

## Cyclo(Phe-Pro) Modulates the Expression of *ompU* in *Vibrio* spp.

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***Vibrio vulnificus* was found to produce a chemical that induced the expression of *Vibrio fischeri lux* genes. Electron spray ionization-mass spectrometry and <sup>1</sup>H nuclear magnetic resonance analyses indicated that the compound was cyclo(L-Phe-L-Pro) (cFP). The compound was produced at a maximal level when cell cultures reached the onset of stationary phase. Sodium dodecyl sulfate-polyacrylamide gel analysis of the total proteins of *V. vulnificus* indicated that expression of OmpU was enhanced by exogenously added synthetic or purified cFP. A *toxR*-null mutant failed to express *ompU* despite the addition of cFP. The related *Vibrio* spp. *V. cholerae*, *V. parahaemolyticus*, and *V. harveyi* also produced cFP, which induced the expression of their own *ompU* genes. cFP also enhanced the expression in *V. cholerae* of the *ctx* genes, which are known to be regulated by ToxR. Our results suggest that cFP is a signal molecule controlling the expression of genes important for the pathogenicity of *Vibrio* spp.**

Communication between cells via diffusible chemicals is a general phenomenon found in virtually all living organisms. However, it is only in the last 2 decades that it has been intensively studied in bacteria. One of the best-known examples is quorum sensing. A number of bacteria associated with eukaryotic hosts employ quorum-sensing systems to sense their population density, thereby modulating the expression of sets of genes involved in physiological responses associated with survival, propagation, and/or virulence (9, 21, 44). *N*-Acyl-homoserine lactones (AHLs) are the most prevalent signal molecules for quorum sensing in gram-negative bacteria, but not all signal molecules belong to this group. For instance, *Vibrio harveyi* employs a furanosyl borate diester molecule in addition to AHL (7). The plant pathogen *Ralstonia solanacearum* uses 3-hydroxypalmitic acid methyl ester together with AHL to control the expression of virulence factors (14), and several gram-positive bacteria utilize peptides or  $\gamma$ -butyrolactone as signals (for a review, see reference 12). *Xanthomonas campestris* also uses non-AHL signal molecules, which have been identified as fatty acid derivatives, to regulate the expression of virulence factors (3, 54). In addition, cyclic dipeptides produced by *Pseudomonas* spp. and some other genera can activate AHL bioindicator strains (22). They probably activate or antagonize signaling components implicated in AHL-dependent quorum sensing by cross talk with the associated sensors. However, the actual biological roles of these cyclic dipeptide molecules remain to be clarified.

*Vibrio vulnificus* is an opportunistic human pathogen that causes severe wound infection and primary septicemia (50). It

has been reported to possess a functional *luxS* and produce a signal molecule that induces bioluminescence in a *V. harveyi* AI-2 reporter strain (25). It is also known to possess *smcR*, a homolog of the positive regulatory gene *luxR* of *V. harveyi* (29). SmcR induces the expression of *vvpE*, encoding a metalloprotease, and represses *vvhAB*, encoding a hemolysin (46). These reports suggest that *V. vulnificus* possesses a *V. harveyi*-type quorum-sensing system rather than the canonical *Vibrio fischeri*-type system, and we attempted to identify a quorum-sensing signal molecule produced by it. We found that it produces a cyclic-dipeptide molecule, which is active on an *Escherichia coli* quorum-sensing bioindicator strain. However, even though this compound induced the expression of a *V. fischeri lux* reporter, it appears that in *V. vulnificus* it does not induce the expression of genes known to be regulated by quorum sensing. Instead, in this and related *Vibrio* spp, it modulates genes whose expression is dependent on ToxR.

### MATERIALS AND METHODS

**Culture conditions and general methods.** *E. coli* was grown in Luria-Bertani (LB) medium (Difco, St. Louis, MO) at 37°C. *V. vulnificus* MO6-24/O strain (57), *V. harveyi* 3392 strain (42), *Vibrio parahaemolyticus* ATCC 27519 strain, and a Korean clinical isolate, *Vibrio cholerae* El-tor, were grown at 28°C in LB or autoinduction bioassay (AB) medium (17). All plasmids were maintained in *E. coli* DH5 $\alpha$ , except for pDM-TKO, which was maintained in *E. coli* SM10 $\lambda$ pir (47). Antibiotics were used at the following concentrations: for *E. coli* strains, ampicillin (Ap) at 50  $\mu$ g/ml, chloramphenicol (Cm) at 25  $\mu$ g/ml, kanamycin (Km) at 25  $\mu$ g/ml, and tetracycline (Tc) at 10  $\mu$ g/ml; for *Vibrio* strains, Ap at 100  $\mu$ g/ml, Cm at 2  $\mu$ g/ml, Km at 100  $\mu$ g/ml, and Tc at 2  $\mu$ g/ml. DNA manipulations, preparation of RNA, and Northern hybridizations were carried out as described previously (45).

