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Original Article

The role of urease in the acid stress response and fimbriae expression in *Klebsiella pneumoniae* CG43

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Ure-1 and Ure-2 operons;
Acid stress response;
Type 1 and type 3 fimbriae

Abstract *Background/purpose:* Two urease operons were identified in *Klebsiella pneumoniae* CG43, *ure-1* and *ure-2*. This study investigates whether a differential regulation of the expression of *ure-1* and *ure-2* exists and how urease activity influences the acid stress response and expression of type 1 and type 3 fimbriae.
Methods: The *ureA1* and *ureA2* gene specific deletion mutants were constructed. Promoter activity was assessed using a LacZ reporter system. The sensitivity to acid stress was determined by assessing the survival after pH 2.5 treatment. The influence on type 1 and type 3 fimbriae expression was assessed using western blotting and mannose-sensitive yeast agglutination and biofilm formation assay, respectively.
Results: Bacterial growth analysis in mM9-U or modified Stuart broth revealed that *ure-1* was the principal urease system, and *ure-2* had a negative effect on *ure-1* activity. Deletion of the *fur* or *nac* gene had no apparent effect on the activity of P_{ure1}, $P_{ure2\text{-}1}$, and $P_{ure2\text{-}2}$. The $P_{ure2\text{-}2}$ activity was enhanced by deletion of the *hns* gene. *ureA1* deletion increased acid stress sensitivity, whereas the deleting effect of *ureA2* was notable without *hns*. Deletion of *ureA1* or *ureA2* significantly induced the expression of type 1 fimbriae but decreased MrkA production and biofilm formation.
Conclusion: *ure-1* is the primary expression system in *K. pneumoniae* CG43, while *ure-2* is active in the absence of *hns*. Impairment of urease activity increases the sensitivity to acid

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stress, and the accumulation of urea induces the expression of type 1 fimbriae but represses type 3 fimbriae expression.

Introduction

Many *Klebsiella pneumoniae* are now recognized as nosocomial pathogens and hypervirulent bacteria, hvKp, because of their ability to cause community-acquired and invasive infections.[1—3] *Klebsiella* liver abscesses (KLA) have been reported in both immunocompromised patients and healthy individuals.[4] Although the pathogenic mechanism of KLA remains to be determined, many virulence traits, including the capsular serotype, RmpA regulator for capsular biosynthesis, siderophore, and adherence factors play a critical role in disease progression.[4,5] However, the role of metabolic activity has not yet been studied. Notably, colonization of the high-alcohol-producing *K. pneumoniae* in the mouse gut has been attributed to its role in nonalcoholic fatty liver disease.[6] Moreover, the urease activity of *Klebsiella* spp. has also been correlated with the development of hepatic encephalopathy.[7]

Urease, the ureolysis enzyme, is an important virulence factor for many bacteria to establish successful infections.[8] Ammonium resulting from ureolysis not only provides bacteria with a nitrogen source but also acts as an acid neutralizer to protect it from acid stress damage.[9] In the stomach, *Helicobacter pylori* urease can tolerate extremely acidic pH.[10] Urease activity is also required for *Yersinia enterocolitica*[11] and *K. pneumoniae*[12] to colonize the mouse gastrointestinal tract. In the urinary tract with abundant urea, urease activity helps the formation of urinary stones, providing a colonization niche for *Proteus mirabilis*.[13]

The *ureDABCEFG* operon encodes a functional urease, of which *ureABC* codes for the structural subunit, and *ureDEFG* for the accessory proteins to incorporate nickel ions for enzyme activation.[14] Many bacteria, such as *Brucella suis* and *Helicobacter felis*, carry additional copies of the urease operon. *ure-1* of *B. suis* is required for acid resistance and in vivo persistence in the animal host, whereas *ure-2* enhances acid resistance.[15] Deletion of *H. felis ureB1* blocks urease activity, whereas inactivation of *ureB2* only causes a decrease in urease activity.[16] In contrast to *K. pneumoniae* MGH78578[17] and NTUH-K2044,[18] CG43 also contains two urease operons *ureDABCEFG* and *ureABCEFGD*, namely *ure-1* and *ure-2* (Fig. 1).

This study reports the deletion effects of *ureA1* and *ureA2* and the differential expression of *ure-1* and *ure-2*. The influence of urease activity on the acid stress response and the expression type 1 and type 3 fimbriae were also studied.

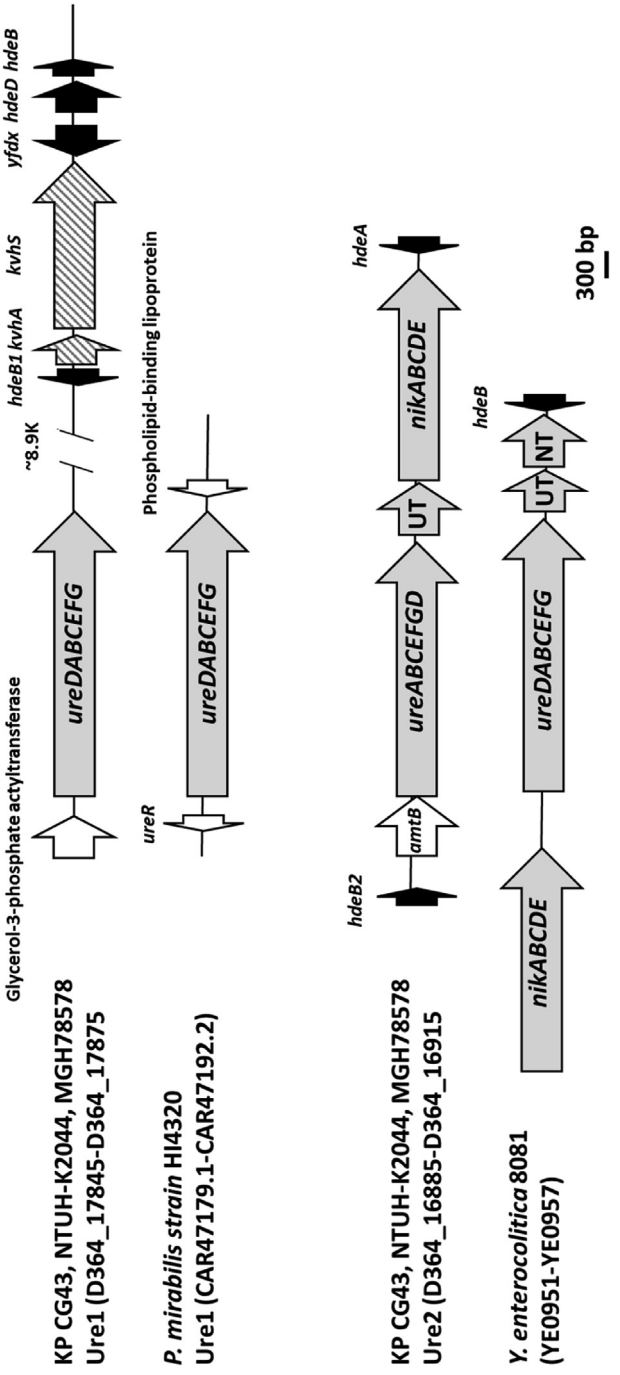

Figure 1. Gene organization of the urease operons of *K. pneumoniae* CG43, NTUH-K2044, and MGH78578, *Proteus mirabilis* HI4320[19] and *Yersinia enterocolitica* 8081.[20] The genes were annotated according to the National Center for Biotechnology Information (version 3.20.3) using BLASTX analysis. The *ure1* gene cluster was identified in all three *K. pneumoniae* genomes and the genome of *P. mirabilis*, whereas *ure2* was identified only in CG43 and *Y. enterocolitica*. NT, nickel transporter; UT, urea transporter.

