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# Persister Formation in *Klebsiella pneumoniae:* Recombinant Expression and Purification of a *hipA* and *hipB* from *K. pneumoniae*

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***Abstract***

***Several bacterial populations are known to be containing some fraction of cells which survive exposure to antibiotics and harsh environment, are called as persister cells. This fraction of cells is very small generally ranging from $10^{-7}$ to $10^{-5}$. The mechanism of persister formation is not yet clearly understood although expression of toxin-antitoxin (TA) pairs of proteins has been found to be associated with persister formation. Klebsiella pneumoniae is also shown to produce persister cells by prolonged exposure to ampicillin. We have identified a pair of proteins, hipA and hipB, of TA system in K. pneumoniae. The proteins have 70% and 60% sequence similarity respectively with their homologous proteins from E. coli. hipA and hipB associate together to regulate survival of persister cells by binding to DNA in unfavourable conditions. Both hipA and hipB proteins from K. pneumoniae were cloned, expressed and purified. The clones were over expressed in fusion with His-tag in E. coli strains BL-21 (DE3). The purification was done using Immobilized metal affinity chromatography having Ni-NTA matrix. Sequence analysis by in-silico methods shows that hipA and hipB from K. pneumoniae may have different pattern of dimer formation and DNA recognition than their counterparts in E. coli.***

***Key Words: Microbial persistence, cloning, expression, toxin-antitoxin system***

## I. Introduction

Bacterial multi drug resistance is majorly responsible for the inactivity of various antibiotics currently used. It has been cited that over 60% infections are caused by bacterial biofilms, which are resistant to antibiotics [1-2]. Most antibiotics act on rapidly growing cells while biofilm grows slowly and remain unaffected by antibacterial agents. The reason for slow growth of biofilm has been attributed to the cells being in persister state. Persisters account for only $10^{-6}$ – $10^{-4}$ cells in a growing population [3,4,5]. Persister cells are phenotypic variants which adopt a transient dormant state which alter the antibiotic effects on the cell [6-7]. These persister cells can switch back to growth phase after removal of antibiotic to allow survival of the bacterial population [8-9]. The bacterial persistence is caused by expression of several toxin-antitoxin pair of genes [10]. Persistence is the capacity of bacterial cells to tolerate a high concentration of antibiotics, as noticed first time by Bigger in 1944 [4]. It is a very well synchronized but poorly understood phenomena used by bacterial cells to survive under stress conditions and to exhibit multi drug resistance. Recent articles have shown that these cells are enriched by presence of proteins called toxin-antitoxin (TA) systems [5, 11, 12].

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TA systems have two genes in single operon, one toxin and another antitoxin counterpart and toxin protein is relatively stable than antitoxin [13]. In normal state both genes are expressed and form dimer whereas under stress condition proteases cleave antitoxin apart and renders toxin to show its effect in form of bacterial cell death or persistence [13,14]. These TA systems have been observed in various bacteria such as *E. coli, Methanococcus jannaschi*, *Streptococcus pneumonae*, *Bacillus thuringiensis, Mycobateriunm tuberculosis* and *Neisseria gonorrhoeae* [15,16]. *hip*B forms a complex with *hip*A to neutralise its effect and also bind to DNA sequence to regulate expression of various genes involved in cell growth and metabolism [17]. *hipAB* system has been categorized as a toxin/antitoxin (TA) module in which *hip*A, the toxin, is neutralized by the antitoxin, *hip*B [17]. It has also been observed that over expression of *hip*A leads to multi drug tolerance in *E. coli.* [18].

*K. pneumoniae* is an important opportunistic pathogen and a frequent cause of nosocomial infections. It has been observed to have persistence against antimicrobial agents [19]. The first TA pair was recognised in *E. coli* was called high persistence A (*hip*A) and its recognizing partner as *hip*B [20]. By using *in silico* homology searches, we have identified similar proteins in the genome of *K. pneumoniae*. The persister cells of *K. pneumoniae* were


Figure 1: (a) Sequence alignment of hipA from K. pneumoniae and E. coli. Cysteine residues are shown in yellow and the regions interacting with hipB in dimer formation are shown in black boxes. Sequence differences are shown in grey shades.

 

induced with high concentration of ampicillin and the genes of *hip*A and *hip*B were identified, cloned and expressed. Here we report cloning, expression and purification of *hip*A and *hip*B, TA pair from *K. pneumoniae*.

