**Functional and structural characterization of YhcB, DUF1043, an unknown protein conserved across proteobacteria**

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**Abstract:**

YhcB, an uncharacterized protein, is composed mostly of a Domain of Unknown Function (DUF 1043). Here, we show that YhcB is a facultative essential, conserved protein across proteobacteria. YhcB interacts with many cell divisome and elongasome proteins including FtsI, FtsQ, RodZ and RodA. The latter protein-protein interactions (PPIs) are conserved in *Yersinia* and *Vibrio*. A full-length protein is required for these YhcB interactions although specific point mutations abolished the interactions of YhcB with FtsI and RodZ. A YhcB deletion strain (Δ*yhcB)* shows an elongation (filamentation) phenotype and viability loss at 4°C and temperatures above X°C. *yhcB* also protects against cell-wall-acting antibiotics A22 and Mecillinam. The *yhcB* deletion results in reduced biofilm formation. Overexpression and deletion of *yhcB* leads to filamentation and abnormal FtsZ ring formation, indicating a role in cell division, septa formation and/or septal PG formation.

**Introduction:**

The sequencing revolution has flooded databases with millions of sequences with many of them annotated as either unknown or hypothetical. Only 1.36% of the ~90 million protein sequences in Uniprot (Aug 30, 2017) are experimentally annotated (0.14%) or having transcript data (1.22%) (**The UniProt Consortium**). Around 24.5% of sequence annotations were inferred by homology. Around 74.11% of sequences were annotated as predicted. Furthermore, in Pfam 31.0 (March 2017), out of 16,712 entries, around 4000 are annotated as Domain of Unknown function (DUF) (**Finn RD, 2013**). Many DUFs have been shown to be essential (“eDUFs”) in at least some species (**Goodacre, 2014)**.

Thus, functions of most of the proteins available in Uniprot (or Pfam), are either computationally predicted or unknown, emphasizing the need for their functional characterization. Hence, functional characterization of unknown proteins, including DUFs, remains a rate-limiting step in molecular biology (**Bastard** K, et al. (2014), **Zhang H**, et al. (2014), **Prakash** A, (2011).

YhcB is an inner membrane protein with a cytoplasmic domain of unknown function (DUF1043). Initially, *E. coli-*YhcB was thought to be a subunit of cytochrome bd which was later found to be dispensable for the assembly of cytochrome bd (**Mogi T, 2006**).

(Need to expand published / databases data)

In the genome, yhcB is located upstream of two periplasmic serine proteases (degQ and degS) and predicted to function as the same transcriptional unit (**Federico M. Lauro,2007**).

Previously, yhcB was captured in some large-scale screening studies. For instance, gene/proteins involved in biofilm formation (), cell envelope integrity (), cold sensitivity (Federico M. Lauro,2007).

In this study, we solved the crystal structure of YhcB followed by identification of interacting proteins. Furthermore, an analysis of mutant phenotypes confirmed that *yhcB* improves viability at low and higher temperature. We also provide evidence for a role of YhcB in cell division. However, the mechanistic details of YhcB function in cell division remain unclear.

**Results:**

**YhcB is conserved in proteobacteria**

Describe phylogenetic distribution

**The structure of YhcB**

xxxxx

**YhcB interacts with proteins of the divisome/elongasome in proteobacteria**

We used a functional interactomics approach to understand the function of YhcB. Bacterial two hybrid (B2H) screening was used to identify the interacting partners of YhcB. About 36 proteins of different functions were screened against YhcB (**Table X**) (**Fig 1**). See materials and methods for details.

Describe which interactions are strongest/most plausible; explain quality scoring.

In order to find out if these interactions are conserved in other proteobacteria, we retested the interactions found among *E. coli* with their homolgos from *Yersinia* *pestis* and *Vibrio cholerae* (**Fig 1**). X and Y (XX% and YY%), respectively, were conserved in these 2 species too (**Table 1**). E-YhcB is only around 40-45% and 80% identical to its homologs in *Vibrio* and *Yersinia,* respectively.

X YhcB interactions are conserved in all three species. For instance, **YhcB** interacted with **HemX** in all 3species (**Table X**). Another interactor was **FtsQ**, which localizes to the division septum and requires other Fts (e.g FtsZ etc) proteins for its localization. Also, the insertion of FtsQ in membrane requires YidC. Both YidC and FtsQ interact with YhcB. This support that these proteins may be functionally associated. Further, YhcB interacts with proteins that are involved in cell divisome/elongasome or cell wall biogenesis. Importantly, YhcB interact with **RodZ, RodA** and **MreB** suggesting an involvement in cell-wall biogenesis.

