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Temperature-Dependency Urease Activity in *Vibrio parahaemolyticus* is Related to Transcriptional Activator UreR

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***Vibrio parahaemolyticus* possessing urease-positive property is relatively rare, but such strains consistently exhibit the TDH-related hemolysin (TRH) gene. In this study, we examined the effects of incubation temperature on urease activity expression, using the TH3996 and AQ4673 strains where the enzyme activity is known to be temperature-dependent and -independent, respectively. In the TH3996 strain, β-galactosidase activity was 4.4-fold lower after 30°C cultivation than after 37°C in a *ureR–lacZ* fusion strain, but temperature dependency was not found in *ureD–* or *nikA–lacZ* fusion strains. However, *ureR–*, *ureD–*, and *nikA–lacZ* fusions of the AQ4673 strain was not influenced by incubation temperature. We compared the promoter sequences of *ureR* between the above two strains. Intriguingly, we detected mismatches of two nucleotides between the two strains located at positions –66 and –108 upstream of the methionine initiation codon for UreR. Additionally, urease activity was not affected by culture temperature at either 30°C or 37°C by allelic introduction of the AQ4673 *ureR* gene into the TH3996 *ureR* deletion mutant. Taken together, our study demonstrates that the transcriptional factor UreR is involved in the temperature dependency of urease activity, and two nucleotides within the *ureR* promoter region are of particular importance for the urease activity dependency of *V. parahaemolyticus*.**

Keywords: *Vibrio parahaemolyticus*, urease, *ureR*, temperature dependency

Urease is an enzyme that catalyzes the hydrolysis of urea to ammonia and carbon dioxide, resulting in net increase in environmental pH [13]. The bacterial urease gene clusters have been reported in several bacteria, such as *Proteus mirabilis* [8], *Klebsiella aerogenes* [14], *Helicobacter pylori* [10], *Yersinia enterocolitica* [4], *Streptococcus salivarius* [3], and *Vibrio parahaemolyticus* [19]. The organization of urease gene clusters in most bacteria is basically similar, comprising structural and accessory genes involved in the incorporation of the nickel ion into an inactive urease apoenzyme [13]. In some cases, accessory genes participate in nickel uptake or in the regulation of urease expression [13]. Two distinct high-affinity nickel transport systems have been reported in several bacteria: the single-component Ni^{2+} permease and the ATP-dependent binding cassette (ABC) transporter system [5]. The nickel ion uptake systems have been identified in *Escherichia coli* [15], *H. pylori* [6], *V. parahaemolyticus* [19], *Brucella suis* [9], *S. salivarius* [2], and *Yersinia pseudotuberculosis* [24], and in all cases, this Ni^{2+} specific transporter is shown to be associated with the ureolytic activity of microorganisms [5]. UreR is a member of the AraC family of transcriptional activators and contains a putative helix-turn-helix in addition to an AraC signature sequence [16, 19]. The dimerization domain of UreR is located in the N-terminal half of the polypeptide, and its DNA-binding domain is located within the C-terminal half [22].

Vibrio parahaemolyticus, one of the human pathogenic vibrios, is a Gram-negative halophilic bacterium that naturally inhabits marine and estuarine environments. The bacterium causes foodborne gastroenteritis that is most frequently associated with the consumption of raw or undercooked seafood [1, 7]. Interestingly, numerous studies reported that thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) are closely related to the pathogenesis of this bacterium, and are considered to be the major virulence factors [7, 25]. *V. parahaemolyticus* strains that are urease

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positive, a relatively rare phenotype, correlate completely with the possession of the *trh* gene [26], thus making urease production a reasonably good clinical diagnostic marker for *trh*-positive *V. parahaemolyticus*. The urease gene clusters of *V. parahaemolyticus* are found as a single copy in the genome. The *ure* cluster consists of eight genes, *ureDABCEFG* and *ureR*. The *ureR* gene is located 5.2 kb upstream of the other seven genes in the opposite direction [19]. Between the *ureR* and *ureD* genes, there are five overlapping genes, the *nik* operon, encoding proteins essential for the incoporation of nickel ion into the metallocenter [19].

In our previous study [19], we demonstrated that urease production of the *V. parahaemolyticus* TH3996 strain is induced in the presence of urea and is modulated by a transcriptional regulator, *ureR*. The *nik* operon also contributes to urease activity. Therefore, in the absence of urea as substrate and after disruption of *ureR* or the *nik* operon, the urease of strain TH3996 was not expressed [19].

In this study, we provide compelling evidence that the urease activity of *V. parahaemolyticus* is intimately related to the transcriptional activity of UreR, and that two nucleotides located at positions −108 and −66 upstream of the methionine initiation codon for UreR play a critical role for temperature-dependent urease activity in strain TH3996.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *V. parahaemolyticus* TH3996 and AQ4673 strains were used for the *ureR*−, *nikA*−, and *ureD*–*lacZ* fusions. All *V. parahaemolyticus* strains used for *ureR* promoter region sequences were obtained from the Laboratory for Culture Collection, Research Institute for Microbial Diseases, Osaka University, Japan *E. coli* DH5α and BL21 (DE3) strains were used for general manipulation of plasmids and expression of recombinant UreB. *V. parahaemolyticus* strains were grown in Luria–Bertani (LB) medium supplemented with 3% NaCl, and *E. coli* was grown in LB medium. Antibiotics were used at the following concentrations: ampicillin (100 μg/ml), chloramphenicol (5 μg/ml), kanamycin (30 μg/ml), and gentamicin (10 μg/ml).