**Bioluminescence assay using a bioindicator.** An overnight culture of the bioindicator strain *E. coli* MT102(pSB403) was washed with fresh LB medium and inoculated in fresh LB medium together with the same volume of a cell-free culture supernatant or ethyl acetate extract of interest. Bioluminescence was measured using a Berthold LB96V luminometer (EG & G, Berthold, England).

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**Purification and identification of compounds from *Vibrio* spp.** The cell-free supernatant of an overnight culture in LB broth at 28°C was extracted twice with the same volume of ethyl acetate. The ethyl acetate extract was concentrated with a rotary evaporator, prepurified using an LC-18 SPE cartridge (Supelco, Bellefonte, PA), and fractionated by high-performance liquid chromatography (HPLC) (Shimadzu; 10AVP series) using a  $C_{18}$  reverse-phase column (Kanto Chemical) with a linear 20 to 100% methanol gradient as the mobile phase. Each fraction was examined for the presence of compounds inducing *lux* expression in the bioindicator MT102(pSB403) (56). Active fractions were combined and further purified using the same HPLC system with 30% methanol as the mobile phase. Active fractions were again combined and characterized by 300 MHz  $^1\text{H}$  nuclear magnetic resonance (NMR) (Gemini300BB; Varian, Palo Alto, CA) and electron spray ionization mass spectrometry (ESI-MS) (4.7 T FTICR-MS; Ion-spec, Lake Forest, CA).

**Cyclo(Phe-Pro)** [(3*s*,9*s*)-hexahydro-3-(phenyl)-pyrrolo-(1,2-*a*)-pyrazine-1,4-dione] was chemically synthesized as described previously (53). To determine the concentration of cyclo(L-Phe-L-Pro) (cFP) in a culture supernatant, cells were grown in LB medium and the culture supernatant was extracted with ethyl acetate as described above. The amount of cFP in the supernatant was estimated by referring the peak height of the HPLC elution profile to a standard curve based on the peak heights of the HPLC elution profiles of ethyl acetate extracts of fresh LB medium containing known concentrations of synthetic cFP.

**SDS-PAGE analysis of membrane proteins containing OmpU.** Cells were harvested by centrifugation (4°C, 7,000 × *g*, 5 min), the pellet was resuspended in ice-cold lysis buffer (20 mM Tris-Cl, pH 7.5, 2 mM EDTA), and the cells were disrupted by two passes through a French press (SLM-AMINCO Instruments, Inc., Rochester, NY). Membrane proteins containing OmpU were prepared as previously described (5, 8) and resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Preparation of ethyl acetate extracts of culture supernatant for analysis of *ompU* induction.** Fifty-milliliter samples of culture supernatants of *V. vulnificus* MO6-24/O at various growth stages were extracted and evaporated as described above and dissolved in 1 ml of methanol per liter of original culture. Three-microliter aliquots of the concentrated extracts were added to 3 ml of a fresh *V. vulnificus* MO6-24/O culture at an  $A_{600}$  of 0.1. After incubation for 2 h with shaking, the cells were harvested and 15 μg of membrane proteins was resolved by 12% SDS-PAGE. The expression of OmpU was then assessed by Western hybridization as described below.

**Expression and purification of *V. vulnificus* OmpU.** A 1,630-bp DNA fragment comprising *ompU* and a 513-bp upstream region was PCR amplified using primers 5'-ATGGGCCAAAGTCATCTTTGG-3' and 5'-GCGGATCCAATCTATCTAGAAT-3' and cloned into the pGEMT-Easy vector (Promega) to yield pDK1. Using two EcoRI sites, one at position 232 from the start codon of *ompU* and the other in the multicloning site downstream of the gene in pDK1, a 791-bp 3'-terminal portion of *ompU* was cloned into pTrcHisB (Invitrogen, Carlsbad, CA) such that *ompU* was translationally fused to the His tag of the vector. The resulting construct was transformed into *E. coli* JM109, and OmpU with a six-His tag at its N terminus was purified with a His-Bind kit (Novagen, Madison, WI).

**Preparation of rabbit antibody against OmpU and Western hybridization.** All immunization procedures were performed as described previously (2). Purified OmpU (400 μg/ml per kg of body weight) emulsified in Freund's adjuvant (Sigma, St. Louis, MO) was injected subcutaneously into a female New Zealand White rabbit. Rabbit immunoglobulin G (IgG) was purified with a Vivapure protein A minispin column (VIVA Science, Hannover, Germany) and used for Western hybridization (5). Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (KPL, Gaithersburg, MD) and a Sense Western blot detection kit (Corebio) were used to visualize hybridization.

**Construction of a *lacZ* fusion of *ompU*.** A 555-bp ApaI-PstI fragment from pDK1 was cloned into pRK $\Omega$ lacZ, a derivative of pRK415 (24) that contains the promoterless *lacZ* gene from pRG970 (51) together with a streptomycin/spectinomycin  $\Omega$  cassette containing the transcription terminator from pHP $\Omega$ 45 (38). The resulting construct, pDK-PVomp, contained the *ompU* promoter region fused to *lacZ*. This construct was mobilized into *V. vulnificus* MO6-24/O by biparental mating using SM10 $\lambda$ pir (32).  $\beta$ -Galactosidase activities were measured as described previously (2).

