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Article

Hemolysin Gene Expression in the *hns* Knockout Mutant of *Klebsiella pneumoniae* UN Strain

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Hemolysin proteins are exotoxins produced by bacteria that can cause lysis of red blood cells *in vitro*. Hemolysin E is a pore-forming hemolysin, one of three types of hemolysin. Generally, hemolysin E is not expressed under standard growth conditions such as growth in LB medium at 37°C. However, in *E. coli*, when the *hns* gene, which encodes H-NS, a global regulatory DNA binding protein, is knocked out, hemolysin E gene is expressed. Because *hns* genes in *E. coli* and *K. pneumoniae* are similar, *K. pneumoniae* UN strain *hns* gene was knocked out in this research. The *hns* knockout mutant of *K. pneumoniae* UN is found to produce a 19 kDa hemolysin protein and shows hemolytic activity.

Key words: hemolysin, H-NS, *Klebsiella pneumoniae* UN

Hemolysins are cytolytic toxins found in a broad diversity of organisms. They are functionally defined by their ability to lyse erythrocytes and have often been associated with virulence for a variety of pathogenic microorganisms [Braun and Focareta, 1991]. Hemolysin has been classified as three types. The first type consists of hemolysins that use enzymatic activity to lyse the membrane. The second type consists of pore-forming hemolysins, which form pores on the cell membrane [Welch, 1991]. The third type of hemolysin consists of surfactants, which are amphiphilic and destroy the cell membrane in the same fashion as detergents [Rowe and Welch, 1994]. The *E. coli* genome database shows that *E. coli* has a hemolysin gene encoding a pore-forming hemolysin, which has recently been reported to be hemolysin E [Wallace *et al*., 2000; Neil *et al*., 2004; Wyborn *et al*., 2004]. It has been speculated that *E. coli* might possess a latent hemolytic activity that is repressed under standard growth conditions such as growth in LB medium at 37°C due to some repressor proteins [Gomez-Gomez *et al*., 1996].

H-NS, which was originally isolated by its ability to bind to compact chromosomal DNA, is a major component of the *E. coli* nucleoid. This small (15.4 kDa), abundant, neutral, and heat-stable protein binds avidly to double-stranded DNA with higher affinity for curved DNA substrates [Donato and Kawula, 1999; Tendeng and Bertin, 2003]. H-NS is a chromatin-associated protein involved in modulating the expression of more than 50 genes in *E. coli* [Bertin *et al*., 2001; Shi and Bennett, 1994]. H-NS is also involved in regulating expression of virulence-associated genes in pathogenic *Shigella* spp., *Salmonella* spp., and enteroinvasive *E. coli* [Wallace *et al*., 2000]. It has been reported that an *hns* mutant strain of *E. coli* JMG100 showed hemolytic activity on blood agar plates [Gomez-Gomez *et al*., 1996].

K. pneumoniae is a Gram-negative, non-motile, encapsulated, lactose fermenting, facultative anaerobic, and rod shaped bacterium found in the normal flora of the mouth, skin, and intestines. It is the clinically most important member of the *Klebsiella* genus of *Enterobacteriaceae*. In humans, *K. pneumoniae* is present as a saprophyte in the nasopharynx and in the intestinal tract [Podschun and Ullmann, 1998]. Host factors significantly affect the outcome of a *K. pneumoniae* infection are reported to be receptors, degree of piliation, and capsular components such as endotoxin [Highsmith and Jarvis, 1985]. *Klebsiellae* probably have two common habitats, one being the mucosal surfaces of mammals such as humans, horses, or swine, which they colonize, and the other being the environment, where they are found in surface water, sewage, and soil and on plants [Matsen *et al*., 1974; Bagley *et al*., 1978; Edberg *et al*., 1986]. In fact, *K. pneumoniae* occurs in the soil, and about 30% of strains can fix nitrogen under anaerobic conditions. The

