Defining Topologically Oriented Transmembrane Helix Contacts in Integral Membrane Protein EmrE by a Modified Two-Hybrid Approach

Burt, Jason, Campbell

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Defining Topologically Oriented Transmembrane Helix Contacts in Integral Membrane Protein EmrE by a Modified Two-Hybrid Approach

by

Jason C. Burt

A THESIS

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Study of integral membrane proteins is comparatively difficult in comparison to soluble proteins, requiring more specialized techniques. Here is presented an application of the Bacterial Adenylate Cyclase Two-Hybrid (BACTH) method to study oligomerization in membrane proteins applied to model integral membrane multidrug transporter EmrE. The system was unable to observe dimerization between full length monomers of EmrE, likely due to the preferential antiparallel nature of EmrE dimers. Further studies required large numbers of samples, so the Miller assay was adapted to work in a small volume microtitre plate format. Using this new higher throughput assay, constructs of individual EmrE transmembrane helices (TMH) were utilized to perform contact mapping of EmrE dimer structure. The results were compiled to construct a transmembrane helix interaction structure. This structure obtained is novel and identifies inconsistencies from previous studies.
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LIST OF ABBREVIATIONS

ATP – Adenosine triphosphate
BACTH – Bacterial adenylate cyclase two-hybrid
BSA – Bovine serum albumin
CAP – Catabolite activating protein
cAMP – Cyclic adenosine monophosphate
Cryo-EM – Cryo electron microscopy
CyaA/cyaA – Protein/gene nomenclature for adenylate cyclase
ddH₂O – Double distilled water
DDM – Dodecyl maltoside
DMSO – Dimethyl sulfoxide

E.coli – *Escherichia coli*

EmrE – Ethidium multidrug resistance E
EPR – Electron paramagnetic resonance
FRET – Förster resonance energy transfer
FTIR – Fourier transform infrared spectroscopy
GG₇ – An amino acid motif characterized by two glycine residues seven residues apart
Hsmr – *Halobacterium salinarum* small multidrug resistance an EmrE homolog

HRP – Horseradish peroxidase
HSQC – Heteronuclear single quantum coherence spectroscopy
IMP – Integral membrane protein
IPTG – Isopropyl β-D-1-thiogalactopyranoside

Kₐ – Dissociation constant

LB – Lysogeny broth
MalE/male – Protein/gene name for maltose binding protein

MBP – Maltose binding protein

NMR – Nuclear magnetic resonance

OD – Optical density, subscript indicates at a particular wavelength

ONPG – Ortho-nitrophenyl-β-galactoside

ONP – Ortho-nitrophenyl anion, chromogenic at 420nm

PAGE – Polyacrylamide gel electrophoresis

PCR – Polymerase chain reaction

PFO – perfluorooctanoic acid

QCC – Quaternary cationic compounds

RbCl₂ – Rubidium chloride

SDS – Sodium dodecyl sulfate

SMR – Small multidrug resistance

TBS – Tris buffered saline buffer

TBST – Tris buffered saline with Tween-20 buffer

TMH – Transmembrane helix/helices

TPP⁺ – Tetraphenylphosphonium

TROSY – Transverse relaxation-optimized spectroscopy

T18 – The adenylate cyclase catalytic domain which is 18kDa after limited proteolysis

T25 – The adenylate cyclase catalytic domain which is 25kDa after limited proteolysis

Zip – Leucine zipper motif from yeast protein GCN4
CHAPTER 1: INTRODUCTION

1.1 Multidrug Resistance

Bacteria must cope with many toxic compounds to make their way in the world, struggling through antibiotics, antiseptics or toxins that would harm them. To this end, bacteria have evolved strategies that allow them to survive a conflict with an antibacterial agent, and these strategies are typically lumped into four main categories (Reviewed by Nakaido et al., 2009)(Figure 1). The first is the simplest, which is that the membranes of the cell itself often act as a permeability barrier, keeping many unwanted chemicals out at the same time as it keeps the cellular components in (Bolla et al., 2011)(Figure 1). When that fails, another survival mechanism is for bacteria to develop and produce enzymes which can catalytically act on dangerous compounds, such as β-lactam antibiotics, nullifying their toxic effects (Korfman et al., 1988)(Figure 1). A third way for bacteria to survive harmful substances is to alter the target of the toxin, either changing characteristics such as binding sites or transition states such that the toxin can no longer exert its effect (Trieber et al., 2002)(Figure 1). Lastly, bacteria can use transporters to simply remove toxins from the cell, which lowers the local concentration in the vicinity of the target so that the local concentration is too small to have a large scale effect (Levy, 1992)(Figure 1).
Figure 1.1 Generalized schematic of the major types of bacterial toxin resistance mechanisms.

Protein transporters are used to help the cell resist toxins such as drugs, which leads them to be referred to as ‘resistance proteins’. Additionally, some of these proteins are capable of recognizing not just one but many drugs, earning them the label ‘multidrug resistance proteins’. The transport of molecules across cell barriers is very important to the life of a cell, and thus the responsibility is taken on by several superfamilies of proteins. At a glance, these are the ATP-binding cassette (ABC) transporters (Licht et al., 2011), the resistance nodulation cell division (RND) family (Blair et al., 2009), the major facilitator superfamily (MFS) (Law et al., 2008), the multidrug and toxin extrusion
(MATE) family (Kuroda et al., 2009), and the Drug/Metabolite Transporter (DMT) superfamily (Putman et al., 2000). In the DMT superfamily is a smaller family of Drug Metabolite Exporters (DME), which is further divided and includes the Small Multidrug Resistance (SMR) proteins (Bay et al., 2008). SMR proteins are found in Gram positive (Jeljaszewicz et al., 2000) and negative bacteria (Yerushalmi et al., 1995) as well as Archea (Ninio et al., 2003). Their widespread nature is unsurprising given that they have been found chromosomally (Morimyo et al., 1992), on plasmids (Grinius et al., 1994) and in integrons (Paulsen et al., 1993). Given the widespread nature of these multidrug resistance proteins, it grows increasingly more important to understand their structure and function.

1.2 Proteins, the Cellular Workhorse

Proteins are a major factor in the function of organisms. Within any kind of organism, whether prokaryotic or eukaryotic, single or multicellular, and irrespective of features such as habitat or lifestyle, proteins are found. Additionally, proteins perform an incredible variety of functions including playing roles in cell structures, signaling, mobility, resistance and the production, transport, and modification of nearly every biologically relevant molecule (Blackman, 1977). Subsequently, developing an understanding of these incredibly versatile macromolecules has remained a subject close to the heart of molecular bioscience since their discovery.

Predictably, being able to perform such a wide variety of functions, proteins exhibit an incredible structural diversity (Boeckmann et al., 2005). While all proteins are composed from the same building blocks, amino acids, some contain cofactors such as
metals or organic molecules which participate functionally as well. Proteins can be classified into groups along many lines, such as their structure, function, or inclusion of cofactors, but one of the most useful is classification according to environment, namely the status of the protein as being a soluble protein or a membrane protein.

Membrane proteins, as the name implies, are in some fashion integrated with the lipid bilayer membranes that compose boundaries such as the membranes of cells and cellular compartments. These proteins, or at least the portions which interact with the membrane, are highly hydrophobic on their exterior, which allows them to reside and operate in what represents a protective hydrophobic barrier for the cell (Engel et al., 2008). Membrane proteins are further divided by the type of their membrane association into two types, integral and peripheral. Peripheral membrane proteins constitute those that are associated with only one membrane leaflet and do not have entire membrane embedded domains (Morozova et al., 2011)(Figure 1.2).

Integral membrane proteins (IMP) differ in that their structure at least partially includes a segment crossing the membrane bilayer itself (van Geest et al., 2000)(Figure 1.2). These are further subdivided in relation to the structural characteristics and type of insertion into monotopic proteins, which only attach to one side of the membrane, polytopic proteins, which span the membrane at least one time utilizing an α-helix (Figure 1.2), and β-barrel proteins, which cross the membrane a number of times utilizing amino acids in a β-strand conformation (van Geest et al., 2000).

Membrane proteins are estimated to account for up to 30% of the genes in Escherichia coli (Wallin et al., 1998) and about 25% in humans (Kozma et al., 2013). Difficulties in studying them due to their different environmental requirements mean that
they are underrepresented in terms of biochemical data and structural information.

Indeed, the protein databank boasts a mere <2% of its structures being membrane protein structures (Kozma et al., 2013).

Figure 1.2 Diagram of a cell showing protein classification by membrane association. Depiction of a cell showing a highlight of a segment of the cell membrane and the locations and membrane associated statuses of a soluble protein (Green) with no membrane attachments, a peripheral membrane protein (Yellow) which associates with one leaflet of the membrane bilayer, and a polytopic membrane protein (Red) which contains secondary structural elements that span the membrane multiple times.
1.3 Methods for the Study of Integral Membrane Proteins

The relative lack of information on IMP is not surprising considering the difficulty involving the study of membrane proteins. Traditional protein purification schemes or techniques such as co-affinity purification assays are not always applicable, as an aqueous environment is substantially more polar and may denature some proteins by exposing regions of lower hydrophobicity, and removal from the membrane can disrupt structure (Engel et al., 2008). Expression in fusion constructs can change protein translocation, folding, insertion, or binding resulting in a non-native form. Removing membrane proteins from the bilayer environment can cause marked changes in structure, even when the protein is reconstituted into artificial membrane mimetic environments, potentially limiting any *in vitro* study (Shen et al., 2013).

While integral membrane proteins are certainly more difficult to study than soluble proteins, there are still many ways in which structural data can be determined. Of particular help is that many IMP share common structural elements due to the constraints of their lipid environment (van Geest et al., 2000). The segments of IMP that cross the cellular membrane tend to be helical, with each transmembrane helix (TMH) being typically at least 20 amino acids such that the helix spans the entire membrane and into the compartments on either side (van Geest et al., 2000). These helices are connected by extramembrane loops can contain soluble domains or structural elements that can often be expressed on their own and adopt their native fold and function. The helices of an IMP tend to be packed closely together within the protein, and may have helical faces that contain hydrophobic sidechains which interact more favorably with the lipid acyl chains
surrounding the protein. Aliphatic or hydrophobic amino acid side chains typically constitute the exterior of IMP, inverse to soluble proteins where hydrophilic side chains often constitute the protein exterior (Rees et al., 1989).

A powerful tool to observe aspects of IMP functionality are site directed mutations, which can be made to membrane proteins such that disruptions of structure and function can be observed or residue placement can be more easily ascertained (Frillingos et al., 1998). Scanning or site directed mutagenesis studies can be useful to determine aspects of membrane protein structure, but often rely on having previous structural data of some kind available either to direct the study or to make sense of it in context of the effects of the mutation, for example loss of function may be due to either structural changes or the loss of important catalytic or binding elements.

Crosslinking has also become a commonly used biochemical technique to determine aspects of membrane protein structure. The idea of crosslinking is that a chemical reagent which can interact with two specific moieties is brought into the environment of the protein and should the protein contain those moieties, they may both react with the reagent which creates a structural bridge between them (Fraenkel-Conrat et al., 1946). Each reagent has a specific length related to its chemical structure, and in order to react, both moieties must come within that specified distance from each other, which gives constraints of distances in the structure (Das et al., 1979). The properties of the chemical linkers can vary wildly, with some being monofunctional where both of the links made occur with the same functional groups, or bifunctional, where the link is made between two specific but different moieties (Middaugh et al., 1983). In addition to this, the linkers can be hydrophilic so that they will not travel into or across membranes, or they may be
hydrophobic, in which case they can enter the membranes and function to crosslink at the membrane surface or interior (Middaugh et al., 1983). The creation of cysteine mutants is commonly utilized here, since many linkers exist which can create disulphide linkages, though reaction with other amino acids such as arginine or even with artificial amino acids are also available. The strength of this technique lies in that it develops specific distance constraints between two particular locations of a protein, which can then be applied in conjunction with or compared to other determined structural information (Middaugh et al., 1983). Concerns about its usage include that any mutant proteins made may have altered structure or functionality, that the measured lengths of the crosslinkers may be inaccurate (Green et al., 2008), and that the development of crosslinks may trap proteins in a non-native state (Iiyama et al., 1994).

X-ray crystallography is a commonly used technique which involves growing a crystallized form of a protein and subjecting it to a beam of X-rays, which will subsequently be scattered by the electron clouds of the atoms to provide a diffraction pattern. This pattern can subsequently be analyzed to generate a map of electron density, which can then be used to infer protein structure (North, 1963). While crystallography has been used to develop structures of IMP, the crystals generated by IMP tend to be poorly ordered, which until recently limited the resolution of the technique to about 4 Å, a level insufficient to assign most amino acids based on the electron density (Arora et al., 2001). Another problem can be related to the nature of the sample, mainly that a crystal does not reflect the dynamic nature of proteins and may instead be represent a non-native state (Muller et al., 2008). Lastly, the quality of the result can depend highly on the processing of data, such that errors in computation or assignment of structural features to
regions of density (which is sometimes accomplished manually) can result in errors (Ma et al., 2007). Thus, structures obtained this way are undoubtedly useful but prone to errors both technical and in interpretation, which require that they should be supported by biochemical data before strong conclusions are drawn.

Nuclear magnetic resonance (NMR), is a technique by which the spins of the nuclei of atoms and the nature of their couplings can be used to generate an idea of structural features in a molecule. The basic idea of the technique is that magnets are used to create a magnetic field in which the sample is placed, which subsequently lines up the molecules and any magnetic nuclei within. Subjecting the sample to controlled radio frequency pulses can knock the nuclei out of their alignment granting them energy at a particular resonance frequency which is radiated outward in return and can be picked up by a detector as they return to alignment with the field (Rabi et al., 1938). The atomic environments and proximity to other nuclei will modulate this effect through shielding (Karplus et al., 1963) or cross relaxation (Anderson et al., 1962) such that individual nuclei can be differentiated by an observed chemical shift and the specifics of their neighboring atoms obtained. Only atoms with a magnetic moment can be observed using NMR, and so the data is gathered essentially around focal points of information where these atoms reside. Fortunately, atoms like protons, phosphorus, carbon-13, and nitrogen-15 can be used with this technique, meaning protein labeling strategies can be used to gather additional spectra or information concerning particular regions. Specific alterations of the radio frequency pulse technique, such as TROSY (transverse relaxation optimized spectroscopy) which is used to generate sharper peaks in the case of large complexes by using the cancelling between two dipolar couplings or between a dipolar
coupling and chemical shift anisotropy (Pervushin et al., 1997). HSQC (heteronuclear single quantum coherence) is another technique that involves the transfer of magnetization between a proton and a second nucleus such as $^{15}$N or $^{13}$C by an insensitive nuclei enhanced by polarization transfer step and back to the proton and can also be applied in order to get information through differing types of structural relationships between the atoms (Bodenhausen et al., 1980). NMR is exceptionally useful in that it can be performed in solution and in vivo, but large or complicated systems can frustrate the analysis due to an overabundance of observed peaks.

### 1.4 The Bacterial Two-Hybrid System

Two-hybrid approaches utilize a protein that has a definitive activity that can easily be quantitatively measured. Reporter domains attached in fusions to proteins of interest consist of domains which are folded properly even when independent of the remainder of their protein of origin, and which have very low tendency to interact with the other reporter domains when expressed on their own. If the proteins of interest do interact, they can bring the reporter domains into a functional proximity which allows the carrying out of their function, selected to be some sort of detectable signal (Figure 1.3) which is typically the expression of a reporter gene. The first two-hybrid system was created for yeast and developed by Fields et al., 1989, though there are now many two-hybrid technologies to choose from all with a general approach that two interacting proteins of interest drive the reassembly of the reporter protein leading to a signal that is proportional to the lifetime of interaction of the protein-protein interaction.
**Figure 1.3 Cartoon general description of the two-hybrid approach for detecting protein-protein interaction.** The two-hybrid approach works on three basic concepts, A) A reporter protein is capable of generating a detectable signal when intact, but B) when its constituent domains are expressed independently they do not self-associate and are incapable of generating the signal. C) Attachment of the domains to two proteins of interest can restore functional proximity to the reporter protein if they interact, causing the signal to be generated.

The yeast two-hybrid system utilized the *Saccharomyces cerevisiae* protein GAL4, a protein that binds DNA regulating genes for glucose metabolism (Fields et al., 1989). GAL4 is constructed of two independently folding domains, UAS$_G$, which binds to particular DNA sequences, and a C-terminal domain which is required for transcription. These domains were made into protein fusions with SNF1 and SNF4, yeast proteins known to interact, and the observation was made that functional proximity of the domains resulted in restoration of expression of the UAS$_G$ regulated genes (Fields et al., 1989). The utility of this method was further expounded upon using the yeast protein SIR4, in which it was shown that it could be used to demonstrate that SIR4 formed homodimers, that a particular domain was the origin of this interaction, and that a library could be constructed for the screening of interactions of proteins (Chein et al., 1991).
An adaptation of the yeast two-hybrid system for bacterial transmembrane proteins was subsequently developed in 1999 based on work done with modular domain substitutions of the *Vibrio cholerae* transcriptional activator ToxR (Kolmar et al., 1995). This system, named TOXCAT, utilized protein constructs wherein the transmembrane domain under study was fused with maltose binding protein (MBP). MBP is a monomeric periplasmic protein which allows periplasmic transport of maltose on one side, and the N-terminal domain of ToxR on the other (Russ et al., 1999). The N-terminal domain of ToxR is responsible for binding DNA to allow the activation of expression of chloramphenicol acetyltransferase (CAT), but only in a dimeric form (Miller et al., 1987). In this system, expression of chloramphenicol acetyltransferase was found to be proportional to the binding equilibrium between constructs using zone of inhibition assays with chloramphenicol (Russ et al., 1999). The drawback to this system was that it is only capable of detecting homodimerization, and further developments were required to be able to screen for heterodimers (Schneider et al., 2002).

This improvement for heterodimerization was made with the introduction of the GALLEX system (Schneider et al., 2002). This system was based on the work of Porte et al., who observed interactions between soluble leucine zipper domains in *E.coli* using the DNA binding domains of the repressor protein LexA. LexA represses the recA or sulA promoters under which they had coded a *lacZ* gene coding for the reporter protein β-galactosidase (Porte et al., 1995). A further discovery was that a LexA mutant with altered DNA binding specificity such that a different binding site was recognized could be used along with a half-native, half-mutant hybrid promoter, causing repression to only occur if a dimer was formed incorporating one native LexA DNA binding domain and a
mutant one (Dmitrova et al., 1997). The implication of this was that it was now possible
to screen for heterodimerization in soluble proteins, even in cases where the proteins of
interest are capable of homodimerization. The application of the technique to IMP was
accomplished in 2002, where a study on interactions of a wild type TMH of glycophorin
A with mutant TMH were observed as well as interactions between mutants.
Simultaneously, associations between α₄ and β₇ integrin were able to form in both a
hetero and homooligomeric manner to show the versatility of the system to any parallel
TMH (Schneider et al., 2002). One caveat to this system resides in its functionality,
namely that if the proteins of interest are IMP, in order for repression of a gene to occur
the DNA has to be in the locality of the repressor domains which are membrane
associated.

The BACTH system was introduced when the catalytic domains of adenylate
cyclase were used as the fusion partners to the proteins of interest (Karimova et al.,
1998). Adenylate cyclase is an enzyme that functions to convert ATP into cyclic
adenosine monophosphate (cAMP), which is involved in a signaling pathway wherein
cAMP interacts with a catabolite activating protein (CAP) to form a CAP/cAMP complex
which can bind to promoters and cause the expression of genes (Ladant et al., 1999). The
catalytic activity of adenylate cyclase resides in two discrete domains, however if these
domains are independently expressed they do not associate (Ladant et al., 1999). In
BACTH, each domain is then fused to the proteins in question and if they associate
cAMP production is restored. A reporter gene can be inserted into a promoter controlled
by the CAP/cAMP complex, which can then be used to evaluate the response (Karimova
et al., 1998). This technique was proven functional for membrane proteins using the Fts
proteins, which catalyze formation of the division septum in *E. coli* (Karimova et al., 2005). This latest version of the two-hybrid system for use in a bacterial host is beneficial in two main ways; first, the interaction does not have to take place near the DNA transcription machinery as in the other systems, and second that the expression level of the reporter gene expressed is concentration dependent on the amount of cAMP produced, which is proportionate to the binding equilibrium, allowing for a degree of quantitation of binding using the assay kinetics (Karmova et al., 1998).

In the application of BACTH methodology, several more tools deserve a special mention since they are enzymes or techniques which are integral to the process established here. The variant of adenylate cyclase typically used is native to *Bordetella pertussis*, and is actually a toxin secreted which harms eukaryotic cells by altering their physiology (Ladant et al., 1999). Its usual mode of action is to enter eukaryotic cells where it is activated by the protein calmodulin, which is only present in eukaryotes, where it converts ATP into cAMP which interacts with a CAP to form a CAP/cAMP complex that acts as a transcriptional activator. Adenylate cyclase has two modular portions, one of which, comprising the first 400 amino acids, is the active site while the rest of the protein is responsible for the proteins haemolytic properties (Ladant et al., 1999). The catalytic domain can be further divided into two self-folding domains which can be obtained independently by limited trypsin proteolysis and are subsequently named T18 and T25, corresponding to their molecular weight in kDa when digested by trypsin (Ladant, 1988). The T25 domain contains the catalytic site and the T18 domain contains the calmodulin binding site, and these two domains do not self-associate when
independently expressed, but when expressed independently in the presence of calmodulin the catalytic activity is restored (Ladant et al., 1989).

β-galactosidase is an enzyme with a long history in biochemistry, with the first recorded mention that yeast cells can hydrolyze lactose (though the term was not used at the time) coming from Beijerinck in 1889 (Rouwenhorst et al., 1989). Interestingly, the enzyme is sufficiently stable that a 90 year old preparation from the Beijerinck’s lab was found to still be active at a few percent of the estimated original activity despite storage with no special care in a temperature that ranged from roughly -10°C to 40°C (Rouwenhorst et al., 1989). The gene for β-galactosidase is lacZ which is under the control of the lac operon, required for the cellular uptake and metabolism of lactose. The lac operon is famous for being the subject of the studies in which the transcriptional regulation of protein synthesis was first described (Jacob et al., 1960). β-galactosidase is used mainly for its catalytic activity, which is to hydrolyze β-galactosides, including lactose, into their component monosaccharides, but which also applies chromogenic substrates such as o-nitrophenol-β-galactoside (ONPG) which is colorless until cleaved, allowing for a colorimetric assay to be performed (Lederberg, 1950). ONPG in particular forms an o-nitrophenol anion when cleaved, which creates a yellow color which can be observed spectrophotometrically at 420nm. β-galactosidase has since been used in numerous biological systems under the control of varied promoters and with a variety of assaying techniques (MacGregor et al., 1991).

The final tool employed is known as the Miller assay. Developed in 1972 by Miller, hence the name, the Miller assay is a relatively simple technique by which the expression of β-galactosidase can be quantitatively compared (Miller, 1972). The idea
behind the assay is that alterations in β-galactosidase expression levels produce proportional cellular concentrations of the enzyme, which will result in concentration-dependent rates of ONPG hydrolysis when excess substrate is added to lysed cell cultures and allowed to react for a specified time. Spectrophotometric measurement of absorbance from the ONP anion at 420nm as well as measurements of scattering for cell density at 600nm and a correction factor at 550nm are applied in the ‘Miller equation’ to generate a value given in ‘Miller units’ which is compared to positive and negative controls. This can be elegantly combined with BACTH methodology when β-galactosidase is produced as a result, such that stronger binding equilibrium between proteins will result in a more consistent production of cAMP, which will then produce more β-galactosidase that can be quantitatively measured in terms of kinetics by use of the Miller assay. The quantitative nature of the BACTH-Miller approach may be particularly valuable for differentiation when analyzing weaker binding kinetics, or in cases where a variety of interactions may be possible such as in nonspecific interactions or variable partners and structures.

