in orientation B. i.e. the opposite orientation to lac. Fig. 4.3 shows the map of plasmid named pYCP5 and also shows the restriction digest of pYCP5 DNA.

4.3 Analysis of proteins encoded by pYCP5

In order to analyse the polypeptides encoded by pYCP5 the plasmid was used to transform the maxicell strain CSH26ΔF6 and the minicell strain DS410 (section 2.20). An attempt was made to prepare maxicells from CSH26ΔF6 (pYCP5) together with pUC12 and pYCP2 for comparison, however, CSH26ΔF6 (pYCP5) was found to be unable to grow in the M9 minimal medium necessary for maxicell preparation. I envisaged that this might be due to increased expression of the his permease in pUC12 causing reduced viability. Minicell preparation of DS410 (pYCP5) created no problem

Figure 4.4

Polypeptide products of plasmid pYCP5 compared to the gene products of the parent plasmids.

Polypeptides produced from maxicells of pUC12 and pYCP2 identify the HisJ protein.

Maxicells were unsuccessful with pYCP5.

Minicells were prepared containing pYCP5 together with pUC12 and pFA13 and the polypeptides labelled with [³⁵S]methionine. The proteins were analysed by 13% SDS PAGE followed by fluorography.

Minicells appear to produce a large amount of precursor β lactamase and HisJ protein creating a large amount of protein around the 27-30,000 molecular weight region of the gel.

An extra band, marked HisP is apparent in the pYCP5 and pFA13 tracks.

There is a further band of interest of 23,000 molecular weight present in pYCP2, pYCP5 and pFA13.

β -lac = β lactamase.

30, 21.5 and 12.5 kD position marked on the gel correspond to positions determined by the low molecular weight markers supplied by Amersham.

maxicell + minicell

pUC12

pYCP2

pUC12

pYCP5

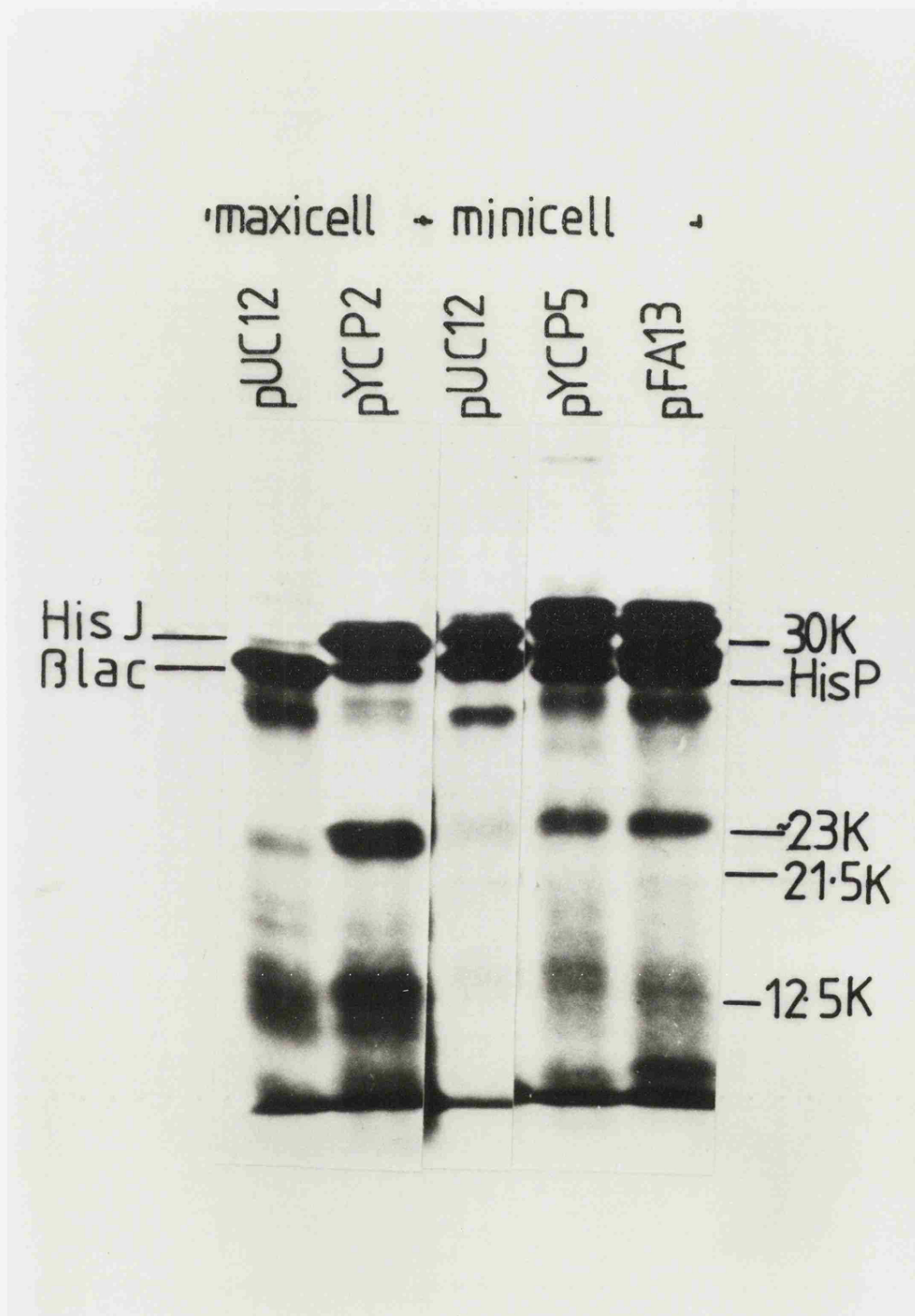
pFA13

His J
Blac

30K
HisP

23K
21.5K

12.5K



since the cells can be grown in LuB; minicells were also prepared from cells containing plasmids pUC12, pYCP2 and pFA13 for comparison.

[³⁵S]-methionine labelled products of maxicells and minicells prepared from CSH26ΔF6 and DS410 containing these plasmids were analysed by SDS PAGE (13%) and fluorography.

Fig. 4.4 shows the results of such an experiment. HisJ is clearly synthesised from plasmid pYCP5. The minicell samples of this plasmid and pFA13 indicate the specific synthesis of at least one other polypeptide. There is a band migrating below β-lactamase in both pYCP5 and pFA13 which is absent in pUC12 and pYCP2; since the latter plasmid only lacks the hisP gene of the his permease this polypeptide, with an apparent molecular weight of 27,000, may be HisP, as labelled in the figure. Subsequent data confirmed this hypothesis (see Chapter 5). It is interesting to note that minicells produce more precursor than maxicells such that when comparing the two pUC12 samples, the minicell sample contains a band localising in the same position as HisJ; this is preβ-lactamase which unfortunately cannot be resolved from HisJ expressed from pYCP2. The presence of a large band above HisJ in the pYCP5 and pFA13 tracks also indicates a precursor; this may be pre HisJ protein.

Both minicells of pYCP5 and pFA13 and maxicells of pYCP2 produce a polypeptide of approximately 23,000 which is absent in pUC12. This polypeptide could represent HisQ or M which from sequence data have molecular weights of 24,500 and 26,400 respectively. However, since both proteins are extremely hydrophobic their apparent molecular weight might be less when analysed by SDS PAGE.

Since it appears to direct the synthesis of the same polypeptide components of the histidine permease as pFA13, pYCP5, with its large number of restriction sites, was a useful source for cloning the his permease into a broad host range vector for subsequent mobilisation into M. methylotrophus (see Chapter 8).

CHAPTER 5

Enhanced expression of the hisP gene

5.1 Introduction

From previous experiments using pUC12 described in Chapter 3 it is clear that lac regulation is not tightly controlled; the genes under lac control are expressed even in the absence of IPTG.

In contrast to overproduction of HisJ, Q and M from pYCP2, enhanced expression of the entire histidine permease operon has some deleterious effects on the cell, as described in section 4.3. It is therefore either overproduction of the hisP gene product or the presence of the entire histidine permease at an elevated level, which is affecting the cells.

In order to enhance hisP gene expression and therefore identify the hisP gene product, I decided to use plasmid pTTQ18 as a vector (M. Stark, 1986). Plasmid pTTQ18 is a derivative of pUC12 in which the lac promoter has been replaced by the more powerful tac promoter. The lacI^Q gene has also been introduced into the plasmid.

In most commonly used strains of E. coli, multiple copies of the lac operator titrate out the lac repressor. This leads to significant expression from the lac promoter present on multicopy plasmids such as pUC12 in the absence of the inducer IPTG. Since pTTQ18 contains the lacI^Q allele of the lac repressor gene, repressor protein is produced

Figure 5.1 Cloning the hisP gene into plasmid pTTQ18.

The hisP gene is contained within a Kpn1/Sal1 fragment of plasmid pFA13. This fragment can conveniently be ligated into the Kpn1/Sal1 sites of the polylinker in pTTQ18 enabling the hisP gene to be expressed from the tac promoter. This construction creates a translational fusion.

Restriction sites:

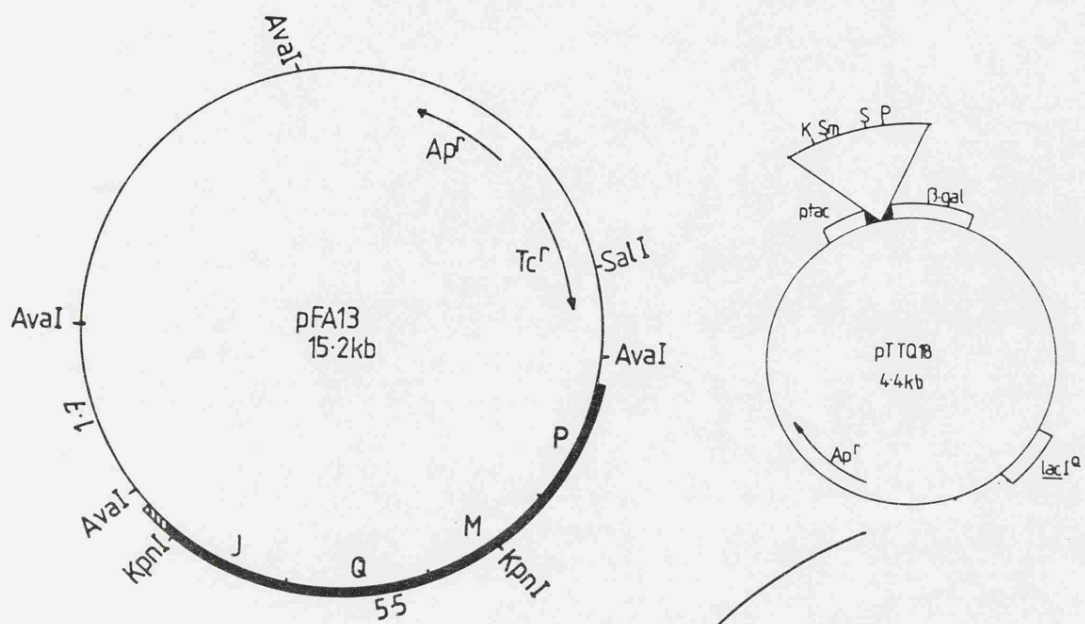
K = Kpn1

Sm = Sma1

S = Sal1

P/Ps = Pst1

These plasmids are not drawn to scale.



1) *KpnI*
2) *SalI*

ligase

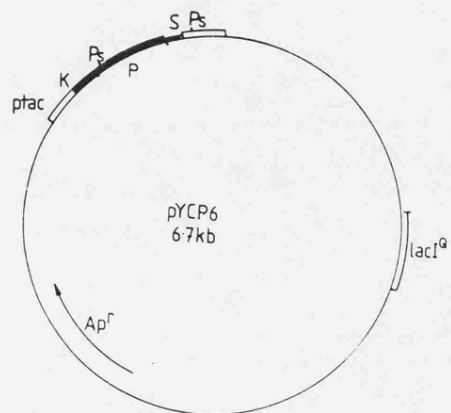


Figure 5.2 Screening for plasmid pYCP6.

(a) The results of a colony hybridisation from transformants of the ligation.

positive control = E. coli pYCP5

negative control = E. coli pTTQ18

Probe = Kpn1/Sst1 fragment of plasmid pYCP5 containing the entire hisP gene and labelled with ^{32}P dCTP by oligolabelling as described in Methods.

(b) Restriction digests of plasmid DNA prepared from sample 6, circled in Fig.(a).

The sizes marked are in Kb.

(c) Restriction map of plasmid pYCP6.

Restriction sites:

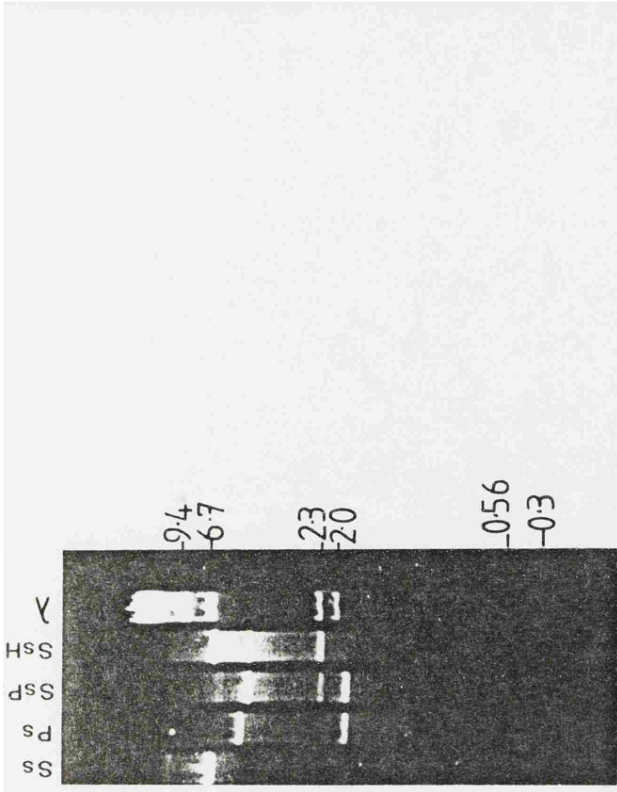
K = Kpn1

Ps = Pst1

S = Sal1

Ss = Sst1

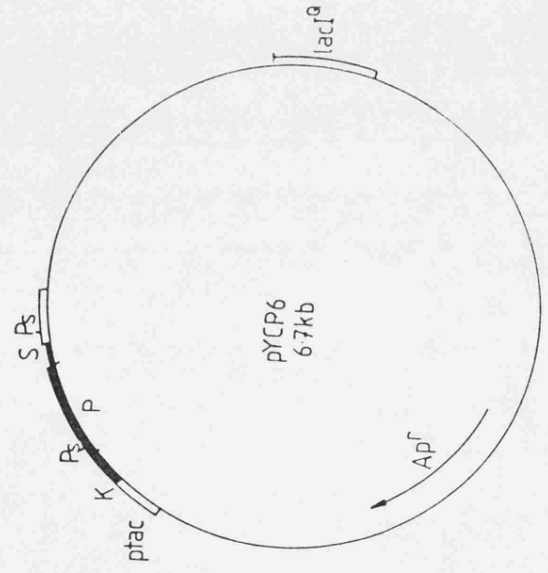
H = HindIII



b)



c)



in sufficient quantities to totally repress expression of the genes cloned under tac control. High level gene expression from the tac promoter can still be obtained however, by the addition of IPTG.

5.2 Cloning of a fragment containing the hisP gene into plasmid pTTQ18

Fig. 5.1 shows the strategy used for cloning the hisP gene contained within a 2.3 kb Kpn1/Sal1 fragment into pTTQ18. This construction forms a transcriptional fusion within the vector. The ligation mixture was used to transform E. coli NM522 but, because of problems experienced in screening using the blue/white insertional inactivation, colony hybridisation with an isolated 1.5 kb Kpn/Sst1 fragment of pYCP5 as a probe, was used to identify pTTQ18 plasmids containing hisP. Fig. 5.2, shows the results of the hybridisation and Fig. 5.2(b) shows restriction digests of DNA prepared from the positive colony encircled. The restriction digests confirmed this to be the expected recombinant and this plasmid was named pYCP6 Fig. 5.2(c).

