IDENTIFICATION OF PROTEINS OF THE HEXOSE PHOSPHATE TRANSPORT SYSTEM OF *ESCHERICHIA COLI*

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PREFACE

"Thus grew the tale of Wonderland
Thus slowly, one by one,
Its quaint events were hammered out-
And now the the tale is done,
And home we steer, a merry crew,
Beneath the setting sun."

- Lewis Carroll in Alice in Wonderland.

This is not the story of a little girl tumbling down the rabbit hole seeing and experiencing incredible things, though it certainly could be one. Nearly five summers ago I embarked on a journey, not much unlike Alice's, into the world of scientific research. Little did I know at the time, of all that was about to be stumbled upon. One had it all – the pleasure and pain of discovery, moments of truth and lots of learning not only of scientific facts but of whole new perspectives on things. And to behold a moment of truth has been a reward in itself.

What would any endeavor such as this be, but the sound of one hand without its key characters? I am deeply grateful to my mentor, Dr. Richard C. Essenberg, who allowed me the freedom and the time in which to follow my own leads, unintrusively guiding my learning with ideas and suggestions. Working with him has been a unique and cherished experience. I am also deeply thankful to Dr. Ulrich Melcher for his confidence in my abilities as a researcher and for many a word of encouragement and support and for help in sorting out many problems. My sincere thanks to the other members of my graduate committee, Dr.

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CHAPTER I

INTRODUCTION

The cell envelope of *Escherichia coli* is a complex structure that consists of three distinct layers, namely the outer membrane, the cell wall and the inner membrane. The space between the outer and inner membranes is known as the periplasm. Despite the complexity of the physical barrier between itself and the outside, *E. coli* is quite capable of acquiring a variety of substances from its environment. The movement of molecules and ions across the membrane barrier is effected by specific transport systems. There are at least three major transport mechanisms other than passive diffusion operative in *E. coli.* They are facilitated diffusion, group translocation and active transport. This classification is based on the mode of energy coupling to the transport process and the actual mechanism of translocation.

Sugar phosphates such as those of glucose and fructose are generally thought of as intermediates of carbon metabolism rather than as immediate sources of carbon. However, it has been known for over twenty years that hexose phosphates can be utilized by *E. coli* (1). The existence of an uptake system for hexose phosphates distinct from that of hexoses was established due to the pioneering work by Lin et al. (2, 3) and Fraenkel et al. (4). Lin and coworkers found that mutants lacking either alkaline phosphatase or double mutants lacking in glucose transport and alkaline phosphatase of *E. coli* were capable of growing on glucose 6-phosphate or

fructose 6-phosphate. Fraenkel et al. demonstrated the ability of glucokinaseless, phosphotransferase system I mutants of *E. coli* to grow on glucose 6-phosphate and not on glucose. The patterns of induction and competition as observed by Winkler (5) suggested that glucose 6-phosphate, fructose 6-phosphate, and mannose 6-phosphate were all substrates of one transport system and that only glucose 6-phosphate was able to induce the system. The K_i for inhibition by fructose 6-phosphate and mannose 6-phosphate when glucose 6-phosphate was the substrate were close to the K_m for transport of glucose 6-phosphate alone. Galactose 6-phosphate had no effect on the uptake of glucose 6-phosphate. Furthermore, Winkler's studies on hexose phosphate transport negative mutants showed that these mutants retained the ability to grow on hexoses and glucose 1-phosphate. The rate of uptake of glucose 6-phosphate by these mutants grown in the presence of the inducer was as low as that of the uninduced parent. Pogell et al. (6) found that induction of an active transport system for glucose 6-phosphate required de novo protein synthesis. They also found that fructose 1,6-di phosphate, glucose, L-α glycerophosphate and glucose 6-sulphate were neither inducers nor substrates of the hexose phosphate uptake system. Studies by Dietz and Heppel (7) showed that there were at least two separate pathways for the uptake of glucose 1-phosphate. In wild type *E. coli* this ester was hydrolyzed before uptake, unless the cells were induced for the uptake of hexose 6-phosphates, whereas in a mutant lacking alkaline phosphatase and constitutive for the uptake of hexose 6-phosphates, glucose 1-phosphate is taken up as such. Studies with 2-deoxy glucose, a nonmetabolizable analog of glucose, showed that intracellular accumulation of 2-deoxy glucose 6-phosphate failed to induce the uptake

of hexose phosphates, whereas extracellular 2-deoxy glucose 6-phosphate did (8). Thus the above mentioned studies clearly established the presence of an inducible uptake system for hexose phosphates and that the uptake itself had broader substrate specificity than the induction process.

The nature of the inducer and the mode of induction of the hexose phosphate uptake system were investigated by Winkler (9) using phosphoglucoisomerase and glucose 6-phosphate dehydrogenase mutants of *E. coli* that were constructed by Fraenkel (10, 11). Such double mutants are unable to grow on glucose or glucose 6-phosphate. Uptake assays on one such double mutant showed that glucose 6-phosphate at extracellular concentrations as low as 10^{-4} M was effective as an inducer. The large intracellular pool of glucose 6-phosphate that is accumulated when this mutant was provided with glucose was ineffective as an inducer of the uptake system for hexose phosphates. Fructose 6-phosphate was unable to induce the double mutant and a phosphoglucoisomerase mutant of *E. coli*, but was able to induce wild type and a glucose 6-phosphate dehydrogenase mutant of *E. coli* for the uptake of hexose phosphates. This was explained by assuming fructose 6-phosphate was converted to glucose 6-phosphate by a membrane-bound phosphoglucoisomerase, which then acted as the true inducer. Friedberg demonstrated the presence of a membrane-bound phosphoglucoisomerase (12). Winkler suggested a model for induction where glucose 6-phosphate had to be present exogenously to induce the system. The nature of the exogenous induction was defined by construction of *E. coli* strains in which *uhp* expression could be measured in the absence of *uhp* function (13). Isogenic strains carrying *UhpT-lac* operon fusions differing only in their uptake activity were found to be induced to the same extent as measured by the

transcription of the operon fusion. This study clearly established that uptake and induction are two different processes and that uptake was not required for induction.

Mutants defective in the uptake system for hexose phosphates were isolated by Kornberg and Smith (14) by a somewhat indirect method based on a really novel idea. An *E. coli* mutant lacking phosphoenolpyruvate carboxylase (ppc) is unable to grow on carbohydrates but is able to grow on acetate. Growth on acetate of this mutant depends on isocitrate lyase of the glyoxylate cycle. This mutant while growing on acetate shows growth inhibition if glucose or glucose 6-phosphate is added, which is reversed by the addition of glyoxylate. PPC mutants that showed resistance to growth inhibition by glucose 6-phosphate and not glucose were isolated. The resistance to growth inhibition by hexose phosphates of such a mutant was assumed to be due to the loss of ability to take up hexose phosphates. Kornberg and Smith coined the mnemonic *uhp* (uptake of hexose phosphates) to describe the genetic locus, which they found to be 50% cotransducible with the *pyr E* (pyrimidine) marker and mapped at 72 min (revised 81 min) on the *E. coli* linkage map. Mutants that were constitutive for the uptake of hexose phosphates were isolated by Ferenci et al. (15), based on their observation that *E. coli* was able to exhibit rapid periods of growth on fructose 1-phosphate if the cells were previously exposed to hexose 6-phosphates. Growth on fructose 1-phosphate decreased after 1 - 1.5 doublings. Fructose 1-phosphate, though not an inducer, was found to be a substrate of the *uhp* system. *E. coli* (KL 16 - 21) that cannot grow on fructose was used to isolate mutants that showed sustained growth on fructose 1-phosphate for many generations. Such mutants had the ability to take up hexose phosphates even in the absence

of prior exposure to the same, and are thus constitutive for the uptake (*Uhp*^{c}), whereas wild type *E. coli* lacked this ability. *Uhp*^{c} mutants mapped at the same locus as uptake negative mutants. A second method of selection for *uhp* mutants is based on the fact that fosfomycin (a bactericidal agent) enters the bacterial cell via the hexose phosphate or L-α glycero phosphate transport systems (16). Kadner and Winkler (17) employed a *glp T* strain (unable to grow on L-α glycero phosphate) of Lin as the parental strain to isolate fosfomycin resistant mutants. These mutants were unable to grow on hexose phosphates. Fine structure genetic mapping of the region in the vicinity of the *uhp* locus yielded the following order of genes in the *E. coli* chromosome: *mtl-cysE-pyrE-uhp-bgl-ilv*. *Uhp* mutants of *E. coli* that showed reduced ability to grow on 1 mM glucose 6-phosphate were also isolated by Essenberg & Kornberg (18) following treatment with ethyl methane sulphonate (19) or nitrous acid (20). These mutants mapped at 81 min in the *E. coli* linkage map. They proposed the following more detailed gene order in the vicinity of 81 min: *mtl-gpsA-pyrE-gltC-uhp-tna-dnaA*.