**Mapping interaction site(s) of YhcB: Conserved residues of YhcB are involved in PPIs:**

YhcB is composed of 2 anti-parallel alpha-helices, so we wondered which helix and which side of it is involved in the interactions we found. Site-directed mutagenesis was used to map and identify the essential residues involved in PPIs of YhcB. We divided the *E. coli*-YhcB protein into 6 different regions based on the conserved residues (**Fig 2**). Only the conserved residues of these regions were mutated (**Fig 2** dotted lines in revised figure). Also, the YhcB protein without transmembrane region (aa 1-21) was constructed to understand the role of long cytoplasmic region (aa 22-132) in PPIs. Each YhcB-mutant had 4-8 amino acid substitutions. Most of the residues were replaced by alanine or glycine. A total of 37 mutations were created and tested. Each YhcB mutant was tested against positive interacting partners of YhcB identified in the previous B2H screens (**Section X**). The amino acids substitutions of YhcB mutants (variants) v1, v4 and v5 were identified as potential PPI sites of YhcB (**Fig. X**). Amino acids X-Y of YhcB-v5 seems to form an interaction site for muiltiple interacting proteins, especially FtsI, RodZ, and YidC (**Fig 2**). The YhcB-v1 includes the conserved residues in TM region only. The rationale behind mutating TM residues was to test if it has any effect on interaction with proteins or whether it was only required for its interactions with membrane. Interestingly, the TM region is required for its interactions with all proteins: when it is deleted, all interactions are lost (v7 in **Fig. 2**). Notably, the mutations in yhcB-v3 result in stronger interactions although the reason remains unclear (but see discussion). We speculate that these a. acids are required for maintaining the structural integrity or increase the affinity (or stickiness) of YhcB vs other proteins. However, we don’t have a better explanation for this observation and needs further investigations.

**Protein-protein docking for YhcB:**

Further protein-protein docking was attempted to get additional support for the interacting residues/site of YhcB **(Fig 2B**). No structure is known for *E. coli* YhcB, therefore, the structure used to dock YhcB was a structure of a homolog of the protein found in *Haemophilus ducreyi*,which is referred to as HDR25. HDR25 shares 21% sequence identity with *E. coli* YhcB, with 42% sequence similarity. HDR25 is a tetramer, however, YhcB in *E. coli* was not found to self-associate in bacterial two-hybrid screens. Therefore, a monomeric structure for YhcB was generated by taking a single chain (A) from the YhcB tetrameric structure in *H. ducreyi*. For all dockings, YhcB tetramer as well as monomer were used and the results were combined to best inform inferences about the actual conformation of binding between YhcB and partners.

As indicated earlier, YhcB was found to interact with the cell divisome and elongasome proteins FtsI, FtsQ, RodZ, RodA and MreB in *E. coli*, and FtsZ in *Yersinia pestis*, additionally. Of these interaction partners, structures are available for FtsI, FtsQ, and MreB. The structures used for FtsI and FtsQ were 4BJP and 2VH1, respectively. 4BJP is a single-chain structure, however, FtsQ is a dimer. A monomeric structure for FtsQ was generated by taking a single chain from 2VH1. HDR25 monomer and HDR25 tetramer were docked to FtsI monomer, FtsQ monomer, and FtsQ dimer. For FtsZ, homologous structures were available with high sequence identity (e.g. 1OFU from *Pseudomonas aeruginosa*, 68% sequence identity), however, these structures were only partial and may lack regions critical for interaction. For MreB, 4CZL was used, which is a structure of *Caulobacter crescentus* MreB (61% sequence identity over 97% of the sequence length).

**Table X Docking experiments performed**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | FtsI (4BJP:A) | FtsQ monomer (2VH1:A) | FtsQ dimer (2VH1:A-B) | RodZ | RodA | MreB (4CZL:A) | FtsZ (1OFU:A) |
| YhcB monomer (HDR25:A) | + | + | + | - | - | + | - |
| YhcB tetramer (HDR25:A-D) | + | + | + | - | - | + | - |

**HDR25 vs *E. coli* YhcB**

As indicated above, HDR25 is 21% sequence identical to *E. coli* YhcB, and 42% sequence-similar. Although conservation information for YhcB across multiple species is already provided (ConsurfDB profile), it is important to note that nearly all of the known and suspect important residues in YhcB *E. coli* are conserved (identical or similar) in HDR25. This includes the His-Phe patch (61, 62, 65, 82, 83) and adjacent residues (86, 87) except for 90, as well as hypothetical interactor residues (69, 76, 80) though not 68 and 75, and suspected transmembrane residues (37, 44).

**YhcB variants**

The six variants cover different stretches of YhcB sequence, in ascending order from V1 (most N-terminal) to V6 (most C-temrinal), with V7 the exception (N-terminal 1-21 deletion). Only V3 has a mutated residue (L62A) in the His-Phe patch or neighboring residues, while V4 has a mutated residue in the suspected interactor residues (D69A), and V5 has two mutated residues among the suspected interactor residues (H76A and S80A). . Because of the apparently greater disruption to YhcB binding in general, and because docking was possible with FtsI but none of the other partners affected by V1 and V4, V5 and the YhcB+FtsI interaction was the focus of docking experiments. Also, V5 contains mutations in residues 76 and 80, which were found to be invariant in all sequences used for the ConsurfDB calculation.