5.3 Identification of the hisP gene product

In order to identify the product of any gene cloned into pTTQ18 under tac control the following procedure was followed:

Duplicate cultures of the plasmid bearing E. coli were grown to an $A_{450} = 0.5$, at which point one culture was induced by the addition of IPTG to a final concentration of 200 μM . Growth was allowed to continue

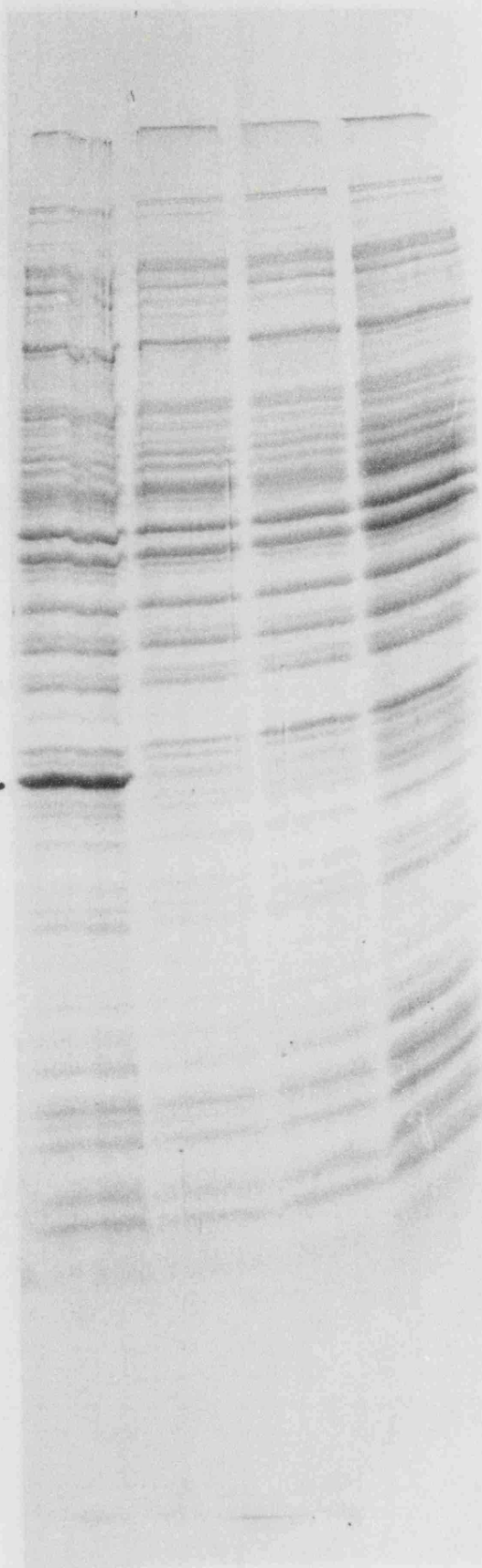
Figure 5.3

Polypeptides prepared from total cell lysates were analysed by 13% SDS PAGE followed by coommasie blue staining. For induction of the tac promoter cultures were incubated for 2 hr with 200 μ M IPTG.

Track 1	NM522(pYCP6)+IPTG
2	NM522(pYCP6)
3	NM522(pTTQ18)+IPTG
4	NM522(pTTQ18)

1 2 3 4

25 000 →



for a further two hr and the cells then harvested. Cell lysates were analysed by SDS PAGE and the proteins stained with Coomassie blue.

In one such experiment NM522 (pTTQ18) and (pYCP6) were compared in order to identify any gene products specific to pYCP6. Fig. 5.3 shows the results of the experiment.

Track 1 (NM522 pYCP6) shows a polypeptide with an apparent molecular weight of 25000 which is absent when the same cells have not been grown in the presence of IPTG (track 2). Tracks 3 and 4 are samples from E. coli containing the parent plasmid and this prominent band is clearly absent. This result suggests that this 25000 polypeptide corresponds to the hisP gene product under control of the tac promoter.

5.4 The 25,000 polypeptide is the HisP protein

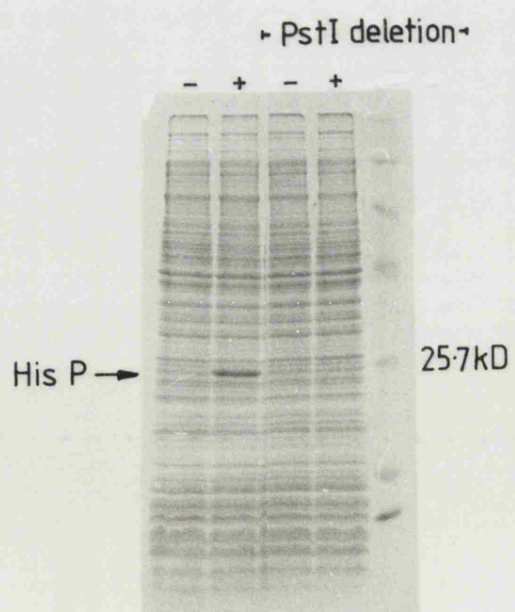
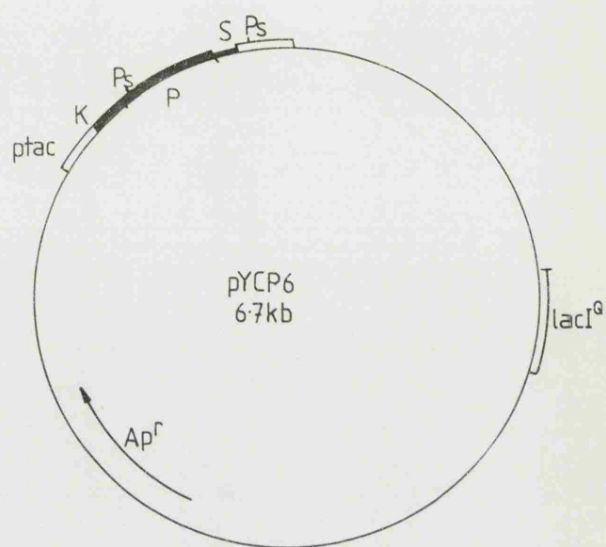
In order to confirm the identity of the hisP gene product a simple deletion of the small Pst1 fragment containing the distal portion of hisP within pYCP6 (see Fig. 8.4a) was carried out.

A total Pst1 digest of pYCP6 was ligated and then prior to transformation the ligation mixture was restricted with Sal1 to disable any pYCP6 plasmids which had been reformed. The resulting plasmid pYCP6ΔP was screened for its lack of digestion by Sal1 and for loss of the Pst1 fragment.

Cell lysates were prepared from E. coli cells containing plasmid pYCP6Δ6 together with the parent pYCP6 both grown in the presence and absence of

Figure 5.4

- (a) Plasmid pYCP6 contains the hisP gene within a Kpn1 Pst1 fragment. Since hisP contains an internal Pst1 site the gene can be mutated in pYCP6 by removal of the Pst1 fragment. This Pst1 fragment contains a restriction site for Sal1 and restriction with the enzyme after ligation will prevent any re-formed pYCP6 plasmids from being transformed into E. coli.
- (b) SDS PAGE analysis of E. coli. Total cell lysates of NM522 (pYCP6) and (pYCP6ΔP) prepared from cells grown for 2 hr with and without IPTG (200 μM), showing clear identification of the hisP gene product. The first two tracks show strain NM522 (pYCP6) and the second two tracks show strain NM522 carrying the PstI deletion in pYCP6. Track 5 contains mol. wt standards.



IPTG. Fig. 5.4(b) shows the polypeptide products produced and clearly indicates that this polypeptide of 25,000 molecular weight is absent from cells containing pYCP6ΔP. This polypeptide is evidently HisP. A truncated HisP protein is not evident in the pYCP6ΔP extracts since this protein would only be approximately 3,500 daltons - too small to be detected under these gel conditions.

5.5 Where is the HisP polypeptide localised within the *E. coli* cell?

For some time similarities have been observed between MalK of the maltose transport system and HisP suggesting that they may have a similar role in transport (see Section 1.7). MalK, like HisP, does not resemble a typical hydrophobic membrane protein and sequence homology has been found between HisP and MalK (Gilson *et al.*, 1982). Recent evidence has shown both HisP and MalK to carry a nucleotide binding site (Hobson *et al.*, 1984) and this suggests that this region of the protein must be exposed to the cytoplasm. On the other hand genetic evidence suggests that HisP interacts with the periplasmic binding protein HisJ (Ames and Spuduch, 1976), indicating that the HisP protein must also have access to the periplasmic side of the cytoplasmic membrane. It is in this respect that HisP and MalK appear to differ. MalK is thought to be a peripheral membrane protein since, although it has been localised in the cytoplasmic membrane (Baviol *et al.*, 1980), it is released upon sonication (Landick *et al.*, 1985). It can also be recovered in the soluble protein fraction in mutants defective in malG (a gene encoding the MalG membrane component), suggesting that it might be anchored to the membrane via the MalG protein (Shuman and Silhavy, 1981). More recently, and whilst my studies were in progress, Ames *et al.* have attempted to repeat these

experiments with HisP using mutations mapping within the hisQ and hisM genes. In these experiments HisP was always found in the cytoplasmic membrane (Ames et al., unpublished results cited in Ames, 1986).

In this study, in order to analyse the localisation of HisP in cells containing pYCP6, cells were fractionated, following sonication, into inner membrane, outer membrane and cytoplasm as described in Section 2.17, and the polypeptides characterised by SDS PAGE. The 25,000 dalton band corresponding to the HisP protein was evident in all three fractions although most of it was within the inner membrane (data not shown). It was noted however that during sonication the cell suspension would not clear in the usual manner. This phenomenon had been observed previously using high expression vectors and was accounted for by the formation of granular like inclusion bodies containing the overproduced protein (M. Stark personal communication, Schoner et al., 1985). Furthermore, microscopic examination showed that the cells over producing HisP were filamenting; this had also been observed by M. Stark; however, no highly refractile cytoplasmic bodies could be observed as had been previously reported.

In another experiment, when the cell suspension cleared, following sonication, the majority of HisP protein was recovered in the soluble fraction; indicating that in the previous result, it was the inclusion body fraction which co-pelleted with the membrane fraction. Schoner et al. (1985) suggested a method for purifying these 'inclusion bodies'. This procedure involved sonication, followed by a slow clearing spin to remove cell debris. The inclusion bodies, together with the cell membranes contained within the supernatant, can then be pelleted at

27,000 x g for 20 min. Successive washing of this pellet in increasing concentrations of urea will solubilise the membrane proteins. The porins of the outer membrane, for example, should be solubilised in 0.5M urea, whilst solubilisation of the inclusion body may require 5.0M urea (Schoner et al., 1985). I repeated this experiment using a cell lysate which did not clear upon sonication, indicating the formation of an inclusion body. The results, shown in Fig. 5.5 show that the HisP protein is partially solubilised by 1M urea, (track 7) although unexpectedly the porin proteins remain insoluble.

This experiment indicated that HisP was not present in inclusion bodies as defined by the results of Schoner et al. The results did not rule out the possibility that HisP was aggregating in some other form and fortuitously sedimenting with the membrane. Unfortunately, the supernatant fraction from the crude extract was not analysed by SDS PAGE; it is therefore unclear whether all of the HisP protein is present in this pellet fraction.

Interestingly, the results shown in Fig. 5.5 indicate a convenient method for purification of the HisP protein, since its solubility in 2M urea clearly isolates the protein from other components of the fraction.

Although inconclusive, these data suggest that upon total sonication of the cells, HisP protein produced in the absence of HisJ, Q and M can be localised within the cytoplasm, however, when the cell lysates remained turbid, HisP protein appeared to localise in the membrane fraction. These contradicting results may be due in part to the absence of the rest of the components of the permease in preventing HisP from assembling

Figure 5.5

Analysis of the solubility of HisP protein (in inclusion bodies) by 13% SDS PAGE upon treatment with increasing concentrations of urea.

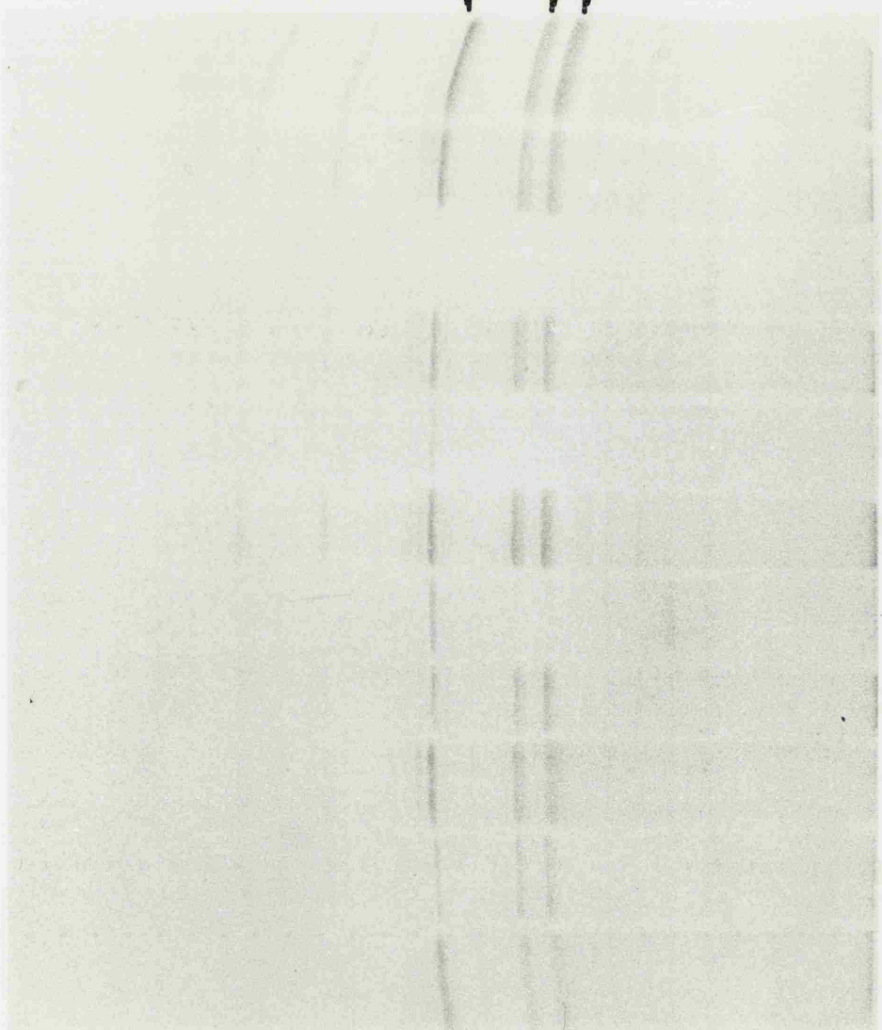
E. coli NM522 cells containing pYCP6 were induced for 2 hr with 200 μ M IPTG. Putative inclusion bodies (crude granule) were prepared by the method of Schoner et al. (1985) as described in the Methods. This involves sonication of the cells followed by pelleting the inclusion bodies together with the cell membranes. The pellet is then washed in increasing concentrations of urea and both soluble (supernatant) and insoluble (pellet) samples collected at each washing.

Lane 1	Crude granule	
2	Pellet	0.5M urea
3	Supernatant	
4	Pellet	1.0M urea
5	Supernatant	
6	Pellet	2M urea
7	Supernatant	
8	Pellet	5M urea
9	Supernatant	
10	Pellet	5M urea + 1% Triton
11	Supernatant	

Method from Schoner et al. (1985).

1 2 3 4 5 6 7 8 9 10 11

OmpF →
OmpA →
HisP →



correctly, together with the high level of production of HisP leading in some experiments to aggregation. I therefore decided to investigate the effects of the presence of HisJ, Q and M on the assembly of HisP.

5.6 Control of HisP synthesis from plasmid pYCP6

The results from section 5.5 suggest that the high level synthesis of HisP protein directed by plasmid pYCP6 may be creating artefacts in attempts to establish the real location of this protein within the cell. In order to overcome these problems an experiment must be designed such that the amount of HisP synthesised is large enough for detection, but small enough to prevent aggregation of the HisP protein. Furthermore suggestions from the Ames laboratory and from my earlier cloning work suggest that overproduction of the entire permease is lethal. The tac promoter of pYCP6 is tightly regulated by the addition of IPTG; to demonstrate this I carried out a titration analysis, whereby NM522(pYCP6) was grown to exponential phase and induced with different concentrations of IPTG for 2 hr. Cell lysates were then analysed by SDS PAGE. Fig. 5.6 shows the results of the experiment.