1.5 EmrE – About as Integral as a Membrane Protein Gets

Found within the SMR family, Ethidium multidrug resistance protein E, or EmrE, is both representative of many of the problems which plague IMP structural analysis and representative of SMR proteins in general, and has been the beneficiary of the majority of research on those proteins (Tate, 2006, Bay et al., 2008). SMR proteins are small proteins, usually 100-140 amino acids and a mere 12kDa per monomer (Paulsen et al., 1996) (Figure 1.4). Their structure is roughly 80% alpha-helical and composed of a
helical bundle with very small extramembrane loops connecting four TMH (Arkin et al., 1996) (Figure 1.4). Structures for EmrE have been proposed based on Cryo electron microscopy (Cryo-EM) (Ubarretxena-Belandia et al., 2003) and X-ray diffraction (Chen et al., 2007) to 7Å and 4Å respectively, but while a few amino acid positions for large residues can be implied, they cannot be directly assigned and thus the identities of the helices are also ambiguous.

**Figure 1.4 Schematic of an EmrE monomer showing the amino acid sequence.** EmrE is a small, highly α-helical, mostly membrane embedded transporter, which exhibits a dual topology in the membrane environment, denoted here as an absence of labels for the cytoplasmic and periplasmic side of the membrane.

SMR proteins have been found to oligomerize, but while consensus generally supports a homodimeric form, experimental conditions have been found to change the dimerization state (Federkeil et al., 2003) and everything from a monomeric (Winstone et al., 2005), dimeric (Schuldiner, 2009), trimeric (Yerushalmi et al., 1996) and tetrameric
(Ubarretxena-Belandia et al., 2004) forms have been observed. The tetrameric state is also suggested by Cryo-EM (Ubarretxena-Belandia et al., 2003) and X-ray crystallography (Chen et al., 2007) experiments. Aside from the suggested trimeric state derived from ligand binding experiments (Muth et al., 2000) being reinterpreted as representative of a mixed population of dimers and ‘dimer of dimers’ tetramers (Elbaz et al., 2004), the biochemical data generally supports a dimeric state and potential for higher order monomers is not yet well understood.

Figure 1.5 Asymmetric structures of EmrE. Dual topology of an EmrE dimer was suggested by asymmetric structures from Cryo-EM (7Å resolution) and X-ray crystallography (4Å resolution), as obtained by A) Ubarretxena-Belandia et al., 2003, Figure 5b, with one monomer represented by helices A-D and the other by F-H and B) Chen et al., 2007, Figure 3c with the two monomers colored blue and yellow and numbered with the proposed identity of the helices respectively. Figure A) and B) taken from Ubarretxena-Belandia et al., 2003 and Chen et al., 2007, with permission.

Until recently, much debate raged over the membrane topology of SMR proteins. While most IMP have a distinct orientation (topology) with respect to the directionality of their insertion, EmrE is one of relatively few ‘dual topology’ proteins which can insert
in either topology due to a small net charge bias, allowing them to resist the application of von Heijne’s positive inside rule (von Heijne, 1986). Furthermore, the asymmetric nature of the Cryo-EM (Ubarrtexena-Belandia et al., 2003) and X-ray (Chen et al., 2007) structures suggested that not only was insertion with either topology possible, but that a dimer of EmrE consisted of one protomer from each orientation, named an ‘antiparallel dimer’ (Figure 1.5). Consensus has now been reached that this is indeed the case, as demonstrated by electron paramagnetic resonance (EPR) (Amadi et al., 2010), experiments with membrane impermeable reagents (Nara et al., 2007), and global topology mapping using green fluorescent protein showing an intermediate signal (Daley et al., 2005). Mutants which altered the charge bias to create single topology monomers showed decreased resistance unless \(N_{in}-C_{in}\) and \(N_{out}-C_{out}\) mutants were co-expressed (Rapp et al., 2007). Tandem genetic fusions of two monomers separated by a non-interacting helix enforcing an antiparallel topology were shown to be active (Nasie et al., 2010). Most recently, it was demonstrated that EmrE energetically prefers antiparallel dimers unless no other option for dimerization is available, in which case parallel dimers are observed to form (Lloris-Garcera et al., 2012).

Unfortunately, while a consensus on the native structure has been reached, the struggle by some researchers to prove a parallel topology has frequently observed active parallel dimers. In a parallel dimer both protomers have the same topology, and any proposed structure of EmrE must now also be able to provide an explanation for parallel dimers unless it accounts for parallel interfaces through a higher oligomeric state. Evidence that supports a parallel topology includes protein fusions which determined the termini as to be in the cytoplasm only (Son et al., 2003), crosslinking of single cysteine
mutants in positions only compatible in parallel topology (Soskine et al., 2006), and functional genetically linked tandem monomers where only parallel topology is possible (Steiner-Mordoch et al., 2008).

In terms of the structural arrangement of the monomers, the first three TMH of EmrE are thought to compose a binding pocket with the active site while dimerization is mediated by an interaction between TMH 4 of each protomer (Rath et al., 2006). This interaction is thought to utilize a GG$_7$ motif, which is defined as two glycine residues separated by six other amino acids, which could be represented as GXXXXXXG. In the specific case of EmrE these glycines occur at positions 90 and 97, and two large hydrophobic residues occur in the center of the motif, positions 93 and 94, making the motif GXX(Large hydrophobic)$_2$XXG for EmrE (Elbaz et al., 2008). Amino acids within SMR proteins are thought to fill multiple roles in binding, stability or oligomerization (Sharoni et al., 2005) but residue Glu-14 is generally considered as the most important active site residue, being the only membrane embedded charged residue and interacting with positively charged substrates (Yerushalmi et al., 2000).

The substrates of EmrE are exceptionally varied, but typically fall into the category of quaternary cationic compounds (QCC), possessing a positive atom (generally N or P) surrounded by four hydrocarbon R groups. These substrates are dyes, antimicrobial agents, some antibiotics, including ethidium bromide, acriflavine, methyl viologen, streptomycin, tobramycin (Nasie et al., 2012), crystal violet, benzalkonium (Kunihiko et al., 2001), osmoprotectants betaine and choline (Bay et al., 2012), and the crystalizing agent tetraphenylphosphonium (Schuldiner et al., 2001). Each SMR protein demonstrates transport of specific subsets of molecules, though the subsets of some SMR proteins
overlap. This strange specificity with plasticity is not yet well understood. Despite the divergent structural data leading to a number of proposed mechanisms (reviewed Bay et al., 2008), the alternating access model of EmrE transport is now the general consensus, supported by electron paramagnetic resonance (Amadi et al., 2010), mutational studies (Poulsen et al., 2009), and most recently solution state NMR (Morrison et al., 2012) and reviewed by Henzler-Wildman in 2012.

While a clearer picture of EmrE structure is slowly emerging, some contradictions in the literature still remain, and the difficulties concerning the size, oligomerization, topology, and membrane embedded nature of EmrE make it an excellent subject of study and a model system for determining the capabilities of techniques to determine structural characteristics of IMP.

1.6 Objectives of Research

The purpose of this research is to develop a method with which to analyze the structural contacts of individual transmembrane helices from integral membrane proteins in vivo through the use of the BACTH system. The developed system will be applied to the model E.coli protein EmrE. The aims are thus defined as:

i) Generate vectors with the elements of the BACTH system that also allow for control of orientation in the membrane.

ii) Develop a high throughput assay for this BACTH system.

iii) Test this approach with EmrE to evaluate its ability to sense dimers and higher order multimers
iv) Use the developed system to make a contact map of the transmembrane helices of EmrE.

Aim (i), the generation of BACTH elements was performed in Chapter 2 and was successful. Plasmid vectors that allow insertion of genes coding for each TMH of interest for future work were developed. Work developing the high throughput assay in accordance with aim (ii) is described in Chapter 4 with details of the protocols outlined in Chapter 2. Aim (iii) is discussed in Chapters 3 and 5, and was unsuccessful while utilizing the whole protomers in Chapter 3, but was able to detect dimerization (though not higher order multimers) as established in Chapter 5. Lastly, aim (iv) is addressed in Chapters 5 and 6, and was successful in that a network of helical contacts is described and its implications towards a structural model of EmrE are discussed both in terms of the data itself (Chapter 5) and in comparison to existing literature (Chapter 6).
CHAPTER 2: METHODS

For a list of buffers, solutions, and their components please refer to Appendix A.

2.1 Restriction Enzyme Digestion

Digests were accomplished by combining 5µL of REact buffer (from Invitrogen, REact buffer 3 for EcoRI and BamHI, and REact buffer 2 for XhoI), 5µL DNA, 0.5µL of each required restriction enzyme and sterile ddH₂O to 50µL, before incubating in a Precision Scientific Gravity Conventional Incubator without shaking for 3h at 37°C. For detection of band shift for inserts of plasmids, 5µL digested plasmids were run on a 1% agarose gel with Orange loading dye at 100V until the dye front was near the bottom of the gel.

2.2 Ligation of Primers Coding EmrE TMH into Digested Plasmids

To anneal and ligate primers coding for the EmrE TMH of interest into digested plasmids, 1µL of digested primer was added to 4µL of both forward and reverse primer each and 2µL of 10x T4 DNA ligase buffer, then made to 19µL with sterile ddH₂O. This mixture was boiled for 5 minutes at 95°C on a salt block to denature the DNA, following which the samples were briefly spun down before the addition of 1µL of T4 DNA ligase. Ligation was allowed to proceed for 2h at room temperature without shaking on the benchtop. Resulting ligation mixture was transformed into appropriate strains (Section 2.3) and grown on LB plates with selective antibiotics overnight at 37°C before single colonies were selected, grown as LB broth cultures, plasmids isolated by Omega e.Z.N.A Miniprep and restriction digested with XhoI (Section 2.1).
2.3 Generation of EmrE Protein Fusions with T18 & T25

The EmrE gene was obtained from the pEmr11 vector using XbaI and HindIII and following a restriction digest protocol (Section 2.1). The excised gene then underwent PCR reaction using the primers BACTH-EmrE-F and BACTH-EmrE-R which introduced a KpnI restriction site at the 3’ end, replacing the HindIII site. Simultaneously, BACTH vectors pUT18, pUT18C, pKT25 and pT25 were digested with KpnI and XbaI using the standard protocol. Ligation was then performed with the EmrE gene as an insert and each of the vectors as per standard protocol (Section 2.2). Resulting from this were constructs containing EmrE fused in turn both N- and C-terminally to both the T18 or T25 adenylate cyclase domain (Figure 2.1-2.4). Transformation was performed into rubidium chloride (RbCl₂) competent *E.coli* DH5α cells to make DMSO storage stocks for the plasmids, which were subsequently kept at -80°C. These stocks were used to isolate plasmids which were subsequently co-transformed into RbCl₂ competent *E.coli* BTH101 strains pairwise. Control plasmids utilizing fusion proteins of yeast transcriptional activator GCN4 (General Control Nonderepressible) leucine zipper motifs attached to the two adenylate cyclase domains, called pZipT18 and pZipT25 were used from existing lab stocks, but newly isolated and transformed into *E.coli* BTH101 simultaneously to ensure uniformity between samples. Constructs were verified through sequencing.
Figure 2.1 Recombinant vector construction schematic for plasmid pEmrET18. The gene for EmrE excised from pEmr-11 was inserted into parental plasmid pUT18 using the restriction enzymes XbaI and KpnI, creating a fusion protein with EmrE expressed N-terminally to the adenylate cyclase T18 catalytic domain. The construct is under the lac promoter and should therefore be IPTG inducible. Plasmid maintenance and strain selectivity is accomplished through the use of ampicillin resistance.
Figure 2.2 Recombinant vector construction schematic for plasmid pT18EmrE. The gene for EmrE excised from pEmr-11 was inserted into parental plasmid pUT18C using the restriction enzymes XbaI and KpnI, creating a fusion protein with EmrE expressed C-terminally to the adenylate cyclase T18 catalytic domain. The construct is under the lac promoter and should therefore be IPTG inducible. Plasmid maintenance and strain selectivity is accomplished through the use of ampicillin resistance.
Figure 2.3 Recombinant vector construction schematic for plasmid pEmrET25. The gene for EmrE excised from pEmr-11 was inserted into parental plasmid pKNT25 using the restriction enzymes XbaI and KpnI, creating a fusion protein with EmrE expressed N-terminally to the adenylate cyclase T25 catalytic domain. The construct is under the lac promoter and should therefore be IPTG inducible. Plasmid maintenance and strain selectivity is accomplished through the use of kanamycin resistance.
Figure 2.4 Recombinant vector construction schematic for plasmid pT25EmrE. The gene for EmrE excised from pEmr-11 was inserted into parental plasmid pKT25 using the restriction enzymes XbaI and KpnI, creating a fusion protein with EmrE expressed C-terminally to the adenylate cyclase T25 catalytic domain. The construct is under the lac promoter and should therefore be IPTG inducible. Plasmid maintenance and strain selectivity is accomplished through the use of kanamycin resistance.
2.4 The Modified Miller Assay

To answer the question of how to pull a quantitative result for interactions from what essentially amounts to a binary state of either proteins interacting or not, an answer lies in the Miller assay (Miller, 1972). For the Miller assay, the secondary reporter protein is β-galactosidase, a protein which converts β-galactosides into galactose and the other component sugar. Hence if cell cultures containing the protein constructs of interest are grown under the same conditions for the same amount of time before lysis, they should release β-galactosidase reporter in amounts relative to their binding equilibrium. O-nitrophenol-β-galactoside (ONPG) is a colorless substrate for β-galactosidase which when enzymatically cleaved results in O-nitrophenol and the formerly bonded monosaccharide. Given a standardized time for β-galactosidase to hydrolyze an excess of ONPG, the pH is increased which both inactivates the β-galactosidase and converts all the O-nitrophenol into the O-nitrophenolate anion, which has a strong yellow color that absorbs light at 420nm. Thus, once cells are lysed, if an excess of substrate is added a timed colorimetric assay may be conducted in which the absorbance is converted to Miller units using the Miller equation.

The Turner lab has used a modified version of the Miller assay previously (Chan et al., 2009). In this assay, co-transformed strains were taken from DMSO stocks stored at -80°C and grown as 3mL LB cultures in culture tubes for 16h at 37°C in the presence of 100µg/mL ampicilin and 50µg/mL kanamycin, with shaking. About 1.5mL of cells were centrifuged at 14000rpm before the supernatant was removed by aspiration. The cell pellet was resuspended in 500µL of Z-buffer and afterwards one 100µL aliquot was added to a new microfuge tube for assaying, while another 100µL aliquot was added to
900µL of ddH$_2$O in a cuvette and measured for scattering at 600nm. To the aliquot intended for assaying, 700µL of Z-buffer containing 2.7µL/mL β-mercaptoethanol was added, then membrane permeabilization was induced by brief vortexing with 30µL of added CHCl$_3$. Following lysis, 160µL of 4mg/mL ONPG in Z-buffer was introduced to each sample, which was subsequently incubated for 30 minutes at 30°C without shaking (due to lack of proper equipment) for β-galactosidase hydrolysis to occur. The pH was then increased using 400µL of 1M Na$_2$CO$_3$ to stop the reaction and convert all hydrolyzed ONPG to the ONP$^-$ form. Measurements of absorbance and 420nm and scattering at 550nm were then taken using cuvettes and a Hitachi U-2000 spectrophotometer. Miller units were determined using the equation (1) set forth in the original publication of the assay (Miller, 1972) where T is the reaction time in minutes and V is the volume of cell culture used in mL, and the scattering as a representation of cell density at OD$_{600}$ is included as a normalizing factor. The OD$_{420}$ is the absorbance of ONP$, and as a correction for the presence of cell debris 1.75$\cdot$OD$_{550}$ is subtracted from that value.

$$\text{Miller Units} = 1000 \cdot \frac{(\text{OD}_{420} - (1.75 \cdot \text{OD}_{550}))}{T \cdot V \cdot (\text{OD}_{600})}$$

2.5 Initial Attempt at a Microtitre Plate Based Miller Assay

The Miller assay was adapted from the modified form previously employed by the Turner lab (Chan et al., 2009) to work with a standard 96-well, 300µL microtitre plate. Cell cultures were taken from stocks frozen at -80°C in LB + 24%v/v DMSO and grown for 16h in LB + 100µg/mL ampicillin and 50µg/mL kanamycin as 0.2mL cultures with
shaking in a microtitre plate. 20μL of culture was added to each of the appropriate wells of a microtitre plate already containing 100μL of Lysis buffer, before incubation without shaking in a 4°C cold room for 30 minutes while OD600 values were obtained using a SpectraMax M2 Softmax Pro 5 plate reader using the original plate. After the cell lysis, 78μL of Z-buffer + 1.64mg/mL ONPG was added to each well using a multichannel pipette and the cells were incubated at 30°C for 30 minutes without shaking. Lastly, 80μL of 1M Na2CO3 was added to each well to raise the pH and stop the reaction. The plates were then taken to a SpectraMax M2 SoftMax Pro 5 microtitre plate reader to record the OD550 and OD420 of each well. The results were calculated using the Miller equation as previously (Equation (1)).

2.6 Addition of a Pre-growth Phase to the Microtitre Plate Assay

When it became apparent that standardization of the microtitreplate assay was required, the protocol was altered such that the day before the assay, 2mL LB + 100μg/mL ampicillin and 50μg/mL kanamycin broth cultures were started from ice crystals of the DMSO stocks of the co-transformed strains stored as -80°C. These cultures were grown for 7 hours at 37°C with shaking before 10μL of culture was taken and used to inoculate 200μL of LB + ampicillin and kanamycin in the appropriate wells of a microtitre plate. The plate was then incubated for 16 hours at 37°C with shaking overnight before 20μL of cultures from each well were transferred to a new microtitre plate to complete the microtitre plate Miller assay as stated above (Section 2.5).

2.7 Standardizing the OD600 of Inoculant Before 16 Hour Incubation

Due to the volume restrictions of the 300μL wells in the microtitre plate, this meant that the amount of inoculants needed to be compatible with the amount of media in
the plates, while still allowing a range of culture growth with $\text{OD}_{600}$ values typically from 0.05 to over 1.0. The assay protocol was modified to that 7h pregrowth cultures were made as before, but the volume of culture added to the wells of the microtitre plate was standardized to be equal to adding 100$\mu$L of a culture with an $\text{OD}_{600}$ of 0.010, determined by the equation:

\[
\frac{1}{\text{OD}_{600}} = \frac{\mu L \text{ Culture added}}
\]

So that

\[
((100\mu L)(0.010)) = ((X\mu L)(\text{OD}_{600}))
\]

This volume was added to all the appropriate wells of a plate for each sample, in replicates stemming from the same 7h pregrowth culture. The plates were then incubated at 37$^\circ$C with shaking and the microtitre plate based Miller assay performed as described in Section 2.5.

**2.8 Growth Curves to Verify Stationary Phase of Samples in the Assay**

To create a growth curve to verify that cell cultures were uniformly in stationary phase by the time the Miller assay was being performed, 7 hour pregrowth cultures were started as before, and following that incubation a standardized amount of cells as derived above (Equations (2) and (3)) was added to 100mL LB + 100$\mu$g/mL ampicillin and 50$\mu$g/mL kanamycin in a microtitre plate. 20$\mu$L of sterile mineral oil was added to the top of the wells as a necessity to avoid evaporation and contamination, though at the cost of limiting oxygen transfer and making the cultures semi-anaerobic. The plate was then incubated at 37$^\circ$C for 26 hours in a PerkinElmer VICTOR X4 2030 multilabel reader.
incubator/microtitre plate reader with shaking, taking readings for each well taken at 2 hour intervals.

2.9 Standardization of Culture OD\textsubscript{600} Immediately Before the Miller Assay

The last change for standardization was to change the point at which the OD\textsubscript{600} for the cultures was standardized. The Miller assay as originally derived attempts to account for differences in cell density by the inclusion of the OD\textsubscript{600} as a denominator (See Equation (1)). In context of the original assay this may be sufficient standardization, but when the conditions of the assay are changed, notably the move to a small volume, aspects such as cell density, aeration or growth rates relative to limiting nutrients can contribute to error in unexpected ways that differ or are absent in larger volumes, which can also take advantage of things like specialized glassware. As such, the next move was to try and increase the consistency of the Miller assay results was to change the timing of the standardization of the culture volume in the assay to right before the assay was performed, potentially diminishing the effects of differential growth rates or error such as a non-linear reading for scattering by the spectrophotometer. The flow of the assay was then to grow 2mL pregrowth cultures using ice crystals from -80°C DMSO stocks for 7 hours at 37°C with shaking in LB + 100μg/mL ampicillin and 50μg/mL kanamycin, and then to use 50μL of those cultures to inoculate each of the wells in a microtitre plate containing 200μL fresh LB with antibiotics. The second plate was incubated for 16 hours at 37°C with shaking and then from those wells, an amount of culture again determined by the equations (2) and (3) above, though this time the culture was added directly to a microtitre plate already containing 100μL of Lysis buffer so that the smaller culture
volumes wouldn’t dry out while the others were being pipetted. From there, the assay proceeded as previously described (Section 2.5).

2.10 Cloning & Transformation of EmrE Single TMH Fusion Constructs

Vectors pMBPT18 & pMBPT25 derived from parent plasmids pUT18 and pKT25 respectively were created by Dr. Limei Chang (Section 2.1). Plasmids were purified using an Omega e.Z.N.A. plasmid mini kit II from overnight cultures, and restriction digested in the multiple cloning site using BamHI and EcoRI for 3h at 37°C. Complimentary forward and reverse primers were constructed constituting the sequence of the TMH region of interest with bounding residues including a restriction site for XhoI or KpnI. For inverted topology constructs, the order of the amino acids coded for was manually reversed in the sequence on the primers (Table 2.1). Plasmid constructs were assembled by combining digested vector with forward and reverse primer, heating to 95°C and ligating. Ligation mixture was then transformed into rubidium chloride (RbCl₂) competent E.coli JM109 and plated on LB with selective antibiotics, with single colonies selected to make overnight cultures. Plasmids were isolated from these overnight cultures and restriction digested using the inserted XhoI site to screen by 1% agarose gel for band shift indicating presence of plasmid containing proper insert. Plasmids with proper insert detected were sequenced to make sure the insert had the correct sequence and stored in 24% DMSO in LB at -80°C.
Table 2.1 Primer sequences coding for the synthetic gene inserts of the individual EmrE transmembrane helices inserted into pMBPT18 and pMBPT25. Forward primer sequence of the primers used as synthetic gene inserts representing individual EmrE transmembrane helices used for pairwise interaction mapping of EmrE, which include sticky ends for BamHI, EcoRI and an engineered XhoI restriction site to test for gene insert presence by band shift of plasmids in agarose gel.