**YhcB protein dynamics is dependent on the growth rate and stress:**

To understand the protein dynamics of YhcB, the protein copies per cell of YhcB and its interactors were extracted from the literature/database (references). We observed increased abundance (copies/cell) of YhcB and its interactors (e.g MreB, FtsZ and RodZ) at higher growth rate (**Fig 3**). These proteins are required for maintaining the rod-shape cell symmetry during cell division. This hints to their functional association. Also, a direct interaction between YhcB and MreB was captured in all three species. However, while we did not see a direct interaction between YhcB and FtsZ in *E. coli,* *Yersinia* YhcB interacted with FtsZ of all three species. For example, higher copies/cell of YhcB were found in rich media compared to selective M9 media. The YhcB abundance was consistent not only in BW25113 (strain used for *E. coli* Keio KOs), but also in other *E. coli* strains (**Fig 3**). Also, even in stationary phase cells YhcB forms a significant part of the proteome (2151 and 2667 copies/cell of YhcB after 1 and 3 days, respectively) compared to 6150 copies in exponentially growing cells (in LB). YhcB abundance in the presence of osmotic, temperature, and pH stress was slightly lower compared to M9-glucose. It may be linked to the response of cell under different conditions or nutrients. In another (genome-wide) independent study (Refe), in response to ethanol stress, the YhcB copy no/cell was increased (Fig) and may indicates some role in stress or may be in protein quality control. Because ethanol is a protein unfolding agent (**Srivastava SK, 2014**).

**Physiological and morphological consequences of *yhcB* gene deletion: Fitness cost of *yhcB* gene**

In previous studies a yhcB deletion showed several phenotypes including reduced biofilm formation (**REF**) and a synthetically lethal phenotype in combination with a rodZ deletion (**Niba, 200**7).

In order to understand the function of the *yhcB* gene better, we determined growth profiles of a Δ*yhcB* deletion strain. We observed that the Δ*yhcB* strain was growing slowly compared to WT and never reached a max OD562 comparable to the WT strain (**Fig 4A**). We also used alternative carbon sources to understand why the Δ*yhcB* strain was growing slowly. In the presence of glucose (in LB), the Δ*yhcB* strain was growing and reaching an OD562 comparable to WT strain in LB only (no glucose). However, morphologically Δ*yhcB* cells were X% broader/wider in LB-glucose than in LB only. This indicates that Δ*yhcB* cells did increased in cell size/volume at higher growth rate. They increase in cell width (**Fig 8B middle**). The supplementary glucose increased the growth rate/mass for both WT and Δ*yhcB* strain. The increased growth rate of Δ*yhcB* strain lead to cell-shape alterations. However, no alterations were observed for WT cells in LB-glucose. The observed growth rate for Δ*yhcB* strain is lowest to highest in M9, LB and LB-glucose, respectively (**Fig 4A**). However, no increase in growth of Δ*yhcB* strain in presence of other carbon sources (e.g lactose) was observed. However, an increase in OD562 was observed in the presence of maltose (2 glucose units/disaccharide) and galactose only after 4 hrs. The presence of glycerol, succinate, sorbitol increased the lag phase (**Supplementary figures**).

The Δ*yhcB* cell-shape alterations observed in LB/glucose were similar to those observed upon treatment with cell-wall acting antibiotics (A22 and Mecillinam, **Fig. xX**). We speculate that may be in presence of LB/glu, the Δ*yhcB* strain wasn’t able to coordinate the rate of cell wall synthesis with cell division. Also, presence of 2-deoxy-glucose (10mM 2-DG) with glucose did not increase cell width. Thus, the changes observed for Δ*yhcB* cells in LB-glucose were glucose-specific. Unexpectedly, Δ*yhcB* did not grow on MacConkey/Kanamycin media plates (**Fig Supplementary**) despite the presence of a kanamycin selection (cassette) marker in the *E. coli* KEIO knockout strains. By contrast, the Δ*yhcB* cells grew on MacConkey media plates in the absence of kanamycin (only MacConkey).

**The *yhcB* gene deletion results in reduced biofilm formation.**

Reduced biofilm formation in Δ*yhcB* strains has been reported previously. We confirmed and quantified this phenotype using a crystal violet staining assay (**O'Toole GA, 2011**). WT and Δ*yhcB* cells were grown for 48 hrs in different conditions and biofilm formation was found to be reduced ~5-fold (see Material & Methods, **Suppl. Fig. X**). We observed that Δ*yhcB* and one of its interactors (Δy*ciB*) caused defective biofilm formation. Also, the presence of glucose in LB suppressed the biofilm formation in WT to a comparable degree as in Δ*yhcB* cells. The inhibition of biofilm formation by glucose catabolite repression is well-known (**Debra W. Jackson, 2002**). However, we don’t know if this is the case in Δ*yhcB* cells.