Methods

Plasmids, bacterial strains, and growth conditions

Bacterial strains, plasmids and primers used in this study are listed in Tables 1 and 2, respectively. *Escherichia coli,*

Table 1 Bacteria and plasmids used in the study.

strain or plasmid	properties	reference or source
E. coli		
S17-1 λ pir	RecA *thi pro hsdR* ⁻M+ [RP4-2-Tc::Mu:KmRTn7]	[21]
K. pneumoniae		
CG43	clinical isolate of K2 serotype	Laboratory stock
CG43-S3	*rspL* mutant, Sm^r	Laboratory stock
Δ*lacZ*	*lacZ* gene removed from CG43-S3	[24]
Δ*ureA1*	*ureA1* gene removed from CG43-S3	This study
Δ*ureA2*	*ureA2* gene removed from CG43-S3	This study
Δ*ureA1*Δ*ureA2*	*ureA1* and *ureA2* genes removed from CG43-S3	This study
Δ*fur*	*fur* gene removed from CG43-S3	[24]
Δnac	*nac* gene removed from CG43-S3	This study
Δcrp	*crp* gene removed from CG43-S3	[42]
ΔcpxAR	*cpxAR* gene removed from CG43-S3	[23]
Δhns	*hns* gene removed from CG43-S3	This study
*Δhns*Δ*ureA1*	*hns* gene removed from Δ*ureA1*	This study
*Δhns*Δ*ureA2*	*hns* gene removed from Δ*ureA2*	This study
plasmid		
pKAS46	suicide vector, *rpsL*, Km^r, Ap^r	[18]
pKAS46+ureA1UD	pKAS46 harboring about 1-kb DNA upstream and downstream of *ureA1*	This study
pKAS46+ureA2UD	pKAS46 harboring about 1-kb DNA upstream and downstream of *ureA2*	This study
yT&A	PCR cloning vector, Ap^r	Laboratory stock
pLacZ15	containing the promoterless *lacZ* from *K. pneumoniae* CG43S3, Cm^r	[20]
pLacZ15-ureD1	pLacZ15 harboring the putative promoter of *ure1*	This study
pLacZ15-ureA2	pLacZ15 harboring the putative promoter of *ure2-1*	This study
pLacZ15-amtB	pLacZ15 harboring the putative promoter of *ure2-2*	This study

K. pneumoniae CG43 and its derivatives were propagated at 37 °C in LB (Luria Broth: tryptone-10 g, yeast extract-5 g and NaCl-10 g/1 L, Cyrusbioscience, Taiwan), M9 and mM9U (Table 3). The antibiotics used include ampicillin (100 μg/ml), chloramphenicol (35 μg/ml), kanamycin (25 μg/ml), tetracycline (12.5 μg/ml) and streptomycin (500 μg/ml).

Construction of the specific gene-deletion mutants

The specific gene deletion was introduced to the chromosome of *K. pneumoniae* CG43S3 by using an allelic-exchange strategy as described.[21] In brief, the DNA fragments of 1 kb flanking both ends of the target gene were amplified using PCR with the primer sets. The two amplicons were cloned into the suicide vector pKAS46 [22], and the resulting plasmid transformed into *E. coli* S17-1 λ*pir* and then transferred to the streptomycin-resistant strain, *Klebsiella pneumonia* CG43S3 via conjugation. Several kanamycin-resistant transconjugants were selected and propagated in 2 ml of LB broth overnight. A small aliquot of the culture was plated on LB agar containing 500 μg/ml of streptomycin. Lastly, the streptomycin-resistant and kanamycin-sensitive colonies were isolated, and the specific gene deletion were verified with PCR analysis.

Urease activity assay

Aliquots of the overnight cultured bacteria in LB were inoculated into the modified Stuart's broth[23] (Table 3), then were cultured at 37 °C for 18-, 24-, and 36-h, and the phenol red color changes were recorded to assess the urease activity qualitatively. To quantitatively determine the urease activity, the bacteria cultured in the modified Stuart's broth without phenol red were harvested by centrifugation at 10,000 g for 3 min. The cell pellets were washed twice and suspended in 50 mM sodium phosphate buffer (pH 7.6) and the urease activity was determined following the instructions of the QuantiChrom Urease Assay Kit (BioAssay Systems, CA, USA). All analyses were conducted in triplicates, and the data calculated from three independent experiments are analyzed using Student's *t*-test.

Measurement of promoter activity

The putative promoter region of *ure1* (P_{ureD1}), *ure2-1* ($P\text{-}_{amtB}$) and *ure2-1* ($P\text{-}_{ureA2}$) were PCR amplified and the amplicons cloned into placZ15 [24]. The generated promoter reporter plasmids placZ15-P_{ure1}-*lacZ*, placZ15-$P_{ure2\text{-}1}$-*lacZ* and placZ15-$P_{ure2\text{-}2}$-*lacZ* from *E. coli* S17-1 λ*pir*[25] were individually mobilized into *K. pneumoniae* CG43S3Δ*lacZ* strains through conjugation. The overnight-grown bacteria diluted 1:10 in broth were incubated in M9*U (Table 3) for 3 h and then the β-galactosidase activity was measured and expressed as Miller units.[26] Each sample assayed in triplicate, and the data calculated from three independent experiments are analyzed using Student's *t*-test.

Table 2 Primer used in this study.

primer	sequence (5′→3′)	
For specific gene-deletion mutants		Target
KOA1F	TGGGTCGGCACTTTGCTGTG	*ureA1*
KOA1R	ACCTCCAGAGGGCCCATGAC	
KOA2F	TGGAAGGCAATGGGATGCAA	*ureA2*
KOA2R	CCAAACGCTTTACTGCGCTC	
Nac-A-F	TCTAGAGATAGTCGATTGCGGCCTC	*nac*
Nac-A-R	GAGCTCGCTGGTCAGTTAGGGTATATTCTC	
Nac-B-F	GAGCTCCTCCTGTAAGGAACGCCAG	
Nac-B-R	GAATTCGCTTCAGTATCCTCGCCGT	
WCC138	CTAGATATATCTGTAGCAAGGAGAGCAGCCATG	*cpxAR*
WCC139	AGATCTGAGCATCCCCGGGAATACTTAATAT	
WCC140	AGATCTTAAATCAACGCTGTCGTCCAGAAG	
WCC141	GAATTCGTTTGTTACTGGGGTTGCAGG	
CY001	GAATTCGTCTGATGACCCAGTTAACC	*fur*
CY002	GGATCCGTTGTCAGTCATGCGGAATC	
CY003	GGATCCACGCGGTGGAAACATAATTC	
CY004	GAATTCACCTCTGGGAGAACGACAATG	
hns-A-R	CTCGAGCGCACGAAGAGTACGGAT	*hns*
hns-A-F	GGTACCCGCTTTCAGCAGGGGTAT	
hns-B-R	TCTAGACTGTCGAAGACGTAACCGCTTATG	
hns-B-F	CTCGAGAAATCACTGGACGATTTCCTG	
For amplifying the promoter fragments		
P-*amtB*-F	GGATCCTCGCTTTTTTACGTGCCTG	
P-*amtB*-R	AGATCTCAGCCCGGGCGTCATCAAC	
P-*ureA2*-F	GGATCCCTTTCTGCCCCATCCCTGT	
P-*ureA2*-R	AGATCTAACGCGACGTCAGCCAGGG	
P-ure1D-F	GGATCCTAATCGCGCCTTTCACCCG	
P-ure1D-R	AGATCTCGTGGCCTGCCAGCCTTTT	
For *fimS* switch assay		
P1	GGGACAGATACGCGTTTGAT	
P2	GGCCTAACTGAACGGTTTGA	

Table 3 Urease activity assay.