Kadner (21) found that his *Uhp*$^{-}$ mutants could revert to either an inducible or a constitutive phenotype for the uptake of hexose phosphates. An assumption was made that those mutants reverting to inducible uptake might have been affected at the structural gene for the transport and those mutants reverting to constitutive uptake might originally carry a lesion at the regulatory locus. All of the assumed regulatory mutations mapped distal to the structural gene mutations, thus implying the presence of at least two regions, a structural region and a regulatory region comprising the *uhp* locus. A whole collection of point, deletion, and transposon insertion mutants in the *uhp* region were isolated (22) and mapped relative

to the *pyrE* or *ilvB* locus on the *E. coli* chromosome by transduction or conjugation crosses. The tentative map positions of the mutant alleles were compared to the reversion properties of the mutants. Tentative gene assignments in the *uhp* locus were made: *gltC-uhpT R A tna.* Mutation in any one of the three genes of the *uhp* region could give rise to an uptake negative phenotype. Mutations reverting only to an inducible phenotype occurred infrequently and mapped at the opposite ends of the *uhp* region, at *uhpT* or *uhpA*. Deletion mutants that lacked *uhpA* did not give rise to *Uhp*$^+$ revertants thus implying a positive regulatory role for the *uhpA* gene product. Mutations reverting to constitutive phenotypes occurred frequently and were clustered between *uhpT* and *uhpA*. The constitutive revertants were thought to be a result of second site mutations thus indicating the possible presence of two regulatory genes in this region, the loss of one of which yields the *Uhp*$^-$ phenotype and the other, *Uhp*^{c} phenotype.

The entire *E. coli* genome is represented in the Clarke & Carbon plasmid library (23), which can be screened for genes of interest if suitable methods of selection are available for them. Essenberg (24) screened the Clarke & Carbon plasmid library for *uhp* genes and found five plasmids that conferred *Uhp*$^+$ phenotype on *Uhp*$^-$ mutants of *E. coli.* These plasmids were grouped according to: 1. The regulatory properties of the plasmid bearing strains and 2. The ability of the plasmids to correct *uhp* deletion and point mutations. It was concluded that plasmids *pLC35-4 & pLC34-43* did not carry *uhpT,* whereas *pLC40-33, pLC17-47* and *pLC14-9* did. A partial restriction map was also constructed for these plasmids. The entire *uhp* region was subcloned from *pLC17-47* and located within a 7.0 kb region by restriction analysis (25). Both *pLC17-*

47 and the subclone derived from it conferred constitutive glucose 6-phosphate uptake on all strains tested. This was thought to be due to the presence of multiple copies of *uhpA*. *pLC40-33* on the other hand conferred inducible expression that required the presence of *uhpA* on the chromosome. This requirement for a functional *uhpA* was also observed in strains carrying *uhp-lac* operon fusion bearing plasmids. A tentative model for regulation of the *uhp* system was proposed which included a structural gene and three regulatory genes. Extensive analysis of transposon insertions in the *uhp* region carried by the subclone and their complementation properties combined with the study of expression of the subclones in *E. coli* maxicells has led to the identification of three regulatory genes and a structural gene designated *uhpA, B, C,* and *T,* respectively (26). Transposon insertion mutations from the subclones were transferred onto the chromosome so that any effect by plasmid copy number is eliminated, thus allowing one to elucidate the effect of the insertion mutations on *uhp* expression. The reversion properties of these strains were similar to the ones observed previously. Those strains that reverted to the *Uhp*^{c} phenotype carried a second site mutation in the *uhpR* region, now known to be the *uhpC* gene. These studies also confirmed the role of *uhpA* as a positive activator of *uhp* expression. A model was proposed in which the products of *uhpB & C* genes prevent that of *uhpA* from activating the transcription of *uhpT* under non inducing conditions. Subsequently the entire *uhp* region has been sequenced (27). The location of open reading frames correlated with the location of *uhp* genes defined by the Weston & Kadner clones. Furthermore, polypeptide sequences deduced from the nucleotide sequence data supported the Weston & Kadner model for *uhp* regulation.

The driving force for the uptake of hexose phosphates was thought to be the proton motive force (28, 29, 30). Recent studies by Ambudkar et al. (31) suggest that a transmembrane phosphate gradient may be responsible for driving the uptake of hexose phosphates.

There are at least two major directions of exploration in the field of membrane transport. The source of energy and how it is coupled to the actual process of transport is one, and the other is, how transport is effected, membranes and all. *E. coli* as an experimental system is well suited for the study of any biochemical problem because the organism is extensively defined at various levels. Thus it is possible to study a transport problem in terms of genes, proteins and membranes. The collective goal of membrane biochemists has been to reconstitute a functional transport system from its constituents in the laboratory.

This study is aimed at characterizing the components of the hexose phosphate uptake system in *Escherichia coli.* As means to an end the following steps were taken: 1. Subcloning of specific restriction fragments carrying *uhp* genes from Clarke & Carbon plasmids in multicopy plasmids. 2. Construction of mutations in the *uhp* genes carried by recombinant plasmids to prove the functionality of the cloned genes. 3. Expression of cloned genes as seen in *E. coli* minicells, leading to a correlation of *uhp* genes and proteins.

CHAPTER II

MATERIALS AND METHODS

Media and Chemicals

LB is Luria-Burrous nutrient medium (32) and Medium A (33) is minimal salts medium. Carbon sources added to the minimal medium were either 0.01 M glucose or 0.005 M glucose 6-phosphate. Amino acids were added at a concentration of 100 μg/ml as required. Bases such as thymine or uracil were added at a concentration of 50 μg/ml. Minimal medium was also normally supplemented with 100 μg/ml thiamine-hydrochloride. Antibiotics used as required were 50 μg/ml ampicillin, 15 μg/ml tetracycline, 50 μg/ml kanamycin, 30 μg/ml chloramphenicol, and 25 μg/ml streptomycin sulphate. Medium H, 1% tryptone and 0.8% sodium chloride or medium R, 1.0% tryptone, 0.1% yeast extract, 0.8% sodium chloride, 0.002M calcium chloride and 0.1% glucose were used for phage plates. Top agar for plating out phages were either F-top 0.8 % agar and 0.8% sodium chloride, or H-top which is F-top with 1.0% tryptone, or R-top is medium R with 0.8% agar. Solid nutrient media plates were made using appropriate medium with 2.0% Difco agar. Plates used for transformation were usually 1.5% LM agar.

Restriction enzymes were from BRL or IBI. ^{3}H amino acid mixture was from ICN. Glucose 6-phosphate was from Sigma Chemical Company. Prestained protein molecular weight standards range 12.3 - 200 kdal, were from BRL. Standards for isoelectric focusing, range 4.6 - 9.6 pI units,

were from Biorad. Ampholytes, range pH 5-8 and pH 3-10, were from Sigma.

Genetic Techniques

Conjugations and P1 mediated transductions were performed as described in Miller (33). Aliquots of the mating mixture were plated out on appropriate nutrient medium. Controls included both parents plated on similar plates. Transductions used a lysate of P1 cml clr100 (Table II) and plated out on selective medium using F-top agar. Plates were normally allowed to grow 24-48 hrs at 37°C.

Transformations with cloned DNA were performed by following the Hanahan procedure (34). Cells grown to a density of $2\text{-}3\text{x}10^8$ cells/ml were extensively treated with buffers containing chlorides of potassium, manganese, rubidium, calcium and cobalt, with dimethylsulfoxide and dithiothreitol. Competent cells thus prepared were incubated with 100-500 ng DNA for 30 mins at 0°C before heat shocking for 90 secs at 42°C. Transformed cells were plated on LM agar plates with ampicillin.

Bacterial Strains and Bacteriophages

The *Escherichia coli* K12 strains and bacteriophages used in this study are listed in Tables I and II.

Construction of Plasmids Carrying *uhp* Genes

The Clarke & Carbon plasmids (35) were obtained from D. Smith (University of California at San Diego). Multicopy plasmids *pUC8* and *pUC9* were obtained from S. Chimie (University of Toronto). Recombinant DNA manipulations were carried out as described in Maniatis

TABLE I

ESCHERICHIA COLI K12 STRAINS

E. coli K12 Strains	Genotype	Origin
RE 74	Hfr(P02) *met*B1, *pyr*E41, *uhp*-40, *glt*C^{c}, *tna*-6, *rel*A1, *ton*A22, T2^{r}, λ+	Essenberg, R.C. & Kornberg, H. (18)
JC10240	Hfr(P045), *srl*C300::Tn10, *rec*A56, *thr*-300, *ilv*-318, *rps*E300	Csonka, L.N. & Clark, A.J. (48)
KL-16-99	Hfr(P045), *rec*A1	CGSC #4026 (49)
DS410	*azi*-8, *ton*A2, *min*A1, *min*B2, *rps*L135, *xyl*-7, *mlt*-2, *thi*-1	CGSC #6486 (50)
ED18	*pur*E, *his*, *str*^{r}, Tc^{r}, *xyl*, *ilv*, *cyc*A^{r}, *cyc*B^{r}, *met* (thr), *lac*Y, *pro*C, T$_3^{r}$, (*min*A), *min*BZcf117::Tn10)	L. Rothfield (51)
χ1178	F^{-}, *ara*-14, *leu*-1, *azi*^{r}, *ton*A, *lac*Y1, Tc^{s}, *min*A, *gal*-12, *min*B, *thy*A31, *str*^{r}, *xyl*-5, *mlt*-5, *thi*-1, *dra*-17, *sup*D, λ^{-}	χ1178 mated with RE74 to *xyl*$^{+}$, temperature cured, transformed to Tc^{r} with JC10240, made Tc^{s}
PE234	F^{-}, *thr*-1, *ara*-14, *leu*-1, *azi*, *ton*A, *lac*Y1, *min*A, *gal*-12, *hy*A31, *str*^{r}, *xyl*-5, *mtl*-5, *thi*-1, *dra*-17, *sup*D, *uhp*-40, *glt*C^{c}, *tna*, *rec*A, Tc^{s}, (Δ*srl*?)	χ1178 mated with RE74 to *xyl*$^{+}$, temperature cured, transformed to Tc^{r} with JC10240, made Tc^{s}
RK4981	*ara*D139, (Δ*arg*F-*lac*)U169, *rel*A1, *rps*L50, *thi*, *gyr*A219, *non*, Δ*uhp*2050, *met*E70, *pyr*E40, *glt*S15	Kadner, R.J. (22)