**The Δ*yhcB* strain results in severe hypersensitivity against cell-wall acting antibiotics**

We tested the sensitivity of the of Δ*yhcB* strain in different conditions and against different antibiotics. Specifically, we tested the cell-wall acting antibiotics (A22, and Mecillinam) because cell-shape and cell division-related proteins (e.g RodZ, RodA, MreB etc) were identified as interactors of YhcB (**Fig/Table**). Proteins of the cell divisome/elongasome such as MreB and PBP2 are direct targets of cell-wall antibiotics. For example, MreB and PBP2 are the target of A22 and Mecillinam, respectively. S-(3,4-Dichlorobenzyl) isothiourea (A22) disrupts the bacterial cytoskeleton by inhibiting ATP binding to MreB (**Bean GJ et al., 2009**). Mecillinam is a β-lactam which specifically inhibits PBP2 (**Ref Cho H, cell 2014**). We found that Δ*yhcB* strain was hypersensitive to both A22 and Mecillinam (**Fig 5**). The IC50 of A22 and Mecillinam was about …… for Δ*yhcB*. A22 and Mecillinam inhibited growth of Δ*yhcB* to ~ X% compared to WT (**supplementary figure X)**.

Also, differences were observed between the overnight grown cells and 2 days old stationary cells. The two days old stationary phase Δ*yhcB* cells didn’t revive in presence of both A22 and Mecillinam after 7 hours (**Fig supplementary**). However, the cells without antibiotics treatment showed similar/normal growth pattern as overnight Δ*yhcB* cells. This indicates that *yhcB* may be required for survival and revival of cells in different stress conditions.

**Central-carbon metabolites suppress the hypersensitivity of the Δ*yhcB* strain against antibiotics**

We observed that the hypersensitivity of Δ*yhcB* strain against cell-wall acting antibiotics (A22 and Mecillinam) was suppressed by glucose both in LB broth and on hard agar (**Fig 5**). Also, Δ*yhcB* cells in TSB media were tolerant to A22 and Mecillinam (**Fig. X**). TSB media is a rich modified version of LB media with glucose and salts (Refer/recipe). We also used 2-deoxyglucose (2DG), a competitive inhibitor of glucose to confirm that the observed phenotypes were glucose-specific. In presence of 2-DG, Glucose did not suppress the hypersensitivity phenotype of Δ*yhcB* strain. This confirmed that the resistance/tolerance in Δ*yhcB* cells conferred against cell-wall acting antibiotics was due to glucose. However, at higher concentrations of Mecillinam, glucose provides tolerance to WT cells only and did not show protection for Δ*yhcB* cells. This might be because of non-specific alterations of the bacterial targets at higher (Mecillinam ?) concentrations. Galactose conferred resistance against A22 only whereas Lactose partially suppress the A22 phenotypes (**Fig 5**). Only glucose conferred resistance to Mecillinam.

Glucose was the only substrate tested in a time dependent manner. We observed protective effect of glucose for WT cells just after 6 h. However, for Δ*yhcB* the tolerance against cell-wall antibiotics was observed after 23 hours. This indicates that may be downstream targets/processes are responsible for the observed phenotypes (**Sylvain Meylan, 2017**).

It has been reported that TCA/carbon sources can affect the sensitivity of antibiotics. TCA cycle intermediates such as succinate conferred protection only to the WT cells but not to the Δ*yhcB* cells at higher concentrations of Mecillinam. We also noticed that fumarate, another TCA substrateprovided tolerance against Mecillinam only. No tolerance in Δ*yhcB* cells was observed against A22 in presence of fumarate. The other TCA substrate i.e (**citrate)** showed no tolerance against Mecillinam but potentiates the efficacy of A22 against both Δ*yhcB* and WT strain. No viable cells were found for Δ*yhcB* strain in LB/citrate. The data also indicates the potential role of citrate as antibiotic adjuvant (**Sylvain Meylan, 2017**). Thus, *YhcB* may be required for tolerance against both bacteriostatic and bactericidal antibiotics directly or indirectly.

**YhcB may be involved in persister cells formation against cell-wall acting antibiotics**

Next, we determined persister cell formation in the Δ*yhcB* deletion strain. Persister formation was determined as colony forming units (CFUs) upon antibiotic treatment. The CFUs were expressed as % survival of treated vs untreated cells (**Fig 6**). We used the cells in the early log and stationary phase. We observed the lower number of persisters in Δ*yhcB* strain compared to WT strain upon A22 treatment. No viable (persister) cell was observed after exposure of exponentially growing cells to Mecillinam alone or in combination with A22 in LB media (**Fig 6**). A higher number of persister cells were observed for stationary phase cells and for the cells grown in the presence of exogenous glucose compared to cells in early exponential phase and grown in LB, respectively. Also, the abundance of YhcB (copies/cell) increased after entry of cells into the persistent stage at different time points (Ref, **Figure 6**). The changes observed for YhcB and its interactors were more prominent during persistence vis-a-vis starvation. Specifically, a significant increase in copies/cell for YhcB and RodZ was observed during persistence only (Ref). However, a decrease in copies/cell for FtsZ was observed in both conditions (Ref). Although, the changes were more robust during persistence.