## II. Materials and Methods

### 2.1 Homology search of *hip*A and *hip*B

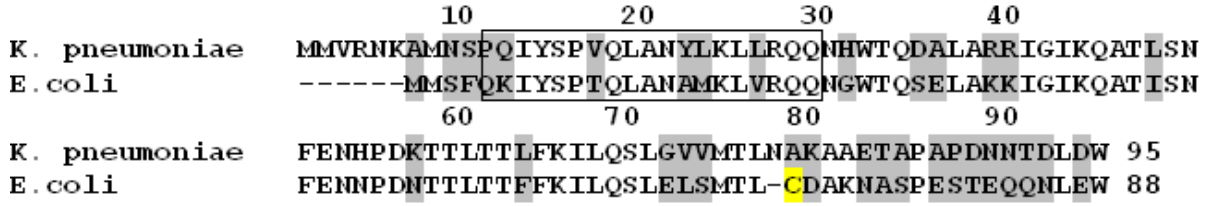


Figure 1(b): Sequence alignment of hipB from K. pneumoniae and E. coli. Cysteine residues are shown in yellow. Regions interacting with hipA in dimer formation are shown in black boxes. Sequence differences are shown in grey shades

*E.coli hip*A and *hip*B were used as search sequences to locate homologous genes in the genome of *K. pneumoniae*. Basic Local Alignment on Search Tool (BLAST) on Expasy server (www.expasy.ch) was used for milking the genomic database, which resulted in following hits in the genome of *K. pneumoniae*: Gene ID: 5340852 and Gene ID: 5340851 having 70% and 60% sequence similarity with *hip*A and *hip*B respectively from *E.coli* (Fig. 1a & 1b).

### 2.2 Gene Cloning

*Escherichia coli* strains DH5$\alpha$ and BL-21 (DE3) (Invitrogen, USA) were used for cloning and expression of the recombinant protein, respectively. The *E. coli* cells harbouring recombinant plasmids were grown aerobically at 37°C in Luria-Bertani broth (Merck, Germany) with 50 $\mu$g/mL kanamycin (Sigma). Plasmid pET-28a (Novagen) was used as an expression vector. *K. pneumoniae* strain was purchased from Microbial Type Culture Collection and gene bank (MTCC), Chandigarh, India. DNA from freshly cultured *K. pneumoniae* was isolated using genomic DNA isolation kit (Qiagen) and used as a template for amplification of *hipA* and *hipB* genes. The full coding sequence of *hipA* and *hipB* was amplified by polymerase chain reaction (PCR) using forward primer *hip*A

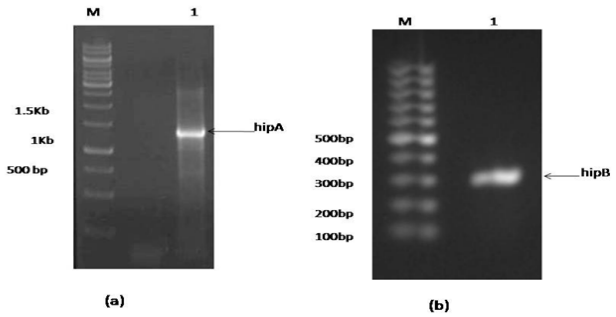


Figure 2: PCR amplification (a) Lane M- marker, Lane 1- hipAamplicon of 1.32 Kb (b) Lane M- marker, Lane 1-hipB amplicon of 285bp.

 

FP:5'GGAATTCCATATGGCGACGCTTACCACCTGG3' and reverse primer RP: 5' CCAAGCTTCTATAGCGCCTGCAGTTG possessing NdeI and *HindIII* restriction enzyme site respectively. *hip*B was amplified using forward primer FP: 5'GGAATTCCATATGATGGTGAGGAACAAGGCG3' and reverse primer RP: 5'CCGCTCGAGTTACCAGTCAAGGTCCGTGTT 3' possessing NdeI and XhoI restriction enzyme site respectively (Fig. 2 a & 2b). The purified *hipA* and *hipB* fragments were treated with the respective restriction enzymes and ligated into prepared pET28a vector, which also added six Histidine residues at the N-terminus of the expressed protein. Recombinant vector pET28a-*hip*A and pET28a-*hip*B were transformed into competent *E. coli* DH5$\alpha$ cells. The integrity of the recovered plasmid was confirmed by colony PCR and restriction endonuclease digestion.

