

**Determining Spatial Organisation and Localization
of a Proposed Enterobactin Metabolon in *Escherichia coli***

Sylvie Ouellette

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By: Sylvie Ouellette

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Doctor of Philosophy Chemistry (Biochemistry)

complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the final examining committee:

_____ Chair
Dr. Malcolm Whiteway

_____ External Examiner
Dr. Natalie Goto

_____ External to Program
Dr. Alisa Piekny

_____ Examiner
Dr. Paul Joyce

_____ Examiner
Dr. David Kwan

_____ Thesis Supervisor
Dr. Peter Pawelek

Approved by

Dr. Louis Cuccia, Graduate Program Director

August 24th, 2023

Dr. Pascale Sicotte, Dean of Faculty of Arts and Science

ABSTRACT

Determining Spatial Organisation and Localization of a Proposed Enterobactin Metabolon in *Escherichia coli*

Sylvie Ouellette. PhD
Concordia University, 2023

To obtain scarce iron from their environment, bacteria synthesize and secrete high affinity siderophores that chelate ferric ions. Enterobactin is a catecholate siderophore produced by *Escherichia coli*. Synthesized in the cytoplasm by the concerted action of seven enzymes, EntCBDAEF and EntH, enterobactin is then transported to the periplasm by the inner-membrane protein EntS. We hypothesize that the Ent proteins assemble in a large multiprotein complex to enhance the metabolic flux of the intermediates and prevent their diffusion, and that this assembly localizes at the inner membrane where it interacts with EntS for secretion. The overarching goal of this thesis is to gather further structural evidence of interactions among the biosynthetic enzymes, to determine the manner in which the proposed assembly interacts with the transporter channel, and to initiate characterization of EntS. Herein we demonstrate an interaction between the first two enzymes of the pathway, EntC and EntB. Through *in vivo* crosslinking with formaldehyde, we captured intracellular complexes comprising both proteins. These results were supported by two-hybrid assays, whereas automated docking of the crystal structures of EntC and EntB resulted in a model where the active sites of EntC and EntB are oriented in apposition and connected by an electropositive surface potentially capable of channeling the negatively charged precursor of enterobactin, isochorismate. We then built on these research outcomes, which suggested that other Ent proteins could interact

simultaneously and form complexes in which both EntC and EntB were detected. Using *in vivo* crosslinking with formaldehyde, we confirmed the presence of proteins EntA and EntE in these higher-order complexes. We also initiated work towards identification of EntS cytosolic interactors, where using an engineered ascorbate peroxidase enzyme fused in frame with EntS as the bait, proximity labeling assays revealed prey proteins localizing within 10 nm of EntS. Finally, we undertook extraction and isolation of EntS in a detergent micelle and in synthetic lipid nanodiscs. We identified two detergents and two copolymers that were efficient in extracting and solubilizing EntS, as well as the optimized conditions for successful extraction. The results obtained also suggest that EntS might assemble as a dimer.

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Contributions of the Authors

Dr Paknoosh Pakarian performed bacterial two-hybrid assays and bacterial growth studies in Chapter Two, as well as having prepared genetic constructs necessary for the chemical crosslinking experiments in Chapter Two and Chapter Three.

Xue Bin generated the EntC-EntB model, performed the CAVER analysis on the model, and superimposed the model onto previously reported EntA-EntB and EntA-EntE models for the published manuscript (here in Appendix One, Figure S1.3A and S1.3C) from which Chapter two is extracted.

Dr Peter Pawelek directed the research and generated Figure 4B for the published manuscript from which Chapter Two is extracted (here Appendix One, Figure S1.3B).

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List of Abbreviations

AASTY	acrylic acid styrene
ACP	acyl carrier protein
Amp ^R	ampicillin resistance
APEX	ascorbate peroxidase
ArCP	aryl carrier protein domain
BACTH	bacterial adenylate cyclase two-hybrid
BiFC	bimolecular fluorescence complementation
Cam ^R	chloramphenicol resistance
CMC	critical micellar concentration
Cryo-EM	cryogenic electron microscopy
DHB	2,3 dihydroxybenzoic acid
DIBMA	di-isobutyl maleic acid
Fe-Ent	ferric enterobactin (or ferrienterobactin)
FRET	fluorescence resonance energy transfer
Fur	ferric uptake regulator
HRP	horseradish peroxidase
Kan ^R	kanamycin resistance
IC	isochorismatase domain
IM	inner membrane
IMP	integral membrane proteins
IPTG	isopropyl β -D-1-thiogalactopyranoside

LB	Luria Bertani broth
M63	Miller's minimal medium
MFS	major facilitator superfamily
MSP	membrane scaffold protein
NMR	nuclear magnetic resonance
NRPS	non-ribosomal peptide synthesis
OM	outer membrane
PALM	photoactivated localization microscopy
PBS	phosphate buffered saline
PIC	protease inhibitor cocktail
PPI	protein-protein interaction
PVDF	polyvinylidene difluoride
RND	resistance-nodulation-cell division
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SMA	styrene maleic acid or anhydride
SMALP	SMA lipid nanodisc
TBS	TRIS buffered saline
TBST	TRIS buffered saline containing 0.05% Tween 20
Tet ^R	tetracycline resistance
TM	transmembrane

Statement of the problem and significance

Iron is essential for bacteria to sustain their growth and metabolic needs. However, in most environments iron is often scarce due to its propensity to form insoluble complexes. In mammalian systems, most circulating iron is bound to serum proteins such as transferrin or ferritin (1). As part of their iron acquisition strategy, bacteria have therefore evolved the ability to produce and secrete compounds called siderophores, molecules that bind ferric ions (Fe^{3+}) with very high affinity.

Enterobactin is one such siderophore produced by *Escherichia coli* and *Salmonella* (2). The molecule is a triserine lactone with three catechol units that confer iron binding properties with a K_D of $\sim 10^{-50}$ M at optimal pH (3). This extraordinarily high affinity enables enterobactin to scavenge essential Fe^{3+} from the extracellular environment for uptake into the bacterial cells. This allows for the growth and proliferation of pathogens in mammalian hosts, making siderophores and the proteins involved in their biosynthesis and secretion virulence factors as they contribute to growth to the detriment of the host (4).

Moreover, bacteria that produce enterobactin are often resistant to antibiotics. Antibiotic resistance is a major concern worldwide, in both agriculture and health (5). The classical path for new antibiotic discovery relies on screening many compounds that exhibit bactericidal or bacteriostatic properties, then elucidating their mode of action. By contrast, the concept of targeted disruption of protein-protein interactions (PPIs) is emerging as a potentially more effective approach: investigate and understand PPI-mediated mechanisms that are essential for survival of bacteria and then devise ways to disrupt them (6-9). Consequently, a greater understanding of how the enzymes involved

in enterobactin biosynthesis interact can lead to the identification of targets for disruption and the development of novel antibiotics aimed at impeding this biosynthesis.

Conversely, the potent iron chelating properties of enterobactin make it an interesting compound that could be of benefit in fields that currently employ siderophores for imaging and therapeutics, as well as for industrial applications and soil bioremediation (10-12). At present, commercially available enterobactin is priced at several hundreds of dollars per milligram (13). Consequently, greater knowledge about the PPIs involved in biosynthesis of enterobactin as well as identification of the key residues involved in these interactions can lead to the development of strategies allowing for their optimization, such as enhancing affinity among the interactors. In an industrial context, this would make bacterial synthesis of enterobactin much more efficient and produce larger yields at lower costs.

For both these reasons, further knowledge of how the biosynthetic machinery of enterobactin assembles in *E. coli* will prove immensely useful. The research presented in this thesis focuses on the assembly of the biosynthetic machinery required for the production of the *E. coli* siderophore, enterobactin. It also provides further insights on its secretion to its surroundings by expanding knowledge of its inner membrane transport channel, EntS.

1. CHAPTER ONE – General Introduction

1.1 *Escherichia coli* in discovery research

The bacterium *Escherichia coli* (*E. coli*) is a ubiquitous model organism that has proven highly valuable for the study of basic cellular processes such as metabolism, DNA replication, transcription and translation, and others too numerous to mention (14). Due to its popularity as a research tool in the decades that followed World War II, *E. coli* laboratory strain K-12 was the first organism to have its genome sequenced in 1997 (15). The natural propensity of *E. coli* to take up exogenous DNA and express its proteins has made it a workhorse of metabolic engineering, in which the bacterium can be genetically modified to produce valuable compounds such as proteins, biofuels, bioplastics, and pharmaceuticals. One of the most well-known examples is the advent of recombinant human insulin (16). More recently, Haushalter *et al.* have shown how oleochemicals can be produced in modified strains of *E. coli* through deregulation of its fatty acid synthase system, prompting the microorganism to constantly secrete the desired compounds while bypassing its endogenous regulatory system (17). Another very recent application was presented by a group that engineered *E. coli* to efficiently produce a key platform chemical, anthranilate (18). This was achieved by optimizing production of anthranilate *via* disruption of the genes that have negative effects on its biosynthesis, thereby overcoming the unstable and expensive supply of anthranilate *via* chemical synthesis from non-renewable resources. At present, efforts are underway worldwide to harness the various capabilities of microbes to use them as a tool in bioremediation, by engineering strains that can degrade pollutants and assist in chelation of heavy metals (19-22).

Despite the body of knowledge amassed over the past decades, several aspects of *E. coli* metabolism remain understudied. Given the multitude of usages derived from past discoveries, it is reasonable to expect that elucidating these lesser-known processes will ultimately yield novel, useful tools for further applications. For instance, for several pathways the metabolic flow of intermediates is not well understood, and methods to measure them are still being developed. As well, the interactions between and within different pathways in *E. coli* remain to be characterized. This is particularly true of complexes known as metabolons, where the enzymes involved in metabolic pathways organise themselves into dynamic, supramolecular assemblies in order to perform substrate channeling (23-24).

The way in which metabolic reactions proceed within metabolons is constantly under study, especially in complex networks within *E. coli*. In recent years, the increasing interest in biofilms and the challenges they pose in healthcare (25), as well as the role of bacteria in environments such as the gut or in soil, have highlighted the dearth of knowledge on the role of *E. coli* as regards the mechanisms governing synthesis and secretion of key secondary metabolites. The interactions within the biosynthetic pathways of natural products, as well as their impact on other pathways and indeed on other microorganisms in the environment deserve greater scrutiny. More research is therefore needed to understand the various aspects that underlie the assembly of the proteins involved in these processes.

1.2 Iron acquisition in *E. coli* – what is known

1.2.1 Need for iron and availability

One crucial bioprocess of *E. coli* worthy of greater attention is that of iron acquisition. This metal, in the form of ferric ions (Fe^{3+}), is essential for *E. coli* growth, a requirement that is exemplified in many pathways among which the tricarboxylic acid cycle and the electron transport chain (26). In addition, iron plays crucial roles in amino acid and in pyrimidine biosynthesis as well as in DNA synthesis and repair (27). Iron is the fourth most abundant element on earth and is required in micromolar concentrations for growth (28-29) yet its availability is notoriously low: in an animal host (*i.e.* during an infection) iron is tightly bound by carrier proteins such as transferrin (1) whereas in soil Fe^{3+} , the oxidation state present under aerobic conditions, forms insoluble ferric hydroxide precipitates.

1.2.2 Siderophores

Given the challenge of acquiring this scarce yet essential micronutrient, many bacteria have evolved mechanisms to scavenge iron from their surroundings through the production and secretion of siderophores, molecules that bind Fe^{3+} with very high affinity. A wide array of siderophores and their uptake systems can be found among a broad spectrum of prokaryotic and eukaryotic microorganisms (30-31).

Most of these systems involve enzymatic biosynthesis followed by secretion of siderophores, as well as subsequent import of the ferric siderophores. It is also worth noting that some species, although they do not produce siderophores, nevertheless possess uptake systems for acquisition of ferric siderophores originating from other species (“highjacking”) (32). Although this can be beneficial for microbial communities

in soil and marine environments, for pathogenic strains siderophores are considered as virulence factors as they support their growth in mammalian hosts and can pose a threat in the context of biofilms where communities of microbes co-exist (4).

More than 500 siderophores produced by bacteria and yeasts have been identified (33-35). They are molecules of low molecular weight (under 1 kDa) that are remarkable in their ability to bind Fe^{3+} with dissociation constants (K_D) ranging from 10^{-25} to 10^{-50} M (3, 34). This large variety of siderophores found among the many microorganisms can be classified from the moieties that coordinate the Fe^{3+} ion. The broad groups of siderophores include the catecholates, phenolates, hydroxamates and carboxylates (*e.g.*, derivatives of citric acid) (3) (Fig. 1.1). Further sub-classification is based on the functional differences between them in terms of their stability and affinity for Fe^{3+} (26).

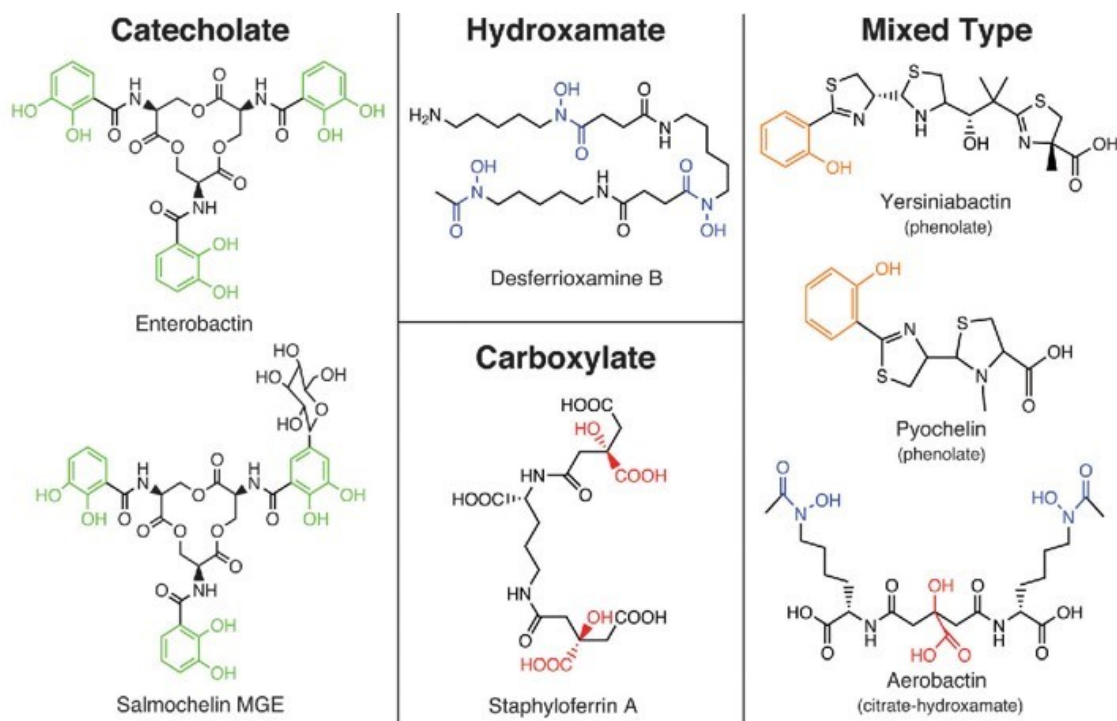


Figure 1.1: Chemical structure of some siderophores (from Holden & Bachman, 2015, with permission (5)). Iron-binding moieties are illustrated by colour. Green: catecholate. Blue: hydroxamate. Red: carboxylate. Orange: phenolate. Mixed type siderophores possess elements of two or more siderophore families.

1.3 Enterobactin

Enterobactin is one of the best characterized and reputedly most potent siderophores (3).

Its high affinity not only makes it a very effective sequestering agent for iron, able to wrest Fe^{3+} from proteins and insoluble iron complexes, it also enables it to advantageously compete against other siderophores in most environments.

With a molecular weight of 67 Da, enterobactin is a triserine lactone classified as a catecholate siderophore, bearing three catechol groups that are responsible for the coordination of the ferric ion (Fig. 1.2).

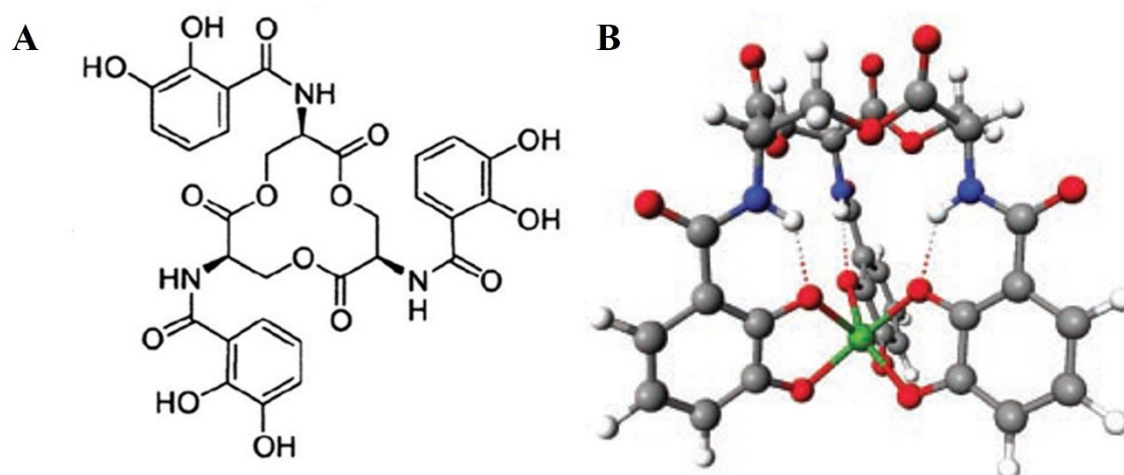


Figure 1.2. The structure of enterobactin (adapted from Raymond *et al.*, 2003, open access (2)). A) Representation of the triserine lactone ring of enterobactin, with its three catechol units. B) Computer-generated structure of enterobactin showing coordination of the Fe^{3+} ion (in green).

1.3.1 Biosynthesis and secretion of enterobactin, import of ferric enterobactin

The pathway through which enterobactin is synthesized in the cytoplasm involves the concerted action of five proteins, EntC, EntB, EntA, EntE and EntF. Two additional accessory proteins, EntD and EntH, are also required. Biosynthesis is divided into two functional modules (Fig. 1.3). In the first module, a chorismic acid precursor is transformed through sequential reactions from EntC, EntB (N-terminal isochorismatase (IC) domain) and EntA to yield 2,3-dihydroxybenzoic acid (DHB). In the second module, DHB is condensed with serine through three cycles of condensation involving non-ribosomal peptide synthetase (NRPS) activities (EntE, EntB (C-terminal aryl carrier protein (ArCP) domain) and EntF), to yield the final product. The accessory protein EntD (phosphopantetheinyl transferase) is responsible for phosphopantetheinylation of both EntB (ArCP) and EntF, converting them from their inactive *apo* forms into to their active *holo* forms (36). EntH (proofreading thioesterase) is another accessory protein that prevents EntB misacylation (C-terminal ArCP domain).

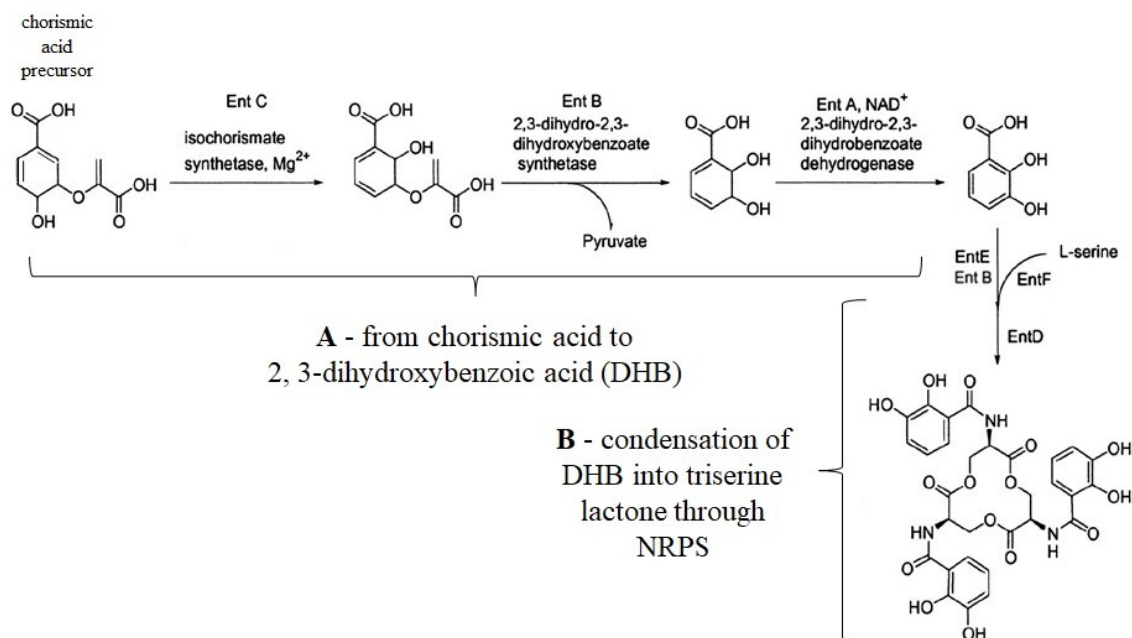


Figure 1.3. The enterobactin biosynthetic pathway (adapted from Raymond *et al.*, 2003, open access (2)). Enterobactin biosynthesis is divided into two modules: A) conversion of a chorismic acid into 2,3 di-hydroxybenzoic acid (DHB). B) non-ribosomal peptide synthesis involving three cycles of condensation of three molecules of DHB with serine to yield enterobactin.

Once synthesis is complete, the final product is first exported out of the cytoplasm *via* the inner membrane transporter EntS (37). From the periplasm, translocation and export are mediated by TolC with the involvement of the AcrB, AcrD, and MdtABC, the multidrug efflux system from the resistance-nodulation-cell division (RND) family (38) (Fig. 1.4, left panel). Having bound Fe^{3+} , the ferric enterobactin (Fe-Ent) molecule is imported *via* FepA (39), the outer membrane receptor that mediates this import from the external milieu into the periplasm, aided by TonB, a periplasmic protein that is anchored in the inner cytoplasmic membrane *via* its N-terminus (Fig. 1.4, middle panel). TonB is flanked by ExbB and ExbD, inner cytoplasmic membrane proteins that mediate proton entry to the cytoplasm, thus supplying energy for TonB to undergo the required

conformational changes (40). In the periplasm, FepB transfers Fe-Ent to a cytoplasmic membrane ABC transport complex comprising FepD, FepG, and FepC to mediate its transport into the cytoplasm (41-42).

Finally, the Fe-Ent is hydrolysed in the cytoplasm by ferric enterobactin esterase Fes back into three molecules of DHB, and the Fe^{3+} ion is released (43).

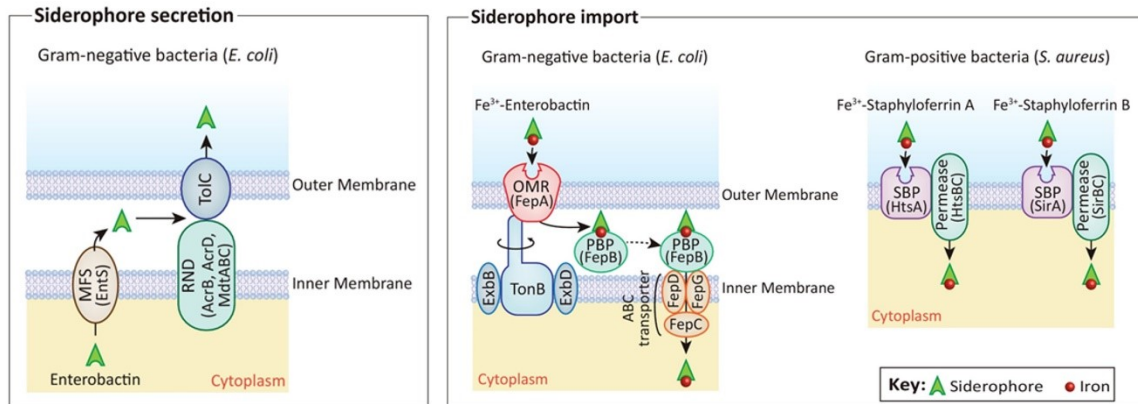


Figure 1.4. Secretion of enterobactin and import of ferric enterobactin (adapted from Wilson *et al.*, 2016, with permission (44)). Left panel: enterobactin is exported from the cytoplasm to the periplasm by channel transporter EntS. There, TolC is responsible for translocation followed by export through the outer membrane in association with RND proteins AcrB, AcrD and MdtABC. Middle panel: FepA is the receptor for ferric enterobactin. Acquisition is mediated by TonB in association with FepB and ABC-transporter proteins FepD, FepG and FepC. Right panel: Comparison with the mechanisms for siderophore secretion/import in Gram-positive bacteria.

1.3.2 Iron homeostasis, control and regulation

Since over-accumulation of iron can lead to oxidative damage and thus be toxic to bacteria, its concentration in the cell must be tightly regulated. Expression of the *ent* genes in the cluster is under control of the ferric uptake regulator, Fur, a histidine-rich protein of approximately 18 kDa (45-47).

The genes encoding these enzymes consists of four polycistronic transcripts expressed from three bidirectional iron-repressive control regions (Fig. 1.5).

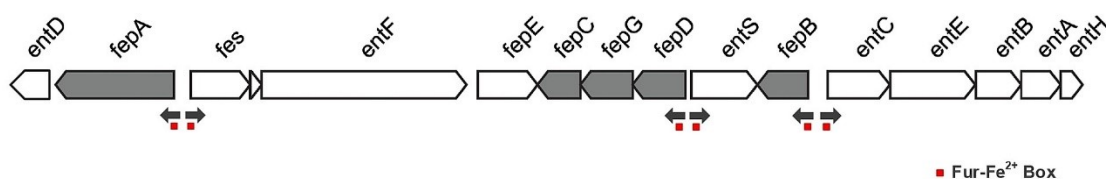


Figure 1.5. Enterobactin gene cluster in *Escherichia coli* (from Peralta *et al.*, 2016, open access (48)). The genes encoding Ent proteins and other proteins involved in ferric enterobactin acquisition and processing are organized in six operons from bidirectional promoter-operator regions under the control of Fur-Fe²⁺ boxes (red squares).

In presence of sufficient iron, Fur binds ferrous ions as a co-repressor and adopts a homodimeric conformation. This allows Fur to bind the promoter region (Fur-Fe²⁺ box) *via* sequence-specific protein-DNA interactions. Binding of the target consensus sequence of the promoter prevents initiation of transcription. In iron-depleted conditions, the lack of ferrous ions triggers the opposite effect: the *apo*-Fur exhibits reduced affinity to the Fur-Fe²⁺ box and dissociates from the promoter region to allow for gene expression (49). In this way, the intracellular iron concentration is tightly regulated, preventing its over-accumulation yet ensuring expression of the Ent biosynthetic enzymes before concentration falls below the threshold of about 10⁻⁶ M that is critical for microbial growth (3).

1.3.3 Implications in health and agriculture

The importance of enterobactin in iron acquisition, hence its role as a virulence factor, cannot be understated. Despite its invaluable contributions and the notoriety *E. coli* has gained as a research tool, several strains and serotypes are considered important pathogens. For instance, *E. coli* O103 is responsible for foodborne illnesses that often occur through the consumption of contaminated food items (50). Pathogenic Shiga toxin-producing *E. coli* strains are amongst the top 10 most common causes of foodborne

illness in Canada (51). The challenges posed by such outbreaks in not only human health but also agriculture are considerable. The possibility of food contamination can occur at almost any step from the farm to the table, raising concern for human health as well as generating huge economic losses worldwide (52). Moreover, siderophore-mediated acquisition is not unique to *E. coli*. Other pathogenic, Gram-negative bacteria that do not produce enterobactin nevertheless express receptors such as FepA on their surface, allowing them to take up ferric enterobactin produced by *E. coli* in milieus harbouring a wide variety of bacterial strains such as in biofilms (32).

More recently, concerns have also been expressed when it was discovered that pathogenic strains that produce enterobactin may contribute to the progression and poor outcome of colorectal cancers, especially in advanced stages (53). In that study, researchers reviewed metagenomic data from stool samples and found that these were highly enriched with microbial siderophores, especially enterobactin and the genes responsible for both its synthesis and its acquisition, thereby suggesting it plays a key role in the evolution of the disease.

1.3.4 Industrial applications

Due to their ability to tightly chelate iron and sequester it from its surroundings, enterobactin and other siderophores are being used or considered as potential agents for a various medical, environmental, and industrial applications. Among others, they have been investigated as a means of recovering iron from iron ores, as a chelator in agriculture, and as drugs to treat iron overload disorders in humans (54). From imaging to biosensors to bioremediation, many other possibilities are currently being considered and explored (10, 55-60).

Up until now, however, the most common siderophores considered for the applications cited above do not include enterobactin. Only a few siderophores are clinically useful since most have dangerous side-effects (61). One important property required of clinically useful chelators is specificity. Since these drugs disperse diffusely in the body, they must bind the target metal ion preferentially over others. Desferrioxamine (Desferal®), for instance, can be used to treat iron overload since the drug binds iron with a large preference over other metal ions such as calcium (61). However, the binding affinity of desferrioxamine for Fe^{3+} is lower than that of enterobactin by many orders of magnitude ($K_D=10^{-31}$ M vs $K_D=10^{-50}$, respectively (62)). Moreover, as a cyclic siderophore, enterobactin is known to form more stable complexes compared to linear siderophores because cyclisation enhances the chemical stability, complex stability, and resistance to enzyme mediated degradation (63). At present there are very few if any known usages for enterobactin in either health or industry. Although being one of the most potent chelators conveys a distinct advantage over other siderophores, its production on a large scale remains costly and prohibitive (13).