<table>
<thead>
<tr>
<th>Helix</th>
<th>Forward Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>GAT CCC ATG AAC CCT TAT ATT TAT CTT GGT GGT GCA ATA CTT GCA GAG GTC ATT GGT ACA ACC TTA ATG AAG TTT TCA GAA GGT CTC GAG TCG</td>
</tr>
<tr>
<td>H1r</td>
<td>GAT CCC GGT GAA TCA TTT AAG ATG TTA ACC ACA GGT ATT GTC GAG GCA CTT ATA GCA GGT GGT CTT TAT ATT TAT CCT AAC ATG CTC GAG TCG</td>
</tr>
<tr>
<td>H2</td>
<td>GAT CCC TTT ACA CGG TTA TGG CCA TCT GTT GGT ACA ATT ATT TGT TAT TGT GCA TCA TTC TGG TTA TTA GCT CAG ACG CTG GCT TAT ATT CCT ACA CTC GAG TCG</td>
</tr>
<tr>
<td>H2r</td>
<td>GAT CCC ACA CCT ATT TAT GCT CTG ACG CAG GCT TTA TTA TGG TTC TCA GCA TGT TAT ATT ATT ACA GGT GTT TCT CCA TGG TTA CGG ACA TTT CTC GAG TCG</td>
</tr>
<tr>
<td>H3</td>
<td>GAT CCC GGG ATT GCT TAT GCT ATC TGG TCA GGA GTC GGT ATT GTC CTG ATT AGC TTA CTG TCA TGG GGA TTT TTC GGC CAA CGG CTG GGT ACC TCG</td>
</tr>
<tr>
<td>H3r</td>
<td>GAT CCC CTG CGG CAA GGC TTC TTT GGA TGG TCA CTG TTA AGC ATT CTG GTC ATT GGT GTC GGA TCA TGG ATC GCT TAT GCT ATT GGG CTC GAG TCG</td>
</tr>
<tr>
<td>H4</td>
<td>GAT CCC GAC CTG CCA GCC ATT ATA GGC ATG ATG TTG ATT TGT GCC GGT GTG TTG ATT ATT AAT TTA TTG TCA CGA AGC ACA CCA CAT GGT ACC TCG</td>
</tr>
<tr>
<td>H4r</td>
<td>GAT CCC CAT CCA ACA AGC CGA TCA TTG TTA AAT ATT ATT TTG GTG GGT GCC TGT ATT TTG ATG ATG GGC ATA ATT GCC CCA CTG GAC CTC GAG TCG</td>
</tr>
</tbody>
</table>
Table 2.2 Plasmids constructed for this work and their features. Plasmids constructed for this work which contain full or partial genes for EmrE, an adenylate cyclase catalytic domain, listed with gene contents.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Resistance Marker</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEmrET18</td>
<td>Ampicillin</td>
<td>Full length EmrE gene, N-terminal to T18 gene</td>
</tr>
<tr>
<td>pT18EmrE</td>
<td>Ampicillin</td>
<td>Full length EmrE gene, C-terminal to T18 gene</td>
</tr>
<tr>
<td>pEmrET25</td>
<td>Kanamycin</td>
<td>Full length EmrE gene, N-terminal to T25 gene</td>
</tr>
<tr>
<td>pT25EmrE</td>
<td>Kanamycin</td>
<td>Full length EmrE gene, C-terminal to T25 gene</td>
</tr>
<tr>
<td>pMBPH1T18</td>
<td>Ampicillin</td>
<td>EmrE TMH 1 gene, C-terminal MBP &amp; N-terminal T18</td>
</tr>
<tr>
<td>pMBPH1T25</td>
<td>Kanamycin</td>
<td>EmrE TMH 1 gene, C-terminal MBP &amp; N-terminal T25</td>
</tr>
<tr>
<td>pMBPH1rT18</td>
<td>Ampicillin</td>
<td>Reversed EmrE TMH 1 gene, C-terminal MBP &amp; N-terminal T18</td>
</tr>
<tr>
<td>pMBPH1rT25</td>
<td>Kanamycin</td>
<td>Reversed EmrE TMH 1 gene, C-terminal MBP &amp; N-terminal T25</td>
</tr>
<tr>
<td>pMBPH2T18</td>
<td>Ampicillin</td>
<td>EmrE TMH 2 gene, C-terminal MBP &amp; N-terminal T18</td>
</tr>
<tr>
<td>pMBPH2T25</td>
<td>Kanamycin</td>
<td>EmrE TMH 2 gene, C-terminal MBP &amp; N-terminal T25</td>
</tr>
<tr>
<td>pMBPH2rT18</td>
<td>Ampicillin</td>
<td>Reversed EmrE TMH 2 gene, C-terminal MBP &amp; N-terminal T18</td>
</tr>
<tr>
<td>pMBPH2rT25</td>
<td>Kanamycin</td>
<td>Reversed EmrE TMH 2 gene, C-terminal MBP &amp; N-terminal T25</td>
</tr>
<tr>
<td>pMBPH3T18</td>
<td>Ampicillin</td>
<td>EmrE TMH 3 gene, C-terminal MBP &amp; N-terminal T18</td>
</tr>
<tr>
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<td>Kanamycin</td>
<td>EmrE TMH 3 gene, C-terminal MBP &amp; N-terminal T25</td>
</tr>
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</tr>
<tr>
<td>pMBPH3rT25</td>
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<td>Reversed EmrE TMH 3 gene, C-terminal MBP &amp; N-terminal T25</td>
</tr>
<tr>
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<td>Ampicillin</td>
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<tr>
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<td>Kanamycin</td>
<td>Reversed EmrE TMH 4 gene, C-terminal MBP &amp; N-terminal T25</td>
</tr>
<tr>
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<td>Ampicillin</td>
<td>Polyalanine sequence, C-terminal MBP &amp; N-terminal T18</td>
</tr>
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<td>Kanamycin</td>
<td>Polyalanine sequence, C-terminal MBP &amp; N-terminal T25</td>
</tr>
<tr>
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<td>Ampicillin</td>
<td>Polyleucine sequence, C-terminal MBP &amp; N-terminal T18</td>
</tr>
<tr>
<td>pMBPLeuT25</td>
<td>Kanamycin</td>
<td>Polyleucine sequence, C-terminal MBP &amp; N-terminal T25</td>
</tr>
</tbody>
</table>
Figure 2.5 Schematic diagram of EmrE full length and single transmembrane helix fusion protein construction. A) an example of the full length EmrE constructs used in Chapter 3 (note the topology may have been inverted, but this topology would be required for a positive response from the assay) compared to B) a generalized single TMH construct which includes not only just the adenylate cyclase catalytic domain fusion, but an N-terminal maltose binding protein which aids to constrain topology further.
Figure 2.6 Plasmid map of the pMBPT18 vector used to clone single transmembrane helix constructs of EmrE. Inserts were composed of complementary annealed primers coding for the sequence of interest with overhangs matching the restriction sites for BamHI and EcoRI, and all inserts added no more than 105bp.
Figure 2.7 Plasmid map of the pMBPT25 vector used to clone the single transmembrane helix constructs of EmrE. Inserts were composed of complementary annealed primers coding for the sequence of interest with overhangs matching the restriction sites for BamHI and EcoRI, and all inserts added no more than 105bp
2.11 Co-transformation of Plasmids into *E.coli BTH101*

To transform plasmids into the correct strain for Miller assay screening, *E.coli* JM109 strains containing the sequenced plasmids of interest were grown 16h overnight at 37°C with shaking as 2mL LB with selective antibiotic cultures before being purified. Plasmids were purified using an Omega e.Z.N.A. plasmid purification kit with the microcentrifuge protocol. Plasmid pairings were simultaneously co-transformed using heat shock at 42°C into RbCl₂ competent *E.coli BTH101* cells. The transformed cells were plated onto MacConkey agar plates with antibiotics selective for both plasmids and grown at 30°C for up to 72 hours pending growth. Single colonies were selected from plates in order to make DMSO stocks for cultures and testing and stored at -80°C.

2.12 Membrane and Cytosol Preparations for Western Blotting

In order to perform Western blotting for construct localization, isolation of membranes from *E.coli* containing plasmids with the constructs had to be undertaken. Small volume membrane preparations were performed under growth conditions as similar to those of the Miller Assay as possible. 2mL LB with antibiotic cultures were grown from DMSO -80°C storage for 7h at 37°C with shaking. The entire 2mL of this culture were used to inoculate a 100mL LB with antibiotic culture which was grown overnight for 16h at 37°C with shaking. Following growth, the cultures were centrifuged at 4000rpm for 10min at 4°C using an SLA-1500 rotor, the supernatant discarded and pellet resuspended in 5mL chilled Lysis Buffer A in 4mL vials. Lysis was accomplished by adding 1mM final concentration PMSF before sonication using a Misonix Microson sonicator on ice in three rounds of 30 second bursts using 40% of the maximum power.
2mL of each sample of the cell lysate was centrifuged at 10000 rpm for 15 min and the low speed pellet was retained for diagnostics. The supernatant was transferred to ultracentrifuge tubes where it was ultracentrifuged at 95000rpm for 30 minutes at 4°C in a TLA 100.3 rotor. The cytoplasm collected as the supernatant was saved separately. The pellets representing the membrane fraction was resuspended in 0.1mL Lysis Buffer A using a homogenizer by hand. Samples were store at -80°C. Whole protein concentration for membrane and cytosolic fractions was determined through use of the Lowry assay.

2.13 The Modified Lowry Assay for Membrane Protein Quantitation

A modified Lowry assay was utilized to quantify the protein in the membrane and cytosol isolates. To prepare, the protein samples were made into 10x dilutions, and 9 parts Lowry Solution A was mixed with 1 part Lowry Solution B in sufficient quantity for the assay. 3mL of Lowry A/B solution was added to a series of test tubes to which the proteins samples and known amounts of Bovine Serum Albumin (BSA), used to make the standard curve were added. The tubes were vortexed and allowed to stand for 5 minutes before 0.3mL of an equal mixture of Folin reagent and Ciocalteu’s Phenol reagent were added to each tube. The tubes were again vortexed and incubated at room temperature for 1 hour to allow color development. Samples were transferred to cuvettes and absorbance at 750nm was recorded on a Hitachi U-2000 spectrophotometer, then a standard curve was constructed and protein concentrations estimated according to it.
2.14 Dot Blotting for Western Detection of Fusion Protein Construct Membrane Insertion

Similar in concept to Western Blotting, dot blotting was used as a control to determine if the constructs were inserting properly into the membrane and in which amounts relative to each other. Using a Bio-Rad BioDot apparatus 5µg of proteins for both cytosolic and membrane fractions were applied to a nitrocellulose membrane. Still inside the apparatus, the exposed portions of the membrane were subsequently washed with TBS and TBST, then the membrane was removed from the apparatus and placed into a 5% skim milk protein blocking solution to prevent nonspecific antibody binding to the membrane for 18 h at room temperature. The membrane was then washed again with TBS and TBST twice for 5 minutes each before primary antibody, polyclonal rabbit anti-CyaA (from Santa Cruz) was added and incubated with the blot for one hour at room temperature with shaking. Following this, the blot was washed twice with TBST for 5 min before the addition of Goat anti-rabbit HRP conjugate secondary antibody (Bio-Rad). A final washing step of two washings with TBST then TBS for 5 min was conducted, following which the blot was developed in Bio-Rad HRP development solution for 1 h before imaging using epi-white light on a Kodak Gel Logic 100 Imaging System.

2.15 Growth on Minimal Media for Topology Determination

In order to ascertain the correct topological insertion of the single-helix constructs, the plasmids containing the constructs were singularly transformed into *E. coli* HS2019, which is ΔmalE, using RbCl₂ methodology. The strains were grown as 200µL LB broth cultures with antibiotics for 16 hours at 37°C with shaking in a microtitre plate,
after which glycerol was added to the plate for storage at -20°C. The recombinant strains were subsequently plated after thawing using a replicator on minimal M9 media with 0.4% maltose and appropriate antibiotic and grown overnight for 16 hours before the plates were imaged using epi-white light on a Kodak Gel logic 100 Imaging System. Several controls were used, including the parent strain to *E.coli* HS2019, *E.coli* MC4100, HS2019 transformed with pMALp, a plasmid containing MBP with a proper signaling sequence targeting it to the periplasm, and HS2019 transformed with pMALc2, a plasmid for which the signaling sequence of MBP is removed, causing it to express in the cytoplasm.

### 2.16 Microtitre Plate Based Miller Assay

The microtitre plate based Miller assay was employed for the studies involving the library of constructs of single TMH co-transformed pairwise for higher throughput. In brief, strains were taken from DMSO stocks stored at -80°C and grown as 2mL cultures in culture tubes for 7h at 37°C in LB with 100mg/mL ampicilin and 50mg/mL kanamycin with shaking. Following that growth, 200µL of LB with ampicilin and kanamycin media in a microtitre plate was inoculated with 50µL of 7h culture, and grown for 16h with shaking at 37°C. The OD<sub>600</sub> of the plate was then examined using a SpectraMax M2 SoftMax Pro 5 plate reader and the cells were added to a microtitre plate already containing 100µL lysozyme lysis buffer in an amount equivalent to adding 42µL of OD<sub>600</sub> = 0.010 following the equation:

\[
((100\mu L)(0.010)) = ((X\mu L)(OD_{600}))
\]

So that
The plates were incubated at 4°C for 30 min, at which time 78 µL of Z-buffer + 1.64 mg/mL ONPG was added and the plates were incubated at 30°C for 30 min. The reaction was stopped and pH increased with 80 µL of 1 M Na₂CO₃ and the OD₅₅₀ and OD₄₂₀ were recorded using the plate reader. Miller units were determined using the Miller equation:

\[
\frac{1}{OD_{600}} = \mu L \text{ Culture added}
\]

\[\text{Miller Units} = 1000 \cdot \frac{(OD_{420} - (1.75 \cdot OD_{550}))}{T \cdot V \cdot (OD_{600})}\]

2.17 Replicates and Statistics

For the purposes of data analysis replicates for all trials of an individual sample in microtitre plates were derived from the same 2 mL culture produced from a stock in DMSO storage, which was pregrown for 7 h before using that culture to inoculate the required number of wells in a microtitre plate, and are thus technical replicates. For the Miller assay in culture tubes, a single culture was grown for each replicate from DMSO cryo storage, giving three biological replicates.

Outliers in the datasets were determined as values being outside two standard deviations of the population mean and were removed, after which the average of the remaining replicates was taken. Error was determined according to standard error of the mean, which is obtained by dividing the value of the standard deviation of the population mean by the square root of the number of samples. To determine if the sample means were significantly different from controls, a paired, two-tailed T-test was used with a 95% confidence interval.
CHAPTER THREE: ASSAYING OF FULL LENGTH EMRE CONSTRUCTS

3.1 Introduction

Reporter systems must be set up carefully because they depend on the cell location, spatial positioning on proteins, and binding equilibrium of the proteins being studied. This can, however, be used to an advantage given that with proper controls, experiments can be set up that clarify given theories about protein structure and interaction. EmrE, a protein for which considerable debate has occurred over topology and mode of interaction is an ideal candidate for such studies. Using a reporter system with EmrE is particularly challenging, given that its membrane embedded nature provides relatively few appropriate sites where a fusion protein could be created, the three options in EmrE being the N- or C-termini, or as part of the small loops between the transmembrane helices. Because of the requirement of spatial proximity, controls may be necessary wherein the fusion takes place in a different location in case the interaction would not bring the domains close enough. Given the proposed nature of EmrE interacting mainly through the fourth transmembrane helix, constructs have been created with the fusion on both the N- and C-terminus, and tested in those combinations. Here is presented studies utilizing N- & C-terminal fusion protein constructs to attempt to determine the mode of interaction between two monomers of EmrE (Figure 3.1).
Figure 3.1. Cartoon schematic of the BACTH system. In the BACTH system, one portion of the adenylate cyclase catalytic domain (T18 or T25) is attached N- or C-terminally to a monomer of EmrE, catalyzing the production of cyclic AMP from ATP, which interacts with a catabolite activating protein to induce the expression of a secondary reporter gene, β-galactosidase, which can be assayed using artificial substrate ONPG.

3.2 Experimental

The modified Miller assay was performed as described in Chapter 2, on pairwise co-transformed samples consisting of EmrE N- or C-terminally fused to the T18 or T25 domain of adenylate cyclase. A co-transformation of vector pUT18 and pKT25 were used as a negative control since they will only produce the T18 and T25 domains on their own, which are not expected to interact. A co-transformation between two Zip motifs was used as the positive control for binding due to its known very low $K_d$ of dimer dissociation.
3.3 Results

In order to characterize contacts in IMP EmrE, the BACTH approach was utilized here with full length monomers of EmrE, with the adenylate cyclase catalytic domains fused N- or C-terminally to search for interactions.

3.3.1 Miller Assay for Full-Length EmrE Constructs

The EmrE gene was cloned into the four vectors pUT18, pUT18C, pKT25 and pT25 to produce fusion proteins of EmrE cloned both either N- or C-terminally to each the adenylate cyclase catalytic T18 and T25 domains singly. These constructs were then co-transformed in all combinations into *E.coli* strain BTH101, as well as in combinations with the pZipT18 and pZipT25 control plasmids. The co-transformational combination of pZipT18 and pZipT25 served as a positive control for the system, utilizing two samples with a leucine zipper motif known to interact with each other in the cytoplasm and with each containing the domains of adenylate cyclase. This known interaction with a very low $K_d$ of dimer dissociation provides a baseline to which other samples can be compared. A co-transformation of plasmids pUT18 and pKT25 was used as a baseline negative control, which would express only T18 and T25 adenylate cyclase domains not in any sort of fusion and having no attachments that would cause them to regain functional proximity.

Having been shown to interact in numerous previous studies by use of techniques such as size exclusion chromatography and analytical ultracentrifugation (Butler et al., 2004), monomer swapping (Rotem et al., 2001), crosslinking (Soskine et al., 2002; Soskine et al., 2006), and the various established crystal structures, EmrE was expected to show a strong equilibrium binding in at least one combination of the fusion proteins.
The positive control for association produced the largest response as expected, showing over 900 Miller units and demonstrating that any lack of association was not due to error in experimental setup. This is no surprise as this is for a soluble subunit interacting in the cytoplasm and includes two proteins with known interaction (Karimova et al., 1998). No significant binding between any of the constructs paired with each other (Fig 3.2) or the negative control was observed, with cross comparison showing all samples occurring near a baseline value of less than 400 Miller units. The four controls utilizing Zip protein constructs behaved in a similar fashion as the samples, eliciting a baseline response (Figure 3.2).
Figure 3.2. Relative binding equilibrium strength of full length EmrE protomers using the Miller assay. N- and C-terminal fusions of adenylate cyclase catalytic domain fragments T18 or T25 were created as protein fusions with full length monomers of EmrE and co-expressed in E.coli BTH101 (Shown in grey), including negative controls for nonspecific interaction (Shown in white) and negative control using coexpressed T18 and T25 by themselves (Shown in black). Genes are listed within the plasmids in the order they occur from N- to C-terminal, with co-transformational partners separated by a slash.

3.4 Discussion

Unexpected when in context of the literature which shows multimeric forms of EmrE through experiments such as SDS-Tricine PAGE (Bay et al., 2010), crosslinking (Soskine et al., 2002), or EPR (Amadi et al., 2010), this BACTH approach to characterize
EmrE interaction using full length monomers failed to observe any strong binding equilibria. Unfortunately, it is difficult to draw any conclusions from this data alone due to the lack of significant binding observed (Appendix B, Table B.1).

There are several possibilities that could explain these results. Firstly, it is possible that the proteins merely did not interact, and this could be proposed for several reasons. Firstly, one must consider the size of the chimeric portions of the fusion protein. EmrE itself is 12kDa in size, which is notably smaller than either the T18 or the T25 domains attached to it, in which the numeral component of the title represents their sizes at 18 and 25kDa respectively. One possibility is that despite EmrE possessing a membrane translocation sequence, the preferences of the T18 and T25 fragments to be expressed cytoplasmically influenced the localization of the construct more than the preference of the smaller EmrE to be buried in the membrane. Deprived of its native cellular environment, especially one which restricts movement to the plane of the membrane and thus decreases the translational space that monomers must search for each other, it would then be unsurprising for a lack of binding to be observed. Another possibility is that the larger size of the reporter domains simply created a steric interference, being too large to allow the proper alignment required for an interaction between the EmrE portions. Given the success of later experiments with smaller protein segments attached to the same reporter domains however, steric hindrance is an unlikely conclusion. Another aspect that may factor into this is the size of the linker between the fused domains. The linkers in the constructs had roughly 6 amino acids between the EmrE and the adenylate cyclase catalytic domains, which is close to the average length of 6.5 amino acids for inter-domain linkers identified in a statistical survey of natural linkers.
(George et al., 2002). This relatively short linker could have been too short to allow either the EmrE monomer or the catalytic reporter domains the translational or rotational freedom required to properly interact with each other, making it possible that any interactions that took place were merely not observed.

Other speculations as towards a lack of EmrE interaction observed relate more strongly to the proposed structure of the EmrE dimer itself in comparison to the nature of the constructs. While data increasingly supports an antiparallel dimer as the native state for EmrE, the constructs, consisting only of N- or C-terminal fusions would only produce a response if the adenylate cyclase domains exist on the same side of the membrane, i.e. in a parallel topology. Furthermore, adenylate cyclase is only active when expressed in the cytoplasm, meaning the parallel dimer produced would be required to have both termini on the inside of the membrane to be effective. Given these restraints, the data could also potentially be interpreted to indicate either an outward facing parallel dimer (unlikely, given the data suggesting antiparallel dimers being the native state and the predilection of the adenylate cyclase fragments to be cytoplasmically expressed) or that an EmrE was being expressed in both topologies and interacting in a parallel manner, leaving the catalytic reporter from one of the constituent protomers in the dimer in the cytoplasm and the other in the periplasm, where no interaction could occur (Figure 3.3).
Figure 3.3. Schematic of potential orientations of EmrE with the corresponding positions of the T18 and T25 adenylate cyclase reporter domains. The separated domains of adenylate cyclase are only active if both are expressed cytoplasmically, potentially creating problems for their use in membrane proteins due if one or both of the domains is localized periplasmically. Here in the case of EmrE fusions, protein termini are not indicated due to the usage of both termini as attachment points in various combinations within the samples. Notably, only orientation C) is capable of allowing the positioning capable of producing a measurable response.
CHAPTER FOUR: DEVELOPMENT AND STANDARDIZATION OF A
MICROTITRE PLATE BASED MILLER ASSAY

4.1 Introduction

While the previous experiments into EmrE interaction (Chapter 3) had not provided the depth of insight hoped for, those experiments represented a starting point into what was proposed to be a more in-depth study of EmrE interactions. At the same time as the assaying for the full length protein fusions was underway, cloning had begun on a more targeted approach wherein individual transmembrane helices of EmrE were to be cloned as used as the bait and prey proteins (Chapter 5). This created an unexpected issue; the β-galactosidase assay defined in 1950 (Lederberg, 1950) which was developed into the Miller assay (Miller, 1972) accommodates a relatively low number of samples at one time, and the large amount of samples intended to be generated along with the need for replicate data made the task somewhat unfeasible to accomplish by manual labor using the assay as written.

The need for a greater sample throughput prompted the modification of the Miller assay to take advantage of 96-well microtitre plates. The Miller assay as defined by Miller in 1972 is conducted in culture tubes (Miller, 1972), and here a modified version which uses 1.5mL microfuge tubes and cuvettes for the assaying portion was applied (Chan et al., 2009)(Chapter 2). While the assay is effective, the number of tubes and cuvettes grows unwieldy with sample size, and the time required to pipette reagents to start and or stop the colorimetric reaction becomes increasingly burdensome to coordinate. Microtitre plates allow for one plate to host multiple samples, cutting down on materials as well as permitting the usage of a multichannel pipette, which greatly
increases the speed at which samples can be processed. The potential disadvantages include even more limited sample volume which can affect oxygen transfer rates (Reviewed by Duetz, 2007) and the requirement of specialized equipment, most notably a microtitre plate reader in order to actually perform the experiment.

The process of converting the assay to a microtitre plate format was relatively simple. The volumes of assay components were downscaled to the point where they would fit into the 300\(\mu\)L well of the microtitre plate, but this also required the concentrations of the reagents such as ONPG to be adjusted so that their final concentrations mirrored those of the traditional assay in the hopes that the results would be comparable (Chan et al., 2009; Miller, 1972). The most significant change to the process was that the traditional Miller assay permeablizes the cell membrane using vortexing with a small amount of toluene or chloroform. Since even the small amount of chloroform used in proportion from the traditional assay melts microtitre plates, a lysozyme lysis procedure for this purpose was implemented instead, which merely required a brief incubation of cell cultures in a lysis buffer (Section 2.5, Appendix A).

Additionally, the first trials of the adapted protocol gave much larger Miller unit responses compared to the traditional, and that there was inconsistency among replicate trials performed on different days forcing standardization of the assays. Several parameters had to be adjusted before consistent results were achieved, including the amount of starting inoculant or including pregrowth phases.

4.2 Experimental

Here, the Miller assay (Miller, 1972) was modified further from a protocol previously used in the Turner lab (Chan et al., 2009) to work with a standard 96-well
microtitre plate. Initially the only change was the substitution of chloroform cell permeablation with a lysozyme lysis procedure (Chapter 2).