**Cloning of *toxRS* from *V. vulnificus* and construction of a *toxR*-null mutant.** To clone *toxRS*, a genomic library of *V. vulnificus* constructed in the cosmid pCP13/B (10) was screened by Southern hybridization using a *toxR* fragment as the probe. The probe was prepared by PCR using primers 5'-CGGAATTCTACCTACGC AAAATGCT-3' and 5'-AACTGCAGTTTTACTGGAGCAGAATC-3'. A 3-kb DNA fragment containing *toxRS* from a selected cosmid clone, pToxR, was subcloned into pBluescript SK(-) (Stratagene, La Jolla, CA) to yield pBS-GRS. A 410-bp fragment was deleted from pBS-GRS by cutting at two HincII sites

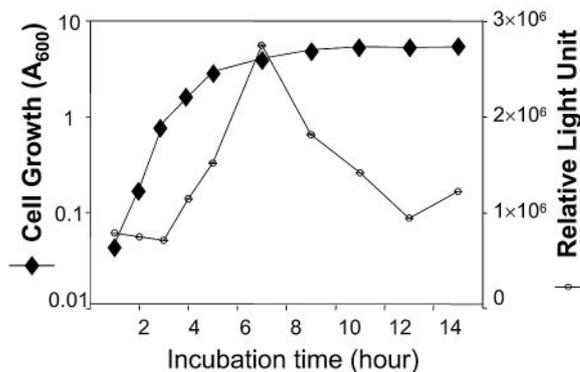


FIG. 1. *V. vulnificus* produces a compound detected by a quorum-sensing bioindicator. (A) Induction of bioluminescence in the quorum-sensing bioindicator MT102(pSB403) by a *V. vulnificus* MO6-24/O supernatant. Relative light units represent the ratio of the luminescence to the  $A_{600}$  of the cell culture.

located within *toxR*, and a 2.6-kb SphI-SpeI fragment was cloned from the resulting plasmid into the suicide vector pDM4 (34). The resulting construct, pDM-TKO, was used to mutate *toxR* by allelic exchange to yield a derivative with the mutant *toxR*, designated TKO.

**Construction of a *ctx-lux* fusion and immunoblotting of cholera toxin.** A 1-kb DNA fragment containing the region upstream of *ctxA* in a Korean clinical isolate of the *V. cholerae* El-tor strain was amplified using primers 5'-GATATC TGCCTAACACGCCTAAC-3' and 5'-AAGTTATATCGGGCAGATTCT AGACC-3' and cloned into pGEMT-Easy vector. From this construct, a 980-bp KpnI-XbaI fragment comprising the upstream region and an 82-bp 5'-terminal segment of *ctxA* was transcriptionally fused to *luxAB* in pHK0011 (23). The resulting construct, pHK-*ctxA*F1, was mobilized into the parental *V. cholerae* by biparental mating. Bioluminescence was measured as described previously (36). For immunoblotting of cholera toxin, a culture supernatant of wild-type *V. cholerae* was collected by centrifugation and filtered through a 0.2-μm membrane filter. The total protein in the cell-free culture supernatant was measured by the Lowry method, and 4 μg of protein was blotted onto an Immobilon polyvinylidene difluoride membrane (Millipore, Billerica, MA) using a Bio-Dot microfiltration apparatus (Bio-Rad, Hercules, CA). Cholera toxin was detected using rabbit anti-cholera toxin antiserum (Sigma, St. Louis, MO).

## RESULTS

***V. vulnificus* produces a chemical that induces expression of *V. fischeri lux* genes in a quorum-sensing bioindicator.** We tested cell-free culture supernatants of *V. vulnificus* MO6-24/O for the presence of a compound(s) which could activate quorum-sensing bioindicators. Both cell-free culture supernatants and ethyl acetate extracts of the supernatants induced bioluminescence in one of the bioindicators tested, *E. coli* MT102 (pSB403) (Fig. 1). Neither the aqueous phase of the ethyl acetate extracts nor an ethyl acetate extract of autoclaved LB medium induced expression of the reporter (data not shown). The level of the active compound increased strongly as the culture entered stationary phase and then declined (Fig. 1).

**Chemical structure of the active compound.** The ethyl acetate extract was concentrated and the active compound purified using a  $C_{18}$  reverse-phase HPLC column. Its proton NMR spectrum was measured in [ $^2\text{H}$ ]chloroform at 300 MHz and gave the following data: [ $\delta_{\text{H}}$ ] 1.982 to 2.051 (2H, m, Pro 4- $\text{H}_2$ ), 2.314 to 2.362 (1 H, m, Pro 3-H), 2.755 to 2.805 (1 H, m, Pro 3-H), 3.625 to 3.665 (4 H, m, Pro 5- $\text{H}_2$  and Phe  $\text{CH}_2$ ), 4.061 to 4.091 (1 H, dt, Pro 2H), 4.264 to 4.280 (1 H, m, Phe  $\alpha$ -H), 5.617 (1 H, bs, NH), 7.219 to 7.367 (5 H, m, Ph) (Fig. 2A and B).