1.4 Iron acquisition in *E. coli* – aspects requiring further investigation

1.4.1 A hypothesized enterobactin metabolon

When taking into considerations points 1.3.3 and 1.3.4, advancing knowledge on how the biosynthetic machinery of enterobactin operates (*i.e.*, by identifying key elements of how the pathway assembles) would help on two fronts. First, it would ultimately reveal targets for disruption that would aid in developing new antibiotics to help defeat infections.

Second, it would also define how either the same or similar targets that can be modified

to optimize the biosynthetic process with a view to industrially produce enterobactin more efficiently and at a lower cost.

Many cellular processes are known to associate into highly dynamic, multienzyme complexes called metabolons, where sequential enzymes of a pathway interact so as to facilitate the reactions they catalize (22, 64-65). As proposed by Bassard, they can be defined as a “Transient and dynamic supramolecular organization of cooperating, often consecutive enzymes of a metabolic pathway, which often is associated with structural elements of the cell (*e.g.*, membrane, cytoskeleton) and non-enzymatic proteins.

Metabolon components can be specific to one metabolon or dynamically shared with other metabolons for swift adaptation of the metabolite profile to environmental changes, challenges and cellular needs” (64).

Determining how a metabolon assembles is perhaps the most crucial step in relating function to structure. Akin to an assembly line, this organization of enzymes and other accessory proteins allows for higher efficiency of production by optimizing metabolic flux (66). Several important metabolons (*e.g.*, fatty acid synthesis, the tricarboxylic acid cycle, photosynthetic complexes in plants) have been extensively studied for their structure and function in prokaryotes(65, 67-70).

We therefore propose that the biosynthetic machinery of enterobactin in *E. coli* also assembles as a metabolon. This hypothesis is based in part on the elucidated assembly of two other catecholate siderophores:

1- Pyoverdine, a siderophore made by *Pseudomonas aeruginosa*, has been well studied (71). Pyoverdine is synthesized in the *P. aeruginosa* cytoplasm by four NRPS enzymes, PvdL, PvdI, PvdJ and PvdD (72). The final product of these sequential reactions is then

transferred to the periplasm to undergo further changes before secretion to the external milieu where it chelates iron. In addition to the NRPS enzymes, enzymes PvdH, PvdA and PvdF are also involved in the biosynthesis of pyoverdine (73). PvdA has been shown to be a membrane bound protein that coordinates the multienzyme complex and anchors it to the membrane (74). As well, colocalization with the NRPS enzymes has also been demonstrated. Investigators have chosen to name this complex “siderosome” and have gathered evidence that such an assembly increases the efficiency of substrate transfer among the biosynthetic enzymes and for secretion of the final product (71, 75).

2- Bacillibactin is a siderophore synthesized and secreted by *Bacillus subtilis* and *Bacillus anthracis* (76). In this instance, biosynthesis is achieved in a pathway that very closely mirrors that of enterobactin in *E. coli*. In a first module, a 3-dehydroshikimic acid precursor is transformed into DHB through the sequential action of three enzymes, DhbC, DhbB (IC domain) and DhbA. In a second module, NRPS enzymes DhbE, DhbB (ArCP domain) and DhbF condense DHB glycine-threonine into bacillibactin, a cyclic trimer (77-78). Just as is seen for enterobactin biosynthesis, this second module of bacillibactin biosynthesis requires obligate protein interactions (77). Finally, secretion of bacillibactin is mediated by YmfE, an MFS-type exporter that contains six transmembrane domains, including a conserved 13-residue domain believed to be involved in substrate channeling (79).

Given what is known from the two examples above, we hypothesize that the enterobactin biosynthetic enzymes of *E. coli* are similarly assembled, in a transient fashion, into a metabolon located in the cytoplasm, localizing to the IM to bring it in direct contact with enterobactin efflux transporter EntS.

1.4.2 Relevance of an enterobactin metabolon

Higher order, multienzyme assemblies are made possible *via* an extensive network of pairwise PPIs. Such PPIs arise from the sum of otherwise weak, non-covalent forces between distinct polypeptide chains. Metabolons have evolved to facilitate substrate channeling, which enhances metabolic flux and helps to protect labile intermediates and to prevent their diffusion in the cytosol (80-81) .

Advantages stemming from the supramolecular assembly of enzymes involved in a pathway are considerable:

1. Increased efficiency: Enzymes within a metabolon can work together in close proximity, which can increase the flux of the metabolic intermediates and prevent their diffusion. This can lead to higher product yields and faster reaction rates.
2. Enhanced regulation: Metabolons can be subject to tight regulation, which can help to control the rate of the metabolic pathway and prevent the accumulation of intermediates that can inhibit the enzymatic reactions.
3. Improved stability: Metabolons can be stabilized by interactions between the enzymes, which can prevent the enzymes from denaturing or being degraded by proteases.
4. Reduced energy consumption: Enzymes within a metabolon can share common substrates and intermediates, which can lower the energy needed to transport these molecules between the enzymes.

Identifying and characterizing metabolons provide a better understanding of the organization and regulation of cellular metabolism, which can be useful for metabolic engineering and biotechnology.

In the case of enterobactin, demonstrating whether its biosynthetic machinery structurally gathers into a metabolon would be the first step towards identifying which residues are involved in the PPIs that orchestrate this assembly. It would also help further investigations as regards substrate channeling.

The interface at which proteins interact presents as landscapes of residues that engage in weak, non-covalent bonding (*e.g.*, hydrogen bonds, Van der Waals forces, electrostatic/ionic interactions, hydrophobic interactions) *vis-à-vis* one another. Yet it is the sum of these bonds that confer the overall affinity. Compounds that compete with this bonding can therefore weaken if not totally disrupt interactions. Conversely, mutations in key residues such that a stronger bond is established would mean an increase in affinity between the interacting proteins. Consequently, the benefits of mapping the landscapes of these PPIs and gaining more knowledge on the mechanisms that govern substrate channeling could paradoxically serve two very different purposes, by revealing targets not only for disruption, but also for optimization of these mechanisms.

1.4.3 Evidence in support of an enterobactin metabolon

Several pairwise PPIs between Ent proteins have been reported in the literature, both from our group and from others: EntE-EntB (82-83) , EntB-EntF (84), EntB-EntD (85), EntD-EntF (85), EntA-EntE (86), and EntA-EntB (86-87). However, until now all confirmed interactions only involve pairs of proteins, and the spatial organization of a higher order multienzyme complex has yet to be demonstrated.

Taken individually, these studies are not enough to conclude that all the Ent proteins assemble simultaneously in a metabolon, or it could be that *in vitro* techniques are not sensitive enough to capture a supramolecular assembly comprising three or more

proteins. An earlier study by Gehring *et al.* into interactions among Ent proteins in the NRPS arm of the pathway had reported that no high-affinity interactions existed between EntB, EntE, and EntF *in vitro* (88). This conclusion was based on a gel filtration experiment in which purified Ent proteins were found to elute as discrete native proteins and not in complexes. Notably, their report focused on Ent proteins in the NRPS arm of the enterobactin biosynthetic pathway, not the DHB arm.

Since then, our lab has extensively reported on interactions between Ent proteins in the [chorismate→ DHB] arm of the pathway, as well as between EntA and EntE, the first enzyme in the NRPS arm of the pathway (86). We found that pairwise interactions (EntA-EntE, EntA-EntB) did in fact occur *in vitro*, albeit at low affinity ($\sim \mu\text{M K}_\text{D}$) (86), consistent with the Gehring study. To that end, it was required to perform *in vivo* studies with bacterial adenylate cyclase two-hybrid (BACTH) assays where we confirmed an interaction occurring within living *E. coli* cells.

This in turn allows us to surmise that it should be possible to capture larger groups of proteins as they interact intracellularly. This assumption is based on two the following arguments:

1- EntB is bifunctional and involved in the two modules of biosynthesis. By virtue of its interaction with EntA and EntC (see Chapter 2) in one arm, and EntE and EntF in the other arm, it might serve as a central element around which the other components of the pathway organise themselves.

2- EntB has also been shown to be membrane-bound or at least membrane-associated (89). Therefore, just as has been confirmed for PvdA in biosynthesis of pyoverdine in *P. aeruginosa*, it might serve not only to anchor our proposed metabolon to the membrane,

but, in support of point 1, and act as the nucleation factor around which other proteins of the pathway assemble.

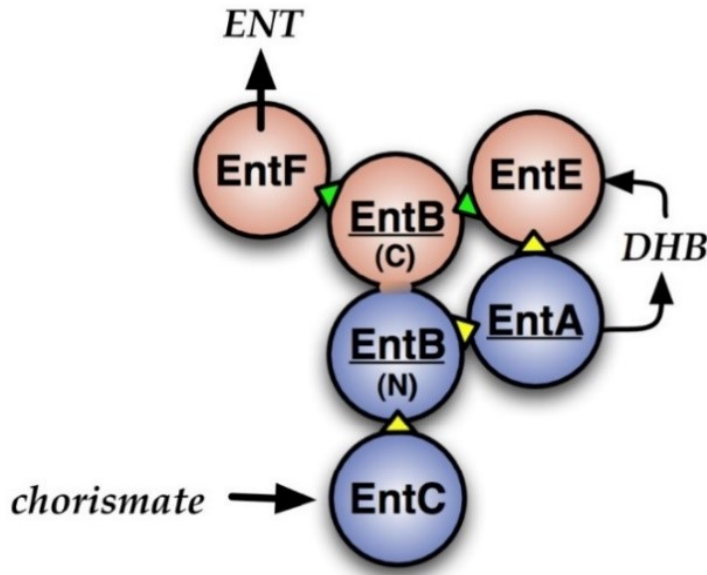


Figure 1.6. A proposed enterobactin metabolon. Schematics of a proposed supramolecular assembly of the enterobactin biosynthetic enzymes. In blue, the first module of enterobactin biosynthesis where chorismate is sequentially transformed into DHB through the sequential action of EntC, EntB (N-terminal domain) and EntA. In red, the enzymes involved in the NRPS module. Yellow triangles: interactions identified in our lab. Green triangles: interactions identified by other groups. This illustration does not account for the fact that EntA has been shown to be tetrameric, and that EntB is dimeric. The respective sizes of the proteins are not representative.

This enables us to propose that there exists an enterobactin metabolon, a supramolecular assembly where the enzymes involved in the biosynthesis of enterobactin gather to perform substrate channeling (Fig. 1.6).

1.5 Interactions involved in secretion

Once biosynthesis is complete, enterobactin is transported out of the cytoplasm through the inner membrane transporter EntS (37). Just as substrate channeling enhances flux and

protects labile or unstable intermediates, there are distinct advantages in a direct interaction between our proposed metabolon and this channel transporter:

1- The final product would be poised for immediate secretion out of the cytoplasm, thereby reducing diffusion of enterobactin, which would delay secretion.

2- Fes, the enzyme responsible for hydrolysis of Fe-Ent is not specific and can also hydrolyse newly synthesized enterobactin (90). In that case, any enterobactin lost to degradation ahead of secretion would be a waste of energy expenditure for the cell.

Furthermore, as previously mentioned, our hypothesis for an enterobactin metabolon is based on the models from the pyoverdine biosynthetic machinery (siderosome) elucidated in *P. aeruginosa* and that of bacillibactin in *B. subtilis*. In those organisms, the identified metabolons have been shown to interact with the membrane. We can therefore predict that the same mechanism operates for enterobactin secretion in *E. coli*.

Finally, we were able to gather further evidence in support of this hypothesis through a collaboration with the group of Dr. Johan Hofkens (KU Leuven, Belgium), who used photoactivated localization microscopy (PALM) to study the colocalization and membrane localization of the Ent proteins. PALM is a super-resolution imaging technique that can be applied to detection of protein interactions in *E. coli* cells with a limit of about 10 nm (91). Their results have shown that EntB localizes near the membrane (Fig. 1.7, unpublished data). Given the central role played by EntB by virtue of its involvement in the two different modules of the biosynthetic pathway, this supports the hypothesis that a proposed enterobactin metabolon would also localize at the membrane.

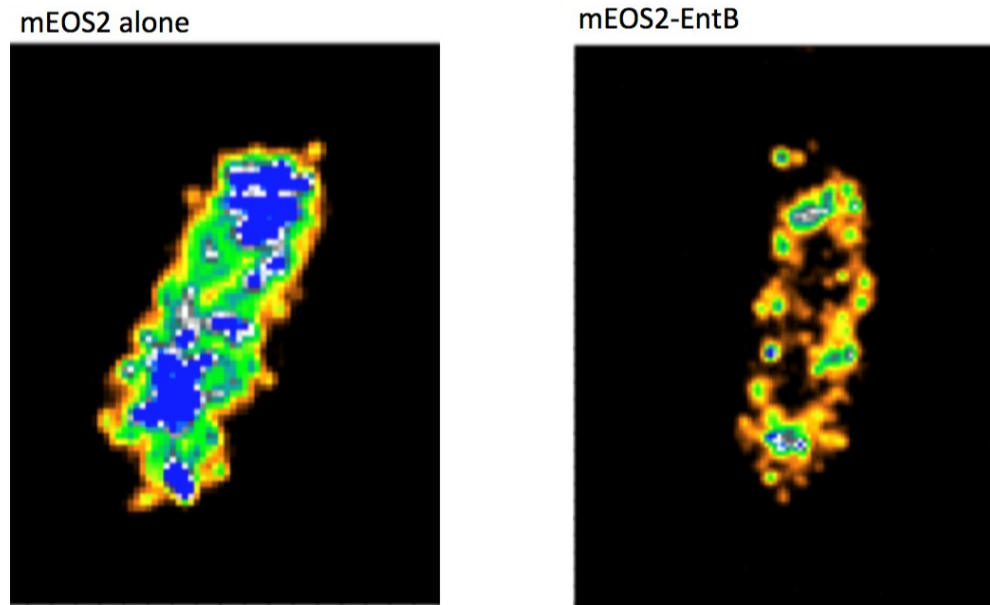


Figure 1.7. EntB localizes to the *E. coli* cell membrane. Super-resolution fluorescence microscopy (PALM) image obtained from collaborators in Belgium (work by Jacek Mika, KU Leuven – unpublished data) showing that EntB fused to the fluorescent reporter protein mEOS2 localizes to the *E. coli* cell envelope. Left panel: control, unfused mEOS2, evenly diffused through the *E. coli* cell. Right panel: mEOS2 fused to EntB.

This does not prove that EntB is the protein directly involved in an interaction with EntS or other components of the inner membrane. At present, only predictions can be offered. However, several possibilities can be considered. Although our model of a proposed enterobactin metabolon is schematic it helps visualise how a supramolecular assembly might be in contact with EntS (Fig. 1.8).

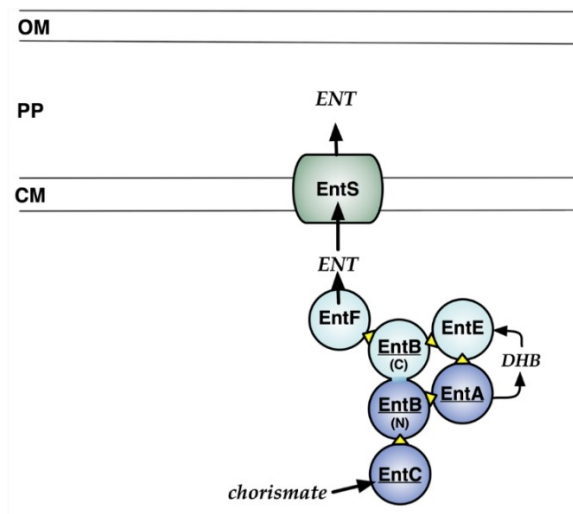


Figure 1.8. A proposed enterobactin metabolon interacting with IM channel transporter EntS. Schematic representation of our hypothetical metabolon, proposing how it could interact with EntS through EntF, the last enzyme of the pathway and therefore a potential interaction partner.

This is supported by results obtained by Hantash and Earhart who observed that all three enzymes of the NRPS modules have an affinity for membranes. Using shockates, the team isolated small fractions of each protein in membrane preparations and showed that these were enriched in a minor membrane fraction of buoyant density intermediate between that of cytoplasmic and outer membranes. This led them to surmise that those enzymes play a role in enterobactin biosynthesis as well as in its secretion (89).

A list of potential interactors can therefore comprise the following proteins, among others:

EntF: as determined by Hantash and Earhart and being the last enzyme involved in the biosynthesis of enterobactin, EntF is the most logical candidate for a direct interaction with EntS so that, upon completion of its synthesis, enterobactin is immediately exported.

EntB: as per Figure 1.7 and also as determined by Hantash and Earhart, EntB localizes at the membrane by means unknown and could also be a direct interactor of EntS.

EntE: also as determined by the study by Hantash & Earhart which showed that all three enzymes of the NRPS module have an affinity for membranes.

EntD: as explained under 1.3.1, EntD is responsible for phosphopantetheinylation of both EntB (ArCP) and EntF, converting them from their inactive *apo* forms into to their active *holo* forms (36). Although not directly involved in the biosynthesis of enterobactin, when overexpressed EntD has been shown to accumulate in the membrane fraction, with EntD domains exposed on the inner leaflet of the membrane (92). Although this does not constitute evidence of an interaction with EntS, there is a possibility that EntD might also play a role in anchoring the hypothesized metabolon to the IM. Within the membrane, proteins are never covalently bound but associate through the sum of otherwise weak interactions with the lipids and head groups and are therefore free to move within the fluid mosaic. How and where EntD localizes relative to EntS remains to be determined.

When querying the STRING database (93), the algorithm also lists other possible interactors. These candidate proteins are suggestions based on data such as the involvement of these proteins either in enterobactin biosynthesis or acquisition of ferric enterobactin, or simply by virtue of their proximity on the gene cluster (Fig. 1.9).

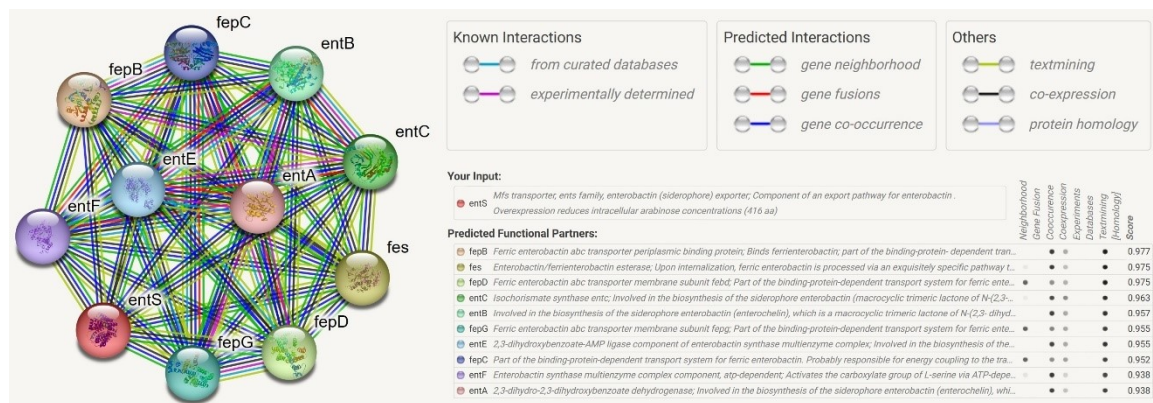


Figure 1.9. Results from the STRING database query for potential interactors of EntS. Results show no demonstrated interactions (turquoise, magenta) but rather predictions based on other parameters such as position within the gene cluster, co-expression or protein homology (from *entS* protein (*Escherichia coli* K12 MG1655) - STRING interaction network (string-db.org) accessed March 26 2023).

There exists no experimental evidence indicating which of these proteins, if any, directly interact with EntS. As well, we cannot discount the fact that more than one protein can participate in either a direct or an indirect interaction. For these reasons, a method aimed at widely probing for potential interactors must be applied.

1.6 Other knowledge to be gained – Secretion of enterobactin

1.6.1 Studying membrane proteins of E. coli

Membrane proteins are involved in a wide range of cellular processes such as transport of molecules across the cell membrane, in energy generation, and in signaling (94). As a Gram-negative bacterium, *E. coli* possesses two membranes, inner (IM) and outer (OM) that delimit a periplasmic space.

Membranes form a barrier segregating contents from surroundings. Relative to the membrane itself, proteins with which it associates can be defined by their location and association: directly or indirectly anchored, trans-membrane. This localization helps explain the many roles that membranes play in addition to their function as a barrier: compartmentalize (*e.g.*, organelles), generate a gradient (*e.g.*, proton pumps) and organise flow of metabolites (*via* transmembrane channels).

Membrane proteins in prokaryotes can be classified as follows:

Class I proteins carry out transport in Gram-negative bacteria in two steps: 1- from cytosol to periplasm, 2- from periplasm to the external environment.

Class II proteins also operate in two steps but here the outer membrane channel is linked to the inner membrane in the periplasm.

Class III proteins carry out transport in one step and in some cases also injects the compound into the host organism.

Class IV proteins are seen in Gram-positive bacteria and comprise various types.

Proteins that mediate transport across membranes are further classified into three categories (Fig. 1.10):

Uniporters: only one type of molecule, in one direction.

Symporters: two molecules, in the same direction.

Antiporters: two molecules, in opposite directions

Antiporters and symporters are known as secondary transporters since the release of free energy from one molecule being transported is used to power the transport of the other.

All transports involve a conformational change in the channel structure.

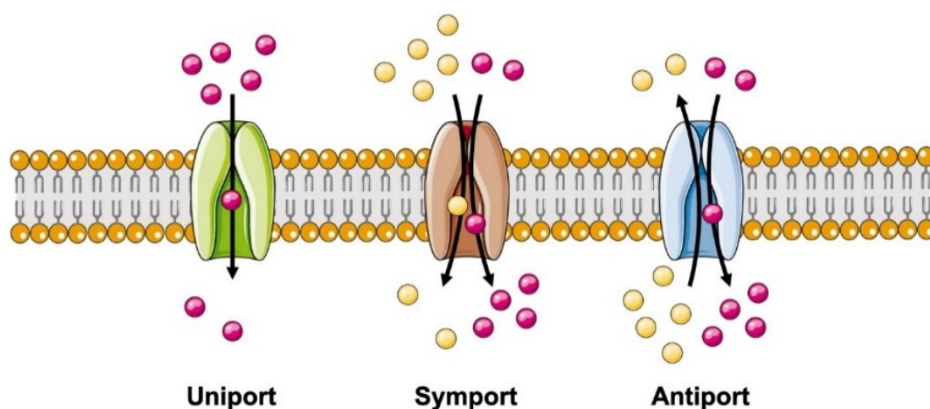


Figure 1.10 Types of active transport across membranes, schematic representation (from Winkelman, 2021, open access (95)). The number of coloured spheres indicate the relative chemical gradient. Image generated from Servier Medical Art under licence CC BY 3.0.

Uniporters that transport glucose and amino acids are among the most extensively studied in *E. coli* (96). They are characterized by their ability to carry or transport a single ion or molecule across a membrane in a single direction. Moreover, this type of transport is achieved down the concentration gradient to facilitate the mode of diffusion and accelerate the reaction that is already thermodynamically favored. The solute is

accommodated by a cavity contained within the interior of a transporter, which must therefore be sufficiently large. This cavity opens in one conformation on one side of the cell membrane, and then switches conformation to open on the opposite side, interchanging these two conformations upon binding and release of the substrate.

A classic example of a symporter is lactose permease LacY, in which transport of lactose across a gradient is aided by transport of protons going down gradient in the same channel (symport). This requires a second pump to export out the protons in order to re-establish this gradient. Other pumps include the P-type ATPase (that has a phosphorylated intermediate to initiate a conformational change opening/closing the channel) and ATP-binding cassettes (ABC) that change conformation upon binding of the molecule to be transported (open to close) thereby recruiting ATP to further continue the changes (close to re-open onto the other side). ABC's are all exporters in higher order organisms (*e.g.*, multidrug resistance phenomenon) whereas in prokaryotes they can also be importers.

In the case of antiporters, transport is achieved in an alternating-access mechanism. During a complete transport cycle, the transporter switches between an inward-facing conformation where the solute to export will bind, to an outward-facing one where this solute be released for the other solute destined to be imported to bind in turn (97). This is illustrated in Figure 1.11. The sodium/proton Na^+/H^+ pumps and the potassium/proton K^+/H^+ pumps are among the most extensively studied antiporters in *E. coli* (98-99).

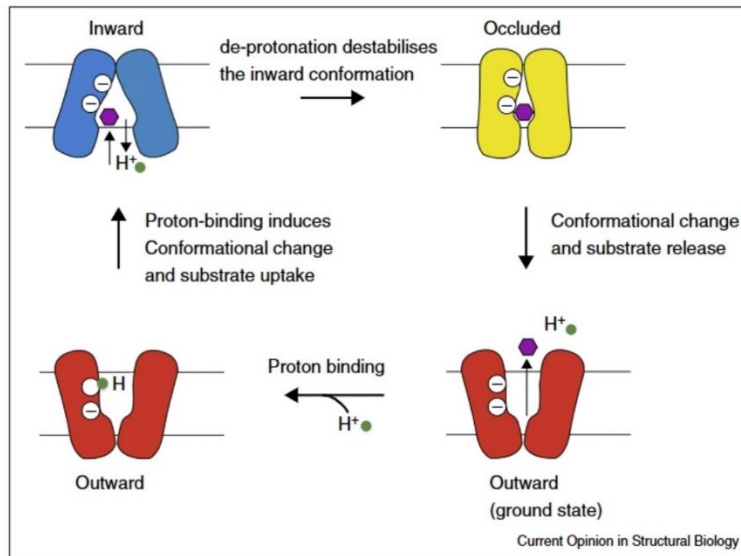


Figure 1.11. Alternating-access mechanism of antiporters (adapted from Du, 2015, open access (100)). Import and subsequent release of a proton induces a conformational change resulting in an inward opening, allowing the solute destined to be exported (in purple) to bind the pocket of the transporter. This leads to another conformational change allowing for an outward opening, where the solute is released. A proton binds the pocket to return the transporter back to an inward-opening conformation, and the cycle repeats.

1.6.2 MFS proteins

Proteins can also be classified in various ways based on their structure, function and composition. Online databases such as the Structural Classification of Proteins (SCOP) (101), the Class, Architecture, Topology, Homology database (CATH) (102) and the Protein Family database (Pfam) (103) each offer their own in the classification of proteins based on parameters such as structural similarities for the first two, and sequence similarities for the latter.

SCOP and CATH use a hierarchical classification system that groups proteins into classes, folds, superfamilies and families based on their overall structural similarities. This provides a standardized way to compare protein structures and can help identify functional relationships between proteins, even among those that are not closely

evolutionary related. This is especially true of superfamilies, a group of homologous proteins that share a common evolutionary ancestor and have similar three-dimensional structures and/or functions, despite having different amino acid sequences. Members of a protein superfamily usually have low sequence identity, yet they share a common structural or functional domain.

The Major Facilitator Superfamily (MFS) is the largest and most diverse group of proteins that play a crucial role in the transport of a wide range of substrates such as drugs, metabolites and ions across biological membranes (104-105). MFS proteins are highly diverse in terms of their amino acid sequences and three-dimensional structures. Despite this diversity, many of them share a common structural motif known as the canonical “MFS fold” comprising two domains, each consisting of six consecutive transmembrane (TM) helices, also known as the “two by six” model (97).

Because of their importance for cellular function as well as their abundance, many MFS transporters have been structurally resolved. One remarkable aspect is that their N and C domains enclose a binding cavity for the substrate they transport, located in the center of the membrane. Amidst this cavity, multiple aromatic residues contribute to the positioning of the substrate and keeping it isolated from the extramembrane environment.

1.6.3 EntS, inner membrane transporter for enterobactin

Through the use of *null* mutants, EntS (previously called YbdA), has been identified as the IM translocation efflux pump for enterobactin (37). At present, there is no resolved structure for EntS. A topology map generated by Furrer *et al.* (Fig. 1.12A) predicted a model in agreement with a canonical TM, 12-helix arrangement shown to contain the characteristic RXXRR(K) motif of MFS proteins (106-107).

The Transporter Classification Database (TCDB) (*104*) has classified EntS in its own family, the enterobactin (siderophore) exporter (EntS) family, related to the larger, sugar porter family that includes known transporters such as those for glucose, lactose, arabinose and xylose, all with 12 TM helices and a similar number of residues. The gene encoding EntS is located within the enterobactin biosynthetic gene cluster (Fig. 1.5) and is therefore under control of Fur, thus upregulated along with the rest of the biosynthetic machinery so that transport can be carried out as biosynthesis is completed.

EntS shares many structural similarities with other proteins of the same superfamily such as lactose permease LacY (PDB: 2CFQ), glycerol-3-phosphate transporter GlpT (PDB: 1PW4) and multi-drug exporter EmrD (PDB: 2GFP). These proteins have often been used as models against which structure-based alignments have been performed, but despite a high correlation there are no means to deduce a definitive structure for EntS.

Recently, a more refined structure has been proposed by DeepMind's AlphaFold Protein Structure Database (*108*) (Fig. 1.12B). The conformation thus generated shows that it possibly allows for the formation of a pore through which enterobactin could be exported (Fig. 1.12C).

