# Intracellular Co-Localization of the *Escherichia coli* Enterobactin Biosynthetic Enzymes EntA, EntB, and EntE

Paknoosh Pakarian¶ and Peter D. Pawelek¶\*

¶ Department of Chemistry and Biochemistry, Concordia University, 7141 Sherbrooke St., W., Montreal, Quebec, Canada, H4B 1R6

Groupe de Recherche Axé sur la Structure des Protéines (GRASP)

\*Correspondence should be addressed to: Peter D. Pawelek, Tel: 514-848-2424 ext. 3118; Fax: 514-848-2868; E-mail: peter.pawelek@concordia.ca.

## Abstract:

Bacteria utilize small-molecule iron chelators called siderophores to support growth in low-iron environments. The *Escherichia coli* catecholate siderophore enterobactin is synthesized in the cytoplasm upon iron starvation. Seven enzymes are required for enterobactin biosynthesis: EntA-F, H. Given that EntB-EntE and EntA-EntE interactions have been reported, we investigated a possible EntA-EntB-EntE interaction in *E. coli* cells. We subcloned the *E. coli* *entA* and *entB* genes into bacterial adenylate cylase two-hybrid (BACTH) vectors allowing for co-expression of EntA and EntB with N-terminal fusions to the adenylate cyclase fragments T18 or T25. BACTH constructs were functionally validated using the CAS assay and growth studies. Co-transformants expressing T18/T25-EntA and T25/T18-EntB exhibited positive two-hybrid signals indicative of an intracellular EntA-EntB interaction. To gain further insights into the interaction interface, we performed computational docking in which an experimentally validated EntA-EntE model was docked to the EntB crystal structure. The resulting model of the EntA-EntB-EntE ternary complex predicted that the IC domain of EntB forms direct contacts with both EntA and EntE. BACTH constructs that expressed the isolated EntB IC domain fused to T18/T25 were prepared in order to investigate interactions with T25/T18-EntA and T25/T18-EntE. CAS assays and growth studies demonstrated that T25-IC co-expressed with the EntB ArCP domain could complement the *E. coli* *entB*- phenotype. In agreement with the ternary complex model, BACTH assays demonstrated that the EntB IC domain interacts with both EntA and EntE.

**Abbreviations**: ArCP: aryl carrier protein domain; BACTH: bacterial adenylate cyclase two-hybrid; CAS: chrome azurol S; DHB: 2,3-dihydroxybenzoic acid; DTT: dithiothreitol; Fur: ferric uptake regulator; HRP: horseradish peroxidase; IC: isochorismatase domain; IPTG: isopropyl β-D-1-thiogalactopyranoside; NRPS: non-ribosomal peptide synthesis; PVDF: polyvinylidene difluoride; SDS-PAGE: SDS-polyacrylamide gel electrophoresis.

**Introduction**

In order to obtain iron from the extracellular environment, many bacteria synthesize and secrete small-molecule chelators known as siderophores. The Gram-negative bacterium *Escherichia coli* produces the catecholate siderophore enterobactin, which is comprised of three 2,3-dihydroxybenzoic acid (DHB) subunits connected to a triserine trilactone core. Seven enzymes are required to synthesize enterobactin in the *E. coli* cytoplasm: EntC, EntB, EntA, EntE, EntF, EntD, and EntH. The DHB precursors are synthesized *via* the sequential activities of EntC, EntB (isochorismatase (IC) domain) and EntA. Three DHB molecules are then condensed with three molecules of L-serine *via* non-ribosomal peptide synthesis (NRPS), the reactions of which are catalyzed by EntE, EntB (aryl carrier protein (ArCP) domain), EntD, and EntF. The DHB biosynthetic enzyme EntA converts 2,3-dihydro-2,3-dihydroxybenzoic acid to DHB in an NAD+-dependent oxidation reaction. The biological assembly of EntA is tetrameric, with four identical 26 kDa subunits [1]. EntB is a dimeric protein with two identical 33 kDa subunits [2]. Each EntB subunit has an N-terminal IC domain that is involved in DHB biosynthesis, and a C-terminal ArCP domain involved in NRPS. EntE is a monomeric 59 kDa protein with a stable N-terminal domain and more mobile C-terminal domain that can undergo conformational change due to domain alternation [3]. EntE is the activity in the NRPS arm of the pathway that activates DHB *via* adenylation.