TABLE I (Continued)

E. coli K12 Strains	Genotype	Origin
RK4983	*ara*D139, (Δ*arg*F-*lac*)U169, *rel*A1, *rps*L150, *thi*, *gyr*A219, *non*, Δ*uhp*2052, *met*E70, *pyr*E40, *glt*S15	Kadner, R.J. (22)
RK4984	*ara*D139, (Δ*arg*F-*lac*)U169, *rel*A1, *rps*l150, *thi*, *gyr*A219, *non*, Δ*uhp*2063, metE70, pyrE40, gltS15	Kadner, R.J. (22)
RK4988	*ara*D139, (Δ*arg*F-*lac*)U169, *rel*A1, *rps*L150, *thi*, *gyr*A219, *non*, Δ*uhp*2089, *met*E70, *pyr*E40, *glt*S15	Kadner, R.J. (22)
RK4986	*ara*D139, (Δ*arg*F-*lac*)U169, *rel*A1, *rps*L150, *thi*, *gyr*A219, *non*, Δ*uhp*2066, *met*E70, *pyr*E40, *glt*S15	Kadner, R.J. (22)
RE213	Hfr(P02), *met* B1, *pyr*E41, *uhp*-40, *glt*C^{c}14, *tna*-6, *rel*A1, *ton*A2, T_2^r, *srl*C300::Tn10, *rec*A56	RE74 transformed to Tc with P1 grown on JC10240

TABLE II

BACTERIOPHAGES USED IN STRAIN CONSTRUCTIONS

Phages	Genotype	Origin
P1 Mortimer		M.C. Jones-
P1 cml clr 100		J.J. Rosner (52)
λ370	b221, cI857, cI171::Tn10, $O_{UGA}261$	N. Kleckner (39)
λ467	b221, rex::Tn5, cI857 $O_{am}29$, P80	N. Kleckner (39)
λ561	b2 b522, cI857::Tn9, $O_{am}29$	N. Kleckner (39)

(36). A list of recombinant plasmids constructed for this study is given in Table III.

The Clarke & Carbon library of plasmids of the *E. coli* genome were screened for *uhp* genes as reflected by the ability to confer *Uhp*$^+$ phenotype on strains that were *Uhp*$^-$ (24). Five such plasmids out of this library were selected for further analysis.

The 1.5 kb Pst I-Hind III fragment from *pLC35-4* was cloned in *pUC9* cut with the same restriction enzymes. The resulting 4.0 kb plasmid is *pRE8*. The 3.2 kb Hpa I fragment from *pLC40-33* was cloned in *pUC9* cut with Sma I. *pRE41* is the resulting 5.8 kb plasmid. The 5.3 kb Pst I-Hind III fragment from *pLC34-43* was cloned into *pUC9* cut with the same enzymes. The resulting 8.0 kb plasmid is *pRE77*. The 7.0 kb Pst I-Hind III fragment from *pLC17-47* was cloned in Pst I-Hind III sites of *pUC8*. *pRE85* is the resulting 9.6 kb plasmid. All the recombinant plasmids were first selected for their ability to confer ampicillin resistance on host strains when transformed into them; secondly for their inability to complement defective β-galactosidase in the host cells as indicated by the failure to hydrolyze a chromogenic substrate, namely X-gal; and thirdly, but most importantly, for their ability to confer *Uhp*$^+$ phenotype on strains that were *Uhp*$^-$. A specific restriction fragment that carried *uhp* genes was sometimes purified from low melting agarose after electrophoresis and used further. The size of the recombinant plasmids that were able to confer *Uhp*$^+$ was quantitated by agarose gel electrophoresis. All the recombinant plasmids were able to confer *Uhp*$^+$ on RE234, a *uhp*-40 mutation.

The recombinant plasmid *pRE41* was mapped using restriction enzymes Hinf I, Rsa I, Bgl II, and Sma I. Aliquots of the plasmid were

TABLE III

RESTRICTION FRAGMENTS, *uhp* GENES CONTAINED IN THEM AND RECOMBINANT PLASMIDS

Recombinant Plasmids	*uhp* Genes Carried by the Recombinant Plasmids	Restriction Fragments Containing the *uhp* Genes
pRE8	*uhp*A	1.5 kb Pst I-Hind III fragment of *pLC35-4*
pRE41	*uhp*T & C	3.0 kb Hpa I fragment of *pLC40-33*
pRE77	*uhp*B & A	5.0 kb Pst I-Hind III fragment of *pLC34-43*
pRE85	*uhp*A, B, C & T	7.0 kb Pst I-Hind III fragment of *pLC17-47*
pRE12	Δ*uhp*A at Hind III end of *pRE8*	
pRE38	*uhp*A::Tn5	
pRE39	*uhp*A::Tn9	
pRE42	*uhp*T^{am}	*pRE41* treated with hydroxylamine, *Uhp*$^-$ in Δ*uhp*T
pRE46	*uhp*R^{am}	*pRE41* treated with hydroxylamine, *Uhp*$^+$ in Δ*uhp*T and *Uhp*$^-$ in
Δ*uhp*TR		
pRE49	Δ*uhp* at BamH I end of *pRE41*	*Uhp*$^-$ deletion
pRE50	Δ*uhp* at EcoR I end of *pRE41*	*Uhp*$^-$ deletion
pRE53	Δ*uhp* at BamH I end of *pRE41*	*Uhp*$^+$ deletion
pRE62	Δ*uhp* at EcoR I end of *pRE41*	*Uhp*$^+$ deletion

incubated with the appropriate restriction enzymes and the fragments were separated on agarose gels by electrophoresis. Suitable single and double digests with the restriction enzymes helped to orient restriction sites with respect to each other.

Construction of Mutants in *uhp* Genes Carried by the Recombinant Plasmids

Mutations made in the recombinant plasmids were either point mutations (37) or deletions (38) or transposon insertions (39). Point mutations were made by incubating aliquots of the plasmids with hydroxylamine for 0-48 hrs at 37°C, then transformed into suitable strains selecting for ampicillin resistance and the loss of ability to confer Uhp^+ on strains that were Uhp^-. Plasmid samples that failed to confer Uhp^+ on strains that were Uhp^- and Sup^+ (RE213), but were able to confer Uhp^+ on strains that were Uhp^- and Sup^- (RE234) were assumed to carry nonsense mutations in the *uhp* gene carried by such a recombinant plasmid.

Deletions in *uhp* genes carried by the recombinant plasmids were isolated by two different methods (38). Aliquots of the plasmid DNA were digested with DNase1 under conditions favoring the formation of single-cut linearized DNA. The plasmid was further treated with either BamH I or EcoR I to generate deletions between these restriction sites and the site at which DNase1 cut. Larger pieces of DNA were precipitated with polyethylene glycol, blunt-ended with Klenow polymerase and self-ligated. Aliquots of the ligated DNA were used to transform appropriate strains. Both Uhp^+ and Uhp^- transformants were isolated. Plasmids

isolated from individual transformants were checked for the insert size in agarose gels.