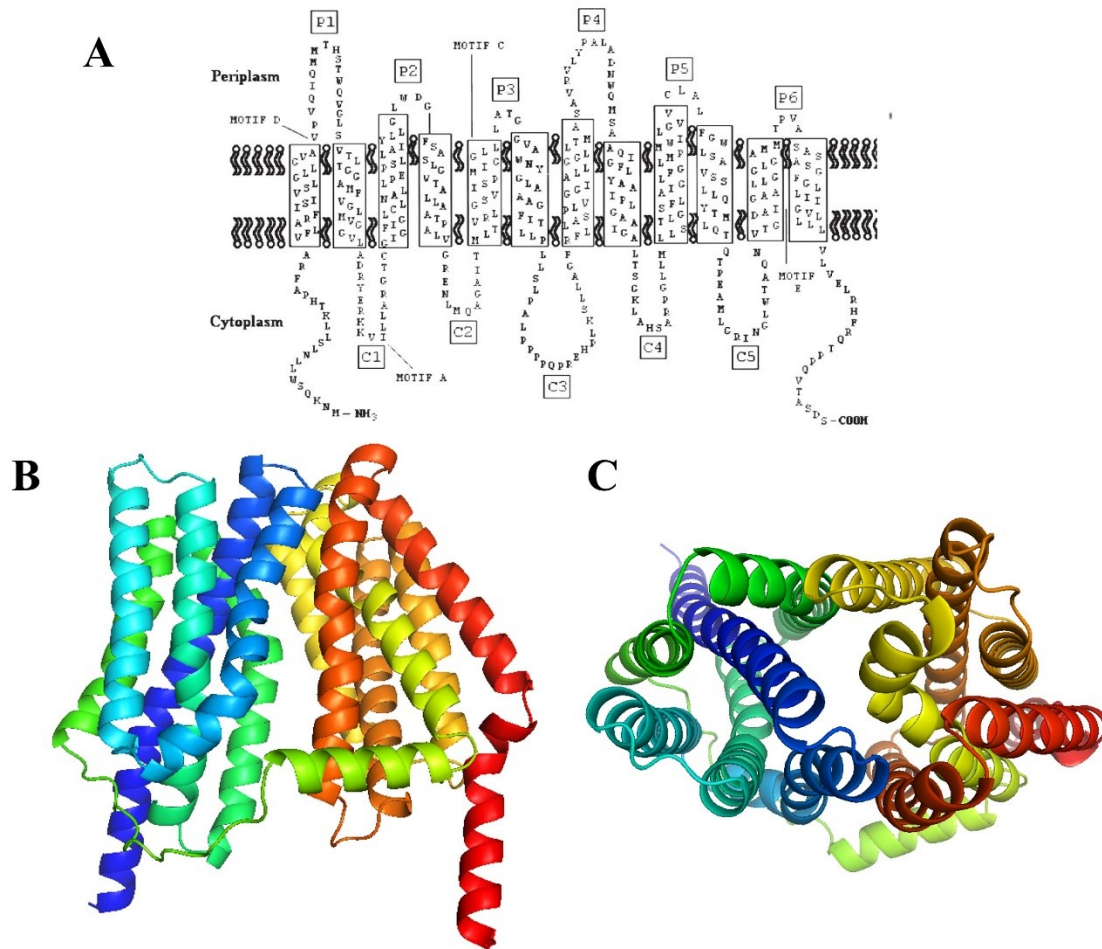


Figure 1.12. Proposed topology map and structure of EntS, the enterobactin transporter channel of *E. coli*. A) Topology map generated by Furrer *et al.*, (open access) (37). B) Predicted structure proposed by AlphaFold. C) Top view of the AlphaFold structure.

EntS has been proposed to be a Class I transport protein following its formal identification as the transporter of enterobactin across the IM. However, there have been very few studies investigating its structure and function. More studies have concluded that transport of enterobactin from the periplasm across the outer membrane is dependent on another system, TolC, that is not encoded locally (109). Although TolC was shown to be responsible for this efflux, further studies using single deletions of pumps known to associate with TolC did not impact export, suggesting that this export was reliant on

another system. More recently an extensive study including several RND transporters looked at the effect on transport of enterobactin by deletion mutants of *acrB*, *acrD*, *mdtABC*, *acrEF*, or *mdtEF* (38). Single deletions did not affect this transport, whereas multiple deletions of *acrB*, *acrD*, and *mdtABC* resulted in a significant decrease in export. Plasmids carrying the *acrAB*, *acrD*, or *mdtABC* genes restored the phenotype, thus indicating that tripartite systems AcrB, AcrD, and MdtABC are required to fulfill the enterobactin export function (Fig. 1.13).

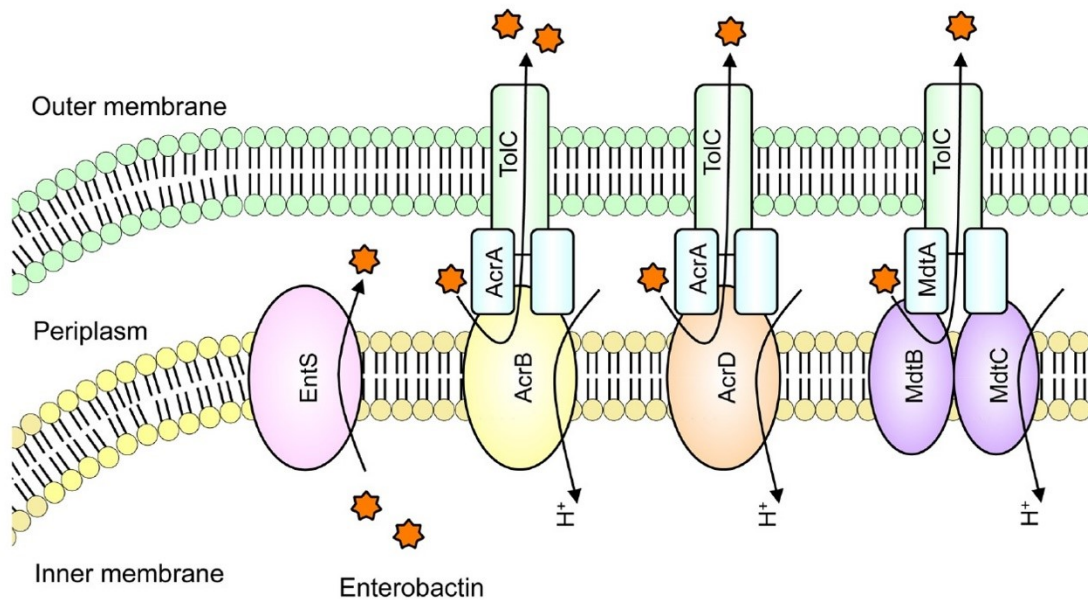


Figure 1.13. Proposed tripartite systems involved in transport of EntS out of the *E. coli* periplasm (adapted from Horiyama & Nishino, 2014, open access (38)). The resistance-nodulation-division (RND) pumps AcrB, AcrD, and MdtABC are involved in enterobactin export function as shown by deletion studies.

In the absence of a resolved structure, detailed information about the mechanism by which EntS carries out transport can only be surmised. Characterizing this protein could yield useful insights into its functionality, by identifying the residues that form the inner and outer opening of the transport pore, presumably where interactions with other

proteins – and with enterobactin itself – could occur. Moreover, based on similarities with other MFS proteins, EntS is predicted to be an antiporter shuttling enterobactin out of the cytoplasm in conjunction with import of protons (37). The mechanism through which this is achieved (*i.e.*, which residues are key to the binding of each solute, how conformational change is achieved to complete a cycle) remains unknown.

As the secretory channel for enterobactin, EntS therefore contributes to virulence in pathogenic strains. Results from characterisation studies could be applied towards efforts at combating disease and in the development of therapeutics. As was also discussed earlier, elucidating the assembly of the biosynthetic machinery of enterobactin could give rise to applications for industrial processes such as biofuel production and bioremediation. Characterizing EntS and gaining a greater knowledge of its role in secretion can therefore help to improve these processes and make them more efficient.

In particular, as a member of the MFS, characterization could address the three fundamental mechanisms commonly shared by all the members of this superfamily: identify the residues responsible for the binding of enterobactin, offer an insight into the conformational changes that take place during transport, and give a general idea of the coupling mechanism involved in antiport, if indeed EntS is demonstrated to be an antiporter (97).

Another noteworthy aspect is that several membrane transporter proteins show a need to assemble as multimers to be functional. One example of a protein known to dimerize is the plant dual-affinity nitrate transporter NRT1.1 from *Arabidopsis thaliana* (PDB: 4OH3) (110), a protein from the proton-dependent oligopeptide transporter family (POT), which shares a conserved structural topology with MFS proteins (111). In this particular

case, the investigators observed that the protein was shown to dimerize upon crystallization. While aiming to achieve monodispersity in solution, they also noted that the dimeric state varied with the pH and depended on the detergent used. As will be seen in Chapter Five, at present there is no evidence that EntS is monomeric, and indeed preliminary results tend to suggest that it possibly presents as a dimer.

1.6.4. Approaches in studying EntS.

The study of integral membrane proteins (IMPs) such as EntS presents numerous challenges, starting with the expression of these proteins, which in excess often leads to misfolding and sequestration in inclusion bodies (112-114). This limits the yield available for isolation and characterization. Extracting IMPs from the lipid bilayer is necessary for further characterization yet constitutes another hurdle as their high hydrophobicity makes them prone to aggregation and precipitation. There is no single extraction protocol for all membrane proteins, and determining the best compounds and conditions is an extensive undertaking.

Detergents

One common strategy for extracting IMPs is through the use of detergents. These amphipathic compounds insert themselves into the lipid bilayer, isolating the proteins into a spherical micelle while retaining some of the native phospholipids that contribute to protein conformation. However, detergent-based protocols require careful optimization of parameters such as temperature, detergent concentration, and duration to prevent delipidation-induced precipitation. Maintaining the stability of the micelle necessitates the proper detergent concentration in all buffers and solutions used in subsequent studies.

This becomes crucial for proteins like EntS, which is predicted to have 12 transmembrane helices of high hydrophobicity.

Detergents are effective in solubilizing and extracting IMPs from membrane bilayers, and various commercially available compounds with different structures and physicochemical properties can be used for this purpose. Despite their effectiveness, the application of detergents can have drawbacks as they may also negatively impact protein stability, function, and structure. Moreover, experimental conditions such as the critical micellar concentration (CMC) can affect the properties of the detergents themselves (115-116).

Lipid nanodiscs (MSP)

Recent technological advances have facilitated the isolation of IMPs without the use of a detergent micelle. In particular, more and more IMPs are being isolated and characterized through the use of lipid nanodiscs (Fig. 1.14A) (117).

Nanodiscs are discoidal assemblies consisting of a lipid bilayer surrounded by membrane scaffolding proteins (MSP) that align in the manner of a double belt to shield the hydrophobic edge of this lipid bilayer from the aqueous environment. Unlike detergent micelles that are mostly spherical, nanodiscs help retain a native-like environment for the solubilization and stabilization of membrane proteins that is more representative of their topology (118).

The self-assembly process begins with the mixture of phospholipids, MSPs, and the protein of interest isolated in a detergent micelle. Upon removal of the detergent, nanoparticles with a diameter in the range of 1-17 nanometers are formed, consisting of

either saturated or unsaturated phospholipids with the protein of interest in their midst, cinched by the MSP belt (119).

This self-assembly method and the stability of the resulting nanodiscs enable the generation of soluble lipid bilayer membranes, providing a platform for incorporation of single membrane proteins into a native-like environment, in a structure that is highly soluble in aqueous solutions. This helps address the challenges posed by the use of detergents as previously described since the protein of interest is only temporarily solubilized with the detergent. Here again, kits are commercially available to aid in the generation of lipid nanodiscs. However, this method still requires that the protein first be extracted and purified in detergent.

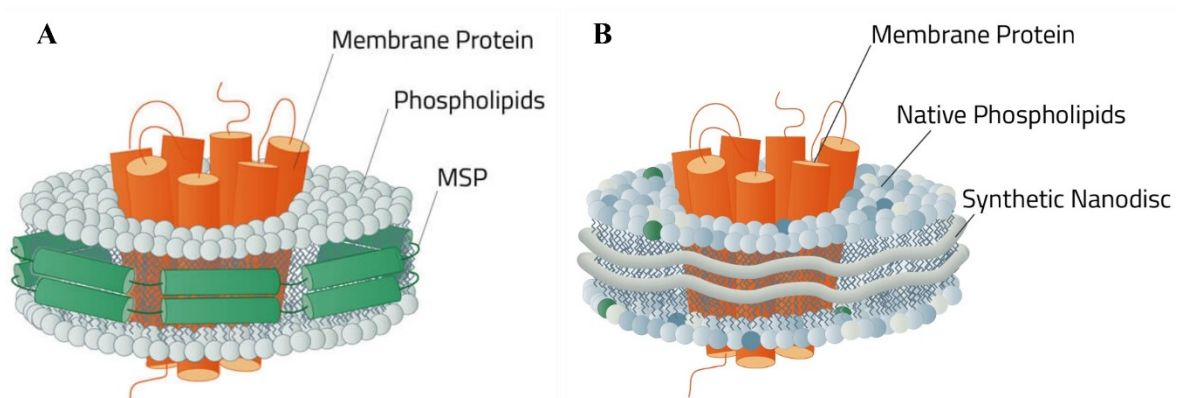


Figure 1.14. Nanodiscs used for isolation and solubilisation of membrane proteins (from CubeBiotech (Germany) website (120)). A) nanodisc assembly held by membrane scaffold proteins (MSP). B) synthetic nanodisc held by synthetic copolymer rings.

Synthetic nanodiscs

More recently, the advent of synthetic lipid nanodiscs (often referred to as SMALPs) has contributed to facilitate the extraction and isolation of IMPs (121-123). Extraction in synthetic nanodiscs, often followed by characterization by nuclear magnetic resonance (NMR) or cryo-electron microscopy (Cryo-EM), has now become the path of choice for

resolving the structure of membrane proteins and can also be advantageously applied for studying EntS (124).

Synthetic nanodiscs differ from MSP nanodiscs in that they are held together by a synthetic copolymer ring (Fig. 1.14B). Styrene-maleic acid copolymers (SMA / SMALPs), diisobutylene-maleic acid (DIBMA) and acrylic acid styrene (AASTY) are the most commonly used at the moment, in formulations where the ratios of acid:styrene vary (120). The main advantage of using such copolymers is that they can extract and solubilize membrane proteins directly from cells or crude extracts, which can be followed by affinity purification, thereby bypassing the need for detergents altogether and avoiding their associated negative effects.

For EntS, in absence of preliminary data to that effect, several approaches need to be explored, each with extensive screening for optimal conditions such as temperature, duration, and concentration of the various compounds utilised.

1.7 Scope of this research

This thesis articulates itself around several questions pertaining to the biosynthesis and secretion of enterobactin in *E. coli*:

1.7.1 Can we demonstrate that the first two enzymes of the enterobactin biosynthetic pathway, EntC and EntB, are directly interacting, and thereby confirm that this pathway is fully networked via protein-protein interactions?

This will be addressed in Chapter Two. Using *in vivo* crosslinking with formaldehyde, supported by evidence previously obtained by a two-hybrid assay and coupled with a proposed docking model, evidence will be presented supporting formation of an EntCB complex can indeed be detected. This will be the first reported confirmation of an

interaction between these proteins and will therefore complete the roster of pairwise interactions among the enzymes involved in all the sequential steps of enterobactin biosynthesis in *E. coli*.

1.7.2 Can we gather evidence suggesting that Ent proteins organise themselves in a dynamic supramolecular complex, i.e., a metabolon, by capturing in vivo a simultaneous, intracellular assembly of several, if not all, Ent proteins?

Chapter Three will discuss efforts at identifying the proteins contained in the higher-order complexes observed as part of the experiments conducted under point 1.7.1. Through the use of clones harbouring several epitope-tagged Ent proteins, and continuing with *in vivo* crosslinking with formaldehyde, this chapter will explain how it was possible to isolate the high-molecular weight bands of interest. Probing these bands before and after boiling (to heat-reverse the chemical crosslink) for the presence of at least four different proteins of the pathway will show how at least two of them, each active in a different arm of the biosynthetic pathway (*i.e.*, EntC in conversion of chorismate into 2,3-DHB *vs* EntE in the NRPS module) are involved in a simultaneous interaction.

1.7.3 Can we determine which, if any, of the Ent biosynthetic proteins closely interact with EntS, the inner membrane secretory channel for enterobactin?

Chapter Four will focus on probing for potential interactors of EntS through proximity labeling. EntS fused to the ascorbate peroxidase (APEX2) enzyme will be used as the bait protein, to achieve biotinylation of proteins with a 10 nm radius. This will yield a list of potential candidate interactors (preys). We expect this list will include not only Ent biosynthetic enzymes, but also structural and accessory proteins involved either in biosynthesis and/or in later steps of secretion out of the cytoplasm and periplasm. The list

will be compared to interactors proposed by platforms such as STRING, thus paving the way for the confirmation of interactors of interest in future work.

1.7.4 Can we efficiently isolate and characterise IM channel EntS so that its role in secretion, hence its role in virulence, can be better studied?

Finally, Chapter Five will detail efforts at extracting and purifying EntS, using both detergents and copolymers needed to engineer synthetic lipid nanodiscs (*e.g.*, SMALPS). The chosen methodology will make use of recently developed tools and compounds that have been optimized for that purpose.

1.8 Rationale for experimental approaches

1.8.1 In vivo crosslinking with formaldehyde

Transient protein assemblies that exhibit weak affinity need to be captured as they occur *in vivo*. Determining whether two or more proteins interact in a live cell can be achieved through several methods.

Should an interaction be suspected between two candidate proteins, popular tools aimed at detecting this interaction include creating fusion proteins where dissected domains or segments of a reporter (*e.g.* an enzyme, a fluorophore, a transcription factor) are brought in close proximity thereby restoring their functionality. In our lab, evidence of interaction was obtained using bacterial adenylate cyclase two-hybrid (BACTH) assays with positive results (87).

However, these methods are sometimes limited due to the steric hindrance brought about by the structure and orientation of the reporters that may prevent the interaction from taking place. In the case of an EntC-EntB interaction, as will be discussed in

Chapter Two, preliminary results obtained in our lab using BACTH needed confirmation *via* another method.

The choice of *in vivo* crosslinking with formaldehyde was based on several properties of this chemical: 1) it is membrane permeable thereby allowing for application in a live cell culture; 2) its short spacer arm (2-3 Å) permits capture of closely interacting proteins; 3) it reacts with primary amines of residues (*i.e.* lysine, tryptophane, the N-terminus) exposed at the surface of the protein in an aqueous milieu; 4) the covalent linkage is reversible by heating the sample at temperatures above 70 °C.

The limitations inherent to the use of formaldehyde however imply that 1) the candidate interactors be oriented in a way that bring in close proximity a suitable number of residues sensitive to formaldehyde; 2) denaturing of the crosslinked sample be done at a temperature below 60 °C if the covalent linkage is to be maintained.

1.8.2 Isolating large, crosslinked complexes

As part of the study conducted under Chapter Three, isolation of crosslinked complexes proved problematic due to their low abundance. Methods requiring large volumes of protein lysates such as gel filtration or dialysis did not yield sufficient amounts of proteins to continue with analysis. However, since it was established in our earlier study (conducted under Chapter Two) that the complex of interest was detected on Western Blot following separation by SDS-PAGE at a molecular weight above 260 kDa, we therefore employed passive elution out of polyacrylamide gel to retrieve it.

This method developed by Kurien (138) consist of excising the portion of the polyacrylamide gel that contains the band of interest and incubating it in buffer so that the proteins elute out of the gel. One caveat is that SDS-PAGE allows for denaturation of

the protein which disallows subsequent analysis should the protein need to remain fully folded. In addition, excising and grinding of the polyacrylamide gel can cause attrition.

1.8.3 Probing for candidate interactors

Should the identity of the interacting proteins be unknown, methods aimed at probing for potential interactors must be used. Again, the transient nature of the proposed assemblies required that the interaction be captured *in vivo*.

As detailed under Chapter Four, we employed proximity labeling with a modified ascorbate peroxidase (146-147) that had been optimized for expression in bacteria. Fusing this enzyme to a bait protein allows for biotinylation of proteins that localise within a 10 nm radius, by transfer of an activated biotin molecule onto electron-rich residues such as tyrosine, tryptophane, cysteine and histidine.

However, it is worth noting that close proximity does not equate to a direct interaction and other methods must therefore be used to determine the extent to which a bait and a detected prey do engage in such an interaction. In addition, although the transfer reaction of the biotin molecule is optimal within 10 nm, this does not constitute an absolute limit and biotinylation may occur (albeit at a lower intensity) for proteins outside of this radius (146-147). Finally, depending on the size of the interactors, the nature of the residues exposed at their surfaces and the orientation in which they position themselves, the transfer of the biotin molecule may not be taking place even if there is an *in vivo* interaction.

1.8.4 Extraction and purification of membrane proteins

As detailed under 1.6.4, several approaches can be used for extraction and solubilisation of IMPs. Our first approach was to create lipid nanodiscs, which required

extraction in detergents. Although two detergents proved successful for the solubilisation step we experienced difficulties in achieving purification. In a later stage we therefore elected to solubilize the protein of interest in copolymers that had become available in the interval. This yielded synthetic lipid nanodiscs that retain the endogenous membrane lipids and contribute to the retention of the protein in its native conformation.

The use of copolymers eliminates the need for detergent, hence the risk of delipidation-induced precipitation. One caveat however is that excess copolymer remaining in the solution post solubilisation may interfere with the binding of the affinity tag on the resin used for the purification step. In addition, the possibility that a solubilised, synthetic nanodisc may contain other proteins in addition to the protein of interest cannot be discounted.

CHAPTER TWO – Evidence of an intracellular interaction between the *Escherichia coli* enzymes EntC and EntB and identification of a potential electrostatic channeling surface.

The work presented in this chapter is extracted and expanded from the published manuscript “Ouellette, S., Pakarian, P., Bin, X., & Pawelek, P. D. (2022). Evidence of an intracellular interaction between the *Escherichia coli* enzymes EntC and EntB and identification of a potential electrostatic channeling surface. *Biochimie*, 202, 159-165.”

Summary

Siderophores are high-affinity, small-molecule chelators employed by bacteria to acquire iron from the extracellular environment. The Gram-negative bacterium *Escherichia coli* synthesizes and secretes enterobactin, a tris-catechol siderophore. Enterobactin is synthesized by six cytoplasmic enzyme activities: EntC, EntB (isochorismatase (IC) domain), EntA, EntE, EntB (aryl carrier protein (ArCP) domain), and EntF. While various pairwise protein-protein interactions have been reported between EntB, EntA, EntE, and EntF, evidence for an interaction between EntC and EntB has remained elusive. Building on preliminary evidence from a bacterial two-hybrid assay, we employed *in vivo* crosslinking to demonstrate an intracellular EntC-EntB interaction. *In vivo* formaldehyde crosslinking of *E. coli* cells co-expressing HA-tagged EntB and H6-tagged EntC resulted in an observable ~ 80 kDa band on Western blots that cross-reacted with anti-HA and anti-H6, corresponding to one HA-EntB monomer (33 kDa) crosslinked with one H6-EntC monomer (45 kDa). This band disappeared upon sample boiling, confirming it to be a formaldehyde-crosslinked species. Bands of molecular masses greater than 80 kDa that cross-reacted with both antibodies were also observed. This finding was further supported by automated docking of the crystal structures of

monomeric EntC and dimeric EntB that resulted in a top-ranked candidate docked ensemble. Automated docking of the crystal structures of monomeric EntC and dimeric EntB resulted in a top-ranked candidate docked ensemble in which the active sites of EntC and EntB were oriented in apposition and connected by an electropositive surface potentially capable of channeling negatively charged isochorismate. These research outcomes provide the first reported evidence of an EntC-EntB interaction, as well as the first experimental evidence of higher-order complexes containing EntC and EntB.

2.1 Introduction

Most bacteria require iron in the form of ferric ions (Fe^{3+}) to sustain their growth and metabolic needs. However, Fe^{3+} in the extracellular environment forms insoluble complexes, and in a mammalian host most circulating iron is bound to serum proteins such as transferrin (1). Given the challenge of acquiring this scarce yet essential micronutrient, many bacteria have evolved mechanisms for high-efficiency iron acquisition through the production of siderophores, which are molecules that bind Fe^{3+} with very high affinity. Enterobactin is a catecholate-type siderophore produced by *Escherichia coli* and *Salmonella* (2). This molecule is a triserine trilactone with three catechol subunits that confer extremely high-affinity iron-binding ($K_D \sim 10^{-50}$ M at physiological pH (1)). This high affinity allows for efficient scavenging of Fe^{3+} from various sources, followed by TonB-dependent uptake into bacterial cells (125). Since iron uptake allows for the growth and proliferation of pathogens in mammalian hosts, siderophores and the proteins involved in their biosynthesis and secretion are recognized as virulence factors in numerous infectious diseases (4).

Biosynthesis of enterobactin in the *E. coli* cytoplasm involves the coordinated activities of five proteins, EntC, EntB, EntA, EntE and EntF. In iron-rich conditions, Fe²⁺-bound ferric uptake repressor protein (Fur) binds to a consensus sequence (*i.e.*, the “fur box”) contained in the promoters of operons containing the genes encoding the Ent proteins, thus repressing transcription. In iron-depleted conditions *apo*-Fur dissociates from these promoter regions, resulting in up-regulation of *ent* gene expression (49).

The enterobactin biosynthetic pathway is divided into two major functional arms. In the first arm of the pathway, a chorismic acid precursor is transformed through sequential reactions of EntC (isochorismate synthase), EntB (N-terminal isochorismatase (IC) domain) and EntA (2,3-dihydro-2,3-dihydroxybenzoic acid dehydrogenase) to yield 2,3-dihydroxybenzoic acid (DHB). In the second arm of the pathway, DHB is condensed with L-serine through three cycles of condensation involving non-ribosomal peptide synthetase (NRPS) activities (EntE, EntB (C-terminal aryl carrier *protein* (*ArCP*) domain) and EntF) (2). Following cyclization, the enterobactin product is then secreted through the inner-membrane efflux transporter EntS (37) in a TolC-dependent manner (109).

Most of the enterobactin biosynthetic enzymes have been reported by our lab and others to engage in pairwise interactions (82-83, 85-86, 126). We hypothesize that these interactions facilitate substrate channeling to enhance the efficiency of the enterobactin biosynthesis and secretion. Substrate channeling has been shown in other systems to limit the diffusion of intermediates into the cytosol as well as to protect labile intermediates from degradation (127). We further hypothesize that all of the Ent biosynthetic enzymes are networked by protein-protein interactions, with higher-order complexes involving

three or more interacting partners occurring within the *E. coli* cell; our previously reported model of an EntBAE complex (87), which is sterically compatible with the pairwise EntA-EntE and EntB-EntE complexes, supports this hypothesis.

One interaction that has so far eluded detection is that involving EntC and EntB, the first two enzymes of the pathway. EntC (isochorismate synthase) is a monomeric enzyme (42 kDa) that catalyses the Mg^{2+} -dependent interconversion of chorismate and isochorismate with K_M values of 14 μM with for chorismate and 5 μM for isochorismate (128-129). EntB (isochorismate lyase) (monomer mass: 32 kDa) is a dimeric bifunctional enzyme that converts isochorismate into 2,3-dihydro-2,3-dihydroxybenzoate at its N-terminal domain (36,82). The 2,3-dihydro-2,3-dihydroxybenzoate is then furthered processed by the other Ent enzymes (including the EntB at its C-terminal, ArCP domain) to yield enterobactin.

Initial studies through a bacterial adenylate cyclase two-hybrid (BACTH) assay (work by P. Pakarian, unpublished data) had provided preliminary evidence for an interaction: when combinatorially co-transformed into *E. coli* BTH101 cells, functional BACTH constructs expressing T18/T25-EntC and T18/T25-EntB showed a positive signal for the pUT18C-*entC* and pKT25-*entB* pair. However, no signal was observed for the pUT18C-*entB* and pKT25-*entC* co-transformants. Fusion of reporters to proteins may interfere with functional activity, cause steric hindrance, or affect stability. Therefore, this lack of signal may be due to any of these factors after fusing the T25 fragment to EntC. Taken together, these results indicate that BACTH positive signal was only observed when EntC and EntB were N-terminally fused to T18 and T25, respectively. Although these results

were highly suggestive of an interaction, confirmation required further experimentation using another, complementary *in vivo* technique.

Through *in vivo* crosslinking with formaldehyde, we demonstrate that EntB and EntC form a specific pairwise interaction within the *E. coli* cell, which supports the BACTH assay. We further report a computational model of the EntB-EntC complex and identify a possible substrate channeling surface connecting the EntB and EntC active sites (work by X. Bin).

2.2 Methods

All reagents were obtained from Bioshop Canada, Inc. (Burlington, Ontario) unless otherwise specified.