### 2.3 Expression and Purification

For expression of the recombinant proteins, pET28a-*hip*A and pET28a-*hip*B plasmid were transformed into competent Bl-21 (DE3) cells. Bl-21 (DE3) cells harbouring the recombinant plasmids were grown in LB medium supplemented with kanamycin (50 $\mu$g/mL) at 37°C with shaking (250rpm) until the absorbance at 600nm reached approximately 0.5. Then, isopropyl-$\beta$-D-thiogalactopyranoside (IPTG) (Sigma) was added to a final concentration of 0.7mmol/L. The cells were incubated for a further 4 hours before being harvested. Pellet of BL-21 cells harbouring *hip*A and *hip*B were suspended by gentle stirring in binding buffer (300 mM sodium chloride, 50 mM sodium dihydrogen phosphate buffer, 10 mM imidazole, pH 8.0) containing lysozyme (1mg/ml).The crude lysate was subjected to sonication at 30% amplitude with 6×10second bursts with 10 second pause and then centrifuged for 20 minutes at 6000g.

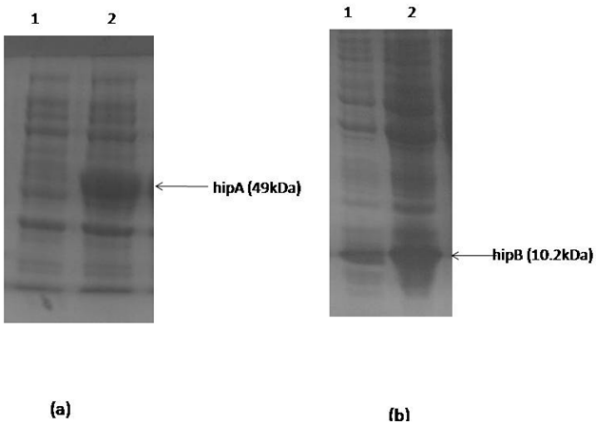


Figure 3:(a) Expression of hipA (49.1kD) :Lane 1- Control, Lane 2- induced with IPTG (0.7mM) at 37°C and (b). Expression of hipB (10.7kD): Lane M-Marker, Lane 1- control, Lane 2-induced with IPTG (0.7mM) at 37°C.

After centrifugation, the supernatant and precipitate were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to verify the expression and solubility of the recombinant proteins (Fig. 3 a & 3b). The soluble fraction was applied directly to 1.5 ml Ni-nitrilotriacetate superflow column (Qiagen) that had been pre-equilibrated with 1× binding buffer and then it was washed with 1× wash buffer (300 mMNaCl, 50 mM sodium phosphate buffer, 20 mM imidazole, pH 8.0). Proteins were eluted with 1× elution buffer (300 mMNaCl, 250 mM imidazole, 50 mM sodium phosphate buffer, pH 8.0) and collected in 2.5 ml fractions. Fractions containing proteins were identified by sodium dodecyl sulfate-polyacrylamide gel

 

electrophoresis (SDS-PAGE). The pooled fractions of eluted sample were extensively dialysed against 50mM sodium phosphate buffer, pH 7.5. *hipB* was expressed in supernatant whereas *hip*B was found to be in pellet of the cellular extract therefore purification of *hipA* was carried out under denaturing conditions in the presence of 8M urea. The purity was determined by SDS-PAGE analysis. Renaturation of *hip*A was carried out by overnight dialysis at 4°C in the refolding buffer containing880mM L-arginine, 55mM Tris, 21mM NaCl and0.88mM KCl. The formation of *hipA-hipB* complex was observed using size exclusion chromatography experiment. A glass column of 200ml bed volume (Sephadex G-100) was used for chromatography. 1:1 mixture of *hip*A and *hip*B solution was loaded and 3 ml fractions were collected. A peak corresponding to the molecular weight of the complex was eluted along with peaks of monomeric *hip*A and *hip*B proteins, which indicate biological activity of both the gene products.

### 2.4 Preparation of Persister Cells

*K.pneumoniae* was grown in LB medium at 37°C in Ehrlenmeyer flasks with agitation (220 rpm). The medium was sterilized by filteration. Culture grown to an OD595= 1. Ampicillin was then immediately added to each flask at a final concentration of 200µg/ml. The culture was incubated for 10 hour at 37°C. Then lysed cells were collected by centrifugation at 14000g at different time intervals. The surviving persisters were plated on LB agar for colony counts. Total cell counts were determined using a colony counter at a1000-fold magnification.

## III. Results and Discussion

Genes encoding *hipA* and *hipB*were identified from genome of *K. pneumoniae*. Their respective products are proteins of 440 and 95 amino acids. *hip*A shares 70% sequence similarity where as *hip*B is 60% similar to their homologues from *E. coli*. The comparative physico-chemical properties of both proteins were calculated using ExPaSy server (www.expasy.ch) as given in Table 1.