***yhcB* is essential for survival at higher temperature and viability at 4°C**

To understand the effect of temperature on Δ*yhcB* strain, the growth of the Δ*yhcB* cells was analyzed (**Fig7B**) at different temperatures (15°C, 25°C, 37°C and 45°C). The cells were analyzed in LB media and in presence of additional supplements (e.g glucose and galactose). The Δ*yhcB* cells seems to grow slowly compared to WT cells at RT (25°C). At higher temperature, the Δ*yhcB* cells didn’t grow. The exogenous glucose and galactose were toxic to both WT and Δ*yhcB* cells at higher temperature (45°C). However, WT cells were growing (colony size in serial dilution) after 48 hrs but no growth was observed for Δ*yhcB* even after 48 hrs. Also, the Δ*yhcB* cells were grown in LB broth at 15°C and 45°C (**Fig** 7A). An increased rate of filamented and chained cells were observed in Δ*yhcB* cells at both low and high temperature after 4 hrs (data not shown or can have in supplementary). Also, we noticed that Δ*yhcB* cells stored at 4°C (in a refrigerator and in cold room) lost the viability after about 4-6 weeks (**Fig7A-ii**). However, WT cells were perfectly viable (Fig 7A-ii). It is possible that the cells die because of the cold stress and didn’t recover upon re-inoculation. Notably, a similar phenotype of been described with *yhcB* being a cold shock gene (**Jonathan M Stokes et al., 2014 elife**).

***yhcB* gene deletion leads to abnormal FtsZ ring and septum formation**

The *E. coli* Δ*yhcB* strain exhibited a significant population of filamented cells (**Fig** 8A). The average cell length of WT cells was 2.2 µm (n=175) but 7.2 µm for Δ*yhcB* cells (n=350). Occasionally, very long filamented cells were also observed (up to 68 µm) for Δ*yhcB* strain including asymmetric and chained cells (**Fig 8B**). Further, we stained the cells using FM4-64 to detect septa formation. No septa formation was observed in most of the filamented cells. Also, the overexpression of yhcB (synthesized and cloned *E. coli* yhcB) in *E. coli* Bl21 resulted in similar phenotypes to that of Δ*yhcB* cells (**Fig 8B bottom**). Induction of YhcB protein expression leads to highly filamented cells with and without formation of septa. Also, chained cells were observed. Similar phenotypes were also seen in Δ*yhcB* cells. These observations indicate that *yhcB* directly or indirectly regulates the bacterial cell divisome/elongasome. Uninduced cells were like WT. Further, to confirm that YhcB could be involved in divisome assembly, FtsZ-ring formation was monitored in Δ*yhcB* strain. We found that the FtsZ-ring looked abnormal in the Δ*yhcB* strain with mis-localization of FtsZ (**Fig 9A-I**, **Table X)**. Interestingly, YhcB localizes in an MreB/RodZ like pattern (**Fig 9A-ii**).

**Peptidoglycan (PG)-labelling showed punctate / incomplete septa and absence of septal PG formation in ΔyhcB filaments / morphotypes.**

YhcB interacts with proteins involved in cell divisome and elongasome complexes which mediate the coordinated steps in cell division or elongation including peptidoglycan (PG) synthesis. The PG-labeling in Δ*yhcB* strain was probed using a non-toxic, fluorescent D-amino acid analog of D-alanine (NADA) that incorporates into the stem peptide of old and newly synthesized PG in living bacteria. W (Kuru et al. PMCID: PMC3589519). In addition, we used another modified, D-amino acid dipeptide, EDA-DA, that incorporates specifically into the stem peptide of newly synthesized PG in the bacterial cytoplasm (Kuru et al.). Thus, EDA-DA labels only newly synthesized PG in the cytoplasm (**Fig 9B bottom)** as opposed to NADA, which probe/label both old and new PG in the periplasm (**Fig 9B top**).  
Interestingly, we observed far fewer labeled septa in the elongated forms of Δ*yhcB*. However, we also noticed a population of WT-like cells (in terms of length and presence of labeled division septa) under both growth conditions. For the filamented forms, we observed what appeared to be septal labeling using the “old PG” probe set and did not see septal labeling with the “new PG” probe set (**Fig 9B).** This indicates that, at least as far as septal PG goes, new synthesis is inhibited, but that PBP proteins still remain at the incomplete septum, allowing the old PG to have the old probe incorporated. We conclude that YhcB … XXX.

**Discussion**

Here, we present a structural and functional characterization of YhcB, a member of the DUF1043 family of proteins which are conserved across proteobacteria. We found that YhcB is a conserved, facultatively essential protein. For instance, *yhcB* is essential for survival at high and low temperature which is also supported by previous observations (Masayuki Murata 2011 plos one, cold response gene reference). Further, YhcB also found as one of CeHSP17-bound proteins in *E. coli*, a heat shock protein required for growth at high temperature (Ezemaduka AN, 2014. Our results were in agreement with previous observations where Δ*yhcB* strain has been identified as a long LTG (lag time of growth) mutant (**Rikiya Takeuchi, 2014, BMC microbiology**) and was deficient in biofilm formation along with Δ*yciB*, an interactor protein of YhcB (**Emma Tabe Eko NIBA, 2007**). Also, *yhcB* gene has been predicted/identified as one of gene required for maintaining cell envelope integrity in a genome-wide screening in an independent study (**Ref**). We also tested this observation using a CPRG assay that tests the integrity of the cell envelope in Δ*yhcB* cells. A minor defect was observed in Δ*yhcB* cells indicating possible/partially defective cell envelope (**Fig. X).**

We determined that conserved residues of YhcB are required for PPIs with proteins of the cell divisome and elangasome complexes.