DNA Manipulations

Chromosomal DNA from *V. parahaemolyticus* species were extracted from overnight cultures of LB broth supplemented with 3% NaCl by a method described previously [28]. Plasmid DNA used for nucleotide sequence analysis was extracted from *E. coli* DH5α using the QIAprep Spin Miniprep Kit according to the manufacturer's recommendations (Qiagen Sciences). Cloning, restriction endonuclease digestion, DNA ligation, bacterial transformation, and agarose gel electrophoresis were performed by standard protocols as described previously [23]. All of the restriction enzymes, alkaline phosphatase, and DNA ligation kit were purchased from Takara Shuzo (Osaka, Japan).

Cloning and Sequencing of *ureR* Promoter Region

The *ureR* promoter region from urease-positive *V. parahaemolyticus* strains was PCR–amplified with a Takara *Ex Taq* kit (Osaka, Japan) using the following oligonucleotide primers: 5'-AAATGCGAACAA TGGCATTTA-3' and 5'-CTTGGATGATTGAATGTTTTT-3'. The PCR product was cloned into a pT7Blue T-vector, and at least three clones per strain were sequenced in both directions to establish error-free consensus sequences. The nucleotide sequences were determined with a Licor (Model 4000L, Lincolin, Nebr.) automated DNA sequencer using universal M13 IRD41 Infrared dye-labeled primers. Computer analysis of DNA sequences was performed with the DNASIS program (Hitachi Software, Tokyo, Japan).

Protein Purification and Antibody Preparation

PCR-amplified DNA harboring the TH3996 *ureB* gene was cloned into pET-30a (+) under the control of a T7lac promoter using the following oligonucleotide primers: 5'-AGTAAACCCATGGTTCCG GG-3' and 5'-ACGCGAAAGTCGACTCATGAT-3'. The His-tag-containing UreB protein was expressed in *E. coli* BL21 (DE3).

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristics	Source or reference
V. parahaemolyticus		
AQ4673	Clinical isolate, *tdh*$^+$, *trh*$^+$, *ure*$^+$	[29]
TH3996	Clinical isolate, *tdh*$^-$, *trh*$^+$, *ure*$^+$	[19]
TH3996-RD	TH3996, *ureR* deletion mutant	This study
TH3996-AE	Allelic introduce of AQ4673 *ureR* intoTH3996-RD	This study
E. coli		
DH5	*hsdR recA lacZYA* π80 *lacZ*ΔM15	Laboratory collection
BL21 (DE3)	F- *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm* (DE3)	Novagen
Plasmids		
pT7Blue T-vector	Multicopy (ColE1 *ori*) TA cloning vector, Amp^r	Novagen
pET30-a(+)	Expression vector, Km^r	Novagen
pHRP309	*lacZ* Transcriptional fusion vector, Gm^r	[18]
pSA19CP	Plasmid vector of *V. parahaemolyticus* origin, Cm^r	[17]
pKS-1	pT7Blue T-vector introduced with the PCR-amplified AQ4673 *ureR* gene containing promoter region	This study
pKS-2	Up-linker ligated into pKS-1	This study
pKS-3	Down-linker ligated into pKS-2	This study

Isopropylthiogalactoside (IPTG) was added to a final concentration of 1 mM, and the culture was grown for an additional 4 h. Bacteria were harvested by centrifugation and lysed by sonication. Recombinant UreB was separated from the cell lysate by performing affinity chromatography with His-Bind affinity resin (Novagen). Recombinant UreB was eluted from the column with an imidazole gradient, and recombinant-UreB-containing fractions were collected and dialyzed into phosphate-buffered saline (PBS; 135 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 8.0). Polyclonal antibody to UreB was raised by immunizing female New Zealand white rabbits as described previously [20]. Specificity of antibody preparations was confirmed by immunoblot analysis of the 15% polyacrylamide SDS–PAGE gel resolved from the whole-cell lysate of *E. coli* BL21 (DE3) expressing the respective target protein.

Western Blot Analysis

Equal volumes of sample and 2× sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 0.004% bromophenol blue, 10% sucrose, 10% 2-mercaptoethanol) were mixed, and the mixture was boiled for 5 min, and loaded onto a 15% polyacrylamide SDS–PAGE gel. Proteins were transferred onto a nitrocellulose membrane (PVDF, Millipore) using a Trans Blot electrophoretic transfer cell (Bio-Rad). The membrane was blocked with 5% skimmed milk in Tris-buffered saline (20 mM Tris, pH 7.5, 137 mM NaCl) containing 0.1% Tween-20 (TBST) and probed with anti-UreB diluted to 1:3,000 for 1 h at room temperature. The secondary antibody was anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Amersham Life Sciences). The blots were developed with an ECL Western blotting kit (Amersham Biosciences) according to the manufacturer's instructions.