Protein-protein interactions are required for siderophore biosynthesis. It has recently been reported that enzymes involved in biosynthesis of the *Pseudomonas aeruginosa* siderophore pyoverdine are organized into a large multi-enzyme complex known as a siderosome [4,5]. Obligate interactions have been reported between the NRPS enzymes in enterobactin biosynthesis: EntE-EntB [3], EntB-EntF [6], EntB-EntD [7], EntD-EntF [8], EntB-EntH [9]. The EntE-EntB interaction has been elucidated at the atomic level by X-ray crystallography using a chimeric protein in which EntE was fused to the ArCP domain of EntB [3]. Since the chimeric protein lacked the N-terminal isochorismatase (IC) domain of EntB, there is currently no information on possible EntB(IC)-EntE contacts in addition to the crystallized EntB(ArCP)-EntE interface. Our laboratory has reported an interaction between EntA, the terminal enzyme in the DHB biosynthetic arm of the pathway, and EntE, the first enzyme in the NRPS arm of the pathway [10,11]. We used the bacterial adenylate cyclase two-hybrid (BACTH) assay to investigate the intracellular context of this interaction, and have determined the relative orientations of EntA and EntE subunits within the complex [11].

Since EntE-EntB and EntA-EntE interactions are known, we were interested in investigating the possibility of an EntA-EntB interaction, or even a ternary EntA-EntB-EntE complex. Using BACTH assays, we demonstrate here that EntA and EntB interact in *E. coli* cells and that the EntB IC domain interacts with both EntA and EntE. Computational docking supports the existence of a ternary EntA-EntB-EntE complex that is consistent with our BACTH outcomes.

**Materials and Methods**

***Reagents***

All chemicals were purchased from Bioshop Canada, Inc. (Burlington, Ontario) unless noted otherwise.

***Preparation of constructs***

The *E. coli* genes encoding EntA and EntB were amplified from pCA24N-*entA* and pCA24N-*entB* templates [11] by PCR using primers containing KpnI and EcoRI restriction sites. A similar approach was used to amplify DNA encoding the EntB IC (EntB residues 1-207) domain. All primers used in this study are summarized in Table 1. Phusion High-Fidelity DNA polymerase (New England Biolabs) was used for amplification of the above-mentioned genes according to the manufacturer's standard protocol. PCR products were subcloned in-frame between KpnI and EcoRI sites of the BACTH vectors pUT18C and pKT25 to produce constructs encoding N-terminally tagged fusion proteins (T18/T25-EntA, T18/T25-EntB, and T18/T25-IC). BACTH vectors were obtained from the BACTH System Kit (Euromedex). In addition to the BACTH constructs, DNA encoding the EntB ArCP domain (residues 213-285) was subcloned into the KpnI and EcoRI sites of the pBR322-based expression vector pFBH1. The pFBH1 vector contains a Fur promoter region upstream of the MCS that allows for iron-controlled protein expression. All constructs were verified by DNA sequencing (Genome Quebec Innovation Centre, McGill University).

***CAS assays and growth studies***

An *E. coli* modified knockout background strain deficient in EntB activity (*entB*-) was prepared as described previously [11]. The competent *entB*- strain was transformed with BACTH constructs encoding T18/T25-EntB. Also, a T25-IC BACTH construct was co-transformed with pFBH1-*ArCP* into the *entB*- strain. Transformants were plated on LB agar containing 100 μg ml-1 ampicillin or 50 μg ml-1 kanamycin, respectively. T25-IC/ArCP co-transformants were plated onto LB agar containing appropriate antibiotics. All plates were incubated overnight at 30 °C. Empty pUT18C, pKT25, and pFBH1 vectors were also transformed into the *entB*- strain as controls. Colonies from transformation plates were used to inoculate LB broth supplemented with appropriate antibiotics. Cultures were incubated with shaking at 30 °C. Overnight cultures were diluted 1:100 in LB broth supplemented with appropriate antibiotics and grown at 30 °C until an A600 between 0.5 – 0.7 was reached. Cultures were then diluted 1:1000 in a 1X M9 modified minimal medium [11] and supplemented with appropriate antibiotics. Cultures diluted in minimal medium were grown at 30 °C overnight. CAS-agar plates were prepared according to Payne *et al.* (1994) [12] and then spotted with 1 μL overnight minimal M9 cultures and incubated at 30 °C for 18 hours. Functionality of constructs was assessed by the formation of orange halos on CAS plates [13]. Plate assays were performed in triplicate to ensure reproducibility.Single colony picks of *E. coli* transformants or co-transformants prepared for CAS assays were also used to perform bacterial growth studies as described previously [11].