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K. pneumoniae UN strain, which originates from the M5a1 strain [Yoch and Pengra, 1966; Ware, 1972], has generally been used to study on nitrogen fixation [John *et al*., 1975] instead of pathogenicity, although *K. pneumoniae* has been reported to show hemolytic activity [Barberis *et al*., 1986]. Considering that *K. pneumoniae* MGH78578 has very similar genomic DNA to *E. coli* [Drancourt *et al*., 2001] and that a hemolysin gene has been reported to be repressed by H-NS in *E. coli* [Gomez-Gomez *et al*., 1996; Wallace *et al*., 2000], hemolysin protein might be produced also in *K. pneumoniae* UN strain without *hns* gene, even though its activity has not been normally detected in *K. pneumoniae* UN in lab culture conditions. Therefore, we tested the possibility that hemolysin is expressed in the *hns* knockout mutant of *K. pneumoniae* UN strain, which is being used for nitrogen fixation research since 1975 [John *et al*., 1975] and that H-NS is a putative cryptic repressor for hemolysin expression in *K. pneumoniae* UN like in *E. coli* K-12.

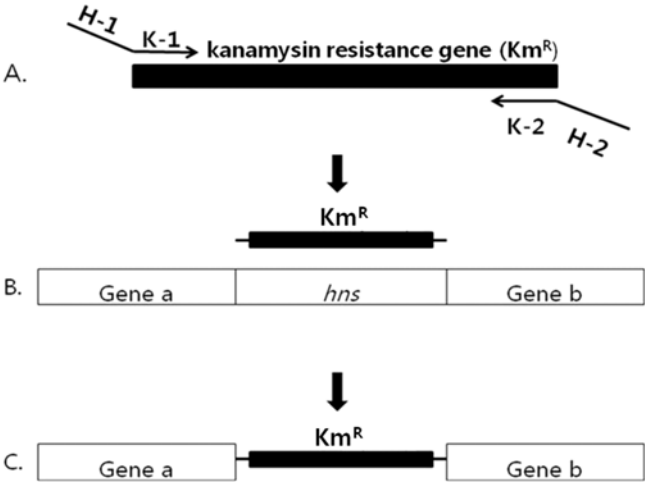

Fig. 1. *Hns* gene knockout system. A, PCR amplification of the kanamysin resistance gene; B, transfomation and homologous recombination; C, selection of antibiotic-resistant transformants; H-1 and H-2, *hns* gene flanking regions; K-1 and K-2, flanking regions for a kanamycin resistance gene; Gene a and Gene b, flanking genes for *hns* gene on the chromosome of *K. pneumoniae* UN strain.

Materials and Methods

Media, chemicals and other reagents. SOB and SOC media were prepared as described [Hanahan, 1983]. Oligonucleotides were purchased from Bioneer (Daejeon, Korea). Enzymes were from New England Biolabs (Beverly, MA) unless otherwise indicated. Taq polymerase was used in the most PCR experiments. Taq and Pfu (Stratagene, La Jolla, CA) polymerases were mixed 10:1 and used according to the manufacturer's instructions to generate DNA used for cloning and mutagenesis. Qiagen (Hilden, Germany) products were used to isolate plasmid DNA, gel-purify fragments, and purify PCR products.

Bacterial strains. The *K. pneumoniae* UN strain was obtained from the Virginia Polytechnique Institute. *K. pneumoniae* UN was grown in Luria-Bertani (LB) medium including ampicillin (100 μg/mL) at 37°C. *K. pneumoniae* cells were evaluated for hemolytic phenotypes on blood agar plates (Hanil komed, Seongnam, Korea).

***Hns* knockout.** The H-NS gene template was used for target gene mutation. This includes a kanamycin resistance gene cassette and *hns* homologous regions. The forward primer (5'-CCTTACATTCCCCCCTATTG CACAACGCATGCTCGTGTAGGCTGGAGCTGCTT C-3'), and the reverse primer (5'-AGCGATCAACGGAG ATTAGATCAGGAAATCGTCCAGTGATATTCCGGG GATCCGTCG-3') were used for PCR. A schematic diagram for the template containing the Km^R used to construct the *hns* gene knockout mutant is shown in Fig. 1. DNA sequences of K-1 and K-2 for kanamycin resistance gene were designed as described by Datsenko and Warner [2000]. H-1 and H-2 are the H-NS inner flanking DNA sequences for homologous recombination. The forward primer consists of K-1 and H-1, and the reverse primer includes K-2 and H-2. Transformants carrying the helper plasmid pKD46 were cultured in 5 mL SOB media containing ampicillin and L-arabinose at 30°C to OD 0.6 at 600 nm and then made electro-competent by concentrating 100-fold after washing three times with ice-cold 10% glycerol. PCR products were gel-purified and suspended in elution buffer (10 mM Tris-HCl, pH 8.0). Electroporation was carried out according to the method described by Datsenko and Warner [2000] with modifications. Electro-porated cells were added to 1 mL SOC medium, incubated for 1 hr at 37°C, and then one-half was spread onto an agar plate to select Km^R transformants. After selection, mutants were maintained on the medium without kanamycin. The mutant selected was confirmed by PCR using internal primers.