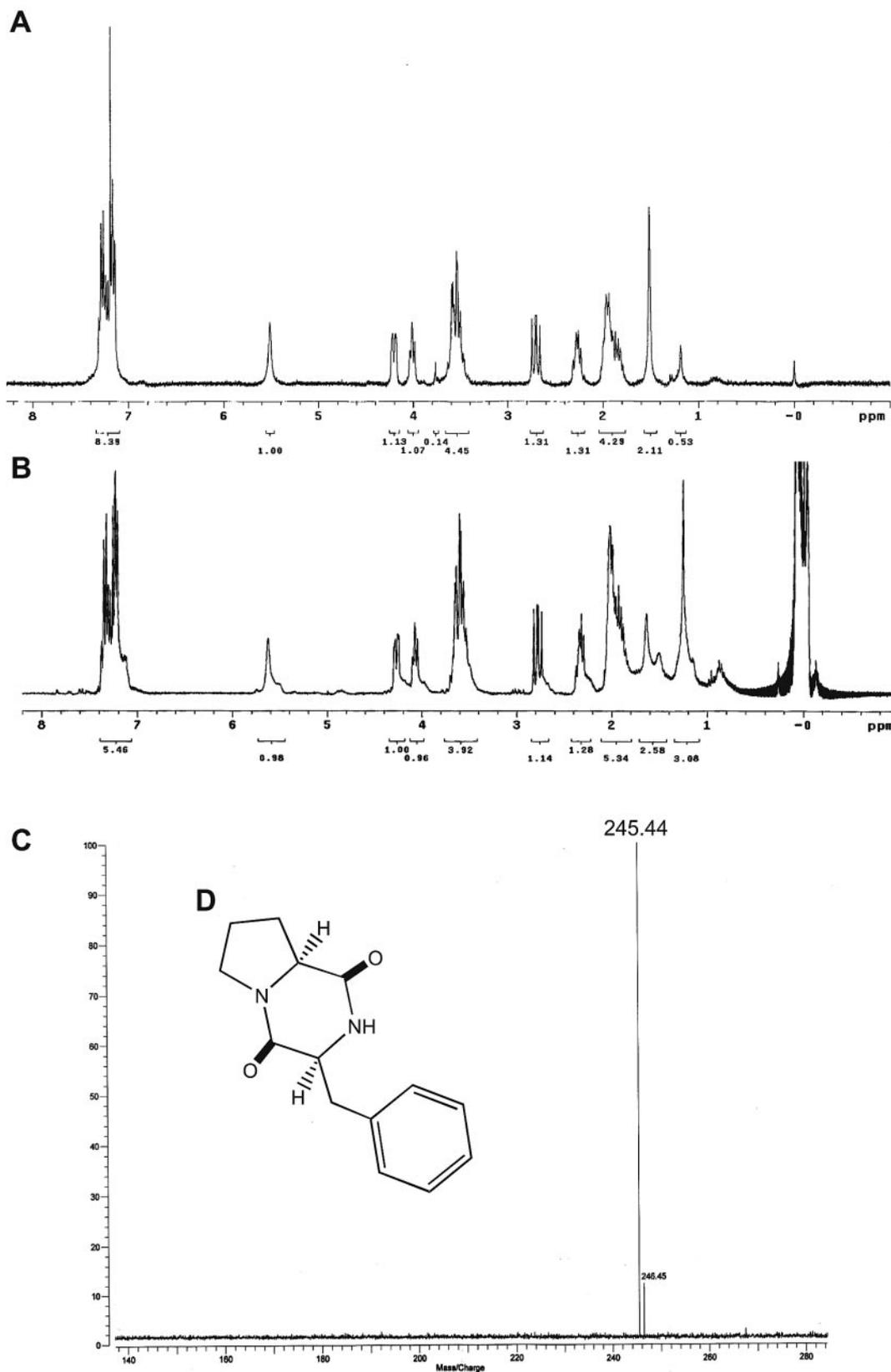


FIG. 2. Chemical structure of the compound in *V. vulnificus* culture supernatants that induces expression of *lux* in MT102(pSB403). Shown are  $^1\text{H}$  NMR spectra (A) of synthetic cFP and (B) of the purified compound. (C) ESI-MS analysis of the purified compound. (D) Chemical structure of cyclic(L-Phe-L-Pro).

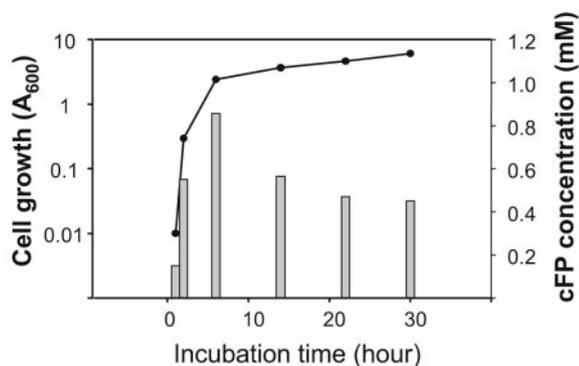


FIG. 3. Concentration of cFP in the supernatant of cultures at various growth stages.