Crosslinking of EntB and EntC clones

E. coli AG-1 cells harboring pCA24N-*entB*-His (Cam^R) and *E. coli* AG-1 cells harboring pCA24N-*entC*-His (Cam^R) were obtained from the ASKA collection (130). Each type of cells was grown in Miller's M63 minimal medium (22 mM KH₂PO₄, 40 mM K₂HPO₄, 15 mM (NH₄)₂SO₄, 0.2% glucose, 60 nM thiamine, and 1 mM MgSO₄, 5 µg l⁻¹ FeSO₄, heptahydrate) at 37 °C in the presence of 34 µg mL⁻¹ chloramphenicol. When OD₆₀₀ reached 0.6-0.7, both cultures were treated with 1 mM IPTG for 3 hours at 37 °C to induce expression of the EntB and EntC proteins encoded on their respective plasmids. Aliquots were taken from the cultures and kept as non-treated samples. The cultures were then treated with formaldehyde (0.5% final concentration) (Sigma) for 20 minutes at 37 °C to initiate crosslinking of interacting proteins. The reaction was quenched by adding equal volumes of 125 mM glycine. Cells were then pelleted and washed in 100 mM PBS prior to lysis with gel-loading buffer (131). Prior to electrophoresis, aliquots were taken

and heated at 46 °C for 10 minutes to preserve crosslinked species. To heat-reverse formaldehyde crosslinking, other aliquots were instead heated at 100 °C at different time intervals (3 minutes vs 6 minutes). All samples were loaded in parallel on two 10% SDS-polyacrylamide gels, one for treatment by Coomassie stain and the other for Western blotting. The proteins were then separated at 200 V on a BioRad Mini-PROTEAN gel electrophoresis apparatus. One gel was rinsed and incubated with Coomassie stain, whereas the other was transferred onto PVDF membrane (BioRad) at 100 volts for 60 minutes, on ice. After rinsing in TBS, the membranes were blocked using the Penta-His HRP conjugate kit (Qiagen), then probed with the Penta-His HRP Conjugate antibody (Qiagen) at 1:2,000 dilution for detection of the His-tagged proteins. Detection was performed with Thermo SuperSignal™ West Pico Chemiluminescent Substrate. Chemiluminescence was recorded using the Amersham Imager 600 system. Exposure times were extended as appropriate in order to observe faint bands indicative of complexed proteins.

Crosslinking of double transformants

E. coli AG-1 cells harboring pCA24N-*entC*-His (Cam^R) used in the previous experiment were grown in Luria-Bertani (LB) broth and transformed with a pBFH1-*entB*-HA (Amp^R) plasmid previously constructed (132). Successful double transformants were grown in a modified version of Miller's M63 minimal medium (22 mM KH₂PO₄, 40 mM K₂HPO₄, 15 mM (NH₄)₂SO₄, 0.15% glucose, 60 nM thiamine, and 1 mM MgSO₄, 5 µg l⁻¹ FeSO₄, heptahydrate) to induce expression the HA-EntB protein (under *fur* control) and adding 0.05% lactose as the carbon source to induce expression of the H6-EntC protein (under *lac* control). Double transformants were cultured in the presence of both 34 µg

mL⁻¹ chloramphenicol and 100 µg mL⁻¹ ampicillin. When OD₆₀₀ reached 0.6-0.7, aliquots were taken to be kept as non-treated samples, and the rest of the culture was treated with formaldehyde (0.5% final concentration) (Sigma) for 20 minutes at 37 °C to initiate crosslinking of interacting proteins. The reaction was quenched by adding an equal volume of 125 mM glycine. Cells were then pelleted and washed in 100 mM PBS prior to lysis with gel-loading buffer (131). Prior to electrophoresis, aliquots were taken and heated at 46 °C for 10 minutes to preserve crosslinked species. To heat-reverse formaldehyde crosslinking, other aliquots were instead heated at 100 °C at different time intervals (1 minute, 2 minutes and 10 minutes). All samples were loaded in parallel on two 4-15% gradient SDS-polyacrylamide gels (BioRad), one for treatment by Coomassie stain and the other for Western blotting. The proteins were then separated at 200 V on BioRad Mini-PROTEAN gel electrophoresis apparatus. One gel was rinsed and incubated with Coomassie stain (supplemental figure S1.1A), whereas the other was transferred onto PVDF membrane (BioRad) at 100 volts for 90 minutes, on ice. The membrane was then recovered and treated with Ponceau stain to assess the quality of the protein transfer (supplemental figure S1.1B). After rinsing in TBS, the membrane was blocked using the Penta-His HRP conjugate kit (Qiagen). The first immunoblot to detect HA-EntB was performed using an anti-HA mouse monoclonal antibody (1:10,000) (Thermo-Pierce), followed by treatment with an m-IgG Fc BP-HRP conjugate (1:2,000) (Santa Cruz Biotechnology). Detection was performed using Thermo SuperSignal™ West Femto Maximum Sensitivity Substrate. After imaging, the membrane was rinsed in TBS for 10 minutes to remove the chemiluminescent developer, then incubated in Restore™ Western Blot Stripping Buffer (Thermo) for 5 minutes at room temperature to

remove the antibodies. The membrane was then incubated overnight at 4 °C in blocking buffer, then re-probed with the Penta-His HRP Conjugate antibody (Qiagen) at 1:2,500 dilution for H6-EntC. Detection was repeated, this time using with Thermo SuperSignal™ West Pico Chemiluminescent Substrate. In both instances, chemiluminescence was recorded using the Amersham Imager 600 system. Exposure times were extended as appropriate in order to observe faint bands indicative of complexed proteins.

2.3 Results

Crosslinking of EntB and EntC clones

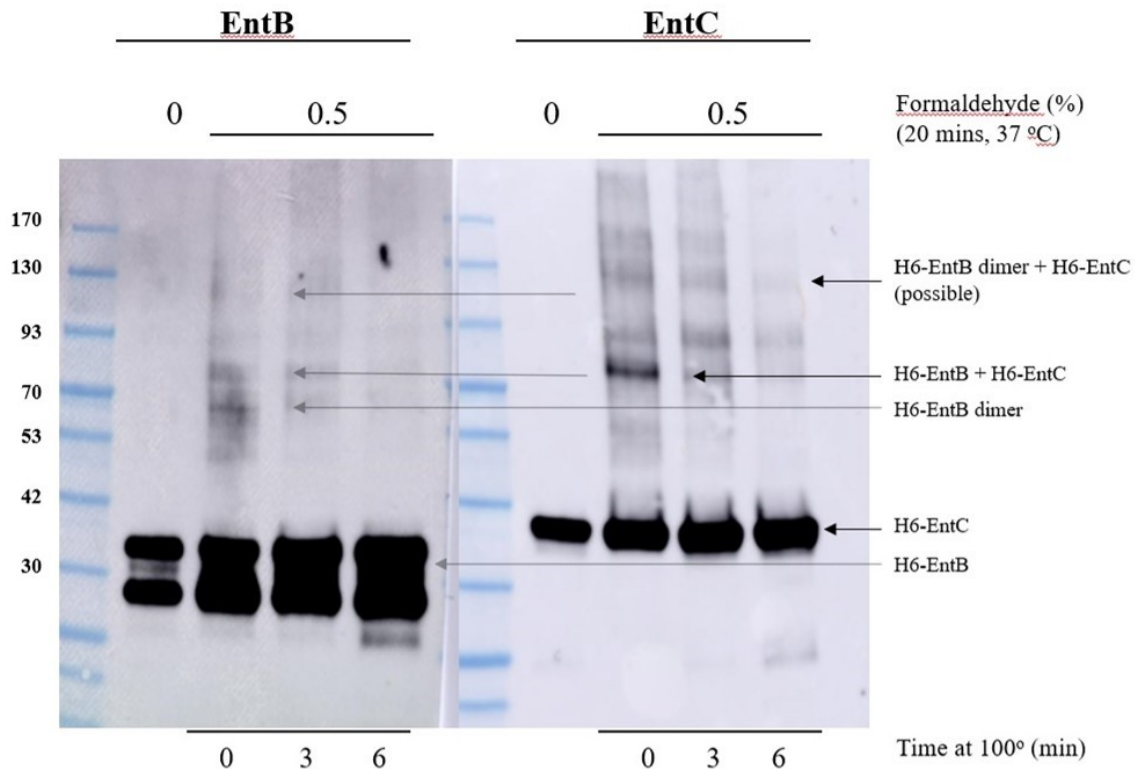


Figure 2.1. *In vivo* crosslinking with formaldehyde – cells harbouring pCA24N-*EntB*-His or pCA24N-*EntC*-His plasmid. Proteins extracted from cells expressing either H6-EntB or H6-EntC, each treated with 0.5% formaldehyde *in vivo*. Immunoblots both probed with the Qiagen Penta-His HRP conjugate antibodies.

Immunodetection of the epitope-tagged N-terminal fusions H6-EntB (Fig. 1, left panel) and H6-EntC (Fig. 1, right panel) demonstrated that following treatment with formaldehyde, bands were detected on Western blots with additive molecular masses corresponding to crosslinked species, whereas samples not treated with formaldehyde did not show additional bands. Coomassie staining of the corresponding SDS-polyacrylamide gel (Supplemental figure S1) demonstrated that all protein samples were loaded to approximately the same amounts, and that protein overexpression had not occurred that could lead to artefactual crosslinking outcomes.

A common band was observed at approximately 75 kDa, consistent with the predicted size of an EntC-EntB complex (one H6-EntC monomer (43 kDa) plus one H6-EntB monomer (32 kDa)). This band was not observed in a control sample not treated with formaldehyde. A band at approximately 65 kDa was also observed for the H6-EntB samples (Fig. 1, left panel) but not in the H6-EntC samples (Fig. 1, right panel). This band likely corresponds to a crosslinked His-tagged EntB dimer. Bands observed at higher molecular masses (> 80 kDa) likely correspond to higher-order complexes containing various combinations of EntC and EntB dimer (*i.e.*, one EntB dimer plus one EntC monomer, one EntB dimer plus two EntC monomers), as well as higher-order complexes containing other proteins (such as EntE and EntA) in addition to EntC and EntB. These bands were observed to disappear after 6 minutes of boiling, demonstrating that they contained crosslinked species and not non-specific contaminants, as expected due to the heat-reversibility of formaldehyde crosslinking. Bands below 30 kDa on the left panel likely correspond to hydrolytic products of degraded EntB protein.

Crosslinking of double transformants

Immunodetection of the epitope-tagged N-terminal fusions H6-EntC (Fig. 2.2A) and HA-EntB (Fig. 2.2B) demonstrated that following treatment with formaldehyde, bands were detected on Western blots with additive molecular masses corresponding to crosslinked species. Coomassie staining of the corresponding SDS-polyacrylamide gel (Appendix 1, Fig. S1.2A), as well as Ponceau staining of the PVDF membrane after electrotransfer (Appendix 1, Fig. S1.2B), demonstrated that all protein samples were loaded to approximately the same amounts, and that protein overexpression had not occurred that could lead to artefactual crosslinking outcomes.

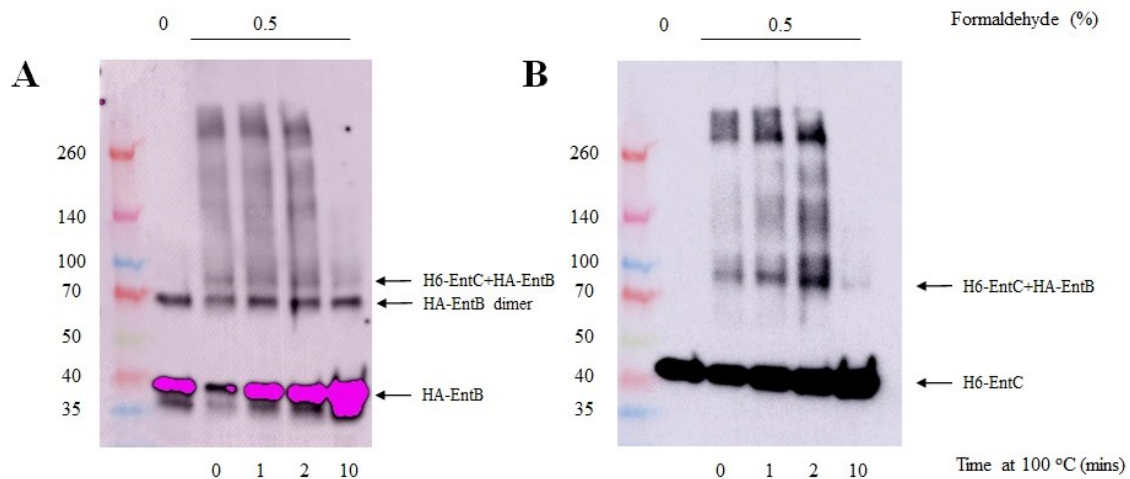


Figure 2.2. *In vivo* crosslinking of double transformants with formaldehyde. Proteins extracted from H6-EntC + HA-EntB double-transformant cells crosslinked with 0.5% formaldehyde. (A) Western blot probed with anti-HA (Thermo Pierce) followed by m-IgG Fc BP-HRP conjugate (Santa Cruz Biotech) secondary antibodies. (B) Same membrane as in (A) stripped and reprobed with the Qiagen Penta-His HRP conjugate antibodies.

For immunoblots in which H6-EntC and HA-EntB were respectively probed with relevant antibodies, a common band was observed at approximately 80 kDa, consistent

with the predicted size of an EntC-EntB complex (one H6-EntC monomer (43 kDa) plus one HA-EntB monomer (37 kDa)). This band was not observed in a control sample not treated with formaldehyde. A band at approximately 70 kDa was also observed in the anti-HA immunoblot (Fig. 2A) but not in the anti-H6 immunoblot (Fig. 2B). This band likely corresponds to a crosslinked HA-tagged EntB dimer. As well, this band is observable in the non-crosslinked sample, which could be due to insufficient denaturing of the protein sample. Bands observed at higher molecular masses (> 80 kDa) in both figures likely correspond to higher-order complexes containing various combinations of EntC and EntB dimer (*i.e.*, one EntB dimer plus one EntC monomer, one EntB dimer plus two EntC monomers), as well as higher-order complexes containing other proteins (such as EntE and EntA) in addition to EntC and EntB. These bands were observed to disappear after 10 minutes of boiling, demonstrating that they contained crosslinked species and not non-specific contaminants, as expected due to the heat-reversibility of formaldehyde crosslinking.

2.4 Discussion

In vivo crosslinking with formaldehyde is a common strategy for confirming interactions among proteins. Its short spacer arm (2 Å) will only react and crosslink interacting proteins that display primary amine residues in close proximity. Furthermore, its membrane permeability makes it a choice reagent for capturing transient, weak interaction as they occur *in vivo*. Finally, the covalent crosslink can be broken simply by heating the complexes at temperatures above 70 °C for a few minutes (133).

In this study, determining the optimal parameters involved first optimizing parameters such as temperatures, concentrations of formaldehyde, and varying length of time. Heat-reversal of crosslinks was also assayed under different timescale (unpublished data).

As with all Ent proteins, expression of endogenous EntC and EntB is Fur-regulated. It was therefore important to grow cultures in minimal, iron-poor media. To that end, Miller's M63 minimal medium proved to be optimal. Initially, the crosslinking protocol was applied to each of the different clones in parallel and the results compared (Fig. 2.1). At this initial stage it was apparent that bands likely containing both tagged proteins migrated in a similar fashion, yet this was no confirmation that these complexes contained the same proteins. Efforts were then directed at capturing complexes that could be probed for the presence of both candidate interactors, each bearing a different epitope tag but expressed in the same cell.

In a previous study our lab had engineered several plasmids encoding various Ent proteins bearing different epitope tags as well as containing a resistance marker to different antibiotics (132). Plasmid pFBH1-*entB* in particular was interesting since it expressed HA-tagged EntB and was resistant to ampicillin. However, since pFBH1-*entB* had been engineered to be *fur*-controlled, this meant that expression of the HA-EntB protein would be initiated as the cultures grew, along with that of the other, endogenous Ent proteins. This posed a problem since pCA24N-*entC*-His needed to be induced using IPTG, which could not proceed until the culture had reached log phase. It was therefore decided to trigger induction at an earlier stage by virtue of using lactose. The medium was modified to contain glucose to initiate cell growth, as well as lactose to be utilised as a carbon source once glucose became depleted. This modified M63 medium thus allowed

not only for adequate growth of the double transformant but also for early, gradual expression of H6-EntC and HA-EntB proteins.

Treatment with formaldehyde intervened at a time point where the cell culture had reached log phase, indicative of maximal metabolic activity, hence expression of the endogenous Ent proteins was synchronous with that of the engineered H6-EntC and HA-EntB proteins. This allowed the recombinant proteins to interact amid the other biosynthetic partners during biosynthesis of enterobactin and be crosslinked as these interactions were happening.

The current study is significant since it provides the first experimental evidence *in vivo* for a pairwise EntC-EntB interaction, as well as confirms the preliminary evidence that had been gathered by previous BACTH assays. This therefore completes the pairwise mapping of interactions involving DHB biosynthetic enzymes and shows that the pathway is fully networked. Furthermore, not only have we demonstrated intracellular interaction between the first two enzymes of the enterobactin biosynthetic pathway, EntC and EntB, but results from our chemical crosslinking experiment suggest the possible involvement of both proteins in higher-order complexes with additional proteins that remain to be identified. Pairwise interactions between EntB at its ArCP domain and the other proteins involved in the NRPS arm (*i.e.*, EntE, EntF) have already been reported (83), suggesting that the observed higher-order complexes may represent larger sub-components of the hypothesized enterobactin biosynthetic metabolon. Ent proteins assembling into higher-order complexes might appear to be inconsistent with previous, *in vitro* observations of low-affinity Ent protein interactions. However, these *in vitro* experiments may not sufficiently replicate the intracellular environment that could

facilitate metabolon formation, either through physical sequestration at the inner membrane or through other mechanisms. In conclusion, the experimental evidence for an EntC-EntB interaction, as reported here, supports our hypothesis that all Ent proteins are interconnected *via* a protein-protein interaction network and are likely assembled into a higher-order complex in the *E. coli* cytoplasm in order to facilitate channeling of labile biosynthetic intermediates as well as the direct efflux of the enterobactin product from the cytoplasm for extracellular secretion.

CHAPTER THREE – Evidence for higher-order complexation of enterobactin biosynthetic enzymes in *Escherichia coli*: expanding the assembly line

The manuscript corresponding to this chapter is in draft for submission to a peer-reviewed journal.

Summary

Many metabolic processes achieve higher efficiency by being organised into dynamic multienzyme complexes to enhance regulation and engage in substrate channeling. We propose that the biosynthetic machinery of the *Escherichia coli* siderophore enterobactin, a small molecule synthesized and secreted by bacteria as part of their iron acquisition strategy, assembles in such a supramolecular complex.

The primary challenge in studying this type of assemblies resides in isolating it for analysis. Previous studies had demonstrated pairwise interactions between the proteins involved in consequential steps of the enterobactin biosynthetic pathway. Some of our findings also suggested that more than two proteins could interact simultaneously and form complexes of high molecular weight. Following *in vivo* crosslinking with formaldehyde, we isolated these higher-order complexes through passive elution out of polyacrylamide gel after electrophoretic separation.

Analysis provided evidence that at least four of the proteins involved in the enterobactin biosynthetic pathway, EntC, EntB, EntA, and EntE, can form higher-order complexes in live *E. coli* cells. Our methodology overcame the challenge posed by the transient nature of these interactions and their weak affinity. Our protocol was able to capture supramolecular complexes comprising multiple Ent proteins. It also provides new insights into the organization of the intracellular enterobactin biosynthetic machinery in

E. coli and suggests that these enzymes may function in a coordinated manner to enhance efficiency.

3.1. Introduction

Many cellular processes are known to associate into dynamic, multienzyme complexes called metabolons (64). The proteins involved in several conserved metabolic pathways (*e.g.*, fatty acid synthesis, the tricarboxylic acid cycle) have been shown to organise themselves into such supramolecular assemblies, whose structural and functional characteristics have been well-studied (65, 67, 69).

The advantages of gathering the enzymes of a pathway into a metabolon are numerous. First, it provides stability through the interactions between the enzymes, which can prevent denaturation or degradation by proteases. This organization also allows for the tight regulation needed to control the rate of the metabolic pathway and prevent the accumulation of intermediates that can inhibit the enzymatic reactions. More importantly, this type of dynamic assembly provides increased efficiency by positioning consecutive enzymes of a pathway in close proximity to engage in substrate channeling, which leads to higher product yields and faster reaction rates (23, 80).

We propose that the biosynthetic machinery of the siderophore enterobactin in *Escherichia coli* (*E. coli*) assembles in such a supramolecular complex, a metabolon. Siderophores are small molecules that bacteria synthesize and secrete as part of their iron acquisition strategy, due to their high affinity for ferric (Fe^{3+}) ions, which enables them to wrest scarce iron from their surroundings.

For the bacteria *E. coli* and *Salmonella typhimurim*, the siderophore produced is enterobactin. This triserine lactone binds iron with an affinity (K_D) of $\sim 10^{-50}$ M at optimal

pH, which makes it one of the most potent chelators of Fe^{3+} (1). The enterobactin biosynthetic pathway consists of two functional modules. The first module involves the sequential reactions of EntC, EntB (N-terminal isochorismatase domain) and EntA to synthesize 2,3-dihydroxybenzoic acid (2,3-DHB) from chorismate. In the second module, three 2,3-DHB molecules are condensed with three L-serine molecules *via* non-ribosomal peptide synthesis (NRPS) to yield enterobactin through the activities of EntE, EntB (C-terminal aryl carrier protein (ArCP) domain) and EntF (2). For this second module, accessory protein EntD (phosphopantetheinyl transferase) is involved for the post-translational modification of both EntB (ArCP domain) and EntF from their *apo*- into their active *holo*- forms (134). Once biosynthesis is complete, enterobactin is then secreted through the inner-membrane efflux transporter EntS (37) and then through the outer membrane in a TolC-dependent manner (38, 109).

Expression of Ent proteins are under the control of the ferric uptake repressor protein, Fur (45). In iron-rich conditions ferrous ions interact with Fur, which then binds with the target consensus sequence of the promoter (Fur- Fe^{2+} box), to prevent initiation of transcription. In iron-depleted conditions the *apo*-Fur exhibits reduced affinity to the Fur- Fe^{2+} box and dissociates from the promoter region to allow for gene expression (49). Given the toxicity stemming from an overload of iron in the cell, we propose that further regulation is achieved *via* the transient, dynamic assembly of the enzymes involved in biosynthesis of enterobactin, *i.e.*, a metabolon.

Among the many examples for such dynamic assemblies, the biosynthetic machinery of pyoverdine, a siderophore made by *Pseudomonas aeruginosa*, and that of bacillibactin in *Bacillus subtilis* have been well characterized (71-73, 76-78). In pathways mirroring

that of enterobactin, the enzymes contained in these assemblies are expressed in low-iron conditions to help modify precursors that are then fed into an NRPS complex to yield the final product that is poised for export *via* a transmembrane system that is also associated with the metabolon. Given what is known from the two examples above, we hypothesize that the enterobactin biosynthetic enzymes of *E. coli* are similarly assembled, in a transient fashion, into a metabolon located in the cytoplasm, localizing to the inner membrane to bring it in direct contact with enterobactin efflux transporter EntS.

The first step in determining whether a biosynthetic pathway forms a supramolecular complex involves characterization of the interactions among its members. Previous work from our group and others has demonstrated that the enterobactin biosynthetic pathway of *E. coli* is fully networked, with proteins engaging in pairwise interactions (82-83, 85-86, 126, 135). We also have preliminary evidence that EntC, EntB and EntA engage in substrate channeling, and that coupling of their reactions is enhanced by EntE (unpublished data).

So far, identified interactions have only been demonstrated for proteins involved in sequential steps of the pathway (*i.e.*, EntC-EntB, EntB-EntA, and so forth). To our knowledge there has been no demonstration that more than two proteins could be observed interacting at once. Efforts at capturing several Ent proteins simultaneously interacting have remained unfruitful. For instance, studies using *in vitro* techniques such as gel filtration and immunoprecipitation have remained inconclusive (88). Several attempts were made to determine whether the proteins involved in the NRPS module, collectively known as Ent synthetase, organise themselves in a stable complex but to no avail. This has prompted authors to surmise that the interactions involved are at best

transient and of weak affinity, thus unable to withstand washing steps (88, 136). It is therefore necessary to capture interactions as they occur intracellularly.

As part of our previous work aimed at confirming an EntC-EntB interaction, we employed *in vivo* crosslinking with formaldehyde (135). In that study, results from the immunoblots revealed that epitope-tagged proteins EntC and EntB were also detected in complexes of molecular weight higher than the sum combining EntC and EntB alone, leading us to conclude that our protocol was able to capture supramolecular complexes comprising other Ent proteins in live cells.

In the present study, we further employed *in vivo* crosslinking with formaldehyde on *E. coli* cells harbouring plasmids expressing EntC, EntB, EntA and EntE. Isolation of the complex thus formed was achieved through passive elution from the polyacrylamide gel. Analysis by immunoblotting revealed that these proteins can be captured as they interact and are present in higher order complexes. As well, it provides empirical evidence supporting our previously predicted model for an EntCBAE assembly (87, 135, 137) in live *E. coli* cells. It further suggests that, as is the case for pyoverdine in *P. aeruginosa* and bacillibactin in *B. subtilis*, a large component of the enterobactin biosynthetic machinery assembles in a supramolecular complex.

3.2. Methods

All reagents were obtained from BioShop Canada (Burlington, ON) unless otherwise specified.

Preparation of co-transformants

E. coli AG-1 cells harboring both pCA24N-*entC*-His (Cam^R) and pBFH1-*entB*-HA (Amp^R) plasmid used in our previous determination of an EntC-EntB interaction (135)

were transformed sequentially with pFCF2-*entA*-FLAG (Kan^R) and pFCF1-*entE*-FLAG (Tet^R and Kan^R), both constructed as previously reported (132).

Crosslinking of co-transformants

Successful co-transformants were grown in a modified version of Miller's M63 minimal medium (22 mM KH₂PO₄, 40 mM K₂HPO₄, 15 mM (NH₄)₂SO₄, 0.1% glucose, 60 nM thiamine, and 1 mM MgSO₄, 5 µg l⁻¹ FeSO₄, heptahydrate) to induce expression of the HA-EntB, FLAG-EntA and FLAG-EntE proteins (under *fur* control) and adding 0.1% lactose as the carbon source to induce expression of the H6-EntC protein (under *lac* control), in the presence of 34 µg mL⁻¹ chloramphenicol, 100 µg mL⁻¹ ampicillin, 13 µg mL⁻¹ tetracycline and 50 µg mL⁻¹ kanamycin. When OD₆₀₀ reached 0.6-0.7, aliquots were taken from the culture and kept as non-treated samples, and the remainder of the culture was treated with formaldehyde (0.5% final concentration) (Sigma) for 20 minutes at 37 °C to initiate crosslinking of interacting proteins. The reaction was quenched by adding an equal volume of 125 mM glycine. Cells were then pelleted and washed in 100 mM PBS, pH 7.6.

Protein separation and elution

Pellets were resuspended in a lysis buffer containing 100 mM PBS, 1 mM EDTA and PIC, then lysed by sonication (10 cycles of 30 seconds, 40% pulse, on ice) on Biologics Model 300 V/T Ultrasonic homogenizer. Unbroken cells and debris were pelleted out by centrifugating at 4,000x g for 15 minutes. The cleared lysate was heated at 46 °C for 60 minutes to denature proteins without reverting crosslinking. The lysate was then loaded in multiple wells of an 8% polyacrylamide gel and electrophoresis was performed at 100 V for 3 hours. Following separation, portions of the gel containing proteins > 260 kDa

were excised, the gel was crushed and incubated overnight in 50 mM Tris HCl, 150 mM NaCl, 0.1 mM EDTA, pH 7.5 at 32 °C, with gentle agitation, to passively elute the proteins out of the gel (138). These complexes were then retrieved by acetone precipitation of the eluate and resuspended in PBS.

Probing with various antibodies

The resuspended sample was separated in two aliquots, one left as was and the other boiled at 100 °C for 15 minutes to completely revert crosslinking of the interacting proteins. These aliquots were separated by SDS-PAGE and stained with Coomassie dye to visualise disassembly of the complex. In a second step, the same samples along with a non-treated control were then loaded in triplicate on a 4-15% gradient SDS-polyacrylamide gels (BioRad) and separated at 200 V on a BioRad Mini-PROTEAN gel electrophoresis apparatus. The gel was rinsed and the proteins were transferred to PVDF membrane (BioRad) at 100 volts for 90 minutes, on ice. The membrane was blocked using the Penta-His HRP conjugate kit (Qiagen) then cut in three. Immunoblots were performed by probing with either the Penta-His HRP Conjugate antibody (Qiagen) at 1:2,500 dilution for H6-EntC, an anti-HA mouse monoclonal antibody (Thermo-Pierce) at 1:10,000 dilution for HA-EntB, or DYKDDDDK tag mouse monoclonal antibody (FG4R, Invitrogen) at 1:1,000 dilution for FLAG-EntA and FLAG-EntE. The two mouse monoclonal immunoblots were both subsequently probed with an m-IgG Fc BP-HRP conjugate (Santa Cruz Biotechnology) at 1:2,000 dilution. Detection was done with Thermo SuperSignal™ West Pico Chemiluminescent Substrate for H6-EntC, and Thermo SuperSignal™ West Femto Maximum Sensitivity Substrate for HA-EntB, FLAG-EntA and FLAG-EntE. In all instances, chemiluminescence was recorded using the Amersham

Imager 600 system. Exposure times were extended as appropriate in order to observe bands indicative of complexed *vs* uncomplexed proteins.

3.3. Results

Coomassie staining of the eluted sample (Fig. 3.1, panel A) showed that passive elution had succeeded in retrieving proteins or complexes of molecular weight > 260 kDa.

Boiling of the sample yielded a large range of lower molecular-weight proteins, confirming that these came from heat-reversal of the crosslinked species.

Immunodetection of the epitope-tagged N-terminal fusions H6-EntC, HA-EntB, FLAG-EntA and FLAG-EntE (Fig. 3.1, panel B) demonstrated that following treatment with formaldehyde, all these proteins were contained within the isolated, high molecular weight complex. The figure also shows how these complexes disassemble after boiling and how the interactors therein revert to the molecular weight prior to cross-linking (third *vs* first lane of each panel).

