Homodimerization and Binding of Specific Domains to the Target DNA Are Essential Requirements for HlyU To Regulate Expression of the Virulence Gene *rtxA1*, Encoding the Repeat-in-Toxin Protein in the Human Pathogen *Vibrio vulnificus*^{∇†}

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The virulence gene *rtxA1*, encoding the repeat-in-toxin protein, plays an essential role in the pathogenesis of *Vibrio vulnificus* infections. Expression of this gene is controlled by the HlyU regulator by direct contact of the DNA upstream of the *rtxA1* toxin operon acting as a derepressor of the H-NS protein. The crystal structure suggests that HlyU forms a homodimer *in vitro*. However, knowledge of the biological implications of these findings *in vivo* is limited. In this work, we endeavored to dissect, using genetic and biochemical approaches, the domains of this protein that are essential for homodimer formation and the interaction of HlyU with the target DNA. We identified that residues L18, N22, R25, S54, Q55, L57, W59, R61, K70, and Y77 are essential for the HlyU protein binding to the DNA and that amino acids L17 and L91 are important for HlyU dimerization. We also determined that HlyU homodimer formation is an essential requirement for binding to the upstream region of the *rtxA1* operon and is the key feature in relieving the H-NS repression of *rtxA1* transcription.

Vibrio vulnificus is an opportunistic human pathogen capable of causing fatal primary septicemia after ingestion of contaminated seafood or necrotizing fasciitis after contamination of wounds (4, 19, 24). Septicemia occurs mainly in patients who are immunocompromised, suffering from hemochromatosis, or have other underlying liver disorders, such as cirrhosis and alcoholic liver disease (8, 25). The common theme in most of these patients is that iron is present at a level higher than the physiological level (27). During *V. vulnificus* infections, the bacterium reaches the intestine and then invades the bloodstream by crossing the intestinal mucosal barrier of the host, which results in a systemic septicemia (9). Using an *in vivo*-induced antigen technology (IVIAT) to study blood from hemochromatosis patients, it was possible to identify antibodies against several *V. vulnificus* proteins, among them, the HlyU protein, that is a homologue of the hemolysin gene regulator in *Vibrio cholerae* (7). It was further demonstrated that this protein is essential for virulence of *V. vulnificus* (7, 12). HlyU belongs to the ArsR/SmtB protein family (26). Members of this family are mostly metal-regulatory transcriptional repressors that normally repress the expression of operons linked to concentrations of stress-inducing heavy metal ions (3, 14, 15). However, sequence analysis shows that HlyU does not possess a metal-binding domain (21). In our previous

work, we demonstrated that HlyU regulates the expression of the RTX (RTX stands for repeats in toxin) toxin *rtxA1* gene by direct contact of the DNA upstream of the *rtxA1* toxin operon (12). RTX toxin is the largest single-polypeptide toxin known, and it is proposed to form a pore in the eukaryotic cell plasma membrane for translocation of the central portion of the secreted toxin to the cytosol (22). Our lab and others demonstrated that the RtxA1 protein is responsible not only for cytotoxicity but that it also plays an essential role in the pathogenesis of *V. vulnificus* infections (2, 5, 10, 12).

In our previous work (13), using a DNase I footprint assay, we identified the binding DNA sequence for HlyU in the promoter region of the *rtxA1* operon. However, *lacZ* fusion experiments demonstrated that HlyU regulation must occur by derepression, rather than by positive regulation, of the *rtxA1* operon. That work also demonstrated that *rtxA1* expression is normally silenced by the master regulator H-NS and that HlyU acts as a derepressor by displacing H-NS from its binding sites, thus relieving the repression of *rtxA1* (13).