β-Galactosidase Assay

The putative promoter regions of *ureR*, *ureD*, and *nikA* of *V. parahaemolyticus* TH3996 and AQ4673 strains were amplified by PCR using the following oligonucleotide primers: for *ureR*, 5'-TCAAAGTCGACCAATGGCATT-3' and 5'-TGTTTTTGAATTCCA TAGTTG-3'; for *ureD*, 5'-ATGACAAGTCGACTGTTT-3' and 5'-ATATCAAGGATCCGAACCTCT-3'; and for *nikA*, 5'-TCTCTGGTC GACATAATACAT-3' and 5'-AACTTTTTGAATTCTTGATTT-3'. Each amplified fragment was cloned into a pT7Blue T-vector and then digested with SalI/EcoRI or SalI/BamHI. These DNA fragments were cloned into a promoterless *lacZ* fusion vector pHRP309 [18] and introduced into strain TH3996 by electroporation (1.5 kV; 1,000 Ω; 25 μF). Bacteria were harvested after growing in LB broth supplemented with 3% NaCl to an OD_{600} of 0.6 to 0.8. β-Galactosidase assays were performed as described previously [11]. Three independent experiments were performed in triplicate when the β-galactosidase activity was assessed.

Urease Assay

Bacteria were grown at 37°C for 14 h in 50 ml of LB broth containing 3% NaCl and 30 mM urea. Bacteria were harvested by centrifugation, washed twice with 20 mM sodium phosphate (pH 7.0), 5 mM dithiothreitol, and 1 mM EDTA, and disrupted by sonication. Urease activity was determined using an ammonia test kit (Wako Pure Chemical Industries, Osaka, Japan) to measure the release of ammonia, as described previously [19]. The amount of ammonia produced was calculated by referring to a standard curve constructed from concentrations of ammonium chloride. Urease activity was expressed as micromoles of urea hydrolyzed per minute per milligram of protein.

Construction of *ureR* Deletion Mutant

The *ureR* deletion mutant of TH3996 strain was constructed using primers with the following sequences by using a method described previously [20]: R-M1 (5'-AACATTCAAGGATCCAAGTCA-3'), R-M2 (5'-ATGCCATTTTCTTTGTTGATGGTGTTTAT-3'), R-M3 (5'-AATAAACACCATCAACAAAGAAAATGGC-3'), and R-M4 (5'-TCGAAACTGCAGTGGTGTTAC-3'). The resulting *ureR* deletion mutant in this manner was a construct of 243-bp deletion within the *ureR* gene. Analysis for the deletion mutant was confirmed by PCR and Southern hybridization.

Allelic Introduction of AQ4673 *ureR* into TH3996 *ureR* Deletion Mutant Strain

The *ureR* gene containing the promoter region from strain AQ4673 was amplified by PCR using the following oligonucleotide primers: 5'-TTAGGCATCGACTAGTTTTTA-3' and 5'-AAGCAGAAAGAG TCGACTAGA-3'. The amplified fragment was cloned into a pT7Blue T-vector and then digested with SpeI and SalI. For construction of up- and down-linkers for allelic introduction, primers with the following sequences were used: for the up-linker, 5'-AAGCCAGCA AGATCTCCACTT-3' and 5'-TTGTTGGAGACTAGTTCACGT-3'; for the down-linker, 5'-ATTTGGTGCGTCGACGTTTTT-3' and 5'-ACA CCTCAACTGCAGCGTACA-3'. These DNA fragments of up- and down-linkers were cloned into the pKS-2 and pKS-3 vectors, and then pKS-3 was digested with PstI and BglII. These DNA fragments were cloned into PstI and BamHI sites using the pYAK vector [20], which contains the *sacB* gene conferring sensitivity to sucrose. This plasmid was introduced into *E. coli* SM10λ*pir* [12], which was then conjugated with the TH3996 *ureR* deletion mutant strain. Allelic-introduced strains were obtained by screening with TCBS agar (Merck) plates containing 5 μg/ml chloramphenicol and then selected on LB plates supplemented with 3% NaCl and 10% sucrose. The allelic-introduced strains were confirmed by Southern hybridization with a probe for the commonly deleted portion of *ureR*. The PCR primers used for probe construction were 5'-TTTAATCACCAACCG ATGTAT-3' and 5'-TGGATCCTGAGTTATCAATCT-3'. The DNA probe was labeled with digoxigenin by a random primer extension method using a DIG DNA labeling kit (Roche Molecular Biochemicals).

