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Characterization of *yhcB* in *E. coli*: *yhcB* Regulates Expression of *degQS*

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Abstract YhcB, a function-unknown protein of *E. coli* was identified as a new inner-membrane factor involved in a large protein-protein interaction network of cell morphology or peptidoglycan biosynthesis. Here we described another character of *yhcB* to speculate its putative function. At first, by analyzing growth course, we divided experimental samples carrying YhcB construct or its vector control into L-arabinose induced group and uninduced group and investigated the effect of over-produced YhcB on cell growth inhibition when compared with positive control YciB overproduced cells. Furthermore, the proteolysis was detected by Western blot analysis to examine the degradation of the redundant YhcB by the protease. Finally, by using YhcB-*lacZ*-fusion constructs and β-Galactosidase activity assay, we analyzed whether *yhcB* was co-transcribed together with the downstream proteases encoding gene, *degQS*. The results indicated: the phenotype of abnormal cell growth inhibition in condition of over-produced YhcB confirmed the relationship of *yhcB* expression with the protease DegQS from two aspects: protein production and genetic level, indicating that *yhcB* regulates expression of protease encoding gene, *degQS*.

Key words *yhcB*; protease; proteolysis; *degQS*; regulation

大肠杆菌内膜因子*yhcB*对*degQS*表达的调节作用的研究

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摘要 YhcB作为大肠杆菌中存在的一种功能未知的内膜因子, 被证实参与细胞骨架以及肽聚糖生物合成。为了对*yhcB*的其他性状进行研究, 以对其未知功能进行推测, 该文检测YhcB过量表达时细胞生长曲线, 实验样品分为诱导组以及未诱导组, 相对于对照组YciB, 分析YhcB过量表达对细胞生长率的影响; Western blot分析检测到过量表达的YhcB被蛋白酶降解, 通过*lacZ*-融合载体构建以及β-半乳糖苷酶活性分析, 对*yhcB*基因及其下游存在的*degQS*基因是否共转录进行研究。结果显示: 通过YhcB过量表达延迟细胞生长阻害这一表现型出发, 从蛋白质表达水平以及遗传水平两方面证实, *yhcB*能够调控蛋白酶编码基因*degQS*的表达水平。

关键词 *yhcB*; 蛋白酶; 蛋白质水解; *degQS*; 调控

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广东省科技厅计划项目(批准号: 2008B060600017)资助的课题

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Escherichia coli (*E. coli*) is a Gram-negative, rod-shaped bacterium which exists in the lower intestine of warm-blooded organisms (including human). Most of *E. coli* are harmless and useful, but some *E. coli* are not friendly which can cause urinary tract infections or diarrheal diseases and contributes to infant mortality. For example, one novel strain of *E. coli* O104:H4 caused a serious outbreak of foodborne illness in northern Germany in 2011 resulting in 810 cases of serious complications and 39 deaths[1]. Thus, understanding how the cell envelope of *E. coli* is developed will help to solve public health and hygienic problems, as well as to develop new medicines.

Previous proteomic analysis of *E. coli* envelop showed that YhcB contains one trans-membrane domain which is located on the inner membrane, but its function is unknown. The primary structure of YhcB deduced from its nucleotide sequence predicted a protein of 132 amino acids with a molecular weight of 15 kDa, and it contains three α-helix domains in super-secondary structure[2-3]. Deletion mutant of *yhcB* exhibited reduced biofilm formation. Moreover, double mutant of *yhcB* and *rodZ* exhibits synthetic lethality. In present study of bacterial two hybrid assay, YhcB was reported to be involved in a large protein-protein interaction network including MreC, MreD, RodA, RodZ and MurG, which determines the cell morphology or biosynthesis of peptidoglycan[4-9]. YhcB was also confirmed to form a homo-oligomeric complex in *in vitro* and *in vivo* studies. In genetics *yhcB* is only found in γ-proteobacteria, however, it conservatively clusters with downstream genes: *degQ* and *degS*. *degQ* and *degS* were reported to encode homologs of the DegP protease, which is one kind of proteases required for degradation of misfolded proteins, the correct localization of secreted proteins, and survival under stressful conditions[10-11]. In this study, we further investigated the expression level of *yhcB* from two aspects: transcription and protein expression, to predict its putative role as an inner membrane protein in *E. coli*.