When the initial protocol failed to give reproducible trends between assays using the two protocols potential sources of error had to be identified. Cold storage and DMSO storage can negatively impact the growth of a cell line while it recovers from the adverse conditions or diminish cell growth (Ansel, 1969). One thought that occurred was that the E.coli strains used contained plasmids with differing constructs in them which might alter the rate of recovery and the resulting growth of the strains before the Miller assay was applied. To try and account for this, a pregrowth phase was added before the 16h growth of samples for the Miller assay (Chapter 2.6).

Given that the addition of a pregrowth alone did not seem to help with standardizing the assay as much as hoped, another variable was attempted being the cell density of the inoculant. Thus the next change implemented was to standardize the amount of cells being inoculated before the 16 hour growth (Chapter 2.7).

In order to verify that the samples were all at a similar stage of growth and that the differences in the assay were not simply due to variability in growth rates due to the expression of the fusion protein constructs, a growth curve for the samples was performed (Chapter 2.9).

Lastly, the point at which the cell density was standardized was changed to consider variability not accounted for by the Miller equation. Instead of standardizing inoculant, the volume of cells added from culture into the Miller assay portion of the protocol was standardized by OD$_{600}$ (Chapter 2.16).
4.3 Results

In order to analyze the large number of samples produced to study the pairwise interactions of IMP TMH in BACTH fusion constructs, the Miller assay was modified here to a higher throughput and lower volume form utilizing microtitre plates.

4.3.1 Microtitre Plate Based Miller Assay

The first thing that needed to be tested was the modified assay developed using the microtitre plates instead of the traditional microfuge tubes to see if the samples were relatively consistent between the two (Fig 4.1). For this initial experiment the negative controls, consisting of the co-transformed parent plasmids pUT18/pKT25 were similar to each other, with the Miller unit reading for the microtitre plate value being 92% of the one from the microfuge tube value. The positive controls, again consisting of the pZipT18/pZipT25 co-transformation are the first and major indicator of a discrepancy between the methods, with the microfuge tube based control providing a value roughly 1200% higher compared to the plate-based assay. Both positive controls maintain their status as possessing the strongest binding equilibria within their respective type of assay, and the negative controls constitute the weakest binding.

Another large difference was observed in that the samples shown to be interacting above a baseline level of the negative controls were inconsistent between the two types of assaying. Using the microfuge tube method, the samples pEmrET18/pMBPH3T25 and pEmrET18/pMBPH4T25 were shown to have the strongest binding equilibrium, both being roughly twice that of the negative control (Figure 4.1). This was curiously shifted in the microtitre plate based assay to show pT18EmrE/pMBPH3T25 and pT18EmrE/pMBPH4T25 as having stronger kinetics, both around 25 fold higher than the
negative control value (Figure 4.1). At the same time pEmrET18/pMBPH3T25 and pEmrET18/pMBPH4T25, which were shown as having the strongest binding kinetics in the microfuge tube based assays were now proportionately among the weaker at about 5 fold the negative control value, still notably higher than observed for the microfuge tube assay. This differential result highlighted the need for some standardization of the microtitre plate based assay.
Figure 4.1. Miller assay comparison between microfuge tube and microtitre plate based assays. Miller assay performed on a variety of different samples including controls, single-helix construct co-transformations and co-transformations between single helix constructs and EmrE full length constructs both by modified Miller assay using microfuge tube based technique (Black) and the new developed microtitre plate based assay (White). Tube based assays n = 3 replicates while the microtitre plate samples n = 11, error bars represent standard error.

The first standardization attempt, which involved only the addition of a 7 hour pregrowth phase for the cultures produced results that were inconsistent even after only two trials. Both the positive and negative controls observed a roughly 20% difference.
between replicates, significant by standard error (Fig 4.2). The samples with the two strongest binding equilibria within the assay remained pT18EmrE/pMBPH3T25 and pT18EmrE/pMBPH4T25, indicating that the trends in strong activity were consistent within the microtitre plate based assay, however pT18EmrE/pMBPH3T25 was shown to be 96 fold higher than the negative control in the first trial and only 78 fold higher in the second, while pT18EmrE/pMBPH4T25 was 163 times larger than the negative for the first trial and only 44 fold higher in the next, showing significant error between the trials indicating that further standardization was required (Appendix B, Table B.2).
Figure 4.2 Replicate trials of the microtitre plate based Miller assay introducing a pregrowth step. Individual sets of Miller assay trials (Black and White for Trials 1 and 2 respectively) performed on a variety of different samples including controls, single-helix construct co-transformations and co-transformations between single helix constructs and EmrE full length constructs by modified Miller assay with an additional 7 hour pregrowth period for recovery from DMSO and cryo storage conditions before the 16h incubation. Sample replicates n = 8 and error bars represent standard error.
The next standardization attempt, which took into account a standardization of the addition of cells before the 16 hour incubation to see if initial inoculant conditions were affecting the end results of the assay gave back more promising but still error prone results (Appendix B, Table B.3). Both the positive and negative controls showed two trials similar to each other while the third was significantly different (Figure 4.3). While pT18EmrE/pMBPH3T25 and pT18EmrE/pMBPH4T25 still provided the strongest response relative to the other samples, even the baseline values were roughly 8 fold higher than for the previous standardization attempt. pT18EmrE/pMBPH3T25 and pT18EmrE/pMBPH4T25 showed two trials which had close values, and a third which was remarkably higher, varying between 9 and 208 fold larger than the negative control (Figure 4.3).
Fig 4.3 Replicate trials of the microtitre plate based Miller assay with standardization of cells before incubation. Replicate trials of the Miller assay (Trials 1, 2 and 3 in Black, Grey, and White respectively) performed on a variety of different samples including controls, single-helix construct co-transformations and co-transformations between single helix constructs and EmrE full length constructs by modified Miller assay with an additional 7 hour pregrowth period and with cells standardized to the equivalent of 100μL of OD$_{600}$ = 0.010 before the 16 hour incubation. Each sample is the result $n = 8$ replicates with error bars representing standard error.
4.3.2 Growth Curve for Representative Co-transformed Samples

The growth curve showed that all the co-transformed samples were leaving exponential growth after no later than 6 hours and were in stationary phase by about 10 hours of incubation, being well into stationary phase before the assay occurs at 16 hours incubation (Figure 4.4). The positive control sample notably stayed in the log phase much later, only leaving exponential growth at about 10 hours and becoming stationary close to the 16 hour mark. It also showed a much slower growth during that period with a more pronounced lag. The negative control showed a similar lag, but hit stationary growth by 10 hours and only grew to an OD$_{600}$ of roughly 0.2. There was much variation of the OD$_{600}$ between the samples, which ranged from about 2.5 to 0.5, and the positive control was stationary at about 0.4. Notably, these values were lower than those frequently obtained during the process of the Miller assay, which routinely range from 0.05 for a blank to over 1.0 for some samples.
Figure 4.4 Growth curves of co-transformed samples of *E.coli* BTH101. To identify phase of growth after 16h of incubation, cultures were observed in 2 hour intervals incubated at 37°C with shaking under a small layer of mineral oil to prevent dehydration over time. Each point represents the average n = 8 replicates.
Lastly, the final standardization attempt involved standardizing the cells directly before the assay took place. In this case the negative controls were consistent as hoped for, but the positive controls showed some amount of variation, being at about 287, 201, and 268 fold larger than the negative controls respectively, but the standard error was large in this case and indicated that the differences could be accounted for within error (Figure 4.5)(Appendix B, Table B.5). The samples with the two strongest binding equilibria were still observed as pT18EmrE/pMBPH3T25 and pT18EmrE/pMBPH4T25, and all replicates were statistically similar by standard error. All other samples were consistent according to standard error as well (Figure 4.5). As is typical for the samples, larger Miller unit values had larger range of standard error and more variance.
Figure 4.5 Replicate trials of the microtitre plate based Miller assay standardizing cells immediately prior to the assay. Replicates of the Miller assay (Trials 1, 2 and 3 in Black, Grey, and White, respectively) performed on a variety of different samples including controls, single-helix construct co-transformations and co-transformations between single helix constructs and EmrE full length constructs by microtitre plate Miller assay with an additional 7 hour pregrowth period and with cells standardized to the equivalent of 100μL of OD_{600} = 0.010 right before the assay was conducted. Each sample is the result n = 8 replicates with error bars representing standard error.
4.4 Discussion

In this chapter a microtitre plate based high throughput version of the Miller assay was developed using a particular molecular system, and undertook several optimization and standardization experiments to enable the generation of internally consistent results using samples from a variety of co-transformants.

The initial step, which included adding a 7 hour pregrowth to ensure that samples had recovered sufficiently from DMSO cryo storage was unsuccessful by itself. It stands to reason that the samples expressing whole EmrE construct compared to cytoplasmic protein controls or single transmembrane helices would exert different physiological effects on the cell even by their presence, and thus could require a different time until they were growing optimally. The large variation even between two trials of this procedure indicates that the differential is arising from another source. This pregrowth phase was maintained in subsequent assays both because it still represents more controlled growth conditions than simply growing using the ice crystals from cryo storage, and because it allowed the cells to grow in a format that could be measured for OD$_{600}$ values which were subsequently used to standardize the amount of inoculants for the 16 hour incubation.

The subsequent experiment, standardizing the OD$_{600}$ of inoculant before the 16 hour incubation, was similarly unsuccessful in that while two of the replicates were similar, a third replicate showed a significant statistical difference as an increase in assay kinetics above its similar trials (Appendix B, Table B.4). This indicated again that there was still variation in the methods which, while it still showed samples positive for interaction to have much stronger binding equilibria than samples which showed a near-
baseline response, were internally inconsistent in the reporting of the results, negating the quantitative aspects of the Miller assay which would promote its usage over more qualitative methods.

The growth curves of EmrE were performed to determine if the timepoint used for the Miller assay was contributing to variation in the assay due to differential rates of cell growth potentially stemming from the presence of the protein constructs. The curves indicate that stationary phase was reached well before the 16 hour time point at which the assay was conducted. Stationary phase was selected as the ideal phase to perform the assay under for several reasons. First, EmrE expression has been shown to be stable independent of the phase of cell (Kobayashi et al., 2006), meaning that EmrE should be present and interacting at any given phase. The second is that the assay requires a 16 hour incubation period, meaning a shorter growth time may not be viable given the lower OD$_{600}$ values of some samples before that time point. For standardization purposes, growth should be allowed to a time after which point all the cells will be in the same phase, instead of using an earlier time point where some samples, notably the controls, may be at different phases of growth. Using the stationary phase therefore provides a larger window during which all samples can be tested during the same phase, as compared to mid log phase, which may occur at a different time point within the same plate as shown in Figure 4.4. Lastly, growth and testing of EmrE after an overnight incubation does have precedent in the literature such as in resistance assays (Elbaz et al, 2008). Of note, work done in the Turner lab indicates EmrE expression behind the $tac$ promoter is constitutive without induction in a ‘leaky’ manner in sufficient quantity to
provide resistance, demonstrating the ability of the constructs to functionally accumulate in the cell (Bay et al., 2012; Unpublished data).

Finally, the experiment where a standardized amount of inoculants was added going into the Miller assay finally gave results where replicate trials were within standard error of each other (Appendix B, Table B.5). A high degree of standard error in samples such as the positive control was observed, but the fact that they were still statistically similar indicated that while the assay protocol can now produce internally consistent results, said results are not quantitative with a high degree of accuracy and may still need to be used for categorization purposes instead of strictly as numerical indicators of binding equilibria. The numbers obtained for interacting samples are still higher by several magnitudes than those seen in a typical Miller assay, but this effect may simply be the result of the denominators of the equation, which account for the volume of culture added to the assay by including that volume in mL as a denominator. Thus, using a smaller volume while obtaining similar OD\textsubscript{600} and OD\textsubscript{420} results would naturally shift that factor upward in an inversely proportional manner. Further optimization or derivation of a modified Miller equation could potentially reconcile the differences with the traditional assays scale, but these purposes merely required a internally standardized assay for high throughput assays (Figure 4.6).
Figure 4.6 Flow diagram of the standardized high throughput variant of the Miller assay.
CHAPTER FIVE: IDENTIFICATION OF CONTACT POINTS IN EMRE BY BACTH OF INDIVIDUAL TRANSMEMBRANE HELICES

5.1 Introduction

With the advent of the functional, standardized microtitre plate Miller Assay attentions were turned to the original intended purpose, which was to characterize the interactions between the transmembrane helices of EmrE in a helix by helix fashion. This would serve to highlight centers of interaction and develop a toolset by which the interactions of membrane embedded proteins could be analyzed. There is some precedent for work using the individual transmembrane helices of SMR family members. Peptides corresponding to EmrE homolog Hsmr were used to look for interactions using Blue-Native PAGE, FRET, and molecular modeling, finding an interaction only between two copies of TMH 4 (Rath et al., 2006). Artificial transmembrane peptides were created for the work which saw through circular dichroism that all peptides save the one representing TMH 2 had helical structure in both aqueous Tris buffer and Tris buffer with 20mM SDS, after which point TMH 2 was excluded from the study (Rath et al., 2006). The remaining three TMH were loaded onto PAGE gels to test for co-migration, both using strongly denaturing SDS gels and PFO gels, which are gentler on the proteins and it was found in the case of both gels that the only co-migration that occurred was between two copies of TMH 4 (Rath et al., 2006). FRET studies on the peptides indicated a three protomer per unit stoichiometry, which they interpreted as being a representation of dimer-tetramer equilibrium, concluding that TMH 4 must interact along two discrete interaction faces (Rath et al., 2006). Further work from the same group used synthetic peptides with identical sequence to Hsmr TMH 4 to disrupt interactions between an
active dimer of Hsmr and inhibit resistance to ethidium in vivo, showing that TMH 4 is capable of competitive interaction as a discrete unit (Paulsen et al., 2009).

Given the known dual topology insertion of EmrE the issue had to be addressed as to how topology of the individual TMH would be properly constrained within and transported to the membrane. To this end a more complicated fusion protein construct for each transmembrane helix in both topologies was created (Figure 2.5).

Maltose binding protein (MBP) is a periplasmic soluble protein that allows for the transport of maltose across the periplasm. The addition of an N-terminal fusion of a maltose binding protein to all constructs would provide the necessary signal sequence for transport across the membrane. It also allows for a topology control, because if the plasmid containing the construct is transformed into a ΔmalE strain growth of that strain should be absent or limited on minimal media with maltose unless the MBP is present and correctly translocated. Additionally, there are antibodies available against MBP and the other fusion partner adenylate cyclase, which allow for Western blotting of isolated membranes and cytosol to track cellular location and proper expression of the protein construct.

At this phase two additional controls were introduced in the form of polyleucine and polyalanine helices. These fusion protein constructs were created in the same form as the EmrE TMH constructs, with both the N-terminal MBP and the C-terminal T18 or T25 catalytic domains. When co-transformed with the other constructs these would control for non-specific interactions.

One objective of this work was to develop a map of the possible contact interactions in EmrE using the BACTH approach that was developed, on a TMH by
TMH basis in both a parallel and antiparallel topology pairing. This will allow for a mapping of helix contacts leading to a further understanding of EmrE monomeric structure and oligomerization.

5.2 Methods and Materials

The microtitre plate Miller assay (Chapter 2) was employed for using E.coli co-transformed pairwise with plasmids encoding the constructs for individual EmrE TMH (Figure 5.1). Controls for insertion and topology by growth on M9 minimal media, and controls for cellular localization and relative expression were done by dot blotting (Chapter 2).
Figure 5.1 Schematic of EmrE divisions of the full length monomer into individual transmembrane helices with amino acid sequence displayed. Note that while in the full protein the N- and C- termini are both on the same side of the membrane and can be located on either side, for all single helix constructs, the N- terminus will be periplasmic due to an N-terminal attachment to maltose binding protein.
5.3 Results

In order to evaluate the ability of the high throughput BACTH system to determine interactions between individual topologically constrained TMH from IMP, the system was applied here to develop a contact map of the TMH in EmrE.

5.3.1 Nomenclature

Several samples based on the same TMH have been developed here, and several variables can be attributed to them that must be distinguished. The helices from EmrE with native sequences are labelled H1, H2, H3, and H4 (Figure 5.1). The samples corresponding to these helices with inverted topology are marked with an r, stemming from the primer samples in which the amino acid order was manually reversed, becoming H1r, H2r, H3r, and H4r, respectively. Additionally each of these eight samples of TMH had to be cloned in constructs with both the adenylate cyclase T18 or T25 catalytic domain as a C-terminal fusion. As such, when the construct with the attachment of a particular domain is discussed, it will be appended to the helix nomenclature, for example, the third TMH of EmrE with inverted topology and the T18 fusion domain is notated as H3rT18. Discussion concerning TMH samples is independent of the particular domain fused to it (for example the combined cases of both H3rT18 and H3rT25) is simply summarized at H3r. Lastly, whereas data concerning these particular constructs is referred to using their sample names, such as H3r, discussion for that particular TMH in context of the entire monomer or data obtained in other experiments not using these particular constructs will refer to them as TMH 1-4 instead.

Two major issues have been raised with regards to the constructs and their designations within the interpretation of the results for single helix Miller assaying. The
first is that the helices within each EmrE monomer have two topologies, with TMH 1 & 3 facing one way and TMH 2 & 4 the other and constructs for all those helices in both topologies (Figure 5.2), so which constructs are the reversed constructs? The second issue it that for an α-helix, the side chains of the amino acids point slightly towards the N-terminus. EmrE has a dual topology, and our constructs force the N-terminus to be pointing toward the periplasm due to the chimeric attachment of a maltose binding protein. This means that even with the order of amino acids manually reversed to simulate the opposing N- or C- terminus inside topology, the side chains will be pointing towards the N-terminus of the construct, which is still facing towards the outside (Figure 5.3).
Figure 5.2. Schematic of EmrE monomers of each topological orientation, showing representative amino acid side chains as oriented towards the N-terminus of the protein. Helices shown in blue have the N-terminus in the periplasm and those in red have their N-terminus in the cytoplasm.

Figure 5.3. Schematic of TMH segments showing that a reversed sequence may contain the amino acids in the same order as a native sequence with inverted topology, but the side chain angles will be facing a non-native orientation for that sequence.

Firstly, with respect to the naming of the helices the nomenclature is assigned such that the helices with native sequence are denoted as H1, H2, H3 & H4. These helices will have the N-terminus facing the periplasm and the side chain angles of a native protein. The helices with reversed sequence, H1r, H2r, H3r and H4r, will also have their N-termini facing the periplasm, but will have the amino acids in the order of helices
where the N-terminus would normally be facing the cytoplasm and the side chains will be at non-native angles. The implications of this are that if the interactions of EmrE rely on specific interactions, such as knob-in-hole associations, the change in side chain angle may alter the structure of the TMH such that false positives or negatives are obtained, so this possibility is addressed in the results.

### 5.3.2 Minimal Maltose as a Control for Topology and Insertion

Given the issues surrounding the nature of EmrE as an IMP and a dual topology protein, it is essential to ensure that the constructs translocated to a position within the membrane and did so in the correct topology. One of the methods utilized to test for this was growth on M9 minimal maltose media, so that recombinant ΔmalE strains transformed with only a single plasmid should only be capable of growth if the MBP from the constructs achieve a periplasmic position (Figure 5.4). The plating on minimal maltose media shows growth after only 16 hours for individual transformants of all single TMH constructs from both the T18 containing constructs and the T25 containing constructs. On the plate with kanamycin, all of the controls failed to grow, which would be expected given that the control plasmids only contain a resistance marker for ampicillin. On the ampicillin plate, both the controls for pMALp and pMALc2 grew successfully, which was not expected given that pMALc2 should be unable to import maltose and be able to grow under these conditions.
To verify the topology of the single helix constructs in vivo, *E. coli* HS2019 transformed with plasmids containing the genes for the fusion protein constructs used for the Miller assay were grown on M9 minimal media + 0.4% maltose and appropriate antibiotics after 16h at 37°C. Growth indicates that maltose binding protein is oriented outward towards the periplasm. **#** in the plasmid name indicates that the construct has either the T18 or T25 adenylate cyclase domain as dictated by the label for the row.

5.3.3 Dot Blotting of Membrane and Cytosol Preps

An important control to verify the presence of the constructs in the *E. coli* membrane is the Western blotting of membrane and cytosol isolates to determine the cellular location since interaction data would be artifactual were it to occur in the cytosol.
or inclusion bodies. C-terminally located in the constructs were the adenylate cyclase T18 and T25 catalytic domains. Each construct needed to be made separately with both the T18 and T25 domains attached so that the samples could be tested using each in case of steric or rotational effects. These effects could potentially limit interaction between the domains if the interaction occurs between particular helical faces which hold the adenylate cyclase T18 and T25 domain active sites facing away from each other. Fortunately, antibodies capable of detecting these domains exist in the form of a polyclonal antibody capable of detecting either domain, making it possible to detect fusion protein cellular location by Western blotting. Since the adenylate cyclase fragments are only active cytoplasmically, combined with the growth on minimal maltose media showing the MBP is periplasmically located, it is certain that the constructs are located in the membrane, effectively bridging the gap across the membrane.

The dot blots for the single transformation samples show that all of the helix constructs are present in the membrane isolates as detected using the anti-CyaA (CyaA being the protein designation for adenylate cyclase based on its gene, cyaA) antibody (Figure 5.5). This is equally true for both samples from with T18 attachments and T25 attachments. The cytosolic isolates, in contrast, show less protein than the membrane isolates, indicating that the majority made it to the membrane (Figure 5.5). Also notably, given a standard mass of protein added to the blots, the resulting developed dots are all of roughly equal intensity, indicating that the expression levels of all constructs were similar (Figure 5.5). The levels detected in the cytosol vary more significantly between samples for the T18 attachments, and samples from T25 attachments appear uniformly weakly in the cytosol, meaning any significant binding must be taking place within the membrane.
The controls, which again consisted of untransformed *E.coli* HS2019, and HS2019 transformed with pMALp and pMALc2 provide a strong signal in the membrane fraction and a weak signal in the cytosol (Figure 5.5).

![Image of Western analysis dots](image.png)

**Figure 5.5 Dot blot Western analysis of membrane and cytosol isolates for identification of protein construct localization.** Isolates of membrane and cytosol fractions from *E.coli* HS2019 transformed with plasmids containing the genes for the constructs used for the Miller assay were screened by Western analysis using anti-CyaA antibodies. 5µg of total protein from the isolates was loaded onto each dot. ## in the plasmid name indicates that the construct has either the T18 or T25 adenylate cyclase domain as dictated by the label for the row.

### 5.3.4 Miller Assay of Control Helices

Effective use of both BACTH and the Miller assay require that controls be performed to provide a basis of comparison for both strong binding equilibria and the
baseline response. The positive and negative control to define strong and baseline binding equilibria were done in triplicate alongside the samples on every microtitre plate assay, both as a representative of positive and baseline binding equilibria, and as a way to ensure the consistency in the assay was maintained as established in Chapter 4. The average strength of binding equilibria represented by Miller units, averaged from the positive controls on every plate is 14991 Miller units, defined as 100% for relative comparisons, while the baseline response is 71 Miller units (Figure 5.6), with the P-value for the difference between the controls being $7 \times 10^{-24}$.

![Figure 5.6 Miller assay results for the positive and negative controls.](image)

**Figure 5.6 Miller assay results for the positive and negative controls.** Microtitre plate based Miller assay results for negative controls containing fusion constructs with maltose binding protein N-terminally fused to adenylate cyclase T18 and T25 domains co-expressed, and positive control for Zip fusion constructs to adenylate cyclase T18 and T25 domains co-expressed. Values represent the average of n = 33 replicates with error bars indicative of standard error.
The results from the polyalanine and polyleucine control constructs co-expressed with the EmrE TMH constructs seldom reached 5000 Miller units (See below), and within the overall data for the TMH construct pairings there tends to be two rough groupings, where many samples show weak binding equilibria represented by values from about 0-4000 Miller units and the majority of the strongest binding equilibria have Miller unit responses from about 6000+ (Section 5.3.5). Subsequently, the cutoff value was set to what was considered a strong binding equilibrium at the middle point of these groupings, at what was about the general maximum value for nonspecific interaction within the controls, which is 5000 Miller units, or 33.4% relative to the positive control, and about 70 fold higher than the negative control. Anything above this value is considered to have a strong binding equilibrium and is the focus of the results. Anything underneath this cutoff, while still valuable data, is generally to be considered as a weak, transient, or absent interaction.