Results

Temperature Dependency of Urease Activity in TH3996 Strain

The urease activity of *V. parahaemolyticus* TH3996 is known to be different when grown at 30°C and 37°C. Thus, we conducted detail analyses to examine the effects of incubation temperature on urease activity in the TH3996 strain. As expected, urease activity was barely detected in strain TH3996 cultured at 30°C, whereas the activity was dramatically increased to at least 25-fold at growth at 37°C. However, urease activity of the AQ4673 strain was already high at 30°C, and did not change further at 37°C (Fig. 1A). The results suggest the possibility that the urease activity of urease-positive *V. parahaemolyticus* strains is dependent or independent upon incubation temperature. To elucidate this hypothesis, we examined

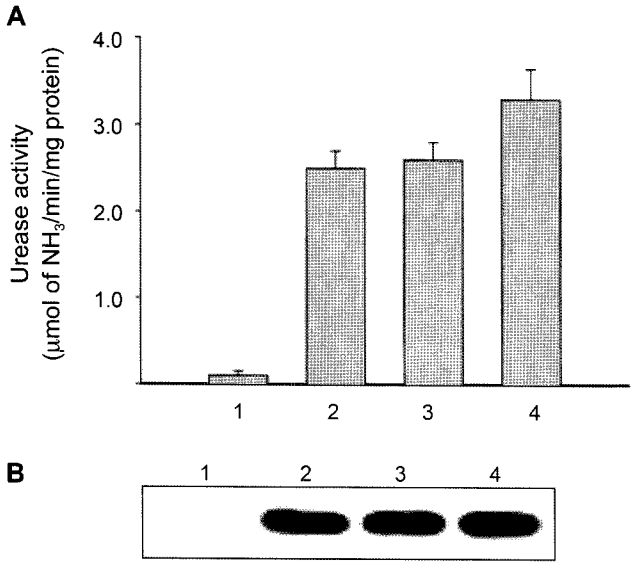

Fig. 1. Urease activity (**A**) and UreB expression (**B**) in *V. parahaemolyticus* TH3996 and AQ4673 strains cultivated at different temperatures.

Bacteria were grown for 14 h at 30 or 37°C with shaking in LB medium containing 3% NaCl and 30 mM urea. **A**. Urease activity assays were performed as described previously [19]. The data shown are the average of three independent experiments. Error bars represent standard deviations. **B**. Whole-cell proteins (10^6 CFU) were loaded onto a 10% polyacrylamide SDS-PAGE gel, transferred to nitrocellulose membrane, and probed with an α-UreB antibody, followed by α-rabbit secondary antibodies, and then developed by ECL. Lanes 1 and 2 from TH3996; Lanes 3 and 4 from AQ4673; Lanes 1 and 3 are from cultures at 30°C; Lanes 2 and 4 are from cultures at 37°C.

whether urease structural subunit (UreA, B, and C) levels are correlated with urease activity by using a polyclonal UreB antibody (Fig. 1B). As shown in Fig. 1B, UreB expression was detected in both the TH3996 and AQ4673 strains when grown at 37°C. When grown at 30°C, however, UreB expression was not detected by UreB antibody in strain TH3996. These data suggest that the urease structural subunit of the TH3996 strain was expressed at 37°C, but not at the lower temperature, 30°C. However, UreB expression in strain AQ4673 was not influenced by incubation temperature. Thus, these data suggest that the temperature dependence in urease activity involves other factor(s) in addition to urease structural subunits.

Table 2. Temperature-dependent expression of *ureR–lacZ* fusions.

Strain	β-Galactosidase activity (U)[a]		
	30°C	37°C	Expression ratio (37°C/30°C)
TH3996			
ureD	647±36	718±46	1.11
nikA	1,604±126	1,805±115	1.13
ureR	101±18	442±33	4.38
AQ4673			
ureD	660±41	760±52	1.15
nikA	1,852±177	2,024±152	1.09
ureR	1,320±104	1,634±144	1.24

[a] Units of β-galactosidase activity are calculated according to Miller [11]. The results represent the average of at least three independent experiments.

Involvement of Transcriptional Activator UreR in Temperature-Dependent Urease Activity

To understand the mechanism for temperature dependence in urease activity, we constructed the *lacZ* fusion vectors containing *ureR*, *nikA*, and *ureD* gene promoter regions from the TH3996 and AQ4673 strains. These plasmids were introduced into the TH3996 strain and then assayed for β-galactosidase levels. The *ureD–* and *nikA–lacZ* fusions of both strains did not show any significant difference in β-galactosidase activity as compared between 30°C and 37°C. With the *ureR–lacZ* fusion of TH3996, however, the enzyme levels were 4.4-fold lower at 30°C compared with when grown at 37°C. In addition, when compared between TH3996 and AQ4673 *ureR–lacZ* fusions, 13.1-fold and 3.7-fold higher activities were observed in the AQ4673 strains at 30°C and 37°C, respectively (Table 2). These results suggest that the temperature-dependent urease activity of strain TH3996 is linked to *ureR* gene but not to the *ureD* and *nikA*.