Peptide SDS-PAGE and native gel electrophoresis. Extracts from wild-type and mutant cells were compared on a 20% peptide gel (Elpis Bio, Daejon, Korea), prepared as described by Shägger [2006]. Native gel electrophoresis was carried out by the method of Shägger and Jagow [1991]. Cells (0.4 mg) were resuspended in 40 μL of 0.75 M aminocaproic acid, 50 mM Bis-Tris, pH 7.0, and 7.5 μL of 10% n-dodecyl-β-D-maltopyranoside. The suspension was incubated for 30 min on ice and centrifuged at 72,000 g for 30 min. To the supernatant, 2.5 μL of 5% Coomassie Blue G in 0.5 M aminocaproic acid was added. Protease inhibitor PMSF was added to a final concentration of 1 mM. Native gels were made with 13% acrylamide and

run at 100 V for 40 min. Gels were then placed on blood agar plates to check hemolytic activity [Albesa, 1989].

Extraction of proteins showing hemolytic activity. Proteins from the region of the native gels that showed hemolytic activity were extracted using a minor modification of the acetone diffusion method described by Jiang *et al*. [2004]. After cutting and grinding the gel, proteins were extracted in 1 mL of 20 mM Tris-HCl buffer, pH 8.0. The extract was concentrated to 250 μL by evaporation and 1 mL of acetone was then added. The mixture was incubated for 1 hr or more at −80°C. After centrifugation at 15,000 rpm for 15 min, the supernatant was removed carefully by pipetting. Fifty μL of distilled water was added to dissolve the remaining precipitate. The extract was analyzed by SDS-PAGE.

Cloning and expression of the hemolysin gene. The hemolysin gene of *K. pneumoniae* UN strain was amplified by PCR. PCR was carried out using the forward primer (GTAGAGCTCCGATGAAACGACCT GA) and the reverse primer (GCTAAGCTTTTACTTTT TCCGGCG), based on the hemolysin gene of *K. pneumoniae* MGH78578. The amplified hemolysin gene for *K. pneumoniae* UN was digested both with *Sac*I and *Hin*dIII restriction enzymes, isolated from agarose gel slices by using a Gene Clean kit (BIO 101, La Jolla, CA), and ligated into pET28c vector predigested both with *Sac*I and *Hin*dIII to generate the recombinant vector, pET28c-hly. The expression vector was transformed into *E. coli* BL21(DE3) cells. *E. coli* harboring pET28c-hly was cultured until the optical density at 600 nm reached 0.6, and then IPTG (isopropyl thiogalactopyranoside) was added to a final concentration of 1 mM. The culture was incubated for another 4 hr and protein expression was determined using SDS-PAGE on a 12% polyacrylamide gel as described by Laemmli [1970]. Polyclonal antibody against *K. pneumoniae* hemolysin was prepared in rabbits according to previously published methods [Moon *et al*., 2006] using the *K. pneumoniae* hemolysin protein produced in *E. coli*.

Western blotting. Cell extracts of wild-type and mutant *K. pneumoniae* were separated by SDS-PAGE on a 12% polyacrylamide gel as described by Laemmli [1970] and then electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes at 14 V for 12 hr in a cold room, according to the method of Burnette [1981] with modifications. Immediately after transfer, following the manufacturer's instructions (Sigma, St. Louis, MO) the PVDF membrane was incubated in TBST blocking solution [50 mM Tris-HCl, 150 mM NaCl, 1 mM HCl, and Tween 20 to 0.1%(v/v)], pH 7.5 containing 5%(w/v) non-fat dry milk at RT for 1 hr on a rocking platform. The blocked membrane was washed four times with TBST, pH 7.5, immersed in non-fat dry milk blocking solution containing *K. pneumoniae* hemolysin antibody (1/10,000 dilution), and then incubated for 30 min at RT on a rocking platform. The membrane was rinsed four times with TBST, pH 7.5 and incubated with 1/10,000-diluted alkaline phosphatase-conjugated goat anti-rabbit antibody (Sigma, St. Louis, MO) in non-fat dry milk blocking solution for 30 min at RT. The membrane was rinsed again four times with TBST, pH 7.5. Finally, a stock solution of NBT/BCIP (Promega, USA) was used to visualize reactive bands.