1 Materials and Methods

1.1 Materials

1.1.1 Strains and plasmids Strain KR0401 and plasmids PACYC184, PUT18 and pJL30 were preserved in our laboratory. Strain JE8471 was purchased from National BioResource Project.

1.1.2 Growth media and antibiotics Bacteria were grown in LB medium [1% Bacto-tryptone (Difco, USA), 0.5% Yeast extract (Difco, USA) and 0.5% NaCl (Nacalai Tesque, Japan)]. Ampicillin (100 g/mL), Chloramphenicol (35 g/mL) and IPTG (1 mmol/L) were added upon requirements.

1.2 Methods

1.2.1 Growth course 200 mL LB medium was inoculated with overnight culture of KR0401 carrying plasmids PACYC184, PACYC184-*yhcB* and PACYC184-*yciB* and incubated at 37 °C. L-arabinose was added at about D_{600} of 0.5. Cell density was monitored at interval of each hour from 0 hour.

1.2.2 Protein expression analysis Cells JE8471 carrying PUT18-YhcB or its variants[5] were cultured in LB medium containing Ampicillin at 30 °C, and subsequently induced by IPTG (0.5 mmol/L) at around D_{600} of 0.6. The cells were collected at 15 000 r/min for 1 minites and resuspended in Bugbuster Protein Extraction Reagent (Novagen). The cell extract was mixed with an equal volume of 2×SDS loading buffer. The mixture was heated and then loaded on to a 12.5% SDS-PAGE. Electrophoresis was performed at room temperature under the constant current of 25 mA using Mini-gel electrophoresis apparatus (ATTO, Japan). After separation by SDS-PAGE analysis, tag fused proteins were transferred to a PVDF membrane using semi-dry blotter Sartorblot II for approximately 1 hour according to manufacturer's protocol. Then, the blotted membrane was soaked into TBST-BSA (Wako, 0.3%) buffer containing properly diluted primary antibody and incubated for 30 minutes at room temperature. Antibody anti-CyaA (3D1, Santa Cruz biotechnology) was used at the concentration of 1~2 mg/mL. The membrane was washed four times with TBST buffer, and soaked into TBST-BSA (0.3%) buffer containing labeled secondary antibody (Anti-mouse IgG) and

incubated for 30 minutes at room temperature. Anti-mouse IgG AP conjugate (Promega, USA) was used at 8 000-fold dilution. The membrane was washed four times with TBST buffer and used in immune-detection of protein by the method appropriate to the secondary antibody. For detection using alkaline phosphatase (AP) conjugated secondary antibody. The washed membrane was soaked in the NBT/BCIP solution.

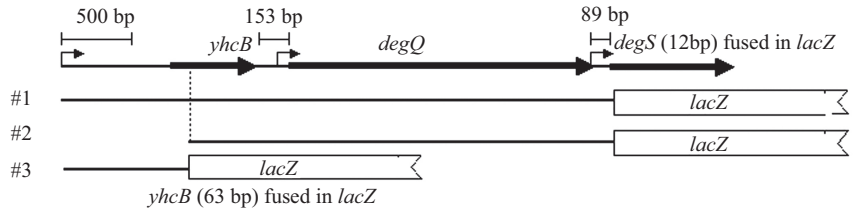
1.2.3 Construction of *lacZ*-fusion plasmids To construct *lacZ* fusions on pJL30 for the expression analysis, fragments with the sequence of interest were PCR-amplified (Fig.1) using appropriate primers (5′-cgg gat ccG GCG CTG TTT CCG CTC TC-3′ and 5′-aac tgc agC TTC ACA AAC ATG ATG GAG GCG-3′ for pJL30-*yhcB*, #1; 5′-cgg gat ccC GTA ATC GTC TGG CAG AGT CTG-3′ and 5′-aac tgc agC TTC ACA AAC ATG ATG GAG GCG-3′ for pJL30-*yhcB*, #2; 5′-cgg gat ccG GCG CTG TTT CCG CTC TC-3′ and 5′-gca agc ttT CCC AGG TCA TGA ACA TCT CCC-3′ for pJL30-*yhcB*, #3), each fragment was inserted into pJL30 *lacZ*-fusion vector[12] respectively. Primers used to construct each plasmid are indicated together with restriction enzymes sites (underline) used for cloning. Each plasmid was introduced into KR0401 by using $CaCl_2$ transformation method.