Comparison of *ureR* Gene Promoter Region Between TH3996 and AQ4673 Strains

We compared the *ureR* gene sequence contained in the promoter region of the two strains. The putative *ureR* promoter in both strains is composed of 146-bp nucleotides of which only two nucleotides are different in the two strains studied: position −108 is T in AQ4673 and C in

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AQ4673 : GCATCGACTAACTTTTATCGATGTAACGTATCATTGCGTTATTTTAGGAAATAAAGCATTAACGCATT
TH3996 : GCATCGACTAACTTTTATCGATGTAACGTATCATTGCGCTATTTTAGGAAATAAAGCATTAACGCATT

         ATATACTTGCTTGGTTTTTAGTCGCATGGATGTTTTACTTTTTACTACCAGACAAGTTTTCAATAAAG
         ATATACTTGCTTAGTTTTTAGTCGCATGGATGTTTTACTTTTTACTACCAGACAAGTTTTCAATAAAG
                  +1
                  ▪
         ATAAACAACTATG
         ATAAACAACTATG
```

Fig. 2. Nucleotide sequence determination and comparison of the *ureR* gene promoter region of the TH3996 and AQ4673 strains.

Positions of the putative −35 and −10 sequences are underlined. The two putative UreR-binding sites are in boldface.

TH3996; position -66 is G in AQ4673 and A in TH3996 (Fig. 2). We found the classical gene promoter motif (-10 and -35 regions) in the *ureR* gene promoter region, but the classical motif for the Shine–Dalgarno site of this bacterium was not found. The consensus UreR-binding site, T(A/G)(T/C)(A/T)(T/G)(C/T)T(A/T)(T/A) ATTG, defined in *P. mirabilis* and in plasmid-encoded *E. coli* [27] was not found in the *ureR* promoter region of the TH3996 and AQ4673 strains. However, two putative UreR-binding sites that partially match the consensus UreR-binding site of *P. mirabilis* and *E. coli* were found to exist. At -112 ~ -103 bp upstream of the methionine initiation codon for *ureR*, there was a TGCGT/CTATTT sequence. At -72 ~ -63 bp, there also was a TGCTTG/AGTTT sequence. Intriguingly, the different two nucleotides in the *ureR* promoter region of both strains exist within the putative UreR-binding sites. Therefore, we suggest the possibility that the two putative UreR-binding sites, either one or both, are involved in the temperature-dependent urease activity.

Allelic Introduction of AQ4673 *ureR* Gene into TH3996 *ureR* Mutant Strain and Urease Activity Changes

We examined whether urease activity can recovery by allelic introduction of the AQ4673 *ureR* gene into the TH3996 *ureR* mutant strain. The allelic construct was prepared by homologous recombination as described in the Materials and Methods section. In strain TH3996, a 4.2-kb DNA fragment was detected when hybridization was carried out with the SpeI digests of chromosomal DNA used as a specific probe. Furthermore, in an allelic introduction where the AQ4673 *ureR* gene was introduced into the TH3996 *ureR* deletion mutant strain, a single 13.0-kb DNA fragment was detected after hybridization with a specific probe. In this hybridization, the resultant insertion in the suicide vector occurred in the *cya* (adenylate cyclase, VP2987) gene used as a linker (Fig. 3).

The recovery of the urease activity of the strain was checked both by enzyme assay and by Western blotting with UreB antibody. When the *ureR*-allelic-introduced strain was grown at either 30°C or 37°C, the level of the urease activity was similar to the wild-type TH3996 strain that was grown at 37°C. However, in the TH3996 *ureR* mutant strain used as the parent strain, no enzyme activity was detected even when grown at 37°C (Fig. 4A). These data suggest that high urease activity, even if grown at 30°C, was due to an overexpression of UreR by the AQ4673 *ureR* gene complementation. Consistent with the results shown in Fig. 4B, UreB expression in the *ureR*-allelic-introduced strain was detected with UreB antibody when grown at either 30°C or 37°C (Fig. 4B). These observations suggest that UreR expression in TH3996 grown at 30°C led to extremely low enzyme activities. It is suggested that the transcriptional activator UreR is normally expressed when grown at 37°C, but its expression at 30°C is absent or minimal at best.

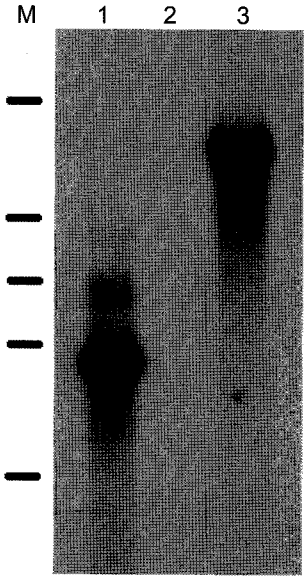