Saha and Chakrabarti developed an *in silico* model of the *V. cholerae* HlyU protein (21), and recently, the crystal structure of the *V. vulnificus* HlyU was solved (18). The monomer of *V. vulnificus* HlyU comprises five α -helices and two β -strands, some of them looking like a typical DNA-binding winged helix-turn-helix (wHTH) motif. Crystal structures, and therefore the models based on them, might not be biologically relevant, and the HlyU dimeric crystal structures might not exist *in vivo*. Thus, in this work, we endeavored to dissect, using genetic and biochemical approaches, the actual domains of this protein that are essential for activity and dimer formation to explore new avenues in the understanding of the mechanism of derepression of H-NS by HlyU.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and primers. The bacterial strains and plasmids used in this work are listed in Table S1 in the supplemental material, and all primers used are listed in Table S2 in the supplemental material. *Escherichia coli* strains were grown in LB broth or on LB containing 1.5% (wt/vol) agar at 37°C. *Vibrio vulnificus* strains were grown aerobically in Trypticase soy broth with 1.5% NaCl (TSBS) at 37°C. When needed, the following antibiotics were added to the medium: 100 µg/ml ampicillin, 30 µg/ml chloramphenicol, and 50 µg/ml kanamycin for *E. coli* and 2 µg/ml chloramphenicol and 400 µg/ml ampicillin for *V. vulnificus*. All plasmids to be conjugated into *V. vulnificus* were first transformed into *E. coli* S17-1 (*Xpir*), which was used as the donor strain. Conjugation from *E. coli* to *V. vulnificus* was carried out by biparental mating (20).

DNA manipulations and sequence analysis. Plasmid DNA was prepared by a Qiaprep miniprep kit (Qiagen). DNA digestion and ligation were performed according to the manufacturer's instructions (New England Biolabs). PCRs were carried out using a Mycycler thermal cycler as specified by the manufacturer (Bio-Rad Laboratories). PCR products were purified by Qiaquick PCR purification kit or Qiaquick gel extraction kit (Qiagen). DNA sequencing reactions were carried out by Oregon Health and Science University MMI Research Core Facility, using an Applied Biosystems 3130xl automated sequence analysis system.

SDM. The desired alanine substitutions were generated by using the QuikChange II site-directed mutagenesis kit (Agilent Technologies). First, the *hlyU* gene was amplified and cloned into pGEM-T Easy vector to generate pGEM-*hlyU* as the template plasmid for site-directed mutagenesis (SDM). The SDM reaction was carried out as follows: (i) 2 min at 95°C; (ii) 18 cycles, with 1 cycle consisting of 1 min at 95°C, 30 s at 57°C, and 4 min at 72°C; (iii) add DpnI and incubate at 37°C for 2 h. The product was transformed into *E. coli* DH5α, and then the plasmids were extracted. After the sequence was confirmed, the *hlyU* gene was subcloned into pMMB208 by XbaI and SacI to generate pMMB-*hlyU*. The plasmid was then conjugated into *V. vulnificus*.

Bacterial two-hybrid system. The bacterial two-hybrid system used in this work is based on the bacterial two-hybrid system of Karimova et al. (6). The *hlyU* gene carrying the mutation was fused to either the T18 or T25 fragment of the catalytic domain of *Bordetella pertussis* adenylate cyclase in plasmid pT18 or pT25. *E. coli* DHP1 was cotransformed with the corresponding plasmids, and the bacteria were plated on MacConkey agar containing maltose supplemented with the appropriate antibiotics to examine the color. β-Galactosidase activity was further measured for these strains.

Purification of the proteins. The *hlyU* gene or a derivative gene was amplified and cloned into pET47 to generate the N-terminal His-tagged HlyU fusion plasmid pET-*hlyU*. The plasmid was then transformed into *E. coli* BL21(DE3). To express the protein, the bacteria were inoculated in LB broth supplemented with 50 µg/ml kanamycin, cultured overnight, and then diluted 1:20 in the same medium. The bacteria were grown until an optical density (OD) reading of 0.5 to 0.7 was reached, and then 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce for another 4 h. The cells were lysed under native conditions, and the protein was purified by using the nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen).