***BACTH assays***

Functional BACTH constructs that could rescue the *entB*- phenotype were co-transformed into competent *E. coli* BTH101 cells (*F-, cya-99, araD139, galE15, galK16, rpsL1 (StrR), hsdR2, relA, mcrA1, mcrB1*) (Euromedex). Co-transformants were incubated on LB plates containing 100 μg ml-1 ampicillin and 50 μg ml-1 kanamycin at 30 °C for 48 hours. Colony picks were used to inoculate 3 ml of LB medium plus appropriate antibiotics as well as 0.5 mM IPTG. Inoculated cultures were grown at 30 °C overnight [14]. β-galactosidase assays were performed as described by Miller [15] except that reactions were incubated at room temperature. For BACTH plate assays, 1 μl of each overnight culture was spotted onto MacConkey agar base (Difco) plates containing approproate antibiotics, 0.5 mM IPTG and 1% maltose. Spotted plates were incubated at 30 °C overnight [14]. A positive two-hybrid signal was indicated by the formation of red colonies on plates and elevated β-galactosidase signal. All BACTH assays (plate-based assays and β-galactosidase) were performed in triplicate.

***Computational docking***

Atomic coordinates of the EntA-EntE docked complex [11] and the dimeric EntB X-ray crystallographic structure (PDB code: 2FQ1; [2]) were submitted to the ClusPro server [16] as a blind docking experiment. Default ClusPro settings were used, and no constraints or prior experimental knowledge was introduced to guide the server towards a docking solution. The selected candidate model of the ternary EntA-EntB-EntE complex was determined as the highest populated cluster in which the docked EntB molecule did not overlap with the EntA-EntE interaction interface. Structural alignments were performed using the Australis server (http://eds.bmc.uu.se/eds/australis.php) that employs the LSQMAN alignment program [17].

**Results & Discussion**

***Intracellular interaction of E. coli EntA and EntB***

In order to investigate a possible EntA-EntB interaction, the *E. coli* *entA* and *entB* genes were subcloned into the BACTH vectors pUT18C and pKT25. These constructs expressed EntA and EntB with N-terminal fusions to the *B. pertussis* adenylate cyclase fragments T18 and T25. To ensure that the T18/T25-EntA/EntB fusions were functional, we performed chrome azurol S (CAS) assays and growth studies on transformants. The CAS assay indicates functional siderophore production in bacteria. Secreted siderophores result in the formation of an orange halo due to iron extraction from the dye in the plate media [13]. We had previously used the CAS assay to determine that T18-EntA, T25-EntA, T18-EntE and T25-EntE were functional for enterobactin biosynthesis [11]. Here, the same approach was used to probe for T18/T25-EntB functionality. Competent *E. coli* BW25113 *entB*- cells transformed with pUT18C alone did not yield a CAS halo, indicating impaired enterobactin biosynthesis in this transformant (Fig. 1A, upper left panel, left spot). In contrast, *entB*- cells transformed with pUT18C-*entB* resulted in the formation of an orange halo, indicative of enterobactin biosynthesis due to complementation of the knockout phenotype by T18-EntB (Fig. 1A, upper left panel, right spot). Similar results were found for *entB*- cells transformed with pKT25 and pKT25-*entB*, indicating that T25-EntB could also rescue the knockout phenotype (Fig. 1A, upper right panel). Bacterial growth studies supported our CAS assay results. Significant growth in minimal media was only observed when *entB*- cells were transformed with either pUT18C-*entB* (Fig. 1A, lower left panel) or pKT25-*entB* (Fig. 1A, lower right panel) due to complementation of the knockout phenotype by T18/T25-EntB expression. Taken together, these results established that fusion of T18 or T25 tags to the N-terminus of EntB did not impair enterobactin biosynthesis.