However, the lower number of PPIs may be because of the protein toxicity in a distantly-related host. For example, *Vibrio* prey proteins may cause severe toxicity compared to *Yersinia* proteins because phylogenetically *Yersinia* is closer to *E. coli*.

We also attempted Y2H screening for the selected proteins from the above prey proteins but not a single interaction was detected for YhcB. This may be because of the many factors, discussed in our previous manuscript (Mehla et al., 2017). One possibility could be that YhcB may be a strictly membrane localizing protein and didn’t expressed/localize in the nucleus which is a prerequisite for Y2H screening.

These results were further supported by hypersensitivity of the Δ*yhcB* strain to antibiotics that target the cell-wall, especially via MreB and Pbp2. MreB was found as direct interactor of YhcB. Furthermore, the formation of persisters in absence of *yhcB* was greatly affected with no or minimal viable cells in presence of both bacteriostatic and bactericidal cell-wall acting antibiotics. For example, no viable cells were found upon treatment with bactericidal antibiotic (e.g Mecillinam) alone or in combination with A22. In another study, YhcB was predicted to be required for colonization of a host by *Vibrio* (John F. Brooks, 2014).

We found several proteins of the PG biosynthesis (MurG, MurF) and scaffolding apparatus (RodA, RodZ, MreB, FtsZ, etc) as interactors of YhcB. The linkage of YhcB with proteins of cell divisome/elongasome suggests a role in cell division. For instance, YhcB interacts with proteins of the rod complex (e.g RodA, RodZ, MreB) which are required for maintaining the rod shape cell symmetry of bacterial cells. The YhcB-RodZ interaction has been reported previously and *yhcB* and *rodZ* were predicted as synthetic lethal (**Li G 2012**). Also, cell shape alterations/defects in Δ*yhcB* cells at higher growth rate were observed.

Also, YhcB was identified as a contaminant protein in bacillus RodA protein purified from E. coli and analyzed by mass spectrometry (**Alexander J. Meeske,2016** Nature).

Δ*yhcB* cells exhibits three different morphotypes: normal length, mid-length filamentous, and elongated filamentous. This further indicates that more likely YhcB may function as a scaffolding complex or act as part of the organizing center. In times of stress, defects in division complex formation are generally exacerbated, particularly if the defects affect PG biosynthesis. Generally, these defects can be overcome by over-expression of other cell division proteins and possibly explain the subset of wild-type cells observed in Δ*yhcB* mutants. While we did not measure the transcript levels of interactor proteins in absence of *yhcB* gene, we observed that the protein expression of interactors such as MreB, FtsZ seems highly coordinated (REF). The over-expression of any of these genes (particularly ftsZ) could potentially overcome elongated cell phenotype, and this might explain the observed phenotypes such as non-filamentous cells mixed in with elongated/filamented forms.

We also speculate that YhcB-YciB interaction may be involved in septa formation. YciB is a predicted intracellular septation protein A (**REFs: Li et al., Baddaluddin et al.**). We also observed that filamented morphotypes of Δ*yhcB* cells were devoid of septa. The PG-labelling studies confirmed the defects in septa formation in Δ*yhcB* and supports its role in cell division. Labelling of new PG was undetectable in Δ*yhcB* cells.

In summary, YhcB is a novel conserved, facultative essential protein for cell survival, viability and cell division in different conditions/stressors. We also predict that YhcB may be required for host colonization given its potential role in PG synthesis, but this needs requires further studies.

The cold sensitivity phenotype was not complemented by plasmid based yhcB expression (for discussion section).

**Materials and Methods:**

**Bacterial strains and reagents:**

All strains were grown in LB (REF) at 37°C unless otherwise mentioned. The *Knock outs (KOs)* were obtained from the *E. coli* Keio collection (REF). PCR was used to confirm the *E. coli* keio KOs using gene specific primers. *E. coli* TOP10 and DH5α were used for cloning. For protein expression, *E. coli* BL21(pLys) cells were used. *E. coli* was selected at 100 μg/ml Ampicillin, 35 μg/ml chloramphenicol for expression in liquid media. All the expression experiments were done at 30°C unless otherwise mentioned. For B2H and Y2H vector details, see Mehla *et al* 2015, 2017. The antibiotics (A22 and Mecillinam) were purchased from ….. (suppliers). The carbon sources and other compounds/reagents details………

**Gateway cloning**

The Gateway cloning was performed according to instructions provided by the manufacturer (Invitrogen). The ORFs as entry clones for test proteins (e.g **Table X**) were obtained from the *E. coli* ORFeome clones assembled into the pDONR221 vector system (**Ref**). Then, the attL-flanked ORFs were cloned into the Gateway-compatible, attR-flanked bacterial two*-*hybrid (BACTH)-DEST plasmids (pST25-DEST, pUT18C-DEST and pUTM18-DEST, REF) using the LR reaction to generate an attB-flanked ORFs in expression vectors. The plasmid preparations were done using Nucleospin column kits (Macherey Nagel). For the details of the B2H vectors and protocol, please refer to Mehla *et al*., 2017.