Gel mobility shift assay. Gel mobility shift assays were performed by using a digoxigenin (DIG) gel shift kit (Roche). The DNA probe was amplified and then labeled with digoxigenin-11-ddUTP by terminal transferase. The binding reaction and detection were performed by the methods of Liu et al. (12).

SDS-PAGE and Western blotting. The anti-HlyU chicken antibody was generated by immunizing the hen with the purified HlyU-His protein (Aves Labs). To verify the levels of expression of the wild-type (WT) and mutant proteins, *V. vulnificus* cells in log phase were harvested by centrifugation. The cells were then resuspended in Tris buffer (10 mM Tris-HCl [pH 7.6]) and sonicated. The same amounts of total proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a 0.2-µm Immobilon P^{SO} (Millipore Corporation) membrane by electroblotting. The membrane was blocked by 5% nonfat milk and incubated with a 1:3,000 dilution of polyclonal anti-HlyU antibody and a 1:50,000 dilution of anti-DnaK antibody (Enzo Life Science). The donkey anti-chicken immunoglobulin Y (Thermo Scientific) and goat anti-mouse immunoglobulin G (Thermo Scientific) secondary antibodies were used at 1:100,000 and 1:10,000 dilutions, respectively. Positive signals were visualized using the Western Lightning chemiluminescence reagent Plus from Perkin Elmer, following the manufacturer's instructions and imaged by using Image Quant LAS4000 (GE).

β-Galactosidase assay. *V. vulnificus* or *E. coli* strains were cultured overnight at 37°C in TSBS (*V. vulnificus*) or LB broth (*E. coli*) supplemented with the appropriate antibiotics. The cell cultures were diluted 1:100 into the same me-

dium supplemented with 0.5 mM IPTG and grown further to the log phase. The β-galactosidase activity of aliquots from the culture was determined by the method of Miller (16). One-way analysis of variance (ANOVA) with Dunnett posttest was used to identify significant differences between each mutant and the positive control. Statistical analyses were performed using GraphPad Prism 5 software.

RESULTS

Mutagenesis of HlyU. The recently solved crystal structure of the *V. vulnificus* HlyU protein reveals that it possesses five α-helices, two β-sheets, and some unstructured regions (18). However, the relationship between the structural organization of HlyU and its biological function remains to be investigated. To characterize this protein and identify the essential amino acids for the biological function of HlyU, we carried out alanine scanning mutagenesis of amino acids in regions that were deemed to be important by examination of the crystal structure of the *V. vulnificus* HlyU protein and the *in silico* model of *V. cholerae* HlyU. We then performed a screen for mutants that were unable to activate transcription of the *rtxA1* gene. Mutant *hlyU* derivatives were cloned in the pMMB208 plasmid and conjugated into the *V. vulnificus* Δ*hlyU* Δ*lacZ* strain, which carries a fusion plasmid in which the promoter of the *rtxA1* operon was fused to the promoterless *lacZ* gene. Thus, β-galactosidase activity was used to ascertain the regulatory property of the HlyU protein. Figure 1 shows the alignment of HlyU from *V. vulnificus* with other members of the ArsR/SmtB protein family as well as the 42 amino acid mutations that we generated in this study.