We used the EntB BACTH constructs in a co-transformation experiment to probe for possible interaction with *E. coli* EntA. The BACTH assay is used for the detection of intracellular protein-protein interactions [18]. Intracellular interaction of the partner proteins brings fused T18 and T25 fragments into proximity, thus reconstituting adenylate cyclase activity that could activate reporter genes. When co-transformed into an *E. coli* strain (BTH101) deficient in adenylate cyclase (*cyaA*-), this results in a positive two-hybrid signal (red bacterial colonies on MacConkey agar plates, or elevated β-galactosidase activity in a liquid assay). Co-transformation of pUT18C-*entA* and pKT25-*entB* resulted in a positive two-hybrid signal on MacConkey agar plates (Fig. 1B, upper panel, left spot), whereas no red colony was observed in BTH101 cells co-transformed with pUT18C-*entA* and pKT25 alone (Fig. 1B, upper panel, right spot). In agreement with the plate-based assay, we observed that the pUT18C-*entA*/pKT25-*entB* co-transformant exhibited elevated β-galactosidase activity in comparison with BTH101 cells co-transformed with pUT18C-*entA* and pKT25 empty vector (Fig. 1B, upper bar graph). Similar results were observed when T18/T25 tags were swapped to investigate co-expression of T25-EntA and T18-EntB. The two-hybrid signal was observed on MacConkey agar for the pKT25-*entA*/pUT18C-*entB* co-transformant, but not for cells co-transformed with pKT25-*entA* and pUT18C empty vector (Fig. 1B, lower panel, left and right spots, respectively). We found that co-expression of T25-EntA and T18-EntB in BTH101 cells resulted in elevated β-galactosidase activity relative to cells co-expressing T25-EntA and the unfused T18 fragment (Fig. 1B, lower bar graph). We also performed BACTH control assays on cells co-expressing T18/T25-EntB with T25/T18 tags fused to proteins unrelated to enterobactin biosynthesis. MobA, a protein involved in molybdenum metabolism that we used previously as a BACTH negative control was N-terminally fused to T18 [11]. Furthermore, a vector encoding T25 N-terminally fused to a leucine zipper domain was also used as a control. BTH101 cells co-expressing T18-MobA with T25-EntB resulted in no detectable BATCH signal on MacConkey agar plates (Fig. 1C, left panel). Similarly, no BACTH signal was observed for the T25-ZIP/T18-EntB co-transformant (Fig. 1C, right panel). Liquid assays on these transformants exhibited β-galactosidase activity similar to our other negative controls (120.2 + 6.0 Miller units and 81.4 + 14.1 Miller units for T18-MobA/T25-EntB and T25-ZIP/T18-EntB co-transformants, respectively). Taken together, these outcomes demonstrate that the positive two-hybrid signal was only observed when EntA and EntB were co-expressed in BTH101 cells, indicative of intracellular EntA-EntB interaction.

***Computational docking of E. coli EntA, EntB, and EntE***

Given evidence for pairwise EntA-EntB (this study), EntE-EntB [3] and EntA-EntE [10][11] interactions, we employed computational docking to investigate a possible EntA-EntB-EntE ternary complex. We used the ClusPro docking server [16] given its reported successes at accurately predicting protein complexes [19]. We had previously used experimental approaches to validate a computational model of the EntA-EntE complex [11]. In the EntA-EntE complex model, one EntE monomer was predicted to interact with one face of the EntA tetramer, such that each EntA subunit was required for the entire EntA-EntE interaction interface. Here we used this experimentally validated model along with the crystal structure of dimeric EntB (PDB code: 2FQ1) in blind docking experiment. No constraints were provided to guide ClusPro towards a correct ternary structure model. ClusPro returned 622 possible ensembles of the ternary complex grouped into 30 clusters. As our candidate model, we selected the top-ranked cluster (29 members; weighted score: -856.7) in which the docked EntB protein did not overlap with the EntE-interacting interface of EntA. The top-ranked candidate of EntB docked to the EntA-EntE complex is shown in Figure 2A. Consistent with the reported X-ray crystallographic structure of EntE-EntB (ArCP), most of the EntB-EntE interaction interface was found between the ArCP domain of EntB and a cleft formed between the N- and C-terminal domains of EntE. The EntA-EntB-EntE model indicates direct regions of contact between the EntB IC domain and EntA as well as EntE. In the model, the C-terminal half of EntB IC α2 (residues 52-71) is found within a pocket formed at the interaction interface between the EntE N-terminal domain and the B-chain of the EntA tetramer. Hydrogen bonding was predicted between EntB Q69 and the main chain amide nitrogen and carbonyl oxygen atoms of EntE E291 (Fig. 2B, left panel). Proximal to EntB α2 is a loop region in the IC domain (residues 110-120) predicted by the model to directly interact *via* a hydrophobic interaction between EntB A116 and residue A60 of the EntA B-chain; additional hydrogen bonding was found between the side-chain of EntB D117 and residue Q64 of the EntA B-chain (Fig. 2B, right panel). To validate the ternary complex model, we performed a structural superposition of the EntE Cα atoms in the EntE-EntB(ArCP) crystal structure (PDB code: 3RG2) with the EntE Cα atoms from the EntA-EntB-EntE ternary complex model. Upon superposition of the EntE backbones, the ArCP domain from the crystal structure superimposed well with the ArCP domain from the ternary complex model (Fig. 2C). Given that the initial docking of EntB to the EntA-EntE complex was blind, the observed superposition of the ArCP domains demonstrates that the ternary complex model obtained by ClusPro is consistent with current knowledge of EntA-EntE and EntE-EntB interactions.