ESI-MS identified a strong quasimolecular  $(M+H)^+$  ion with an  $m/z$  of 245.44 (Fig. 2C). This corresponded to the chemical composition  $C_{14}H_{16}N_2O_2$ . These data suggested that the chemical was cyclo(Phe-Pro) (Fig. 2D). Confirmation was achieved by chemical synthesis of the predicted chemical. As this compound can exist as two enantiomers, cyclo(L-Phe-L-Pro) and cyclo(D-Phe-D-Pro), we tested which of the two chemically synthesized enantiomers was active in the bioindicator assay. Only cyclo(L-Phe-L-Pro) activated the bioindicator MT102(pSB403) (data not shown), indicating that the active compound was cyclo(L-Phe-L-Pro). Based on the height of the peak of the HPLC elution profile, the maximal concentration of cFP in the *V. vulnificus* supernatant was estimated to be 0.9 mM (Fig. 3).

**cFP up-regulates the expression of *ompU* in *V. vulnificus*.** To identify possible changes in gene expression in response to cFP, we examined the expression of total *V. vulnificus* proteins in response to a 2-h incubation with synthetic cFP by denatured-protein PAGE. When cFP at a concentration of 1 mM, which is approximately the concentration found in late culture supernatants of *V. vulnificus*, was added to the medium, the expression of numerous proteins appeared to be affected; of these, that of a 35-kDa membrane protein appeared to be the most strongly affected (Fig. 4A). The 35-kDa protein band was excised, and sequencing identified its N terminus as AELYN QDGTSLD. This is identical to the deduced N-terminal sequence of the *V. vulnificus* OmpU homolog (VV11686). The overall amino acid sequence of the protein (NCBI accession number NP760575) was 68% identical (76% homologous) to that of the *V. cholerae* protein. Its expected molecular mass, excluding a leader sequence, was 34.7 kDa, consistent with the size of the protein by PAGE, confirming that it is OmpU.

Cell-free supernatants of *V. vulnificus* cultures at various growth stages were extracted with ethyl acetate, the extracts were added to *V. vulnificus* cultures in early log phase, and the expression of OmpU was monitored by Western hybridization using rabbit antibody against purified OmpU (Fig. 4B). Methanol or ethyl acetate extracts of autoclaved LB medium did not enhance the expression of OmpU. Ethyl acetate extracts of culture supernatants of *V. vulnificus* in early- and mid-exponential phases also did not significantly induce expression. However, extracts of supernatants of cells in stationary phase ( $A_{600} = 2.42$ ) did enhance the expression of OmpU (Fig. 4B).

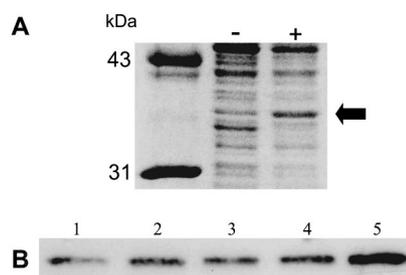


FIG. 4. cFP enhances OmpU expression in *V. vulnificus*. (A) Protein expression profiles of *V. vulnificus* in the presence and absence of chemically synthesized cFP. *V. vulnificus* cells were grown for 2 h with (+) or without (-) 1 mM cFP, membrane proteins were prepared as described in Materials and Methods, and 15  $\mu$ g protein was resolved by 12% SDS-PAGE. The arrow denotes the position of OmpU. (B) Western hybridization of OmpU expressed in *V. vulnificus* cells in the early-stationary-phase response to the addition of 1% methanol (lane 1); ethyl acetate extract of fresh LB medium (lane 2); and ethyl acetate extract of culture supernatants of *V. vulnificus* at  $A_{600}$  values of 0.01 (lane 3), 0.29 (lane 4), and 2.42 (lane 5). Preparation of ethyl acetate extracts of spent medium and Western hybridization were carried out as described in Materials and Methods.

These results show that cFP produced in stationary phase induces the expression of *ompU* in *V. vulnificus*.

To confirm the induction of *V. vulnificus ompU* by cFP, we also measured its transcription using an *ompU-lacZ* fusion. Expression of  $\beta$ -galactosidase from the fusion was increased up to threefold by 1 mM cFP. The same concentration of Pro or Phe, or a mixture of the two amino acids, had no effect, and a 3% NaCl or 1 mM mannitol solution also did not significantly affect  $\beta$ -galactosidase expression (Fig. 5). These results indicated that cFP enhances the transcription of *ompU* in *V. vulnificus* and that the effect of cFP is not simply due to high osmolarity or ionic strength.