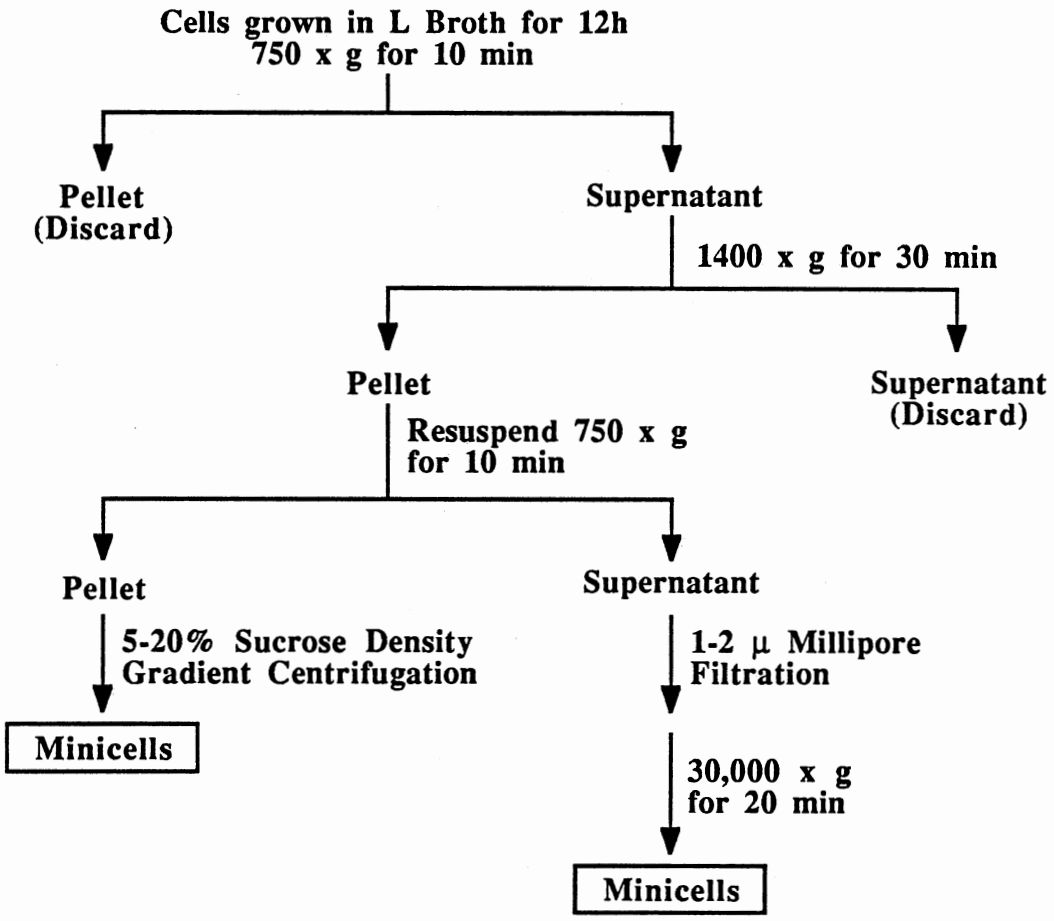
Deletions in the plasmid were also made by cutting separate portions of the plasmid with Pst I and Hind III, then digesting with Bal-31 to remove nucleotides around the cloning sites. These mixtures were ligated back together in the presence of Hind III or Pst I linkers and transformed into a strain with uhp-40 lesion. Both *Uhp*$^+$ and *Uhp*$^-$ transformants were isolated.

Transposon insertions inactivating the *uhpA* gene carried by *pRE8* were selected as plasmids transferring drug resistance but failing to confer *Uhp*$^+$ phenotype on *Uhp*$^-$ strains (39). Plasmid carrying strains were grown to a cell density of 2-3 x 10^{8}/ml, infected with λ 467 (*b221 cI857 rex::Tn5 Oam29 P80*) or λ 577 (*b2 b522 cI857 Tn9 Oam 29*) (10^{10} phages/ml), incubated at 37°C for 20 min and plated out on LB medium plates containing kanamycin or chloramphenicol. Colonies growing in these plates were scraped off into liquid broth. Plasmids isolated from these pools were used for transforming RE234, a strain with the *uhp*-40 lesion, selecting for ampicillin^r and kanamycin^r or chloramphenicol^r and failure to grow on glucose 6-phosphate as sole carbon source.

Expression of Plasmids Carrying *uhp* Genes

Escherichia coli minicell was the expression system of choice. Minicells were isolated by 1.2μ filtration to separate parent cells from minicells (40) or 5-20% sucrose density gradient centrifugations (41). Modifications in the above procedures were made as shown in Scheme 1, so that clean minicells in sufficient numbers could be obtained consistently. Minicells thus isolated were used immediately or stored at

Scheme I. Isolation of Minicells



-20°C. The protein synthesizing capability of such frozen minicells was not affected to any appreciable extent even after six months.

Minicells were suspended to 250 μg protein/ml in 1.0 ml of medium A containing 10 mM glucose or 7.5 mM glucose 6-phosphate, 1.0 mM cAMP, 100 μg/ml of ampicillin and incubated at 37°C for 20 mins. 10 μCi/ml of ^{3}H amino acid mixture was added and incubation continued for 2 hrs. Minicells were pelleted by centrifuging at 30,000xg for 20 mins and washed twice with 0.2M tris-hydrochloride, pH 8.0 or sodium phosphate buffer pH 7.0. Such labelled minicells were either solubilized in sample dissolving buffer (0.5M tris HCl pH 6.8, 1% SDS, 1% β-ME) by heating at 100°C for 5 mins, or fractionated into membrane and soluble fractions following sonication in a cell disintegrator or freeze-thaw cycles in dry ice acetone bath, followed by centrifugation at 100,000xg for 60 mins. The membrane fractions were further separated into inner and outer membranes following solubilization of inner membrane with 0.5% sodium lauryl sarcosinate at 30°C for 30 mins and centrifuging at 100, 000xg for 60 mins (42).

Proteins synthesized in *E. coli* minicells carrying plasmids were analyzed on one dimensional SDS-polyacrylamide gradient gels (10-20%) (43) or two dimensional gels of O'Farrell (44) with modifications for membrane proteins by Ames & Nikaido (45). The first dimension isoelectric focusing gels were tube gels which were loaded on 15% polyacrylamide second dimension gels. Gels were fixed by immersing in water-isopropanol-acetic acid (65:25:10) for an hour. The gels were then dehydrated using dimethylsulfoxide and finally impregnated with PPO (46) and dried. The dried gel was autoradiographed for the required length of

time. A modified Lowry protein assay (47) was used for quantitating membrane proteins.

CHAPTER III

RESULTS

Location of *uhp* Genes in the Plasmids of the Clarke & Carbon Collection

The Clarke and Carbon plasmid library of *Escherichia coli* genome was screened for *uhp* genes and five plasmids, namely *pLC17-47, pLC40-33, pLC14-9, pLC34-43, and pLC35-4,* were found that were able to confer *Uhp*$^+$ on *Uhp*$^-$ mutants of *E. coli.* When these plasmids were tested for their ability to correct Δ*gltS-uhp-223,* only *pLC17-47, pLC40-33,* and *pLC14-9* conferred *Uhp*$^+$, whereas the others did not. It was concluded that *pLC35-4* and *pLC34-43* lacked a portion of the *uhp* region closer to the *gltS,* which was described as the *uhpTR* region by Kadner & Shattuck-Eidens (22). The five plasmids were classified into three groups: *uhpTR (pLC40-33), uhp A (pLC35-4 & pLC34-43) and uhpTRA (pLC17-47 & pLC14-9).* Restriction maps were also constructed for these plasmids as shown in Figure 1 (Essenberg, R. C, unpublished observations).

Subcloning of *uhp* Genes

Three specific problems were encountered when the expression of *pLC* plasmids carrying *uhp* genes was sought. 1. The size of the *pLC* plasmids were of the order 25-30 kb, carrying not only the *uhp* genes but also some other genes. This complicated the protein patterns on SDS-

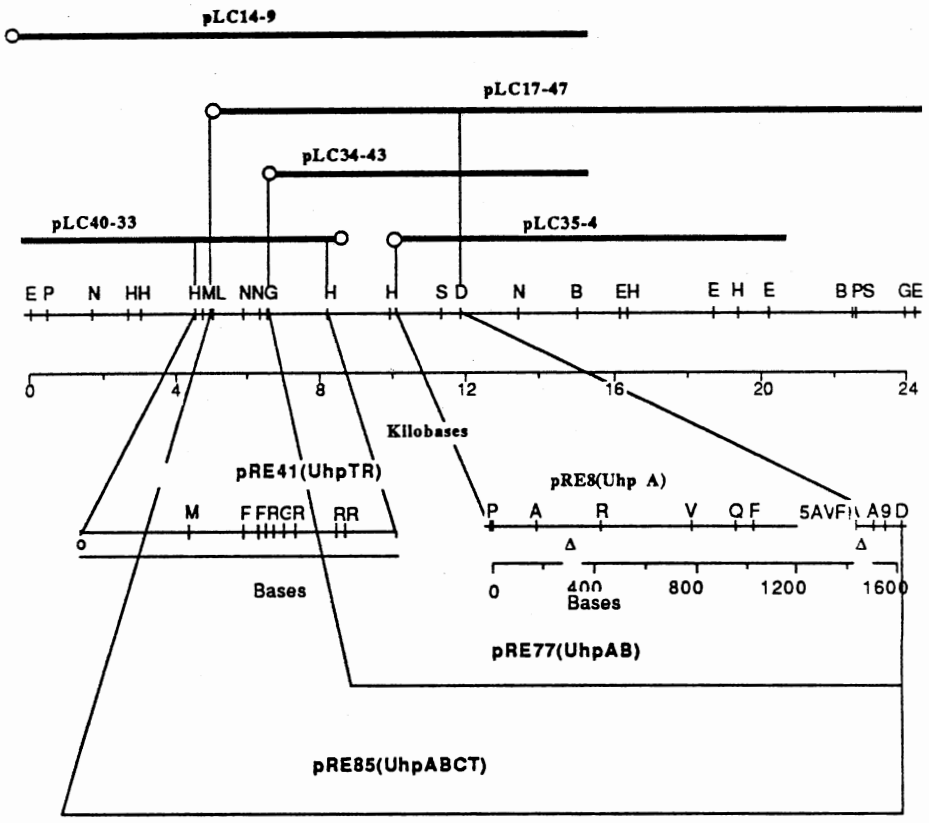


Figure 1. **Restriction map of Clarke and Carbon plasmids showing *uhp* genes on specific restriction fragments. Restriction sites are indicated as A. Hae III; B. BamH I; D. Hind III; E. EcoR I; F. Hinf I; G. Bgl II; H. Hpa I; M. Sma I; N. Hinc II; P. Pst I; Q. Taq I; R. Rsa I; S. Sst I. Δ indicates deletion end points. Numbers 5 & 9 indicate location of transposon 5 & 9.**

polyacrylamide gels in that there were too many bands that could be thought of as likely candidates for *uhp* gene products. 2. The *pLC* plasmids were of low copy number that could not be sufficiently amplified for expression. 3. Selection for the presence of *pLC* plasmids had to be based on the marginally effective method of selection for colicin resistance. These problems necessitated that the *uhp* genes be subcloned in multicopy plasmids which could be selected readily.