***Interaction of EntA and EntE with the EntB IC domain***

Since the model of EntA-EntB-EntE complex predicted direct interaction of the EntB IC domain with both EntA and EntE, we prepared a BACTH construct expressing the EntB IC domain alone in order to experimentally investigate EntB IC domain interactions. DNA encoding the EntB IC domain (residues 1-207) was cloned into pUT18C and pKT25 to produce BACTH constructs expressing T18-IC and T25-IC. CAS assays and growth studies were used to validate the functionality of the discretely expressed EntB IC domain. To test for functionality of the isolated IC domain, it was co-expressed in *entB*- cells with the isolated EntB ArCP domain. We co-transformed the *entB*- strain with pKT25-*IC* and pFBH1-*ArCP* (a protein expression construct under the control of the *E. coli* iron regulator Fur). When grown on CAS agar plates, the co-transformant exhibited an orange halo (Fig. 3A, left spot). In contrast, no CAS halo was observed for *entB*- cells co-transformed with the vector control pKT25 and pFBH1-*ArCP* (Fig. 3A, middle spot), or with pKT25-*IC* and the vector control pFBH1 (Fig. 3A, right spot). Bacterial growth studies were in agreement with our CAS assay outcomes. *E. coli* *entB*- cells co-expressing T25-IC/ArCP grew at wild-type levels in iron-deprived minimal media (Fig 3B, column 1). In contrast, no significant growth was observed when constructs encoding T25-IC or ArCP were co-transformed with respective empty vector controls (Fig. 3B, columns 2 and 3). These outcomes demonstrated that the EntB IC and ArCP domains do not have to be connected in a single polypeptide in order to support intracellular enterobactin biosynthesis. Furthermore, we showed that the IC domain remained functional when N-terminally fused with a BACTH fragment.

BACTH constructs expressing the isolated EntB IC domain were used in co-transformation experiments with EntA and EntE. Figure 4A shows BACTH outcomes of EntA co-expressed with the EntB IC domain. Co-transformation of pUT18C-*entA* with pKT25-*IC* resulted in a red colony on MacConkey agar media, indicative of a positive two-hybrid signal (Fig. 4A, upper panel, left spot). In contrast, co-transformation of pUT18C empty vector with pKT25-*IC* did not result in a two-hybrid signal (Fig. 4A, upper panel, right spot). Co-transformation of pUT18C-*IC* with pKT25-*entA* also yielded a two-hybrid signal (Fig. 4A, lower panel, left spot), whereas co-transformation of pUT18C-IC with pKT25 did not (Fig. 4A, lower panel, right spot). β-galactosidase assays supported the plate-based assays in that elevated β-galactosidase activity was only observed when T18/T25-EntA was co-expressed with T25/T18-IC, in comparison with vector controls (Fig. 4A, upper and lower bar graphs). We then investigated potential interaction of EntE with the EntB IC domain (Fig. 4B). Co-transformation of pKT25-*entE* with pUT18C-*IC* in *E. coli* BTH101 cells resulted in a positive two-hybrid signal (Fig. 4B, upper panel, left spot), whereas transformation of pKT25 empty vector with pUT18C-*IC* did not (Fig. 4B, upper panel, right spot). Similarly, the pUT18C-*entE*/pKT25-*IC* co-transformant yielded a positive two-hybrid signal unlike the pUT18C/pKT25-*IC* control (Fig. 4B, lower panel, left and right spots, respectively). Corresponding β-galactosidase assays of co-transformants indicated elevated activity upon co-expression of T25-EntE with T18-IC (Fig. 4B, upper bar graph) or co-expression of T18-EntE with T25-IC (Fig. 4B, lower bar graph). Taken together, the BACTH outcomes support the computational model of the EntA-EntB-EntE complex since EntB IC was found by BACTH to interact with both EntA and EntE within *E. coli* cells. Since we showed that the EntB IC and ArCP domains are functionally independent, this suggests that they are organized within the EntB protein to structurally anchor an intracellular EntA-EntB-EntE ternary complex. Further experiments are now being performed to directly investigate the ternary complex as well as the role of EntB as a hub protein, since it is known to also interact with EntD, EntF and EntH [6][7][9].