Essential amino acids for HlyU-DNA binding. According to the crystal structure and the results of computational analysis of the HlyU family proteins, helix α4 of the HlyU protein is predicted to be the DNA recognition domain. In the crystalline state, HlyU from *V. vulnificus* is reported to be a homodimer, and two helices, α1 and α5 in the dimer are oriented in the same direction. Furthermore, in that structure, several conserved amino acids, R24, R25, H56, R61, and K70, are supposed to form a positively charged patch to contact the DNA (18). In the *in silico* model of the HlyU-DNA complex, the amino acids on the α4 helix, Q50, S51, S54, Q55, H56, W59, R61, and R62, the amino acids on the α1 and α2 helices, K18, R24, and R25, as well as Q73, the amino acid located in the winged region, are close enough for a favorable interaction with the negatively charged DNA. In addition, amino acids Q50, S51, Q55, R61, and Q73 are thought to define the sequence specificity of HlyU in *V. vulnificus* by comparing the structural model of HlyU in *V. vulnificus* to that of the other ArsR/SmtB proteins (18). To evaluate the possible contribution of the HlyU amino acids identified in the crystal structure to HlyU-DNA binding, we performed site-directed mutagenesis to generate the mutations K18A, R24A, R25A, Q50A, S51A, S54A, Q55A, H56A, W59A, R61A, R62A, K70A, and Q73A in *V. vulnificus*. On the basis of the *in silico* analysis of the *V. cholerae* HlyU protein, we also generated N22A, E23A, S38A, G40A, S49A, T68A, T74A, and V75A, which are likely to be involved in HlyU-DNA binding. Since L57 and L60 are highly conserved residues in the DNA recognition domain, we also included them in the mutant list. First, we examined whether those mutations affected the HlyU regulatory function by comparison of the β-galactosidase activities of strains with

FIG. 1. Amino acid sequence of HlyU aligned with sequences from representative members of the ArsR/SmtB family. VvHlyU is HlyU from *V. vulnificus* CMCP6, and VcHlyU is HlyU from *V. cholerae* N16961. ArsR is from *E. coli* HS, SmtB is from *Synechococcus* sp. strain PCC7942, CzrA is from *Staphylococcus aureus*, and CadC is from *S. aureus* p1258. α -Helices are shown as rectangles, and β -strands are shown as arrows. Identical residues (*), conserved residues (:), and semiconserved residues (.) are shown below the sequence alignment. Gaps introduced to maximize sequence alignment (dashes) are indicated. The symbols above the sequence alignment indicate alanine replacements that did not affect HlyU function (open circle), that affected DNA binding (black square), or that affected HlyU dimerization (black triangle). The numbers above the sequence represent the amino acid order in the sequence of VvHlyU.

the *rtxA1* promoter-*lacZ* fusion. As shown in Fig. 2A, compared with the wild type, strains with HlyU mutations K18A, N22A, S54A, H56A, L57A, W59A, R61A, and K70A exhibited values between the values for the positive and negative controls, indicating that these residues are important for the biological function of HlyU, possibly by affecting the HlyU-DNA interaction. It is of interest that strains with the R25A and Q55A mutations showed a very low level of β -galactosidase activity, suggesting that these two residues must be critical for the HlyU-DNA interaction. From the crystal structure, residue S51 in this domain is predicted to take part in the interaction with the negatively charged DNA and to define the sequence specificity of the *V. vulnificus* HlyU (18). Surprisingly, the S51A mutation did not change the β -galactosidase activity from that of the wild-type HlyU.

To exclude the possibility that low β -galactosidase activity is due to the loss of the pMMB-*hlyU* plasmid or to the instability of the HlyU protein in the mutants, all the mutants were also examined for the presence of the HlyU proteins using a Western blot assay with an HlyU-specific antibody. As shown in Fig. 2B, all the mutants showing a low β -galactosidase activity still expressed HlyU proteins at levels similar to those seen in the wild type. To analyze whether the *in vitro* binding of the mutant HlyU proteins to the promoter DNA was affected, we purified each of the mutant HlyU proteins with the K18A, N22A, R25A, S54A, Q55A, H56A, L57A, W59A, R61A, and K70A mutations and performed a gel mobility shift assay with each of them. As shown in Fig. 3, with the exception of the mutant proteins with the S54A, Q55A, and L57A mutations, we did

not observe any DNA shift with these mutant proteins at the protein concentrations tested. As for HlyU with the S54A or Q55A mutation, there was no specific shift observed; however, with increasing protein amounts, some nonspecific shift was observed. For the HlyU protein with the L57A mutation, the protein exhibited much less DNA binding ability than the wild-type HlyU did. Thus, these *in vitro* protein-DNA binding results are consistent with the *in vivo* results, in which a lower β -galactosidase activity was detected in the *V. vulnificus* strains carrying the corresponding amino acid mutations, demonstrating that these amino acids are essential for the interaction between the HlyU protein and DNA.