It is clear from this experiment, that the amount of HisP synthesised is proportional to the amount of inducer added. This result provides useful information for the IPTG concentration required to produce HisP at a detectable amount, without greatly overproducing the protein.

Figure 5.6

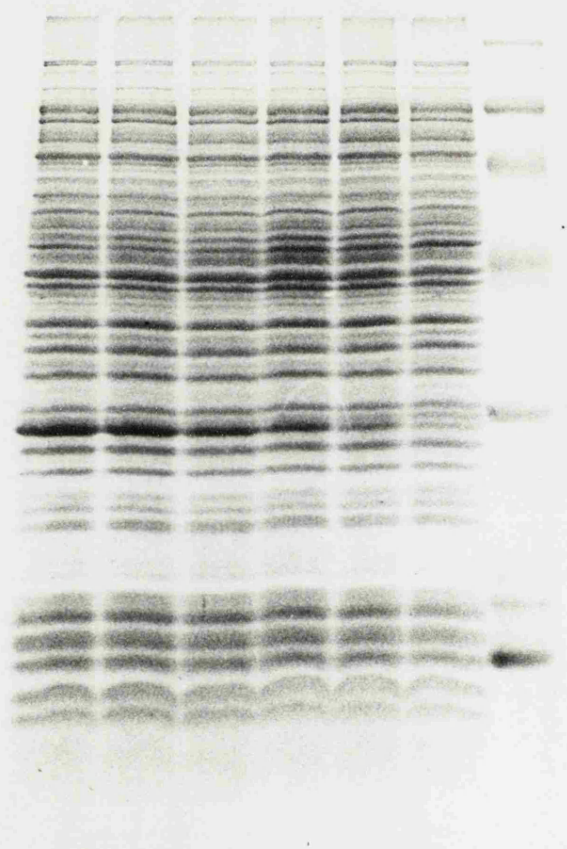
SDS PAGE analysis of NM522 (pYCP6) grown for two hr in the presence of IPTG of decreasing concentrations.

Track 1		200 μ M
"	2	100 μ M
"	3	50 μ M
"	4	25 μ M
"	5	12.5 μ M
"	6	NO IPTG

His P →

25.7kD

[IPTG] 200 μ M → 0



5.7 Assembly of the HisP protein into the membrane

The experiments outlined in section 5.5 suggested that in the absence of HisJ, Q and M, HisP was unable to assemble into the membrane at least under some conditions. This however does not necessarily confirm that HisP is a cytoplasmic protein which may, during histidine uptake, form a complex with the other components of the permease; although this would explain the genetic evidence for interaction with HisJ (Ames and Spudich, 1976). In the presence of stoichiometric amounts of HisM and Q, HisP may behave differently, such that it may assemble into the membrane under all conditions.

To test these ideas I set up experiments to assay the dependence of membrane localisation of HisP on the presence of HisJ, Q and M. In order to begin these experiments genes encoding HisJ, Q and M must be available on a vector selectable with a different antibiotic to pYCP6; for this reason plasmid pYCP3 was constructed which contained HisJ, Q and M and the dhua promoter within pBR325. The ligation of this fragment causes insertional inactivation of the βlac gene however the plasmid can be selected using the tetracycline and chloramphenicol resistance markers.

Plasmids pYCP6 and pYCP6/pYCP3 were transformed into DS410 (recA) in order that minicells could be prepared, fractionated, and the plasmid-encoded polypeptides assayed easily. The experiment was designed so that hisJ, Q and M genes are constitutively expressed and hopefully able to assemble correctly into the periplasm and cytoplasmic membrane of the minicells. HisP protein synthesis remains repressed until such time as IPTG is added. At time intervals after induction samples are taken,

pulse labelled, fractionated and analysed. If HisP is dependent on the rest of the permease for assembly, the polypeptide will remain cytoplasmic in the absence of pYCP3. However, when the membrane components of histidine permease are present HisP would assemble into the membrane shortly after induction. After several minutes of induction HisP might be located both in the cytoplasm and the membrane since all the binding sites would be occupied, however, the protein is still being synthesised, now at a greater rate than HisQ and M proteins.

In an attempt to obtain stoichiometric amounts of HisP, HisQ and HisM only 60 μ M IPTG was used for the induction of HisP synthesis; a lower concentration than this impaired HisP detection.

Minicells were prepared of both DS410 (pYCP6) and (pYCP6 pYCP3) and, prior to addition of IPTG, a sample was taken and labelled for 2 min using 15 μ Ci [35 S]-methionine followed by a 5 min chase with 2.5 μ g/ml 'cold' methionine. After addition of IPTG samples were taken at the time intervals indicated in Fig. 5.7, pulsed for 2 min with [35 S]-methionine and chased for 5 min. The minicells were then fractionated as outlined in Section 2.21 and the polypeptide components of the cytoplasm/periplasm and the membranes analysed by SDS PAGE and fluorography (Fig. 5.7).

The figure indicates that even after nine min induction (track 2) HisP is already accumulating in the cytoplasm as well as in association with the membrane in both the presence and absence of the other permease components encoded by pYCP3. This result indicates that HisP is not an

Figure 5.7

Minicells prepared from DS410 (pYCP3pYCP6 : hisJ, Q, M, P) and (pYCP6; hisP) pulse labelled for 2 min with ^{32}S -methionine and chased for 5 min. Plasmid gene products are shown fractionated into membrane and soluble (cytoplasm/periplasm) fractions.

Tracks 1-5 DS410 (pYCP3/pYCP6)

Tracks 6-10 DS410 (pYCP6)

Tracks	Sample taken	Induction time
1, 6	No IPTG induction	0
2, 7	2 min after induction	9
3, 8	5 min after induction	12
4, 9	10 min after induction	17
5, 10	30 min after induction	37

NB. Induction time = Time after IPTG addition ($60\text{ }\mu\text{M}$) + 2 min labelling time + 5 min chase,

This experiment shows the location of the hisP gene product at a specific time after induction (induction time) of gene expression.

The polypeptide labelled LacI is the expected position of the 43,000 polypeptide presumed to be the lacI gene product.

soluble
fraction

1 2 3 4 5 6 7 8 9 10

LacI → -46

HisJ →
HisP → -30
cat →

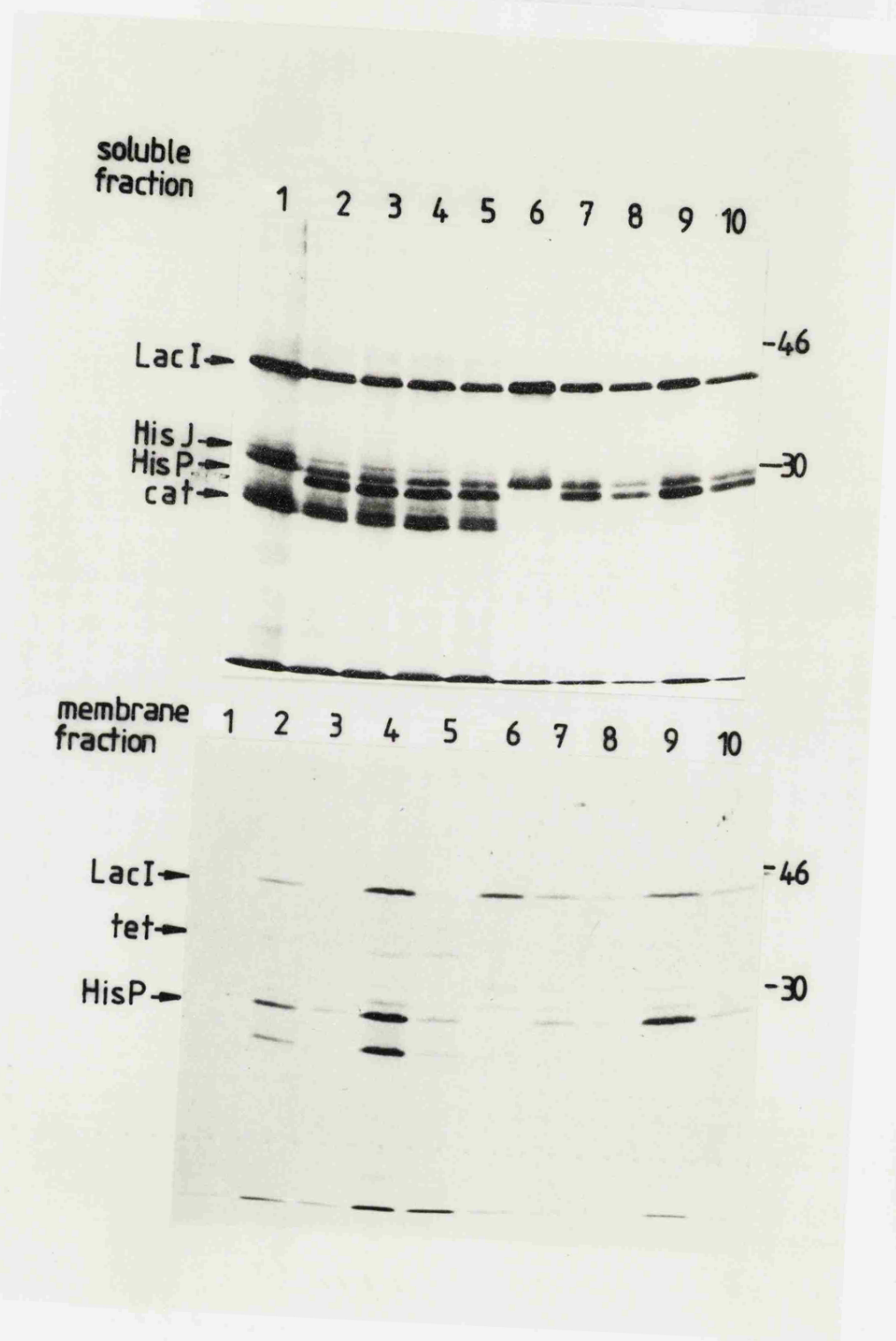
membrane
fraction

1 2 3 4 5 6 7 8 9 10

LacI → -46

tet →

HisP → -30



integral membrane protein and that if the protein associates with membrane through phospholipid or association with other membrane components, these must be relatively few in number and therefore quickly saturated.

Several other plasmid encoded gene products are also evident on the autoradiograph shown in Fig. 5.7; these proteins may be used as internal standards. LacI protein, synthesised from the LacI^Q gene on pYCP6 is a cytoplasmic protein with an apparent molecular weight of 43,000; chloramphenicol acetyl transferase synthesised from plasmid pBR325 is also a cytoplasmic protein although it tends to stick to membrane components.

On more detailed examination of the membrane fractions shown in Fig. 5.7 it is clear that the fractionation procedure does not produce an unequivocal result since both LacI and Cat proteins are evident in the membrane. However, comparison of the amount of LacI and HisP in tracks 7 to 10 indicate that with exception of track 9, both proteins are present in approximately equal amounts indicating that in the absence of HisJ, HisQ and HisM, significant amounts of HisP protein may not actually be present in the membrane. Comparison of the amount of LacI and HisP in tracks 2 to 5 however indicate that HisP is present in excess over LacI indicating that in the presence of HisJ, Q and M HisP is able to associate with the membrane. It is unclear from this experiment whether stoichiometric amounts of each of the permease components was achieved since the hisJ, Q and M genes are being constitutively expressed throughout the experiment, the amount synthesised during the short labelling period is very small and therefore very little protein is seen

on the autoradiograph. Furthermore, HisJ protein is normally synthesised in 30-fold excess to HisP (unpublished results cited in Ames, 1986), making the achievement of cellular conditions even more difficult.

Although the design of this experiment did not permit any distinction to be made between the assembly of HisP in the presence of pre-existing HisJ, Q and M and the assembly of the protein with co-synthesised HisJ, Q and M, since the amount of the latter synthesised during the labelling period is very small, this may account for the small amount of HisP apparently enriched in the membrane fraction since this protein may only be able to assemble stoichiometrically with newly synthesised HisQ and M. In order to separate these two phenomena, experiments must be designed whereby both HisJ, Q and M; and HisP synthesis; can be induced by different systems therefore providing a totally controllable system. No histidine substrate was present in this experiment, since the cells were labelled in minimal medium, therefore the need for a functional histidine permease in determining HisP binding to membrane was not addressed. An experiment designed to compare assembly both in the presence and absence of histidine would be interesting since this may be the key to the location of HisP; when inactive it may well be cytoplasmic, whereas when transporting histidine it may associate with the membrane.

CHAPTER 6

Identification of *hisQ/M* gene product(s)

6.1 Introduction

The success of identifying the *hisP* gene product by cloning into the pTTQ18 vector led me to undertake an analogous exercise, cloning a fragment containing the *hisQ* and *hisM* genes. Plasmid pYCP2 has a convenient 1.6 kb *HincII*/*Pst*I fragment containing the *hisQ* and *hisM* coding sequences. The *HincII* enzyme cuts within the non-coding region between the *hisJ* and *hisQ* genes, whilst the *Pst*I site is at the promoter-proximal end of the *hisP* gene as described in Chapter 3. This fragment can be introduced into plasmid pTTQ18 such that the genes are expressed from the *tac* promoter as outlined in Fig. 6.1. Transformants were again screened by colony hybridisation using the 1.6 kb *HincII*/*Pst*I fragment of pYCP2 as the probe. The authenticity of the positive colonies was then confirmed by restriction enzyme analysis. The recombinant plasmid was called pYCP7.

6.2 Identification of one gene product from pYCP7

Cell lysates were prepared from *E. coli* NM522 containing pYCP7 grown with and without two hr induction with 200 mM IPTG and the polypeptides analysed by SDS PAGE and Coomassie blue staining (Fig. 6.2). Tracks 1 and 2 of Fig. 6.2 show the results of such an experiment. Unfortunately track 1, corresponding to uninduced cells, was overloaded, however, it was clearer in the original gel. There is an overproduced

Figure 6.1

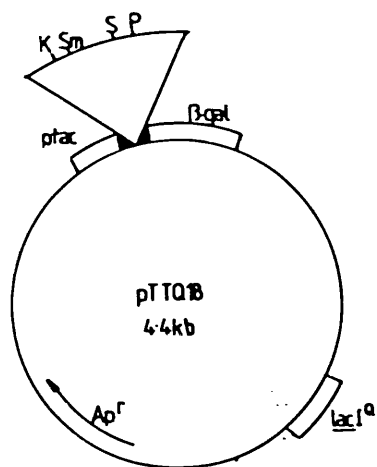
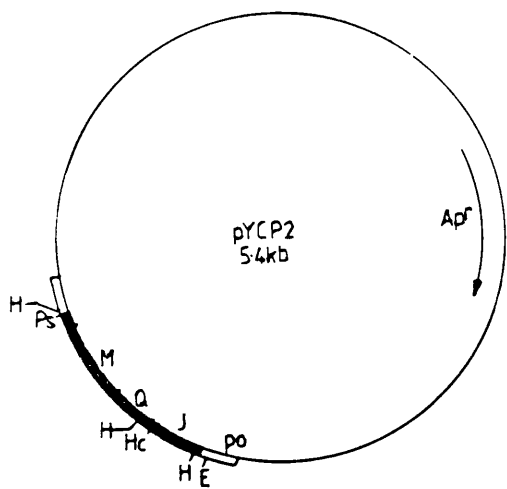
Cloning hisQ and hisM into pTTQ18.

The entire hisQ and hisM genes, together with the first 26 codons of the hisP gene is located on a HincII/PstI fragment of pYCP2. This is easily ligated into the SmaI/PstI restriction sites of pTTQ18 since both HincII and SmaI produce blunt ends.

The resulting plasmid, pYCP7 provides a translational fusion of hisQ and hisM to the tac promoter.

Restriction sites :	H	=	<u>HindIII</u>
	P/Ps	=	<u>PstI</u>
	Hc	=	<u>HincII</u>
	E	=	<u>EcoRI</u>
	Sm	=	<u>SmaI</u>
	S	=	<u>SalI</u>
	K	=	<u>KpnI</u>
	Bs	=	<u>BstEII</u>
	Ss	=	<u>SstI</u>

These plasmids are not drawn to scale.