Composition (g/l)	M9	M9U	mM9U	M9*-U	Stuart's	Modified Stuart's
Yeast extract	—	—	—	—	0.1	0.1
Glucose	4	4	4	4	—	4
NaCl	0.5	0.5	0.5	0.5	—	—
Na_2HPO_4	6.78	6.78	6.78	6.78	—	—
KH_2PO_4	3	3	3	3	9.5	9.5
K_2HPO_4	—	—	—	—	9.1	9.1
Urea	—	0.36 (6 mM)	0.36	0.36	20 (333 mM)	20
Phenol red	—	—	—	—	0.012	0.012
NH_4Cl	0.5	0.5	0.005	0	—	—
$MgSO_4$	0.24	0.24	0.24	0.24	—	0.24
$CaCl_2$	0.011	0.011	0.011	0.011	—	0.011

Acid stress survival assessment

Overnight-grown bacteria diluted 1:20 in M9 broth were incubated at 37 °C to OD_{600} of 1.0—1.1. An aliquot of the bacteria was collected by centrifugation, suspended in pH 5 M9∗-U for adaptation 2 h and then subjected to pH 2.5 M9∗-U for acid stress challenge 35 min, or subjected to pH 2.5 M9∗-U for acid stress challenge 1 h. The bacteria were

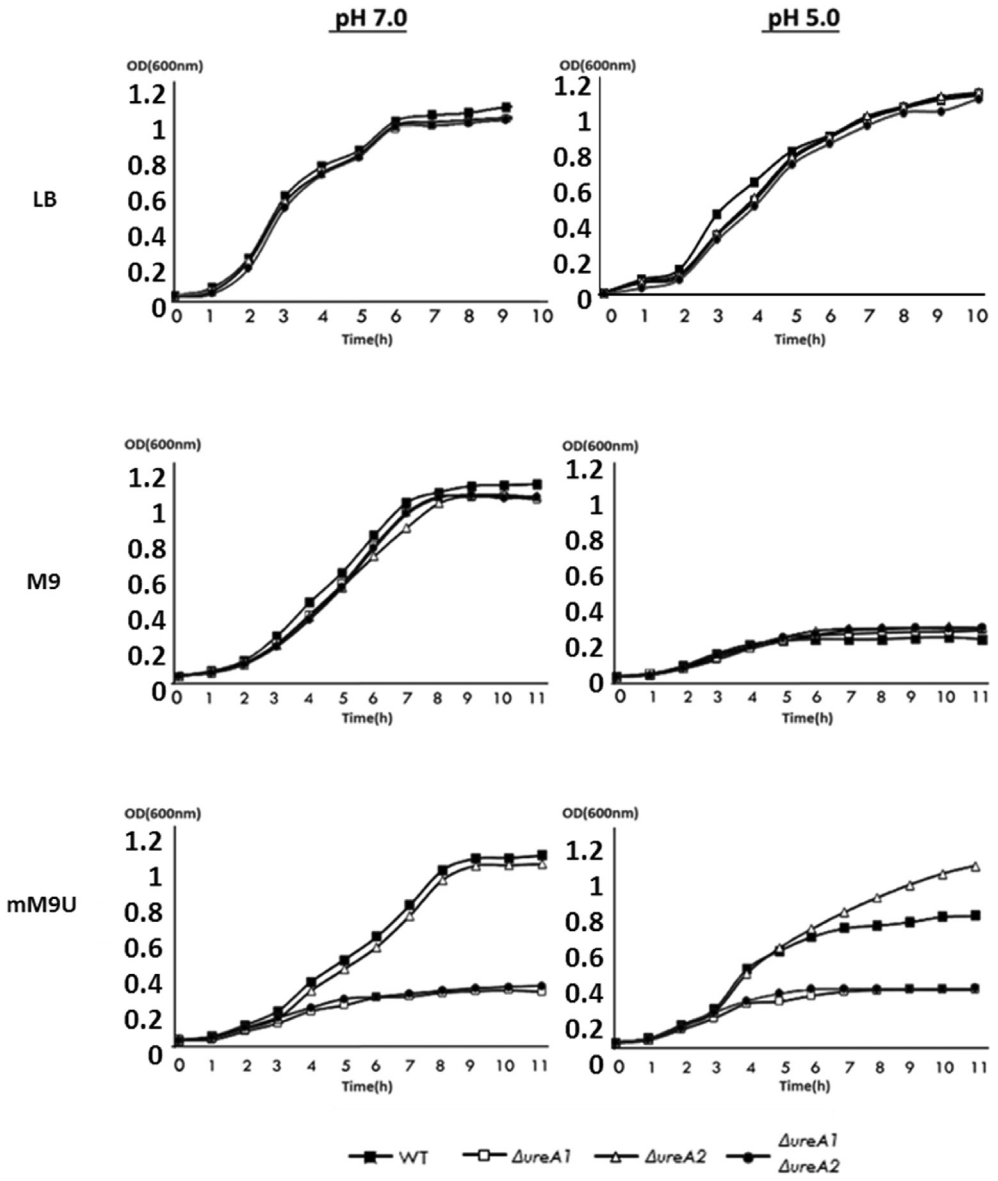

Figure 2. Growth curve of CG43S3 (WT), Δ*ureA1*, Δ*ureA2*, and Δ*ureA1*Δ*ureA2* at pH 7 or pH 5 individually in LB, M9, and mM9U. Growth of the bacteria was cultured was at 37 °C and each data point reading was taken every 1 h.

then ten-fold diluted serially to 10^{-6} and 5 μl of each sample was spotted onto LB agar plate and incubated at 37 °C overnight.

Western blot analysis

Aliquots of total cellular lysates were resolved by SDS-PAGE, and the proteins were electrophoretically transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). After incubation with 5% skimmed milk at room temperature for 1 h, the membrane was washed three times in PBS with Tween 20 (PBST). The membrane was then incubated with anti-GAPDH (GeneTex Inc.), anti-FimA,[27] or anti-MrkA[28] antiserum at room temperature for 2 h. After three washes with 1X PBST, the PVDF membrane was incubated with a 1: 5000 dilution of the secondary antibody, alkaline phosphatase conjugated anti rabbit immunoglobulin G (Millipore,AP132A) at room temperature for 1 h. Finally, the blot was washed and the antibody complex detected using chromogenic reagents 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

Yeast-cell agglutination

The agglutination of yeast *Saccharomyces cerevisiae* AH109 was conducted as described.[29] Bacteria cultured at 37 °C for 18 h in LB broth (0—500 mM urea) was suspended in 0.85% saline with or without 5% mannose and then mixed with yeast suspended in saline (10 mg/ml) into each well of a 24-well microtiter plate (Orange Scientific, Catalogue#4430300). The degree of clumping was assessed by naked-eye observation.