**Bacterial Two Hybrid screening:**

For bacterial two hybrid screens, please refer to Mehla *et al*., 2017. Briefly, the expression constructs/(ORFs) of test proteins encoding the T25-X (or X-T25) and T18-Y (or Y-T18) fusions are co-transformed into an adenylate cyclase (cya) deficient *E. coli* strain (BTH101). The competent cells were prepared using standard protocol (Refe). The co-transformants were selected on LB plates containing 100 ug/ml ampicillin and 100 ug/ml spectinomycin at 30°C after 48 h. The selected co-transformants were screened on indicator plates at 30°C for 36-48 hours. The positive interactions were detected by specific phenotypes on indicator plates, i.e., blue colonies on LB-X-Gal-IPTG or red on MacConkey-Maltose medium. For quantification of PPIs (where required), the β-galactosidase assay was used (**Ref**).

**Yeast two hybrid screening** was done as described previously (Mehla et al., JB).

**Mapping Protein-protein interaction site (s): Mutagenesis of yhcB**

To map interaction site(s), mutants of YhcB were constructed. YhcB sequence was divided into 7 different regions and in each region 3-4 site specific mutations were inserted (**Table/figure**). Only the conserved residues of YhcB were mutated (figure). The mutant DNA sequences encoding specific mutants, were synthesized as full gene sequences by Geneart (ThermoFisher pvt Ltd). These sequences were further cloned into pDNOR/Zeo using BP clonase reaction of Gateway cloning (Invitrogen). The transformants with correct sequences were confirmed by sequencing at least 2 different clones. The ORFs were further sub-cloned into bacterial two hybrid vector (pUT18C) followed by co-transformation and screening for interactions against prey proteins (**Section B2H**).

**Protein-protein docking:**

**Overexpression of YhcB:**

The ORFs encoding YhcB and its mutants were cloned into expression vector (pDEST14). For expression in *E. coli* BL21(pLys), the cells were transformed with expression constructs. The protein expression was induced using IPTG (0.5 mM) at 30°C and the cells were analyzed under microscope as described **in section….**

**Growth Inhibition/sensitivity against drugs**

The growth of cultures of Δ*yhcB* strain and WT was monitored in different media and in different conditions. For example, in presence of exogenous carbon sources, antibiotics as well as both in rich and selective media. Cultures of *E. coli*, were grown in 96-well microplates at 37 °C. The bacterial growth was measured as the optical density (OD) at 562 nm using a plate reader (**Refer**). The % inhibition (or survival) was calculated as described somewhere else (Mehla *et al.,* 2011).

**Antibiotic susceptibility testing (Serial dilution assay)**

An overnight culture of the *E. coli* strains (both WT and yhcB KO) was used for testing susceptibility towards cell wall antibiotics and in other conditions using serial dilutions. The cells were serially diluted, and 5 μl of each dilution was spotted on LB with or without added antibiotic or other compounds (e.g carbon sources). For MacConkey plates, 3 μl of each dilution was used. The plates were then imaged after 24 hours or at specific time mentioned in the figure.

**Persister cells/Persistence assay (PNAS reference):**

Persistence was determined by determining the number of colony-forming units (CFUs) upon exposure to A22 and Mecillinam (Conc). The overnight culture was sub-cultured at 37C for 2 hrs and the cells in early log phase were treated with antibiotics. The overnight cells were used as stationary phase cells. For determination of CFUs, 2 ul of culture was resuspended in fresh medium, serially diluted, and plated on solid LB medium. Persisters were calculated as the % survival of cells in treated vs untreated samples as per following formula. This was calculated by dividing the number of CFUs in the culture after 6 h of incubation with the antibiotic by the number of CFUs in the culture before adding the antibiotic.

**Peptidoglycan (PG)- labelling using probes/** **Probing of Peptidoglycan (PG) localization:**

The PG labelling studies were done as previously reported. Briefly, experimental cultures were begun from overnight cultures and we used double the amount of YhcB mutant for the starter culture (50ul vs 100ul in 5 mLs) in order to get the ODs as close as possible after 2 and a half hours of growth (.8 and .7, respectively). The reason being that the time required to back-dilute to achieve exactly equal OD readings will have an effect on the rate of PG synthesis / turnover.  
We took logarithmic growing cultures (WT in LB and YhcB in LB + 1% glucose) and conducted a short pulse with our 1st gen probes (NADA) for 45 seconds.  After the short pulse, bacteria cultures were fixed immediately in 70% (final concentration) ice-cold ethanol for 20 minutes. They were washed three times in PBS and mounted on 1% agar pads for imaging.

**FtsZ ring assay:**

**CFP-YhcB localization:**

**Light microscopy and image analysis**

The cells were stained and imaged to visualize cell membrane and nucleoid using FM4-64 (Synapto Red C2, Biotium Inc.) and DAPI respectively. The cells were imaged on an Olympus BX41 microscope at 100x in a dark room. Images were captured with a microscope digital camera AmScope MU1400. The ImageJ software was used for measuring cells dimensions/length (National Institute of Health).