**Acknowledgements**

This work was supported by Discovery Grant 341983 from the Natural Sciences and Engineering Research Council of Canada to PDP. Financial support for P. Pakarian was provided by an FQRNT PBEEE/MELS Scholarship and GRASP. We acknowledge NBRP-E. coli at NIG (Japan) for providing KEIO strains.

**References**

[1] J.A. Sundlov, J.A. Garringer, J.M. Carney, A.S. Reger, E.J. Drake, W.L. Duax, A.M. Gulick, Determination of the crystal structure of EntA, a 2,3-dihydro-2,3-dihydroxybenzoic acid dehydrogenase from *Escherichia coli*, Acta Crystallogr D Biol Crystallogr 62 (2006) 734-740.

[2] E.J. Drake, D.A. Nicolai, A.M. Gulick, Structure of the EntB multidomain nonribosomal peptide synthetase and functional analysis of its interaction with the EntE adenylation domain, Chem Biol 13 (2006) 409-419.

[3] J.A. Sundlov, C. Shi, D.J. Wilson, C.C. Aldrich, A.M. Gulick, Structural and functional investigation of the intermolecular interaction between NRPS adenylation and carrier protein domains, Chem Biol 19 (2012) 188-198.

[4] F. Imperi, P. Visca, Subcellular localization of the pyoverdine biogenesis machinery of *Pseudomonas aeruginosa*: a membrane-associated "siderosome", FEBS Lett 587 (2013) 3387-3391.

[5] V. Gasser, L. Guillon, O. Cunrath, I.J. Schalk, Cellular organization of siderophore biosynthesis in *Pseudomonas aeruginosa*: Evidence for siderosomes, J Inorg Biochem 148 (2015) 27-34.

[6] J.R. Lai, M.A. Fischbach, D.R. Liu, C.T. Walsh, A protein interaction surface in nonribosomal peptide synthesis mapped by combinatorial mutagenesis and selection, Proc Natl Acad Sci U S A 103 (2006) 5314-5319.

[7] J.R. Lai, M.A. Fischbach, D.R. Liu, C.T. Walsh, Localized Protein Interaction Surfaces on the EntB Carrier Protein Revealed by Combinatorial Mutagenesis and Selection, J Am Chem Soc 128 (2006) 11002-11003.

[8] R.H. Lambalot, A.M. Gehring, R.S. Flugel, P. Zuber, M. LaCelle, M.A. Marahiel, R. Reid, C. Khosla, C.T. Walsh, A new enzyme superfamily - the phosphopantetheinyl transferases, Chem Biol 3 (1996) 923-936.

[9] D. Leduc, A. Battesti, E. Bouveret, The hotdog thioesterase EntH (YbdB) plays a role *in vivo* in optimal enterobactin biosynthesis by interacting with the ArCP domain of EntB, J Bacteriol 189 (2007) 7112-7126.

[10] S. Khalil, P.D. Pawelek, Enzymatic adenylation of 2,3-dihydroxybenzoate is enhanced by a protein-protein interaction between *Escherichia coli* 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (EntA) and 2,3-dihydroxybenzoate-AMP ligase (EntE), Biochemistry 50 (2011) 533-545.

[11] P. Pakarian, P.D. Pawelek, Subunit orientation in the *Escherichia coli* enterobactin biosynthetic EntA-EntE complex revealed by a two-hybrid approach, Biochimie (2016).