1) HincII
2) PstI

1) SmaI
2) PstI

ligase

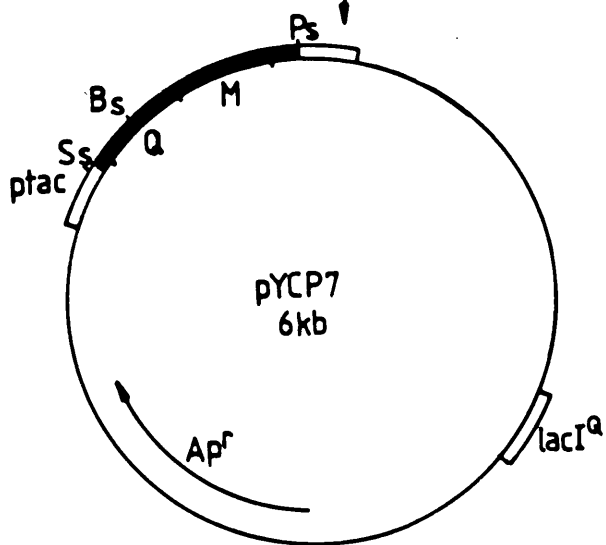


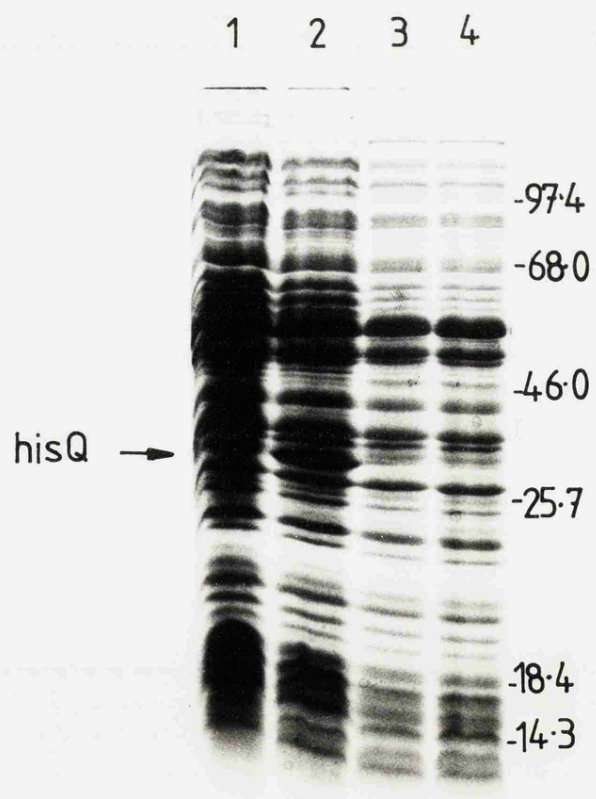
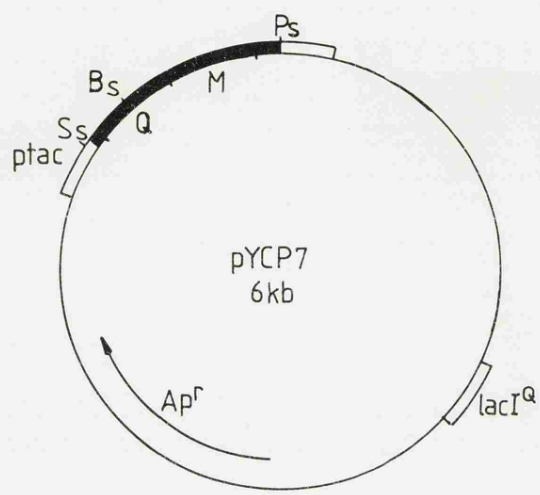
Figure 6.2

- (a) Restriction map of plasmid pYCP7.
- (b) 15% SDS PAGE and Coomassie blue stained gel of total cell lysates grown with and without induction.

Track 1	NM522	pYCP7
2	NM522	pYCP7 + IPTG
3	NM522	pYCP7ΔB
4	NM522	pYCP7ΔB + IPTG

The arrow indicates a possible hisQ or hisM gene product corresponding to a molecular weight of approximately 30,000.

pYCP7ΔB indicates pYCP7 plasmid having the unique BstEII site filled in as described in the text.



polypeptide band evident in track 2 with an apparent molecular weight of 30,000 as indicated by the arrow. There is no other overproduced band apparent. This band could correspond to HisQ or HisM protein or both proteins co-migrating under these gel conditions; the sequence data from the genes encoding the HisQ and HisM proteins predict a molecular weight of 24,000 and 26,500 respectively. The possibility that the inducible protein produced by plasmid pYCP7 was a fusion protein was ruled out by analysing the sequence data, both the fusion created between the tac promoter and the hisJ/hisQ intergenic region and between the hisP gene fragment and lacZ α should terminate shortly after initiation. Further manipulation of the plasmid is needed to confirm the identity of this protein of 30,000 molecular weight.

6.3 A frame shift mutation confirms the identity of HisQ

The hisQ gene contains a unique BstEII restriction site (as shown in Fig. 6.2). Restriction with this enzyme creates a 5 base overhang at the 5' end which can be filled in using the Klenow fragment of DNA polymerase 1 with the necessary dNTPs. Subsequent ligation will create a frame shift mutation (Fig. 6.3).

In order that only mutated DNA was used for transformation the ligation mix was again restricted with BstEII prior to transformation in E. coli NM522. Resulting transformants were screened for their acquired resistance to be cut by BstEII. One such plasmid was called pYCP7 Δ B.

Figure 6.3

The codons around the BstEII site within the hisQ gene.

The arrows indicate the cutting sites of BstEII.

The lower sequence shows the results of filling in the recessed 3' ends of this BstEII restricted fragment, indicating the change in reading frame from that of the hisQ gene.

This frameshift mutation causes transcriptional termination seven codons downstream of this BstEII site.

AAC GTG' GTG ACC GAT TCG
TTG CAC CAC TGG CTA AGC

AAC GTG GTG ACG TGA CCG ATT CG
TTG CAC CAC TGC ACT GGC TAA GC

6.4 The 30,000 molecular weight polypeptide is not encoded by
pYCP7ΔB

Cell lysates were prepared from NM522 (pYCP7) and NM522 (pYCP7ΔB) grown with or without IPTG induction. The polypeptides were analysed by SDS PAGE and Coomassie blue staining.

Fig. 6.2 shows the results; by comparing tracks 1 and 2 (pYCP7, as discussed in section 6.2), with tracks 3 and 4 (pYCP7ΔB). The induced gene product with an apparent molecular weight of 30,000 is clearly deleted in the samples containing the frame shift mutation. This provides evidence that this 30,000 molecular weight polypeptide is HisQ. Unfortunately, no other polypeptide is evident, suggesting that although the hisM gene is read from a different reading frame to hisQ the mutation may also be preventing synthesis from the downstream hisM gene. A similar frame shift mutation within the hisM gene would enable confirmation of the identity of this 30,000 polypeptide. Alternatively, a final identification of HisQ would be to introduce an in frame DNA insertion into the BstEII site within the hisQ gene, such that a size increase in the gene product could be detected. Since this polypeptide has the same apparent molecular weight as HisJ, this gene product would not be evident when expressed from plasmid pYCP2.

CHAPTER 7

Hybridisation analysis between genes from *E. coli* and

M. methylotrophus

7.1 Introduction

The overall ambition of this project was to construct a functional histidine permease within *M. methylotrophus*. There are two major prerequisites which might be met before a functional permease can be established. The first is the introduction and expression of the histidine permease genes in *M. methylotrophus* and the second is the correct assembly of the proteins. One would expect that both the periplasmic and membrane proteins need to assemble themselves correctly in order to transport histidine efficiently.

The previous chapters have identified a number of the components of the histidine permease in *E. coli*; this simplifies their identification and localisation in *M. methylotrophus*. The following chapters describe the preliminary experiments required in order to establish a functional permease within *M. methylotrophus*.

Since little data was available to compare the cellular and membrane components of *M. methylotrophus* and *E. coli*, I set up a simple cellular fractionation of exponentially growing *E. coli* K12 and *M. methylotrophus* and analysed the protein components by SDS PAGE (Fig. 7.1).

The gel shows the major components of the *E. coli* outer membrane are the porin proteins OmpF/C (which are not resolved) and the 35,000 dalton OmpA.

Figure 7.1

Comparison between the outer membranes, inner membranes and soluble fractions of E. coli and M. methylotrophus.

13% SDS polyacrylamide gels stained with coomassie blue. The E. coli and M. methylotrophus samples were not run on the same gels and both gels ran differently.

Sizes shown are $\times 10^3$ daltons.

M. methylotrophus

E. coli

OM IM C/P

C/P IM OM

RNApol

200

200

RNApol

58

Mdh

43

OmpF 38
OmpA 35

14.3

-

In contrast, M. methylotrophus appears to possess only one major outer membrane protein with an apparent molecular weight of 43,000. The protein, although larger than any other studied (Lugtenberg et al., 1977), is probably a porin and its presence in the inner membrane fraction of the cell is probably due to its partial solubility in Sarkosyl.

The cytoplasmic/periplasmic fraction of M. methylotrophus contains a major protein of 50,000. This protein corresponds to methanol dehydrogenase which is located in the periplasmic fraction after sonication but may be loosely attached to the inner membrane, probably via the mediation of Mg^{2+} (Carver et al., 1984). Another protein of approximately 150,000 daltons is also evident in this cytoplasmic fraction; this corresponds to the RNA polymerase band in the E. coli cytoplasmic fraction and may therefore be the M. methylotrophus RNA polymerase.

Other proteins which have been identified in the periplasm of M. methylotrophus are the 28,000 and 18,000 dalton subunits of cytochrome C_L and the 12,000 and 10,000 dalton subunits of cytochrome C_H (Quilter and Jones, 1984), however, these are not resolved under these gel conditions.

Fig. 7.1 establishes that E. coli and M. methylotrophus possess very different protein components whereas S. typhimurium and E. coli are known to be very similar. It was nevertheless hoped that the ability for the histidine permease of S. typhimurium to function in E. coli extends to its functionality in any Gram negative organism and hence that the permease will be able to assemble within this very different organism.

7.2 There are no sequences in the *M. methylotrophus* genome homologous to the *S. typhimurium* histidine permease

M. methylotrophus is thought not to possess any amino acid permeases (A. Charles personal communication). This deduction had been made from the indirect evidence that it has been difficult to isolate auxotrophs and when histidine requiring auxotrophs were isolated the concentration of histidine required was very large (1 mg ml⁻¹) (D. Byrom, personal communication).

In order to investigate this further, I carried out a Southern hybridisation analysis, assessing homology between the *S. typhimurium* histidine permeases and chromosomal DNA isolated from both *E. coli* and *M. methylotrophus*. This DNA was probed with [³²P] labelled plasmid pFA13, which contains the whole of the histidine permease operon from *S. typhimurium* using hybridisation conditions of 3 x SSC at 65°C.

The results in Fig.7.2A show no detectable DNA sequence homology lending support to the prediction that *M. methylotrophus* does not possess a similar histidine permease to that found in *S. typhimurium*. Homology is found between *E. coli* and *S. typhimurium* as expected (Ardeshir and Ames 1980).

7.3 *M. methylotrophus* shows sequence homology to an *E. coli* gene

In order to demonstrate that it was possible to detect DNA sequence homology between genes from *M. methylotrophus* and *E. coli*, I decided to carry out a similar experiment using an essential *E. coli* gene.

Figure 7.2 Southern hybridisation of M. methylotrophus and E. coli chromosomal DNA.

A. Chromosomal DNA probed with the his permease genes of S. typhimurium.

B. Chromosomal DNA probed with the lep gene of E. coli.

Restriction enzymes.

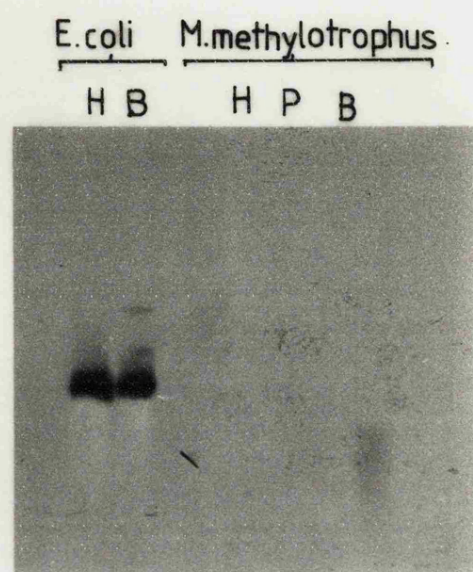
H = HindIII

B = BamHI

P = PstI

E = EcoRI

A)



B)



One essential protein in any organism is likely to be leader peptidase. This protease catalyses the proteolytic removal of the amino-terminal signal sequence and without such, no outer membrane or periplasmic protein could be assembled correctly. M. methylotrophus is expected to possess a leader peptidase, since it is able to localise methanol dehydrogenase towards the periplasmic side of the cytoplasmic membrane (Quilter and Jones, 1984); it is also able to process β -lactamase to its mature size and localise the protein within the periplasmic space (A. Charles personal communication). Finally, as shown in section 7.1 the organism is known to possess at least one porin-like protein within the outer membrane.

Leader peptidase is encoded in E. coli by the lep gene and this gene has been shown to cross hybridise with chromosomal DNA from a wide range of bacteria and also from eukaryotes such as yeast and honeybee (W. Wickner, personal communication). Since this lep gene was available to me on plasmid pTD101 (Date and Wickner, 1981) I decided to screen M. methylotrophus chromosomal DNA for lep homology using Southern hybridisation. The results, shown on Fig. 7.1B clearly indicate that the E. coli leader peptidase gene shows homology to the M. methylotrophus chromosome. Each sample of chromosomal M. methylotrophus DNA digested with a different enzyme shows a region of homology with pTD101. However, when this experiment was repeated using the parental plasmid pBR322 as a probe no homology was seen (data not shown). This result shows that M. methylotrophus does possess a homologous sequence to lep.

The finding that M. methylotrophus appears to possess a lep gene led me to attempt to isolate the gene for possible further characterisation and

functional analysis. I constructed a λ library of M. methylotrophus chromosomal DNA in the λ vector λ DB287. This vector will accept donor DNA with a size range of 10-25 Kb ligated into the BamHI restriction sites.

The previous Southern hybridisation result of M. methylotrophus with the lep gene, detected this positive region at approximately 15 Kb when restricted with BamHI. λ DB287 and M. methylotrophus chromosomal DNA were both restricted with BamHI and then mixed and ligated. The ligation mixture was then packaged using a λ Packaging Kit (P & S Biochemicals). The size limitation of λ created a selection for λ containing foreign DNA of between 10-25 Kb; this ought to include λ clones carrying the M. methylotrophus lep gene. Attempts to isolate a positive λ clone however, failed, and I did not proceed further.

7.4 Discussion

The fact that homology was found between M. methylotrophus and the E. coli lep gene but was not found with the S. typhimurium his permease suggests strongly that M. methylotrophus does not possess genetic information for the his permease. This finding encouraged me to introduce this permease into M. methylotrophus in an attempt to understand the M. methylotrophus membrane structure:- can proteins from one Gram negative bacterium assemble into another Gram negative envelope despite the fact that the protein composition of the bacterial envelopes are vastly different? Furthermore, these experiments may aid in the elucidation of the functioning of the histidine permease.

CHAPTER 8

Expression of the histidine permease in *M. methylotrophus*

8.1 Introduction

In order that the function of the *S. typhimurium* histidine permease could be studied in *M. methylotrophus* the corresponding genes were cloned into a broad host range vector such that the plasmid could be maintained in both *E. coli* and *M. methylotrophus*. Sharpe (1984) constructed several useful cloning vectors from plasmid R300B. This is a member of the IncQ group of plasmids which have a host range including most Gram-negative organisms. R300B has several advantages over the alternative vector RP4 which made it a suitable choice for the development of a cloning vector. It is relatively small (8.7 kb, Meyer *et al.*, 1982) and is multicopy (9-12 copies per chromosome in *E. coli*; Barth and Grinter, 1974). It is however non-conjugative, but it can be efficiently mobilised by a variety of conjugative plasmids. Since R300B has relatively few useful restriction sites, it was manipulated using restriction fragments from pBR322 and pBR328 to produce a series of cloning vectors including pGSS33. Plasmid pGSS33 is 13.4 kb in size and carries genes for resistance to 4 antibiotics, ampicillin, streptomycin, chloramphenicol and tetracycline all of which contain unique restriction sites for insertional inactivation.