We included the additional controls consisting of a polyalanine and polyleucine helix constructs as well as the Zip constructs to test for non-specific and aberrant interactions among the samples such as aggregation due to the hydrophobic effect which would create a false positive in the results. Interactions between two helices where one has shown an interaction with a nonspecific control may still represent a biologically relevant structural feature in EmrE, potentially even related to dual topology capability. Interactions between helices that have both shown interactions with a nonspecific binding control may not be biologically relevant and should be evaluated in context. The control samples are compared to the same controls as the single TMH samples to give a relative
strength of the binding equilibrium, with a cutoff for strong binding kinetics at 70 fold higher than the negative control.

Figure 5.7 BACTH assay of single helix constructs towards control polyalanine helices with the T18 fusion domain. Miller assay for EmrE and control single transmembrane helix fusion constructs co-transformed with constructs containing a polyalanine helix with N-terminal maltose binding protein and C-terminal adenylate cyclase domain T18, in microtitre plate based assays as described in Section 2.16. Each sample is the result of n = 8 replicates with error bars representing standard error.

The polyalanine T18 fusion control helix showed either weak or baseline binding equilibrium with all other constructs (Figure 5.7). Several samples with binding equilibrium above baseline values were observed, but none of the others were larger than the cutoff value. In the case of the polyalanine control with the T25 fusion (Figure 5.8),
H1rT18 showed a response above the cutoff that indicated strong binding kinetics at about 81 fold higher than the baseline value. H1 provided the next strongest response, occurring at just below the cutoff value at a 69 fold increase from the negative control.

Figure 5.8 BACTH assay of single helix constructs towards control polyalanine helices with the T25 fusion domain. Miller assay for EmrE and control single transmembrane helix fusion constructs co-transformed with constructs containing a polyalanine helix with N-terminal maltose binding protein and C-terminal adenylate cyclase domain T25, in microtitre plate based assays as described in Section 2.16. Each sample is the result of n = 8 replicates with error bars representing standard error.
Figure 5.9 BACTH assay of single helix constructs towards control polyleucine helices with the T18 fusion domain. Miller assay for EmrE and control single transmembrane helix fusion constructs co-transformed with constructs containing a polyleucine helix with N-terminal maltose binding protein and C-terminal adenylate cyclase domain T18, in microtitre plate based assays as described in Section 2.16. Each sample is the result of n = 8 replicates with error bars representing standard error.

The polyleucine T18 control helix only showed one occurrence of a strong binding equilibrium with any of the sample helices (Figure 5.9). Again, this was with H1 where a Miller unit response of 121 times the baseline value was observed. H1r was only 66 fold higher than the negative control, being below of the cutoff of 70 fold higher.
Figure 5.10 BACTH assay of single helix constructs towards control polyleucine helices with the T25 fusion domain. Miller assay for EmrE and control single transmembrane helix fusion constructs co-transformed with constructs containing a polyleucine helix with N-terminal maltose binding protein and C-terminal adenylate cyclase domain T25, in microtitre plate based assays as described in Section 2.16. Each sample is the result of n = 8 replicates with error bars representing standard error.

The other polyleucine control with the T25 adenylate cyclase fusion provided a more subdued response across the entire sample range, with no samples providing any response larger than 50 times the baseline, under the required value of 70 times the baseline to be judged a strong binding equilibrium (Figure 5.10).
Figure 5.11 BACTH assay of single helix constructs towards control Zip helices with the T18 fusion domain. Miller assay for EmrE and control single transmembrane helix fusion constructs co-transformed with constructs containing a Zip control with C-terminal adenylate cyclase domain T18, in microtitre plate based assays as described in Section 2.16. Each sample is the result of n = 8 replicates with error bars representing standard error.

The ZipT18 control construct showed a strong binding equilibrium with the ZipT25 control, as has been repeatedly observed and expected (Figure 5.11). None of the other samples however, cross the threshold value of 70 times the baseline response to be considered significant, though H1r and H2 approach the value, being 63 and 60 fold, respectively.
Figure 5.12 BACTH assay of single helix constructs towards control Zip helices with the T25 fusion domain. Miller assay for EmrE and control single transmembrane helix fusion constructs co-transformed with constructs containing a Zip control with C-terminal adenylate cyclase domain T25, in microtitre plate based assays as described in Section 2.16. Each sample is the result of \( n = 8 \) replicates with error bars representing standard error.

Finally, in the ZipT25 control, while the usual expected control response from the ZipT18/ZipT25 interaction took place, two other sample helices observed exceptionally large binding equilibria (Figure 5.12). H1 interacted even stronger than the positive control at 239 times the baseline value and H1r reported well above the cutoff at 159 times, only 70 fold being required to be considered a strong binding equilibrium. None of
the other samples provided a response considered significant, making the finding particularly of note.

5.3.5 Miller Assays for Single Helix Constructs

In order to develop a contact map of EmrE, BACTH constructs containing the individual TMH that make up the vast majority of EmrE structure were co-expressed pairwise and evaluated using the BACTH/Miller assay methodology. The largest issue with the resulting data from the single helix construct assays was how to effectively present it. The dataset is difficult to show trends in by more traditional graphs such as a 3D bar graph or a series of 2D ones, creating a need for a display through alternatives (Fig 5.13, Table 5.1). Thus the data here is also presented using figures created through use of the program Circos (Krzywinski, 2009). While not particularly effective at displaying trends in the data, one thing the 3D graph does highlight is that some of the samples show weak but nonzero binding equilibriums within the data and a smaller amount of samples with comparatively strong binding equilibriums, which are the ones of particular concern, hence the establishment of the cutoff value (Section 5.3.4).
Figure 5.13 3D graph for the results of the Miller assay for helix-helix interactions using EmrE single transmembrane helix constructs. Miller assay values for constructs containing individual EmrE transmembrane helices with either the T18 or T25 domain of the adenylate cyclase catalytic domain, co-expressed with each of the other constructs pairwise in order to gauge the binding equilibria via a modified microtitre plate Miller assay. The X and Y-axes represent the constructs in the format X-X where the X is the T18 then T25 construct being tested respectively. Values are the result of n = 8 replicates.
Table 5.1. Normalized Miller activities for all possible EmrE transmembrane helix interactions.\textsuperscript{1,2} Tabular results for the pairwise determination of BACTH response using a modified microtitre plate based Miller assay for constructs containing the individual transmembrane helices of EmrE with fusions of the adenylate cyclase T18 or T25 domain where values represent the fold change relative to a negative baseline control\textsuperscript{3}. Interactions above threshold value of 70 fold above the negative control for strong binding equilibria are colored blue.

<table>
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<th>1</th>
<th>2r</th>
<th>3</th>
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<td>161</td>
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\textsuperscript{1}Averages are a result of 11 technical replicates
\textsuperscript{2}Negative values represent samples where the colorimetric response was sufficiently low that the cell scattering correction in the Miller equation is greater, and should be treated as insignificantly different from baseline.
\textsuperscript{3}P-values for statistical difference from negative control are located in Appendix B, Table B.7

As a brief explanation for how to interpret Circos graphs, the categories from a table are plotted in a radial manner, with connections between the rows and columns on a graph represented as a connection between them on the circle, with thickness corresponding to their proportional component of the sum of the relative strength of all interactions (here, the Miller unit response of the interaction to the 100% set maximal signal). With the settings used here, thicker lines represent stronger kinetics and are placed overtop of other, weaker kinetics, and the samples have all been highlighted one by one in subsequent figures in red while the samples and interactions not being discussed in that figure are colored grey (they would have been rendered invisible for easier viewing of the relevant trends, but the program lacks that functionality as of this
writing). Also present in each figure is a ‘wheel and spoke’ diagram, showing the Miller unit response of one helix sample (represented at the center of the ‘wheel’) with the opposing helix samples containing the appropriate T18 or T25 represented as the ‘spokes’ and drawn radially with the associated number being the relative fold of the Miller unit values compared to the negative control.
Figure 5.14 Interaction diagrams of EmrE H1 construct with T18 fusion. A) Circos generated map with red traces showing the relative strength of binding equilibria of H1 having the T18 adenylate cyclase fragment with all other helices, weighted as a portion of the total Miller units observed. B) Wheel and spoke diagram showing the fold values of the interactions of H1 with all of the other helices relative to the negative control. Results of n = 8 replicates.
Figure 5.15 Interaction diagrams of EmrE H1 construct with T25 fusion. A) Circos generated map with red traces showing the relative strength of binding equilibria of H1 having the T25 adenylate cyclase fragment with all other helices, weighted as a portion of the total Miller units observed. B) Wheel and spoke diagram showing the fold values of the interactions of H1 with all of the other helices relative to the negative control. Results of n = 8 replicates.
Figures 5.14 and 5.15 represent the binding equilibrium of H1 with the gene sequence as occurs naturally in EmrE, and with the T18 and T25 adenylate cyclase domains, respectively. Figure 5.14 shows that the H1T18 sample has only one sample with a strong binding equilibrium, that being with H2r and therefore in antiparallel topology providing a Miller unit response of 134 times the baseline response. There are three other samples with weaker binding equilibria in antiparallel interactions with the inverted sequence samples H1r, H2 and H4, none of which reach the threshold cutoff at 70 times the negative control for strong binding equilibria. The corresponding interactions of H1T25 in Figure 5.13 show three samples with strong binding equilibria, those being a antiparallel interaction with H3 at 110 fold of the negative response and one with H4 being 125 fold. Only one sample with a strong binding equilibrium in an antiparallel interaction was found, with H4r at 147 times the baseline control value.
Figure 5.16 Interaction diagrams of EmrE H1r construct with T18 fusion. A) Circos generated map with red traces showing the relative strength of binding equilibria of H1r having the T18 adenylate cyclase fragment with all other helices, weighted as a portion of the total Miller units observed. B) Wheel and spoke diagram showing the fold values of the interactions of H1r with all of the other helices relative to the negative control. Results of n = 8 replicates.
Figure 5.17 Interaction diagrams of EmrE H1r construct with T25 fusion. A) Circos generated map with red traces showing the relative strength of binding equilibria of H1r having the T25 adenylate cyclase fragment with all other helices, weighted as a portion of the total Miller units observed. B) Wheel and spoke diagram showing the fold values of the interactions of H1r with all of the other helices relative to the negative control. Results of n = 8 replicates.
The results for the H1rT18 samples show an association with H4r at 174 fold relative value to the negative control, which is the only strong binding equilibrium in a parallel interaction observed for this sample group. An antiparallel interaction with a strong binding equilibrium to H2 was also observed at 133 fold above baseline (Figure 5.16). While the sample construct H1rT25 shows numerous weaker binding equilibrium values with H1, H2r, H3, H2 and H3r, none of those meet the strong binding equilibria cutoff. One antiparallel sample, H4 shows a binding equilibrium represented at 76 fold above the negative control value, and a strong binding equilibrium is observed with H4r in a parallel fashion (Figure 5.17). Interestingly, this means that for the H1 and H1r samples the most consistently strong interaction is a parallel one with the corresponding H4r or H4, respectively. Antiparallel interactions with strong binding equilibria between H1 and H2r as well as between H1r and H2 are only observed in the samples where H1 (in either topology) is attached to the T18 domain and T25 to the respective H2 sample.
Figure 5.18 Interaction diagrams of EmrE H2 construct with T18 fusion. A) Circos generated map with red traces showing the relative strength of binding equilibria of H2 having the T18 adenylate cyclase fragment with all other helices, weighted as a portion of the total Miller units observed. B) Wheel and spoke diagram showing the fold values of the interactions of H2 with all of the other helices relative to the negative control. Results of n = 8 replicates.
Figure 5.19 Interaction diagrams of EmrE H2 construct with T25 fusion. A) Circos generated map with red traces showing the relative strength of binding equilibria of H2 having the T25 adenylate cyclase fragment with all other helices, weighted as a portion of the total Miller units observed. B) Wheel and spoke diagram showing the fold values of the interactions of H2 with all of the other helices relative to the negative control. Results of n = 8 replicates.
The construct containing H2 with the T18 fusion showed a strong binding equilibrium with a parallel copy of itself, producing a response equal to 125 times the negative control (Figure 5.18). The remainder of the binding equilibria were quite weak by comparison, none being more than 25 times the baseline, and the binding equilibrium with H3 even occurring at baseline value. The T25 fusion construct again performed much better, displaying strong binding equilibria with helices H1r, H2, H3r and H4 having Miller unit values of 133, 125, 127, and 161 fold respectively, relative to the baseline control (Figure 5.19).
Figure 5.20 Interaction diagrams of EmrE H2r construct with T18 fusion. A) Circos generated map with red traces showing the relative strength of binding equilibria of H2r having the T18 adenylate cyclase fragment with all other helices, weighted as a portion of the total Miller units observed. B) Wheel and spoke diagram showing the fold values of the interactions of H2r with all of the other helices relative to the negative control. Results of n = 8 replicates.
Figure 5.21 Interaction diagrams of EmrE H2r construct with T25 fusion. A) Circos generated map with red traces showing the relative strength of binding equilibria of H2r having the T25 adenylate cyclase fragment with all other helices, weighted as a portion of the total Miller units observed. B) Wheel and spoke diagram showing the fold values of the interactions of H2r with all of the other helices relative to the negative control. Results of n = 8 replicates.
The results in Figure 5.20 show that H2r has two interactions with strong binding equilibria, one with another sample of H2r at 87 times the baseline control and one with H3 showing 99 times baseline. It also shows a weaker binding equilibrium with H4r under the cutoff at 60 times baseline response. A few weaker interactions with H1r, 2r and 4r were observed under the baseline. The corresponding T25 affixed sample for H2r has the most numerous and strong binding equilibria observed of any sample (Figure 5.21). H2r with T25 interacts with H1, H2r, H3 and H4r having binding equilibria of 134, 87, 169, and 109 fold higher than the baseline control respectively. It shows weak interactions for H1r, H2 and H4, but a strong binding equilibrium with H3r at 144 fold higher relative to the negative control.
Figure 5.22 Interaction diagrams of EmrE H3 construct with T18 fusion. A) Circos generated map with red traces showing the relative strength of binding equilibria of H3 having the T18 adenylate cyclase fragment with all other helices, weighted as a portion of the total Miller units observed. B) Wheel and spoke diagram showing the fold values of the interactions of H3 with all of the other helices relative to the negative control. Results of n = 8 replicates.
Figure 5.23 Interaction diagrams of EmrE H3 construct with T25 fusion. A) Circos generated map with red traces showing the relative strength of binding equilibria of H3 having the T25 adenylate cyclase fragment with all other helices, weighted as a portion of the total Miller units observed. B) Wheel and spoke diagram showing the fold unit values of the interactions of H3 with all of the other helices relative to the negative control. Results of n = 8 replicates.
H3 proved to have the weakest binding equilibria overall for all TMH. Figure 5.22 shows that only two interactions with strong binding equilibria were observed, one with H2r constituting 169 times baseline response and the other with H1 generating 110 times baseline value. Similarly, Figure 5.23 shows that the T25 version of H3 constructs has only one antiparallel interaction with a strong binding equilibrium, associating H2r at 99 fold negative control response, and is otherwise the lowest overall interacting sample, with some of the Miller unit values even reaching slightly into negative values (possible only if the light scattering correction using OD\textsubscript{550} in the Miller equation outweighs the OD\textsubscript{420} value produced) and all being statistically no different than the baseline value.
Figure 5.24 Interaction diagrams of EmrE H3r construct with T18 fusion. A) Circos generated map with red traces showing the relative strength of binding equilibria of H3r having the T18 adenylate cyclase fragment with all other helices, weighted as a portion of the total Miller units observed. B) Wheel and spoke diagram showing the fold values of the interactions of H3r with all of the other helices relative to the negative control. Results of n = 8 replicates.
Figure 5.25 Interaction diagrams of EmrE H3r construct with T25 fusion. A) Circos generated map with red traces showing the relative strength of binding equilibria of H3r having the T25 adenylate cyclase fragment with all other helices, weighted as a portion of the total Miller units observed. B) Wheel and spoke diagram showing the fold values of the interactions of H3r with all of the other helices relative to the negative control. Results of n = 8 replicates.
The inverse topology H3r samples followed much the same pattern as the native sequence ones did. The sample H3rT18 had only two interactions with strong binding equilibria, one with H2r at 144 times and the other with H2 at 127 times relative to the negative control binding equilibrium (Figure 5.24). All other samples fell well below the cutoff value. The sample with a T25 attachment had no binding equilibria stronger than 10 fold the baseline value, and thus seemed to be particularly poor in terms of interactions, with most again being indistinct from the baseline by standard error (Figure 5.25). Overall, between all the samples for H3 in both topologies, the only sample with a routinely strong binding equilibrium seems to be with H2, and generally a slight preference for antiparallel topology was observed.
Figure 5.26 Interaction diagrams of EmrE H4 construct with T18 fusion. A) Circos generated map with red traces showing the relative strength of binding equilibria of H4 having the T18 adenylate cyclase fragment with all other helices, weighted as a portion of the total Miller units observed. B) Wheel and spoke diagram showing the fold values of the interactions of H4 with all of the other helices relative to the negative control. Results of n = 8 replicates.
Figure 5.27 Interaction diagrams of EmrE H4 construct with T25 fusion. A) Circos generated map with red traces showing the relative strength of binding equilibria of H4 having the T25 adenylate cyclase fragment with all other helices, weighted as a portion of the total Miller units observed. B) Wheel and spoke diagram showing the fold values of the interactions of H4 with all of the other helices relative to the negative control. Results of n = 8 replicates.
The data for the native H4 TMH with the T18 attachment shows two strong parallel attachments, one to H1 at 125 fold, one to H2 at 162 fold, and one antiparallel attachment to H1r at 76 fold of the negative control value (Figure 5.26). The remainder of TMH pairing provided signals of interaction showing weak binding equilibria with values under 12 fold if not insignificantly different from the baseline. Figure 5.27 shows no interactions that occur above the cutoff value labeled as strong binding equilibria, but it may still be noteworthy that the strongest binding equilibria still occur between H1, H2r, and H1r. Overall for all of the H4 constructs, binding equilibria are shown to be strong with H1 in either topological configuration as a common thread between the samples, and a strong binding equilibrium between H4 and H2 is observed, but only in a parallel manner, and only when the H4 construct uses the T18 domain and the H2 construct is attached to the T25 domain.
Figure 5.28 Interaction diagrams of EmrE H4r construct with T18 fusion. A) Circos generated map with red traces showing the relative strength of binding equilibria of H4r having the T18 adenylate cyclase fragment with all other helices, weighted as a portion of the total Miller units observed. B) Wheel and spoke diagram showing the fold values of the interactions of H4r with all of the other helices relative to the negative control. Results of n = 8 replicates.
Figure 5.29 Interaction diagrams of EmrE H4r construct with T25 fusion. A) Circos generated map with red traces showing the relative strength of binding equilibria of H4r having the T25 adenylate cyclase fragment with all other helices, weighted as a portion of the total Miller units observed. B) Wheel and spoke diagram showing the fold values of the interactions of H4r with all of the other helices relative to the negative control. Results of n = 8 replicates.
Figure 5.28 shows the data for constructs of H4r using the T18 catalytic domain. In this figure, strong binding equilibria are observed between H4r and H1, at 147 and H2r, at 109 fold compared to the negative control. One other interaction with a strong binding equilibrium is seen, between H4r and the inverted topology H1r sequence, producing 105 fold the baseline value. The remainder of the interactions show weak binding equilibria, some even being statistically indistinguishable from the baseline values. The corresponding T25 fusion construct shows only one interaction with a binding equilibrium above the cutoff, that being an parallel interaction with H1r, coming out at 174 times the negative control value (Figure 5.29). The parallel interaction with H2r at 60 times the baseline had the next strongest binding equilibrium, but was still under the cutoff value, and the rest were much lower even than that.

5.4 Discussion

In this chapter, the BACTH system was utilized on the TMH of IMP EmrE to develop a contact map of the individual TMH in a pairwise fashion and to demonstrate the efficacy of the developed high throughput approach in the determination of IMP contacts.

5.4.1 Topology and Expression Controls

There were some inconsistencies observed with the controls chosen. Several factors may be involved in this. The M9 minimal media with maltose plates indicated that even without a signal sequence, growth on minimal maltose media could occur, albeit more slowly in a ΔmalE strain. This makes it difficult to support any conclusions concerning topology given that the only selection occurring seems to be from the
antibiotics present. One possible explanation is that the amount of maltose was insufficiently stringent to restrict growth, and simply slowing it. The fast growth after only 16 hours supports this interpretation.

The dot blots show their controls as occurring in the isolated membrane fractions, a result which is again troubling due to the use of anti-CyaA antibodies for detection. HS2019 is not ΔcyaA as is the BACTH strain, *E.coli* BTH101, and is likely to show a baseline amount of the protein expressed at a normal level for the cells, located in the cytoplasm where it is expected to be found. The blotting shows that CyaA is located in the membrane in significant concentration, though lower than the samples transformed with plasmids encoding fusion proteins containing the constructs and little located in the cytoplasm. The dots on the blot occur at similar intensities, meaning that the use of similar plasmids and constructs was sufficient to express proteins in similar amounts for these experiments, as opposed to having a large surplus of one construct or the other in a co-expression.

The blotting results are counterintuitive given the nature of the Miller assay results. Strong binding equilibria found within the co-transformed strains indicate by default that the T18 and T25 domains must be located cytoplasmically in an active form attached to the fusion constructs. Work with circular dichroism has shown that H2 from EmrE homolog Hsmr is in random coil conformation outside of the membrane, meaning that any interactions seen using it indicate that it is present in its native environment to be able to fold and interact (Rath et al., 2006), else a signal could not be produced. Growth on minimal media supports that the MBP must be at least transported to the periplasm.
5.4.2 Control Helices for Nonspecific Interactions

The inclusion of polyalanine and polyleucine helices as controls was done to detect if the constructs were associating merely due to hydrophobic effects instead of a true interaction. Using these control helices, binding equilibria were detected ranging from baseline to a 113% of the positive control value. The cutoff threshold was partially determined from this data as well, and set so that the all of these interactions barring a few notable exceptions fell below this value, determined to be 5000 Miller units (33.4% of the positive control value, and 70 fold higher than the negative baseline control). Aside from ZipT18/ZipT25 having a strong binding equilibrium, which was expected since they also represent the positive control because of their very low $K_d$ of dimer dissociation, only one particular helix occurred above the cutoff value with any consistency. H1r was observed to have a strong binding equilibrium at 81 fold above the negative control resulting from interaction with polyalanine with the T25 domain attachment, and H1 close to the cutoff value with 69 fold, which while technically under the threshold value is sufficiently close to be worth noting (Figure 5.8). A similar trend with these was seen for the polyleucine, where the T18 domain fusion H1 provided 121 times higher than the baseline, and H1r a value of 66 times higher (Figure 5.9). Expressed with the ZipT25 construct using the T18 fusion, both helices showed 239 and 159 fold increase over the negative control, H1 appearing to have an even stronger binding equilibrium than the positive control. No other helix provided similar results, especially against the Zip controls. Given that the binding equilibria with either topology of H1 occur fairly strongly with all of the controls, the data here seems to indicate that any interactions involving H1 may be through a nonspecific mechanism, potentially driven through
hydrophobic interactions. In context of the data from the co-expressed TMH constructs H1 which interacted in both a parallel and antiparallel manner with H4, and in this context the data may indicate that the driving force for the interaction is indeed nonspecific and hydrophobic, which would potentially provide and explanation for how the helices are able to interact in either topology to form a dimer.

One argument against nonspecific interaction is put forward by White and Wimley in their 1999 review, who state that partial charges on the termini of helices that extend beyond the hydrophobic core decrease the attractive force to a point where nonspecific interactions are unlikely. This would certainly be the case in constructs such as these, where the TMH are bounded by entire protein domains with relatively short linkers. It can be seen in the sequence in Figure 5.1 that EmrE is host to both small and larger hydrophobic residues, and it is not impossible that these could be arranged into motifs capable of interacting other helices, which could be probed using this methodology. Unfortunately, such experiments were beyond the scope of the present work.