Multicopy plasmids *pUC9* or *pUC8* (Fig. 2) were the vectors of choice for subcloning (53). These plasmids are derived from *pBR322*, and contain the *lac* promoter-operator sequences, part of *lacZ*, an origin of replication, several unique restriction sites and a gene coding for ampicillin resistance. These plasmids in a transformed cell can be selected for their ability to confer ampicillin resistance. Initial selection for the cloned genes is based on the lack of complementation of a defective β-galactosidase in the host as reflected by the failure to hydrolyze X-gal, a chromogenic substrate. Table III shows the restriction fragments, the genes contained within and the recombinant plasmids derived thereof.

Complementation Properties of the Cloned *uhp* Genes

Table IV summarizes the results of complementation studies. All the *pRE* plasmids carrying different *uhp* genes were able to complement *uhp*-40 (a chromosomal *Uhp*$^-$ point mutation thought to be in *uhp*A or the end of *uhp*B), i.e. confer *Uhp*$^+$ on strains carrying *uhp*-40. This result is similar to Essenberg's observations with *pLC* plasmids. This kind of behavior is expected of all the *pRE* plasmids except *pRE41* which carries only *uhpT & C* and hence is unable to complement a mutation in *uhpA*.

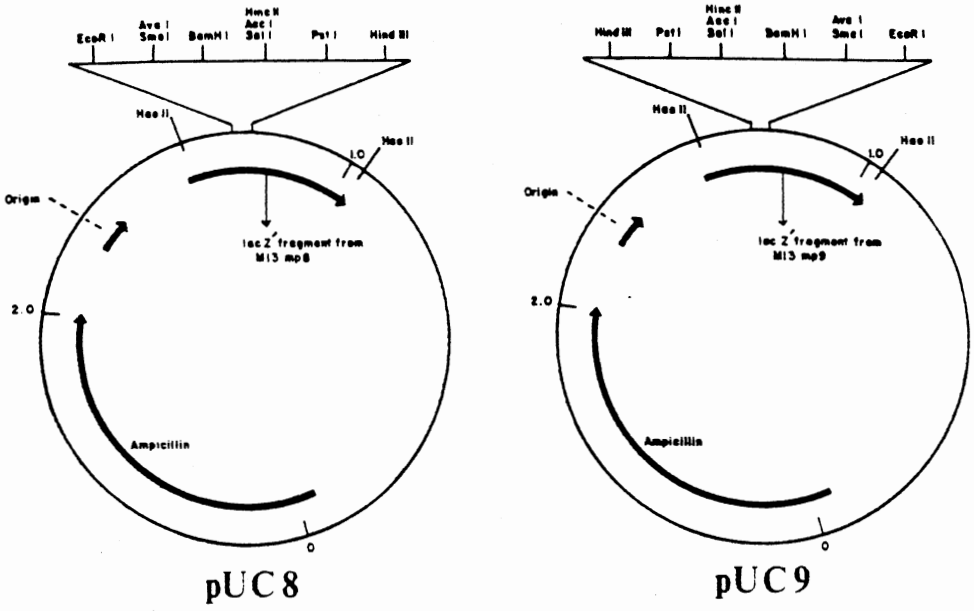


Figure 2. Diagramatic representation of pUC8 and pUC9.

TABLE IV

COMPLEMENTATION PATTERNS OF *uhp* PLASMIDS

E. coli K12 Strains	*pRE8*	*pRE41*	*pRE77*	*pRE85*
RE322 (ΔT)	-	+	+	+
RE324 (ΔTR)	-	+	-	+
RE325 (ΔRA)	-	-	-	+
RE326 (ΔA)	-	+	-	+
RE327 (ΔTRA)	-	-	-	+
RE234 (*uhp*-40)	+	+*	+	+

**pRE41* was not expected to complement *uhp*-40.

pLC40-33, the parent plasmid of *pRE41*, also shows similar complementation behavior with respect to *uhp*-40. The positive complementation of *uhp*-40 by *pRE41* may be attributed to the copy number. It may be that the presence of multiple copies of *uhpT* overcomes the need for the presence of *uhpA*. However, in a strain with pRE41 there are also multiple copies of *uhpC*, which is considered a repressor of *uhp* expression. Two kinds of *uhp* transformants were obtained when *pRE41* was used to transform RE234, namely *Uhp*$^+$ and *Uhp*$^-$. Plasmids from the *Uhp*$^+$ transformants, when retransformed into the same strain gave rise to *Uhp*$^+$ transformants. The *uhp*-40 mutation in RE234 may be leaky or the strain carrying *pRE41* may be able to undergo some mutational event that it is able to overcome the effect of *uhpC*. The behavior of *pRE85* in RE234 was similar to that observed with *pRE41* in that both *Uhp*$^+$ and *Uhp*$^-$ transformants were obtained. However, initial transformation of this plasmid did not yield *Uhp*$^+$ transformants in this strain. Plasmids extracted from these clones had the expected 7.0 kb Pst I-Hind III fragment, when digests of these were run on agarose gels, in addition to a smaller fragment of unknown origin. This plasmid when transformed into RE323 (*rec A* derivative of strain RK4983) and RE437 (genetic background similar to RE74 with the addition of *min B*) complemented the Δ*uhp* in the former and failed to do so the *uhp*-40 mutation in the latter. However, plasmids extracted from RE323 *Uhp*$^+$ transformants when retransformed into RE234 yielded both *Uhp*$^+$ and *Uhp*$^-$ clones. The complementation patterns of *pRE* plasmids when transformed into strains with different chromosomal *uhp* deletions were as expected, except *pRE8* and *pRE77* did not complement Δ*uhpA*. *pRE41* complemented this strain. *pRE77* also failed to complement Δ*uhpRA*.

Mutations in the Cloned *uhp* Genes

Deletion mutations as well as transposon insertion mutations were constructed in *uhpA* carried by *pRE8*. All these mutations failed to complement *uhp*-40. The deletion end points and the transposon insertions were mapped within the *uhpA* gene as shown in Figure 1. Two kinds of mutations were made in the *uhp* genes carried by *pRE41*, namely deletions and nonsense mutations. The nonsense mutations were first selected in a Δ*uhp*T strain. Both *Uhp*$^+$ and *Uhp*$^-$ transformants were obtained. Plasmids from the *Uhp*$^+$ transformants were extracted and retransformed into a Δ*uhp*TC strain. Those plasmids that failed to confer *Uhp*$^+$ in this strain were thought to have a mutation in *uhp*C. A similar strategy was used for the deletion mutants. After making the deletions the plasmids cut with suitable restriction enzymes were run on agarose gels for size determination. Those that showed fragments smaller than the 3.0 kb *uhp* fragment in *pRE41* were used for transformations. *Uhp*$^-$ deletions coming from the BamH I and EcoR I ends are likely to be in *uhp*C and *uhp*T, respectively. Deletions from either end that still conferred *Uhp*$^+$ were also isolated.

Expression of pRE Plasmids in *E. coli* Minicells

There are three possible expression systems for cloned genes in *E. coli*. They are: 1. Maxicells, 2. In vitro transcription-translation and 3. Minicells. Maxicells are prepared by uv irradiation of a particularly sensitive strain of *E. coli* in an attempt to damage the cellular DNA. Any plasmid carried by the strain is unaffected simply because the target is much smaller than the chromosomal DNA. Proteins synthesized in such cells are predominantly coded by the genes carried by the plasmid.

Though the method appears to be simple enough, it takes a lot of effort to get it to work satisfactorily. In vitro transcription-translation system employs a suitable template DNA for expression. The composition of such systems is complex which means that there are at least as many possibilities for error as there are components. *E. coli* cells normally divide in the center to make daughter cells. *Min B* is a mutation that causes these cells to divide at the poles as well as in the center. Minicells are the result of such polar division and are devoid of any chromosomal DNA. Plasmid-bearing min B strains make minicells that carry only the plasmid DNA. Minicells can be isolated from a culture of *E. coli* and used as an expression system for cloned genes. This is as reliable a method as any for the expression of plasmid coded genes and was the method of choice. The trick was to get a relatively pure preparation of minicells as assessed by microscopic observation, since even a very small number of whole cells led to a large number of labelled proteins as seen in SDS-PAGE. Once pure minicells were obtained, this expression system yielded clear cut protein patterns.