Table 1: Physico-chemical properties of hipA and hipB from fromE. coli and K. pneumoniae

| Property | hipA | | hipB | |
|---|---|---|---|---|
| | E. coli | K. pneumonia | E. coli | K. pneumoniae |
| No. of Amino acids | 440 | 440 | 88 | 95 |
| Molecular weight (Da) | 49070 | 49171 | 10016 | 10737 |
| No. of Acidic residues | 50 | 46 | 8 | 7 |
| No. of Basic residues | 53 | 46 | 8 | 10 |
| Theoretical pI | 8.57 | 7.17 | 6.17 | 9.52 |
| Hydropathy index | -0.199 | -0.166 | -0.542 | -0.415 |

The sequence of both *hip*A and *hip*B indicates that their structures may be essentially similar to their homologues from *E. coli* although differences in their amino acid sequences may produce drastic difference in nature of the surfaces of protein molecules. *hipA* and *hipB* genes specific primers were designed. The genes were amplified using PCR reactions which was confirmed by digesting the vector with restriction enzymes. Both of the clones were further confirmed by DNA sequencing. The clones were transfected to *E. coli* Bl-21(DE3) cells. The expression was induced using IPTG (0.7 mmol/L) at 37°C for 4 hours [Fig.3].

 

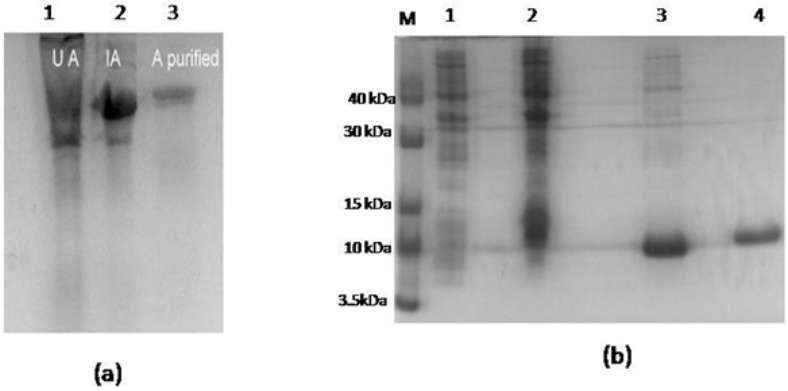


Figure 4: Sodium dodecyl sulphate (SDS) electrophoresis. Purified proteins after Ni-NTA Chromatography.(a) Purified hipA. Lane 1- UA-uninduced /control, Lane 2- induced with IPTG, Lane 3-purified hipA (15% gel). (b) Purified hipB. M-Marker, Lane 1-Control, Lane 2- induced with IPTG, Lane 3-lysate applied to column, Lane 4-purified hipB.

The cells were grown and lysed to obtain the cell extracts. The cell extracts was centrifuged and the supernatant and pellet were tested for presence of proteins. The recombinant proteins were purified by immobilized metal affinity (IMAC) column containing Ni-NTA matrix [Fig. 4]. It is noteworthy here that the culture growth and the yield of *hip* A was found to be relatively low, that could be because of its toxic effects on the cell. *K. pneumoniae* culture was treated with high concentration of ampicillin i.e. 200µg/ml to isolate persister cells. Treatment with the antibiotic resulted in initial rapid killing of cells followed by slow killing, leaving behind surviving cells aka persisters.

Comparatively more acidic nature of *hip*A and highly basic nature of *hip*B from *K. Pneumoniae* suggest that their dimer formation and recognition of nucleotide sequence may have different pattern than their homologues from *E. coli*. (Table 1)

The crystal structures of *hip*A, *hip*B and their complexes have been reported from *E. coli* [17]. The structure of *hip*A-*hip*B complex from *E. Coli* [17] shows that the N-terminal helix (region Gln 5 – Gln 23) of *hip*B interacts with three major loops of *hip*A (regions Pro 43 - Arg 49, Gly284-Glu287, Ala321-Tyr325) to form a dimer which can interact with DNA to regulate transcription. Sequence analysis show that the regions of *hip*A forming complex with *hip*B dimer are conserved in both *E. Coli* and *K. pneumoniae,* whereas regions in *hip*B are drastically different. The sequence differences have been observed in the surface and DNA binding regions. The regions which bind to DNA are *hip*A (Ala 378-Asn 382), *hip*B (Gln18- Tyr22, Gln 29-Ser31, Lys38-Asn48) as well. The critical mutations are Gln29 to Asp, Phe51 to Lys57 (*hip*B), Arg 382 to Asn382 (*hip*A). These sequence difference present in *hip*A and *hip*B from *K. Pneumoniae* indicate that the dimer formation and DNA recognition may have different pattern in *K. Pneumoniae* and *E. coli*, which can be further cleared by solving the three dimension structures of these proteins from *K. pneumoniae*.