[12] S.M. Payne, Detection, isolation, and characterization of siderophores, Methods Enzymol 235 (1994) 329-344.

[13] B. Schwyn, J.B. Neilands, Universal chemical assay for the detection and determination of siderophores, Anal Biochem 160 (1987) 47-56.

[14] A. Battesti, E. Bouveret, The bacterial two-hybrid system based on adenylate cyclase reconstitution in *Escherichia coli*, Methods 58 (2012) 325-334.

[15] J.H. Miller, A short course in bacterial genetics : a laboratory manual and handbook for *Escherichia coli* and related bacteria, Cold Spring Harbor Laboratory Press, Plainview, N.Y., 1992.

[16] S.R. Comeau, D.W. Gatchell, S. Vajda, C.J. Camacho, ClusPro: an automated docking and discrimination method for the prediction of protein complexes, Bioinformatics 20 (2004) 45-50.

[17] G.J. Kleywegt, Use of non-crystallographic symmetry in protein structure refinement, Acta Crystallographica Section D: Biological Crystallography 52 (1996) 842-857.

[18] G. Karimova, J. Pidoux, A. Ullmann, D. Ladant, A bacterial two-hybrid system based on a reconstituted signal transduction pathway, Proc Natl Acad Sci USA 95 (1998) 5752-5756.

[19] M.F. Lensink, S.J. Wodak, Docking, scoring, and affinity prediction in CAPRI, Proteins 81 (2013) 2082-2095.

**Table 1: PCR primers used in this study**

|  |  |  |
| --- | --- | --- |
| Vector | Gene | Primer Sequence (5’-3’) |
| pUT18C, pKT25  pFHB1 | *entA* | F: TAGGGGTACCTATGGATTTCAGCGGTAAAAATGTCTGGG |
| R: CTACGGAATTCTTATGCCCCCAGCGTTGAGCC |
| *entB* | F: TAGGGGTACCTATGGCTATTCCAAAATTACAGGCTTACGC |
| R: CTACGGAATTCTTATTTCACCTCGCGGGAGAGTAGC |
| *IC* | F: TAGGGGTACCTATGGCTATTCCAAAATTACAG |
| R: CTACGGAATTCTTACAGTAATTCTTCAGTCATC |
| *ArCP* | F: TAGGGGTACCAGCCAGCAAAGCG |
| R: CTACGGAATTCTTATTTCACCTCGC |
| F = forward, R = reverse. Underlined sequences indicate *Kpn*I (forward) and *Eco*RI (reverse) restriction sites. | | |

**Figure Legends**

**Figure 1. Bacterial two-hybrid assays of EntA with full-length EntB.**

(A) Validation of BACTH constructs. Upper left panel: Photograph of CAS agar plate spotted with *E. coli* *entB*- cells transformed with pUT18C (left) and pUT18C-*entB* (right). Lower left bar graph: Bacterial growth study of *E. coli* *entB*- cells transformed with pUT18C (left) and pUT18C-*entB* (right). Upper right panel: Photograph of CAS agar plate spotted with *E. coli* *entB*- cells transformed with pKT25 (left) and pKT25-*entB* (right). Lower left bar graph: Bacterial growth study of *E. coli* *entB*- cells transformed with pKT25 (left) and pKT25-*entB* (right). Growth studies were performed in iron-depleted minimal medium at 30 oC. Cell densities measured as absorbances at 600 nm (A600). Orange bars indicate transformants that exhibited a positive CAS signal; error bars represent standard deviations from mean values. All CAS assays and growth studies were performed in triplicate.

(B) BACTH assays of T18/T25-EntA co-expressed with T25/T18-EntB. Photographs are of *E. coli* BTH101 co-transformants spotted onto MacConkey agar plates. Bar graphs represent BACTH β-galactosidase assays. *E. coli* BTH101 cells co-transformed with BACTH vectors are indicated as follows. Upper left panel: pUT18C-*entA*/pKT25-*entB*. Upper right panel: pUT18C-*entA*/pKT25. Upper bar graph: β-galactosidase assays of pUT18C-*entA*/pKT25-*entB* co-transformant (upper) and pUT18C-*entA*/pKT25 co-transformant (lower). Lower left panel: pKT25-*entA*/pUT18C-*entB*. Lower right panel: pKT25-*entA*/pUT18C. Lower bar graph: β-galactosidase assays of pKT25-*entA*/pUT18C-*entB* co-transformant (upper) and pKT25-*entA*/pUT18C co-transformant (lower). Error bars represent standard deviations from mean values (n=3).