**Phylogenetic analysis (itol and coevolution):**

**References:**

**Tables:**

**Table X:** Table 1: FtsZ localization in WT or Δ*yhcB* cells. WT pattern contains cells that showed a central Z-ring or helix. Diffuse indicates that cells did not show any discernable pattern of FtsZ localization. Abnormal indicates cells with bright foci, multiple Z-rings, or off center Z-rings. Error is 90% CI.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Strain | Total Cells | WT pattern | Diffuse | Abnormal |
| RM586 (WT) | 675 | 88% ± 4.6 | 10% ± 0.41 | 2% ± 0.10 |
| RM588 (Δ*yhcB*) | 793 | 72.4 % ± 3.4 | 15.4% ± 0.60 | 12.2% ± 0.50 |

**Supplementary tables:**

Table S1: List of strains/plasmids/primers used in the study.

**Figure legends:**

**Figure legends:**

**Figure** 1 **PPIs**: A B2H screens of protobacterial proteins with YhcB of E. coli, Yersinia and Vibrio. The positive control (MalF and MalG) is shown as….. (B) A network of YhcB protein-protein interactions with a subsection of network of proteins involved in cell division. See methods for technical details.

**Figure 2** A, B: **Interaction site mapping and docking**

**Figure 3: YhcB and interactors protein dynamics**. The abundance (copies/cell) for YhcB and interactors is shown at different growth rate. Also, the YhcB dynamics in different E. coli strain is shown. Further, the YhcB dynamics under stress is shown.

**Figure 4: YhcB growth kinetics and serial dilutions**. Δ*yhcB* is required for the optimal growth of the E.coli however, supplementary glucose lead to increased growth rate comparable to that of WT in LB only. The growth rate of Δ*yhcB* shown in presence of different carbon sources. Further, Δ*yhcB* in LB and LB-glucose on hard agar plates.

**Figure 5** A,B,C,D: YhcB KO hypersensitivity against cell wall antibiotics in presence and absence of carbon sources (TCA cycle intermediates).

**Figure 6:** Persister cell assay. Persistence expressed as CFU and % survival. **Figure 6B:** Comparison of exponential and stationary phase cells.

**Figure 7:** Effect of temperature on Δ*yhcB* cells (both broth and hard agar) and viability loss at 4C.

**Figure 8:** Rate of filamentation and the increased cell width of Δ*yhcB* cells in presence of glucose. Micrographs are shown for Δ*yhcB* cells in LB, LB-glucose. Also overexpression in Bl21 is shown.

**Figure 9:** i) Δ*yhcB* cells display an increase in abnormal FtsZ localization. A) Representative image of WT cells expressing FtsZ-GFPsw. B) Representative image of Δ*yhcB* cells expressing FtsZ-GFPsw. Different classes of FtsZ localization are indacted as follows: arrow head- Z-ring, double arrowhead- helix, star-diffuse, bar= bright foci.

ii) YhcB localizes in an MreB/RodZ like pattern. Left phase image, Right YhcB-CFP.

**Figure 9B.** PG labelling/septa formation affected in Δ*yhcB* cells. Figure panel containing *yhcB* mutant labeled with EDA-DA. This is showing punctate looks similar to FtsZ panel. A long filamentous morphotype is shown which does not appear to have incorporated any of the probe into newly forming septa. Green panels show images as they appear in the FITC channel and blue panels show corresponding pixel intensity maps that range from 0 (blue) to 255 (red)." The green is the raw data from the scope in the 488 channel (green). The blue panels are a 'heat map' of pixel intensity values for the images to the left. It's pretty much allowing you to better distinguish between areas of low and high intensity labeling. All you are seeing in either image is EDA-DA probe incorporation or background fluorescence, which is a by-product of the click-chemistry based labeling methodology (so background Alexa 488). The background fluorescence is actually a good thing, because it allows us to determine where the outlines of our bacteria are (as there is no bright field or DIC on our Zeiss ELYRA imaging system).

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**Acknowledgement:**

We are thankful to Dr. Scot…… for providing us the bacterial two vectors.

**Supplementary text:**

**Multiple alignment of YhcB and homologs:**

**The aggregation seen in Δ*yhcB* cells were not the inclusion bodies:**

One of the Δ*yhcB* phenotype was appearance of small dots in filamented cells organized in a pattern. These spots may be of inclusion bodies formed in the cells with aging and revejuscence. It has been shown that ageing cells form inclusion bodies (reference). These inclusion bodies were formed because of aggregation of proteins and the cells lost the protein folding and quality control system. To rule out formation of inclusion body formation in Δ*yhcB* cells, the ibpA protein was expressed and we found that the spots observed on Δ*yhcB* cells, were not colocalized with the ibpA. IbpA expression was used as biomarker for formation of inclusion bodies. We confirmed that the spots observed here were not because of inclusion bodies.

**Add itol/phylogenetic analysis/coevolution**

**Pattern of YhcB localization is similar to RodZ and MreB:**

A CFP-YhcB fusion protein showed that the localization of YhcB is similar to the cell wall/divisome associated proteins RodZ and MreB. We see the punctate expression of the YhcB all around the cell membrane (check it).