Minicells carrying plasmids were labelled with ^{3}H amino acid mixture and the proteins synthesized were resolved in SDS-polyacrylamide gels. Two dimensional gels were also run on these mixtures to obtain better resolution. Tritium-labelled minicells fractionated into soluble and membrane (inner & outer) fractions and their protein patterns helped assign the *uhp* proteins to different cellular locations. β-lactamase, a known extracellular enzyme synthesized by the *pUC* derived plasmids, occurred in the membrane fraction which acted as an internal standard for this separation.

pRE8 codes for a 18.0 kdal membrane protein in addition to β-lactamase as seen in Figure 3 and Figure 4. This protein is specifically lacking in deletion (*pRE12*) and transposon insertion (*pRE38, uhp*A::Tn5 & *pRE39, uhp*A::Tn9) mutants as seen in Figures 3 & 5.

pRE41 codes for a 32.0 kdal membrane protein and a 23.0 kdal protein along with β-lactamase as seen in Figure 6 and Figure 7. The 23.0 kdal protein did not appear as a distinct band on SDS-PAGE. This protein had an unusual isoelectric point of 4.70. Since this plasmid is able to complement Δ*uhp*T as well as Δ*uhp*TC, it is likely to carry both these genes. To resolve the question of which protein is which, SDS-PAGE was run on deletion and nonsense mutants of *pRE41* and is shown in Figure 8. In this experiment, the host strain was RE480, a *recA minB* derivative of RK4981. *pRE49* deleted for *uhp*C lacks the 23.0 kdal protein, but has the 32.0 kdal protein. This result indicates that the 23.0 kdal protein is probably *uhp*C. Both *pRE53* and *pRE62* (*Uhp*$^+$ deletions, Table III) shows *uhp*T. It seems that *uhp* C is lacking in *pRE62* though the deletion in this case comes from the *uhp*T end. The location of these proteins in this particular and numerous other experiments were complicated by the inability to obtain clean minicell preparations. As seen in Figure 8 there are quite a number of extraneous proteins that are probably not coded by the plasmid since many of these are similar to the ones seen in minicells with no plasmid (Lane 1, Figure 8).

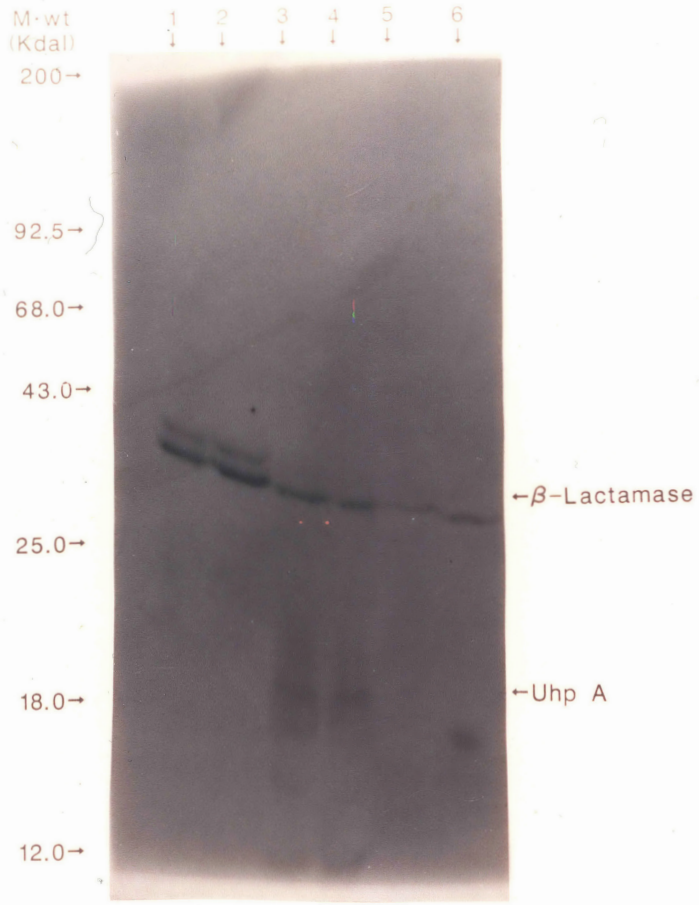


Figure 3. Proteins synthesized in *E. coli* minicells with *pUC9* and *pRE8*. Lanes 1 & 2 *pUC9*; 3 & 4 *pRE8*; 5 & 6 *pRE12*.

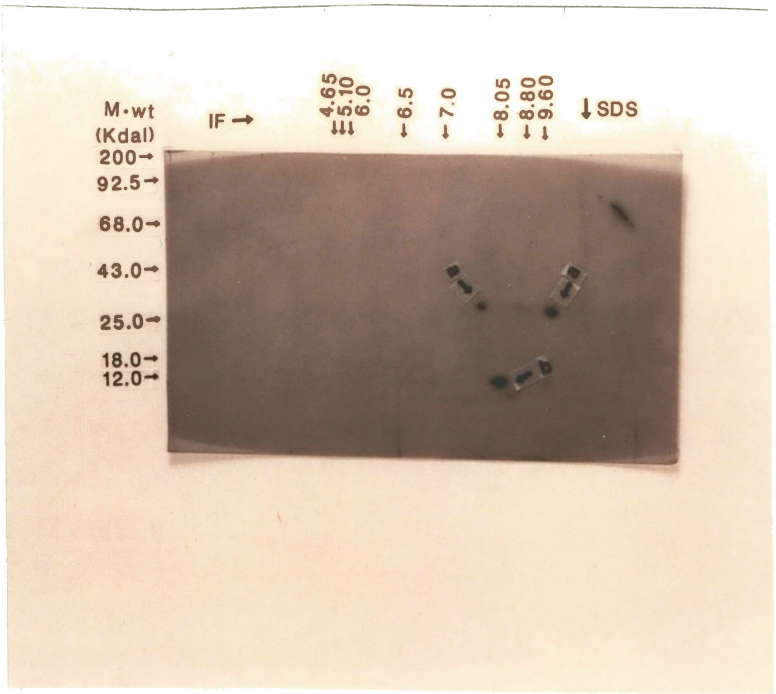


Figure 4. 2-D gel pattern of proteins synthesized in *E. coli* minicells with *pRE8*. a. β-lactamase; b. *uhp*A.

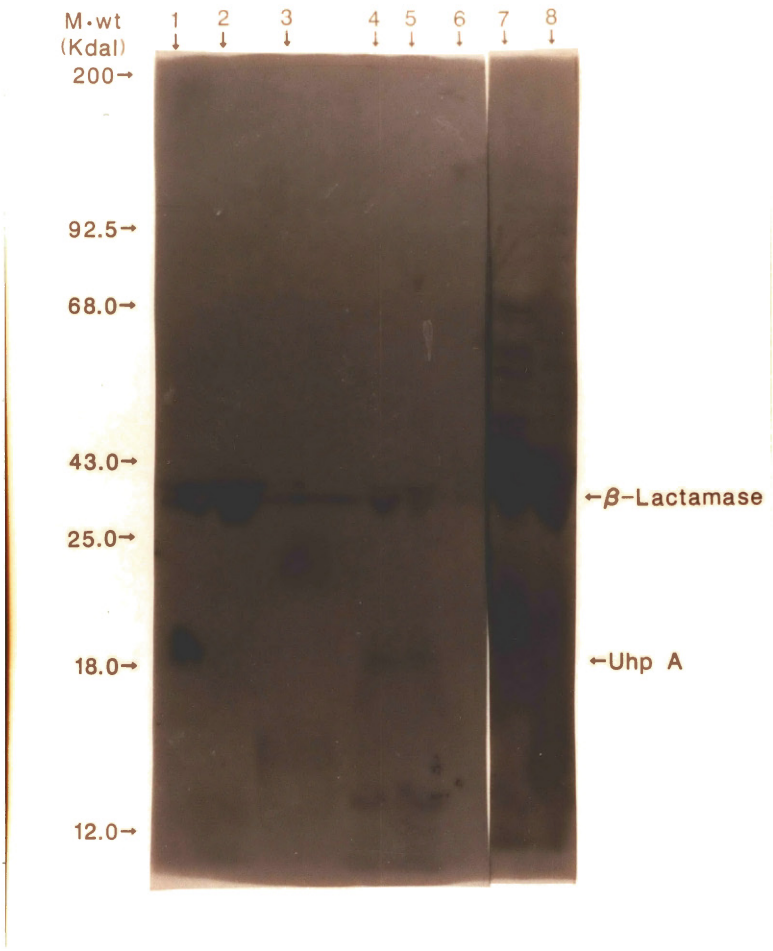


Figure 5. Proteins synthesized in *E. coli* minicells with *pUC9*, *pRE8*, *pRE38* and *pRE39*. Lanes 1, 2, & 3 *pUC9* total, membrane and soluble fractions, respectively; Lanes 4, 5 & 6 *pRE8* total, membrane and soluble fractions; Lane 7 *pRE38* (*uhp*A::Tn5); Lane 8 *pRE39* (*uhp*A::Tn9); Lanes 7 & 8 were longer exposure of the same gel.