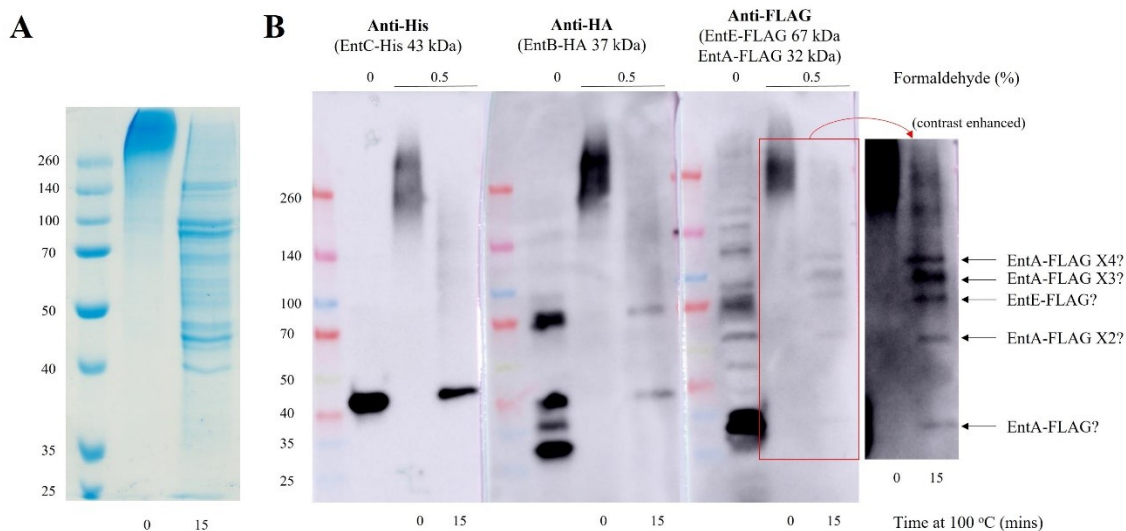


Figure 3.1. *In vivo* crosslinking with formaldehyde of quadruple transformants.

Proteins extracted from H6-EntC + HA-EntB + FLAG-EntA + FLAG-EntE transformed cells crosslinked with 0.5% formaldehyde, separated by SDS-PAGE and passively eluted out of the gel for the portion above 260 kDa. A) Coomassie stain following SDS-PAGE separation of the concentrated eluate, before and after boiling. B) Western blot in triplicate. Immunoblotting was performed with Qiagen Penta-His HRP conjugate antibodies (left panel), anti-HA (Thermo Pierce) followed by m-IgG Fc BP-HRP conjugate (Santa Cruz Biotech) secondary antibodies (middle panel) and anti-FLAG (Invitrogen) followed by m-IgG Fc BP-HRP conjugate (Santa Cruz Biotech) secondary antibodies (right panel). For each panel, first lane is the non-treated sample, second lane is isolated high molecular weight band not boiled, and third lane is boiled for 15 minutes.

On the left panel, H6-EntC is detected at 43 kDa in both the untreated sample and in the crosslinked/heat-reverted sample (1st and 3rd lanes). The middle lane reveals the presence of two large complexes, one at about the 260 kDa mark and the other at an even higher molecular weight, beyond the scale provided by the ladder. This could be due to manipulation error at the time of excising the first SDS-polyacrylamide gel or poor resolution on that first separation. This would have left the two complexes tangled, with the smaller one not migrating as fast as it should have in that first separation.

The middle panel reveals detection of HA-EntB in multiple bands. On the untreated sample (first lane), HA-EntB is detected as expected at 37 kDa and in a higher band just above 70 kDa. This latter band is due to the propensity of EntB for dimerization as illustrated in our previous study (82, 135). The bands of lower molecular weight correspond to degraded EntB protein, which has been shown to be prone to hydrolysis (unpublished data). In the middle lane, HA-EntB is detected in bands at and above 260 kDa which correspond to those seen when probing for H6-EntC. In the last lane, the crosslinked/heat-reverted sample also reveals HA-EntB at 37 kDa and just above 70 kDa for the dimer, as detected in the untreated sample.

The right panel shows the results of probing for both FLAG-EntA and FLAG-EntE. In this instance the results shown in the first lane (non-treated sample) might be considered inconclusive due to the high number of bands exhibiting non-specific binding. FLAG-EntA should be visible at 32 kDa and FLAG-EntE at 67 kDa. However, it is known that EntA adopts a tetrameric conformation (139) and if not completely denatured could therefore also be detected as multimers (dimer of 64 kDa, dimer + monomer of 96 kDa, tetramer of 128 kDa). The intensity of the band at above 260 kDa in the middle lane, as well as the absence of other bands in that lane reveals that EntA and/or EntE are present in this high molecular weight complex. For the crosslinked/heat-reverted sample in the third lane, the lack of non-specific binding eases identification. There, five bands with the lowest at 32 kDa (faint) and one close to 67 kDa are in agreement with expectations. Three other bands are detected, at about 60 kDa, slightly above 100 kDa and at approximately 120 kDa, which could correspond to EntA dimer, dimer + monomer, and

tetramer, respectively. Enhanced contrast on this panel offers a better view of these bands.

3.4. Discussion

Our previous study demonstrating an EntC-EntB interaction had revealed bands of molecular weight beyond what would be expected from a complex corresponding to two monomers of EntC + dimeric EntB complex (predicted at 158 kDa), including several other complexes, some well above the 260 kDa mark. We surmised that these possibly contained other Ent proteins, but due to the upper limits of the molecular markers, the exact size of these complexes could not be established. Considering the individual molecular weights of the Ent proteins involved in synthesis (Table 3.1), a complete enterobactin metabolon as hypothesized would weigh in excess of 450 kDa. However such large complexes are difficult to isolate by SDS-PAGE and migration could be anomalous due to possible entanglement of the crosslinked species.

Protein	Molecular weight (kDa)
EntC	43
EntB (in dimeric form)	66
EntA (in tetrameric form)	100
EntE	60
EntF	140
EntD (accessory protein)	25
Total molecular weight of a complete assembly:	≈ 435

Table 3.1. Molecular weights of the biosynthetic Ent proteins.

Based on that study, the strategy chosen was to pursue *in vivo* crosslinking efforts since parameters such as growth and induction regimens for cultures, formaldehyde treatment protocol and its subsequent reversal, and immunoblotting were all already optimized. Efforts at engineering co-transformants harbouring several plasmids made use of engineered constructs already available (132). This resulted in an *E. coli* AG1 transformant clone harboring His-EntC, HA-EntB, FLAG-EntA and FLAG-EntE that could be cultured in minimal medium supplemented with chloramphenicol, ampicillin, tetracycline and kanamycin.

Following *in vivo* crosslinking with formaldehyde, several strategies were employed with a view to isolate and enrich this complex. Gel filtration, dialysis using membranes with 100 kDa and 300 kDa pore size, centrifugal concentrators with 100 kDa and 300 kDa cut off size were all used without success (data not shown).

Isolation of high-molecular weight complexes was finally achieved through a simple yet under-utilised technique of excising, post-separation, the relevant section of the polyacrylamide gel. This was taking into consideration that the complex of interest consistently appeared on immunoblots in the space between the 260 kDa band of the molecular marker and the top of the resolving gel. Passively eluting the bands of interest out of the gel yielded a concentrated sample suitable for analysis.

This analysis showed that complexes around or in excess of 260 kDa contain at least EntC, and EntB, as well as EntA and/or EntE as demonstrated by the detection of a signal brought about by the respective antibodies used for probing.

Accounting for their respective molecular weights, a complex comprising all four proteins (EntC monomer, EntB dimer, EntA tetramer and EntE monomer) should run at around 311 kDa. However, considering that the interactions are transient, we cannot surmise whether the EntB dimer and the EntA tetramer are stable or transient. Moreover, considering that the molecular weight ladder used here cannot be reliable beyond 260 kDa and that the rate of migration of proteins on SDS-PAGE follows a logarithmic pattern, ascertaining the exact weight of higher-order complexes remained problematic.

Our results show that at least four of the proteins involved in biosynthesis of enterobactin, EntC, EntB, EntA and EntE, form a complex that can be captured through *in vivo* crosslinking with formaldehyde. The presence of the last enzyme of the pathway, EntF, remains to be confirmed. Application of a similar protocol to probe for the presence of EntF would require the engineering a clone harbouring an additional plasmid, with a different antibiotic marker, which would be very difficult, if not impossible to achieve.

Therefore, the identity of all the interactors contained in the complexes remains to be analysed in another fashion.

Moreover, we must consider that if *in vivo* crosslinking with formaldehyde is effective at capturing our proposed enterobactin metabolon, in all likelihood it would also crosslink the proteins interacting in other, similar supramolecular complexes that would show at a molecular weight of over 260 kDa. For those reasons, we suggest further isolation and purification of the proteins retrieved by passive elution. In the present study, the first enzyme of the pathway, EntC, bears a H6 tag at its N-terminus. Ongoing efforts include using Ni-NTA resin to eliminate other, irrelevant large protein complexes. Following boiling to revert the chemical crosslink, the resulting mix of proteins contained in the eluate will then be analysed by mass spectrometry.

Nevertheless, the present study indicates that it is possible to capture a complex as it occurs intracellularly, and where at least four enzymes involved in biosynthesis of enterobactin have been found to simultaneously interact. This adds empirical evidence to our previously predicted models for an EntCBAE assembly. It also supports our hypothesis that the whole machinery assembles into a metabolon, which we wish to name enterobactome (Fig. 3.2). Efforts are also ongoing to elucidate mechanisms that govern substrate channeling, especially between enzymes involved in the first arm of the pathway, EntC, EntB and EntA.

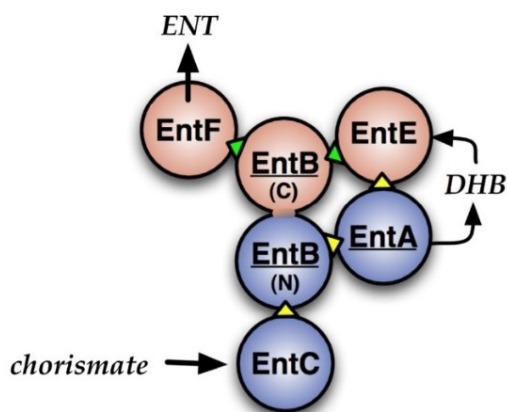


Figure 3.2. A proposed enterobactin metabolon. Schematics of a proposed supramolecular assembly of the enterobactin biosynthetic enzymes. In blue, the first module of enterobactin biosynthesis where chorismate is sequentially transformed into DHB through the sequential action of EntC, EntB (N-terminal domain) and EntA. In red, the enzymes involved in the NRPS module. Yellow triangles: interactions identified in our lab. Green triangles: interactions identified by other groups.

In addition, the mechanism through which assembly is orchestrated remains to be elucidated. We are currently trying to establish the possible role of EntB not only as a nucleation factor for this assembly but also as a means through which the enterobactome is anchored or associated with the membrane, as is seen in other, well-studied metabolons (140).

CHAPTER FOUR – Probing for interactors to EntS, the membrane exporter of the siderophore enterobactin in *Escherichia coli*

The manuscript corresponding to this chapter is in preparation for submission to a peer-reviewed journal.

Summary

In *Escherichia coli*, the siderophore enterobactin is produced in the cytoplasm through the concerted action of five proteins, EntC, EntB, EntA, EntE, and EntF, aided by accessory proteins EntD and EntH. Enterobactin is then secreted to the periplasm by the transporter channel EntS, a member of the Major Facilitator Superfamily. Localization studies have shown that several Ent proteins localize to the membrane, suggesting a direct transfer mechanism to EntS upon completion of synthesis, presumably to facilitate secretion and to avoid premature hydrolysis of *apo*-enterobactin by the ferric enterobactin esterase, Fes. To probe for potential interactors, we performed proximity bio-labeling using engineered ascorbate peroxidase APEX2, with an EntS-APEX2 fusion protein as the bait. The APEX2 gene used for this study has been optimized for expression in the cytosol of bacteria (www.addgene.org/72558/). Preliminary results from immunoblotting suggest that several Ent proteins colocalize with EntS during enterobactin biosynthesis and/or secretion. Further studies are required to determine the precise manner in which these proteins interact with EntS and contribute to the biosynthetic metabolon assembly.

4.1 Introduction

Iron is essential for the growth of microorganisms, however its poor solubility in soil, as well as complexation to proteins such as ferritin and transferrin in mammals make it difficult to acquire (1). Bacteria such as *Escherichia coli* (*E. coli*), *Salmonella* and others

have therefore evolved the capacity to produce and secrete siderophores, molecules that bind iron with great affinity (2, 141-143). For *E. coli* in particular, enterobactin is reputedly the most potent siderophore (3).

The pathway through which enterobactin is synthesized in the cytoplasm involves the concerted action of five proteins, EntC, EntB, EntA, EntE and EntF. Two additional accessory proteins, EntD and EntH, are also required. Expression of the Ent genes (*entCEBAH*, *entF*, *entD*) is under the control of the ferric uptake repressor protein, Fur (45). In iron-rich conditions ferrous ions interact with Fur, which then binds with the target consensus sequence of the promoter (fur box), to prevent initiation of transcription. In iron-depleted conditions the *apo*-Fur exhibits reduced affinity to the fur box and dissociates from the promoter region to allow for gene expression (49).

Another important aspect of siderophore-mediated iron uptake is the secretion of newly synthesized enterobactin through its efflux transporter channel, EntS. Although not structurally characterized to date, EntS (42 kDa) is a member of the Major Facilitator Superfamily (MFS) with 12 predicted transmembrane helices (37). MFS proteins are important for controlling the efflux of several compounds in Gram-negative bacteria *via* symport or antiport mechanisms (144).

In the case of enterobactin, EntS has been identified as the transport channel through the use of *null* mutants (37). EntS also tightly regulates enterobactin transport to prevent over-accumulation of iron in the cell. Many complexes that synthesize secretory compounds have been shown to localize near the inner membrane (145). In the case of the biosynthetic machinery of enterobactin in *E. coli*, the final stages of synthesis involve non ribosomal peptide synthesis (NRPS) performed by the enzymes EntE, EntB (Aryl-

carrier protein (ArCP) domain) and EntF. These had been previously shown to be membrane-bound or membrane-associated (89). Using photoactivated localization microscopy (PALM), in conjunction with our collaborators (Johan Hofkens, KU Leuven, Belgium) we observed that other Ent biosynthetic enzymes, EntB (isochorismatase domain) and EntC are not freely diffused in the cytoplasm but instead localize at the *E. coli* cell membrane (unpublished data). Such localization would facilitate direct transfer of newly-synthesized enterobactin from the cytoplasm to the periplasm *via* EntS-mediated efflux.

Upon retrieval of enterobactin bound to ferric ions, this ferrienterobactin is cleaved by the ferric enterobactin esterase Fes. However, the activity of Fes is not specific to ferrienterobactin (90), and it can also hydrolyse newly synthesized molecules of enterobactin that were ready for secretion. For that reason, it could be expected that the Ent biosynthetic machinery interacts closely with EntS to avoid diffusion and hydrolysis of enterobactin and to assist in its swift transport through EntS.

Previous studies in our lab have provided evidence that the biosynthetic machinery of enterobactin assembles in a supramolecular complex (manuscripts in preparation). To date our data do not allow determination of the manner in which this assembly interacts with EntS. PPIs can be confirmed through a variety of methods such as *in vivo* or *in vitro* crosslinking, two-hybrid assays and various fluorescence techniques (*e.g.*, bimolecular fluorescence complementation (BiFC), fluorescence resonance energy transfer (FRET)). Before applying these techniques, it is advisable to proceed with a wider probing technique aimed at generating a list of candidates.

A query submitted to STRING (string-db.org) reveals predicted interactions based on gene co-occurrence or co-expression, but no known, experimentally determined interactions (Fig. 4.1). By virtue of their co-expression and related roles in enterobactin biosynthesis, secretion and acquisition, the list of predicted functional partners includes the biosynthetic enzymes (EntC, EntB, EntA, EntE and EntF), the proteins responsible for uptake and processing of ferric enterobactin (FepB, FepD, FepG and FepC), and the ferric enterobactin esterase Fes. The latter proteins are not known to have a role in synthesis or secretion of enterobactin, and their presence on the list may simply highlight the limitations of the database query. Also absent from this list is EntD (4'-phosphopantetheinyl transferase) that is needed for catalyzing the transfer of the 4'-phosphopantetheine moiety from coenzyme A to the *apo*-domains of both EntB (ArCP domain) and EntF (PCP domain) to yield their *holo*-forms (36, 134). A reverse query of the database for interactors of EntD does however list EntS as a predicted interactor, without experimental confirmation. Moreover, there is evidence that over-expressed EntD will accumulate in the membrane, thereby supporting its importance in the very last stages of biosynthesis as well as suggesting a role either in anchoring the biosynthetic machinery to the membrane, or even for secretion (92).

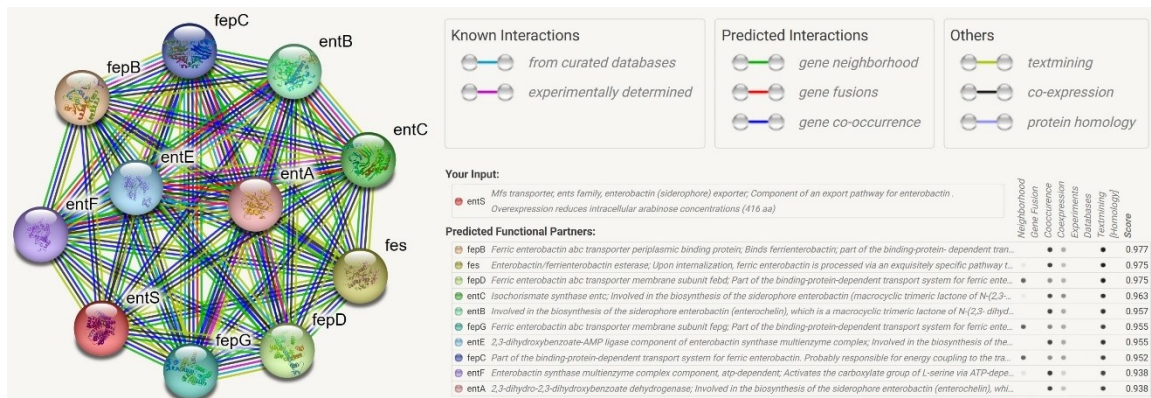


Figure 4.1. Results from the STRING database query for potential interactors of EntS. Results show no demonstrated interactions (turquoise, magenta) but rather predictions based on other parameters such as position within the gene cluster, co-expression or protein homology (from *entS* protein (*Escherichia coli* K12 MG1655) - STRING interaction network (string-db.org) accessed March 26 2023).

To empirically determine which of the Ent biosynthetic proteins may interact with EntS, we have employed proximity bio-labeling with an engineered ascorbate peroxidase enzyme (APEX2), derived from soybean and that has been optimized for bacterial expression (146-147). When fused to the protein of interest (*i.e.*, the bait), APEX2 uses hydrogen peroxide (H₂O₂) as an oxidant to catalyze the one-electron oxidation of a biotin phenol (BP) substrate. This oxidized product, the biotin-phenoxy radical, is a highly reactive species that conjugates to electron-rich residues (*e.g.*, tyr, trp, cys and his) of proteins that are in close proximity (*i.e.*, the preys). Although there is no fixed radius for the biotinylation, it has been shown to be most efficient under 10 nm and rapidly decreasing in intensity beyond that distance.

Since we already have identified interactors for EntE (2,3-dihydroxybenzoate-AMP ligase) (82-83, 85-86), we have obtained an EntE-APEX fusion protein as a bait which will be used as a control for the proximity bio-labeling assay.

Preliminary results from Western blotting showed biotinylation of several proteins, most with molecular weights corresponding to that of various purified Ent proteins, suggesting that these colocalize with EntS during enterobactin biosynthesis and/or secretion. We further suggest that other proteins not corresponding to Ent biosynthetic enzymes, but which been detected in both assays could be structural or scaffold proteins.

4.2 Materials and Methods

All reagents were obtained from Bioshop Canada, Inc. (Burlington, Ontario) unless otherwise specified.

Engineering of constructs

E. coli AG-1 cells harboring pCA24N-*entS*-His (Cam^R) and *E. coli* AG-1 cells harboring pCA24N-*entE*-His (Cam^R) were obtained from the ASKA collection (130). The pTRC-APEX2 plasmid was a gift from Alice Ting (Addgene plasmid # 72558) (147). The plasmids were extracted from the cells and sent to TopGene (St-Laurent, Canada) to create the two constructs, encoding either EntS or EntE, with the APEX2 enzyme N-terminally fused.

Assessment of APEX2 activity of fusion proteins

The engineered pCA24N-*entS*-APEX2-His (Cam^R) and pCA24N-*entE*-APEX2-His (Cam^R) plasmids were successfully transformed into competent *E. coli* AG1 cells and grown in Miller's M63 minimal medium (22 mM KH₂PO₄, 40 mM K₂HPO₄, 15 mM (NH₄)₂SO₄, 0.2% glucose, 60 nM thiamine, and 1 mM MgSO₄, 5 µg l⁻¹ FeSO₄, heptahydrate) until their optical density at 600 nm (OD₆₀₀) reached 0.6-0.7 units. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (1 mM) was added to an aliquot from each culture, overnight, on rotator, at room temperature to induce expression of the fusion

proteins. APEX2 activity was qualitatively assayed using guaiacol, a phenolic, colorless compound that turns into tetraguaiacol, a brick red compound, when oxidized, based on a protocol previously described (148-149) but adapted as follows: 10 μ L of guaiacol (Sigma) was added to 1 mL of induced culture and left to incubate on rotator for 30 mins at room temperature. In parallel, aliquots of the same cultures not treated with IPTG to were used as controls. Subsequently, 33 μ L of 10 M H_2O_2 were added to all the microfuge tubes and briefly mixed to observe development of colour. The aliquots were then centrifugated for two minutes at maximum speed in a microcentrifuge to pellet the cells in order to better observe the change in colour.

Ascertaining membrane localization of the S-APEX2 fusion protein

Clones harbouring either the pCA24N-*entS*-APEX2-His (Cam^R) or the pCA24N-*entE*-APEX2-His (Cam^R) construct were grown in 50 mL of Miller's M63 medium until their optical density at 600 nm (OD_{600}) reached 0.6-0.7 units. IPTG (1 mM) was added, and the cultures were incubated overnight, on rotator, at room temperature to induce expression of the EntS-APEX2 and EntE-APEX2 fusion proteins, respectively. The cells were pelleted and lysed by sonication in 700 μ L of 100 mM PBS with PIC, followed by centrifugation at 5,000 xg for 30 minutes to pellet out unbroken cells and debris. The supernatants were then centrifugated at 21,000 xg for 1 hour at 4 °C to isolate the membrane fraction. The supernatants were decanted and the membrane fractions were resuspended in 300 μ L of 100 mM PBS. APEX2 activity was assayed in both the supernatants and the resuspended membrane fractions using a protocol developed by Murshed *et al.* (150) and modified as follows: 1.9 mL of reaction mix (50 mM KPO_4 buffer, 0.25 mM ascorbic acid, 5 mM H_2O_2 , pH 7.3) was used to blank the

spectrophotometer at 290 nm. Then 100 μ L of the sample was added to trigger the oxidation reaction and the decrease in absorbance (due to peroxide-dependent oxidation of ascorbate) was recorded for five minutes. All samples were processed in triplicates.

Proximity labeling assay

E. coli AG-1 cells harboring pCA24N-*entS-APEX2*-His (Cam^R) and pCA24N-*entE-APEX2*-His (Cam^R) were grown in Miller's M63 medium supplemented with 34 μ g mL⁻¹ chloramphenicol until their optical density at 600 nm (OD₆₀₀) reached 0.6-0.7 units. IPTG (1 mM) was added and the cultures were incubated overnight, on rotator, at room temperature to induce expression of the fusion proteins.

The proximity labeling step was then performed as previously described (146). Briefly, 1 mM biotin phenol (Sigma) substrate was added to 1 mL of the test cultures, and incubated at 37 °C for 30 mins, on rotator. In parallel, control cultures were not supplemented with the substrate. The proximity labeling reaction was initiated in all samples by the addition of 1 mM H₂O₂. The reaction was quenched after one minute using a 2x quenching solution (20 mM sodium ascorbate (Sigma), 20 mM sodium azide, 10 mM Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma) in PBS with 0.05% Tween-20). The cells were then repeatedly washed with 1x quenching solution, and then with PBS-Tw20, to finally be resuspended in 1 mL of PBS.

Initial protein separation, transfer and probing with avidin-HRP

After the cells were washed and resuspended in PBS, the samples were split in 200 μ L and 800 μ L aliquots and pelleted by centrifugation. The cells from the 800 μ L aliquots were kept in the freezer at -20 °C for future analysis. The cells from the 200 μ L aliquots were lysed with gel-loading buffer (131) and denatured at 100 °C for 10 minutes. All

samples were loaded for protein separation on a 10% SDS-polyacrylamide gel and the proteins were separated at 200 V on a BioRad Mini-PROTEAN gel electrophoresis apparatus. The gel was rinsed in distilled water and proteins were transferred onto PVDF membrane (BioRad) at 100 volts for 60 minutes, on ice. The membrane was blocked with 0.5% bovine serum albumin (BSA), rinsed with TBS and then probed with avidin-HRP (Invitrogen) at a 1:600 dilution for the detection of biotinylated proteins. Detection was performed with Thermo SuperSignalTM West Pico Chemiluminescent Substrate. Chemiluminescence was recorded using the Amersham Imager 600 system.

Preparation of a mix of purified Ent proteins

Six *E. coli* AG-1 clones harboring pCA24N (Cam^R) plasmids expressing either His-tagged EntC, EntB, EntA, EntE, Ent F or EntD were obtained from the ASKA collection (130). All the clones were cultured in Luria Bertani broth at 37 °C in the presence of 34 µg mL⁻¹ chloramphenicol. When OD₆₀₀ reached 0.6-0.7, all cultures were treated with 1 mM IPTG overnight at room temperature to induce expression of the proteins encoded on their respective plasmids. All cultures were pelleted by centrifugation and the cell pellets were lysed through three cycles of freeze/thaw (-80 °C vs room temperature) in 100 mM PBS supplemented with EDTA and PIC. The crude lysates were briefly centrifuged to pellet out unbroken cells and debris, and the supernatants were loaded onto Qiagen Ni-NTA Spin System columns (catalog# 31014). Washing and elution of the proteins was performed as per the manufacturer's standard protocol. Aliquots from each sample were loaded on a 10% polyacrylamide gel and separated by SDS-PAGE at 200 V. Gels were then rinsed in distilled water and incubated in Coomassie stain to assess yield and purity.

Aliquots of each protein were incorporated into a mix to use as a control on immunoblot for comparison purposes.

4.3 Results

Assessment of APEX2 activity of fusion proteins

Results from the whole cell activity assay using guaiacol (Fig. 4.2) showed that following incubation with this compound, the addition of hydrogen peroxide triggered a change in colour (tubes 2 and 4). This effect was not observed in the control samples where protein expression was not induced with IPTG (tubes 1 and 3), indicative of APEX2 activity in the former. This allowed us to confirm that subcloning of the *APEX2* gene into the pCA24N-*EntS* and pCA24N-*EntE* plasmids was in frame, allowing for proper expression of the fusion protein hence a fully functional enzyme.

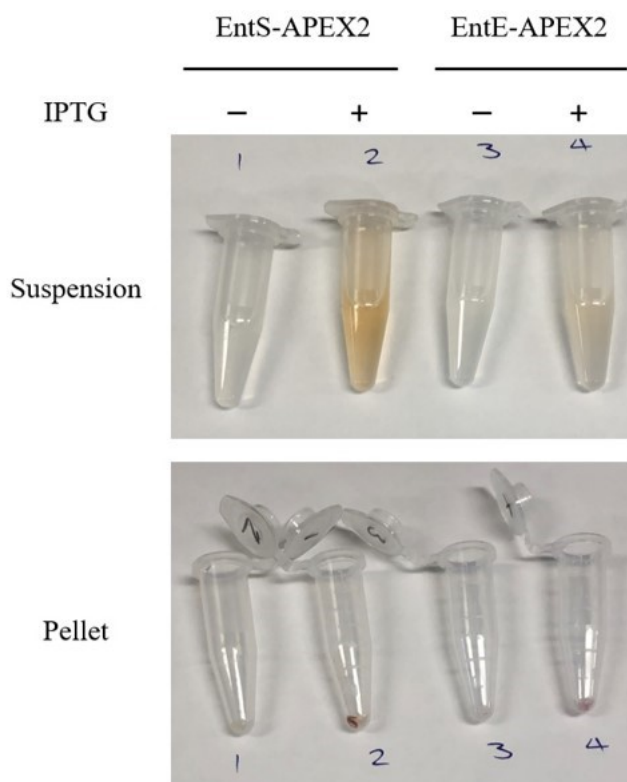


Figure 4.2. APEX2 activity assay using guaiacol. Qualitative colorimetric assay on cultures harbouring either pCA24N-*EntS-APEX2*-His or pCA24N-*EntE-APEX2*-His plasmids for qualitative assessment of APEX2 activity in whole cells. Cultures grown in Miller's M63 medium, with or without the addition of IPTG to induce protein expression. Incubation with guaiacol substrate, followed by treatment with hydrogen peroxide to initiate the peroxidase reaction, reveals activity in samples where expression of the fusion proteins was induced with IPTG (tubes 2 and 4) but not in untreated samples (tubes 1 and 3), shown by the appearance of a reddish brown colour.