Does HlyU need to be a dimer to control expression of the *rtxA1* gene? The crystal structure of HlyU suggests that the two terminal helices $\alpha 1$ and $\alpha 5$ are involved in forming a homodimer and are located in the dimeric interface. However, *in vivo* evidence supporting the dimer status of HlyU is not available. To determine whether HlyU dimerizes *in vivo*, we first used the bacterial two-hybrid system to assess dimerization in *E. coli*. For a positive control for this system, we used a strain harboring the pT18-*zip* and pT25-*zip* plasmids encoding the T18 and T25 fragments, respectively, fused in frame with a DNA fragment encoding a 35-amino-acid leucine zipper. The strain harboring the plasmids pT18 and pT25 was used as a negative control. As described in Materials and Methods, the wild-type *hlyU* gene was fused to pT18 and pT25, and the two plasmids were cotransformed into *E. coli* DHP1. As shown in Fig. 4A, the HlyU-fused strain exhibited a high level of β -ga-

FIG. 2. Site-directed mutagenesis analysis of putative DNA-binding residues. (A) Effects of replacing amino acids on the HlyU protein with alanine on the transcriptional regulation of the *rtxA1* operon. The positive control is *V. vulnificus* MQ4 harboring vector pTL-PrtxA and pMMB-*hlyU*. The negative control is *V. vulnificus* MQ4 carrying vector pTL-PrtxA and empty vector pMMB208. The values for all the mutants are normalized against the value for the positive control. β -Galactosidase activities were measured using cultures grown in log phase. Values are means plus standard deviations (error bars) of relative activity from 3 independent experiments. One-way analysis of variance with Dunnett test was used to compare the values for mutants with the results for the positive control; values that were significantly different from the value for the positive control are indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (B) Levels of expression of the mutant HlyU proteins in *V. vulnificus* by Western blot analysis. DnaK was used as an internal control.

lactosidase expression, indicating that HlyU does indeed form a dimer *in vivo*.

The crystal structure of HlyU suggests that the N-terminal helix $\alpha 1$ and C-terminal helix $\alpha 5$ are two dimer interfaces of this protein (18). Thus, we constructed *in vivo* bacterial two-hybrid strains with HlyU derivatives in which either helix $\alpha 1$ or helix $\alpha 5$ was deleted to examine the effect of each helical region on the dimerization process. As shown in Fig. 4A, the strain with helix $\alpha 1$ deleted ($\Delta \alpha 1$) showed about 61% β -galactosidase activity; however, it still has much higher activity than the negative control, suggesting that deletion of the $\alpha 1$ dimer interface reduces but does not abolish formation of the HlyU dimer *in vivo*. Figure 4A also shows that when helix $\alpha 5$ was deleted ($\Delta \alpha 5$), very low β -galactosidase activity was detected, indicating that the protein has lost a significant amount of its ability to form a dimer.

The crystal structure of HlyU also suggests that amino acids M6, E7, S10, V14, L17, K18, L16A, A21, E23, R24, Q27, E83, M87, L90, L91, and L94 could be involved in the dimer interface (18). Thus, we performed site-directed mutagenesis to change these residues to alanine, then fused the mutated *hlyU* genes to the two-hybrid plasmids, and cotransformed into the *E. coli* DHP1 strain. The experiments showed that mutations L17A located in helix $\alpha 1$ and L91A located in helix $\alpha 5$ resulted in a significantly lower β -galactosidase activity compared to the wild type, indicating that these two residues provide some contribution to HlyU dimerization (Fig. 4A). Furthermore, changing both amino acids L17 and L91 to alanine resulted in a very low β -galactosidase activity (13% of the wild-type HlyU), indicating that together these two amino acids are essential for dimer formation.