1.2.4 β-Galactosidase activity assay From over-night culture, 0.1 mL cells containing each *lacZ*-fusion were sub-cultured into 5 mL LB medium and grown at 37 °C to a D_{600} of approximately 0.4 to 0.6. 1 mL of the culture was taken and the D_{600} used for calculation of β-galactosidase activity was measured. The sample was kept at –20 °C. The frozen sample was thawed and up to 0.3 mL of the culture depending on its activity was added into 350 μL of Z buffer containing 0.2% of sarkosyl. The final volume was adjusted to be 0.3 mL by adding extra medium for the sample with less volume. The mixture of the sample and Z-buffer was incubated at 30 °C for 30 minites before 150 μL ONPG (O-nitrophenyl galactopyranase) solution was added and incubation was continued until a yellow color was developed. The reaction time was measured and the reaction was stopped by adding 375 μL of 1 mol/L Na_2CO_3 when sufficient color was observed.

2 Results

2.1 Effect of over-produced YhcB on cell growth inhibition

Firstly we analyzed the effect of the tagged YhcB produced in bacteria carrying pACYC184-*yhcB* with and without L-arabinose induction on the growth of cells. The plasmid pACYC184-*yhcB* and its vector control were transformed into wild type bacteria. The bacteria were grown overnight without arabinose and appropriate antibiotic to sustain the plasmid, and then subcultured in medium with or without arabinose (0.1%). Growth was monitored at an hourly interval in liquid medium. Without induction the growth of WT cells carrying pACYC184-*yhcB* was completely showing almost the same growth rate to the WT cells carrying the vector only. Here *yciB* was used as a positive control, that its over-expressed products stop cell growth immediately (Genobase) because of its toxicity, as also shown in Fig.2. Interestingly the growth of cells carrying plasmid pACYC184-*yhcB* didn't stop immediately after L-arabinose induction, but increased to some extent (D≈0.9), and then stopped (Fig.2). It strongly suggests that over-



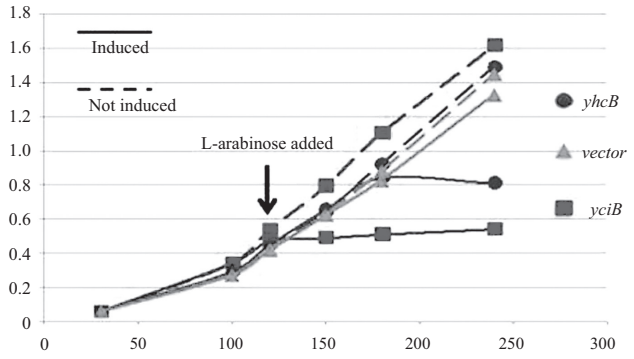
Genetic organization of *yhcB*, *degQ* and *degS* genes and predicted promoters are depicted. The regions fused to *lacZ* gene on pJL30 plasmid are depicted (thick bar).

Fig.1 Construction of *lacZ* fusion for expression of *yhcB* gene

production of YhcB, as an inner membrane protein delays the cell growth inhibition.