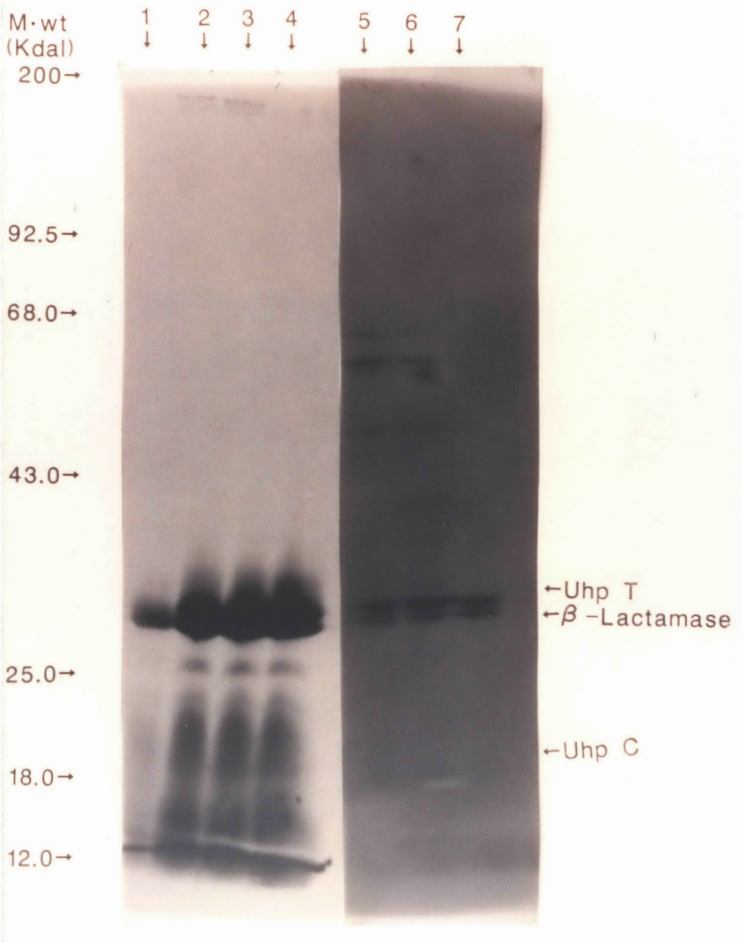


Figure 6. Proteins synthesized in *E. coli* minicells with *pUC9* and *pRE41*. Lanes 1 & 7 *pUC9*; Lanes 2, 3 and 4 *pRE41* (total); Lane 5. *pRE41* (membrane); Lane 6. *pRE41* (soluble).

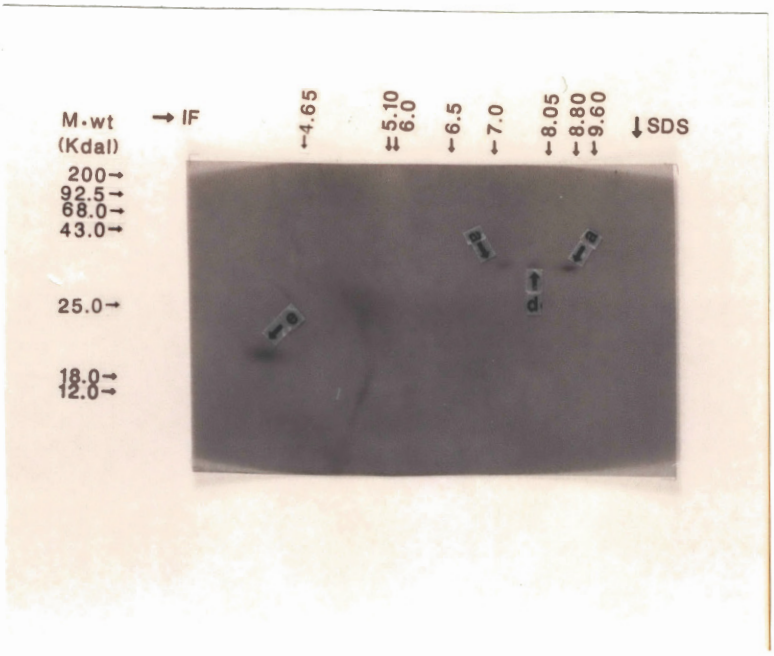


Figure 7. 2-D gel pattern of proteins synthesized in *E. coli* minicells with *pRE41*. a. β-lactamase; d. *uhp*T, e. *uhp*C.

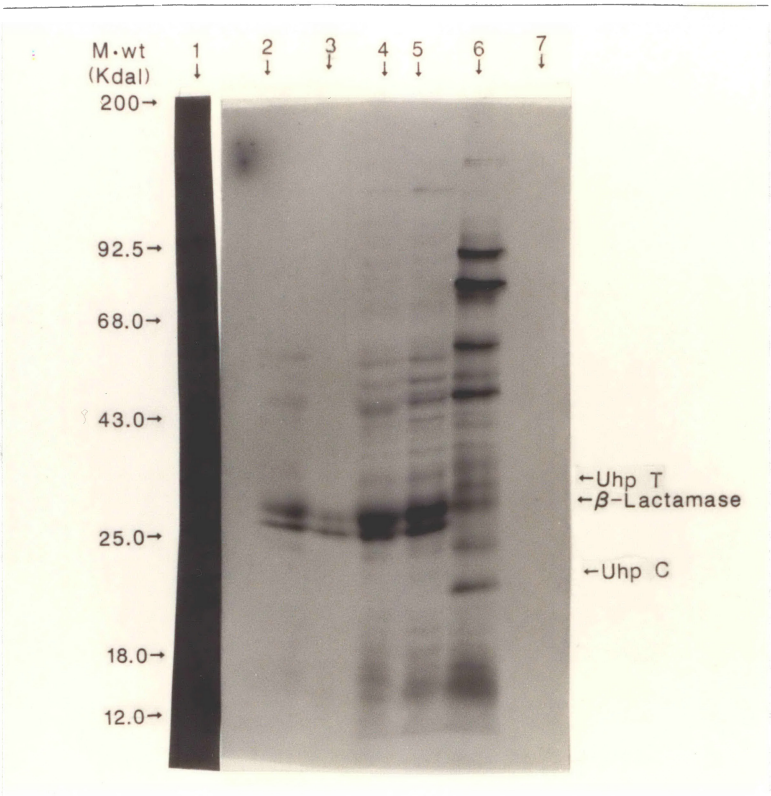


Figure 8. **Proteins synthesized in *E. coli* minicells with *pRE41* and mutants. Lane 1. No plasmid; 2. *pRE62*; 3. *pRE46*; 4. *pRE53*; 5. *pRE49*; 6. *pRE41*; 7. *pUC9*.**

pRE77 **codes for a 43.0 kdal protein and a 18.0 kdal membrane protein in addition to β-lactamase as shown in Figure 9 and Figure 10. The 18.0 kdal protein is the same as *uhpA*. The 43.0 kdal protein is probably *uhpB*. The position of this protein relative to β-lactamase is different in SDS-PAGE and 2-D gels. Proteolysis of the labelled proteins before solubilization could be a possible cause for this difference. SDS-PAGE of proteins synthesized by *pRE77* shows a protein at a position similar to that seen in 2-D gel in addition to the 43.0 kdal protein. The labelled samples used for these two were prepared on different occasions. The position of all the proteins relative to the molecular weight standards were quite different in SDS-PAGE and 2-D gels. 2 D-gel pattern of proteins synthesized from pRE85 is shown in Figure 11. In addition to β-lactamase and *uhp*A, a protein of m.wt. 43 kdal is seen which could be thought of as *uhp*B. The pI of this protein is 9.1. The presence of *uhp*T & C is not immediately apparent from this Figure. Table V summarizes the molecular weights, isoelectric points and cellular locations of the different *uhp* proteins.**

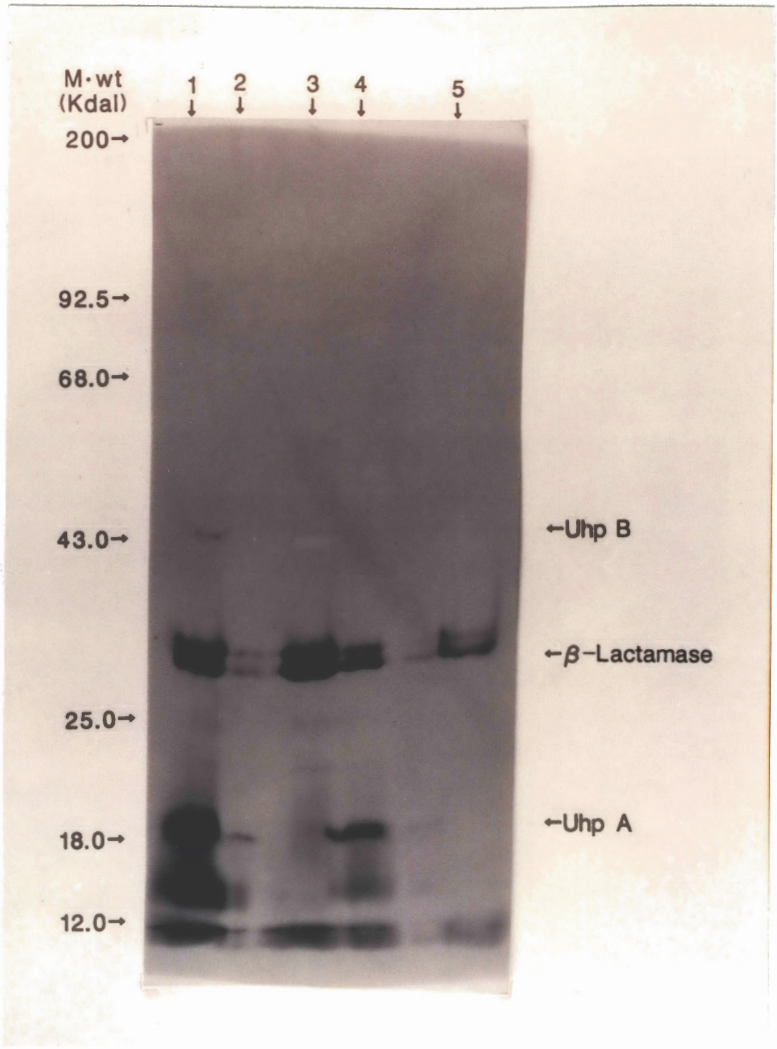


Figure 9. Proteins synthesized in *E. coli* minicells with *pUC9* and *pRE77*. Lane 1. *pRE77* total; 2. *pRE77* soluble; 3. *pRE77* membrane; 4. *pRE8*; 5. *pUC9*.