Is HlyU dimerization essential for its biological function?
To understand the biological implications of the *in vivo* dimerization of HlyU, we examined whether L17A and L91A affect HlyU activity in *V. vulnificus*. Figure 4B shows that with either the L17A or L91A mutation alone, we did not observe an obvious difference in the HlyU activity of each of the mutants compared to the activity of the wild type. However, *V. vulnificus* harboring the HlyU protein with both substitutions L17A and L91A exhibited significantly lower β -galactosidase activity concomitant with similar protein amounts compared to the wild type by Western blot assays (Fig. 4C). Thus, the abolishment of HlyU dimer formation dramatically affected

FIG. 3. Gel mobility shift assay for binding of HlyU mutants to the *rtxA1* operon promoter regulatory region. A biotin-labeled 246-bp DNA fragment was mixed with increasing amounts of the wild-type (WT) or mutant proteins (50, 100, 150, and 500 nM indicated by the height of the black triangle above the lanes). The leftmost lane for each HlyU protein contains only the probe. The DNAs were resolved on a 6% DNA retardation gel (Invitrogen). Visualization of the DNA band was performed per the manufacturer's instructions (Roche).

FIG. 4. Dimerization of HlyU and its biological effects. (A) Site-directed mutagenesis analysis of putative residues essential for HlyU dimerization using the bacterial two-hybrid system in *E. coli* DHB1. 18+25 represents the strain harboring the empty vectors pT18 and pT25. $\Delta\alpha 1$ indicates that the entire helix $\alpha 1$ of HlyU is deleted (from N2 to M20), and $\Delta\alpha 5$ indicates that the entire helix $\alpha 5$ of HlyU is deleted (from E82 to Y95). L17A/L91A represents the strain with both the L17A and L91A mutations. The cellular β -galactosidase activities were measured using cultures in log phase by the method of Miller (16). All the mutants are normalized against the wild-type HlyU strain. Values are means plus standard deviations (error bars) from 3 independent experiments. One-way analysis of variance with the Dunnett test was used to compare the values for mutants with the results for the wild type; values that were significantly from the value for the wild type are indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (B) Effects of replacing L17 and L91 on HlyU with alanine on the transcriptional regulation of *rtxA1* operon in *V. vulnificus* by comparing the β -galactosidase activity. The positive control is *V. vulnificus* MQ4 harboring plasmid pTL-PrtxA and pMMB-*hlyU*. The negative control is *V. vulnificus* MQ4 harboring plasmid pTL-PrtxA and empty vector pMMB208. All the mutants are normalized against the positive control. The cellular β -galactosidase activities were measured using cultures in log phase by the method of Miller (16). Values are means plus standard deviations (error bars) from 3 independent experiments. In this case, we also used the same statistical approach to compare the values for mutants with the results for the positive control (**, $P < 0.01$). (C) Expression levels of mutant HlyU proteins in *V. vulnificus* by Western blot analysis. DnaK was used as an internal control.

the biological function of HlyU. For this particular double mutant, we also examined whether the purified HlyU with the L17A/L91A mutations affected the HlyU dimerization status *in vitro*. The N-terminal His tags of the purified HlyU proteins were cleaved off and then examined in a native protein gel. Figure 5A shows that for the wild-type HlyU protein, we could observe a band of approximately 22 kDa that corresponds to a dimer. However, there was no dimer band for the HlyU harboring the double mutation L17A/L91A. To investigate whether the lower activity of the mutant HlyU is due to the loss of DNA binding ability, we performed the gel mobility shift assay using the mutated HlyU protein. As shown in Fig. 5B, the shift is considerably reduced in the double mutant, indicating that HlyU dimerization is also essential for HlyU-DNA binding.