8.2 Cloning of the histidine permease operon into pGSS33

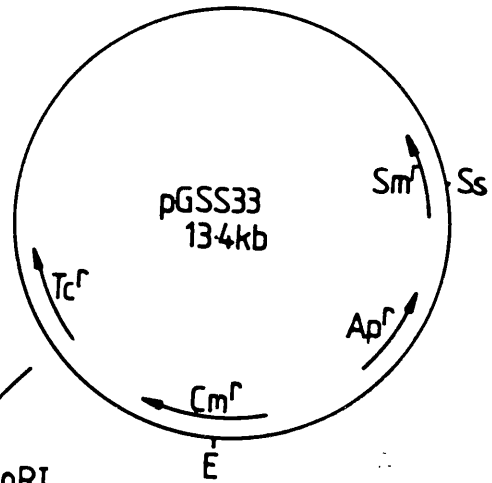
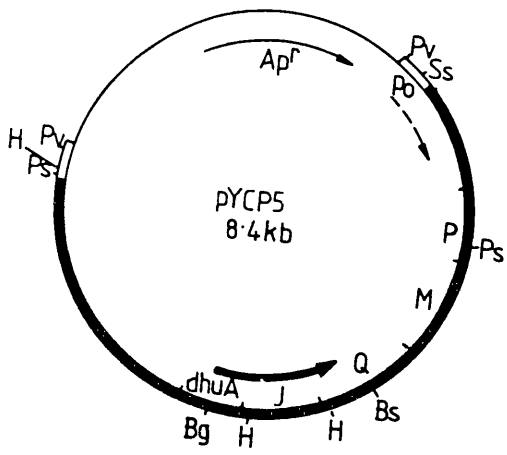
Fig. 8.1 outlines the strategy used for introducing the 6 kb 'blunt' ended PvuII fragment of pYCP5 containing the whole of the histidine

Figure 8.1 Construction of plasmid pYCP15

Plasmid pYCP15 contains the entire histidine permease operon of S. typhimurium, including the dhuA promoter (hashed area). The plasmid is able to replicate in both E. coli and M. methylotrophus.

Restriction sites

Bg	=	<u>Bgl</u> III
Bs	=	<u>Bst</u> EII
E	=	<u>Eco</u> RI
H	=	<u>Hind</u> III
Ps	=	<u>Pst</u> I
Pv	=	<u>Pvu</u> II
Ss	=	<u>Sst</u> I



PvuII

1) EcoRI
2) klenow

ligase

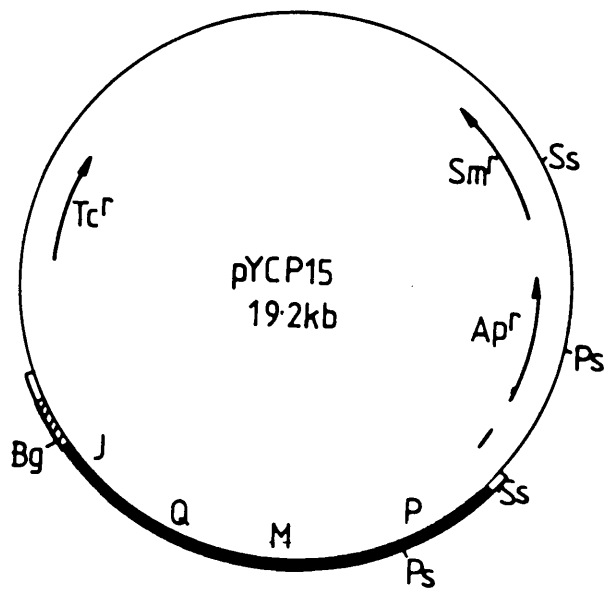


Figure 8.2

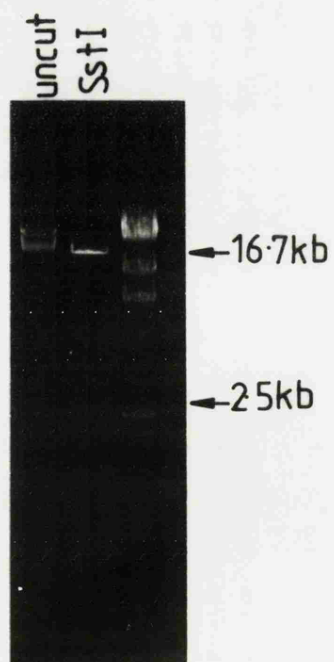
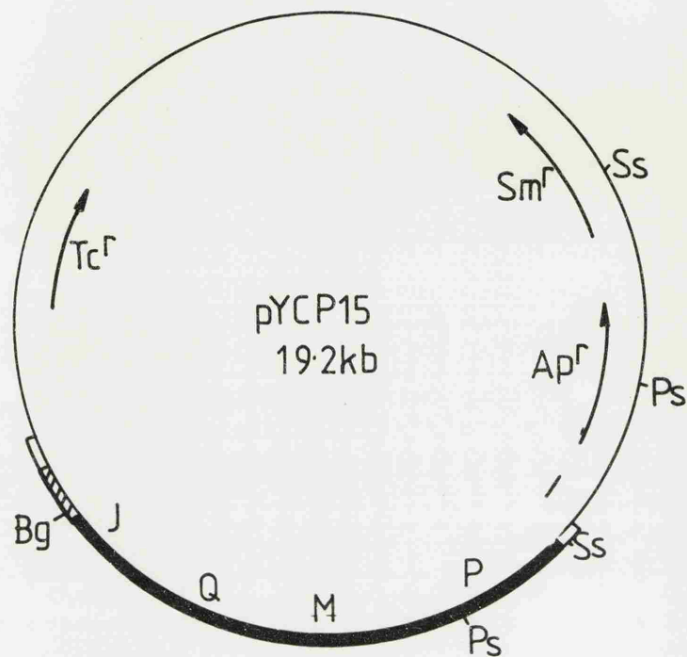
Plasmid pYCP15 contains the histidine permease operon in the opposite orientation to the cat promoter, this is determined by an SstI digest producing fragments of 16.7 kb and 2.5 kb as shown on the gel. (The 2.5 kb band is very faint in this figure, however was clearly present on the original).

Restriction sites

Ss = SstI

Ps = PstI

Bg = BglII



permease operon into pGSS33. Since there are no unique blunt ended restriction sites in pGSS33, this vector was linearised with EcoR1, thus inactivating the chloramphenicol resistance gene, and the ends filled in using the Klenow fragment of DNA polymerase 1 prior to ligation with the PvuII fragment. Amongst the tetracycline resistant transformants obtained, those which were chloramphenicol sensitive were screened with restriction enzymes to determine the orientation of the inserted Salmonella DNA. One such recombinant plasmid, pYCP15 contained the permease operon in the opposite orientation to the cat promoter; this was determined by digestion with Sst1 which produced two fragments of 16.7 kb and 2.5 kb (Fig. 8.2). This plasmid is used for all the following investigations with M. methylotrophus. Any recombinant found in the same orientation as cat was not used due to the problems caused by overproduction observed earlier (Chapter 4).

8.3 Identification of gene products encoded by pYCP15

(a) In *E. coli*

In order to identify the gene products of pYCP15 cell lysates of *E. coli* DS410 (pYCP15) and (pGSS33) were prepared together with cell lysates from NM522 (pUC12), (pYCP2) and (pYCP6), the latter grown with and without IPTG induction. The polypeptides were analysed by SDS PAGE and Coomassie blue staining. Fig. 8.3 shows the results of this experiment and comparing pGSS33 with pYCP15, it is apparent that the CAT protein is no longer synthesised by cells possessing pYCP15. It is also apparent that two gene products with apparent molecular weights of 26,000 and 30,000 are synthesised by cells containing this recombinant plasmid. Comparison of these bands with the polypeptides of pYCP6 and pYCP2 shows that these

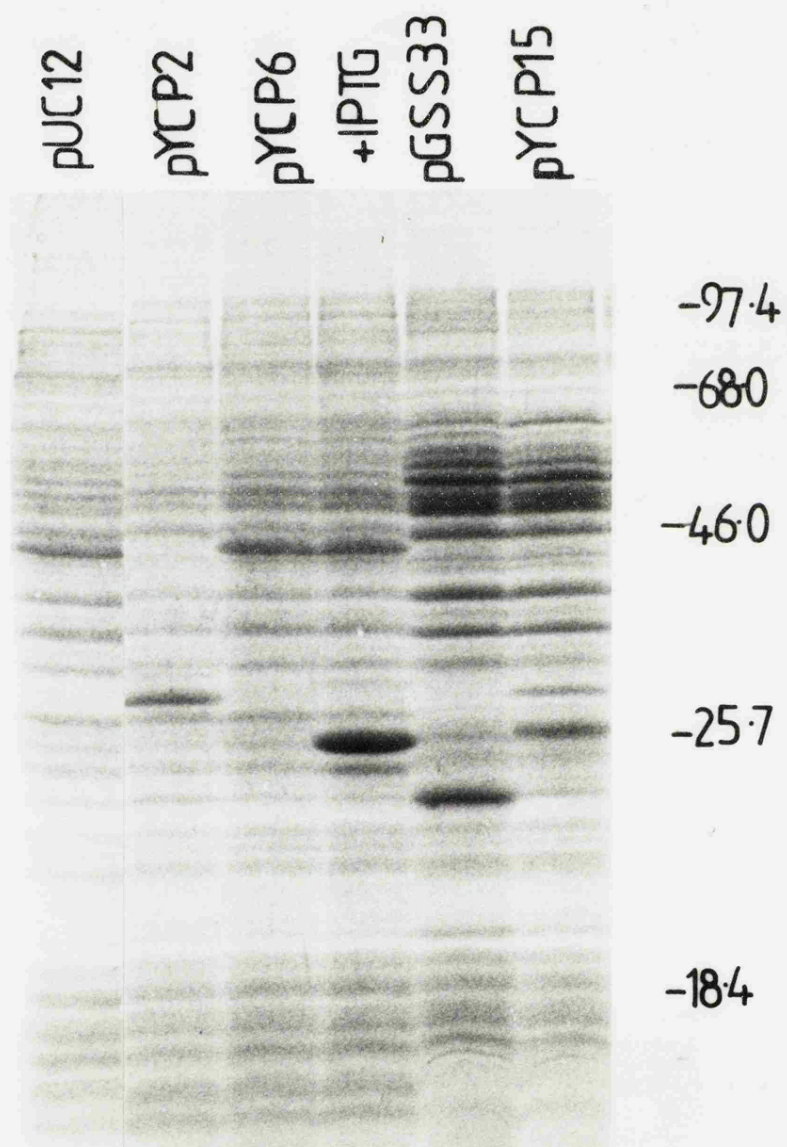
Figure 8.3

Total cell lysates of E. coli containing the following plasmids:-

<u>Plasmid</u>	<u>his</u> genotype
pUC12	-
pYCP2	<u>hisJ</u> , Q, M
pYCP6	<u>hisP</u> - IPTG
pYCP6	<u>hisP</u> + IPTG
pGSS33	-
pYCP15	<u>hisJ</u> , Q, M, P

The cell lysates were separated by SDS PAGE and stained with Coomassie blue.

His J -
HisP -
Cat -



bands represent HisP and HisJ respectively. It is therefore assumed that the histidine permease operon is being expressed from pYCP15 in E. coli.

(b) In *M. methylotrophus*

In order to introduce pYCP15 or pGSS33 into *M. methylotrophus* they were first used to transform an *E. coli* strain containing a conjugative plasmid which enabled mobilisation into *M. methylotrophus*. *E. coli* BW162 containing plasmid R64drd11 was used for such purposes and mobilisation was carried out as described in Section 2.6. This plasmid, belonging to the IncI α group, is especially advantageous since it has been observed that such plasmids are not maintained in the recipient *M. methylotrophus*.

Following confirmation that the histidine permease genes were being expressed by pYCP15 in *E. coli*, I then proceeded to investigate if these genes were being expressed from pYCP15 in *M. methylotrophus*.

Cell lysates were prepared from *E. coli* (pGSS33) and (pYCP15) and *M. methylotrophus* containing the same plasmids. Equivalent A₄₅₀ units were analysed by SDS PAGE loading approximately 3.5 units per track, and the polypeptides were stained with Coomassie blue.

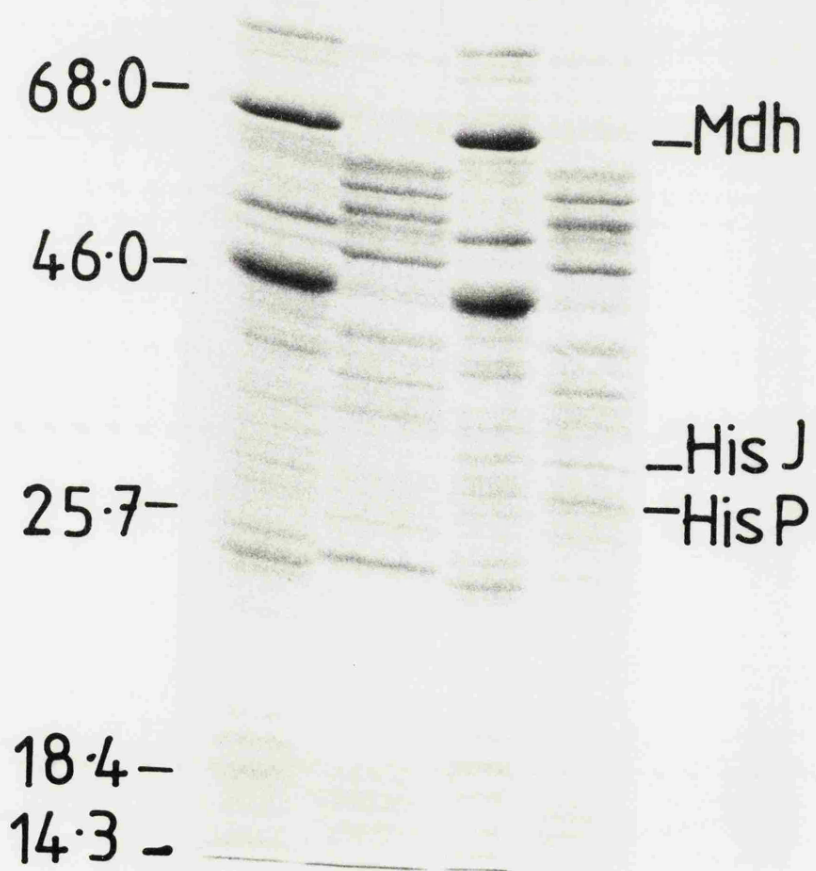
The results shown on Fig. 8.4 indicate that the polypeptide corresponding to HisJ synthesised by *E. coli* (pYCP15) is also evident in the *M. methylotrophus* (pYCP15) track; although the sensitivity of detection from total cell lysates is low. This indicates that the dhua promoter appears to function in *M. methylotrophus*, promoting synthesis of at least one of the proteins comprising histidine permease. In order to confirm this observation a more sensitive detection system is required.

Figure 8.4

Comparison of total cell lysates from E. coli and M. methylotrophus containing pYCP15 or the parent plasmid pGSS33. Each sample contained 3.5A₄₅₀ units. Samples were separated by 13% SDS PAGE and stained with Coomassie blue.

pGSS33 pYCP15

M E M E



(c) Western blot analysis of pYCP15 gene products

Immunological techniques provide a sensitive detection system for the synthesis of polypeptides within cell preparations and fractionations. Without such sensitive systems many polypeptide products remain undetectable due to their low abundance within the cell or fraction under study. The availability of antiserum to the HisJ protein enabled me to carry out the Western blot procedure to confirm the synthesis of HisJ protein under the direction of pYCP15 in both E. coli and M. methylotrophus cells. Total cell lysates from both E. coli and M. methylotrophus blotted against HisJ antibody confirmed that the HisJ protein is being synthesised by M. methylotrophus (pYCP15) as shown in Fig. 8.5. Lane 4 shows that there is no HisJ protein synthesised from cells containing pGSS33 whilst lane 5 shows HisJ synthesis from cells containing pYCP15. An analogous result was obtained with the E. coli samples (data not shown) although small amounts of antibody binding were apparent in E. coli pGSS33 since the strain used (BW162) contains chromosomal histidine permease.