5.4.3 Interaction Mapping of EmrE Based on Helical Contacts

Characterization of interactions with EmrE was much more effective using constructs of individual EmrE helices than it proved for constructs including full length protein. Not only were strong binding equilibria identified between the helix constructs compared to the general lack of such among full length EmrE constructs, but factors such as topology and more specific sites of interaction can be addressed through this methodology. However, one unfortunate aspect to this particular methodology is again that without extensive controls only positive results can be properly interpreted, since
negative results can be related to a variety of factors (See Section 3.4). In the cases where an interaction is observed between helices with the T18 and T25 domains attached in one combination but not the other, the unobserved interaction is interpreted as a false negative due to factors such as position or rotation of the helices only permitting interaction in one combination. In the discussion for this chapter, the results will be interpreted for their intrinsic meaning, and context with the existing literature will be the focus of the more general discussion in Chapter 6.

The study of EmrE interactions in this manner should either support an existing structure or provide enough structural elements that an alternative can be proposed. To date, and based on the available structural information EmrE is typically thought of as associating by means of a GG7 motif occurring on the TMH 4, with the other three constituting a binding pocket and not strongly contributing to dimerization. (Figure 5.30)(Fleishman et al., 2006; Rath et al., 2006; Bay et al., 2008; Korkov et al., 2009). Importantly, while they establish the positions of the helices in the structure, at 7Å and 4Å for the Cryo-EM and X-ray data respectively, they cannot specifically assign the identities to the helices they define (Ubarretxena-Belandia et al., 2003; Chen et al., 2007).
Figure 5.30 Published structures of EmrE proposing two differing helical arrangements. The assignment of the helices within an EmrE dimer is different in the structures as presented by A) Fleishman et al., 2006, derived from Cryo-EM electron density modeling at 7 Å, from which the helices could not be defined but were assigned such that helices A-D represent one EmrE protomer and helices E-H as the other, which is currently the more generally accepted model, and B) an alternative model from Gottschalk et al., 2004 created through computational derivation, showing a dimer structure composed of two helical bundles. Part A) reprinted from Fleishman et al., 2006 and part B) from Gottschalk et al., 2004, with permission.

From the data presented here, H1 was shown to interact strongly with H2 when they exist in a dissimilar topological state. This could be the result of two possibilities; either TMH 1 is at the dimer interface of a parallel dimer which has both TMH 1 and TMH 2 as part of the interaction face, or that TMH 1 and TMH 2 associate within a single monomer in a stable fashion. Being connected by only a short loop, the answer that makes more logical sense is that this interaction represents an intraprotomer connection between the two helices, or else a structure must be proposed that provides an association between the two TMH which only explains a parallel dimer. The fact that strong binding equilibria are only seen when the H1 helix, in either topology, is within a construct with
the T18 active domain, may indicate that there is a particular facial interaction that is either rotationally or sterically disallowed when the reporter domains are reversed.

More interesting is the other major interaction for H1, which is with H4. These binding equilibria are strong in both a parallel and antiparallel fashion, which is notable given the propensity for EmrE to dimerize in both these topological conformations. Whereas an interaction only observed in an antiparallel topology could be more easily interpreted to be occurring within a protomer than between them, those involved strongly in both topologies may account for a site of interaction between two protomers. This data then suggests that an interaction between H1 and H4 may constitute a major dimerization interface within a dimer of EmrE, which requires a model in which these helices associate unlike the currently accepted structure, which shows them as separate and with H3 between them.

The binding equilibria for H2 support the notion that it interacts with other helices within a single protomer. The vast majority of interactions for H2 were found for a topology matching what would be found in a monomer, and they were found for every other helix, but only in that topology save one antiparallel interaction between H2r and H3r. Given that EmrE is considered to be a helical bundle as determined by FTIR spectroscopy, it makes more sense for TMH 2 to be a central point within each protomer to which all the other helices interact than it does for TMH 2 to constitute part of an interaction face (Arkin et al., 1996). This does leave the question, however, of the parallel H2 interaction observed with H2. No easy answer can be pulled from this dataset alone, and this interaction may be the result of two nonspecific interaction sites used with other helices that can interact when their usual partners aren’t present, or perhaps it may hint
towards a potential interaction point with a second dimer, to create a dimer-of-dimers
tetramer as initially proposed by Ubarretxena-Belandia & Tate in 2004, especially given
that this interaction only occurs in a parallel fashion. TMH 2 of Hsmr was shown
previously to be in random coil conformation when studied using circular dichroism
methods, but strong binding equilibria here indicate that it must have attained a helical
structure, supporting the observations of environment being a strong factor in EmrE
structural characteristics and highlighting the further need for in vivo work or appropriate
mimetics in membrane protein work (Rath et al., 2006).

H3 is slightly more troublesome to place than those others, largely in part to the
fact that very few cases of strong binding equilibria were observed between it and the
other TMH. The observed interactions were all with H2, save for one parallel interaction
between it and H1, and the interactions with H2 tended also to occur more frequently in
the antiparallel topology. Given the proposed nature of TMH 2 to be a major contact
point within monomers, this interaction is likely of limited importance within the EmrE
structure, and an alternative conclusion could be that TMH 3 provides other important
roles, such as contributing important amino acid side chains to the binding site or even as
a structural spacer, allowing the pocket to be larger while other helices perform the bulk
of the interacting and transporting work.

The most puzzling result of the experiments was the notable absence of any
strong binding equilibria between H4 with H4 or H4r, representing either topological
combination. There is an abundance of literature which describes interaction between
these two helices as being the central driving dimerization face using alternative
techniques such as cross-linking, and the presence of this interaction has been noted on
many occasions (such as Rath et al., 2006; Poulsen et al., 2009; Poulsen et al., 2011).
Given the positive-only nature of the interpretation of BACTH results, it is difficult to be
sure as to why this was not observed. One possibility is that the constructs didn’t allow
the helices to tilt in a way required for them to interact. Given that the helices are
presumed to possess specific interaction faces, it is possible they did interact, but the
adenylate cyclase domains were not positioned properly while they were doing so,
perhaps being held with their own catalytic sites held apart (Rath et al., 2006). It is also
possible that under these particular experimental conditions, two copies of H4 simply did
not self associate, though the body of literature supporting this contention makes this
unlikely. Keeping the above factors in mind, provided that the helix mapping based on
this data allows the interaction between TMH 4 from each monomer observed by others,
the lack of such an interaction in this dataset provides no concerns towards validity in the
rest of the data.

Equally interesting are the interactions that H4 was found to take part in. It is not
surprising that H4 had strong binding equilibria with H2, that having been seen
previously. In this case it occurs only when H4 is attached to the T18 adenylate cyclase
domain, again suggesting that there may be a specific interaction on a particular position
between the two, perhaps on a particular helix face. The unexpected interaction was
between H1 and H4. These strong binding equilibria occur in both the parallel and
antiparallel modes when H4 has the T18 domain, and in the parallel mode when H4 is in
a construct with T25. While this interaction has been observed by crosslinking
previously, PFO PAGE failed to see association in Hsmr, and the commonly accepted
structure of EmrE places these helices such that an interaction is difficult to explain using
it (Rath et al., 2006). The ability to associate in both a parallel and antiparallel fashion observed here supports that this association likely makes up the dimerization interface. This arrangement allows two protomers to associate in either of the observed topological mixtures, and is supported by cross-linking data indicating that TMH 1 and 4 should occur in close contact (Soskine et al., 2002).

Given the nature of the interactions observed, the data then gives a set of constraints around which a model for interaction faces between protomers can be developed. A short summary of these constraints consists of several important points; 1) All helices within a single protomer should be in a position such that they can contact TMH 2, 2) TMH 1 and TMH 4 should be on the interaction face, and 3) TMH 1 and TMH 4 should be positioned such that they can interact in both a parallel or antiparallel fashion. Given the interpretation of the Cryo-EM structure of EmrE, the dimer is presented as two linear protomers, pressed together with one face interacting (Figure 5.30). This form is impossible under the constraints established, notably because of the isolated position of TMH 2 and the distance separating TMH 1 and TMH 4 (Figure 5.31A). Notably, a look at the paper which initially establishes this structure proposes it over other possibilities solely on the basis that their helices F and H are in close proximity and one end and therefore must be joined by one of the short extramembrane linkers (Ubarretxena-Belandia et al., 2003). Much of the subsequent structural research has been conducted on the basis that this assignment is correct.
Figure 5.31 Diagram of the results of the contact mapping data applied to the two proposed helical arrangements of EmrE. Top down schematic diagrams of helical arrangements of antiparallel EmrE dimers perpendicular to the plane of the membrane, displaying observed strong parallel and antiparallel interactions observed in this work, A) as suggested by cryo-EM and X-ray techniques (Ubarretxena-Belandia et al., 2003; Chen et al., 2007) and B) as in Gottschalk et al., 2004 suggested by in silico techniques without substrate. Double bars indicate the interactions found with both potential combinations of T18 and T25 fusions.

EmrE has been noted, however, to consist of a helical bundle as a monomer as detected by FTIR, which is inconsistent with the linear helix arrangement proposed when considering EmrE in the monomeric form (Arkin et al., 1996). Another structure, in which a parallel dimer was proposed consisting of two helical bundle conformations of EmrE protomers was proposed by Gottschalk et al. based on their computational work in
2004 (Figure 5.30B). Importantly, the Cryo-EM paper considered such a structure before ruling it out only on the basis of their unproven linkage between the F and H helices (Fleishman et al., 2006). Thus there is another model to which this data can be applied for relevance.

Looking at this model (Figure 5.30B) the helices are not as tilted as the FTIR data from Arkin et al. 1996 indicates, which means that the physical positions of the helices may differ in vivo, but the helical assignments should still be considered due to observed plasticity in EmrE. In this model the helical assignments for TMH 1 and 4 appear at the interface between the dimers, direct contacts existing between them. TMH 2 appears as part of the helical bundle in such a manner that every other TMH could potentially contact with a part of it. An antiparallel structure would have TMH 4 from each monomer in contact, but still in a fashion that would allow them to be spatially close to TMH 1.

The alternative structure would satisfy all of the constraints established in this work, as well as maintaining EmrE’s structure as a helical bundle. Furthermore, this structure can still be applied to the Cryo-EM structure if the assumption of a link between helices F and H is disregarded, leading to the conclusion based on this work and others that the identities of the TMH segments in the commonly accepted structures of EmrE have been misassigned.

Further analysis can be accomplished on the models of helical arrangement by looking at the placement of the helices in the models using a heat map table constructed based on the placements of the helices of which interactions could potentially be allowed based on their proximity to certain other helices. Assuming each helix is only capable of interacting with its direct neighbors, the heat map shown for the linear helices model and
the helical bundle model supported by the data are shown as Table 5.2 and 5.3, respectively.

Table 5.2. Heat map overlay of the table for expected helical interactions observed in the linear helix model of EmrE, normalized to a fold increase over the negative control. Green squares indicate expected potential interactions and red squares indicate interactions disallowed by lack of helical proximity in the model.

<table>
<thead>
<tr>
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<th>T25 Construct Helix</th>
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<tbody>
<tr>
<td></td>
<td>1 2r 3 4r 1r 2 3r 4</td>
</tr>
<tr>
<td>1</td>
<td>7 134 -1 3 48 39 2 46</td>
</tr>
<tr>
<td>2r</td>
<td>18 87 99 60 45 49 2 69</td>
</tr>
<tr>
<td>3</td>
<td>110 169 10 15 53 30 6 1</td>
</tr>
<tr>
<td>4r</td>
<td>147 109 2 10 105 29 10 3</td>
</tr>
<tr>
<td>1r</td>
<td>16 19 2 174 11 133 1 40</td>
</tr>
<tr>
<td>2</td>
<td>15 0 0 21 25 125 1 12</td>
</tr>
<tr>
<td>3r</td>
<td>8 144 -1 7 35 127 1 1</td>
</tr>
<tr>
<td>4</td>
<td>125 12 0 3 76 161 1 1</td>
</tr>
</tbody>
</table>

Table 5.3. Heat map overlay of the table for expected helical interactions in the helical bundle model of EmrE, normalized to a fold increase over the negative control. Green squares indicate expected potential interactions and red squares indicate interactions disallowed by lack of helical proximity in the model.

<table>
<thead>
<tr>
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<th>T25 Construct Helix</th>
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<tbody>
<tr>
<td></td>
<td>1 2r 3 4r 1r 2 3r 4</td>
</tr>
<tr>
<td>1</td>
<td>7 134 -1 3 48 39 2 46</td>
</tr>
<tr>
<td>2r</td>
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<td>3</td>
<td>110 169 10 15 53 30 6 1</td>
</tr>
<tr>
<td>4r</td>
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</tr>
<tr>
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</tr>
<tr>
<td>2</td>
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</tr>
<tr>
<td>3r</td>
<td>8 144 -1 7 35 127 1 1</td>
</tr>
<tr>
<td>4</td>
<td>125 12 0 3 76 161 1 1</td>
</tr>
</tbody>
</table>

The heat maps for potential interactions show a similar number of potential interactions, with the linear helix model having 32 possibilities and the helical bundle model having 28. In both cases, a dimer is assumed as the structural unit, and in all
subsequent analysis the potential for interaction between identical helices (i.e. H1-H1, H2r-H2r, etc) is ignored due to lack of biological relevance in an antiparallel dimer of EmrE.

Tables 5.4 & 5.5 show the heat maps of the interactions observed in the experiments colored based on whether those interactions are allowed in the linear helix and the helical bundle models, respectively.

Table 5.4. Heat map overlay of the table for helical interactions observed experimentally with expected interactions of the linear helix model of EmrE, normalized to a fold increase over the negative control. Green squares show expected potential interactions and red squares show interactions disallowed by lack of helical proximity in the model.

<table>
<thead>
<tr>
<th>T25 Construct Helix</th>
<th>1</th>
<th>2r</th>
<th>3</th>
<th>4r</th>
<th>1r</th>
<th>2</th>
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<tr>
<td>1</td>
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<tr>
<td>2r</td>
<td>18</td>
<td>87</td>
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<td>4r</td>
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<td>109</td>
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<td>105</td>
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<td>10</td>
<td>3</td>
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<tr>
<td>1r</td>
<td>16</td>
<td>19</td>
<td>2</td>
<td>174</td>
<td>11</td>
<td>133</td>
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<tr>
<td>2</td>
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<td>3r</td>
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<td>4</td>
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<td>12</td>
<td>0</td>
<td>3</td>
<td>76</td>
<td>161</td>
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</table>
Table 5.5. Heat map overlay of the table for our helical interactions observed experimentally with the expected interactions helical bundle model of EmrE, normalized to a fold increase over the negative control. Green squares show expected potential interactions and red squares show interactions disallowed by lack of helical proximity in the model.

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<tr>
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<th>T25 Construct Helix</th>
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<td>18  87  99  60  45  49  2  69</td>
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<td>110 169  10  15  53  30  6  1</td>
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<td>147 109  2   10  105 29  10 3</td>
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<td>1r</td>
<td>16  19  2   174 11  133 1  40</td>
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<td>15  0   0   21  25  125 1  12</td>
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<tr>
<td>4</td>
<td>125 12  0   3   76  161 1  1</td>
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Table 5.4 shows that the linear helix model allows only three of the sixteen identified interactions, demonstrating that it is a poor fit for the dataset. Table 5.5 meanwhile indicates only three interactions that are disallowed, two of which constituting a TMH 2 - TMH 2 interaction merely due to it being biologically irrelevant to an antiparallel EmrE dimer. The only interaction which cannot be explained directly in the helical bundle model is H2r-H3r, which does not fit well into either model.

Lastly, to address the issue of potential false positives or negatives due to changes in side chain angles as discussed in Section 5.3.1, Table 5.6 shows highlighted the samples in which the binding equilibrium provided values above a cutoff threshold determined through the use of polyalanine and polyleucine controls. In this table sixteen interactions are identified, consisting of pairings of two constructs with native sequence, pairs with two reversed sequence helices and dimers of a native sequence helix with a reversed helix.
Table 5.6: Normalized Miller activities for all possible EmrE transmembrane helix interactions. Associations between two native sequence or two reversed sequence helices highlighted in green, and associations between a native and reversed sequence highlighted in red.

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<tr>
<th>T25 Construct Helix</th>
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<td>3r</td>
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Table 5.6 shows that among the interactions identified by the experiments, there is no strong bias favoring either interactions between pairs of native or reversed sequence helices or pairs with a combination of them. Interactions between helices with both native and non-native side chain angles were found for nearly all samples. When just the interactions between the native sequence helices are considered, interactions between H1-H3, H1-H4, and H2-H4 are present, and by themselves indicate that the linear helix arrangement is implausible given the helical placements. When the associations between just the reverse samples with non-native side chains are considered, binding between H1r-H4r, H2r-H3r, and H2r-H4r, are found, mirroring most of the interactions found in the native sequence samples and supporting the interactions of samples utilizing reversed sequences as legitimate. The remainder of the binding between construct pairs with both a native and a reversed sequence fits well within the context of the model supported by the interactions from only native sequences, suggesting that the difference in the angle of the sidechains did not significantly impact the results.
Summarily, based on the data obtained here and structure models available of EmrE, it is proposed that EmrE protomers retain their helical bundle nature even within a dimer instead of existing in a more linear form, and that the dimer interface involves TMH 1 and 4 of each protomer (Figure 5.31B). Given the broader implications of this claim, fitting it into the larger volume of available data will be the subject of larger discussion (Chapter 6).
CHAPTER 6: LITERATURE COMPARISON

6.1 A Broader Context

Previous chapters discussed the data in terms of its own meaning and implications. The aims were initially defined as;

i) Generate vectors with the elements of the BACTH system that also allow for control of orientation in the membrane described in Chapters 2 & 5

ii) Develop a High throughput assay as described in Chapter 4

iii) Test the approach with EmrE to evaluate its ability to sense dimers and higher order multimers as described in chapter 4

iv) Use the developed system to make a contact map of the transmembrane helices of EmrE as demonstrated in chapter 5.

The specificity of helix-helix contacts for IMP is an elusive question, especially for small protein systems such as EmrE where amino acid residues may be forced to take on multiple roles such as binding substrate and providing structural stability (Sharoni et al., 2005). The approach here, which has been the development of a two-hybrid system for application with IMP in a high throughput manner to allow for helix contact mapping has allowed the observation of some of these specific interactions, but how do they compare to existing data?

6.2 The Peg is Square, What Shape is the Hole?

Much of the work accomplished has been with the aim of developing a tool with which the interactions within and between integral membrane proteins can be examined. EmrE is useful as a model system in this endeavor because of its highly membrane
embedded state and sensitivity in terms of multimerization, function or topology to alteration by protein fusion (Rapp et al., 2007), mutation (Poulsen et al., 2011), or experimental conditions (Bay et al., 2010). The usefulness of any scientific tool should be judged from the accuracy of the data and predictions, and so the model of the helical contacts of EmrE developed must be evaluated based on data in relation to the broader literature to see how well it correlates.

The data presented here strongly presents a particular picture of EmrE structure, and while there is precedent in literature for such a structure (Section 6.4), it differs from the commonly accepted one. The fact that the majority of subsequent experiments on EmrE base their design and interpretation on a different structure means that the helical arrangement proposed here must be reconciled with more than a decade of work. Subsequently, the focus of the discussion presented here is not just to summarize the conclusions reached here but to further discuss how they are affected by the major experiments in the established literature.

### 6.3 The Helical Bundle Model Based on Pairwise Interactions

The proposed model was built based on pairwise interactions between individual TMH from EmrE, in both parallel and antiparallel combinations using the BACTH approach *in vivo* to determine which helices were capable of association (Chapter 5, Figure 6.2c below). This has the marked advantage of providing localized interaction information as well as removing any questions regarding topology. The resulting proposition is that the structure of an EmrE dimer is composed of two antiparallel
protomers, each maintaining a helical bundle configuration, interacting on the face of the bundle which contains both TMH 1 and TMH 4 (Figure 6.1c, Figure 6.2c).

![Diagram](image)

**Figure 6.1. Electron density helix identification and proposed assignments of the transmembrane helices in EmrE.** A) Electron density projection of EmrE with helices modeled in and labeled nonsequentially as published in 2003 by Ubarretxena-Belandia et al., Figure 5b, along with B) their proposed assignments of the helices in each protomer labeled a different color and C) the identities of the helices as proposed here. Part A) reprinted from Ubarretxena-Belandia et al., 2003, with permission.

The structure proposed originally from Cryo-EM electron density (Ubarretxena-Belandia et al., 2003)(Figure 6.1a) is significantly different, being a roughly linear arrangement of helices in the order 2-1-3-4 (corresponding to A-B-C-D in Figure 6.1 by their helix labeling conventions in which A is TMH 2 and B is TMH 1) which interacts with the other monomer along one side (Figure 6.1b, Figure 6.2b). The structure proposed here can still utilize the electron density mapping and merely suggests a different assignment of the TMH identities, proposing a helical bundle arrangement of the helices in both monomers (Figure 6.1c, Figure 6.2c). It is also worth noting that the arrangement of helices proposed in this work assumes a dimeric state of EmrE which is
the minimal functional unit, but other multimeric states are possible (Chapter 1). While
this data cannot assess oligomerization beyond a dimeric state, observations such as
parallel interactions between H2 with another H2 (Chapter 5) do not exclude a higher
order multimer and in fact may even contribute to a tetrameric state as has been observed
in literature (Rath et al, 2006; Tate et al., 2003).

Figure 6.2 Structures of Antiparallel EmrE with differing helical assigments. Here,
the arrangements of the helices in EmrE provided by Cryo-EM electron density maps are
shown A) without assigning helix identities and merely showing the arrangement, B)
with helical assignments as originally given by Ubarretxena-Belandia et al., 2003, with
one protomer colored blue and the other red, and C) the structure proposed here as
supported by interaction mapping data from BACTH (Chapter 5) again with each
protomer colored separately.

6.4 Structures of EmrE

The first actual structure of EmrE to be developed was in 2001 by Tate et al.. The
structure was done by Cryo-EM, on EmrE purified and reconstituted into bilayers in
dimyristoylphosphatidylcholine from which hydrated crystals were created. This was
used to create a 2D projection structure with a resolution of only 7Å, which was
interpreted as two arcs of highly tilted helices across from each other and nearly
perpendicular to the membrane (Figure 6.3a). Interpretation of this density map was
based off the structure of bacteriorhodopsin, and is consistent with the helix tilt angles from FTIR data (Arkin et al., 1996).

![Cryo-EM electron densities for EmrE in apo and TPP bound forms.](image)

**Figure 6.3** Cryo-EM electron densities for EmrE in apo and TPP bound forms. Cryo-EM work on EmrE has presented structures based on A) 2D projection map of EmrE in dimyristoylphosphatidylcholine crystals as observed by Tate et al., 2001., Figure 3b B) Electron density difference map between native and TPP+ bound structures of EmrE obtained by Tate et al., 2003., Figure 6. In both structures, P represents their interpretation of transmembrane helices perpendicular to the membrane, T represents a tilted helix, and A represents what they interpret as an arc of 4 tilted helices. Reprinted with permission from Tate et al., 2001 and Tate et al., 2003, respectively.