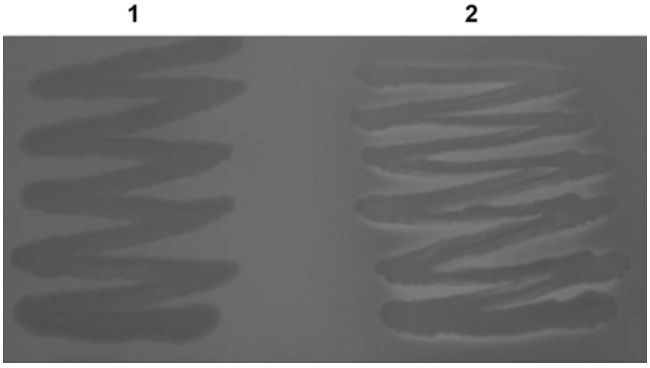

Fig. 2. Hemolytic activity on a blood agar plate. Region 1, *K. pneumoniae* wild-type cell; region 2, *K. pneumoniae hns* knockout mutant cell. The two types of cells were streaked on a blood agar plate to check hemolytic activity. The wild-type cell does not show hemolytic activity, but the mutant strain shows hemolytic activity on the blood agar plate.

Results and Discussion

Hemolytic activity in an *hns* knockout mutant of *K. pneumoniae*. To confirm that that *hns* gene was deleted from the *hns* knockout strain, PCR against *hns* gene was utilized. The hemolytic activity of the isolated *hns* knockout mutant of *K. pneumoniae* UN was compared to the wild-type cell on blood agar plates as shown in Fig. 2. Compared to no hemolytic activity in the case of *K. pneumoniae* wild-type (region 1 of Fig. 2), the *K. pneumoniae hns* knockout mutant (region 2) showed prominent hemolytic activity. This result indicates that the hemolysin gene in *K. pneumoniae* UN might be derepressed in the absence of the *hns* gene, much like *hlyE* in *E. coli*. It has been reported that the *hns* mutant strain of *E. coli* JMG100 (MC4100 *hns*$^{-}$90::Tn10) showed hemolysis on blood agar compared to MC4100 [Gomez-Gomez *et al*., 1996]. The hemolytic ability of the JMG100 mutant strain indicated that *E. coli* K-12 can produce a hemolysin, and the cryptic hemolytic activity of *E. coli* K-12 is repressed by H-NS. In fact, upstream

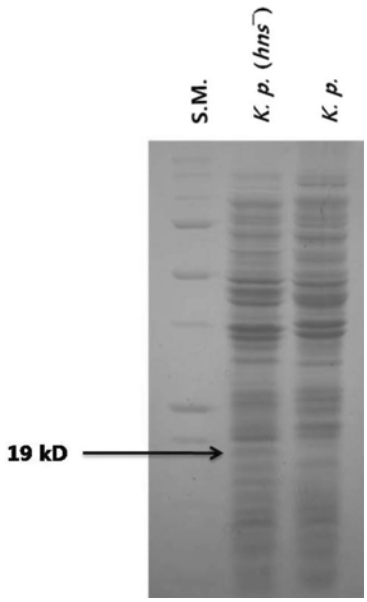

Fig. 3. Peptide gel for extracts from *K. pneumoniae* wild-type and *hns* knockout. Left lane, molecular weight markers (S.M.); middle lane, cell extracts of *hns* knockout mutant of the *K. pneumoniae*; right lane, extracts of *K. pneumoniae* wild-type cells.