Membrane localization of the S-APEX2 fusion protein

In vitro APEX assays (Fig. 4.3) were performed on the supernatants as well as on the membrane fractions from cells expressing either the EntS-APEX2 or the EntE-APEX2 fusion proteins. For cells expressing the EntS-APEX2 protein, peroxidase activity was detected in the membrane fraction but was nearly absent in the cytosol. Conversely, for cells expressing the EntE-APEX2 protein, activity was detected in the cytosol but was very weak in the resuspended membrane fraction. A very low change in absorbance was

recorded for the EntE-APEX membrane fraction and in the EntS-APEX cytosol (< 0.010 units). This might be due to the generation of bubbles by the hydrogen peroxide. This assay allowed us to confirm that the EntS-APEX2 fusion protein localizes to the inner membrane in such a fashion that the activity of the APEX2 enzyme is unhindered.

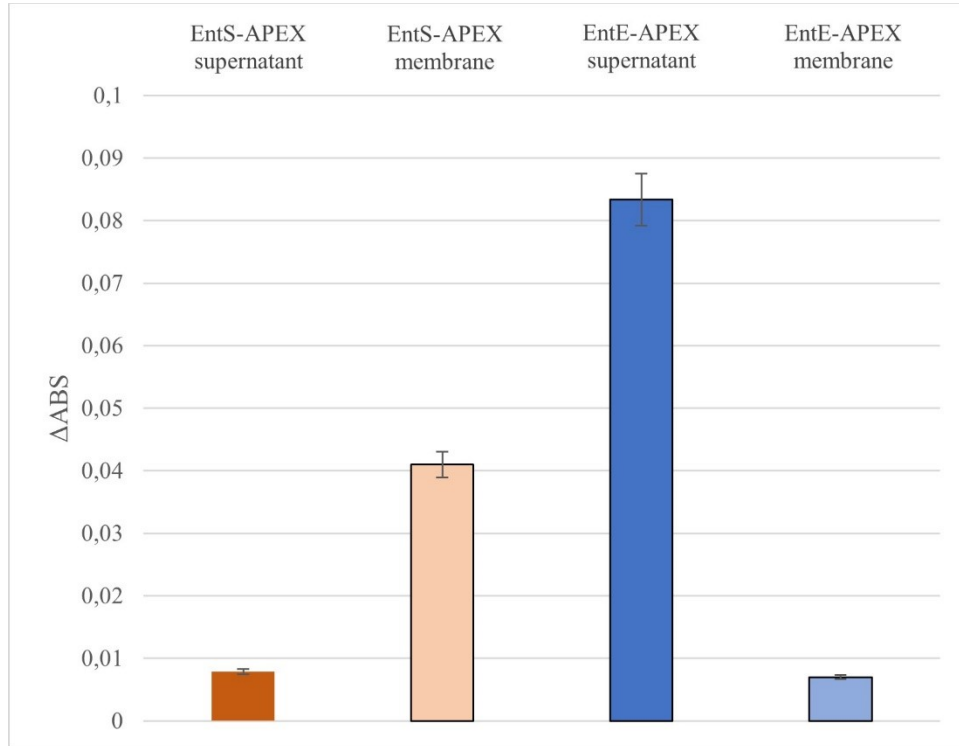


Figure 4.3. Comparative APEX activity in cytosol vs membrane in cells expressing EntS-APEX or EntE-APEX fusion proteins. For cells expressing EntS-APEX, a change in absorbance at 290 nm, due to peroxide-dependent oxidation of ascorbate shows that activity was confined to the resuspended membrane fraction, whereas for cells expressing EntE-APEX, activity was predominant in the cytosol.

Proximity labeling assay

Cell cultures expressing EntS-APEX and EntE-APEX fusion proteins were incubated with a biotin phenol substrate and then treated with hydrogen peroxide to initiate the

transfer of the biotin moiety from the substrate to electron-rich residues of proteins in close proximity (< 10 nm). Figure 4.4 shows results from the immunoblot when probing the membrane with avidin-HRP to detect biotinylated proteins.

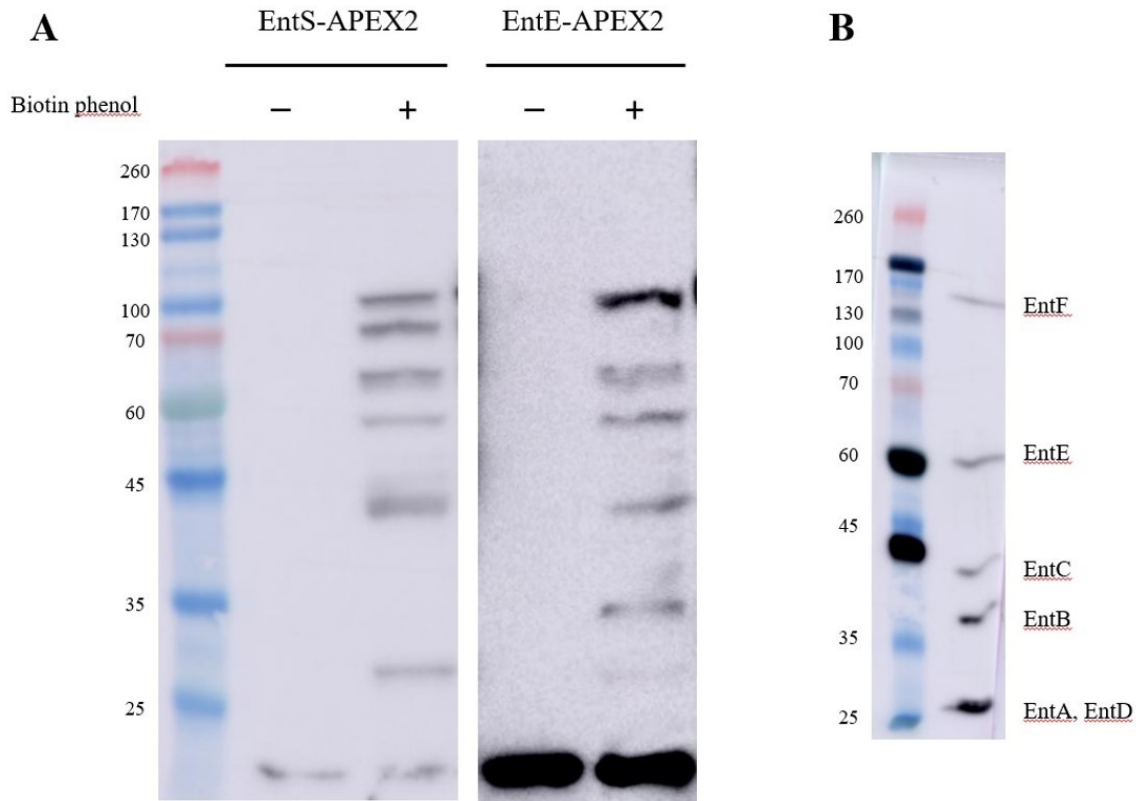


Figure 4.4. Proximity labeling assay with APEX2. A) Cultures expressing EntS-APEX2 or EntE-APEX2 showed biotinylation of several proteins in samples where the biotin phenol substrate was added, but not in untreated cultures. B) Purified Ent proteins separated on 10% polyacrylamide gel reveal migration pattern for size comparison.

Following the proximity labeling assay using EntS-APEX2 (Fig. 4.4 A-left panel), bands appeared at just above 100 kDa, around 70 kDa, above and below 60 kDa, around 43 kDa and around 30 kDa. Several of these bands also appeared when proximity labeling was performed using the EntE-APEX2 fusion protein (Fig. 4.4 A-right panel). Across all samples, a lower molecular weight band (between 25 and 15 kDa) consistently

appeared even in absence of the biotin phenol substrate, which could be the biotin carboxyl carrier protein (BCCP) of acetyl-CoA carboxylase (Unitprot access P0ABD8), which in *E. coli* is the only readily biotinylated protein and forms a homodimer of 17 kDa (151).

There were notable differences between the two assays: 1) a band of about 70 kDa was visible when proximity labeling was performed with EntS-APEX2 but not with EntE-APEX2 and, 2) conversely, a band slightly above 35 kDa was visible when proximity labeling was performed with EntE-APEX2 but not with EntS-APEX2.

These results validated that the protocol for proximity labeling with APEX2 was efficient and detection by avidin-HRP was adequate.

4.4 Discussion

Probing for transiently interacting proteins that exhibit weak affinity poses challenges that limit the use of *in vitro* techniques such as co-immunoprecipitation and pulldown assays. Proximity labeling is an *in vivo* technique that allows for detection of interactions as they occur in live cells. In the present study we fused our protein of interest, enterobactin exporter EntS with the APEX2 enzyme to probe for possible interactors.

When reacting with hydrogen peroxide, APEX2 catalyses the transfer of the biotin moiety from a biotin phenol substrate onto exposed, electron-rich residues of neighbouring proteins within a 10 nm radius. Our results show that using EntS-APEX2 as the bait protein leads to the biotinylation of several proteins. In a parallel reaction using EntE-APEX2 as the bait, several proteins of similar molecular weight were also biotinylated. This further supports to our hypothesis that the Ent biosynthetic machinery

(EntCBAEF) assembles in a supramolecular complex that localizes at the membrane where it interacts with EntS.

Colocalization of proteins in proximity to the prey protein does not signify that a direct interaction is occurring. Nonetheless, our results have yielded a list of six possible interactors for EntS, many of which could be individually investigated through other methods such as two-hybrid assays or *in vivo* chemical crosslinking.

Comparison with the migration pattern observed upon separation of the six Ent proteins purified for that purpose (Fig. 4.4B) allows us to suggest that proteins being detected on both panels of Figure 4.4A include EntE at just above 60 kDa, EntC slightly below 45 kDa, and either EntA or EntD slightly above 25 kDa. For EntE, we have to consider the possibility that the APEX2 reaction might result in “self-labeling” where the biotin moiety is transferred to the fusion protein itself.

Table 4.1 lists the molecular weights of the Ent biosynthetic proteins and indicates the likelihood that these proteins were biotinylated and hence detected by immunoblotting.

Protein	Molecular weight (kDa)	Biotinylated by EntS-APEX2?	Biotinylated by EntE-APEX2?
EntC	43	Likely	Likely
EntB (in monomeric form)	32	No	Likely
EntA (in monomeric form)	25	Possible	Possible
EntE	60	Likely	*Likely
EntF	140	No	No
EntD (accessory protein)	25	Possible	Possible
EntH (accessory protein)	15	N/A	N/A

* if we consider the possibility of self-labeling

Table 4.1. Molecular weights of the biosynthetic Ent proteins. Detection of bands of corresponding molecular weight allows for presumption that biotinylated Ent proteins are being detected.

This allows us to infer that EntB might be the band detected around 35 kDa on the EntE-APEX2 assay, but which does not appear for the EntS-APEX2 assay. This would mean that the orientation and arrangement of the proteins in our proposed assembly are such that whereas there are exposed residues on EntB in close proximity and amenable to biotinylation by EntE-APEX2, it is not the case when the APEX2 enzyme is fused to EntS.

It is also worth noting that EntF (140 kDa), one of the key proposed interacting partners of EntS and confirmed interacting partner of EntE, did not appear upon detection. This could be due to the fact that here, transfer of the proteins onto PVDF following separation by SDS-PAGE was performed for only 60 minutes, and that proteins of higher molecular weight (> 100 kDa) require a longer transfer time.

Moreover, we have to consider whether EntS would get biotinylated by EntE-APEX2. With a molecular weight similar to that of EntC (42 kDa), there might be superposition of the two proteins during migration. It could also be that EntS, being a membrane protein, might migrate anomalously (152) or undergo aggregation at the denaturing stage which would make it undetectable on the gel.

There also remains uncertainty about the identity of the 70 kDa band seen only on the EntS-APEX2 panel, as well as the bands at 100 kDa, and below 60 kDa on both panels. The association of proteins other than those directly involved in biosynthesis of enterobactin cannot be discounted, as exemplified in the results from the STRING query in Figure 4.1 (*e.g.*, scaffold, membrane or other accessory proteins).

This work therefore presents preliminary data, and more work is warranted. As a next step, the biotinylated proteins will be subjected to peptide mass fingerprinting to obtain formal identification.

CHAPTER FIVE – Solubilization screens of enterobactin exporter protein EntS in *Escherichia coli* reveals possible dimerization.

The manuscript corresponding to this chapter is in preparation for submission to a peer-reviewed journal.

Summary

Enterobactin is a siderophore synthesized and secreted by bacteria such as *Escherichia coli* and *Salmonella* as part of their iron acquisition strategy. The biosynthesis and secretion of enterobactin involve multiple enzymes and accessory proteins, regulated by the ferric uptake repressor protein, Fur. Enterobactin is transported from the cytoplasm to the periplasm by the inner membrane protein EntS, which is predicted to be a member of the Major Facilitator Superfamily with 12 transmembrane helices. However, the exact structure and mechanism of EntS remain unknown and require experimental confirmation. In order to isolate and study EntS, we performed a solubility screen using both detergents and copolymers to extract the protein from the membrane, with a view to achieve purification. Results yielded a list of compounds showing potential for solubilization and also suggest that EntS likely adopts a dimeric conformation. This research sheds light on the structure and solubility of EntS, providing insights into the mechanism of enterobactin transport and iron acquisition in bacteria. Further studies are needed to fully understand the structure, function and regulation of EntS in bacterial iron acquisition strategies.

5.1 Introduction

Iron, in the form of Fe^{3+} ions, is essential for the metabolic functions of most micro-organisms. Despite its abundance, iron is generally unavailable as it is bound by proteins such as transferrin in mammalian hosts and forms insoluble precipitates in soil (1). As

part of their iron acquisition strategies, many bacteria have therefore evolved the ability to synthesize molecules known as siderophores, chelators that scavenge iron and bind it with very high affinity. In bacteria such as *Escherichia coli* (*E. coli*) and *Salmonella*, the siderophore produced is enterobactin, a triserine trilactone with three catechol units that confer iron binding properties with a K_D of $\sim 10^{-50}$ M at optimal pH (3). This extraordinarily high affinity allows for drawing of essential Fe^{3+} out of the extracellular environment, followed by uptake of the ferric enterobactin into bacterial cells. Since iron uptake allows for the growth and proliferation of pathogens in mammalian hosts, siderophores and the proteins involved in their biosynthesis and secretion are recognized as virulence factors (4).

The pathway through which enterobactin is synthesized in the cytoplasm involves sequential reactions from five enzymes, EntC, EntB, EntA, EntE and EntF, as well as accessory proteins EntD and EntH (2). Expression of the Ent genes (*entCEBAH*, *entF*, *entD*) as well as other proteins required for secretion and uptake of the ferric enterobactin, is under control of the ferric uptake repressor protein, Fur (45). In iron-rich conditions ferrous ions interact with Fur, which then binds with the target consensus sequence of the promoter (fur box), to prevent initiation of transcription. In iron-depleted conditions the *apo*-Fur exhibits reduced affinity to the fur box and dissociates from the promoter region to allow for gene expression (49).

Upon completion of biosynthesis, enterobactin is transported from the cytoplasm to the periplasm by a 42-kDa inner membrane (IM) protein identified as EntS (37), which is also encoded by the gene cluster regulated by Fur. There, EntS interacts with TolC *via* a

tripartite system comprising AcrB, AcrD and MdtABC for secretion through the outer membrane (OM) (38).

At present, very little is known about EntS and its structure has not been experimentally resolved. A topology map constructed by Furrer *et al.* suggests EntS is a member of the Major Facilitator Superfamily (MFS) with 12 predicted transmembrane (TM) helices (37). A prediction of EntS structure using AlphaFold (108) suggests a structure in agreement with the 12 TM helix model, however confirmation of the exact structure of EntS requires experimental data. MFS proteins are important for controlling the efflux of several compounds in Gram-negative bacteria *via* symport or antiport mechanisms (144). The manner in which EntS transports enterobactin out of the cytoplasm has yet to be elucidated.

The first step in studying membrane proteins is to extract and isolate them from the membrane, and to solubilize them while maintaining their conformation as close to native as possible. The most common way to achieve this has long been the use of detergents. In recent years, the advent of lipid nanodiscs and SMALP assemblies made from copolymers has helped streamline the extraction process and alleviate the challenges such as delipidation and aggregation stemming from the use of detergents (121).

Using both detergents and copolymers, we performed a solubility screen for EntS, which allowed us to determine which compounds showed the best potential for solubilization and subsequent purification. In the course of our experimentation, results have revealed that EntS likely adopts a dimeric conformation. Whether dimerization is needed for functionality remains to be investigated.

5.2 Methods

All reagents were obtained from Bioshop Canada, Inc. (Burlington, Ontario) unless otherwise specified.

5.2.1 Detergent screen

Protein preparation and detergent screen

E. coli AG-1 cells harboring pCA24N-*entS*-His (Cam^R) were obtained from the ASKA collection (130). Cells were grown in Luria-Bertani (LB) broth supplemented with 34 $\mu\text{g mL}^{-1}$ chloramphenicol at 37 °C until optical density at 600 nm (OD_{600}) reached 0.6-0.7 units. The culture was then treated with 1 mM IPTG to induce expression of the H6-EntS protein at room temperature overnight. The cells were pelleted and lysed in buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole, lysozyme 10 mg mL^{-1} , DNAase, RNase and protease inhibitor cocktail (PIC), pH 8.0) at 10 mL per gram of wet pellet and then submitted to three cycles of freeze/thaw (freezing at -80 °C for 30 minutes each cycle). The lysates were centrifugated at 7,000x g for 30 mins at 4 °C to pellet out unbroken cells and debris. The cleared lysates were centrifugated on the Thermo Heraeus Pico 21 microcentrifuge at maximum speed (21,000x g) for one hour at 4 °C to isolate the membrane fraction. These membrane fractions were resuspended in 100 mM PBS at one quarter of the initial lysate volume. The detergent screen was performed using the Hampton Research kits HR2-410, HR2-411 and HR2-412, for a total of 72 detergents (Hampton Research Corp., USA) (Supplemental Table S2.1).

For the first round, aliquots of resuspended membrane fraction were mixed with the detergents from the screening kits at 1.5X critical micellar concentration (CMC) and incubated for 6 hours at 37 °C with gentle rotation as per the manufacturer's

recommendations. Samples were centrifuged on the Thermo Heraeus Pico 21 microcentrifuge at maximum speed (21,000x g) for one hour at 4 °C. The supernatants containing the solubilized proteins were denatured at 46 °C for 10 minutes and then loaded in parallel on two 10% SDS-polyacrylamide gels, one for treatment by Coomassie stain and the other for Western blotting. The proteins were then separated at 200 volts on BioRad Mini-PROTEAN gel electrophoresis apparatus. One gel was rinsed and incubated with Coomassie stain, whereas the other was transferred onto PVDF membrane (BioRad) at 100 volts for 60 minutes, on ice. After rinsing in TBS, the membrane was blocked using the Penta-His HRP conjugate kit (Qiagen). After rinsing, the membrane was probed for presence of EntS-His with the Penta-His HRP Conjugate antibody (Qiagen) at 1:2,500 dilution for one hour on rotator at room temperature. Detection was performed using Thermo SuperSignalTM West Pico Chemiluminescent Substrate. Chemiluminescence was recorded using the Amersham Imager 600 system. Exposure times were extended as appropriate in order to observe fainter bands where applicable.

Further optimization was undertaken using only n-Decyl- β -D-thiomaltoside (DtM), FosCholine12[®] and DDMAB. For this second round, extraction was performed varying concentrations of detergent at 1.5X CMC, 2.0X CMC and 2.5X CMC (results not shown).

In a third round, the same three detergents were compared for efficiency of extraction at 2.0X CMC by loading both the supernatant and the resuspended pellet after the microcentrifugation (21,000x g) step. The protocol for protein separation and immunoblotting was repeated as above.

5.2.1.1 Purification of EntS solubilized in n-Decyl- β -D-thiomaltoside, FosCholine12[®] and DDMAB

Larger volumes of protein lysate were produced as above and extracted in n-Decyl- β -D-thiomaltoside, FosCholine12[®] or DDMAB at 2.0X CMC to proceed with affinity purification on Ni-NTA agarose resin (Qiagen). In all cases, 10 mM imidazole was added to the solubilized protein solution. In two different attempts, capture on the resin was performed either on gravity, flow-through column or by batch binding on rotator, overnight at 4 °C. The resin bed was washed three times with washing buffer (NaH₂PO₄ 50mM, 300 mM NaCl, 50 mM imidazole, pH 8) supplemented with the appropriate detergent, and then eluted successively with the same buffer with increasing imidazole concentrations (100 mM, 250 mM), also supplemented with the appropriate detergent.

Aliquots from all steps were loaded onto SDS-PAGE for either Coomassie staining or to be transferred onto PVDF membrane for immunoblotting as described above.

5.2.2 Copolymer solubility screen

Two screening kits containing copolymers were obtained from Cube Biotech (Germany), one containing four formulations of styrene maleic anhydride (SMA) along with four formulations of diisobutylene-maleic acid (DIBMA), and the other containing six formulations of poly acrylic acid-co-styrene (AASTY) (Table S2.3). Cultures were grown and protein expression was induced as described above. Pelleted cells were resuspended in PBS with the addition of PIC and 1 mM EDTA at 10 mL per gram of wet pellet. Cell lysis was performed on French press at 16,000 psi for three cycles. The resulting lysate was centrifugated at 7,000x g for 30 minutes at 4 °C to pellet out unbroken cells and debris. The cleared lysate was then centrifugated at 60,000x g for one hour at 4 °C to

isolate the membrane fraction. This membrane fraction was resuspended in 100 mM PBS with PIC at 1 mL for each 40 mg of wet pellet. Samples of the resuspended membrane were used to reconstitute aliquots of either SMA or DIBMA at 4%, 2%, 1% and 0.5% or AASTY at 2%, 1%, 0.5% and 0.1% as per the manufacturer's protocol. All aliquots were incubated at 4 °C on rotator overnight (18 hrs), after which they were centrifugated at 38,000x g to pellet unsolubilized membrane proteins. All samples were denatured at 46 °C for 30 minutes. Protein separation and immunoblot were performed as described above, except for samples containing DIBMA which required a step of precipitation in acetone followed by resuspension in PBS at 1X initial volume prior to denaturation and separation.

5.2.2.1 Purification of SMALPS (EntS solubilized in SMA 200)

EntS-SMA lipid nanodiscs (EntS-SMALPs) in a solution of 1% were diluted 1:40 in 20 mM HEPES, 100 mM NaCl, pH 8 to dilute out excess free copolymer, as suggested by the manufacturer. Then, 10 mM imidazole was added and the solution was left to bind rinsed PureCube Ni-NTA resin beads (Cube Biotech, Germany) overnight, on rotator at 4 °C. The solution was then poured onto a gravity column and left to settle prior to decanting excess liquid. The resin bed was washed three times with washing buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8), and then eluted successively with the same buffer with increasing imidazole concentrations (100 mM, 250 mM, 500 mM). Aliquots were loaded on 10% polyacrylamide SDS gels for PAGE separation at 200V, then stained with Coomassie dye.

In an attempt to increase binding efficiency, another batch of extracted proteins was divided into three and treated as above, except for the increase of NaCl in the dilution,

wash and elution buffer at 600 mM, 900 mM and 1000 mM, respectively. All other parameters and subsequent treatment remained unchanged.

5.3 Results

5.3.1 Results from the detergent screen using the Hampton Research kit.

Figure 5.1 shows selected results from the immunoblots following probing with Penta-His antibody. Out of the 72 detergents supplied in the screening kits, only a few demonstrated any potential for adequate extraction. Forty-three detergents were eliminated due to absence of detectible protein on immunoblot (Appendix Two, Table S2.2). Seventeen detergents performed poorly in their ability to extract EntS which could only be detected on the immunoblot if exposure and contrast were enhanced on the imager (Fig. 5.1A). A third group of twelve detergents showed the best potential for extraction (Fig. 5.1B). Of this latter group, only three detergents, FOS-Choline[®] 12, DDMAB and n-Decyl- β -D-thiomaltoside (DtM), appeared markedly better for extraction.

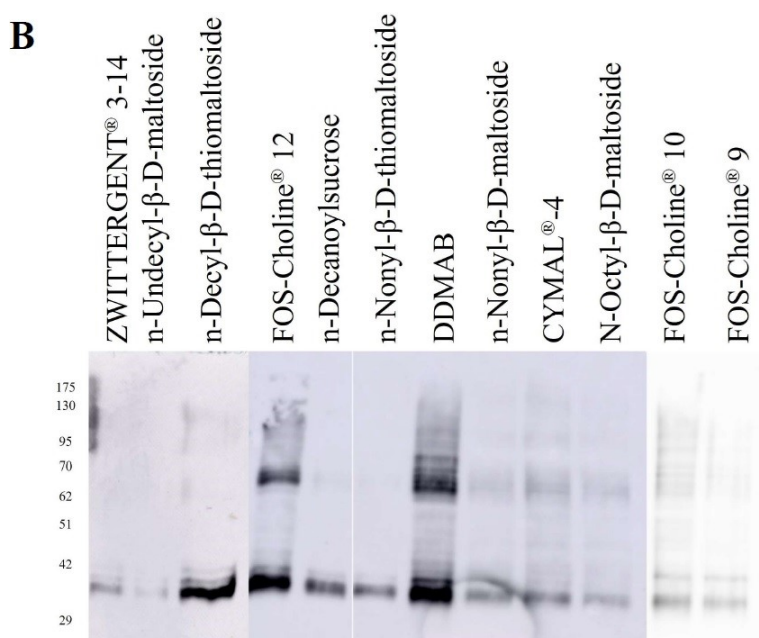
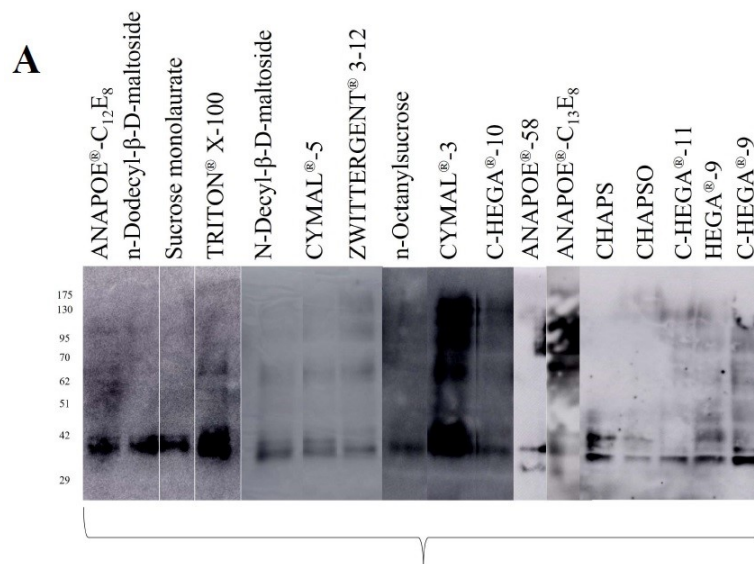


Figure 5.1 Selected results from detergent screen using Hampton Research Detergent Screen kits HR2-410, HR-411 and HR-412. A) Results from detergents with which extraction was poor and could only be detected on the immunoblot if exposure and contrast were enhanced on the imager. B) Results from detergents with which extraction was deemed the best.

It is worth noting that although EntS is predicted to weigh 42 kDa, the immunoblots show the protein running at a much lower molecular weight, closer to approximately 35 kDa. This could be due to gel shift, an anomaly commonly encountered when membrane proteins are separated on SDS-PAGE (152).

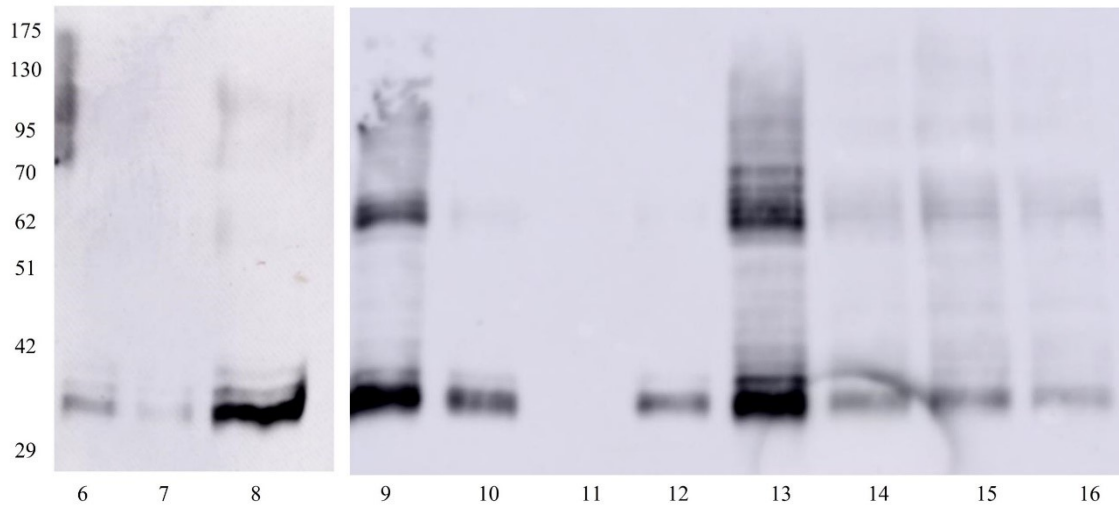


Figure 5.2: Extraction of EntS with detergents #6-16 from Hampton Research Detergent Screen kit HR-412. 6) Zwittergent® 3-14; 7) n-Undecyl- β -D-maltoside; 8) n-Decyl- β -D-thiomaltoside; 9) FOS-Choline® 12; 10) n-Decanoylsucrose; 11) n-Nonyl- β -D-thioglucoiside; 12) n-Nonyl- β -D-thiomaltoside; 13) DDMAB 14) n-Nonyl- β -D-maltoside 15) CYMAL®-4 16) n-Octyl- β -D-thiomaltoside.