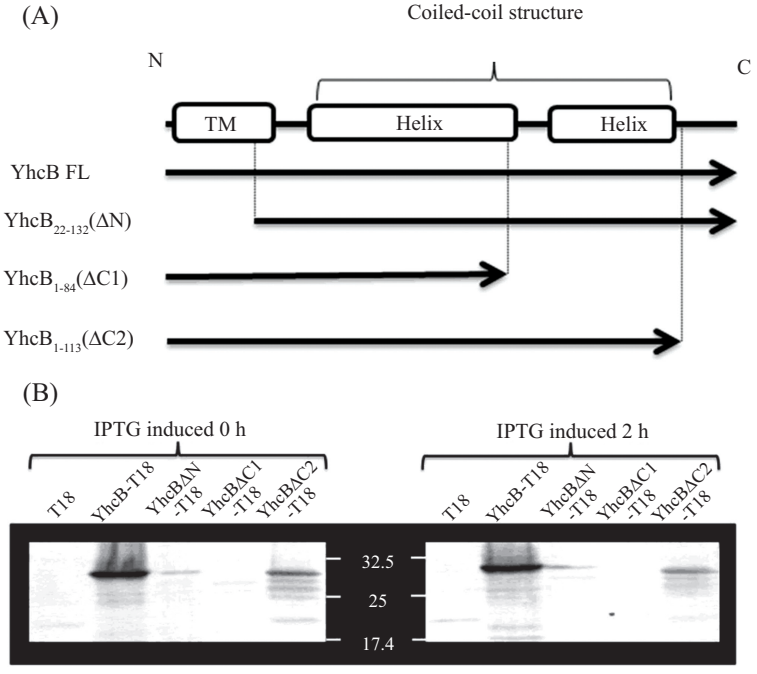
2.2 Over-produced YhcB and its variants are degraded by protease

To confirm the role of YhcB in cell growth inhibition, we further investigated the protein stability of YhcB. YhcB is predicted to contain two specific domains, a TM (trans-membrane) domain which extends from residue 4 to 25 and a large putative coiled-coil structure from amino acid residue 28 to 99 (COILS-Prediction of Coiled Coil Regions in Proteins)[13]. Lacking these two motifs makes YhcB lose the ability of interacting with other inner membrane proteins[14]. Here, to confirm the role of these two domains for YhcB's activity, we expressed YhcB and its variants with different regions (Fig.3A) in *E.coli*. The plasmid PUT18 includes an IPTG-inducible Plac promoter and a T18 tag at the C-terminal end which can be detected using anti-CyaA antibody. Western blot assays were performed on the total extracts from cells grown at 30 °C without or with IPTG induction. As shown in Fig.3B, regardless whether induced or not by IPTG, YhcB-T18 fusion protein was well expressed. The molecular mass of detected protein (approximately 30 kDa) is as expected for the fusion protein, which is 15 kDa of YhcB and 17 kDa of the T18 tag. However, the membrane space is finite, redundant over-produced protein was detected to be degraded. On the other hand, the expression of detected YhcB deletion



Wild type cells carrying vector plasmid, with cloned *yhcB* were grown in LB medium containing chloramphenicol. At indicated time L-arabinose was added in a portion of the culture. *yciB* was used as positive control.

Fig.2 Effects of YhcB overproduction on cell growth



A: schematic representation of YhcB and its truncated derivatives. Predicted transmembrane domain (TM) and cytoplasmic helical structures (helix) are indicated.Thick lines show regions cloned into pUT18. B: Western blot assays were performed on total extracts from cells grown with or without 2 h of induction of the T18 fusion construct with 0.5mmol/L IPTG, and then probed with anti-CyaA (T18) antibody as described in Materials and Methods. Arrow is indicated as negative control (T18).

Fig.3 The expression of YhcB and its variants with different length in *E. coli*

mutants was drastically reduced. The large C-terminal deletion protein (YhcBΔC1) was disappeared after 2 hours of induction with IPTG, although it could slightly be detected before induction. The protein with a very C-terminal deletion (YhcBΔC2) seemed to be subject to the proteolysis, because various shorter bands were observed. These results indicated that truncated YhcB-T18 fusion proteins were unstable.