The next structure to be published was again by Tate et al. in 2003, this time including the presence of the non-natural substrate TTP+. The structure remained largely the same even when bound to TPP+, showing largely significant differences in electron density only where the TPP+ was proposed to be binding and one of the tilted helices interpreted to be rotating away from the center of the structure (Figure 6.3b). Using these maps, a subsequent paper attempted to assign identities to the helices they observed, making the very relevant decisions to ignore existing crosslinking data (discussed in Section 6.6) because of the then-unknown oligomeric state, and to presume the position of one of the extramembrane linkers due to a small band of electron density connecting...
two helices (Ubarretxena-Belandia et al., 2003) (Figure 6.1a). Now that the dimeric state of EmrE has been established as the minimal functional unit (Butler et al., 2004) and antiparallel topology is considered to be the native, stable state for a dimer (Lloris-Garcera et al., 2012) the decision to ignore the crosslinking can no longer be justified. The model put forward is analogous to the second model presented by the paper (Figure 6.1c, Figure 6.4a) which was discarded only because of the proposed helix F-H connection (Ubarretxena-Belandia et al., 2003). While the presence of a bridge in the electron density cannot be denied, it is important to note that similar connections also exist in other places such as between helices C-B, D-E, or G-H, and none of the models they put forward (Figure 6.4) can be presumed without separating at least one other linkage, making the presumption of a linker a particularly poor criterion from which to judge helical associations.
Figure 6.4 Schematics of EmrE possible helix assignments proposed by Cryo-EM and the helix mapping onto electron density used for selection of a model. Schematics from Ubarretxena-Belandia et al., 2003, Figure 5a and 5b respectively, of the possible identities of the helices of EmrE for the structure derived from Cryo-EM microscopy. A) Models of the potential dimer, where a red dot indicates the binding place of TPP and green curves indicate a putative dimer interfaces. B) View of their model derived from the electron density map, with a solid white arrow indicating electron density between helices F and H, and a dashed arrow indicating the location of binding for TPP\(^+\). Reprinted from Ubarretxena-Belandia et al., 2003, with permission.

A computational model was produced by Gottschalk et al. (2004) who created monomers using canonical helices using EmrE sequence which were subsequently minimized in terms of free energy through molecular dynamic simulation with helical tilt applied as the average from the 1996 FTIR experiments by Arkin at al., which was 27°. Dimerization was simulated using a slow cool-simulated annealing/molecular dynamics procedure in which the monomers were separated by 12Å and the simulation was cooled to 0°K. Fifty structures were generated and clustered, and the largest cluster taken for evaluation.
The end result was a parallel symmetric dimer (Figure 6.5), and antiparallel
dimerization was not attempted because the paper was in collaboration with the
Schuldiner group who were among the strongest proponents of a native parallel dimer at
that time (Reviewed by Schuldiner in Schuldiner, 2007). Even though it has since been
established that the native topology of the dimer is antiparallel, this structure is still
useful because active parallel dimers have been shown multiple times, and must be
explained in context of a structure, something which a linear arrangement of helices has
conceptual difficulties. The helical bundle model proposes that the Gottschalk structure
may represent a more accurate picture of parallel EmrE because of incorrect assignment
of the helix identities in the other proposed structures.
X-ray crystallography structural analysis of EmrE was initially done in 2004 by Ma and Chang and repeated in 2005 by Pornillos et al., but both structures were subsequently retracted due to computational errors resulting from a sign change in data processing. A final, corrected, structure was published in 2007 which was similar to the Cryo-EM structures and furthermore strongly supported the dual topology model of EmrE structure before it became widely accepted (Chen et al., 2007). They interpreted the diffraction pattern to a 4.5Å resolution, showing a similar proposed arrangement of helices, with TMH 1, 2, and 3 forming a binding pocket and TMH 4 interacting with the TMH 4 from the other monomer (Figure 6.6c). Some issues with this structure result from details that cannot be reconciled with biochemical data, such as TMH 4 from both protomers kinking and sticking out laterally from the dimer (Figure 6.6a), and even the authors conclude that the structure may be artifactual resulting from acid-induced partial denaturation.
Chen et al provided structures for EmrE bound to TPP⁺ in three different crystal forms, in this case obtaining a resolution as high as 3.8 Å (Figure 6.6b), which allowed the determination of visible loop densities for the assignment of the helices, as well as obtaining the locations of TMH 1 and TMH 4 by using protein containing selenomethionine for positioning. This model aligns very well with the Cryo-EM model, and much of the sequence conservation and residue placement data available (Fleishman et al., 2006). Similarly to the Cryo-EM model however, this model has difficulty reconciling with biochemical data to explain interactions such as between TMH 1 and
TMH 4 obtained by crosslinking data, and chooses to address this by arguing that crosslinking data underestimates distances in dynamic structures rather than to reconcile them within the model they propose. Even if this supposition is true, the crosslinks observed are between TMH 1 and TMH 4, which would still have to link and interact across the entirety of TMH 3 in their model, which would likely disable protein function. Lastly, and as noted by Nasie et al. in a 2010 article, the detergents used to obtain these structures, N-nonyl-β-D-glucoside & octyl glucoside, were observed previously to disrupt dimerization and inactivate the protein, indicating that the structure represented may be denatured or non-native and not truly representative of EmrE (Soskine et al., 2006).

The most recent Cryo-EM work to be done on EmrE was again by Korkhov and Tate in 2008. This time the work focused on changes to the binding site upon binding of three different substrates and revealed that a large amount of plasticity is found in EmrE depending on the substrate being transported. Otherwise the structures were largely similar in the placement of the helices, but importantly, the connection between the helices which they had previously labeled F & H is not seen in any of their subsequent projections, further determining it as a poor criterion for the assignment of a linker as discussed above. Overall, the same issues with helix assignment existing in the previous structures still exists here, and these structures should serve as an additional reminder of the large degree of plasticity within EmrE indicating the need for biochemical data to corroborate aspects of structures derived from crystals.

Overall, the history of EmrE structure is largely based off the assumptions made by Ubarretxena-Belandia et al. in 2003, whose helix assignments were made outright ignoring some of the established biochemical data of the time. The TPP bound X-ray
structures are of sufficient resolution that some large amino acid side chains can begin to be implied, which match up well to mappings of the amino acid structure onto the Cα backbone. The Cryo-EM structures thus far are unable to determine helix identity conclusively by themselves, and the lack of a map of amino acid residues onto the electron density following the proposed model means that even the resolution advantage, which was only sufficient for the assignment of Met residues and some large aromatic amino acids, cannot strongly imply that the proposed helical assignments based on biochemical data are incorrect.

An NMR study on EmrE was performed using solution NMR on EmrE solubilized in isotropic bicelles by Morrison et al. in 2012, who observed two sets of peaks representing an asymmetric dimer of an antiparallel topology, backed by FRET and crosslinking experiments (Morrison et al., 2012). Additional work using a soluble, chelated, paramagnetic gadolinium compound was able to determine water accessibility, noting that residues on the ends of the TMH of EmrE have differential water accessibility, consistent with their transport model (Morison et al., 2012). While their interpretation of their results supports the model put forward be Fleishman et al. in 2006, their results are acceptable with the structure proposed here, though mapping of residues onto the structure according to the helical bundle arrangement has not yet been performed and cannot be compared. The latest NMR work was a study by Banigan et al., 2013 which identified that the TPP bound form of EmrE shows less conformational fluctuation than the unbound form due to a lack of inter-monomer packing in all helices save TMH 4. This illustrates that conformational changes occur not just upon transport but upon binding as well, and serves to highlight that membrane proteins are highly dynamic.
structures and care should be taken to think of any models as a snapshot, and not an immobile structure.

6.5 Hydropathy Analysis and Scanning Mutagenesis Experiments

In 1998 Edwards and Turner analyzed EmrE as well as five other members of the SMR family using the average membrane preference scale (AMP07) and alpha periodicity (Degli Eposti et al., 1990). They identified six zones, five of which face lipid on one side and protein on the other and interestingly, the remaining zone which was located on TMH 2 was identified as not exposed to lipid, and interpreted as involved in other contacts. Comparing this to the data presented here, in which H2 strongly interacted with all other helices that would compose a monomer and this would account for such a region. Also interesting is that the analysis identified regions that contain zones where the there are 3.0 or 3.9 amino acid residues per turn, which means that any structural assignment using a canonical α-helices with 3.6 residues per turn may misassign helical faces. This is a particular concern for the Cryo-EM maps, in which idealized helices were manually placed, but no refinement was done (Ubarretxena-Belandia et al., 2003).

Among the most recent experiments concerning EmrE was Trp scanning mutagenesis, wherein 60 different residue replacements were made with Trp and analyzed by *in vivo* growth and dimerization assays in order to examine the effect of a bulky disruptive reside at potentially sensitive locations (Lloris-Garcera et al., 2013). The normalized growth data demonstrates a periodicity consistent with that observed in the more controversial X-ray structure from Chen et al. in 2007, supporting that it may be a more accurate representation of native EmrE structure than the Cryo-EM. Using blue-
native PAGE, no strong effects on dimerization were found for any of the mutants in any helix aside from a G67W mutant, which demonstrated that any effects from the mutations were likely due to effects within one protomer as opposed to affecting the ability of the protomers to dimerize.

The model put forward by Gottschalk et al. in 2004 is assessed in terms of the Trp scanning data to discuss the implications of a parallel topology as well. In doing so they discuss that an interface between TMH 1 and TMH 4 is inconsistent with their data, given that a number of residues along a TMH which would make up a part of that interface can be mutated without disturbing in vivo activity. This may be able to be explained in the model proposed here because the nature of the dimer interface would still potentially allow for interactions along the TMH 4 – TMH 4 faces which contain the GG7 motifs and on a TMH 1 – TMH 1 interface, allowing for association while the mutations sites may only be causing local effects in a single protomer they propose from their data. Further comparisons to this data will be useful when an atomistic structure for EmrE using the helical bundle model is constructed, particularly if the side chains are added.

6.6 Crosslinking Data and EmrE

Some of the most important work that the model put forward here must fit in with is the crosslinking work done in 2002 by Steiner-Mordoch et al.. In their work they used single cysteine mutants of EmrE to apply HgCl2/HMDC (hexamethylene diisocyanate) and o-phenylene diamine (o-PDM) as crosslinkers to identify contact points between two protomers which crosslink with estimated distances of 4-5Å and 8Å, respectively. Residue C95 between two monomers was found to cross-link with HgCl2/HMDC (but not
with o-PDM, interpreted as being because of the lower crosslinking efficiency of o-PDM in a low dielectric medium), which fits well into the helical bundle model and which still supports TMH 4 being close to the TMH 4 from the corresponding monomer (Figure 6.1c, Figure 6.2). The residue C39, which would be located midway down TMH 2 was shown not to crosslink, as would be expected in the spatial separation provided by the helical bundle model, but could potentially be close enough to crosslink if helix rotation were correct in the currently accepted structure (Figure 6.1b, Figure 6.2).

Crosslinks between single cysteine substitution mutants at positions 88, 92, 95, 98, 99, and 102, were found when the mutants were singly expressed indicating that these positions must be spatially close to the identical residue on another protomer in the dimer. Substitutions made at positions 94, 96 and 97 failed to produce a crosslink, and can therefore be interpreted as either too far apart, rotated incorrectly, or involved in interactions with another helical partner. While the data did not identify any strong binding equilibria between two copies of TMH 4, it does not preclude close proximity between them in a dimeric form, and in fact the model proposed here still maintains the helices as being located such that they can directly associate with each other in the structure (Figure 6.1c, Figure 6.2).

The only reliable crosslink they obtained in TMH 1 was between E14C mutant dimers. Reproducible but low yield dimers were obtained using replacements at residues 7, 10, 11, and 18, and none at all were observed for residues 12 and 16. It makes sense that the residue Glu14 should be facing the interior of the protein given its involvement in the transport mechanism, and its proximity to the Glu14 of the other protomer indicates that TMH 1 should be reasonably close to TMH 1. This is seen in both the Gottschalk and
in the linear helix arrangement, where the helices are close to one another, and while the low yield of dimers provides an interesting question given the close association of the helices in either proposed model the BACTH data reported here supports the general lack of association.

Few crosslinks were observed between cysteine residue replacements in the loop region. Links were observed at replacements of positions 22, 57 and 110, but not for position 83. These crosslinks are difficult to explain in the antiparallel dimer, however the experiments were performed after solublization in DDM which may have scrambled the topology from native state and allowed the formation of parallel dimers.

The most interesting data to result from this crosslinking study were the interactions they observed between TMH 1 and TMH 4. When two types of mutant monomers were tested simultaneously, crosslinks were observed between mutants of G14C and I88C as well and between G14C and C95, L99C or N102C (Rath et al., 2006). Perhaps one of the most important aspects of this paper, is that despite not accounting for helix tilt, the authors use their data to propose a helix packing model of EmrE similar to the proposed model save that they assume a parallel topology, while EmrE has since been shown to prefer antiparallel topology (Lloris-Garcera et al., 2012). Regardless, the crosslinking data does not support the original helix assignments as proposed by Ubarretxena-Belandia et al., 2003 due to interactions between TMH 1 and TMH 4 which are impossible given their placement of TMH 3 (Figure 6.1).

In response the emergence of contradictory data, Soskine et al. did more crosslinking experiments in 2006 to support their view of a parallel topology. In these experiments they were able to crosslink EmrE mutants with a D84C mutation, as well as
ones with a T108C mutation, though they did not attempt to crosslink between the two mutants. Additionally, they tested these mutants for activity, and were able to determine that the crosslinked monomers are capable of transport. This is relevant in several fashions, showing that parallel topology is functional, that a crosslink on TMH 4 does not impede function and therefore that TMH 4 conformational changes during substrate translocation are either permitted within the space requirements of the linker or that TMH 4 does not contribute to the conformational changes upon translocation. Considering the NMR study by Morrison et al. in 2012 which concludes from peak doubling that the entire protein is involved in the conformational exchange process, this data also supports the helical bundle model. Given a linear arrangement of helices, there is no logical way in which TMH 4 can be in close proximity with TMH 4 from the other protomer in both topologies. Looking at the helices in the structure, assigning them as helical bundles would place TMH 4 in a position where the TMH 4 from the other protomer crosses it at an angle and near the center of the dimer, meaning that a translocation event would involve the movement of TMH 4 as seen in the NMR studies while simultaneously allowing for a crosslink in TMH 4 to be maintained while both helices were moving (Fig 6.1a).

Crosslinking was also done on a smaller scale by Morrison et al., 2012 for their study of the transport mechanism of EmrE. In this case they used the bifunctional crosslinker sulfo-(N-[g-maleimidobutyryloxy]succinimide) (S-GMBS) which crosslinks between a cysteine and a primary amine at approximately 7.3Å. Crosslinking was observed using this linker using single cysteine mutant S107C to link between residue S107C and K22, showing a strong crosslink between antiparallel dimers and a proximity
of those residues of within 7.3Å. Given that such a link is impossible within a single protomer because S107C and K22 are located on opposite sides of the membrane at a distance much larger than 7.3Å, and that a linear arrangement of helices separates TMH 1 and TMH 4 with TMH 3 in between them, this data also supports the proposed model (Figure 6.1, Figure 6.2).

6.7 Implications of the Transport Mechanism of EmrE

Any model of EmrE structure needs to take into account the transport mechanism, which complicates the issue further. Work in 2012 by Morrison et al. using solution state NMR to examine global conformation state changes of EmrE in isotropic bicelles identified two states, one being an inward facing antiparallel monomer, and the other being the corresponding identical but outward facing monomer. While their main objective was to show the antiparallel nature of the dimer as it applies to the alternating access model, as part of their data they map chemical shift differences to the structure of antiparallel EmrE. Their data corresponds very nicely with the accepted model by their explanation and unfortunately because the helical bundle model currently has no structure with residues mapped to it, it is difficult to propose an alternate explanation meaning this data presented in their paper should be revisited upon completion of such a structure.

However, an alternating access model has serious implications for a structure of EmrE. Identical inward and outward facing structures implies that a monomer must be able to move in a fashion such that it directly mirrors the conformation that the other monomer has just occupied. This capability exists in the antiparallel structures of both the model put forward here and the existing one, but the complication lies in addressing how
parallel dimers could then be functional in a similar capacity to antiparallel ones. Simply put, in a linear arrangement of the helices of the EmrE parallel monomer, in order for the similar functional residues of the second protomer to be positioned towards the interior of the protein the only arrangement that is possible disrupts the TMH 4 – TMH 4 contacts considered integral to dimerization. (Figure 6.7).

The BACTH data presented here and crosslinking data (Soskine et al., 2002) indicate that interactions between TMH 1 – TMH 4 are possible, and found occurring, albeit less consistently, with a strong binding equilibrium in a parallel orientation. The way a linear arrangement would be required to interact to satisfy those constraints makes no logical sense in the context of an alternating access model of transport or in terms of a functional substrate binding pocket. The model proposed here addresses this concern in that no matter the orientation of the protomers, the binding pocket is still composed of the same helices, all with the same residues still facing inward with observed helical interactions (Figure 6.7). While it is still consensus that EmrE prefers and natively forms an antiparallel dimer, parallel dimers have been observed with sufficient frequency in the literature that the structural model must by nature consider them.
Figure 6.7 Diagram of effects of transformations of EmrE protomers within the dimer applied to proposed helical arrangements. Schematics of the required transformations of an antiparallel EmrE dimer to become a parallel dimer with proposed helical assignments of EmrE for both the commonly accepted linear helix arrangement and the proposed helical bundle arrangement showing the rough position of functionally important amino acid residue Glu14 in green, and the two protomers in blue and red.

6.8 Other Data and its Implications

While of lesser import to the question of the helical bundle model, several other experiments should be mentioned as to how they apply. Electron paramagnetic resonance (EPR) was originally done on only TMH 1 of EmrE in 2003, when only the original Cryo-EM structure was available (Koteiche et al., 2003). The reconstituted single cysteine mutants for residues 3–28 of EmrE into lipsomes and determined through spin labeling determined that TMH 1 constituted part of the dimer interface, and that there was also a population which held E14 substitutions at a distance greater than 20Å. The
data also suggests that TMH 1 packs into a scissor-like structure with TMH 1 from the other protomer, with residues 14 and 18 in close proximity. This arrangement can be seen in both the commonly accepted structure and in the structure proposed here. Interestingly, they propose that the two-component spectrum the observed may be due to conformational heterogeneity, which may represent the reconstitution into liposomes scrambling the topology, resulting in a mix of dimers in both the single and dual topology which would potentially have a different spacing between the residues.

Recent EPR experimentation supports the structures proposed by the X-ray and Cryo-EM experiments showing each of TMH 1-3 as being packed closely to its counterpart in a dimer as derived from the dipolar coupling in spin labeled mutants but only weak dipolar coupling for TMH 4 (Amadi et al., 2010). The data they obtained contains two populations only one of which is in close proximity, and they interpret the other as being a different packing arrangement of the dimer meaning it is possible that the process of purifying or reconstituting their protein into liposomes may have significantly altered the dimer structures. Similar results are obtained for EmrE bound to TPP, and their conclusions note that flexibility, dynamics and low resolution of the structures with which to compare mean that residue assignments may be off as far as an entire helical turn, which suggests that extreme caution should be used in the application of this data for structural parameters.

A particularly interesting dataset results from the efforts of the Schuldiner lab while trying to determine the native topological state. In one experiment they genetically fused to EmrE monomers such that a short linker existed between them which was insufficiently long enough to cross the membrane and thus enforced a parallel topology
(Steiner-Mordoch et al., 2008). Strains expressing the parallel dimers produced this way were shown to be able to grow at roughly wild type levels when challenged with ethidium. Subsequently, another experiment saw them again fuse monomers, but this time including a ninth TMH between them from human glycophorin A, which does not interact and enforces an antiparallel topology between the dimers, which were shown to be active (Nasie et al., 2010). While valuable on its own merit as a demonstration of the activity in EmrE for either topology, it also can provide some unintended constraints as to relative helical positions. Enforcing an antiparallel topology in the fashion they did means that the ability of the monomers to diffuse in three dimensional space is severely limited within the plane of the membrane. Short linkers to the ninth helix mean that the corresponding N-terminus of one monomer and the C-terminus of the other, TMH 1 and TMH 4 respectively, are by obligation going to be occurring next to each other in the structure (Figure 6.8a). Additionally, the only way that a TMH 4 – TMH 4 interaction could be observed with these specific constructs would be if the arrangement of the helices were not linear as commonly proposed, but if the individual monomers were helical bundles such that the ends could come together though this is in a diagonal fashion across the dimer interface (Figure 6.7a). The remaining issue would be the placement of the glycophorin A helix, but since that data results from a standalone experiment, no structural data concerning it is available. The other case is equally compelling, wherein a parallel topology was enforced. In this case either a long but very hydrophilic 22 amino acid linker was used, or very short 2, 4, and 6 amino acid linkers were used. These constructs were found to be functional, but the forced juxtaposition of TMH 4 from one monomer with the TMH 1 of the other means that the linear
arrangement of helices is again strictly impossible while maintaining the order suggested by the commonly accepted structure and a TMH 4-TMH 4 interaction (Figure 6.8b). Again, this constraint is removed by the model proposed here in which each monomer can form its own helical bundle facing the other bundle, and still allowing much of the other structural data to remain valid.
Figure 6.8 Schematic diagrams of the helical arrangements in Genetically linked EmrE monomers. Implications of proposed helical arrangements based on the work done with genetically linked monomers with A) with short linkers to a non-interacting Human glycophorin A helix, forcing an antiparallel topology and (Nasie et al., 2010) B) with only a short 2-6 amino acid linker enforcing a parallel topology (Steiner-Mordoch et al., 2008).
6.9 About TMH 4 Interactions

It is impossible to discuss EmrE interaction without addressing the research concerning the associations found between TMH 4 from both monomers. Perhaps one of the most important papers which determined in some ways the direction of the entire field was the work done by Rath et al. in 2006 which suggested that the TMH 4 of EmrE homolog Hsmr interacts using two distinct faces. Similar to this research, they utilized synthetic peptides corresponding to each of the four TMH of Hsmr and subjected them to a battery of experiments including circular dichroism, SDS and PFO PAGE, FRET and molecular modeling. While it is of note that the initial circular dichroism experiments revealed Hsmr TMH 2 to be in a random coil conformation and it was therefore not used in any of their subsequent experiments, the major relevant finding was that only TMH 4 was found to oligomerize, and its oligomeric state was dimeric under SDS-PAGE but tetrameric under the more permissive PFO PAGE. From this, the authors concluded that TMH 4 must self associate, and furthermore must do so using two discrete facial interactions. Their work continued by mutating residues on the TMH to identify the two faces, which they called the Ala-Val face and the Gly face (Fig. 6.9), and mutants of both of them were able to still dimerize with wild type TMH 4 when mutations disrupting the interactions of one of the faces were performed.
Figure 6.9 Helical wheel diagrams of the fourth transmembrane helix in SMR proteins Hsmr and EmrE. Helical wheel diagrams of A) Hsmr and B) EmrE TMH 4 respectively, as put forward by Rath et al., 2006, Figure 6, and Soskine et al., 2002, Figure 5a, showing the locations of the putative Ala-Val and Gly faces. Included is a partial diagram of amino acids sequence among the two proteins and the conservation sequence for SMR family members. Reprinted from Rath et al., 2006 and Soskine et al., 2002, respectively with permission.

The authors go on to relate that while they cannot account for topology in their experiments, it is certain that TMH 4 has multiple interaction surfaces, and additionally the helix interactions in SMR proteins are variable, so any complete description of SMR structure must therefore include the contributions of the other TMH, especially the TMH 1 – TMH 1 interactions previously observed (Soskine et al., 2002). Their data strikes a stark contrast with the interaction data presented in Chapter 5, in which a TMH 4 – TMH 4 interaction is not at all observed, but interaction mapping for all the other helices are determined, including topological data (described in Chapter 5). Also, whereas they were unable to get information on the interactions regarding TMH 2, here it was found to be a highly involved helix, interacting with each other helix inside a single protomer, information which may have drastically changed subsequent experiments which were based off only knowing the interaction between TMH 4 – TMH 4. While the data
presented here did not identify a H4 – H4 interaction, it certainly does not preclude the existence of one, and while these experiments would have been unable to detect the presence of any multimeric state larger than a dimer, the premise that TMH 4 has two potential interaction faces could help to explain other interactions within a dimer, such as with TMH 1 suggested by the helical bundle model. This could easily be tested by a systematic mutagenesis of the amino acid residues in the TMH 4 portion of the constructs to determine if the mutation of specific residue positions, or residues along the Ala-Val or Gly face in particular diminish the interactions observed here (Chapter 5).