 

## IV. Acknowledgements

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## Bibliography

[1] K. Lewis, Persister cells, dormancy and infectious disease, Nat. Rev. Microbiol. 5 (2007) 48-56.

[2] K. Lewis, Persister cells and the riddle of biofilm survival, Biochemistry (Mosc.). 70 (2005) 267-274.

[3] N.Q. Balaban, J. Merrin, R. Chait, L. Kowalik ,S. Leibler, Bacterial persistence as a phenotypic switch, Science. 305 (2004) 1622-1625.

[4] J.W. Bigger, Treatment of staphylococcal infections with penicillin, Lancet. 11(1944) 497–500.

[5] I. Keren, D. Shah, A. Spoering , N. Kaldalu, K. Lewis, Specialized persister cells and the mechanism of multidrug tolerance in Escherichia coli, J. Bacteriol. 186(2004) 8172-8180.

[6] I. Keren, N. Kaldalu, A. Spoering, Y. Wang, K. Lewis, Persister cells and tolerance to antimicrobials, FEMS Microbiol. Lett. 230 (2004)13-18.

[7] D. Shah, Z. Zhang, A. Khodursky, N. Kaldalu, K. Kurg, K. Lewis, Persisters: a distinct physiological state of E. coli.B.M.C,Microbiol.6 (2006)53.

[8] K. Lewis, Pathogen resistance as the origin of kin altruism, J. Theor. Biol.193(1998) 359-363.

[9] K. Lewis, Persister cells, Annu. Rev. Microbiol. 64 (2010) 357-372.

[10] Xiaoxue Wang, Thomas K. Wood, Toxin-Antitoxin Systems Influence Biofilm and Persister Cell Formation and the General Stress Response, Appl Environ Microbiol. 77 (2011) 5577–5583.

[11] F. Hayes, Toxins-antitoxins: plasmid maintenance, programmed cell death, and cell cycle arrest, Science. 301 (2003) 1496-1499.

[12] J.M. Brown, and J.K. Shaw, A novel family of Escherichia coli toxin-antitoxin gene pairs, J. Bacteriol.185 (2003) 6600-6608.

[13] D. S. Black, B. Irwin, H. S. Moyed, Autoregulation of *hip*, and operon that affects lethality due to inhibition of peptidoglycan or DNA synthesis, J. Bacteriol. 176 (1994) 4081–4091.

[14] D. S. Black, A. J. Kelly, M. J. Mardis, H. S. Moyed, Structure and organization of *hip*, an operon that effects lethality due to inhibition of peptidoglycan or DNA synthesis, J. Bacteriol. 173 (1991) 5732–5739.

[15] Y. Xia, L. Cui, J. Zhang, Characterization of toxin-antitoxin systems in Mycobacterium tuberculosis,Wei Sheng Wu XueBao. 51 (2011) 214-223.

[16] Y. Yamaguchi, J.H. Park, M. Inouye, Toxin-antitoxin systems in bacteria and archaea, Annu Rev Genet.45 (2011) 61-79.

[17] M.A. Schumacher, K.M. Piro, W. Xu , S. Hansen, K. Lewis , R.G. Brennan, Molecular mechanisms of *Hip*A-mediated multidrug tolerance and its neutralization by *Hip*B, Science. 323(2009) 396-401.

[18] F. Correia, A. D'Onofrio, T. Rejtar , L. Li, B.L. Karger, K. Makarova, E. Koonin, K. Lewis, Kinase Activity of Overexpressed *Hip*A Is Required for Growth Arrest and Multidrug Tolerance in Escherichia coli, J. Bacteriol. 188 (2006) 8360-8367.

[19] J.N. Anderl, M.J. Franklin, P.S. Stewart, Role of antibiotic penetration limitation in Klebsiella pneumoniae biofilm resistance to ampicillin and ciprofloxacin, Antimicrob Agents Chemother. 44 (2000) 1818-1824.

[20] H.S. Moyed, K. P. Bertrand, *hip*A, a newly recognized gene of Escherichia coli K-12 that affects frequency of persistence after inhibition of murein synthesis, J. Bacteriol. 155(1983) 768–775.

 