Previous workers have shown that the expression of OmpU in *V. cholerae* and in *V. vulnificus* requires ToxR, a transmembrane transcriptional activator (8, 27). To see whether the

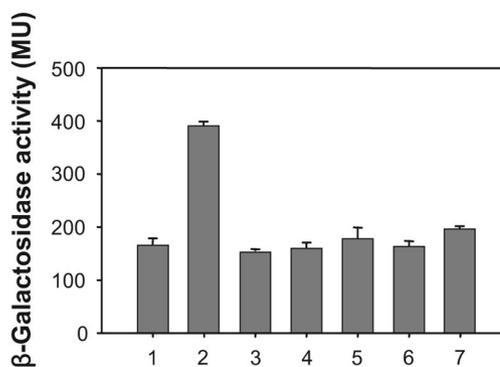


FIG. 5. *ompU* transcription is induced by cFP. Overnight cultures of *V. vulnificus* were inoculated into fresh AB medium supplemented with AB minimal medium only (lane 1), 1 mM cFP (lane 2), 1 mM Pro (lane 3), 1 mM Phe (lane 4), 1 mM Phe and Pro (lane 5), 3% NaCl (lane 6), or 1 mM mannitol (lane 7). After a 2-h incubation, cells were harvested and  $\beta$ -galactosidase activity was measured. Error bars denote standard deviations of three independent experiments; MU, Miller units.

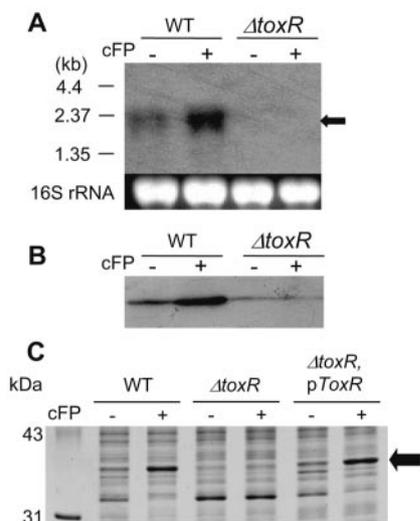


FIG. 6. Effect of cFP and ToxR on the expression of *ompU*. (A) Northern hybridization of total RNA using an *ompU* probe. Twelve micrograms of total RNA was resolved by a 1.2% agarose gel. (B) Western hybridization of total proteins from wild-type *V. vulnificus* and a *toxR* mutant using the anti-OmpU antibody. (C) Total protein expression of wild-type *V. vulnificus*, a *toxR* deletion derivative, and a *toxR* deletion derivative transformed with a *toxR* clone. Arrows indicate the position of OmpU.

enhancement of *ompU* expression by cFP in *V. vulnificus* was dependent on ToxR, we compared the effects of cFP on the transcription of *ompU* in the wild type and an isogenic *toxR*-null mutant by Northern hybridization. When wild-type *V. vulnificus* was cultured for 2 h in AB minimal medium containing 1 mM cFP, the intensity of a 2.3-kb transcript increased (Fig. 6A). The size of this transcript coincided with the estimated size of the *ompU* transcript, which contains a cotranscribed gene, VV11685. However, no transcript was detectable in the *toxR*-null strain with or without addition of cFP. Expression of OmpU as monitored by SDS-PAGE confirmed this result (Fig. 6B) and also showed that introduction of pToxR, encoding ToxR, complemented the defect in the *toxR*-null mutant (Fig. 6C). These results indicate that cFP induces the expression of *ompU* at the transcriptional level and that its expression requires ToxR.

**cFP is also produced by related *Vibrio* spp.** We extended our survey of the ability to produce cFP to related *Vibrio* spp.: *V.*

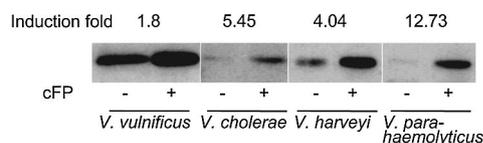


FIG. 7. Expression of OmpU homologs in related *Vibrio* spp. is induced by cFP. Shown is Western hybridization of membrane fractions of *V. vulnificus*, *V. cholerae*, *V. harveyi*, and *V. parahaemolyticus* using anti-OmpU rabbit IgG. Overnight cultures were diluted into fresh AB medium with or without 1 mM cFP and the cells harvested in log phase. Membrane fractions were prepared as described in Materials and Methods, and samples of 12  $\mu$ g protein were resolved by 12% SDS-PAGE. Induction was calculated from the band intensities measured with an image analyzer (BAS-1500; Fujifilm, Tokyo, Japan).

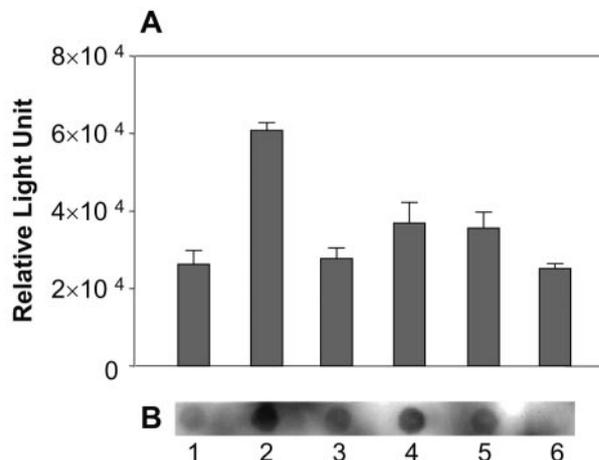


FIG. 8. cFP induces the expression of *ctx* in *V. cholerae*. (A) An overnight culture of *V. cholerae* containing a *ctx-lux* fusion was inoculated into fresh AB medium supplemented with AB minimal medium only (lane 1), 1 mM cFP (lane 2), 1 mM Pro (lane 3), 1 mM Phe (lane 4), 1 mM Phe and Pro (lane 5), or 3% NaCl (lane 6). After 3 h, cells were harvested and the luciferase activity of each sample was measured as described in Materials and Methods. Error bars denote standard deviations of independent assays in triplicate. (B) Culture supernatants of wild-type *V. cholerae* grown in the presence of the above chemicals were blotted, and the production of Ctx was detected using rabbit anti-cholera toxin antiserum.