8.4 Assembly of histidine binding protein in M. methylotrophus

The knowledge that at least the HisJ protein of the cloned histidine permease system is synthesised in M. methylotrophus led me to investigate whether this foreign permease could assemble in this organism in a

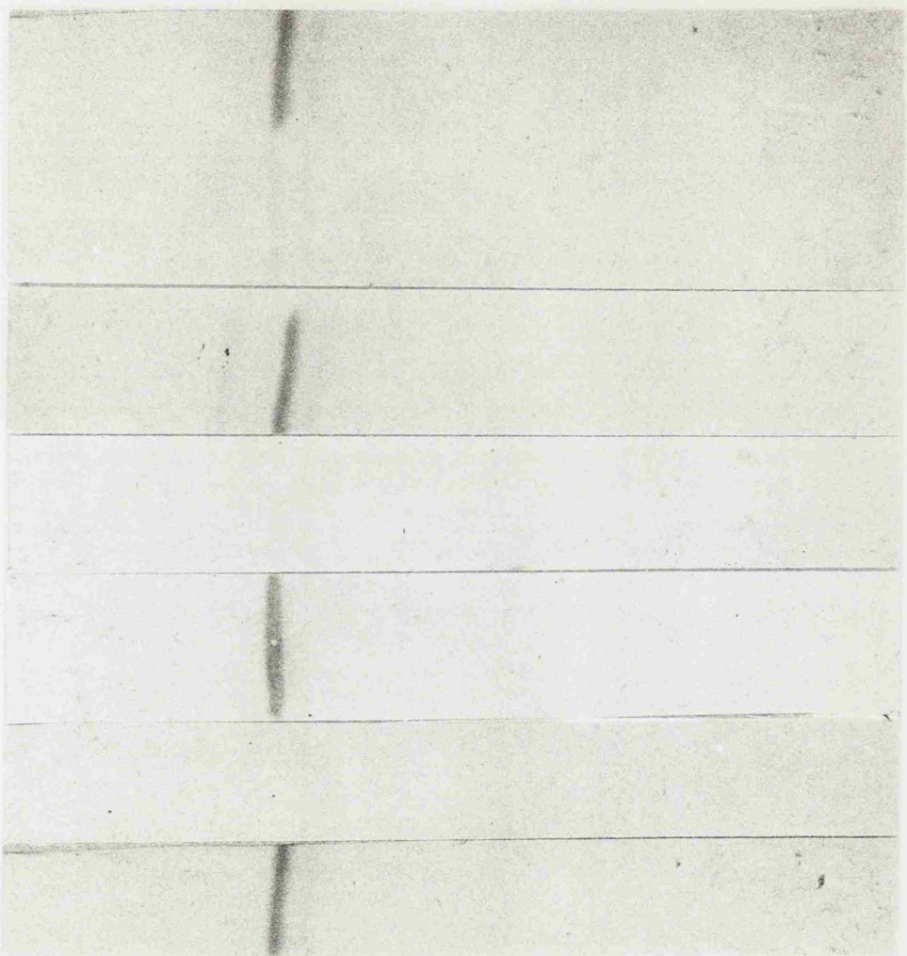
Figure 8.5

Western blot of protein samples blotted against HisJ protein antibody.

	<u>Total cell lysate</u>
Lane 1	<u>E. coli</u> pYCP2
2	<u>E. coli</u> pGSS33
3	<u>E. coli</u> pYCP15
4	<u>M. methylotrophus</u> (pGSS33)
5	<u>M. methylotrophus</u> (pYCP15)
	<u>Osmotic shock</u>
6	<u>M. methylotrophus</u> (pYCP15) cell pellet after osmotic shock
7	<u>M. methylotrophus</u> (pYCP15) osmotic shockate.

All these positive bands corresponded to the 30,000 molecular weight band of the expected position of the HisJ protein on a Coomassie stained portion of the gel.

1 2 3 4 5 6 7



-HisJ
-25.7kD

functional form. HisJ, being a periplasmic protein, is easily detectable in osmotic shock fluid of E. coli. An exponential culture of M. methylotrophus is very sensitive to osmotic shock such that it is merely necessary to resuspend the cells in 10% sucrose solution in the presence of 25 mM Tris-HCl and 1 mM EDTA to release the contents of the periplasm into the supernatant (A. Charles, personal communication).

This procedure was carried out on M. methylotrophus (pYCP15) and (pGSS33) and the proteins analysed by SDS PAGE. Fig. 8.6 shows the results of the experiment. The 30,000 molecular weight polypeptide corresponding to HisJ is clearly evident above the polypeptide for β -lactamase (molecular weight 27,000) in the pYCP15 shockate, whereas this extra polypeptide band is absent in the pGSS33 track.

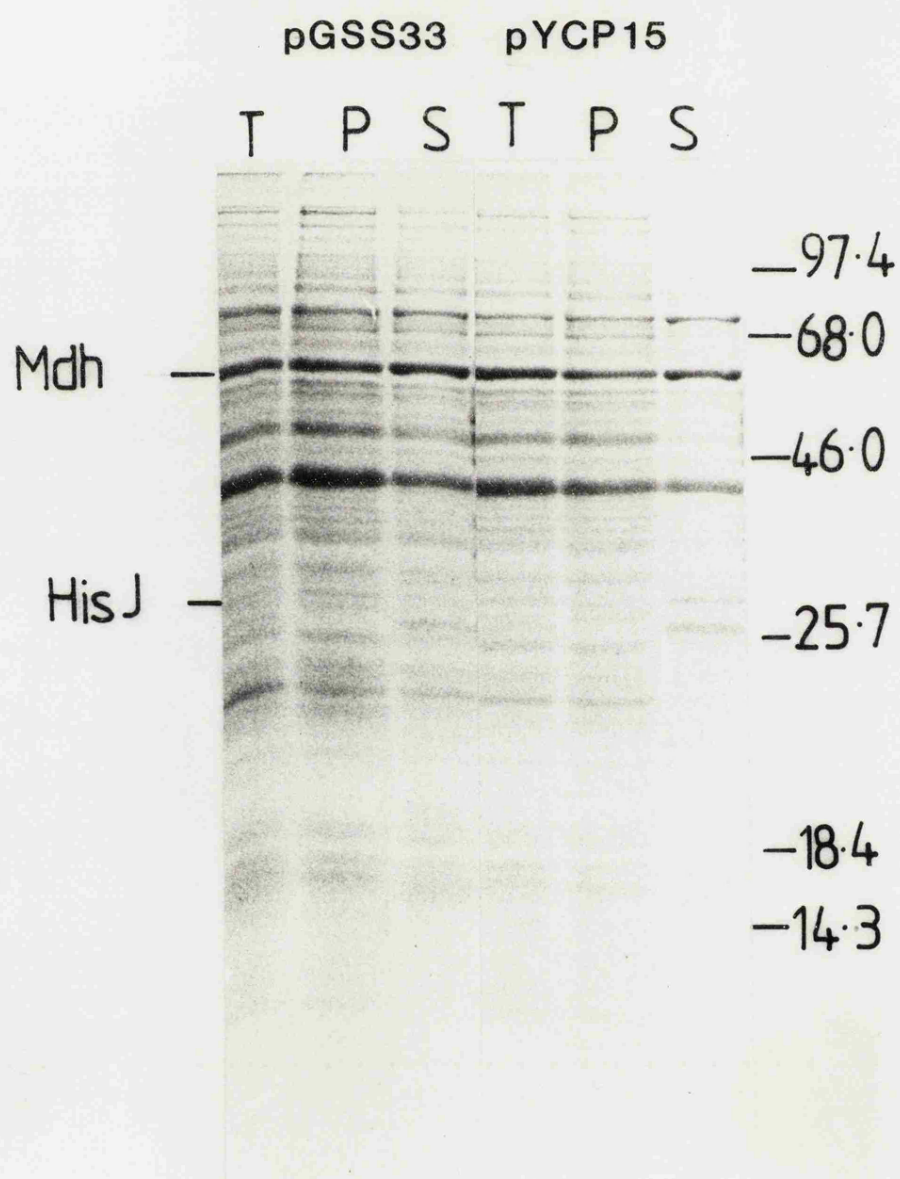
To confirm these observations a Western blot using HisJ antibody was carried out on these samples, together with osmotic shockates from E. coli (pYCP2) (pGSS33) and (pYCP15), and the results are also shown on Fig. 8.5. Tracks 1, 2 and 3 show the E. coli samples which clearly show that HisJ antibody does not react with the gene products of E. coli pGSS33 but reacts specifically with protein products of pYCP2 and pYCP15. Tracks 7 and 6 compare M. methylotrophus osmotic shockate and cell pellet respectively and clearly indicate the HisJ protein is exclusively assembled in the cell's periplasm.

It is clear from these experiments that the Gram negative organism M. methylotrophus is able to express a foreign gene, synthesise the protein and at least in the case of HisJ assemble the synthesised protein correctly. In addition, in order to assemble HisJ the preprotein must be

Figure 8.6 Osmotic shock of M. methylotrophus (pGSS33) and (pYCP15)

Total (T), pellet (P) and supernatant (S) fractions were analysed by 13% SDS PAGE. Total corresponds to total cell lysates; supernatant corresponds to the contents of the periplasm released into the medium and pellet corresponds to cells lacking the periplasm. An exponential culture at A_{450} was harvested and fractionated.

The gel was stained using Coomassie blue.



processed by the removal of the signal sequence; I have already found that M. methylotrophus possesses a gene encoding a leader peptidase and I have now provided evidence that presumably this M. methylotrophus encoded leader peptidase is able to process the cloned HisJ gene product. M. methylotrophus had already been shown to process β -lactamase presumably by the same mechanism (A. Charles, personal communication).

The lack of antibody to either HisP, HisQ or HisM prevented me from investigating the assembly of the other components of the histidine permease in M. methylotrophus. To conclude these investigations the functionality of the permease in M. methylotrophus must be analysed and this is described in the next chapter.

CHAPTER 9

Is the histidine permease functional in *M. methylotrophus*?

9.1 Introduction

Since it is clear that at least the HisJ protein is both expressed and apparently translocated correctly in *M. methylotrophus* it was then important to assay the possible functional activity of the histidine permease. Initially, I planned to attempt to test for a reduction in the external concentration of histidine required by the auxotrophs isolated by D. Byrom. These mutants required supplementation with 1mg ml^{-1} histidine for growth. The plasmids pYCP15 and pGSS33 were mobilised into such strains and cultures plated onto MMA containing $0\mu\text{g}$, $100\mu\text{g}$ and 1mg histidine per ml to assess whether the histidine concentration required for growth was reduced in the mutant strains containing pYCP15, compared to those containing pGSS33.

Although initial results indicated that the cells containing pYCP15 were able to grow on lower concentrations of histidine, it was also observed that under these conditions cells containing pGSS33 were also able to grow and, after several generations showed a similar growth rate to wild-type cells indicating that the cells were reverting to wild-type. Attempts to obtain stable histidine requiring mutants by transposon mutagenesis were unsuccessful. I therefore decided to measure the function of the permease in wild-type *M. methylotrophus* by direct measurement of histidine uptake.

9.2 Histidine uptake must be assayed in wild-type *M. methylotrophus*

In order to compare the effects of histidine permease on wild-type *M. methylotrophus* (pYCP15) and (pGSS33) experiments were set up to measure the uptake of radioactive histidine into the cells. Since *M. methylotrophus* is thought to possess no permease for histidine it was anticipated that there would be very little amino acid uptake. However, if the *S. typhimurium* permease was functional in this organism its presence, in the form of expression from pYCP15, should stimulate histidine uptake into the cells. If this was found to be so, a further experiment could be carried out to assay whether this radioactive histidine was being incorporated into cell protein by TCA precipitation.

9.3 Measurement of histidine uptake in *M. methylotrophus*

Histidine uptake was assayed in exponentially growing cells by measuring uptake of [^{14}C]-L-histidine. Cells were spun down and resuspended in MMM to give a final $A_{680}=1$ in 2ml. The culture was incubated in special apparatus within the laboratory of Alex Cornish in the Department of Biochemistry. This apparatus consists of a 37°C constant temperature chamber equipped with an air supply which provided sufficient aeration for the cells. For *M. methylotrophus*, samples were taken every 5 min over a 30 min period after the addition of [^{14}C]-histidine to a concentration of 0.6 μM . The samples were washed with MMM (37°C) on filters and dried before the radioactivity incorporated was measured. If incorporation into protein was being measured the cell sample was added to TCA to give a final concentration of 10% and incubated on ice for 30 min. The samples were then filtered, washed with 10% TCA and the amount of radioactivity incorporated was measured.

9.4 Histidine is transported by *M. methylotrophus* (pYCP15)

Histidine uptake was compared in *M. methylotrophus* (pYCP15) with (pGSS33) over a 30 min time period. Fig. 9.1A shows clearly that the presence of the *S. typhimurium* histidine permease (closed circles) enhances the rate of uptake by approximately 10-fold such that after 30 min over 30% of the radioactive histidine has been taken up into *M. methylotrophus* (pYCP15). In contrast, cells containing the parent vector plasmid pGSS33 (open circles) have only taken up approximately 4% of the added histidine.

9.5 Histidine uptake is dependent upon proton motive force in *M. methylotrophus*.

Higgins et al. (1985) reported that shock-sensitive transport systems such as the histidine permease are relatively insensitive to uncouplers of oxidative phosphorylation but are sensitive to arsenate. This suggests that transport is energised directly by hydrolysis of ATP. In order to determine the effects of an uncoupler on histidine uptake in *M. methylotrophus* (pYCP15) FCCP was added 5 min after the addition of ^{14}C -histidine and samples taken for a further 10 min.

Fig. 9.1A (open boxes) shows the effects of such an uncoupler on *M. methylotrophus* indicating clearly that uptake of histidine no longer takes place. FCCP is known to prevent ATP synthesis in *M. methylotrophus* via respiratory chain phosphorylation, such that the proton motive force is broken down on addition of the uncoupler preventing further ATP synthesis (Patchett et al., 1985; Anthony, 1982). *M. methylotrophus*,

Figure 9.1

The uptake of histidine by M. methylotrophus and E. coli shown by the amount of histidine taken from the medium both in p mol quantities and as a percentage of the total added. The A_{680} of each culture was measured and concentration adjusted to give $A_{680}=1$ in 2ml culture.

In order to plot this data, the following information was required.

Histidine concentration $50 \mu\text{Ci ml}^{-1}$.

Volume used in each incubation $8 \mu\text{l}$ ie $0.4 \mu\text{Ci} = 1.19 \text{ nmol}$ Histidine in each incubation.

[This is equivalent to $0.6 \mu\text{M}$ Histidine].

$[2.22 \times 10^9 \text{ dpm Ci}^{-1} = 745920 \text{ dpm in } 1.19 \text{ nmol histidine}]$.

Data was plotted as pmol Histidine taken up per time interval (in min) and as a percentage incorporated against time.

Plot A. M. methylotrophus

Open circles pGSS33

Closed circles pYCP15

boxes + pYCP15 with the addition of FCCP.