The immunoblot shown on Figure 5.2 corresponds in greater detail to the extractions obtained using the detergents #6-16 of kit HR2-412. The results obtained using FOS-Choline® 12 and DDMAB (lanes 9 and 13) showed the best yield for extraction of EntS ($\approx$ 35 kDa) and revealed the presence of a more intense band at approximately 65 kDa, or twice the expected molecular weight, which initially was suggestive of incomplete extraction. A similar band is observable in lanes 14-16. A third detergent, n-Decyl- β -D-thiomaltoside (lane 8), also showed a good potential for extraction but without the

presence of higher MW band. These three detergents were therefore selected for further attempts at extraction by varying different parameters.

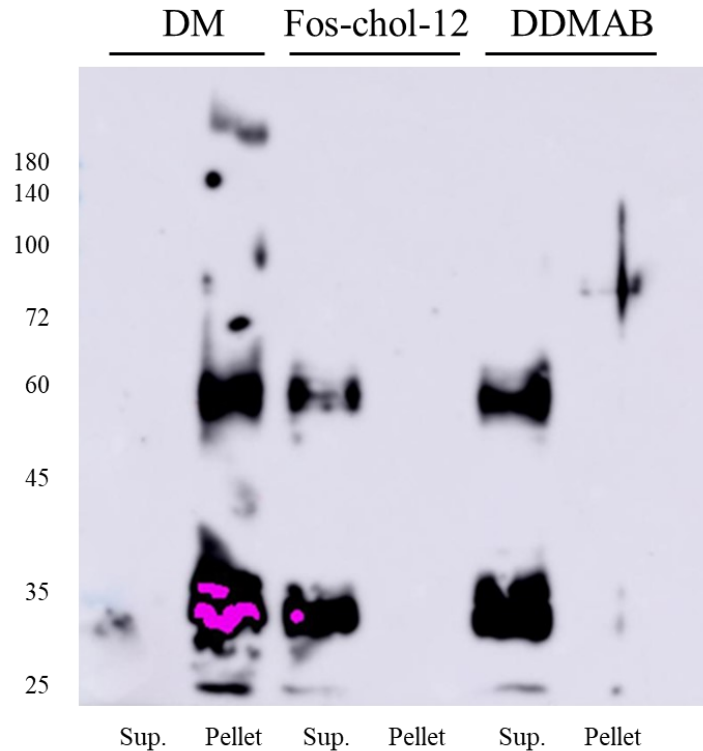


Figure 5.3. Extraction of EntS using n-Decyl- β -D-thiomaltoside, FOS-Choline[®] 12 and DDMAB at 2X CMC. Both the supernatant (sup.) and the resuspended pellet were loaded for comparison.

The immunoblot shown on Figure 5.3 corresponds to the extractions obtained using the three detergents cited above at a concentration of 2X CMC. Both the supernatants and the resuspended pellets were loaded on the gel to compare the efficiency of the extraction. For n-Decyl- β -D-thiomaltoside, extraction was minimal, and the bulk of the protein remained in the pellet. For FOS-Choline[®] 12 and DDMAB, extraction proved efficient, particularly for the latter.

5.3.1.1 Purification of EntS solubilised in detergents on Ni-NTA agarose.

Purification by gravity flow did not yield results for any of the detergents on trial. For FOS-Choline[®] 12 and DDMAB, although extraction was efficient, Coomassie stain of the various aliquots and eluates post purification by overnight batch binding showed that most of the protein remained in the flowthrough. Following immunoblotting the proteins were only faintly detectible in the eluates (results not shown). Purification of EntS solubilized in n-Decyl- β -D-thiomaltoside did not show high yield on Coomassie stain but could only be detected by immunoblotting (Fig. 5.4), albeit faintly.

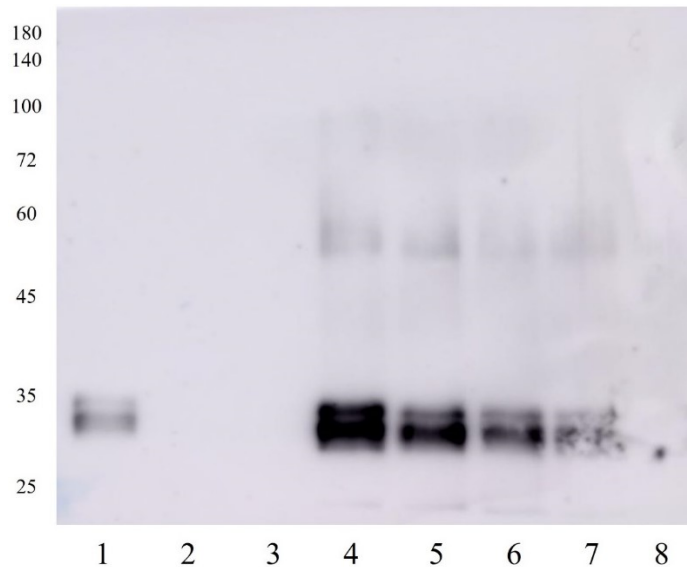


Figure 5.4. Batch binding purification of EntS solubilized in n-Decyl- β -D-thiomaltoside at 2X CMC, immunoblot probed with Qiagen Penta-His HRP Conjugate antibody. Lane numbers indicate: 1) Flow through from column after packing. 2) Wash with buffer containing 50 mM imidazole. 3) Wash with buffer containing 100 mM imidazole. 4-8) Elution with buffer containing 250 mM imidazole.

It is worth noting that purification of EntS solubilized in n-Decyl- β -D-thiomaltoside revealed the presence of a band at about twice the molecular weight of the protein (Fig.

5.4, lanes 4-8), which had not been observable during the initial detergent screen and optimization steps.

5.3.2 Results from the solubility screen using SMA, DIBMA and AASTY.

Figure 5.5 shows the results from the detection immunoblots probed with anti-His antibody. Out of the 14 copolymers supplied in the screening kit, only a few demonstrated any potential for adequate extraction. Incubation with DIBMA did not result in successful extraction (immunoblots not shown). For the SMA formulations, only SMA200 and SMA300 showed potential for extraction, although in the latter case detection was only achieved if the exposure time for imaging of the immunoblot was extended to four minutes, compared to 30 seconds for the former (Fig. 5.5, panel A).

Results obtained for the AASTY formulations revealed that AASTY 11-55 showed the best capacity for extraction (Fig. 5.5, panel B). Efforts towards achieving purification of synthetic nanodiscs generated with this compound are currently underway.

The repeated detection of a band 70 kDa (Figs. 5.2, 5.3, 5.4 and 5.5) was unexpected. Initially discounted as incomplete extraction out of the membrane, this observation under different conditions led to the suggestion that EntS dimer could dimerize (Fig. 5.6).

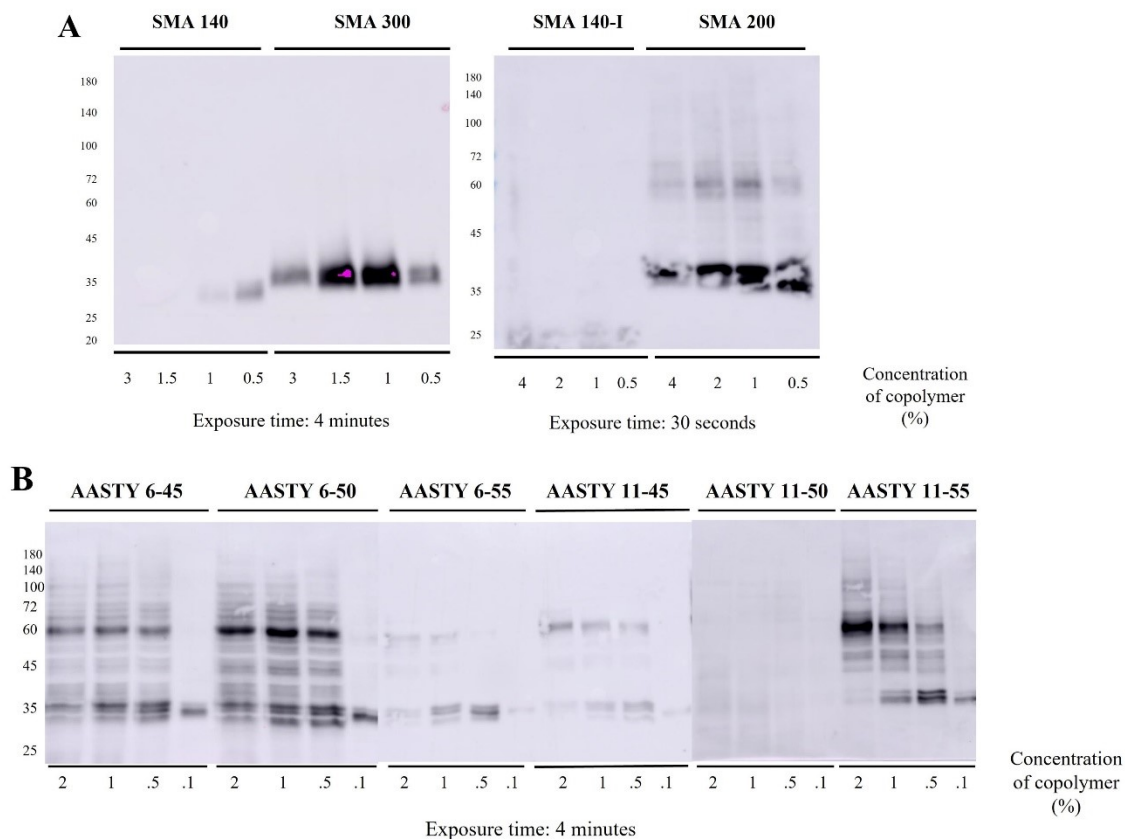


Figure 5.5 Immunoblotting results from copolymer solubility screen using CubeBiotech copolymers SMA and AASTY. A) Obtained using the various formulations of styrene maleic acid (SMA) copolymers. B) Obtained using the various formulations of acrylic acid styrene (AASTY) copolymers.

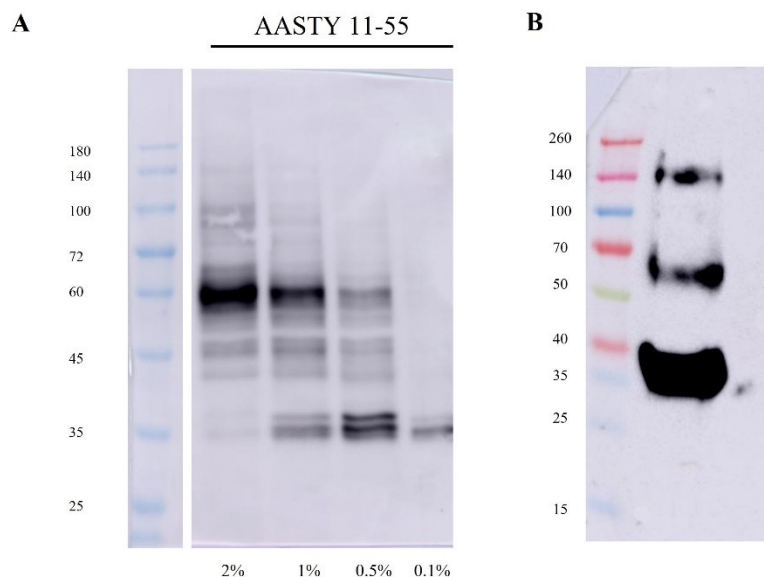


Figure 5.6: Further evidence for EntS dimerization. A) From Figure 5.5, panel B, concentration of protein varies according to the concentration of AASTY 11-55 copolymer used for extraction. B) EntS protein extracted in 0.5% AASTY 11-55, left in the refrigerator for several days, shows that protein is dispersed in three species at around 35 kDa (monodispersed), 60 kDa (presumed to be dimeric) and around 140 kDa (possibly tetrameric).

5.3.2.1 Purification of EntS solubilised in SMA200 (EntS-SMALPS) on Ni NTA agarose.

Figure 5.7 shows that although binding on the Ni NTA agarose beads appears adequate when following the protocol recommended by the supplier, elution of the EntS-SMALPS can be performed with a very low concentration of imidazole, at which point the eluate still contains a considerable amount of non-specific proteins.

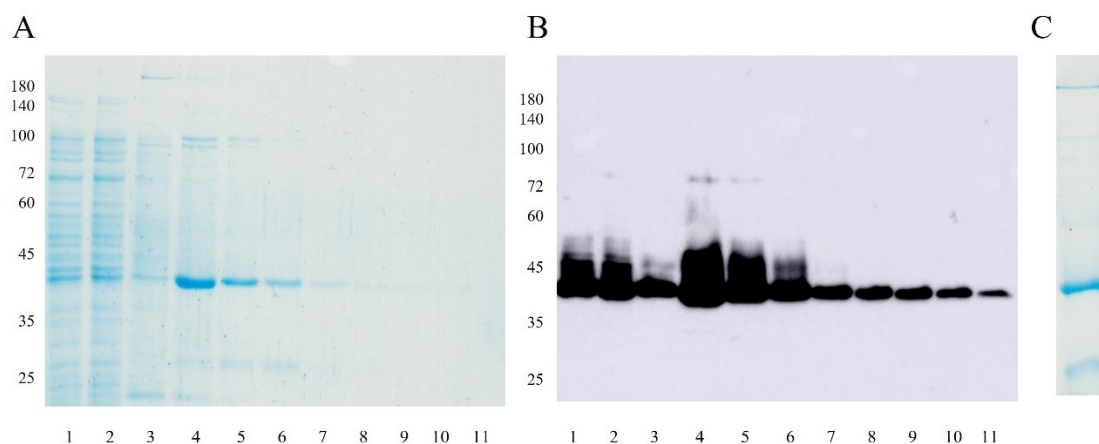


Figure 5.7: Batch binding purification of EntS solubilized in 1% SMA200. A) Coomassie stain. B) Immunoblot probed with Qiagen Penta-His HRP Conjugate antibody. Lane numbers indicate: 1) Aliquot from extraction product diluted 1:20 in HEPES 20 mM, NaCl 100 mM, pH 8.3. 2) Flow through from column after allowing the resin to settle. 3) Wash with buffer containing 20 mM imidazole. 4) Wash with buffer containing 50 mM imidazole. 5-6) Elution with buffer containing 100 mM imidazole. 7-8) Elution with buffer containing 250 mM imidazole. 9-12) Elution with buffer containing 500 mM imidazole. C) Samples from lanes 4, 5 and 6 pooled and concentrated.

Attempts at optimizing the protocol included diluting out the extracted protein in at 1:40 in HEPES/NaCl buffer (results not shown) as suggested by the manufacturer, as well as using Ni NTA agarose beads from different suppliers (Qiagen, PureCube Indigo™) (results not shown). Shown on figure 5.8 are the results from increasing the concentration of NaCl in the dilution, wash and elution buffers.

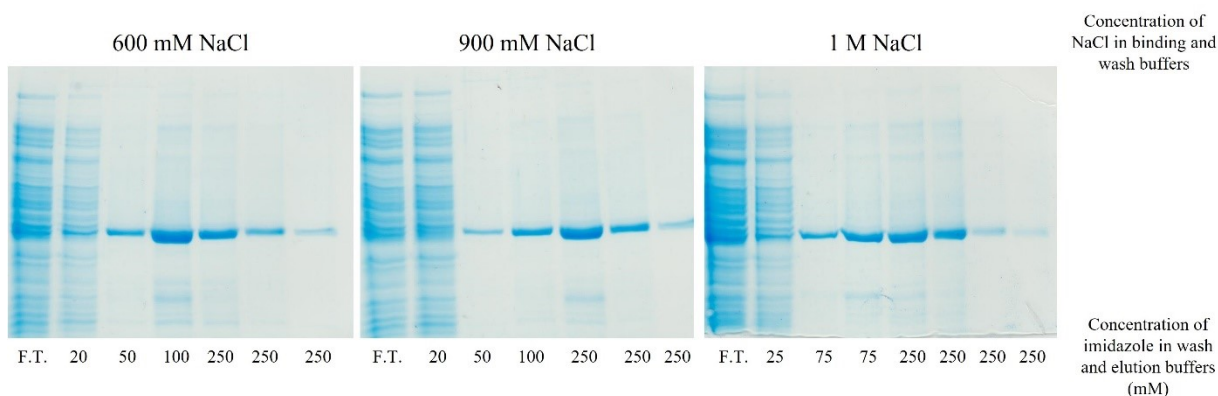


Figure 5.8: Comparison of efficacy of binding from EntS-SMALPS on PureCube Ni NTA beads when varying the concentration of NaCl in buffers. Same sample distributed in binding buffer (F.T.: flow-through)

5.4 Discussion

Membrane proteins adopt their functional conformations not only through interactions among the residues that support their tertiary or quaternary structure, but also due to interactions between the amino acids and the phospholipids of the membrane. Studying membrane proteins should therefore be attempted in conditions that preserve this milieu to best represent the native structure. This is especially true of MFS transporters that undergo various degrees of conformational change, and thus for which the surrounding lipids form an integral component for the structure and function (97).

During extraction of membrane proteins and their subsequent purification, this native environment is often destroyed or relatively adulterated. This long remained a challenge when extraction was done using detergents. In a detergent micelle that readily assumes a spherical shape, many domains of the protein were put under strain and thus less likely to adopt their native configuration. Moreover, prolonged incubation in detergents often lead to de-lipidation and degradation of the protein. Finally, during all subsequent procedures

such as purification and analytical measurements, the concentration of detergent has to be maintained, which was often problematic and cost prohibitive.

Here we initially performed a classic detergent screen. Out of 72 formulations tested, only three showed any potential for extraction. Further assays aimed at optimizing parameters such as temperature, concentration and time raised other challenges such as compromised stability without increasing yield. Purification also proved inadequate and time consuming. Using both Fos-Choline 12 and DDMAB, purification was impossible to achieve: there was no binding of the Ni-NTA agarose affinity beads, which we presume could be due to either a shielding of the affinity 6His affinity tag in the detergent micelle, or to other parameters owing to the type of detergent that precluded this binding. When using DtM, purification seemed possible, but yield was so poor that the eluted protein could only be detected by immunoblotting. For all those reasons, efforts towards solubilisation in detergents was abandoned.

The advent of tools such as lipid nanodiscs has greatly helped address these challenges and constitutes a major advancement towards the characterization of membrane proteins (121). Here, we tested solubility using copolymers, compounds that form a ring holding together the membrane protein along with the associated phospholipids, assembling into synthetic nanodiscs, or SMALPs. The ability of copolymers to isolate the protein of interest from the membrane while conserving the native phospholipids helps to reproduce the environment of the cell membrane. In doing so, they also circumvent the challenges faced when using detergents and provide a stable and mobile assembly of the membrane in its active state. In addition, optimization of protocols for extraction and purification having been optimized by the manufacturer.

In a first round of solubilisation, extraction with copolymers revealed that two compounds SMA 200 and AASTY 11-55, exhibited the best potential. Proceeding with purification using SMA 200 showed that binding efficiency was adequate, and the elution step yielded a considerable amount of SMALPS. However yield and purity were too low to allow for further characterization. As per the manufacturer's recommendations, achieving a high degree of purity often requires the application of a second method (*e.g.*, size exclusion chromatography, ion exchange chromatography) which is currently being investigated.

One unexpected outcome from these efforts, whether using detergents or copolymers, is that upon separation of the extracted proteins by SDS-PAGE, two bands were often clearly visible, one corresponding to the molecular weight of EntS, the other at approximately twice that size, suggestive of a dimer. Several MFS proteins are known to dimerize, and this conformation is required for functionality. Whether this is the case for EntS, or if the bands observed at a higher molecular weight are artefactual warrants closer scrutiny.

Membrane proteins that act as channels must adopt a conformation that allows for formation of a pore through which the metabolite is shuttled. Monomeric channel proteins such as lactose permease achieve this simply by virtue of the topology of their residues, where polar groups in the midst of the transmembrane helices line the channel while hydrophobic groups interact with the lipids of the membrane. In other known cases, such as dimeric nitrate transporter NRT1.1 from *Arabidopsis thaliana* (PDB: 4OH3) or the mammalian polycystic kidney disease channel PKD2 (PDB: 5T4D), formation of the

transport channel is reliant on multimerization where the interface of the protomers brings polar residues into proximity within the interface of the multimer to form a pore.

In the case of EntS in a first instance attempts were made to modify denaturation parameters so as to yield monodisperse vs dimeric assemblies in parallel. These included denaturation by various duration and/or temperature regimens as well as the use of other denaturants such as urea and guanidine hydrochloride. These efforts did not yield satisfactory results. So far, the most compelling evidence has been the use of copolymer AASTY 11-55 under different concentrations as explained in Figure 5.6, as well as an additional band appearing in a sample left in the refrigerator for several days. Ongoing work includes using chemical crosslinkers to capture the proposed dimer prior to extraction, then using different concentrations of AASTY 11-55 (*i.e.*, 2% vs 0.5%) to compare the variation in monodispersity in crosslinked relative to non-crosslinked samples.

5.5 Future work

The use of copolymers to synthesize lipid nanodiscs poses a challenge for subsequent purification due to the presence of excess free copolymer that interferes with binding of the His-tagged protein to the Ni-NTA resin beads. The manufacturers initially recommended either diluting out the supernatants to a maximum of 0.025% copolymer (about 1:40). Although this step improved the yield (Fig 5.7), working with the large volumes required resulted in attrition due to the loss of some of the resin upon transfer to the gravity column.

So far, attempts at further purifying EntS-SMALPs using either size exclusion chromatography or ion exchange chromatography have been unsuccessful. Recently,

Cube Biotech has introduced a new its proprietary product PolyHunter™ that removes excess free copolymer following extraction. Efforts towards optimizing purification as per the manufacturer's recommended protocol are ongoing, focussing on the use of copolymers SMA200 and AASTY 11-55.

CHAPTER SIX – General discussion

6.1 Answers to the initial research questions

The studies detailed in this thesis set out to answer four research questions, as posed under Section 1.7. Briefly, the overarching goal of this work was to gather additional evidence for the intracellular assembly of the proteins involved in biosynthesis and secretion of the siderophore enterobactin in *E. coli*.

In some instances, the results obtained were sufficient to conclude that the goals had been reached, whereas in other cases more work is still needed. Unexpected observations also gave rise to further questions that could become the topic of future studies.

6.1.1 Can we demonstrate that the first two enzymes of the enterobactin biosynthetic pathway, EntC and EntB, are directly interacting, and thereby confirm that this pathway is fully networked via protein-protein interactions?

As per our publication in *Biochimie* (135) from which Chapter Two was extracted, this study was successful. Using *in vivo* crosslinking with formaldehyde, supported by previously gathered preliminary evidence from a bacterial two-hybrid assay and combined with a proposed docking model (Fig. S1.3), we demonstrated that the first two enzymes of the enterobactin biosynthetic pathway, EntC and EntB, interact. This was the first reported confirmation of an interaction between these proteins and therefore completed the roster of pairwise interactions among the enzymes involved in all the sequential steps of enterobactin biosynthesis in *E. coli*.

This study was significant for three reasons: A) it reported *in vivo* evidence for an intracellular, pairwise interaction between the first two enzymes of the enterobactin biosynthetic pathway. This was supported by molecular modeling of the complex thus

formed. This demonstration completed the pairwise mapping of interactions involving DHB biosynthetic enzymes; B) the docking model gave rise to a mechanism by which the isochorismate precursor could proceed to substrate chaneling, thereby strengthening our hypothesis of a metabolon assembly; C) in addition to this demonstration of an interaction between EntC and EntB, results from our chemical crosslinking experiment suggested the possible involvement of both proteins in higher-order complexes with additional proteins that remained to be identified. This latter finding paved the way for the study described in Chapter Three.

6.1.2 Can we gather evidence suggesting that Ent proteins organise themselves in a supramolecular complex, i.e., a metabolon, by capturing in vivo a simultaneous, intracellular assembly of several, if not all, Ent proteins?

The study detailed under Chapter Three demonstrated that it was possible to isolate an intracellular complex containing at least four Ent proteins. Through the use of clones harbouring several epitope-tagged Ent proteins and continuing with the *in vivo* crosslinking with formaldehyde protocol developed for the previous study, it was possible to isolate and to initiate analysis of the high-molecular weight bands of interest. Probing these bands before and after boiling (to heat reverse the chemical crosslink) revealed the presence of at least four different proteins of the pathway, two of which are active in a different arm (*i.e.*, EntC in conversion of chorismate into 2,3-DHB vs EntE in the NRPS module, see Fig. 1.3), capturing elements from the two biosynthetic modules engaging into a simultaneous interaction.

Further work is still needed if we wish to confirm that this large complex comprises other, if not all Ent biosynthetic proteins. At this stage it has not been possible to

ascertain whether other proteins involved in biosynthesis (*e.g.*, EntF, EntD) were also contained in this higher-order complex. More efforts to that end are required, as will be discussed later.

This study was interesting for two reasons. First, it built on the observations from our previous EntC-EntB study and succeeded in demonstrating that the higher order intracellular complexes detected upon the crosslinking treatment included other Ent proteins, as per our hypothesis. Second, not only did it allow us to further optimize the *in vivo* protocol we had developed for that study, but it also allowed for the application of another protocol whereby the complexes of interest were passively eluted out of the SDS-PA gel for further analysis. This method of isolating proteins of interest might prove useful in future.

6.1.3 Can we determine which, if any, of the Ent biosynthetic proteins closely interact with EntS, the inner membrane secretory channel for enterobactin?

Efforts aimed at answering this question yielded preliminary yet compelling results. To empirically determine which of the Ent biosynthetic proteins interact with EntS, we employed proximity bio-labeling with an engineered ascorbate peroxidase enzyme APEX2 that has been optimized for bacterial expression. Another biosynthetic Ent protein, EntE was also fused with the APEX2 reporter, to be used as a control since most interactors of EntE have already been identified.

Assays upstream of the proximity labeling attempts showed that our fusion products were functional and localized within the bacterial cell as expected, *i.e.*, in the membrane for EntS-APEX2, and in the cytosol for EntE-APEX2. Considering that the clones with which we work were all obtained from the ASKA collection and are borne on the same

vector, it would be reasonable to presume that other Ent proteins could therefore be used to create similar, functional fusion products for future studies.

Initial immunoblotting results allow us to suggest not only that many Ent proteins were biotinylated upon treatment of APEX2 with hydrogen peroxide, but that there could be other interactors of interest whose identity should be formally established by other means. For instance, those proteins might be scaffold or accessory proteins that are not encoded on the *ent* gene cluster yet are essential for proper assembly and localization. Future work to confirm this includes purification and enrichment with a view to proceed with peptide mass fingerprinting for formal identification.

Also of interest were the prey proteins biotinylated by both the EntS-APEX2 and the EntE-APEX2 bait proteins. Although not formally identified, several proteins of similar molecular weight were detected in both assays, which further supports our hypothesis of a large complex of many Ent proteins that assemble at the inner membrane.

6.1.4 Can we efficiently isolate and characterise IM channel EntS so that its role in secretion, hence its virulence, can be better studied?

This study under Chapter Five was the most extensive and time-intensive part of this thesis. Although still incomplete, it nevertheless yielded very interesting, unexpected data.

Through a detergent screen and a copolymer screen, at least two detergents and two copolymers were found effective in extracting EntS out of the membrane and isolating it in either a detergent micelle or a synthetic lipid nanodisc (SMALP), respectively.

Efforts at purifying EntS need to be pursued. The challenges inherent to purification of proteins contained in detergent micelles (*i.e.*, instability of the protein, cost of reagents)

have led to the decision to forego this avenue to the benefit of working with SMALPs. So far purification has only been attempted using copolymer SMA 200. A similar effort using copolymer AASTY 11-55 should now be attempted.

At present, a major caveat relating to the use of copolymers is their interference with the binding of the protein to the Ni-NTA affinity resin. Extra steps must therefore be undertaken to alleviate this effect which causes attrition and affects yield. The manufacturer (Cube Biotech, Germany) is aware of this situation and currently offers various options to circumvent this problem. In particular, a resin that binds and removes excess copolymer (Polyhunter®) is now available.

One unexpected finding that was consistent for both the detergent and the copolymer extraction was the detection of a band corresponding to twice the molecular weight of EntS. This effect was not systematically observed but seemed to vary depending on conditions. This observation allows us to suggest a possible dimerization of EntS, although the possibility of an artifact cannot be discounted at this stage. Should dimerization of EntS be demonstrated, further studies would be needed to determine whether it is essential for function.

6.2. New evidence for an enterobactin metabolon that interacts with the IM transporter EntS

Evolution tends towards optimization of bioprocesses by selecting for mutations that confer an advantage. To that end, many biosynthetic processes have evolved the ability to organise themselves into metabolons to increase efficiency of the pathway, to provide a mechanism for its regulation, and to improve the stability and the assembly of the enzymes involved (65, 67-70).

Determining whether a metabolic pathway assembles into a metabolon is an extensive, multi-faceted undertaking (23, 64). From a structural perspective, it must first be established that enzymes are interacting intracellularly, be it pairwise or in complexes. In parallel, in keeping with the notion that a metabolon is a dynamic, transient assembly that performs substrate channeling, such functional aspects must also be explored. Bringing together these two aspects, additional information such as binding affinity between the interactors, mapping of the interfaces and other structural properties, can then be gathered to obtain a more complete picture.

Given the importance of iron acquisition for the survival of *E. coli* in limiting conditions, the primary focus of research in our lab is to determine whether the enzymes that synthesize enterobactin assemble into a metabolon. Our hypothesis is based on two proven assemblies, namely biosynthesis of pyoverdine in *P. aeruginosa* (71-75) and bacillibactin in *Bacillus* (76-79). Just as in the case of enterobactin, siderophore biosynthesis in both these species involves assembly of the proteins that perform sequential reactions, as well as an NRPS step. Both assemblies also engage in a close interaction with a TM exporter protein.