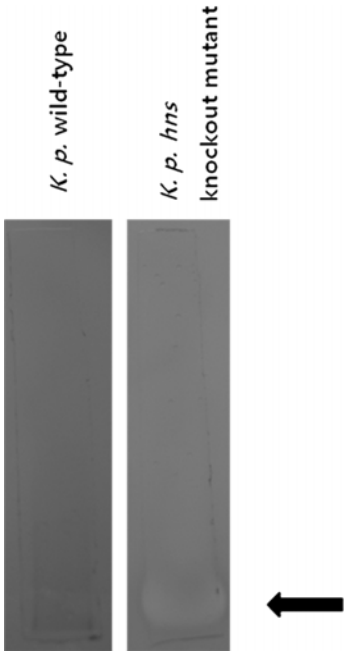

Fig. 4. Hemolytic activity on the native gel (pH 7.0). Left lane, extracts of *K. pneumoniae* wild-type cells; right lane, extracts of *K. pneumoniae hns* knockout mutant cells. Hemolytic activity of the cell extracts is indicated by an arrow.

DNA of the *K. pneumoniae* hemolysin gene contains the region including 5 T's, which might have curvature and be the candidate site for H-NS binding like *E. coli* hemolysin E gene upstream DNA. Gel mobility shift assay could confirm the H-NS binding in the future experiment. It has been also reported that *K. pneumoniae* CMC-1 has a gene that encodes a hemolysin and *E. coli* expressing the hemolysin gene of *K pneumoniae* CMC-1 produced a unique polypeptide of approximately 20 KDa, showing hemolytic activity [Chuang *et al*., 2002]. Our result shows that the *K. pneumoniae* UN strain has at least one cryptic hemolysin gene, which may indicate that nonpathogenic *K. pneumoniae* UN could produce a hemolysin under certain growth conditions. Determining pathogenic potential and identifying genes that affect hemolysin gene activation could be elucidated after genomic DNA sequence of *K. pneumoaniae* UN strain is completed.

Peptide SDS-PAGE and native gel electrophoresis. The proteins found in extracts from *K. pneumoniae* and those from the *K. pneumoniae hns* knockout mutant were visualized on a 20% SDS-PAGE gel as shown in Fig. 3. Many differentially produced proteins were observed. Among them, a 19 kDa protein was observed in the *hns* knockout mutant but not in the wild-type *K. pneumoniae* strain, as indicated by an arrow in Fig. 3. In fact, the *K. pneumoniae* MGH78578 genome database (NCBI) contains a hemolysin gene product with a predicted size of 19 kDa. In order to retrieve the hemolysin candidate in a native form, extracts of *K. pneumoniae* and *K. pneumoniae hns* knockout mutant were run on a native gel as described in the Materials and Methods section. The native gel was cut vertically and put on a blood agar plate to determine where hemolysin protein runs on the gel. One band shows hemolytic activity as indicated by an arrow on the lane for *K. pneumoniae hns* knockout mutant in Fig. 4. This might indicate that *K. pneumoniae* UN expresses a gene for hemolysin that is not expressed under standard culture conditions such as growth in LB medium at 37°C. Barberis *et al*. [1986] purified 2 forms of *K. pneumoniae* hemolysin, which are 8.4 and 19 kDa, using Sepahdex G-100 and DEAE-Sepahadex. They speculated that the 8.4 kDa hemolysin resulted from the breaking of the 19 kDa hemolysin during culture or purification based on their antipain treatment experimental results. Therefore, the 19 kDa protein observed may be the product of a hemolysin gene that is derepressed in the case of *hns* knockout mutant of *K. pneumoniae* UN strain.

Extraction of hemolytic activity region proteins. After concentration of extracts from portion of the gel slice showing hemolytic activity, the concentrate was analyzed on a 15% SDS-polyacrylamide gel (Fig. 5). In the case of the more concentrated samples (lanes 4 and 5 of Fig. 5), 4 gel slices were used and 25 μL of distilled water instead of 50 μL was used to dissolve the pellet.

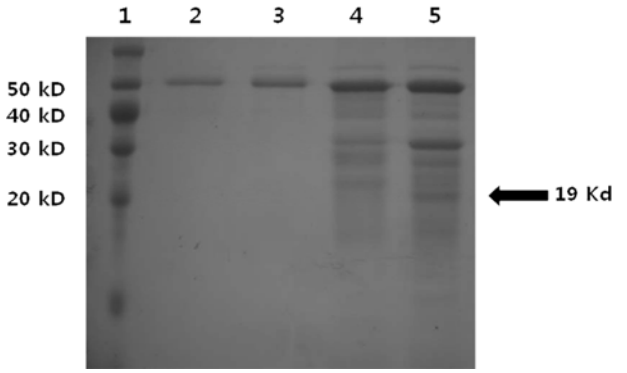

Fig. 5. SDS-polyacrylamide gel for the extracts showing hemolytic activity region. Lane 1, molecular weight markers; lane 2, wild-type gel extracts from the same migration position as the region showing hemolytic activity for the *hns* knockout mutant (seen in Fig. 4); lane 3, *hns* knockout mutant gel extracts from the hemolytic activity position; lane 4, same as in lane 2, but purified separately to 8-fold higher concentrations; lane 5, same as in lane 3, but purified separately to 8-fold higher concentrations.