Fig. 3. Confirmation of allelic introduction of the AQ4673 *ureR* gene into the TH3996 *ureR* deletion mutant strain by Southern blot analysis.

The chromosome DNA of each strain was digested with SpeI enzyme. Lane M, Lambda DNA/HindIII marker; Lane 1, total DNA of TH3996; Lane 2, total DNA of TH3996 *ureR* deletion mutant; Lane 3, total DNA of allelic introduction of the AQ4673 *ureR* gene into the TH3996 *ureR* deletion mutant strain.

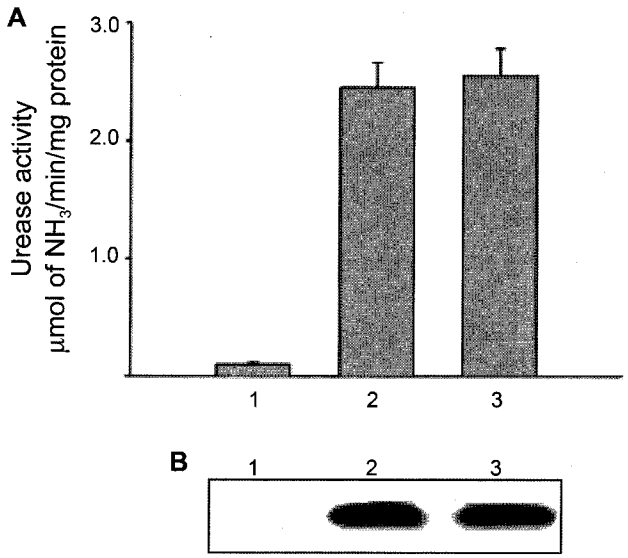

Fig. 4. Urease activity (**A**) and UreB expression (**B**) by allelic introduction of the AQ4673 *ureR* gene into the TH3996 *ureR* deletion mutant strain.

Bacteria were grown for 14 h at 30 or 37°C with shaking in LB medium containing 3% NaCl and 30 mM urea. Lane 1 is TH3996 *ureR* deletion mutant; Lanes 2 and 3 from AQ4673 *ureR*-allelic-introduced strain. Lanes 1 and 3 are from cultures at 37°C; Lane 2 is from cultures at 30°C.

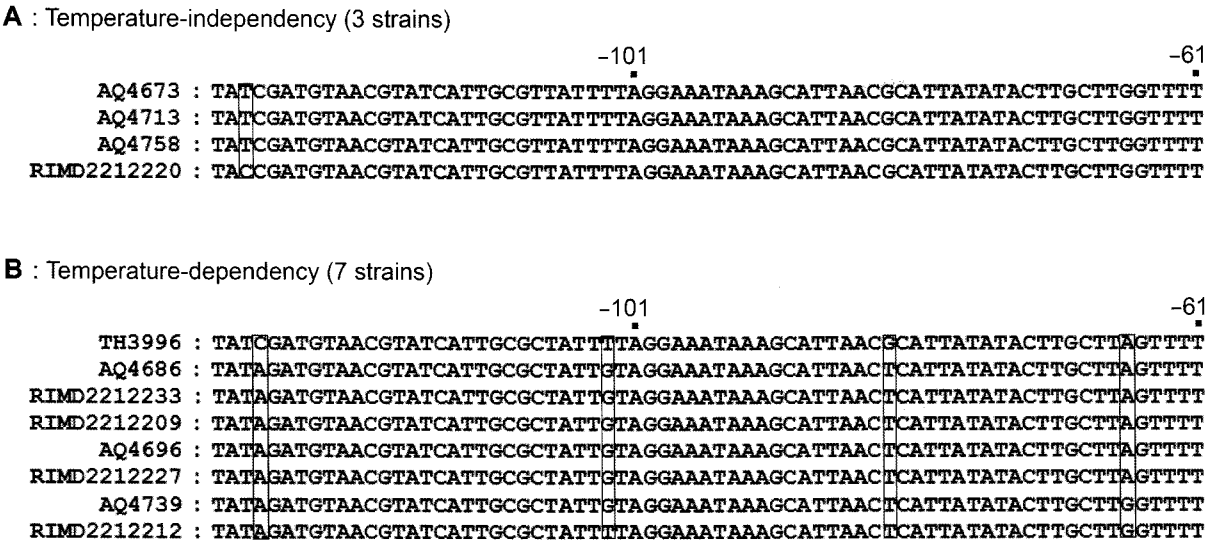

Fig. 5. Temperature-independent (**A**) and temperature-dependent (**B**) strains.
Nucleotide sequence determination and comparison of the *ureR* gene promoter region of urease-positive *V. parahaemolyticus* strains.

Sequences of *ureR* Promoter Region in Urease-Positive *V. parahaemolyticus* Strains

To determine the relationship of the *ureR* promoter regions with the temperature-dependent urease activity, we cloned and sequenced the *ureR* promoter regions derived from various urease-positive *V. parahaemolyticus* strains. We first examined urease expression of the 10 different urease-positive strains by measuring UreB expression. In 3 out of 10, we found that UreB expression was temperature-independent. In the remaining seven, UreB expression was observed only at 37°C but not at 30°C (data not shown). This dissimilarity is somewhat similar to the phenomenon observed between the AQ4673 and TH3996 strains. Therefore, we cloned and sequenced the *ureR* promoter region of the 10 strains for further analysis. The three temperature-independent strains were found to have nucleotides T and G at positions −108 and −66, respectively, as observed in strain AQ4673. However, in one strain, there was a base replacement with C at position −129, whereas it was T in the other two strains (Fig. 5A). In the other seven temperature-dependent strains, we consistently found C at position −108, as was the case in strain TH3996. However, at position −66, the nucleotide was found to be replaced with either A or G (Fig. 5B). Together, our promoter region sequence analyses suggest that nucleotides placed at positions −108 and −66 are critical for temperature independency of urease activity.

DISCUSSION

It is generally accepted that urease-positive *V. parahaemolyticus* strains are rare, but these strains are always associated with the possession of the *trh* gene [26]. In previous studies [19], we cloned and sequenced a DNA region containing the urease gene clusters and *trh* gene from *V. parahaemolyticus* strain TH3996. This DNA region seems to have been introduced into *V. parahaemolyticus* in the past through a mechanism mediated by insertion sequences, because at the end of the region, an insertion sequence-like element was confirmed that was not normally found in Kanagawa phenomenon-positive and environmental strains [19]. The enzymatic property found in the urease of *V. parahaemolyticus* is similar to that of *P. mirabilis* in many ways, such as the organization of gene cluster, induction, and mediation pattern of expression [19]. UreR is a member of the AraC family of transcriptional activators, and it promotes transcription of genes required for the synthesis of urease structural and accessory proteins in *V. parahaemolyticus* and *P. mirabilis*. The *V. parahaemolyticus* UreR sequence (282 aa, 32.9 kDa) is 53% identical to the *P. mirabilis* UreR. In this study, we demonstrated that the expression of the gene in some *V. parahaemolyticus* strains is controlled by the *ureR* gene in a manner dependent upon incubation temperature. *V. parahaemolyticus* TH3996 cultured at 37°C had 25-fold higher urease activity than when cultured at 30°C in the presence of urea. In the urease-positive strain AQ4673, however, the activity was not different between the two culture temperatures of 30°C and 37°C (Fig. 1A). In *P. mirabilis*, expression of the urease gene cluster was observed to be thermoregulated, and its enzyme activity in the presence of urea at 37°C was 2-fold higher than at 25°C [21]. However, the mechanism for temperature dependency in urease activity has not yet been elucidated.