*harveyi* strain 3392, Korean clinical isolate *V. cholerae* El-tor strain, and *V. parahaemolyticus* strain ATCC 27519. Culture supernatants of each of these representative *Vibrio* spp. contained cFP as shown by NMR analysis of HPLC-purified fractions (data not shown). The concentration of cFP in culture supernatants of these strains ranged from approximately 0.3 mM (*V. harveyi*) to 0.8 mM (*V. cholerae*). We employed Western hybridization of membrane fractions to test whether cFP also enhanced the expression of OmpU in these *Vibrio* spp. The intensity of the hybridizing bands increased in each case (Fig. 7), demonstrating that cFP also induces the expression of OmpU in these species.

**cFP induces the expression of *ctxA* in *V. cholerae*.** It is well documented that the virulence genes *ctxA* and *ctxB*, encoding subunits of the cholera toxin of *V. cholerae*, are regulated by ToxR (11, 32, 33). As shown above, *ompU* is also a component gene of the ToxR regulon. To test whether the *ctx* genes are regulated by cFP, we measured the bioluminescence generated by a *ctx-lux* fusion in *V. cholerae* in the presence and absence of cFP. Bioluminescence was enhanced more than twofold in the presence of cFP (Fig. 8A). As controls, 3% NaCl and 1 mM proline did not significantly enhance expression of the *ctx* genes, and 1 mM phenylalanine, or a mixture of the two amino acids, had only slight effects. The presence of Ctx in culture supernatant as detected using rabbit antiserum to cholera toxin confirmed these findings (Fig. 8B).

## DISCUSSION

In this study, we showed that *V. vulnificus* produces cFP, which is detected by a quorum-sensing bioindicator and affects the expression of the ToxR-dependent genes *ompU* and *ctxAB* in *Vibrio* strains. At an early stage of this study, we assumed

that this pathogen would produce an AHL as do *V. fisheri* and *V. harveyi*, and we employed various bioindicators sensitive to AHL molecules to detect a signal responsible for quorum sensing affecting expression of *vvp* and *vvh* in *V. vulnificus*. To our surprise, the active compound from *V. vulnificus* which activated the quorum-sensing bioindicator was a cyclic dipeptide molecule. This result was consistent with the report that pseudomonads produce this same compound and that it induces the expression of a *V. fisheri lux* reporter system (22). It is likely that cFP cross-reacts with a sensor in the bioindicator cells.

Our efforts to identify an AHL molecule in *V. vulnificus* were unsuccessful. While we were carrying out this work, the genome sequence of *V. vulnificus* was released (6). The genome sequence data showed that this pathogen does not have a canonical LuxIR-type quorum-sensing system like *V. fisheri*. Moreover, examination of the sequence revealed that it does not appear to have a homolog of *luxM* of *V. harveyi*, which encodes an AHL synthase, or a homolog of *luxN*, which encodes the sensor protein for AHL (4), suggesting that *V. vulnificus* lacks not only the ability to produce a typical AHL molecule but also an AHL-mediated signaling system. *V. vulnificus* possesses a functional *luxS* homolog (25), and analysis of the genome sequence revealed that it also contains a homolog of *cqsA* of *V. cholerae*, which encodes a function responsible for production of another quorum-sensing signal molecule (31). We tested whether either of these two genes was responsible for the production of cFP in *V. vulnificus* by examining supernatants of mutants for each of the two genes. Both of the mutants produced normal levels of cFP (data not shown), indicating that cFP is not a product of the *V. vulnificus luxS* or *cqsA* homologs. We have also tested the effect of cFP on the expression of *vvp*, which encodes a metalloprotease and is positively regulated by quorum sensing via a LuxR homolog, SmcR, in *V. vulnificus* (46). Exogenous cFP did not significantly affect expression of *vvp* (data not shown), suggesting that cFP is not a signal for quorum sensing affecting the expression of this gene in *V. vulnificus*. At the moment, a genuine signal for the quorum-sensing pathway in *V. vulnificus* remains to be identified.

It is possible for cyclic dipeptides to form spontaneously in fermentation broth (37). Therefore, to avoid possible artifacts originating from such spontaneously formed cFP, we paid special attention in our choice of culture medium or included appropriate medium-only controls in each experiment using LB medium. We also examined autoclaved LB medium from different manufacturers (Sigma, Difco, and Conda) for the presence of compounds with an HPLC retention time similar to that of synthetic cFP. Such compounds, if any, were present at less than 0.07 mM, whereas the concentration of cFP in the spent medium of *V. vulnificus* cultures was as much as 0.9 mM, showing that cFP was produced by the cells.