Importance of the Y77 amino acid in the β -sheet-2. Y77 is a highly conserved amino acid in the β -sheet-2 domain of the ArsR/SmtB family of proteins. We changed this residue from tyrosine to alanine and then examined whether the change affects the function of HlyU. As shown in Fig. 2A, this substitution dramatically affected the β -galactosidase activity in *V. vulnificus*. Since this amino acid is located in the β -sheet-2 domain that is next to one of the dimer interfaces, helix $\alpha 5$, and is also close to the DNA recognition domain helix $\alpha 4$, we first examined whether this mutation affected dimer formation by

using the bacterial two-hybrid system. Figure 4A shows that it behaved like the wild-type HlyU, demonstrating that Y77 is not essential for HlyU dimer formation. We then purified the HlyU protein with the Y77A change and examined whether the mutation affects the ability of HlyU to bind DNA. Figure 3 shows that the HlyU protein with the Y77A mutation could not bind to the promoter DNA at the protein concentration tested, which suggests either that this residue is involved in HlyU-DNA interaction or that the mutation changes the protein structure, somehow affecting the binding of HlyU to the DNA.

DISCUSSION

We and others demonstrated that the *rtxA1* gene, encoding the repeat-in-toxin protein, is an important virulence factor of *V. vulnificus* (2, 5, 10, 12). We also showed that the HlyU protein plays an essential role in its regulation. In its normal state, *rtxA1* expression is repressed by the H-NS protein, and HlyU acts as a derepressor by binding to a region upstream of the *rtxA1* operon promoter, resulting in the removal of H-NS and allowing *rtxA1* expression and bacterial invasion to occur (13). However, an intimate knowledge of the intrinsic steps involved in the biological mechanism of action of HlyU is limited to predictions based on the recently solved crystal

FIG. 5. HlyU dimerization status *in vitro* and effect of HlyU dimerization on DNA binding. (A) Proteins in the native blue protein gel. The N-terminal His tag was cleaved off the wild-type HlyU protein and the HlyU protein with L17A and L91A mutations [HlyU(L17A/L91A)] by human rhinovirus (HRV) 3C protease. Then 5 μ g of each protein was mixed with 2 μ l of 5 $\times$ sample buffer (310 mM Tris-HCl [pH 6.8], 0.05% [wt/vol] bromophenol blue, 50% glycerol). The resulting mixture was then loaded in the native PAGE Novex 4 to 16% Bis-Tris gel (Invitrogen). α -Lactalbumin (14.2 kDa) (lane 1) and carbonic anhydrase (29 kDa) (lane 2) were used as molecular size controls. Lane 3 contains HlyU protein, and lane 4 contains HlyU with the L17A and L91A mutations. (B) Gel mobility shift assay for binding of HlyU(L17A/L91A) to the *rtxA1* operon promoter regulatory region. A biotin-labeled 246-bp DNA fragment was mixed with increasing amounts of the wild-type or mutant proteins (50, 100, 150, and 500 nM [indicated by the height of the black triangle above the lanes]). The leftmost lane for each protein contains only the probe. The DNAs were resolved on a 6% DNA retardation gel. Visualization was performed per the manufacturer's instructions (Roche).

structure of *V. vulnificus* HlyU and on the *in silico* model of *V. cholerae* HlyU (18, 21).

From the crystal structure of *V. vulnificus* HlyU, helix α 4 is predicted to be a DNA recognition domain, and it can also be deduced that amino acids R24, R25, H56, R61, and K70 are supposed to form a positively charged patch for allowing the DNA contact. Here, we demonstrate that with the exception of R24, most of these amino acids do contribute to the HlyU-DNA interaction at certain levels. In the *in silico*-modeled structure of the DNA-HlyU complex, the Q50, S51, S54, Q55, H56, W59, R61, and R62 amino acids on the recognition helix, the L18, R24, and R25 amino acids on the α 1 and α 2 helices, and the Q73 amino acid on the winged region are close enough to make favorable interaction with the negatively charged DNA (18). We show that except for R24A, Q50A, S51A, R62A, and Q73A, replacing the rest of the amino acids with alanine significantly affected the function of HlyU, indicating their involvement in the HlyU-DNA interaction. Saha and Chakrabarti (21) built an *in silico* three-dimensional structure of the *V. cholerae* HlyU protein. From this analysis, we deduced that the corresponding amino acids on the *V. vulnificus* HlyU protein, namely, N22, E23, S38, G40, S49, T68, T74, and V75, are probably involved in HlyU-DNA binding. However, with the exception of N22, we demonstrated in this work that none of these amino acids are essential for the HlyU function in *V. vulnificus*.