Therefore, the samples in lanes 4 and 5 of Fig. 5 are assumed to be approximately 8 times more concentrated than the samples in lanes 2 and 3 of Fig. 5. *Hns* knockout mutant cell extracts from the gel region of the hemolytic activity showed a distinct 19 kDa protein in the case of the 8-fold concentrated sample as indicated by an arrow (lane 5) compared to no distinct protein band at the corresponding position (lane 4) for the wild-type cell extracts. This result indicates that hemolytic activity may be caused by 19 kDa hemolysin in the *K. pneumoniae* UN *hns* knockout mutant strain. This protein has a very similar molecular weight as the 20 kDa hemolysin previously found from *K. pneumoniae* CMC-1 [Chuang *et al*. 2002]. Presumably, the *hns* knockout mutant of *K. pneumoniae* UN strain produces a hemolysin related to the hemolysin E expressed by the *E. coli hns* knockout mutant.

Western blotting for the hemolysin in the *hns* knockout mutant of *K. pneumoniae* UN. Polyclonal antibodies were generated against the *K. pneumoniae* hemolysin produced in *E. coli* harboring the plasmid pET28c-hly containing *K. pneumoniae* hemolysin gene. The *K. pneumoniae* hemolysin above showed hemolytic activity (data not shown) like the *K. pneumoniae* CMC-1 hemolysin produced in *E. coli* [Chuang *et al.*, 2002]. The polyclonal antibodies detected the hemolysin protein produced in the *hns* knockout mutant of *K. pneumoniae* UN strain as shown in lane 2 of Fig. 6 (arrow). In the case of extracts from the wild-type *K. pneumoniae* UN strain (lane 1), there is no reactive band with the polyclonal antibodies. This Western blot confirms that *K. pneumoniae*

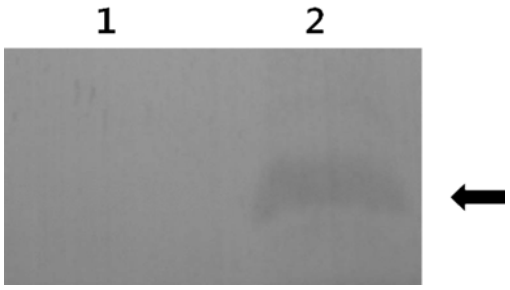

Fig. 6. Western blot of cell extracts from wild-type and mutant *K. pneumoniae* using polyclonal antibodies against *K. pneumoniae* hemolysin produced in *E. coli*. Lane 1, cell extracts from wild-type *K. pneumoniae*; lane 2, cell extracts from the *K. pneumoniae hns* knockout mutant. Hemolysin band was seen only in extracts from the *K. pneumoniae hns* knockout strain.

hemolysin gene is also derepressed in the *hns* knockout mutant of *K. pneumoniae* UN, like in the *E. coli hns* knockout mutant [Gomez-Gomez *et al*. 1996]. Thus, the *K. pneumoniae* UN strain, which is nonpathogenic and does not normally produce hemolysin, could be switched to express hemolysin gene under certain conditions. H-NS protein in *Salmonella enterica serovar Typhimurium* has a key role in selectively silencing the transcription of large numbers of horizontally acquired AT-rich genes including its major pathogenicity islands [Dorman, 2007]. Therefore, it is possible that H-NS may repress other pathogenic genes in *K. pneumoniae* UN strain, which is being used mainly in nitrogen fixation research, in addition to hemolysin gene in this experiment, even though hemolysin is not classified as pathogenic factors in *K. pneumoniae*. Future studies will detail, the role of H-NS on the expression of potential pathogenesis marker proteins such as hemolysin, and on determining what conditions might allow expression of hemolysin in the presence of H-NS.

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