Work was done by Poulsen et al. in 2011 which correlated mutations in the GG7 motif conserved in the SMR family on TMH 4 to oligomerization strength obtained by SDS-PAGE and substrate binding recorded using the fluorescent properties of ethidium bromide for Hsmr in DDM micelles. The results of mutagenesis along this motif led to the conclusions that oligomerization strength is related, though not exclusively, to substrate binding efficiency and that mutations in TMH 4 that do not disrupt the dimerization strength (and in fact some that increase it) can still negatively impact efflux activity. From this they put forward that a ‘pivot point’ may be located centrally on TMH 4 which allows optimization of the binding site on a per substrate basis and may provide for mechanistic assistance of efflux. The placement of TMH 4 in the helical bundle model proposed here allows for this explanation, and may equally explain some of the oddities found in the paper such as why dimeric mutants of TMH 4 lead to nonfunctionality and why β-branched residues may be required at the Hsmr Val98 position, because the placement of TMH 4 may implicate that some of those residues are near the binding pocket instead of relatively distant as previously supposed. Notably, any
experiments utilizing growth phenotype in media with EmrE substrates as an indicator of EmrE activity would be unable to differentiate between prevention of dimerization or changes to the binding pockets resulting from changes in TMH 4 since either case could potentially disrupt substrate extrusion.

6.10 Potential for Higher Multimeric States

The question persists as to the prospects for a tetramer form of EmrE in our data. Because the minimal functional unit is a dimer, sensibly a tetramer would have to be in the ‘dimer of dimers’ form. A recent thesis by Jun Wang at the University of Toronto also identifies interactions between peptides of TMH 2, and posits that they might make up the interface for a tetramer in one of two potential configurations, laid out below (Figure 6.10) (Wang, 2013).
Figure 6.10. Schematic of potential EmrE dimer of dimers tetramer using TMH 2 interfaces in A) an end-on-end arrangement and B) a side-by-side arrangement. Based on studies of EmrE helices by Wang, 2013.

Notably, there are only two options for tetramerization using the linear helix model of EmrE, either with the dimers side-by-side or end-on-end, with TMH 2 providing the interface. The side-by-side arrangement would be supported by the Cryo-EM structure of EmrE. Our data identified H2-H2 and H2r-H2r interactions, but cannot support the linear helix arrangement and thus cannot support either of these tetrameric structures.
Figure 6.11. Schematics of the four potential tetrameric arrangements of EmrE for the helical bundle model of EmrE structure.
On the other hand, the helical bundle arrangement the data presented here proposes allows three different tetrameric arrangements, shown in Figure 6.11. Figure 6.11 A shows the end-on-end arrangement of the dimers, with the interface made up by TMH 2 & 3 of each monomer. This structure would explain the H2-H2 & H2r-H2r interactions and could imply that a H2-H3r or H2r-H3 interaction exists between dimers either in place of or in addition to contributing to interactions within a monomer. None of the structures of EmrE published based on electron density maps support an end-on-end arrangement and neutron scattering experiments that observed higher order multimers suggest a cylindrical structure for both the dimeric and tetrameric form (Bay et al., 2010).

The other alternative is the side-by-side arrangement shown in Figure 6.11 B & C. Within this arrangement, an individual dimer has a face with helices 1 & 2 and a face with helices 3 & 4 that it would interact with, and the difference between structures B and C is which face the tetramerization takes place on. None of the specific interactions on either of those faces were identified in my experiments.

A trimer could be created in either case by the addition of a third protomer to an existing dimer in a fashion which would allow binding of substrate, but no additional transport. Since binding of substrate to EmrE was found to be in a 1:1 ratio (Sikora et al., 2005) this could explain some of the discrepancies in binding and transport. The dimer being the minimal functional unit still supports that a trimeric state may be part of an equilibrium between a dimer and tetrameric state (Elbaz et al., 2004). This is further compounded, the lack of support for a trimer from the Cryo-EM (Ubarretxena-Belandia et al., 2003) and X-ray structures (Chen et al., 2007), as well as work concerning the
disruption and reformation of complexes which showed dimeric and tetrameric complexes only (Lloris-Garcera et al., 2012).

In regards to the higher order oligomeric forms observed for EmrE, the scenario shown in Figure 6.11 A which would result in the lengthening of a long chain of EmrE monomers. This is less likely than the scenarios shown in parts B and C because clustering of EmrE instead of elongation has been observed using Brewster angle microscopy (Nathoo et al., 2013). In the cases of forms larger than a tetramer, the possibility exists that a combination of the two modes shown in Figure 6.11 could exist, adding individual protomers or dimers to already existing complexes to form states such the hexamer to decamer forms observed in SDS-PAGE (Bay et al., 2010). The work using *E.coli* lipids further demonstrates that the experimental parameters have important effects even in highly similar membrane mimetic environments, and functional higher order multimers have yet to be conclusively demonstrated *in vivo*.

Oligomerization of EmrE may be more complex than a problem of helical arrangement considering detergents have been shown to affect complex formation using SDS (Bay et al., 2010), and *E.coli* lipids in varying concentration (Gröger et al, 2012; Nathoo et al., 2013). While the speculation for Figure 6.11 specifically concerns the possibilities derived from the association of the single helix constructs we tested, association of higher oligomeric states mediated by substrates or lipids is possible but outside the scope of this research. The data shows weak indications of a potential tetrameric state, but the only interaction our data identifies that could really be used to explain tetramerization also supports a configuration of helices that hasn’t previously been observed and can also be used to explain intra-protomer interactions, which is a
more likely explanation. The evidence from the Deber lab that supports two interaction faces on TMH 4 could be explained by interaction with two helices in the dimeric model we propose, and does not necessarily indicate a native tetrameric form (Rath et al., 2006).

6.11 Conclusions

As seems to be perpetually the case with EmrE structural features, the evidence never fully supports one particular model, whether it be the topology, contacts, or even the positions of the helices as discussed here. A model is presented which can account for a vast majority of the biochemical data in both the parallel and antiparallel topological conformations, including the new data presented here (Chapter 5). Much of the energy invested in EmrE structure was spent on determining the native membrane topology, and while the field has finally come to a consensus on the matter, their efforts have also developed a wealth of other structural information that provides the additional constraints discussed. One of the main difficulties with studying EmrE, and which resounds throughout the literature, is that the structure is highly mobile, easy to influence and prone to artifacts from experimental conditions. In the case of the common structures, the helices were suggested to be able to rotate as much as a full helical turn from what was presented (Amadi et al., 2010). It should therefore be stressed again that assumptions based on crystal structures, especially depending on the nature of their solvation, may not accurately represent the wide range of conformations available to proteins, even those constrained within a membrane. The model for EmrE structure that best fits the data present here and the majority of the existing literature is a reassignment of the TMH as identified from electron density such that the linear arrangement of the TMH is now an
association of two protomers more resembling a helical bundle conformation (Figure 6.2c).

6.12 Summary

Here is presented data on an application of the BACTH methodology to determine contact points of the integral membrane protein EmrE. To do this a way to detect integral membrane protein interaction in a method which can detect homo- or hetero- IMP interactions in a fashion which is not constrained to be localized near the protein transcriptional machinery or subject to errors involved with transcription factor- based methods, and which can be performed in a high throughput fashion was developed.

Within this system, the development of protein TMH constructs with constrained membrane topology, notably including a sample set in which the membrane topology of the TMH segment was reversed was explored. Attempts to gather data using fusions of the full length protein were largely unsuccessful, potentially due to factors such as the topology or arrangements of the fusion constructs. A second approach utilizing individual TMH of EmrE was able to define numerous structural contacts with no topological ambiguities, identifying interactions not previously seen while largely corresponding to important biochemical data. In order to accomplish this, the colorimetric Miller assay adaptation for detection of membrane protein association was further adapted to a smaller volume higher throughput methodology which was standardized. Lastly, using the data collected, a model for EmrE structure was proposed which differs from the structure commonly accepted in the literature but which makes sense in terms of much of the
biochemical data, proving the effectiveness of this BACTH method as a structural determination tool which could be applied to other IMP systems.

6.13 Future Work

Some of the most frequent questions asked of researchers during dissemination are ‘So what?’ and ‘Now what?’. Fortunately, this research offers some clear directions as to potential applications and directions to follow. Firstly, with the development of a higher throughput Miller assay the transmembrane domains of integral membrane proteins which are typically difficult to study in an *in vivo* environment due to accessibility and other environmental challenges can now be addressed with greater rigor, shown by an ability to use data from EmrE to propose a distinct structural arrangement. EmrE is a good model for this case because it is small with few places to apply tags or other detection or characterization methods, easily influenced by any methods applied to it and a complex case for both membrane topology and structural dynamics. Especially when used in combination with the technique of examining individual segments of a protein such as individual TMHs, it was demonstrated that robust and definitive structural information can be obtained, though not as qualitatively as was hoped.

The data obtained for EmrE does not so much close a chapter of discussion regarding the structure as open a new one, where data will have to be evaluated in light of a different arrangement of helices. The first objective to forward evidence towards or against the proposed structure would be to map the helices as assigned in the helical bundle model at an amino acid level to make sense of data such as residue conservation and orientation within the structure using the currently available electron density maps.
Such evaluation would again be wise to keep in mind the potential variation in helix rotation by up to 20° as well as other potential structural factors such as the deviation from the canonical α-helix packing of 3.6 amino acid residues per turn in parts of the structure predicted by Edwards and Turner (1998).

Having the assay system set up also provides the opportunity to do several other studies using it. Single residue substitutions of the TMH in the constructs could be performed to determine which residues are involved in the various TMH associations, with potential for a rough indication of the strength of their contribution given the numerical data provided by the Miller assay. Mutations could be made to cysteine residues for which crosslinking experiments could be performed to generate a more specific set of constraints, especially if crosslinkers with a variety of minimal distances are used, and crosslinking efficiency could potentially be correlated with binding equilibria. Additional constructs could be developed that include specific motifs in polyalanine or polyleucine helices to examine the binding mechanisms of observed interactions. Another type of experiment that could be performed now that some of the interactions have been observed would be to do the experiments in the presence of a number of chemicals with potential to disrupt those interactions, such as Non-steroidal anti-inflammatory drugs (NSAIDS) which are known to disrupt GG7 motifs, to see if a disruption of the binding equilibria could be observed. Such data might be useful for the development of adjuvants that might aid in anti-microbial action by disrupting SMR dimerization and thereby removing a layer of protection in resistant bacteria.

Furthering a dataset with more potential applicability than simple structural details of EmrE, a continuation of the interesting experiment performed by Poulsen and
Deber recently in 2012 wherein an artificial peptide consisting of Hsmr TMH 4 was seen to interact with and disrupt active Hsmr dimers *in vivo* could be performed. The data they produced again demonstrates the importance of a TMH 4 – TMH 4 interaction as a major dimerization site of SMR proteins and its potential utility for use in disruption of dimerization for clinical purposes, but they only use TMH 4 in the experiment. Using this system to repeat the experiment in EmrE and with all the other helices included could potentially provide provocative results with applicability for potential therapeutic purposes. A partial library of co-transformed full-length EmrE and single TMH construct co-transformant strains has already been built for this purpose.
REFERENCES


APPENDIX A

Solutions and Buffers

LB Media

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<tr>
<th>Reagent</th>
<th>Final Concentration</th>
<th>Notes</th>
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<tr>
<td>5g Yeast extract</td>
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<tr>
<td>10g Tryptone</td>
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<tr>
<td>5g sodium chloride</td>
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Make up to 1L using ddH2O, store at room temp

Lowry Solution A

<table>
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<tr>
<td>20g sodium carbonate</td>
<td>0.16mM</td>
<td>Na2CO3•H2O MW = 124.0g/mol</td>
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<td>0.5g sodium potassium tartrate</td>
<td>1.8mM</td>
<td>NaKTartrate • 4H2O MW =282.23 g/mol</td>
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<tr>
<td>4.0g sodium hydroxide</td>
<td>0.1M</td>
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<tr>
<td>10g sodium dodecyl sulfate</td>
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Make up to 1L with ddH2O, store at room temp

Lowry Solution B

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<tr>
<td>0.1g copper sulfate</td>
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<td>CuSO4•5H2O MW=249.68g/mol</td>
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Make up to 100mL with ddH2O, store at room temp

Lysis Buffer A

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<td>100mL Glycerol</td>
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<td>1mL Triton X-100</td>
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<td>1.46g EDTA</td>
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<td>Make as separate solution</td>
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<tr>
<td>0.174g PMSF</td>
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Make up to 1L with ddH2O, store at RT

Add EDTA and PMSF from stock solutions as indicated in protocol
Lysozyme Lysis Buffer (For Miller assay)

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<td>0.0008g DNase</td>
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<td>0.0015g Lysozyme</td>
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<td>14µL Triton X-100</td>
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<tr>
<td>57µL β-mercaptoethanol</td>
<td>3.8µL/mL</td>
<td>Add day of, before lysis</td>
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<tr>
<td>ddH₂O</td>
<td>To 15mL</td>
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Make to 15mL with ddH₂O
15mL should provide enough for one plate
Solution contains enzymes, try to make fresh.

MacConkey Agar plates

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<tr>
<td>7.5g Agar</td>
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Make up to 500mL with ddH₂O, autoclave mixture and stir while it cools.
If antibiotics required, add when cooling so they don’t degrade
Store plates at 4°C

Minimal M9 Media + 0.4% Maltose

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<td>MgSO₄</td>
<td>1mM</td>
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<tr>
<td>CaCl₂</td>
<td>0.1mM</td>
<td>Make as separate solution</td>
</tr>
<tr>
<td>Maltose</td>
<td>0.4%w/v</td>
<td>Make as separate solution</td>
</tr>
</tbody>
</table>

Make to desired volume in ddH₂O leaving room for maltose, which must be made
separate sterile, and added after solution has cooled from autoclaving, for plates add
1%w/v agar and autoclave, pour while cooling.

M9 Salts Solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>64g dibasic sodium phosphate heptahydrate</td>
<td>Na₂HPO₄•7H₂O MW = 268.07g/mol</td>
</tr>
<tr>
<td>15g monopotassium phosphate</td>
<td>KH₂PO₄ MW = 136.09g/mol</td>
</tr>
<tr>
<td>2.5g sodium chloride</td>
<td>NaCl MW = 58.44g/mol</td>
</tr>
<tr>
<td>5g ammonium chloride</td>
<td>NH₄Cl MW = 53.49g/mol</td>
</tr>
</tbody>
</table>

Make to 1L in ddH₂O, store at room temp.
### 6x Orange Loading Dye

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris·HCl pH 7.5</td>
<td>100µL</td>
<td>10mM</td>
</tr>
<tr>
<td>Orange G dye</td>
<td>0.015g</td>
<td>0.15% w/v</td>
</tr>
<tr>
<td>Xylene cyanol FF</td>
<td>0.003g</td>
<td>0.03% w/v</td>
</tr>
<tr>
<td>Glycerol</td>
<td>6mL</td>
<td>60% v/v</td>
</tr>
<tr>
<td>0.5M EDTA</td>
<td>1.2mL</td>
<td>60mM</td>
</tr>
</tbody>
</table>

Make to 10mL in ddH₂O, store at -20°C.

### REact Buffer 2 (Obtained from Invitrogen)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris·HCl pH 8.0</td>
<td>50mM</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>10mM</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>50mM</td>
</tr>
</tbody>
</table>

### REact Buffer 3 (Obtained from Invitrogen)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris·HCl pH 8.0</td>
<td>50mM</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>10mM</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>100mM</td>
</tr>
</tbody>
</table>

### 10x Tris-buffered saline (TBS)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>60.6g Tris·HCl, pH 7.5</td>
<td>0.5M</td>
</tr>
<tr>
<td>116.88g sodium chloride</td>
<td>2M</td>
</tr>
</tbody>
</table>

Make up to 1L with ddH₂O, store at room temp

### Tris-buffered saline + Tween-20 (TBST)

Dilute 10x TBS to 1x, and add Tween-20 to 5%v/v (5mL of 10% Tween-20 per 1L)

### Transfer Buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>3.03g/L</td>
</tr>
<tr>
<td>Glycine</td>
<td>14.4g/L</td>
</tr>
<tr>
<td>100% Methanol</td>
<td>20%v/v</td>
</tr>
</tbody>
</table>

Make up to 1L with ddH₂O and store at room temp
### Z-Buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.426g dibasic sodium phosphate</td>
<td>0.06M</td>
<td>$\text{Na}_2\text{HPO}_4 \text{ MW} = 141.96\text{g/mol}$</td>
</tr>
<tr>
<td>0.275g sodium dihydrogen phosphate</td>
<td>0.04M</td>
<td>$\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O} \text{ MW} = 137.99\text{g/mol}$</td>
</tr>
<tr>
<td>0.0375g potassium chloride</td>
<td>0.01M</td>
<td>$\text{KCl} \text{ MW} = 74.55\text{g/mol}$</td>
</tr>
<tr>
<td>0.0123g magnesium sulfate</td>
<td>0.001M</td>
<td>$\text{MgSO}_4\cdot(\cdot\text{7H}_2\text{O}) \text{ MW} = 246.48\text{g/mol}$</td>
</tr>
</tbody>
</table>

Make up to 50mL with ddH$_2$O, store at room temp
APPENDIX B

Statistical values for Miller Assays

Table B.1 P-values Figure 3.2 relative to empty plasmid negative control, with n = 3.

<table>
<thead>
<tr>
<th>Sample</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EmrET18-EmrET25</td>
<td>0.11</td>
</tr>
<tr>
<td>T18EmrE-EmrET25</td>
<td>0.08</td>
</tr>
<tr>
<td>EmrET18-T25EmrE</td>
<td>0.11</td>
</tr>
<tr>
<td>T18EmrE-T25EmrE</td>
<td>0.72</td>
</tr>
<tr>
<td>ZipT18-EmrET25</td>
<td>0.74</td>
</tr>
<tr>
<td>ZipT18-T25EmrE</td>
<td>0.88</td>
</tr>
<tr>
<td>EmrET18-ZipT25</td>
<td>0.45</td>
</tr>
<tr>
<td>T18EmrE-ZipT25</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Table B.2 P-values for samples in Figure 4.1 compared between sample populations of microfuge tube based and microtitre plate based Miller assays, where n =3 and n = 8 respectively.

<table>
<thead>
<tr>
<th>Sample</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUT18-pKT25</td>
<td>0.017</td>
</tr>
<tr>
<td>pZipT18-pZipT25</td>
<td>0.000</td>
</tr>
<tr>
<td>H2r-H3</td>
<td>0.242</td>
</tr>
<tr>
<td>H4-H3</td>
<td>0.587</td>
</tr>
<tr>
<td>ET18-H3</td>
<td>0.005</td>
</tr>
<tr>
<td>T18E-H3</td>
<td>0.000</td>
</tr>
<tr>
<td>ET18-H4</td>
<td>0.012</td>
</tr>
<tr>
<td>T18E-H4</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Table B.3 P-values for samples in Figure 4.2 compared between the two replicate trials (n = 8) of the Miller assay in microtitre plates where a pregrowth period for recovery from DMSO storage was added.

<table>
<thead>
<tr>
<th>Sample</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUT18-pKT25</td>
<td>0.52</td>
</tr>
<tr>
<td>pZipT18-pZipT25</td>
<td>0.00</td>
</tr>
<tr>
<td>H1-H3</td>
<td>0.66</td>
</tr>
<tr>
<td>H2r-H3</td>
<td>0.16</td>
</tr>
<tr>
<td>H4-H3</td>
<td>0.50</td>
</tr>
<tr>
<td>ET18-H3</td>
<td>0.00</td>
</tr>
<tr>
<td>T18E-H3</td>
<td>0.13</td>
</tr>
<tr>
<td>ET18-H4</td>
<td>0.05</td>
</tr>
<tr>
<td>T18E-H4</td>
<td>0.00</td>
</tr>
<tr>
<td>Ala-Ala</td>
<td>0.00</td>
</tr>
<tr>
<td>Leu-Leu</td>
<td>0.13</td>
</tr>
</tbody>
</table>
Table B.4 P-values for Figure 4.3 comparing pairwise between the three replicate trials (n = 8) of Miller assays where the OD$_{600}$ of the inoculant is standardized before 16h incubation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>P-value</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trial 1 vs 2</td>
<td>Trial 1 vs 3</td>
<td>Trial 2 vs 3</td>
</tr>
<tr>
<td>pUT18-pKT25</td>
<td>0.82</td>
<td>0.15</td>
<td>0.08</td>
</tr>
<tr>
<td>pZipT18-pZipT25</td>
<td>0.01</td>
<td>0.00</td>
<td>0.73</td>
</tr>
<tr>
<td>H1-H3</td>
<td>0.58</td>
<td>0.38</td>
<td>0.06</td>
</tr>
<tr>
<td>H2r-H3</td>
<td>0.24</td>
<td>0.64</td>
<td>0.03</td>
</tr>
<tr>
<td>H4-H3</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>ET18-H3</td>
<td>0.20</td>
<td>0.66</td>
<td>0.00</td>
</tr>
<tr>
<td>T18E-H3</td>
<td>0.12</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>ET18-H4</td>
<td>0.03</td>
<td>0.24</td>
<td>0.03</td>
</tr>
<tr>
<td>T18E-H4</td>
<td>0.69</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Ala-Ala</td>
<td>0.02</td>
<td>0.35</td>
<td>0.00</td>
</tr>
<tr>
<td>Leu-Leu</td>
<td>0.01</td>
<td>0.00</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Table B.5 P-values for Figure 4.5 comparing pairwise between the three replicate trials (n = 8) of Miller assays where the OD$_{600}$ of the samples is standardized just prior to the Miller assay.

<table>
<thead>
<tr>
<th>Sample</th>
<th>P-value</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trial 1 vs 2</td>
<td>Trial 1 vs 3</td>
<td>Trial 2 vs 3</td>
</tr>
<tr>
<td>pUT18-pKT25</td>
<td>0.00</td>
<td>0.00</td>
<td>0.04</td>
</tr>
<tr>
<td>pZipT18-pZipT25</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>H1-H3</td>
<td>0.01</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>H2r-H3</td>
<td>0.10</td>
<td>0.09</td>
<td>0.08</td>
</tr>
<tr>
<td>H4-H3</td>
<td>0.00</td>
<td>0.00</td>
<td>0.30</td>
</tr>
<tr>
<td>ET18-H3</td>
<td>0.00</td>
<td>0.00</td>
<td>0.24</td>
</tr>
<tr>
<td>T18E-H3</td>
<td>0.51</td>
<td>0.52</td>
<td>0.99</td>
</tr>
<tr>
<td>ET18-H4</td>
<td>0.00</td>
<td>0.00</td>
<td>0.71</td>
</tr>
<tr>
<td>T18E-H4</td>
<td>0.64</td>
<td>0.77</td>
<td>0.67</td>
</tr>
<tr>
<td>Ala-Ala</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Leu-Leu</td>
<td>0.00</td>
<td>0.00</td>
<td>0.17</td>
</tr>
</tbody>
</table>
Table B.6 P-values for Miller assay binding of polyalanine, polyleucine and Zip control helices compared to the negative controls within their given microtitre plate samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AlaT18</td>
</tr>
<tr>
<td>H1</td>
<td>0.00</td>
</tr>
<tr>
<td>H1r</td>
<td>0.00</td>
</tr>
<tr>
<td>H2</td>
<td>0.00</td>
</tr>
<tr>
<td>H2r</td>
<td>0.00</td>
</tr>
<tr>
<td>H3</td>
<td>0.63</td>
</tr>
<tr>
<td>H4</td>
<td>0.01</td>
</tr>
<tr>
<td>H4r</td>
<td>0.28</td>
</tr>
<tr>
<td>Ala</td>
<td>0.15</td>
</tr>
<tr>
<td>Leu</td>
<td>0.00</td>
</tr>
<tr>
<td>Zip</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Table B.7 P-values for the pairwise interactions of EmrE single TMH constructs compared against the respective negative controls that accompanied each microtitre plate.

<table>
<thead>
<tr>
<th>Sample</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T25</td>
</tr>
<tr>
<td></td>
<td>H1</td>
</tr>
<tr>
<td>T18</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.21</td>
</tr>
</tbody>
</table>