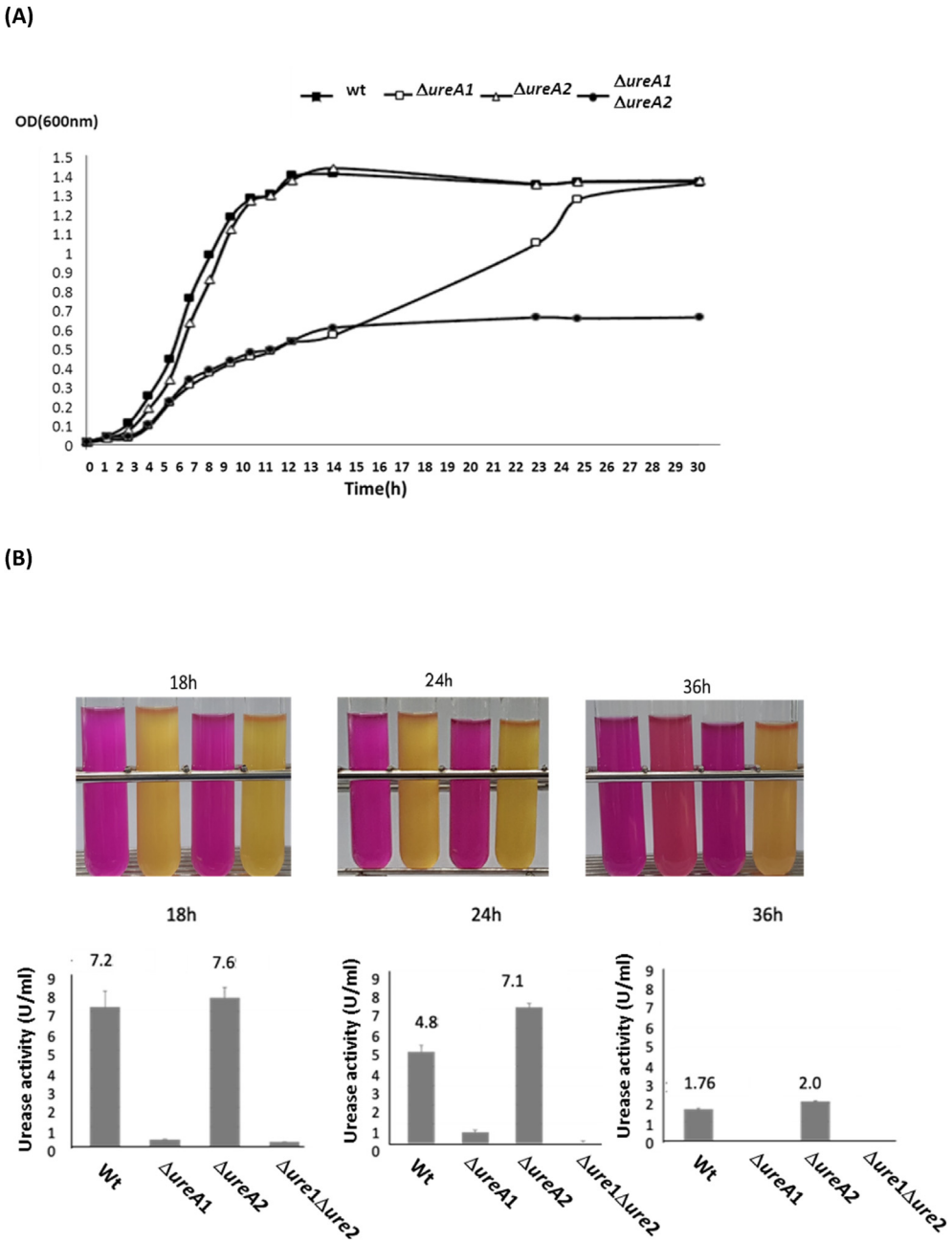

Figure 3. Growth curve and urease activity of CG43S3 (WT), Δ*ureA1*, Δ*ureA2*, and Δ*ureA1*Δ*ureA2* in the modified Stuart's broth. (A) Growth of the bacteria was cultured at 37 °C in the medium without phenol red and each data point reading was taken every 1 h till 14 h of incubation and at the 23rd, 25th, and 30th h. (B) Qualitative and quantitative analysis of the urease activity. The urease activity was qualitatively determined by observing the color changes while quantitatively measured for the bacteria cultured in the medium without adding phenol red using the urease assay kit following the manufacturer's instructions. One unit urease is defined as the catalysis resulting in 1 μmole ammonia formation per min at pH 7.0 under the assay condition.

Biofilm formation assay

Bacteria diluted 1:100 in LB broth were inoculated into each well of a 96-well dish (Orange Scientific) and statically incubated at 37 °C for 24 h. Planktonic cells discarded, and the wells were washed once with distilled water to remove unattached cells. Crystal violet (0.1%, Sigma) was used to stain the attached cells for 30 min and the stained biomass was solubilized in 1% SDS. Adsorption at 595 nm was measured and the biofilm-forming activity was calculated.

fimS switching assay

The sequence containing *fimS* was PCR-amplified with the primer pair P1/P2 (Table 2) and then the amplicon digested by restriction enzyme *Hin*fI. The phased status of *fimS* was

represented with two sets of different sized fragments, 605- and 212-bp for ON-phase while 496- and 321-bp for OFF-phase.

Results

Ure-1 is the major expressed system in urea medium

The *ureA1* and *ureA2* gene deletion effects were first examined to determine whether both *ure* operons were functionally expressed. Fig. 2 shows that CG43S3, Δ*ureA1*, Δ*ureA2*, and Δ*ureA1*Δ*ureA2* exert similar growth patterns in LB (pH 7 or pH 5) and M9 (pH 7 or pH 5). In pH 7-mM9-U, however, Δ*ureA2* and CG43S3 grew more than Δ*ureA1* and Δ*ureA1*Δ*ureA2*, indicating that *ure-1* is required for urea growth. Notably, Δ*ureA2* exerted better growth, with approximately 2-fold increase of the number (Fig. S1), than CG43S3 at pH 5-mM9-U after 6 h of incubation, suggesting a negative role of *ure*-2 in the expression of *ure-1*.

The growth pattern in modified Stuart's broth was also determined. As shown in Fig. 3A, Δ*ureA1* regained its growth after 12 h incubation and reached a growth point similar to that of CG43S3 and Δ*ureA2* at 30 h. This implies that *ure-2* is functional and that its expression may have been activated after 12 h of incubation.

Ure-2 may have an inhibitory effect on Ure-1 activity

As shown in the upper panel of Fig. 3B, the broth color of CG43S3 and Δ*ureA2* changed from yellow to bright red, while the color of Δ*ureA1* and Δ*ureA1*Δ*ureA2* remained yellow after 24 h of incubation. Nevertheless, the broth color of Δ*ureA1* turned red after 36-h of incubation. The urease activity was also quantitatively determined in modified Stuart's broth without phenol red. As shown in the bottom panel of Fig. 3B, the urease activity level of Δ*ureA2* was approximately the same as that of CG43S3 after 18 h of incubation. However, the urease activity level of CG43S3 decreased after 24 h of incubation, suggesting that *ure*-2 is effect at the later growth stage and that *ure*-2 may have an inhibitory role on *ure*-1 expression. No urease activity was detected in Δ*ureA1* after 36-h of incubation, indicating that the broth color change may be due to some unknown alkaline metabolites.

P_{ure-1}, $P_{ure-2-1}$ and $P_{ure-2-2}$ are differentially expressed

The putative promoters of Ure-1, P_{ure1}, Ure-2, P_{ure2-1}, and P_{ure2-2} were isolated and cloned into the promoter-less *lacZ* gene, and the β-galactosidase activity was measured. Fig. 4A shows that P_{ure1} activity significantly higher when the pH 7-M9∗-U or pH 5-M9∗-U medium was used instead of pH 7-M9 or pH 5-M9, and the activity level in pH 5-M9∗-U was higher than that in pH 7-M9∗-U. These results indicate that *ure-1* expression is induced by urea or weak acid (pH 5). In contrast, the activity of P_{ure2-1} or P_{ure2-2} was not