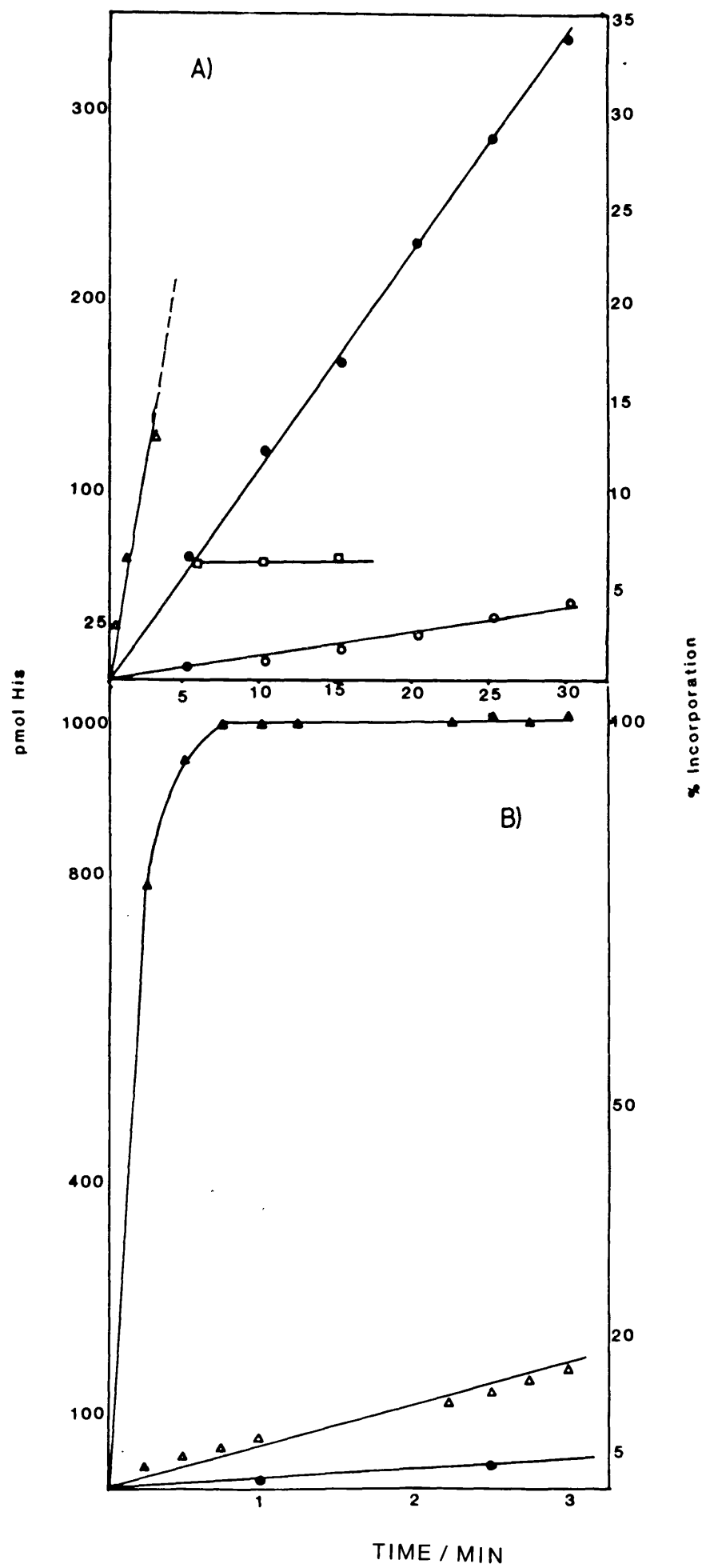
[open triangles E. coli pGSS33 data from Plot B].

Plot B. E. coli

Open triangles pGSS33

Closed triangles pYCP15

[closed circles M. methylotrophus pYCP15 data from Plot A].



unlike E. coli probably generates little ATP by substrate level phosphorylation therefore it is unclear from this experiment whether the cessation of histidine uptake is due to the loss of proton motive force per se or the consequent unavailability of ATP.

In order to distinguish between the effect of respiration on uptake and the effect of ATP starvation I looked at the effects of starving M. methylotrophus (pYCP15) of methanol. Cell pellets were prepared, washed and resuspended in minimal medium without methanol. The rate of respiration was measured using an oxygen electrode and the results clearly showed that respiration is dependent on methanol (data not shown).

A similar experiment was then set up using methanol starved cells to measure the amount of histidine taken up. Histidine uptake was similar to that observed in Fig. 9.1 suggesting that histidine uptake is independent of respiration.

9.6 Comparison of histidine uptake by E. coli BW162 (pGSS33) and (pYCP15)

E. coli BW162 possesses a chromosomal copy of the genes encoding the high affinity histidine permease operon and is therefore able to take up histidine via this route. The rate of histidine uptake in these cells containing plasmid pGSS33 was assayed and compared to the rate of uptake when the organism contained several copies of the Salmonella histidine permease genes through the presence of plasmid pYCP15.

The uptake of histidine by E. coli is very rapid and the experiment was therefore carried out over a two min time period taking samples every 15 seconds.

Fig. 9.1B shows the results of the experiment and clearly indicates that the rate of histidine uptake increases approximately 20-fold in the presence of plasmid pYCP15 (closed triangles).

The graph shows histidine uptake by BW162 (pYCP15) reaches 100% incorporation after 45 seconds after which the graph plateaus since there is no more radioactive label available. Therefore, when the histidine permease operon is present in approximately 10 copies per cell it is able to take up histidine at an approximately 10-fold greater rate than when present in only one copy (BW162). This is shown on the graph by comparing 100% incorporation after 45 seconds by BW162 (pYCP15) with 10% incorporation after the same time period by BW162 (pGSS33). Since the copy number of pGSS33 derivatives in M. methylotrophus has not been established its relevance to the efficiency of histidine uptake in this organism carrying pYCP15 cannot be assessed.

The observation that histidine uptake is extremely rapid in E. coli (pYCP15) could explain the deleterious effects observed in E. coli (Chapter 4), when the permease is cloned into the high copy vector pUC12. When E. coli CSH26ΔF6 carrying the recombinant plasmid pYCP5 is grown in minimal medium it was noted that the cells lysed; this could be explained by the high concentration of histidine inside the cell with respect to outside creating an osmotic effect which might lead to an efflux of

Table 9.1

A comparison of the rate of uptake of histidine per μg cells for both M. methylotrophus and E. coli.

Cell densities were calculated from the A_{680} reading assuming that an

$$A_{680} \text{ of } 1 = 0.4 \mu\text{gml}^{-1} \text{ } \underline{\text{E. coli}}$$

$$A_{680} \text{ of } 1 = 0.63 \mu\text{gml}^{-1} \text{ } \underline{\text{M. methylotrophus}}$$

Approximately 1 A_{680} unit of cells was incubated in each experiment.

Table 9.1

	p mol His min ⁻¹ μg cells	
	pYCP15	pGSS33
<u>M. methylotrophus</u>	7	1
<u>E. coli</u> BW162	1150	76

histidine out of the cells, disabling the cells. Alternatively, simply high levels of histidine or the membrane components of the histidine permease may be toxic to the cells.

9.7 Comparison of the efficiency of histidine uptake between M. methylotrophus and E. coli

The two plots in Fig. 9.1 show the relative rates of histidine uptake by E. coli and M. methylotrophus carrying pYCP15. Plot A shows the rate of E. coli BW162 (pGSS33) (open triangles) as an indication of the greater rate of histidine uptake by E. coli even when only one copy per chromosome of histidine permease is available. Plot B indicates the same observations by comparing the rate of uptake by M. methylotrophus (pYCP15) with the rate of uptake by E. coli. However, in these experiments the culture densities of the two bacteria was not the same and therefore the rate of uptake per minute was calculated and expressed per A₆₈₀ unit and the data are shown in Table 9.1.

These results clearly show that E. coli is able to transport histidine at a much faster rate than M. methylotrophus. The possible basis for this difference will be discussed below.

9.8 Transported histidine is incorporated into cellular protein in M. methylotrophus

In order to compare the amount of [¹⁴C]-histidine taken into the cell by M. methylotrophus (pYCP15) with the amount actually transported across the inner membrane and possibly incorporated into cellular protein, a

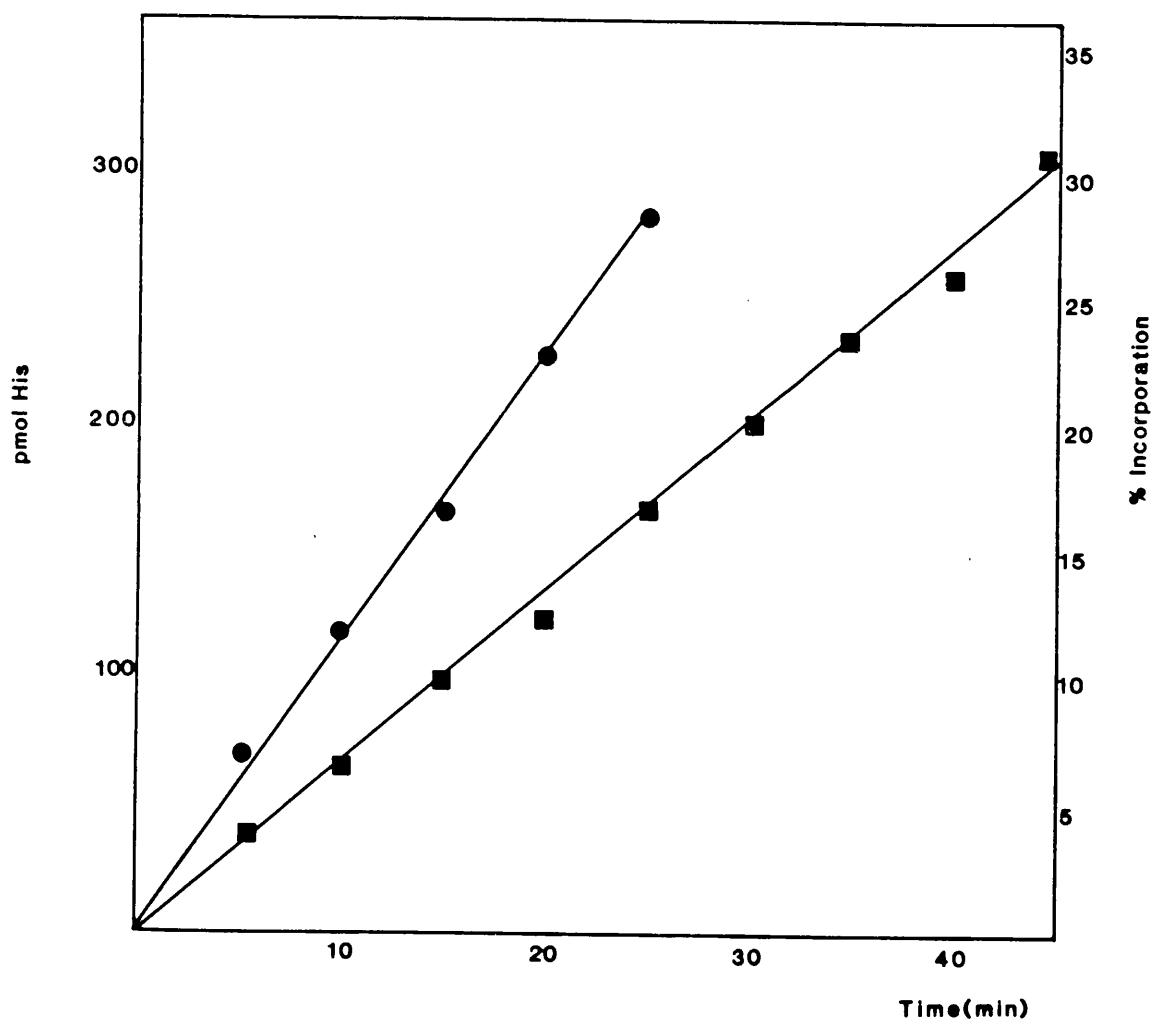
Figure 9.2

The incorporation of transported histidine into cellular protein
in M. methylotrophus

Closed circles represent the amount of histidine taken up into the cell.

Closed boxes represent the amount of histidine incorporated into TCA
precipitable material.

Conditions in this experiment were the same as those indicated in Fig.
9.1.



parallel experiment was set up whereby samples were assayed for both the total amount of cellular [^{14}C]-histidine and the amount incorporated into TCA precipitable material (representing intracellular protein) (Fig. 9.2).

The results shown in Fig. 9.2 clearly indicate that in M. methylotrophus the rate of uptake is greater than the incorporation into intracellular protein indicating that the cells cannot incorporate as much histidine as the permease is transporting. The increase of intracellular histidine over the 45 min labelling, however, confirms that this amino acid is being transported across the inner membrane and not merely binding HisJ protein and accumulating in the periplasmic space.

9.9 The presence of histidine permease in M. methylotrophus does not facilitate leucine uptake

To confirm that the observed stimulation of uptake of histidine in the presence of histidine permease was specific for histidine and not some general effect on amino acid transport, leucine uptake was also measured. No significant increase in incorporation, compared to the control (pGSS33) could be detected (data not shown) indicating that pYCP15 does not provide a general permease. However, since every amino acid was not tested we can only assume that this permease remains histidine specific.

The membrane components of the S. typhimurium histidine permease are known to transport lysine, arginine and ornithine provided that the 'LAO' binding protein is present (Kushi and Ames, 1973; Kushi et al., 1979). The argT gene encoding this binding protein is located upstream of the

dhuA promoter of the histidine permease operon and is present within plasmid pYCP15. An interesting extension to these studies would be to analyse the uptake of lysine, arginine or ornithine in M. methylotrophus to determine whether the histidine permease membrane components are again able to undertake their dual role in transporting the amino acids specified by the LAO protein.

9.10 Discussion

The introduction of the histidine permease into M. methylotrophus has provided an in vivo system with no other such permease present for studying further details of this amino acid uptake system. It has also provided further knowledge as to the potential of M. methylotrophus in biotechnology since these experiments have shown that it can be manipulated genetically.

The experiments have shown that the genes encoding the histidine permease system of S. typhimurium can be introduced into M. methylotrophus and that this organism can apparently express these foreign genes, synthesise the polypeptide products and process and assemble these proteins correctly to form a permease which is functional. M. methylotrophus is known to possess a gene encoding the enzyme leader peptidase (this study) and such an enzyme should be necessary for the correct processing and export of the HisJ histidine binding protein to the periplasm.

The results of Chapter 8 demonstrate that the HisJ protein is localised within the periplasm in M. methylotrophus. However, lack of antibody to HisP prevented the detection of HisP within M. methylotrophus, or localising this protein within the cell.

It is clear from the results in E. coli (Appendix 1) that the whole of the histidine permease must be present for transportation of histidine, we must therefore assume that at least a proportion of all the proteins comprising histidine permease must be assembled correctly in M. methylotrophus to enable histidine to be imported into the cell. The results comparing the amount of histidine taken from the medium with the amount incorporated into the cell suggest that not all the histidine reaches the cytoplasm, indicating that the histidine permease is not as efficient in M. methylotrophus as it is in its native S. typhimurium or E. coli.

Moreover, the results comparing the overall uptake of histidine in E. coli and M. methylotrophus, clearly show a large difference in efficiency. This is not surprising since it is obvious that these two organisms are very different both in their polypeptide composition and their natural environments. We must therefore consider the implications of these differences on the results obtained; firstly the high affinity histidine transport system of E. coli is only one of several routes used for histidine uptake within this organism; therefore E. coli BW162 (pYCP15) is able to take up [^{14}C]-histidine via a number of routes, whereas M. methylotrophus (pYCP15) is limited to the plasmid encoded permease proteins. The level of expression of all these permease components has not been assessed within M. methylotrophus, therefore although the results of Chapter 8 indicate that hisJ gene is expressed, there is not guarantee that hisQ, M and P genes are expressed as efficiently. Factors affecting expression could of course include the copy number of pYCP15 in M. methylotrophus which has not been determined. Even if this his operon is expressed, the proteins then have to assemble

into the inner membrane. The lipid structure of the cytoplasmic membrane of M. methylotrophus has not been determined and may not be able to accommodate these foreign proteins in their most optimal position for transport. HisQ and M proteins must assemble in such a way presumably to interact with both HisJ and HisP, any deviation in their normal assembly pattern may greatly reduce the efficiency of histidine transport. Not only will the composition of the inner membrane be expected to affect the uptake of histidine, but the composition of the outer membrane must also be considered. This membrane may be less permeable to histidine than the outer membrane of E. coli or S. tyhimurium. Evidence in support of this is provided by experiments using the M. methylotrophus histidine mutants; these mutants required a concentration of 1mg ml^{-1} histidine in order to grow; this is a very high concentration of amino acid suggesting that diffusion of the substrate into the cell is very limited. In E. coli with OmpF and OmpC deleted [^{35}S]-methionine uptake is reduced ten fold (A. Boyd, personal communication). M. methylotrophus may or may not possess such proteins or their ability to transport amino acids maybe extremely limited.