We also proved that amino acid S51 in the DNA recognition domain does not contribute to the HlyU-DNA interaction. However, in the case of the *Staphylococcus aureus* CsrA protein (a member of the ArsR/SmtB family), it was reported that amino acid S54, which is equivalent to S51 of HlyU in *V. vulnificus*, makes a key intermolecular contact to DNA (1). It is of interest that L60, in the DNA recognition domain, that is highly conserved among members of the same protein family, is not essential for the function of the HlyU protein in *V. vulnificus*. Furthermore, other highly conserved residues of HlyU such as L32, E36, V39, and L79 are also nonessential for

HlyU function, since replacing these residues with alanine did not affect HlyU activity (data not shown). We also defined key determinants of the HlyU dimer interface demonstrating that homodimer formation is critical for the HlyU biological function *in vivo* and identified the essential amino acids that are crucial for homodimerization. Our results indicate that deletion of the entire α 1 helix slightly affects the HlyU homodimerization process, but the homodimeric protein is still stable. Conversely, when helix α 5 of HlyU was deleted, the homodimer was barely detected. Like SmtB, another member of this family of proteins, hydrophobic interactions of HlyU are also involved in the homodimerization (18). Our results demonstrated that nonpolar amino acids M87, L90, and L94 from helix α 5 and L16 from helix α 1 may not contribute to the interactions, although the prediction from the crystal structure analysis suggested that they might (18). In this work, we showed that single mutations L17A and L91A in the HlyU helices α 1 and α 5 caused significantly less dimerization compared with the wild-type HlyU. When both amino acids L17 and L91 were replaced by alanine, obtaining the double mutant derivative, the HlyU protein completely lost its homodimeric structure and its ability to bind to the target DNA. These genetic changes were reflected in a loss of the biological function, i.e., relieving the repression of *rtxA1* by H-NS.

Amino acid Y77 is highly conserved in the β -sheet-2 domain that is next to one of the dimer interfaces, helix α 5, and is also close to the DNA recognition domain helix α 4. Our results suggest that this residue does not contribute to HlyU dimerization. However, when Y77 is replaced by alanine, the mutant protein HlyU (Y77A) no longer binds to the promoter DNA on the basis of the *in vitro* gel mobility shift assay results, and the protein is no longer functional, indicating that Y77 is indeed essential for HlyU. It was suggested that like most proteins from the ArsR/SmtB family, the binding of the HlyU homodimer to the DNA might require bending of the DNA helix. Amino acid Y77 might actually be able to make direct contact with the DNA due to the target DNA bending, alter-

natively Y77 may cause either a structural change in a region of the protein or might affect the entire protein, interrupting the interaction between the DNA backbone and the protein.

The nuclear magnetic resonance (NMR) structure of another member of the ArsR/SmtB family, the CzrA protein in the DNA-bound state has been solved (1). The quaternary structure of DNA-bound CzrA reveals that Zn(II) binding drives a “closed to open” protein conformational change. The results of comparing the sequences of HlyU and other proteins from the ArsR/SmtB family suggest that HlyU does not contain any typical metal-binding residues. The solution of the structure obtained by DNA-HlyU cocrystallization will likely provide accurate information to dissect the molecular mechanism of regulation by HlyU.

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