2.3 *yhcB* regulates the expression of *degQS* encoding protease

The reason of heterologous protein, and finite or misfolded proteins degraded by protease was unclear now. Here, we further investigated the relationship of *yhcB* gene with protease encoding genes. The genomic context can often reveal clues to the function of genes, since genes coding for proteins with related cellular function often cluster together to allow co-regulation. YhcB exists only in γ-proteobacteria, and it is conservatively arranged in chromosomal genome with downstream genes, *degQ* and *degS* encoding homologs of the DegP protease. The *degQ* and *degS* were reported as operons[15]. To assess whether *yhcB* is co-regulated with *degQS*, DNA fragments containing indicated regions (Fig.1) were cloned and fused with *lacZ* as described in MATERIALS AND METHODS so that the examination of *degQS* expression was replaced by β-galactosidase activity. As shown in Table 1, #1 showed higher β-galactosidase activity than #2, suggesting there was regulator for *degQS* from the 500 bp upstream of *yhcB*, namely, the promoter of *yhcB* gene effects on the expression of *degQS* was detected. However, the reason why the expression of *yhcB* (#3) was found to be much higher than that of *degQS*, is that over-produced *degQS* is toxic to cell growth but *yhcB* can be over-expressed safely.

Table 1 β-galactivities of the regions fused on *lacZ* for *yhcB* gene expression

Sample	β-galactivity (Miller units)
Empty vector	2±1.7
#1	13±0.9
#2	6±0.4
#3	92±16.0

Cell of strain KR0401 carrying individual plasmids were grown in LB medium containing ampicillin (100 μg/mL) at 37 °C to a D_{600} of around 0.5, their β-galactosidase activities (Miller units) were measured, and average values with standard deviation of three or more independent experiments are shown.

3 Discussion

YhcB was firstly identified from the protein complexes of cell envelope in recent proteomics studies on the *E. coli* membrane proteins. It was described as a putative conserved protein, but its function was unknown. YhcB has no phenotype in its deletion mutant, the most significant phenotype is cell growth inhibition when over-expression. However, the inhibition is not as usual as the other toxic proteins, such as YciB. Over-produced YhcB prevent the cell growth at certain point of log phase. In addition, it was reported recently that most of membrane proteins inhibit the cell growth when over produced even though themselves are not toxic, because overproduced membrane proteins would inhibit the transport of other membrane proteins or periplasmic proteins and proper localization[16], and it might be a reason of the delayed growth inhibition by the induction of *yhcB*. Many recombinant proteins produced in *E. coli* are unable to obtain their native conformation and tend to be degraded rapidly by cell proteases. Cell proteases, such as DegP are an important members of the quality control system, in which they act in cooperation with folding assistant proteins to survey conformational quality. Over produced YhcB are not permitted because of the cause for poor cell growth and thus degraded by protease. YhcB protein has two special motifs: trans-membrane domain and coiled-coil domain. Both domains are confirmed to be crucial for protein stability and are also necessary for interaction with other shape proteins. The YhcB lacking the TM domain or coiled-coil domain probably has lost the intrinsic ability to be inserted into its original target or misfolded, and remains in the cytoplasm, which could be harmful to the cell. So these abnormal proteins will be degraded by DegQ and DegS, homologs of the DegP. In this research, we found *yhcB* gene clusters together with protease encoding gene, *degQS*, and examined the expression of *yhcB* together

with its chromosomal neighbors. The transcription level of *yhcB* was high, and its promoter has effect on the downstream gene *degQS* operon, suggesting *yhcB* regulate the expression of *degQS* gene. On the other hand, redundant YhcB products induce DegQS to proteolysis. Moreover, it is still conceivable that YhcB and DegQS are somehow functionally related because DegQS are periplasmic proteases and involved in the protein quality control in the cell envelope. This result gives crucial basis on further research on biotechnological application of *E. coli*, as a new way other than antibiotics to inhibit pathogenic bacteria.

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