A difference in the composition of the periplasmic space could also affect the efficiency of histidine uptake since HisJ protein may not be able to 'scavenge' histidine as efficiently in M. methylotrophus as it is able to do in E. coli and S. typhimurium. Finally, differences in the availability of energy sources in M. methylotrophus may reduce the function of the energy requiring histidine permease, thus reducing the efficiency of uptake. The experiments carried out under methanol starvation (Section 9.5) suggest that uptake is independent of respiration, however, caution must be taken in interpreting these

results since the [^{14}C]-histidine used was made up in aqueous solution containing 2% ethanol. M. methylotrophus is known to be able to use ethanol as a carbon source (A. Cornish, personal communication). Therefore, although very little ethanol is present (0.008%) in the incubation mixtures it might have been sufficient to enable the cells to respire and this possibility should be eliminated before a final conclusion can be drawn.

An interesting extension to this study would have been to compare the uptake (or lack of) histidine by the E. coli mutants lacking the histidine permease (as described in Appendix I), together with such E. coli strains transformed with plasmid pYCP15. These E. coli mutants are still able to transport L-histidine via a general aromatic permease and would therefore act as a control in assessing the amount of histidine taken up via the histidine permease alone in E. coli. This would have provided a better base line for comparison with histidine. It is nevertheless quite likely that one or more of the other factors discussed above will have a major effect on the efficiency of the permease in the heterologous organism, M. methylotrophus.

CHAPTER 10

DISCUSSION

The broad aim of this project was to investigate the effects of introducing the histidine permease of S. typhimurium into M. methylotrophus, a Gram negative bacterium with no known protein transport mechanism. The fact that the operon comprising this permease had already been sequenced (Higgins et al., 1982) provided extensive data and an excellent starting point, for me to commence this study. However, the precise identity of a number of the histidine permease proteins had not been established. For the purpose of this study it was first essential that firstly, the protein components must be easily identifiable by convenient electrophoretic methods. I therefore constructed plasmids containing one of several of the histidine permease genes in order to overproduce and consequently identify the gene products both in vivo and in vitro. Problems with lethality in overproduction of the whole histidine permease led to the construct vectors containing only a portion of the permease genes. The experiments described in Chapters 3 to 6 outline the strategies for identifying, by conventional SDS PAGE, HisJ, HisP and HisQ proteins.

The construction of these plasmid vectors assisted in the second stage of the study; the introduction of the histidine permease operon into M. methylotrophus. Furthermore, with three out of the four protein components formally identified in E. coli these could then be compared in M. methylotrophus. Chapter 8 described the expression of the histidine permease in M. methylotrophus. The results clearly showed not only that at least HisJ was synthesised but also apparently processed and localised

correctly to the periplasm of M. methylotrophus. Finally, I set out to test whether M. methylotrophus containing a histidine permease would now transport histidine. The results of Chapter 9 demonstrate clearly that the cells are now capable of histidine transport although the overall efficiency was much less than that observed in E. coli or S. typhimurium.

Throughout the course of this work papers were published which helped to clarify the possible mechanism of these periplasmic permeases. Firstly, HisP was identified as a nucleotide binding protein (Hobson et al., 1984) then more recently an entire family of nucleotide binding proteins has been identified all with similar structure and function. These, together with experiences from my work, for example attempts to confirm the location of HisP, has enabled me to bring together more ideas towards deducing the mechanism for amino acid uptake via a periplasmic permease.

In the Introduction (section 1.9) I discussed the first models put forward by Ames and co-workers. The two opposing models put forward suggest either that a pore is formed by HisQ and M enabling histidine molecules to pass into the cell or that a cascade mechanism is set up whereby histidine binds sequentially to each of the permease components as it traverses the membrane. Both models require energy presumed to be supplied from ATP hydrolysis by HisP (Hobson et al., 1984). Genetic evidence has suggested that direct interaction exists between the periplasmic J protein and HisP since mutations in P could suppress defects in J (Ames and Spudich, 1976; Kushu and Ames, 1974). In order to interact in this way the P protein must protrude into the periplasmic

space. However, since ATP hydrolysis presumably takes place in the cytoplasm, the ATP binding region in P must be at the cytoplasmic side of the inner membrane. In complete contrast, the hydropathicity of HisP (Fig. 1.6) suggests that this protein is not a membrane protein; its extensive hydrophilic nature resembles a cytoplasmic protein. This extreme hydrophilicity need not be taken as evidence that the protein cannot cross the membrane however, since the protein OmpF also has a very hydrophilic composition (Fig. 1.6) although it has been shown to be embedded in its eventual location the outer membrane. Nevertheless, there are no known examples of hydrophilic inner membrane proteins. The evidence that HisP binds HisJ stems from genetic suppression experiments prior to the identification of HisQ or M (Ames and Spudich, 1976). Therefore, although the data suggests that a mutant HisJ protein with a defective interaction site can still function if there is an appropriate compensating mutation introduced in the P protein, the precise location of this mutation may well have been within the upstream hisM gene. In line with this interpretation, comparison with the similar maltose permease demonstrates that the periplasmic protein MalE interacts with both MalG and MalF proteins; the integral membrane proteins equivalent to HisQ and HisM (Treptow and Shuman, 1985).

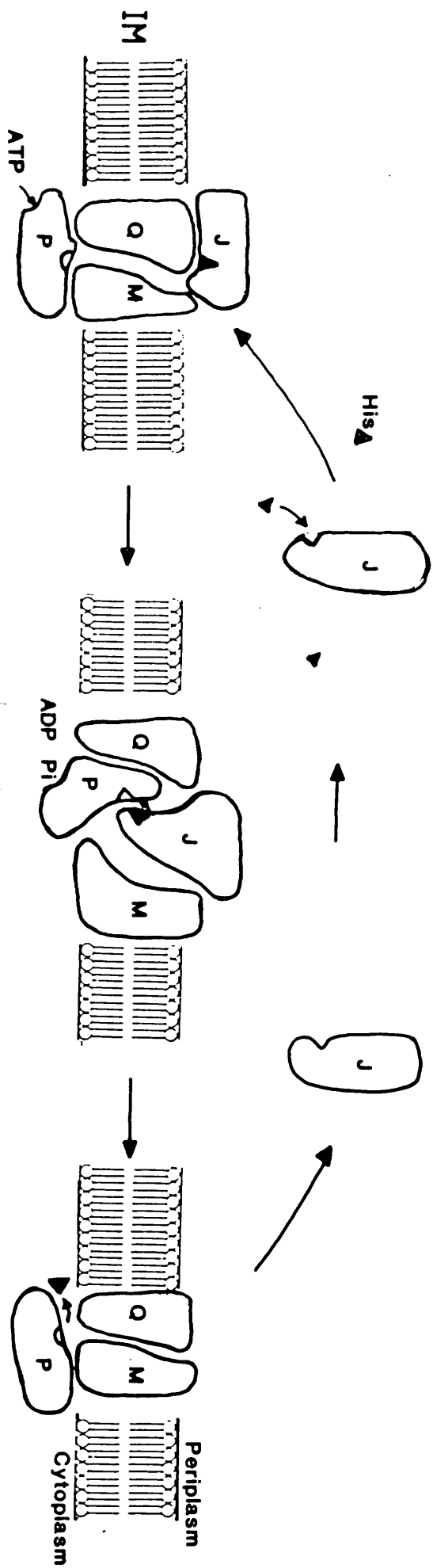
The possibility that HisP is a membrane protein at all in fact remains controversial. Experiments to localise the P protein to a particular cellular function have proved inconclusive. Ames (1986) found that HisP cannot be released from the membrane fraction upon sonication; nor can it be recovered in the soluble protein fraction in mutants defective in HisQ or HisM suggesting that HisP is firmly located in the cytoplasmic membrane. MalK on the other hand is released upon sonication and is also

soluble in mutants defective in MalG, suggesting that this is a peripheral membrane protein (Treptow and Shuman, 1985). Experiments carried out in this thesis (Chapter 5) predominantly suggest a cytoplasmic location for HisP although some association with the inner membrane was also observed. Although these results were inconsistent with the tight association with the inner membrane reported by Ames, the absence of several of the histidine permease components or the overproduction of HisP may have masked any other forms of peripheral binding. Unfortunately, the majority of work carried out by Ames on the localisation of HisP remains unpublished and details of the experiments are unknown making comparisons difficult to assess. Early work involving two-dimensional electrophoresis was published and suggested a membrane location for HisP, however, HisP was also detected within the cytoplasmic fraction (Ames and Nikaido, 1978).

In an attempt to clarify the possible dependence of the membrane assembly of HisP upon other constituents of the histidine permease the experiment described in section 5.7 was carried out. Unfortunately, the experiment was unable to distinguish between the assembly of HisP in the presence of pre-existing HisJ, Q and M and assembly dependent upon cosynthesis with HisJ, Q and M. Furthermore, stoichiometric amounts of P, Q and M were not obtained and the amount of HisP far exceeded that of J. In contrast, under normal conditions HisJ is thought to be present in a 30-fold excess over P. Finally, the absence of histidine may well have affected the assembly of HisP. In the presence of substrate, transport will be active and therefore the components are more likely to mimic their cellular role in vitro.

Figure 10.1 An alternative model for transport by the periplasmic permeases

HisJ, the substrate binding protein binds to one or more of the membrane components. HisQ and M are typical hydrophobic membrane proteins which form a pore within the cytoplasmic membrane. HisP is a peripheral membrane protein with a nucleotide binding site. The binding of the histidine bound HisJ to the membrane triggers a conformational change in HisQ which triggers ATP hydrolysis by HisP, this energy is sufficient to cause the pore to open and the histidine substrate to be transferred from HisJ to HisP. HisP then releases the histidine within the cytoplasm of the cell and the pore closes.



With the data obtained from this thesis together with the previous data in the literature I have suggested an alternative model for the transport of histidine, put forward in Fig. 10.1. The HisP protein is depicted as a peripheral membrane protein, forming a functional complex with HisQ or M. On binding histidine HisJ interacts with the permease complex in the membrane. This binding triggers conformational changes with HisQ which are transmitted to HisP which in turn releases energy in the form of ATP hydrolysis. This drives the pore formed by His Q and M, into opening sufficiently to allow HisJ and HisP to interact and exchange the histidine molecule. HisP then carries the molecule to the cytoplasm. Energy for this entire mechanism may be supplied, not only by ATP but also by membrane potential since energy is expended both in the pore opening mechanism and the binding between HisJ and HisP.

Genetic suppression analysis suggests that HisP and HisQ are able to interact with each other (Ames and Nikaido, 1978); furthermore a review of this data in the light of the discovery of the fourth permease component, HisM, suggests that this protein can also interact with HisQ and HisP (Higgins et al., 1982). Further direct evidence about this interaction is lacking, however the model put forward in Fig. 10.1 would allow for this interaction since HisP is proposed as peripherally bound to the membrane, presumably via a loose interaction between either HisQ or M or both proteins.

The model is also consistent with the properties of HisJ mutants which indicate that HisJ interacts with a membrane component of the permease since a mutant HisJ cannot function in transport despite possessing an intact histidine-binding site (Kustu and Ames, 1974). Moreover, experiments using nuclear magnetic resonance show that such a mutant J

protein is unable to undergo a normal ligand-induced conformational change suggesting that this conformational change is ultimately involved in the functioning of binding proteins in transport.

The interaction of HisJ and HisP deduced from genetic data; suggest that the mutations in the J protein described above can be suppressed by a mutation in hisP. This interaction is visualised in this model as an interaction due to conformational changes taking place within the permease pore complex allowing access to the HisP protein without the necessity that HisP must entirely cross the cytoplasmic membrane in the manner as an integral or trans-membrane protein.

Finally, HisP is shown to interact with the membrane components of the permease HisQ and HisM in the model; this interaction was suggested from the results of complementation studies during which the hisQ gene was discovered, explaining the apparent intragenic complementation noted within regions of the hisP gene. This hisP gene was therefore divided into hisP and hisQ.

The closely related lysine-arginine-ornithine (LAO) binding protein shows over 90% homology with HisJ in the region of the putative P-interaction site; (Higgins and Ames, 1981). Since the two proteins require the same Q, M and P proteins in order to transport their specific amino acid we can assume that they have an identical function, that of substrate binding and the interaction with a common membrane component (Higgins and Ames, 1981; Ames and Higgins, 1983).

In order to prove the correctness of the various aspects of any of these models for the structure and function of the histidine permease it will be necessary to isolate, purify and characterise each of the membrane-bound components and, for example, to reconstitute the entire system in liposome vesicles. This will be a very difficult task, however, the synthesis of each component using the techniques outlined in this thesis allows efficient overproduction of at least three of the four proteins, and hopefully will facilitate purification of the products. This in turn should provide for detailed analysis of the interactions between each of the components.

APPENDIX

Functional test for histidine permease

In order to test each of the plasmids constructed in this thesis for full permease activity, DNA from each was used to transform E. coli TA3476 and the resulting transformants plated onto M9 minimal agar plates as described in Table AI.

E. coli TA3476 contains the deletion his Δ (pta-ack-dhuA-hisJ-hisQ-hisP) but although the high affinity histidine permease is mutated, L-histidine is still able to be transported via a general aromatic permease (Ames and Lever, 1970). The high affinity histidine permease is also able to transport D-histidine into the cell; therefore whilst these mutants cannot grow on minimal medium lacking L- or D-histidine, nor can they grow on MM and 10 $\mu\text{g ml}^{-1}$ D-histidine (Grynkeiwicz et al., 1971). In contrast, they can grow in minimal medium supplemented with 4 $\mu\text{g ml}^{-1}$ L-histidine. The addition of the permease to these mutant cells by a plasmid vector will restore their ability to grow on 10 $\mu\text{g ml}^{-1}$ D-histidine.

Table AI

The results of E. coli TA3476 and TA3476 transformed with plasmids described in this thesis grown on M9 minimal agar plates. Plates were prepared without histidine, supplemented with 10 $\mu\text{g ml}^{-1}$ D-histidine or with 10 $\mu\text{g ml}^{-1}$ D-histidine and 4 $\mu\text{g ml}^{-1}$ L-histidine.

Plates were incubated at 37°C for 24 hr.

- = no growth

+ = growth

N.T. = not tested

TABLE AI

Strain	Plasmid	No. His	L+D His	D-His
TA3476	none	-	+	-
TA3476	pFA13	-	+	+
TA3476	pUC12	-	+	-
TA3476	pYCP2	-	+	-
TA3476	pYCP5	-	+	+
TA3476	pGSS33	-	+	-
TA3476	pYCP15	-	+	+
TA3476	pYCP6	N.T	N.T	N.T
TA3476	pYCP7	N.T	N.T	N.T
TA3476	pYCP3	N.T	N.T	N.T
TA3476	pYCP3 + pYCP6	N.T	N.T	N.T

The results indicate that the permease is not functional unless the whole complement of histidine permease proteins is present; even with pYCP2 when only the 3' (COOH) end of HisP is absent the permease remains non-functional.

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Studies of the Histidine permease of S.typhimurium in E.coli
and M.methylotrophus

The broad aim of this project was to investigate the effects of introducing the Histidine permease of S.typhimurium into M.methylotrophus, a Gram negative bacterium with no known protein transport mechanism. This should not only contribute to the elucidation of the mechanism by which these periplasmic permeases operate, but also help in the understanding of M.methylotrophus an organism of commercial importance whose biochemistry is not well characterised.

Although the entire operon encoding this permease had recently been sequenced prior to the commencement of this study, the precise identity of the components had not been established; furthermore, since each component is produced in very small amounts, simple identification by electrophoretic techniques was not possible.

My initial aim, therefore was to introduce the structural genes of the operon under the control of a strong, but inducible promotor, in order that each gene product could be identified and the location of the protein within the cell established. The His P protein was identified in this manner, however discrepancies in overproduction prevented the precise location of this polypeptide within the cell from being established. Induced expression of the hisQ gene also identified an overproduced polypeptide of 30,000 molecular weight, suggesting this to be the His Q protein, however I was unable to establish a precise identification.

The construction of these new plasmid vectors assisted in the second stage of this study; the introduction of the histidine permease into M.methylotrophus. Furthermore, with three out of four protein components formally identified in E.coli these could then be confirmed in M.methylotrophus. The results obtained clearly show, not only that at least His J was synthesised, but also apparently processed and localised correctly into the periplasm of M.methylotrophus. Furthermore, the permease system was found to be functional in M.methylotrophus demonstrating active histidine uptake into the cell.