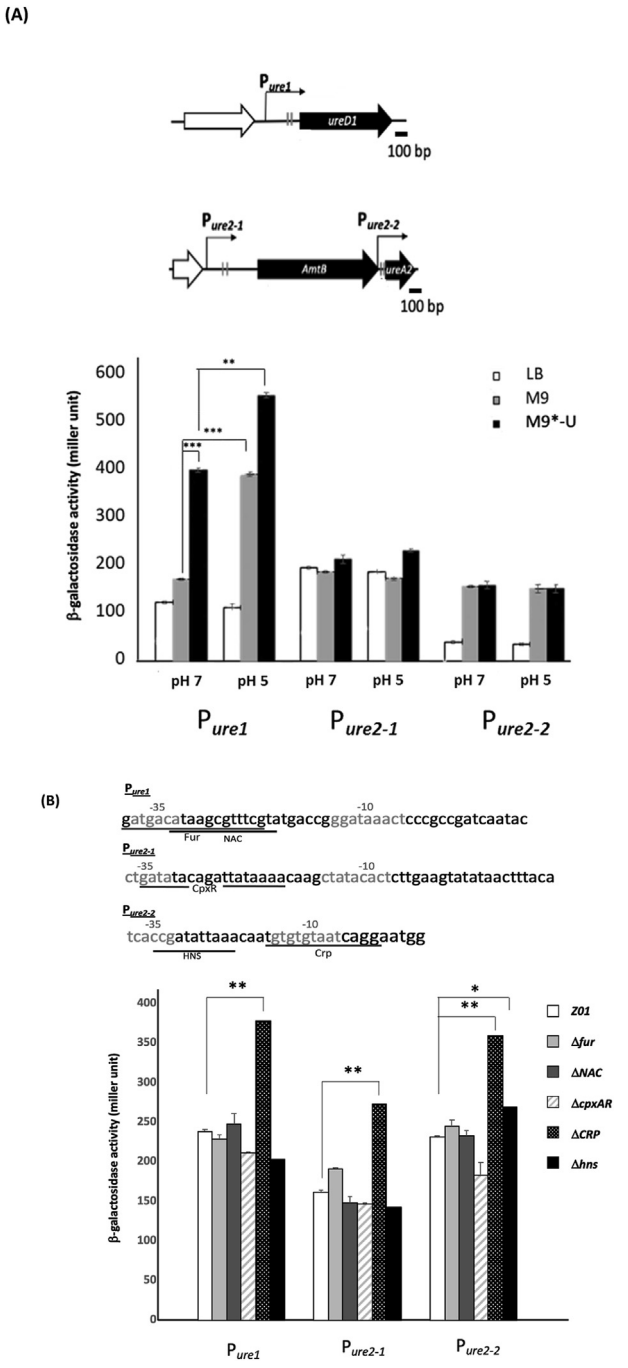

Figure 4. Promoter activity measurement. (A) Schematic representation of P_{ure1}, P_{ure2-1} and P_{ure2-2} location, and the β-galactosidase activities of the 3 h-cultures in different medium were determined. (B) The putative Fur, NAC, CpxR, Crp, and H-NS binding box are marked respectively on P_{ure1}, P_{ure2-1} and P_{ure2-2}. The specific gene deletion effect on the activity of P_{ure1}, P_{ure2-1} and P_{ure2-2} of the 3 h-cultures grown in M9-U (pH 6.8) broth 3 h were determined. Error bars indicate standard deviations of three independent experiments done in triplicate. −10 box and −35 box were predicted promoters by BPROM software. All reported p values were two-tailed, and statistical significance was set to *$p < 0.01$, **$p < 0.05$ and ***$p < 0.001$.

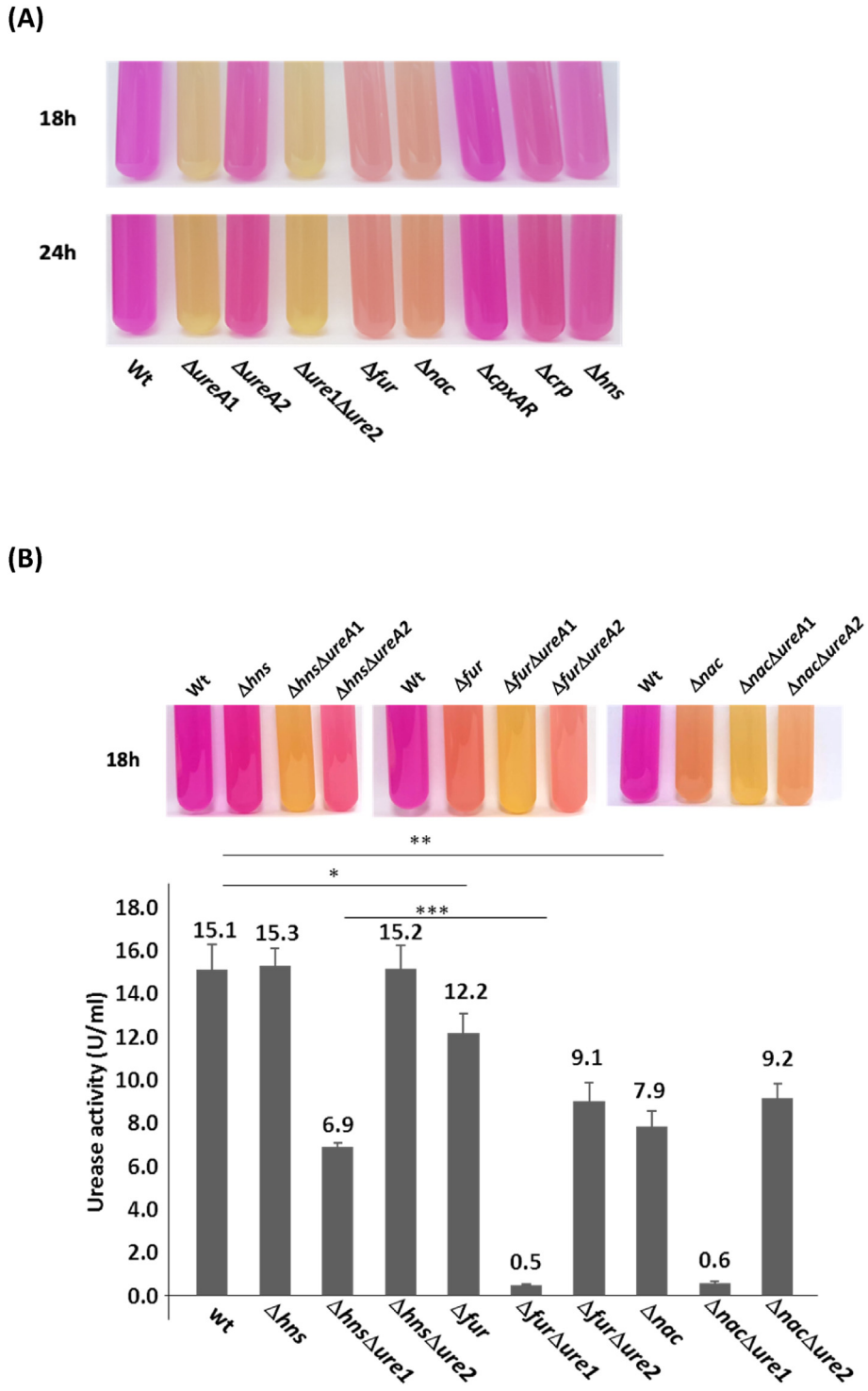

Figure 5. Influences of Fur, NAC, and H-NS on the urease activity. The urease activity of WT and the specific gene deletion mutants were qualitatively determined by observing the color changes in the modified Stuart's broth (A) and quantitatively measured after 18 h of incubation using the urease assay kit, according to the manufacturer's instructions(B). The reported p values were two-tailed, and statistical significance was set to *$p < 0.01$, **$p < 0.05$ and ***$p < 0.001$.

affected by the addition of urea or pH changes. Nevertheless, the activity of $P_{ure2\text{-}2}$ in M9 or M9∗-U medium was higher than that in LB, implying a starvation-inducible expression.