Thomas and Collins [27] identified two distinct UreR-binding sites, T(A/G)(T/C)(A/T)(T/G)(C/T)T(A/T)(T/A)ATTG, in the *ureR*p–*ureD*p intergenic region of *Enterobacteriaceae*

plasmid-encoded and *P. mirabilis* urease operons by employing electrophoretic mobility shift assay (EMSA) and DNase I footprint assays. In addition, the affinity of UreR for DNA-binding sites increases significantly in the presence of urea [27]. In the case of urease-positive *V. parahaemolyticus* strains, two distinct putative UreR-binding sites were located in the *ureR* promoter region, at −112 ~ −103 bp and −72 ~ −63 bp upstream of the methionine initiation codon for *ureR* (Fig. 2). These DNA region sequences were different between temperature-dependent and -independent strains. In the temperature-independent strains, nucleotide sequences of two putative UreR-binding sites were identical, whereas they were different in temperature-dependent strains (Fig. 5A and 5B). Therefore, we propose that the difference in urease activity in cultures grown at different temperatures derives from the change in affinity of the UreR binding in urease-positive *V. parahaemolyticus*. In other words, UreR binds, at 37°C but not at 30°C, to two putative sites in the promoter region of *ureR* in all urease-positive *V. parahaemolyticus* strains. However, UreR binds loosely at 30°C to either one or two sites responsible for temperature dependency. As the final consequence, regulation of urease expression is incomplete, resulting in a low level of urease activity. In fact, urease activity between the TH3996 and AQ4673 strains at 37°C showed no distinguishable difference. Moreover, urease activity in the TH3996 *ureR* mutant strain was fully restored even at 30°C following complementation of the AQ4673 *ureR* gene. These data are consistent with the hypothesis mentioned above. The intriguing issue of how UreR can regulate urease expression in a temperature-dependency manner should be addressed through future experiments.

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References

1. Blake, P. A., R. E. Weaver, and D. G. Hollis. 1980. Diseases of humans (other than cholerae) caused by vibrios. *Annu. Rev. Microbiol.* **34:** 341–367.
2. Chen, Y. Y. and R. A. Burne. 2003. Identification and characterization of the nickel uptake system for urease biogenesis in *Streptococcus salivarius* 57.I. *J. Bacteriol.* **185:** 6773–6779.
3. Chen, Y. Y., K. A. Clancy, and R. A. Burne. 1996. *Streptococcus salivarius* urease: Genetic and biochemical characterization and expression in a dental plaque *Streptococcus*. *Infect. Immun.* **64:** 585–592.
4. De Koning-Ward, T. F., A. C. Ward, and R. M. Robins-Browne. 1994. Characterisation of the urease-encoding gene complex of *Yersinia enterocolitica*. *Gene* **145:** 25–32.
5. Eitinger, T. and M. A. Mandrand-Berthelot. 2000. Nickel transport systems in microorganisms. *Arch. Microbiol.* **173:** 1–9.
6. Hendricks, J. K. and H. L. Mobley. 1997. *Helicobacter pylori* ABC transporter: Effect of allelic exchange mutagenesis on urease activity. *J. Bacteriol.* **179:** 5892–5902.
7. Honda, T. and T. Iida. 1993. The pathogenicity of *Vibrio parahaemolyticus* and the role of the thermostable direct haemolysin and related haemolysin. *Rev. Med. Microbiol.* **4:** 106–113.
8. Jones, B. D. and H. L. Mobley. 1989. *Proteus mirabilis* urease: Nucleotide sequence determination and comparison with Jack bean urease. *J. Bacteriol.* **171:** 6414–6422.
9. Jubier-Maurin, V., A. Rodrigue, S. Ouahrani-Bettache, M. Layssac, M. A. Mandrand-Berthelot, S. Kohler, and J. P. Liautard. 2001. Identification of the *nik* gene cluster of *Brucella suis*: Regulation and contribution to urease activity. *J. Bacteriol.* **183:** 426–434.