The extent of the induction of target genes such as *ompU* and *ctxA* by cFP is moderate (at most threefold) compared to those of target genes affected by other known quorum-sensing signal molecules such as AHL. It is possible that the level of induction observed is high enough to alter a physiological or pathological function(s) in the pathogen. Alternatively, it may be due to the fact that the genes responsible for the biosynthesis of the cyclic dipeptide are functional in the strains we

employed in this study such that there is a high basal level of expression of the cyclic dipeptide. More-pronounced responses might occur in a mutant. Recently, studies have been carried out on the biosynthesis of cyclic dipeptides in gram-positive bacteria and fungi (15, 16, 18, 19, 20, 35), and it was shown that these compounds are all produced nonribosomally by multi-step biosynthesis. The genome sequences of *V. vulnificus* and *V. cholerae* do not appear to contain homologs of these fungal and gram-positive genes. Hence *Vibrio* spp. may employ a different pathway for the production of cFP. Investigation of the gene(s) responsible for cFP production in *Vibrio* spp. and further evaluation of the biological roles of cFP could provide a rationale for the moderate induction level.

Is cFP a signal molecule involved in cell-to-cell signaling in *V. vulnificus*? cFP is produced preferentially in stationary phase, accumulates extracellularly, and affects the expression of certain genes (*ompU* and *ctx*). Hence, in view of the consensus definition of quorum sensing, we suggest that the signaling exerted by cFP can be considered a form of quorum sensing. We have recently identified a *lysR*-type regulator whose expression is modulated by cFP (unpublished results). This regulator appears to affect a series of genes involved in various functions including those related to pathogenicity. This suggests that cFP may be a signal molecule modulating a regulon that controls the physiology and virulence of the pathogen. Related *Vibrio* species also produce cFP at concentrations similar to that in *V. vulnificus*, and, furthermore, cFP also enhances the expression of OmpU proteins in these species, suggesting that cFP is a signal molecule common to members of the genus *Vibrio*. In *Vibrio* spp., OmpU is implicated in various activities associated with pathogenicity, such as resistance to antimicrobial peptides and bile acids, organic acid tolerance, biofilm formation, attachment to host cells in normal symbiotic relationships, and, possibly, adhesion (1, 28, 30, 40, 48, 55). Moreover, the expression of OmpU is enhanced by bile and is positively correlated with maximal induction of virulence factors such as cholera toxin and the coregulated pili and with intestinal colonization (39, 41). Therefore, the enhanced expression of OmpU at high cell density may well contribute substantially to its pathogenicity. Taken together, our observations indicate that the cFP accumulated at high cell density enhances the expression of OmpU as well as *ctx*, the major virulence factor in *V. cholerae*, suggesting that cFP is a signal affecting virulence in pathogenic *Vibrio* spp.

At the moment the pathway by which the expression of ToxR-dependent genes is affected by cFP is not known. Mutation in *toxR* completely abolished the expression of *ompU* irrespective of the addition of cFP. This compound may therefore act directly on ToxR to stimulate the induction of its target genes, or ToxR is essential for the expression of the gene and cFP may exert its effect downstream of ToxR in some signaling pathway.

Quorum sensing mediated by AHL and a furanosyl borate diester molecule (commonly referred to as AI-1 and -2, respectively) is known to affect the expression of the ToxR regulon in *V. cholerae* (26, 58), and, although regulation of *ctx* expression itself by quorum sensing has not been clearly demonstrated, expression of ToxT, the positive regulator of *ctx*, is negatively regulated by quorum sensing (58), which implies that *ctx* is down-regulated by quorum-sensing signaling. If that is the

case, the action of cFP on the expression of *ctx* antagonizes the effect of quorum sensing mediated by AI-1 and AI-2. A more detailed study of the relationship between cFP and the quorum-sensing circuit associated with AI-1 and -2 is needed to test this idea.

Cyclic-dipeptide compounds have been widely investigated, mainly in connection with antibiotic activities and biological effects on animal physiology (for a review, see reference 37). Cyclo(Leu-Gly) produced by the fungus *Rosellinia necatrix* inhibits the development of physical dependence on morphine (52), and cFP produced by *Lactobacillus plantarum* has antifungal activity (49), while a stereoisomer, cyclic(D-Phe-D-Pro), produced by marine bacteria, inhibits the growth of the fish pathogen *Vibrio anguillarum* (13). cFP together with cyclo(L-leucine-L-proline) inhibits the growth of pathogenic yeasts and is antimutagenic in a *Salmonella* mutation assay (43). Considering the relatively high concentration of cFP produced by *Vibrio* spp. compared to other signal molecules associated with quorum sensing, it may affect the host as well as the pathogen itself and also other microorganisms sensitive to this compound. We suggest that the effects of cyclic-dipeptide compounds on host-microbe interactions deserve more attention.

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