As shown in the upper panel of Fig. 4B, the sequence analysis revealed a relatively conserved sequences to the reported binding element of Fur (GATAATGATWATCATTATC)[30] and NAC (ATAA-N_5-TGNTAT)[31] on P*ure1*, as well as

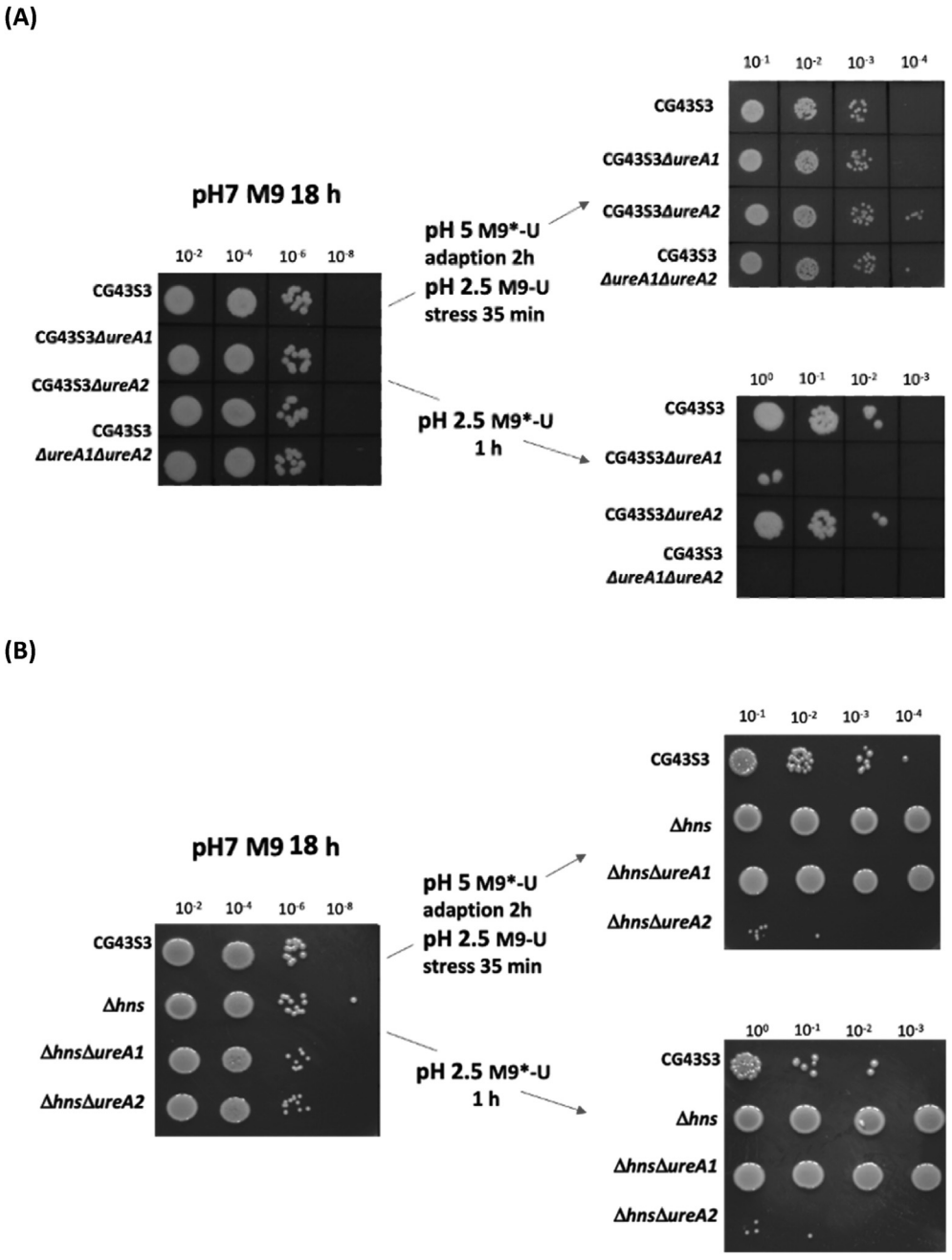

Figure 6. Effect of *ureA1* or *ureA2* deletion on the acid survivals. Bacteria were grown to the stationary phase (OD_{600} 1.0—1.1), and an aliquot of the bacteria was subjected to the acid stress treatment as described in Materials and Methods.

CpxR ($GAAATN_{5-10}GTAAAA$),[32] HNS (TCGATATATT),[33] and Crp ($TGTGAN_6TCACA$),[34] respectively, on P*ure2-1* and P*ure2-2*. The specific gene deletion effects of Fur, NAC, CpxAR, CRP, and H-NS were then analyzed. As shown in Fig. 4B, the activity of $P_{ure1,}$ P_{ure2-1}, and P_{ure2-2} activity were all increased by the deletion of the *crp* gene, while only P_{ure2-2} activity increased with the removal of the *hns* gene.

Fur, NAC, and H-NS play a regulatory role on the urease activity

As shown in Fig. 5A, deletion of *fur* or *nac* confers the culture an orange color after 18 h and 24 h of incubation suggesting a positive role of *fur* and *nac* on urease activity. The urease activity after 18 h of incubation was also quantitatively