(C) BACTH assays of T25/T18-EntB co-expressed with T18/T25-fused to control proteins. Photographs are of *E. coli* BTH101 co-transformants spotted onto MacConkey agar plates. Left panel: pUT18C-*mobA*/pKT25-*entB*; Right panel: pKT25-*zip*/pUT18C-*entB*.

**Figure 2. Computational modeling of the *E. coli* EntA-EntB-EntE complex.**

(A) ClusPro model of EntA-EntB-EntE ternary complex showing EntB dimer (coils, colored from N-terminus (blue) to C-terminus (red)) docked to the EntA-EntE model reported previously [11] (protein subunits shown as van der Waals surfaces; light blue: EntE, dark grey: B-chain of EntA tetramer, light grey: A-chain of EntA tetramer; the D-chain of the EntA tetramer is below the B-chain and also colored light grey; the C chain of the EntA tetramer is not visible in this representation).

(B) Left panel: interaction of EntE E291 with EntB Q69; EntE: light blue, EntB: cyan. Right panel: interaction of A60 and Q64 from the B subunit of EntA with EntB residues A116 and D117, respectively; EntA: dark grey, EntB: green. Atoms are shown in stick representations and colored by element. Dotted lines represent predicted contacts; numbers represent distances in Ångstrom units.

(C) Superposition of the EntE-EntB(ArCP) crystal structure (yellow) with the EntE (blue) and EntB (red) chains from the ternary complex model. Only one EntB subunit from the model is shown. Protein chains are shown in the coil representation.

**Figure 3. Functional validation of EntB IC BACTH construct.**

(A) Photograph of CAS agar plates spotted with *E. coli* *entB*- cells co-transformed with pKT25-*IC*/pFBH1-*ArCP*(left spot); pKT25/pFHB1-*ArCP* (middle spot); pKT25-*IC*/pFBH1 (right spot).

(B) Bacterial growth studies of *E. coli* *entB*- co-transformants. Growth studies were performed in iron-depleted minimal medium at 30 oC. Cell densities measured as absorbances at 600 nm (A600). Orange bar indicates transformant that exhibited a positive CAS signal; error bars represent standard deviations from mean values. Left to right: pKT25-*IC*/pFBH1-*ArCP;* pKT25/pFHB1-*ArCP;* pKT25-*IC*/pFBH1.

**Figure 4. Intracellular co-localization of EntB IC domain with EntA and EntE.**

Photographs are of *E. coli* BTH101 co-transformants spotted onto MacConkey agar plates. Bar graphs represent BACTH β-galactosidase assays.

(A) Interaction of EntB IC domain with EntA. Upper left panel: pUT18C-*entA*/pKT25-*IC*. Upper right panel: pUT18C/pKT25-*IC*. Upper bar graph: β-galactosidase assays of pUT18C-*entA*/pKT25-*IC* co-transformant (upper) and pUT18C/pKT25-*IC* co-transformant (lower). Lower left panel: pKT25-*entA*/pUT18C-*IC*. Lower right panel: pKT25-*entA*/pUT18C. Lower bar graph: β-galactosidase assays of pKT25-*entA*/pUT18C-*IC* co-transformant (upper) and pKT25-*entA*/pUT18C co-transformant (lower).

(B) Interaction of EntB IC domain with EntE. Upper left panel: pKT25-*entE*/pUT18C-*IC*. Upper right panel: pKT25/pUT18C-*IC*. Upper right bar graph: β-galactosidase assays of pKT25-*entE*/pUT18C-*IC* co-transformant (upper) and pKT25/pUT18C-*IC* co-transformant (lower). Lower left panel: pUT18C-*entE*/pKT25-*IC*. Lower right panel: pUT18C/pKT25-*IC*. Lower right bar graph: β-galactosidase assays of pUT18C-*entE*/pKT25-*IC* co-transformant (upper) and pUT18C/pKT25-*IC* co-transformant (lower).

**Pakarian & Pawelek, Figure 1.**



**Pakarian & Pawelek, Figure 2.**



**Pakarian & Pawelek, Figure 3.**



**Pakarian & Pawelek, Figure 4.**