To investigate the extent to which biosynthesis of enterobactin mirrors these processes, it was necessary to gain further knowledge and evidence of interactions for many of the components of the pathway. Although our lab had already obtained extensive evidence for functionality, several components with regards to structure remained to be elucidated.

Towards that goal, the evidence presented in this work has provided useful data on which to build further investigations:

- 1- By identifying an intracellular interaction between EntC and EntB (Chapter Two), we confirmed that the enterobactin biosynthetic pathway is fully networked *via* pairwise interactions. This provides a basis to determine, through functional studies, the extent to which the chorismate precursor is then committed to the pathway and whether this constitutes the initiating step in substrate channeling.
- 2- We demonstrated that at least four Ent proteins, including enzymes involved in the two different arms of the biosynthetic pathway, can be captured in a simultaneous, intracellular interaction in live *E. coli* cells (Chapter Three). This shows that although transient, there is a moment during which many of the biosynthetic enzymes assemble simultaneously in a large complex, effectively linking the two modules of the pathway. This could serve as a basis to investigate which factor(s) orchestrate(s) this assembly.
- 3- We have observed that several proteins, some of which could likely be Ent proteins based on their molecular weights, localize within a 10 nm radius of the enterobactin transporter EntS. Other proteins whose molecular weight does not correlate with any Ent proteins also appear to colocalize. We presume the latter could play a structural role and therefore further investigation should be undertaken. Finally, we also observed that several proteins colocalize with both EntS and EntE. This supports our hypothesis of a large complex localizing at the inner membrane, comprising not only the enterobactin biosynthetic machinery but also structural or scaffold elements.
- 4- We identified the most efficient compounds with which to extract and solubilize enterobactin transporter EntS, paving the way for future work such as purification

and characterization of that protein. We also obtained evidence that EntS might be dimeric. This can serve as the basis from which the exact mechanism for transport can be elucidated.

The research outcomes of the current thesis will help support ongoing efforts by other lab members who are investigating the possibility of substrate channeling, thereby determining whether this assembly can be considered a metabolon, which we would name enterobactome. They can also serve to construct, through docking models, a representation of what our proposed enterobactome that would be more accurate than our schematics presented in Figure 1.6.

In particular, most identified metabolons are known to assemble around a central protein that is considered a nucleation factor (24). Having established that EntB is involved in the two modules of the biosynthetic pathway may suggest not only that it could serve as a nucleation factor, but also that it might contribute to anchor the complex to the inner membrane and thus play a key role in localization.

Based on the previous study by Hantash and Earhart who had shown that EntB was likely membrane-bound or membrane-associated (89), preliminary work has been undertaken to see whether it would be possible to isolate a membrane-bound EntB in a lipid nanodisc as per the method described in Chapter Five.

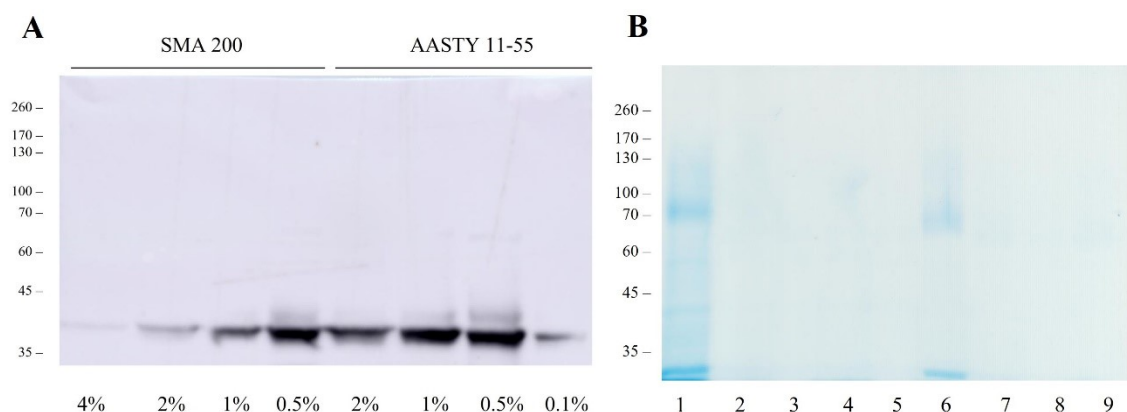


Figure 6.1: Extraction and purification of EntB from a washed membrane fraction.

A) the membrane fraction was prepared as for EntS as described in Chapter Five (see 5.2) and incubated with SMA 200 and AASTY 11-55 at various concentrations. B) the sample in 0.5% AASTY 11-55 was concentrated, and excess copolymer was removed before purification was attempted on Qiagen Ni-NTA Spin Columns: 1- flow through from column; 2- wash with 50 mM imidazole; 3-5- elution with 100 mM imidazole; 6-9 elution with 250 mM imisazole.

Figure 6.1A shows that extraction of EntB in both SMA 200 and AASTY 11-55 from a membrane fraction that had been washed twice in PBS to remove all cytosolic proteins was successful. EntB in AASTY 11-55 at 0.5% was then purified on a Qiagen Ni-NTA spin column (Fig. 6.1B). This allows us to suggest that there remained a significant amount of EntB associated with the membrane in a manner that prevented it from being washed away. Here again, we can refer to the similarities with the biosynthetic machinery for pyoverdine in *P. aeruginosa*, where PvdA was shown to be anchored to the inner membrane through a single N-terminal domain while a large part of the enzyme remained cytosolic (74). Whether this is also the case for EntB requires further investigation. By repeating extraction, solubilization and purification on a larger scale to obtain a greater yield of protein, it can then be characterized by cryo-EM.

The resolved structure of EntB solubilized in a lipid nanodisc can then be compared to the structure of EntB purified from the cell lysate (PDB: 2FQ1 (82)). This will allow us to determine whether a domain of the protein is bound to the membrane and evaluate the extent to which a conformational change might occur between a cytosolic and a membrane bound EntB.

6.3 Impact of these findings on health and industry

The body of evidence presented in this thesis cannot be readily translated into applications. Nevertheless, the research outcomes detailed herein provide a strong basis on which future studies can be undertaken.

Protein-protein interactions and assembly into supramolecular complexes are essential for a wide array of cellular functions, including those that confer virulence to pathogenic strains of bacteria. One of these processes is the biosynthesis of siderophores, which is essential for growth and survival of most bacteria.

A critical component of research projects studying biological and biochemical processes is to first establish whether and how the machinery involved in these processes interacts or assembles itself. Therefore, any additional knowledge on these interactions can eventually lead to the identification of druggable targets or disruption by other means. As detailed in Chapter One, for eventual industrial applications, the same targets can also be subjected to optimization with a view to commercially synthesize enterobactin.

Whether the ultimate goal is to find targets for disruption or optimization, be it for health, industrial or commercial purposes, the research presented here has advanced our knowledge of synthesis and secretion of enterobactin so that it can benefit research

groups that wish to pursue this further. The research outcomes presented in this work should therefore be considered a stepping stone for further research by our lab and others.

6.4 Contribution of this research to the body of knowledge – technical aspects and future applications

One immediate contribution resulting from the research presented here is the development, adaptation and/or optimization of new or established protocols.

6.4.1 In vivo crosslinking with formaldehyde (Chapters Two and Three)

Although not novel, this protocol required intense optimization so that it could best serve our particular research goals. In doing so, it established yet another variation on a proven technique. This was shown to be efficient on the strain we used, including double and quadruple transformants. In addition, it made use of a simple method for passive elution of proteins out of polyacrylamide gel. This protocol by Kurien (138) which requires very few reagents and manipulations is paradoxically under-utilised.

6.4.2 Proximity labeling protocol using APEX2 fusion proteins (Chapter Four)

Our work showed that it was possible to fuse at least two Ent proteins, one cytosolic and one membrane-spanning with the APEX2 enzyme so that the activity of the latter was maintained. This permits us to predict that similar fusions can now be attempted with other Ent proteins subcloned into the pCA24N vector used for establishing the ASKA library and thus further generate a list of potential interactors for other enzymes and accessory proteins in that collection.

6.4.3 Extraction and solubilisation of enterobactin transporter EntS in detergents and synthetic nanodiscs

Adapting the protocols supplied by Cube Biotech, we have established which compounds are most suitable for the study of EntS. This knowledge can therefore be of use to other researchers interested in further pursuing this avenue of research. As explained under 6.2, the use of lipid nanodiscs as per the protocols optimized for EntS has already been proven applicable to EntB, and could also be put to use for other proteins that may be associated or bound to the membrane.

6.5 Knowledge gaps to be filled

Research outcomes from the work described in this thesis contribute to elucidating a lesser-known metabolic process of *E. coli*, that of iron acquisition through the siderophore enterobactin. Although not conserved across all bacteria, this process is nevertheless crucial for not only *E. coli* but also species that possess the ability to acquire (*i.e.*, “highjack”) enterobactin. Adding to the elucidated mechanisms for synthesis and secretion of pyoverdine in *Pseudomonas* and bacillibactin in *Bacillus*, our research can inspire other groups to model their own endeavours towards studies of other siderophores.

6.5.1 Work in progress – short term

In the course of the research presented here, several goals were not met, mostly due to lack of time and resources. In addition, a fair number of observations arose that have prompted more questions and highlighted possible avenues for future exploration.

1- As described in Chapter Three, we successfully isolated crosslinked complexes of high molecular weight that contained several Ent proteins. To determine whether other or even all Ent biosynthetic proteins assemble simultaneously, we must still demonstrate whether

EntF and possibly accessory protein EntD are also part of the complex captured *in vivo*.

For formal identification, purification and enrichment of these complexes should be performed in preparation for peptide mass fingerprinting.

2- As shown on Figure 6.1, preliminary work on EntB has resulted in successful extraction and solubilization in a synthetic lipid nanodisc using AASTY 11-55 from Cube Biotech. The first attempt at purification has also proven successful and can now be performed on a larger scale for resolution of the structure of membrane bound EntB.

3- Considering that EntE and EntF were also shown in the Hantash study to be membrane associated or membrane bound, these proteins should also be tested for extraction and isolation in a synthetic lipid nanodisc. Although we propose that EntB could be responsible for anchoring our proposed metabolon to the membrane, we cannot discount the possibility that EntE and EntF might also play a similar role. As an initial step, efforts towards extraction and solubilization of EntE and EntF from a washed membrane fraction should be attempted as we did for EntB.

4- Proximity labeling assays performed as described in Chapter Four revealed that it was possible to biotinylate proteins localizing within 10 nm of EntS. As an immediate next step, two-dimensional gel electrophoresis is ongoing to further identify these potential interactors by comparing their molecular weights and isoelectric points to those of Ent proteins. Following this, purification and enrichment of these proposed interactors can be performed with a view to proceed with peptide mass fingerprinting.

5- Extraction and solubilisation of EntS in detergents and in synthetic lipid nanodiscs have provided evidence that this protein might be dimeric. At present, we are developing a protocol using *in vivo* crosslinking followed by extraction under various parameters that

favour monodispersal as opposed to the retention of a dimeric structure to observe whether a chemically crosslinked dimer would be detected.

6.5.2 Future directions – long term

1- We have determined that it was possible to isolate EntB in a SMALP. Considering that it is a bifunctional protein involved in the two distinct steps of enterobactin synthesis, we should investigate whether EntB might act as the nucleation factor, the protein around which other Ent proteins assemble. Here again, this is based on the pyoverdine model where the biosynthetic enzyme PvdA was shown to fulfill that role (74).

2- Should it be possible to extract and solubilize EntE and/or EntF from an isolated and washed membrane fraction, further efforts towards large scale purification should be undertaken to obtain a resolved structure of the membrane bound protein(s).

3- Efforts at isolating EntS to high purity and yield, and resolution of its structure by crystallization, cryo-EM or other means should be pursued.

4- As detailed above, should EntS prove to be dimeric, functional studies should be undertaken to establish whether dimerization is necessary for function.

5- We have gathered extensive evidence that many Ent proteins are simultaneously interacting in live *E. coli* cells. Other efforts in our lab are being deployed towards defining the mechanisms of substrate channeling. Using the combined results from these efforts, mapping the interfaces of those interactions should be further pursued to identify all the key residues involved.

6.6 Conclusion

The bacterium *E. coli* has proven highly valuable not only as a model organism for the study of many bioprocesses, but also as a tool for countless health and industrial

applications. There remain several aspects, metabolic or structural, that have yet to be elucidated. Additional evidence such as the research outcomes presented in this thesis could eventually be expanded towards the development of other applications or used as models for research involving other Gram-negative species.

This thesis aimed to answer four questions relating to a lesser-known bioprocess in *E. coli*, the biosynthesis of the siderophore enterobactin, with a focus on its molecular aspects. The ability to synthesize and secrete siderophores has been further elucidated in a few Gram-negative bacteria (*e.g.*, *Pseudomonas*, *Bacillus*), yet for *E. coli* that process still requires investigation. The studies undertaken herein sought to provide additional evidence in support of our hypothesis that the mechanisms underlying assembly of biosynthetic machinery of enterobactin mirrors that of these other species. We continue to obtain evidence that biosynthetic Ent proteins, and possibly other accessory proteins, assemble as a metabolon that closely interacts *via* one or more of its components with the inner membrane transporter protein. The research outcomes detailed in this work have either elucidated or contributed compelling evidence for several aspects of this proposed assembly.

Any such evidence can ultimately be used to provide a complete map of this supramolecular assembly, identify residues that are key to the stability of the complex, and in so doing reveal targets for either disruption or optimization of the interactions contributing to the assembly of our proposed enterobactin metabolon.

This constitutes another contribution to the study of *E. coli* and its metabolism, particularly in as regards complex networks and their assembly. There is a significant knowledge gap regarding the assembly of proteins involved in these several processes,

and although enterobactin biosynthesis is only one bioprocess among others that remain to be elucidated, the importance of iron acquisition by *E. coli* in biofilms, healthcare challenges and impact on agriculture cannot be overstated.

As previously indicated, antibiotic resistance is a global concern and bacteria that produce enterobactin, as well as those that are able to acquire it, often exhibit resistance to antibiotics. The work described in this thesis will contribute to the development of tools for targeted disruption by demonstrating which PPIs occur *in vivo* and should be submitted to further investigation. Conversely, the potent iron chelating properties of enterobactin can lead to applications in imaging, therapeutics, industrial processes, and soil bioremediation. For both these reasons, further research on metabolons, the assembly of proteins, and their interactions in *E. coli* such as are detailed in this thesis contributes novel data from which to continue building future investigations.

This will add to the knowledge on bacterial metabolism in *E. coli* which can be harnessed and applied to future developments to benefit the research community, the health sector, industry, and eventually the population as a whole.

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APPENDIX ONE

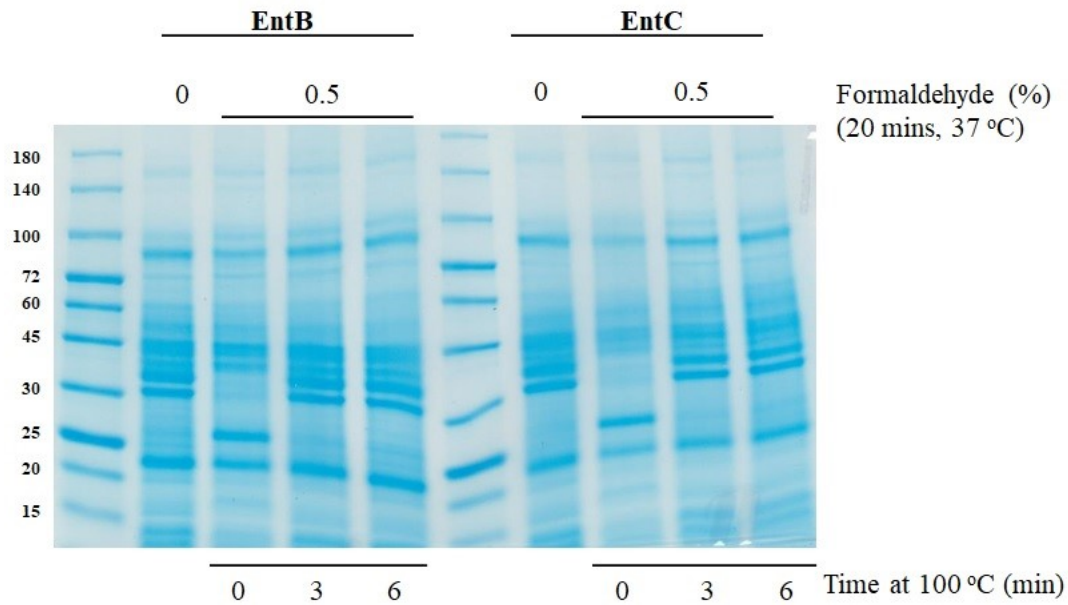


Figure S1.1 *In vivo* crosslinking with formaldehyde of cells harbouring pCA24N-*EntB*-His and pCA24N-*EntC*-His plasmids. Proteins extracted from H6-EntB and H6-EntC cells each crosslinked with 0.5% formaldehyde. Compassie stain revealing relative levels of protein expression.

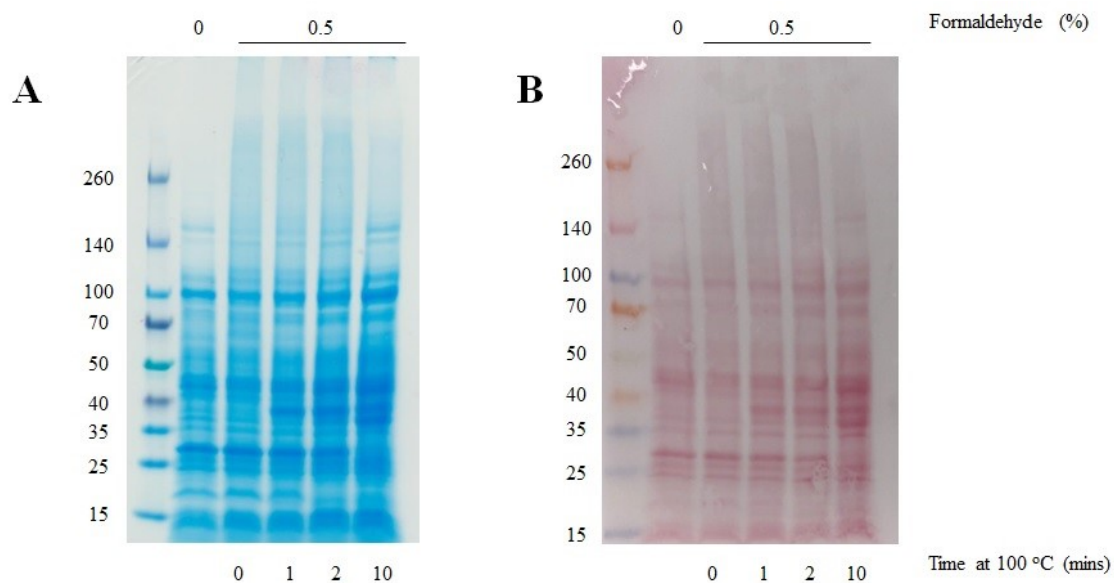


Figure S1.2. Visualization of total protein loads before and after transfer onto PVDF membrane.

A) Coomassie staining of pre-transfer SDS-polyacrylamide gel of H6-EntC and HA-EntB double-transformant cells crosslinked with 0.5% formaldehyde. B) Ponceau staining of PVDF membrane following transfer of gel for 90 minutes at 100 V.

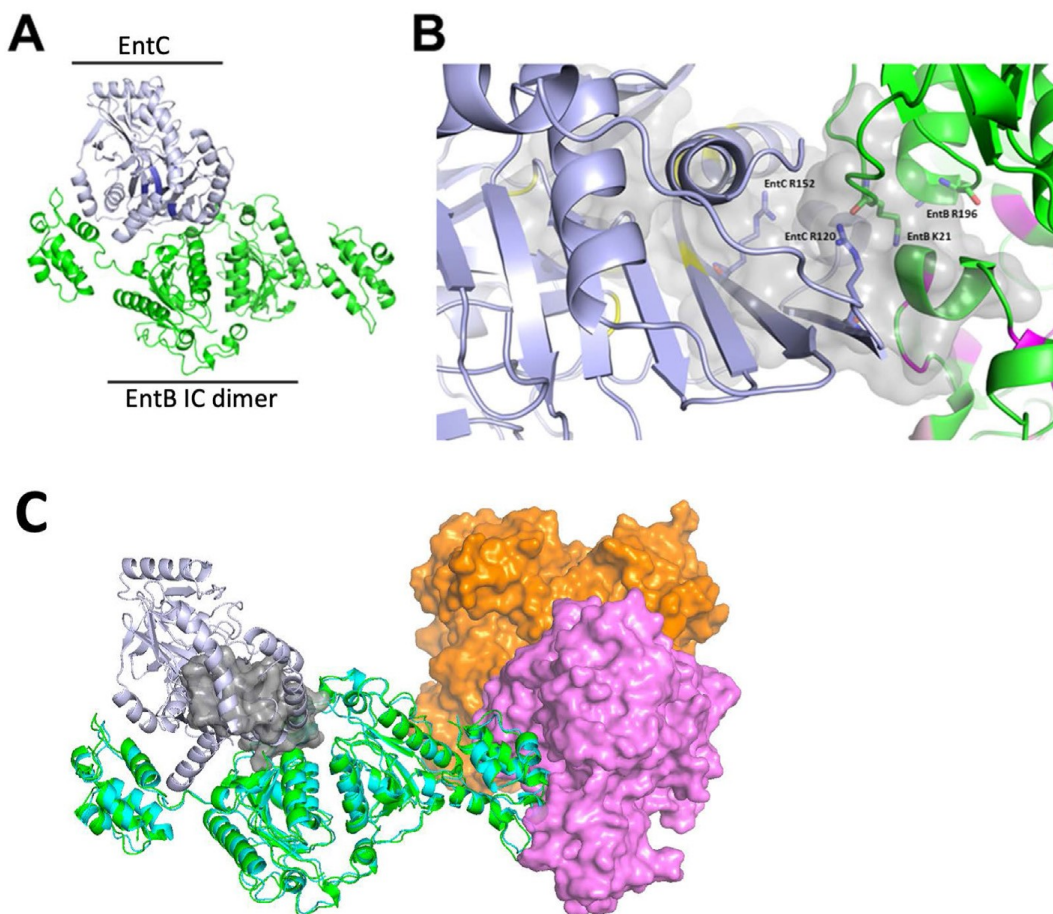


Figure S1.3 EntCB model generated by CLUSPRO (from Ouellette *et al.* (135)). (A) Top-ranked CLUSPRO model of monomeric EntC (lilac) docked to dimeric EntB (green). (B) Proposed EntC-EntB channeling pathway. EntC (lilac) and EntB (green) shown in cartoon representation. Previously reported active site residues for EntC and EntB shaded yellow and magenta, respectively. CAVER-predicted tunnel connecting EntC and EntB active sites shown as surface representation (light grey). Positively charged residues linking tunnel shown as sticks coloured by atom. (C) Alignment of the model shown in (A) with the previously reported EntBAE model (EntB dimer: cyan, EntA tetramer: orange, EntE: magenta). CAVER-predicted tunnel connecting EntC and EntB active sites shown as surface representation (light grey)

APPENDIX TWO

KIT	Detergent number	Name
Hampton Research Detergent Screen 1™ HR2- 410	1	ANAPOE®-C ₁₂ E ₉
	2	ANAPOE®-C ₁₂ E ₈
	3	n-Dodecyl-β-D-maltoside
	4	Sucrose monolaurate
	5	CYMAL®-6
	6	TRITON® X-100
	7	CTAB
	8	Big CHAP, deoxy
	9	n-Decyl-β-D-maltoside
	10	LDAO
	11	CYMAL®-5
	12	ZWITTERGENT® 3-12
	13	n-Nonyl-β-D-glucoside
	14	n-Octyl-β-D-thioglucoside
	15	DDAO
	16	HECAMEG®
	17	n-Octanoylsucrose
	18	n-Heptyl-β-D-thioglucopyranoside
	19	n-Octyl-β-D-glucoside

	20	CYMAL [®] -3
	21	C-HEGA [®] -10
	22	ZWITTERGENT [®] 3-10
	23	MEGA [®] -8
	24	n-Hexyl- β -D-glucopyranoside
Hampton Research Detergent Screen 2 [™] HR2-411	1	Pluronic [®] F-68
	2	ANAPOE [®] -35
	3	2,6-Dimethyl-4-heptyl- β -D-maltopyranoside
	4	ANAPOE [®] -58
	5	ANAPOE [®] -X-114
	6	ANAPOE [®] -X-305
	7	ANAPOE [®] -X-405
	8	ANAPOE [®] -20
	9	ANAPOE [®] -80
	10	ANAPOE [®] -C ₁₀ E ₆
	11	ANAPOE [®] -C ₁₀ E ₉
	12	ANAPOE [®] -C ₁₂ E ₁₀
	13	ANAPOE [®] -C ₁₃ E ₈
	14	IPTG
	15	n-Dodecyl-N,N-dimethylglycine
	16	HEG [®] -10

	17	C ₈ E ₅
	18	CHAPS
	19	CHAPSO
	20	C-HEGA [®] -11
	21	HEGA [®] -9
	22	C-HEGA [®] -9
	23	HEGA [®] -8
	24	C-HEGA [®] -8
Hampton Research Detergent Screen 3 [™] HR2-412	1	BAM
	2	n-Hexadecyl- β -D-maltoside
	3	n-Tetradecyl- β -D-maltoside
	4	n-Tridecyl- β -D-maltoside
	5	Thesit [®]
	6	ZWITTERGENT [®] 3-14
	7	n-Undecyl- β -D-maltoside
	8	n-Decyl- β -D-thiomaltoside
	9	FOS-Choline [®] -12
	10	n-Decanoylsucrose
	11	n-Nonyl- β -D-thioglucoside
	12	n-Nonyl- β -D-thiomaltoside
	13	DDMAB
	14	n-Nonyl- β -D-maltoside

	15	CYMAL [®] -4
	16	n-Octyl- β -D-thiomaltoside
	17	FOS-Choline [®] -10
	18	FOS-Choline [®] -9
	19	MEGA-9
	20	n-Heptyl- β -D-thioglucopyranoside
	21	FOS-Choline [®] -8
	22	CYMAL [®] -2
	23	ZWITTERGENT [®] 3-08
	24	CYMAL [®] -1

Table S2.1. Detergents from Hampton Research Detergent Screen kits HR2-410, HR-411 and HR-412.

ANAPOE [®] -C ₁₂ E ₉	ANAPOE [®] -20
CYMAL [®] -6	ANAPOE [®] -80
CTAB	ANAPOE [®] -C ₁₀ E ₆
Big CHAP, deoxy	ANAPOE [®] -C ₁₀ E ₉
LDAO	ANAPOE [®] -C ₁₂ E ₁₀
n-Nonyl-β-D-glucoside	IPTG
n-Octyl-β-D-thioglucoside	n-Dodecyl-N,N-dimethylglycine
DDAO	HEG [®] -10
HECAMEG [®]	C ₈ E ₅
n-Heptyl-β-D-thioglucopyranoside	HEGA [®] -8
n-Octyl-β-D-glucoside	BAM
ZWITTERGENT [®] 3-10	n-Hexadecyl-β-D-maltoside
MEGA [®] -8	n-Tetradecyl-β-D-maltoside
n-Hexyl-β-D-glucopyranoside	n-Tridecyl-β-D-maltoside
Pluronic [®] F-68	Thesit [®]
ANAPOE [®] -35	n-Nonyl-β-D-thioglucoside
2,6-Dimethyl-4-heptyl-β-D-maltopyranoside	MEGA-9
ANAPOE [®] -58	n-Heptyl-β-D-thioglucopyranoside
ANAPOE [®] -X-114	FOS-Choline [®] -8
ANAPOE [®] -X-305	CYMAL [®] -2
ANAPOE [®] -X-405	ZWITTERGENT [®] 3-08
	CYMAL [®] -1

Table S2.2. Detergents from Hampton Research Detergent Screen kits with which extraction was unsuccessful.

Name	Structure	Polymer-to-Maleic- Anhydride Ratio [X:Y]	Molecular Weight
SMA 140		1.4:1	5 kDa
SMA 300		3:1	10 kDa
SMA 140-I (Styrene maleimide copolymer)		1.4:1	5 kDa
SMA 200		2:1	6.5 kDa
DIBMA 10		Not available	10 kDa
DIBMA 12		Not available	12 kDa
DIBMA Glycerol		Not available	>13 kDa
DIBMA Glucosamine		Not available	>15 kDa

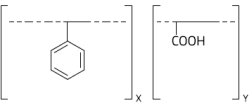
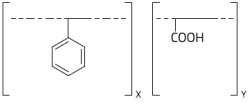
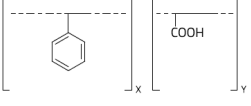
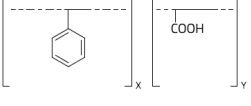
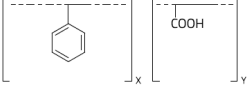
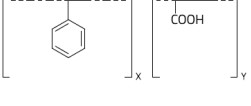
AA:STY ratio			
AASTY 6-45		45:50	5.5 kDa
AASTY 6-50		50:50	5.5 kDa
AASTY 6-55		55:45	5.5 kDa
AASTY 11-45		45:55	10-11 kDa
AASTY 11-50		50:50	10-11 kDa
AASTY 11-55		55:45	10-11 kDa

Table S2.3. List of copolymers obtained from Cube Biotech (Germany) tested as part of the copolymer screen. SMA: Styrene Maleic Acid copolymer. DIBMA: Diisobutylene Maleic Acid copolymer. AASTY: Styrene Acrylic Acid copolymer.