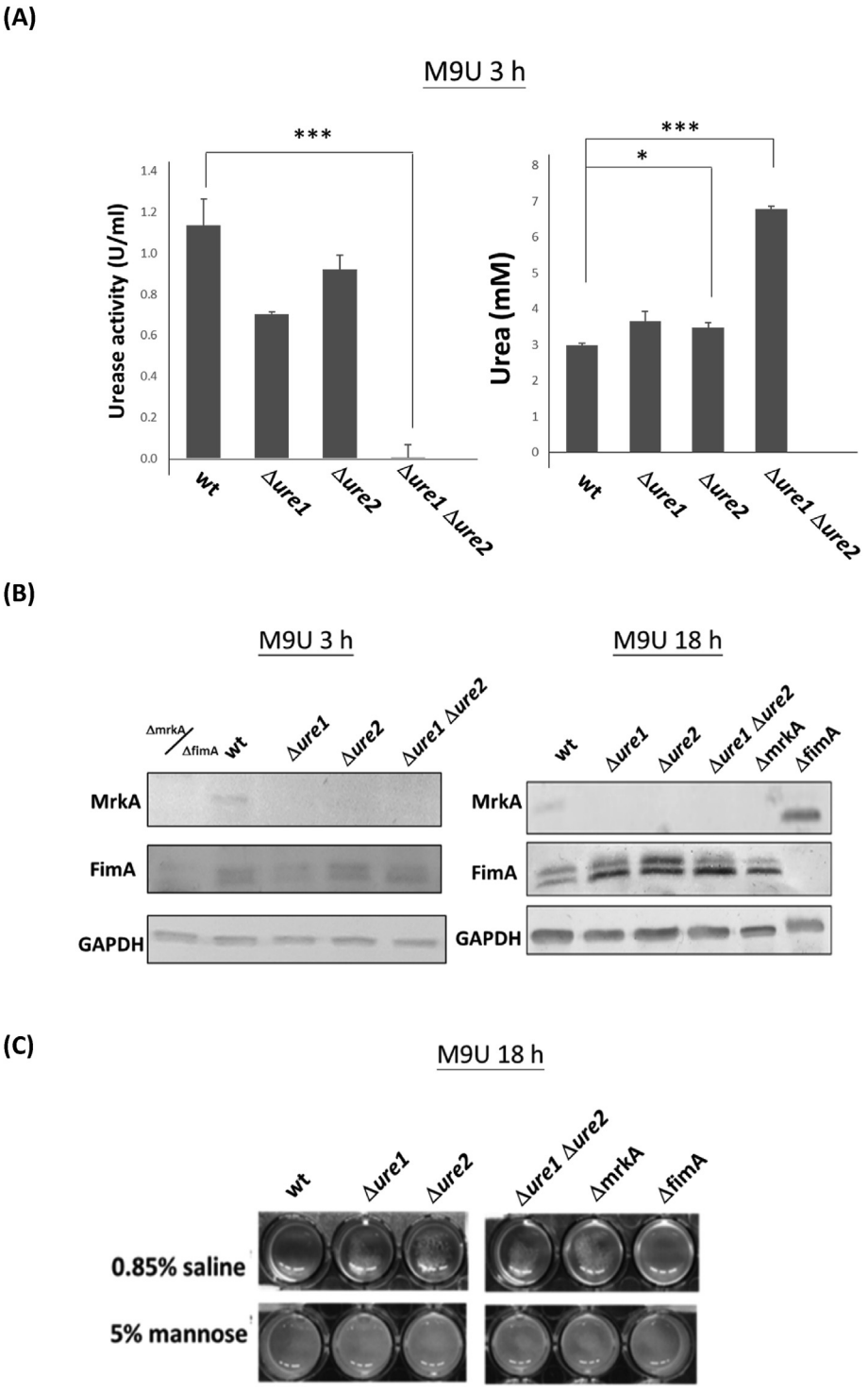

Figure 7. Effect of *ureA1* or *ureA2* deletion on the urea consumption, urease activity, and the expression type 1 and type 3 fimbriae. (A) The urease activity was quantitatively measured after the bacteria grown in M9U broth for 3 h. Urea concentration was determined using the assay kit (BioAssay Systems, DIUR-100, CA, USA). (B) The expression of type 1 and type 3 fimbriae were determined using western blotting against anti-FimA and anti-MrkA antibody, and (C) mannose-sensitive yeast agglutination (MSYA) assay. The bacteria Δ*mrkA*/Δ*fimA* were used as negative control respectively for the expression of MrkA and FimA. The reported p values were two-tailed, and statistical significance was set to *$p < 0.01$, **$p < 0.05$ and ***$p < 0.001$.

examined. Fig. 5B shows that deletion of *fur* or *nac* decreases urease activity. Although deletion of *hns* confers no apparent effect on urease activity, Δ*hns*Δ*ureA1* exhibits a significantly increased level of urease activity when compared with that of Δ*fur*Δ*ureA1* or Δ*nac*Δ*ureA1*. This suggests that H-NS negatively affects the expression of *ure-2*.

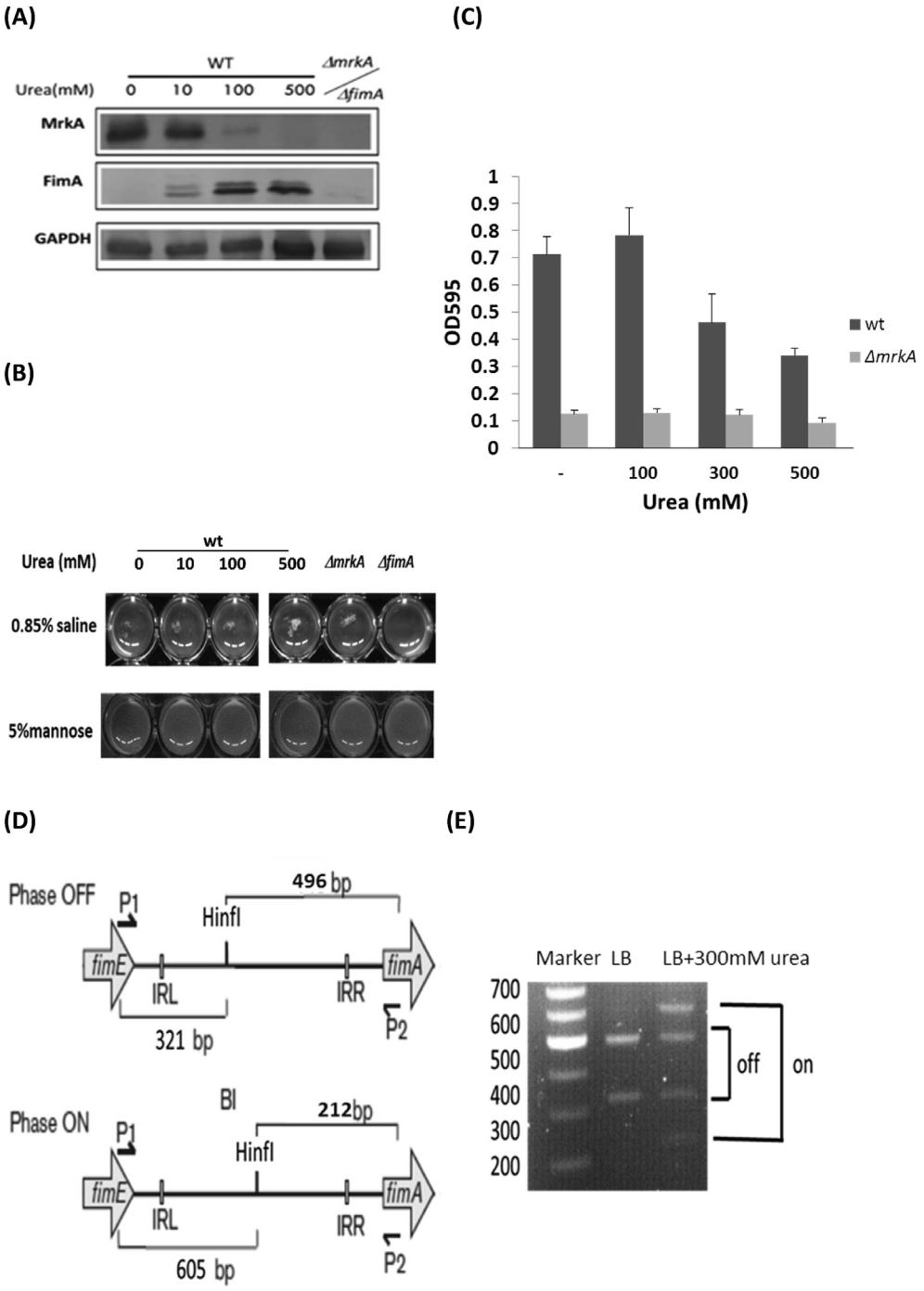

Figure 8. Urea effect on type I and type 3 fimbriae expression. (A) *K. pneumoniae* CG43S3 was grown in LB with 10, 100, and 500 mM urea for 18 h at 37 °C, and the expression of type 1 and type 3 fimbriae was determined using the analysis of Western blotting, (B)MSYA, and (C) biofilm formation. (D) Diagrammatic representation of the *fimS* promoter analysis. The bacteria Δ*mrkA*/Δ*fimA* were used as negative control respectively for the expression of MrkA and FimA. The PCR primer pair P1 and P2 (Table 2) were used for amplifying the region containing *fimS*. (E) Digested PCR products were electrophoresed on 2% agarose gels.

Ure-1 and Ure-2 are both required for the acid stress response

To assess the role of *ure*-1 and *ure*-2 in the acid stress response, acid survival analysis was performed. As shown in Fig. 6A, when compared with CG43S3, a significant decrease in survival of Δ*ureA1* was observed after the treatment with pH 2.5 for 1 h. By contrast, the *ureA1* deletion effect was not observed with pH 5 adaptation prior to the acid stress treatment. This suggests that *ure*-1 is the primary system responding to an extreme acidic stress.

H-NS has been reported to play an important role in regulating the acid stress response.[35] Fig. 6B shows that the deletion of *hns* from CG43S3 significantly increases acid survival, revealing a negative role of H-NS in the acid stress response. This is consistent with the report in *E. coli*[35] and *P. mirabilis*[36] that H-NS acts to repress the transcription of urease. In the absence of *hns*, the deletion effect of *ureA2* was significant which again supporting a negative role of H-NS in the expression of *ure-2*. However, in the absence of *hns*, deletion of *ureA1* has no apparent effect on the acid stress survival. Whether H-NS also plays a role in regulating the expression of *ure-1* upon acid stress treatment remains to be investigated.