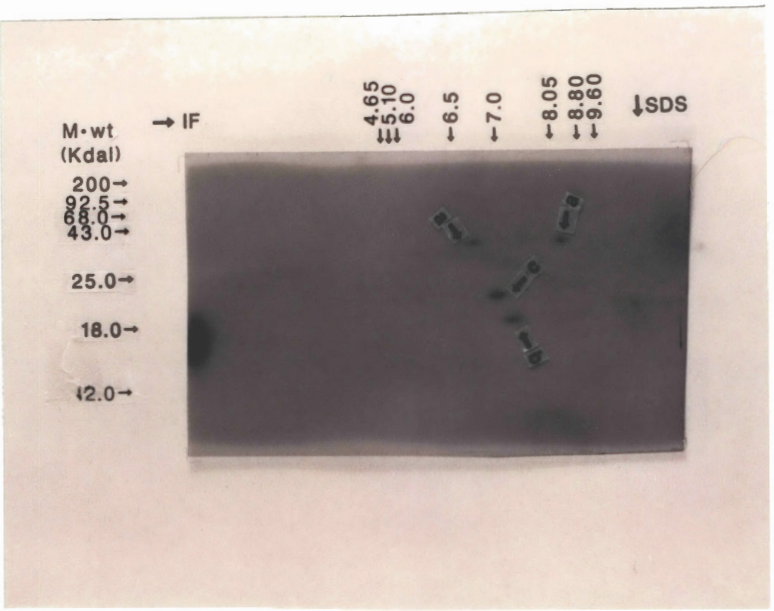


Figure 10. 2-D gel pattern of proteins synthesized in *E. coli* minicells with *pRE77*. a. β-lactamase; b. *uhp*A; c. *uhp*B.

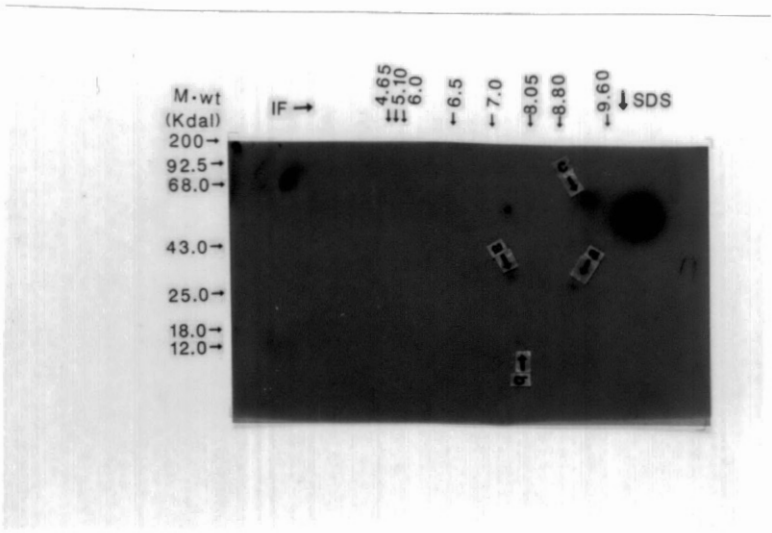


Figure 11. 2-D gel pattern of proteins synthesized in *E. coli* minicells with *pRE85*. a. β-lactamase; b.*uhp*A; c. *uhp*B.

TABLE V

uhp PROTEINS, MOLECULAR WEIGHT, pI AND CELLULAR LOCATIONS

uhp Proteins	M. Wt.	pI	Cellular Locations
*uhp*A	18	8.25	membrane
*uhp*T	32	8.5	membrane
*uhp*B	43	8.15	membrane (?)
*uhp*C	23	4.7	soluble (?)

CHAPTER IV

DISCUSSION

Four recombinant plasmids each carrying different portions of the *uhp* region were constructed, the expression of which led to the identification of four *uhp* gene products. *UhpT and A* were found to be membrane proteins. *UhpB* is probably a membrane protein. Taking into consideration 1. exogenous induction shown by the *uhp* system, 2. involvement of both positive and negative control elements in the regulation of *uhp* expression as described by Kadner, the simplest model for regulation that can be proposed consists of a membrane bound element capable of sensing external inducer, which then conveys the information to another membrane bound component, that may be freed to bind to the repressor, thus effecting the transcription of the transporter itself. According to this model *uhpB* can be assigned the role of the transmembrane protein capable of sensing external inducer, *uhpA* the membrane bound mediator that conveys the induction signal to the intracellular milieu, *uhpC,* the repressor and *uhpT*, the transporter. It is likely that *uhpA* is complexed with *uhpB* under noninducing conditions. The binding of the inducer to *uhpB* may release *uhpA*, which now is free to bind to *uhpC,* thus activating the transcription of *uhpT*. The implication here is that *uhpC* is directly bound to the *uhpT* promoter preventing its transcription. However *uhpC* has an unusual pI of 4.70 which is suggestive of a model where it could be complexed either to *uhpB* or *A* or

both in the membrane. This model would explain the observations of Kadner (21, 22) and some of our own. 1. That mutation in *uhpA* will lead to the *Uhp*$^-$ phenotype, that is not easily reversed except by second site mutations in *uhpC*. 2. Mutations in *uhpC* may lead to the *Uhp*^{c} or *Uhp*$^-$ phenotype, though it is a little hard to explain the occurrence of the latter type of mutation. 3. Mutations in *uhpB* may lead to the *Uhp*$^-$ phenotype, that can be reversed by second site mutations in *uhpC*. 4. That mutiple copies of *uhpA* lead to constitutive expression can be explained as due to complete titration of *uhpC*, and that *uhpA* may be able to directly bind to the *uhpT* promoter thus activating its expression. 5. Mutations that confer constitutivity may also be occurring in the *uhpT* promoter such that it is no longer sensitive to the repressor. Uptake negative mutations that cannot be reversed can also occur in *uhpT*, though it might be possible for the membrane-bound sensor to act as the transporter since the inducer binding site and substrate binding site must be similar. 6. This model would call for *uhpA* to be able to be membrane-bound as well as membrane-unbound. Though the author has always found *uhpA* to be associated with the membrane fraction except on one occasion, Kadner has found it to be present in both membrane and soluble fractions, a higher proportion in soluble fraction.

The molecular weights of the different uhp proteins found in this study (Table V) are different from those obtained by Weston & Kadner. 25, 48, 20 and 38 kdals are the molecular weights of *uhpA, B, C* and *T* respectively quoted in the above mentioned reference. The sequence data obtained by Friedrich and Kadner (27) shows that *uhpB* has the characteristics of a transmembrane protein. *uhpA* sequence has regions homologous to the DNA binding regions of activators and repressors.

UhpT & C share substantial sequence homology. Their model for regulation implies that *uhpC* is in the membrane along with *B* and *A*. It is interesting that the system is regulated by both an activator and a repressor and that the induction process is more specific than the transport itself. The nature and mode of communication of the induction signal between the sensor and mediator is another intriguing problem.

As the author sees it the future of this project is in 1. Constructing suitable mutations in the cloned *uhp* genes and testing them in strains with different *uhp* backgrounds, so that a plausible function can be assigned to each of the four *uhp* genes. One likely problem with this approach may arise due to the effect of copy number of a particular cloned *uhp* gene on *uhp* expression, since stoichiometry of different *uhp* proteins may be exacting, that can be overcome with recloning in low copy number plasmids or transferring the genes to the chromosome. 2. Isolating the different *uhp* proteins and reconstituting them into a functional in vitro system can be seen as another major direction of exploration. One might then be able to observe in such a synthetic system changes occurring on binding the inducer, how the signal is conveyed to the mediator, how transport is effected, the stoichiometric relationships between the different regulatory elements, the source of energy and its coupling to the the transport, all of which depends upon the availability of suitable analytical methods.

The processes occurring at the biological membranes are essential to keep the cell informed about happenings elsewhere, be it the immediate environment as in the case of microbes, or in another cellular location as in the case of multicellular organisms. It is thus important to define the physical nature of biological membranes and elucidate the mechanisms of

membrane processes, so that basic themes can be found enabling towards a better understanding of not only the processes occurring at the membranes but also the overall functioning of a cell.

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