10. Labigne, A., V. Cussac, and P. Courcoux. 1991. Shuttle cloning and nucleotide sequences of *Helicobacter pylori* genes responsible for urease activity. *J. Bacteriol.* **173:** 1920–1931.
11. Miller, J. H. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
12. Miller, V. L. and J. J. Mekalanos. 1988. A novel suicide vector and its use in construction of insertion mutations: Osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J. Bacteriol.* **170:** 2575–2583.
13. Mobley, H. L., M. D. Island, and R. P. Hausinger. 1995. Molecular biology of microbial ureases. *Microbiol. Rev.* **59:** 451–480.
14. Mulrooney, S. B. and R. P. Hausinger. 1990. Sequence of the *Klebsiella aerogenes* urease genes and evidence for accessory proteins facilitating nickel incorporation. *J. Bacteriol.* **172:** 5837–5843.
15. Navarro, C., L. F. Wu, and M. A. Mandrand-Berthelot. 1993. The *nik* operon of *Escherichia coli* encodes a periplasmic binding-protein-dependent transport system for nickel. *Mol. Microbiol.* **9:** 1181–1191.
16. Nicholson, E. B., E. A. Concaugh, P. A. Foxall, M. D. Island, and H. L. Mobley. 1993. *Proteus mirabilis* urease: Transcriptional regulation by UreR. *J. Bacteriol.* **175:** 465–473.
17. Nomura, T., H. Hamashima, and K. Okamoto. 2000. Carboxy terminal region of haemolysin of *Aeromonas sobria* triggers dimerization. *Microb. Pathog.* **28:** 25–36.
18. Parales, R. E. and C. S. Harwood. 1993. Construction and use of a new broad-host-range *lacZ* transcriptional fusion vector, pHRP309, for Gram− bacteria. *Gene* **133:** 23–30.
19. Park, K.-S., T. Iida, Y. Yamaichi, T. Oyagi, K. Yamamoto, and T. Honda. 2000. Genetic characterization of DNA region containing the *trh* and *ure* genes of *Vibrio parahaemolyticus*. *Infect. Immun.* **68:** 5742–5748.
20. Park, K.-S., T. Ono, M. Rokuda, M.-H. Jang, K. Okada, T. Iida, and T. Honda. 2004. Functional characterization of two type III secretion systems of *Vibrio parahaemolyticus*. *Infect. Immun.* **72:** 6659–6665.

21. Poore, C. A. and H. L. Mobley. 2003. Differential regulation of the *Proteus mirabilis* urease gene cluster by UreR and H-NS. *Microbiology* **149:** 3383–3394.
22. Poore, C. A., C. Coker, J. D. Dattelbaum, and H. L. Mobley. 2001. Identification of the domains of UreR, an AraC-like transcriptional regulator of the urease gene cluster in *Proteus mirabilis*. *J. Bacteriol.* **183:** 4526–4535.
23. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual,* 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
24. Sebbane, F., M. A. Mandrand-Berthelot, and M. Simonet. 2002. Genes encoding specific nickel transport systems flank the chromosomal urease locus of pathogenic *Yersiniae*. *J. Bacteriol.* **184:** 5706–5713.
25. Shirai, H., H. Ito, T. Hirayama, Y. Nakabayashi, K. Kumagai, Y. Takeda, and M. Nishibuchi. 1990. Molecular epidermiologic evidence for association of thermostable direct hemolysin (TDH) and TDH-related hemolysin of *Vibrio parahaemolyticus* with gastroenteritis. *Infect. Immun.* **58:** 3568–3573.
26. Suthienkul, O., M. Ishibashi, T. Iida, N. Nettip, S. Supavej, B. Eampokalap, M. Makino, and T. Honda. 1995. Urease production correlates with possession of the *trh* gene in *Vibrio parahaemolyticus* strains isolated in Thailand. *J. Infect. Dis.* **172:** 1405–1408.
27. Thomas, V. J. and C. M. Collins. 1999. Identification of UreR binding sites in the *Enterobacteriaceae* plasmid-encoded and *Proteus mirabilis* urease gene operons. *Mol. Microbiol.* **31:** 1417–1428.
28. Wilson, K. 1987. Preparation of genomic DNA from bacteria, pp. 2.4.1–2.4.2. *In* F. M. Ausubel, R. Brent and R. E. Kingston *et al.* (eds.). *Current Protocols in Molecular Biology,* Vol. 1. John Wiley & Sons, Inc., New York, NY.
29. Yamaichi, Y., T. Iida, K.-S. Park, K. Yamamoto, and T. Honda. 1999. Physical and genetic map of the genome of *Vibrio parahaemolyticus*: Presence of two chromosomes in *Vibrio* species. *Mol. Microbiol.* **31:** 1513–1521.