Deletion of *ureA1* or *ureA2* affects the expression of type 1 and type 3 fimbriae

M9U containing 6 mM urea was used to substitute the modified Stuart's broth in order to quantitatively measure the urea consumption. As shown on the left panel of Fig. 7A, the culture extract of Δ*ureA1* or Δ*ureA2* after 3 h incubation in M9U had similar levels of urea to that of wt strain indicating urea consumption. The urease activity measurement shown on the right panel of Fig. 7A also demonstrated that Ure-2 as well as Ure-1 exerts a urease activity in M9U at the early growth stag, although with a lower activity level than that of wt.

Whether the urease activity influences the expression of type 1 fimbriae and type 3 fimbriae is then examined. As shown on the left panel of Fig. 7B, in M9U 3 h, deletion of either *ureA1* or *ureA2* blocked the production the major pilin of type 3 fimbriae MrkA. The deletion effect on the expression of type 1 fimbriae was much more apparent after 18 h growth in M9U. As shown in Fig. 7B, either deletion of *ureA1* or *ureA2* or double deletion has increased the production of FimA, the major pilin of type 1 fimbriae, and also the mannose sensitive yeast agglutination (MSYA) activity (Fig. 7C). These results suggest that the impairment of urease activity may result in the accumulation of urea leading to influence the expression of type 1 and type 3 fimbriae.

Urea induces the phase-ON expression of *fimS*

As shown in Fig. 8, increasing urea concentration in LB to 100 mM blocked MrkA production or to 300 mM reduced the biofilm formation, which indicating an inhibitory effect of urea on the expression of type 3 fimbriae. By contrast, 10 mM urea was able to induce FimA production and MSYA activity. The PCR analysis shown in Fig. 8E revealed that 300 mM urea was able to increase the switch-ON expression of *fimS*.

Discussion

The *ure1* gene cluster is conserved in the genomes of *K. pneumoniae* CG43, NTUH K2044, and MGH78578, whereas *ure2* is only present in CG43 (Fig. 1). The GC content analysis revealed that the GC ratio of the *ure1* operon ranged from 59.5% to 64%, while that of the *ure2* operon ranged from 45.6% to 54.6% (Fig. S2). The GC content difference may represent a different evolutionary history of *ure1* and *ure2*. The similar gene organization of *ure2* to that of the *Y. enterocolitica* urease gene cluster suggests a common ancestor.

Deletion of *ureA1* significantly inhibited cell growth in mM9-U, indicating a major role of *ure1* in urea catalysis. As shown in Fig. 2, Δ*ureA2* exhibited better growth than CG43S3 after 6 h incubation in mM9-U pH 5. As shown in Fig. S1, the growth difference assessed using colony counts between CG43S3 and Δ*ureA2* could be observed after 6 h or 12 h incubation supporting a negative role of *ure-2* on the expression of *ure-1*. In the modified Stuart's broth, the pH value increasing, from 8.27, 9.27, to 9.53 for wt and 8.3, 9.02, to 9.41 for Δ*ureA2*, over the incubation time, from 18 h, 24 h—36 h, was observed (Fig. S3). Notably, no colony count was obtained for both wt and Δ*ureA2* after 36-h incubation further demonstrating that the color change of Δ*ureA2* was due to some unknown alkaline metabolites.

Similar to the report in which the urease activity of *P. mirabilis* is urea- and acid-inducible,[36] promoter activity analysis revealed that Ure-1 activity is also induced by urea and weak acids. In contrast, the activity of $P_{ure2\text{-}2}$ was higher in M9 than in LB suggesting that *ure*-2 may be induced in minimal medium (Fig. 4A). *K. pneumoniae* W70 urease is not urea-inducible but is activated under nitrogen-limited conditions.[31] Whether *ure*-2 carries the same property as W70 urease remains to be studied.

The urease activity of *Helicobacter hepaticus* is negatively affected by iron.[3] This is similar to the urease operon regulation in *H. pylori*, where urease activity is modulated by iron availability. Deletion of *fur* or *nac* had no apparent effect on the expression of *ure-1* or *ure-2* (Fig. 4), suggesting that neither *fur* nor *nac* affects the expression of *ure-1* or *ure-2* at the transcriptional level. Nevertheless, either *fur* or *nac* deletion caused a significant reduction of the urease activity (Fig. 5). We speculate that the urease activity regulated by *fur* or *nac* is through an indirect control of the iron homeostasis or nitrogen metabolic activity.

As shown in Fig. 1, the *ure-1* and *ure-2* operons are located respectively next to the genes *hdeB1-yfdX-hdeDhdeB* and *hdeB2-hdeA*, which codes for the H-NS-dependent expression of the acid stress chaperone proteins.[37] The acid survival analysis showed that H-NS plays a negative role in the acid stress response, possibly through repression of *ure*-2 expression (Fig. 6B). As shown in Fig. 5B, Δ*hns*Δ*ureA2* exerted a relative high level of urease activity in the modified Stuart's broth. In pH 2.5 M9∗U, however, Δ*hns*Δ*ureA2* was sensitive to the acid stress treatment (Fig. 6B). We speculate that under the extreme acidic environments, pH 2.5 M9∗U, *ure-1* expression is inactive in the absence of *hns*. This suggests H-NS plays a positive role in affecting *ure-1* expression in pH 2.5 M9∗U. How H-NS differentially regulates the expression of *ure-1* and *ure*-2 requires further study.

As shown in Fig. 7A, in M9U 3 h, Δ*ureA1*Δ*ureA2* grown has no detectable urease activity while Δ*ureA1* or Δ*ureA2* still carrying certain levels of activity. Nevertheless, the residual urea was able to induce the expression of type 1 fimbriae but repress the type 3 fimbriae expression after 18 h incubation (Fig. 7B). The urea effect was further demonstrated in Fig. 8 showing that urea may serves as an inducer to increase the phase-ON switch of *fimS*. It has

been reported in *P. mirabilis* that the *ure* activator UreR could be activated upon binding to urea.[38] However, there is no *ureR* homologous gene in *K. pneumoniae* genome. Whether an unknown urea effector exists to affect the expression of type 1 and type 3 fimbriae remains to be investigated.

In summary, we have shown that *ure-1*, under nitrogen limitation conditions, is the major urease system and its activity is induced by urea or weak acids. Fur and NAC indirectly may play a positive role in the expression of *ure-1*, probably by controlling intracellular iron and nitrogen concentrations. On the other hand, the expression of *ure*-2 is induced in nutrient-limited conditions or in the absence of *the hns* gene (Fig. 4). Under the acid stress environment, in the absence of *hns* and the presence of urea, the expression of *ure-1* is inhibited by unknown factor and hence *ure-2* becomes the major urease system to respond to the acid stress (Fig. 6).

Here, we conclude with a model of that in the human intestinal tract, where the pH is approximately 5.5 and the urea concentration ranges from 1 to 10 mM,[39,40] the urease activity is induced to metabolize urea and the decreasing concentration of urea may in turn increase the expression of type 3 fimbriae to enhance the adherence activity for the bacteria to bind to the epithelial cells. In contrast, the urea concentration in urine may reach 500 mM,[41] the urease activity may be inhibited, and the accumulated urea enhances the expression of type 1 fimbriae, while the type 3 fimbriae expression is reduced.

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Authors' contributions

Conceived and designed the experiments: Wei-Feng Lin, Rong-Yu Hu, Hwan-You Chang and Hwei-Ling Peng. Performed the experiments: Wei-Feng Lin, Rong-Yu Hu, Fan-Yu Lin, Chih-Hao Kuo and Li-Hsin Su. Analyzed and interpreted the data: Wei-Feng Lin, Rong-Yu Hu, and Hwei-Ling Peng. Wrote the paper: Wei-Feng Lin and Hwei-Ling Peng. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2022.02.002.