

The Cyclic Dipeptide Cyclo(Phe-Pro) Inhibits Cholera Toxin and Toxin-Coregulated Pilus Production in O1 El Tor *Vibrio cholerae*[∇]

Xiaowen R. Bina and James E. Bina*

The University of Tennessee Health Sciences Center, Department of Molecular Sciences, 858 Madison Avenue, Memphis, Tennessee 38163

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Cyclo(Phe-Pro) is a cyclic dipeptide produced by multiple *Vibrio* species. In this work, we present evidence that cyclo(Phe-Pro) inhibits the production of the virulence factors cholera toxin (CT) and toxin-coregulated pilus (TCP) in O1 El Tor *Vibrio cholerae* strain N16961 during growth under virulence gene-inducing conditions. The cyclo(Phe-Pro) inhibition of CT and TCP production correlated with reduced transcription of the virulence regulator *tcpPH* and was alleviated by overexpression of *tcpPH*.

Vibrio cholerae is an aquatic organism and a facultative human pathogen that causes the disease cholera. Cholera is an acute diarrheal disease that is endemic in many parts of the world. Cholera is acquired by ingestion of *V. cholerae*-contaminated food or water. Following *V. cholerae* ingestion, a complicated regulatory cascade is initiated in the proximal small intestine which leads to the production of a number of important virulence factors, including toxin-coregulated pilus (TCP) and cholera toxin (CT) (4). TCP is an adhesin that is required for colonization of the intestinal tract. CT is an enterotoxin that is responsible for the secretory diarrhea, which can rapidly lead to hypotensive shock and death.

CT and TCP production in *V. cholerae* is under the control of a hierarchical regulatory system known as the ToxR regulon (reviewed in reference 4). There are three primary regulatory proteins in this regulon, ToxR (19), TcpP (8), and ToxT (10). In response to unknown environmental cues, ToxR and TcpP, acting with their respective membrane-associated partners ToxS and TcpH, bind together at the *toxT* promoter to activate *toxT* transcription (8, 14, 17). ToxT then directly binds to the promoter of the *tcpA-tcpF* operon to activate the expression of the genes required for TCP production and to the *ctxAB* promoter to activate the production of CT (6, 10). ToxR also regulates the expression of additional genes that are important for pathogenesis, including the OmpU and OmpT porins, independently of TcpP and ToxT (18).

Recently, it was reported that *V. cholerae* produced a cyclic dipeptide (CDP), cyclo(Phe-Pro) (cFP), that exhibited characteristics that were consistent with it being a novel signaling molecule (20). This included the finding that cFP accumulated in culture media in a growth-dependent fashion and that the addition of cFP to the culture medium of an O1 El Tor *V. cholerae* strain grown in AB broth at 28°C resulted in enhanced OmpU and CT production (20). The induction of CT production by cFP in AB broth was intriguing, as virulence gene expression in El Tor vibrios is usually only induced under very

specific growth conditions that are referred to as AKI conditions (defined below) (11). In light of these data, we sought to assess the effect of cFP on CT and TCP production in *V. cholerae* O1 El Tor strain N16961 (9) during growth under *in vitro* virulence gene-inducing conditions (i.e., AKI conditions).

We first tested the hypothesis that cFP was an inducer of CT and TCP production by adding synthetic cFP to AKI broth prior to growth of strain N16961 under AKI growth conditions. cFP had been reported to accumulate in *V. cholerae* culture supernatants to a concentration of ~0.8 mM (20). We therefore added cFP to the AKI broth at 0, 0.5, 0.8, and 1 mM concentrations. Control cultures received an equivalent volume of the solvent dimethyl sulfoxide (DMSO). AKI conditions were as follows. A saturated overnight *V. cholerae* culture was diluted 1:10,000 into 10 ml of AKI broth (1.5% Bacto peptone, 0.4% Difco yeast extract, 0.5% NaCl, pH 7.4) in a 15-by 150-mm test tube. The inoculated culture was then incubated statically at 37°C for 4 h before being transferred to a 125 ml-Erlenmeyer flask and incubated at 37°C with shaking. Following growth under AKI conditions, the cultures were assayed for virulence factor production by CT enzyme-linked immunosorbent assay (ELISA) and TcpA Western blot analyses, as previously described (3). These experiments showed that the presence of cFP at 0.5 and 0.8 mM resulted in an approximately 2-fold reduction in CT production (Fig. 1A). The presence of cFP at 1 mM resulted in an ~4.5-fold reduction in CT production. The results of the CT assays were confirmed by a TcpA Western blot analysis (Fig. 1B), which showed a cFP concentration-dependent reduction in TcpA production. Samples from the DMSO control cultures did not show any changes in CT or TcpA production, as previously documented (2) (data not shown). In contrast to the previous report, our data showed that cFP inhibited CT and TCP production in a concentration-dependent manner during growth under virulence gene-inducing conditions. One possible explanation for the disparate CT results of these two studies could be the culture conditions. To test this, we quantified CT and TcpA production in strain N16961 and O1 El Tor strain C6706 following growth in the presence and absence of cFP in AB broth at 28°C as described in the previous study (20). Using these growth conditions, we did not detect CT or TcpA production in either of these two strains (data not shown). In agreement with

* Corresponding author. Mailing address: The University of Tennessee Health Sciences Center, Department of Molecular Sciences, 858 Madison Avenue, Memphis, TN 38163. Phone: (901) 448-1786. Fax: (901) 448-7360. E-mail: JBina@uthsc.edu.

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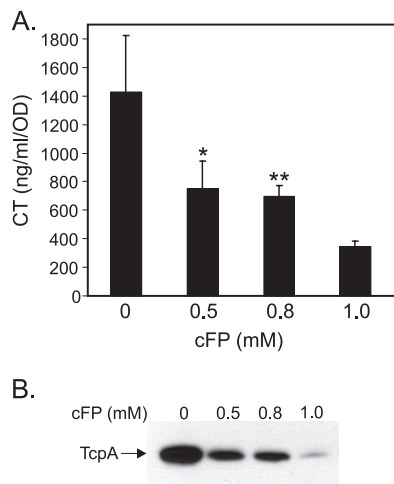


FIG. 1. cFP inhibits CT and TCP production. *V. cholerae* was cultured under AKI conditions in the presence of the indicated concentrations of cFP before CT and TcpA production was assayed. (A) Cholera toxin production. The results are the averages of three or more independent experiments \pm standard deviations. Statistical analysis was performed using a Tukey-Kramer multiple comparison test. *, $P < 0.05$; **, $P < 0.01$. (B) Results of TcpA Western blot analysis of whole-cell lysates.

the published data (20), we did observe increased *ompU* expression in N16961 during growth under AKI conditions in AKI broth supplemented with 1 mM cFP (data not shown). This observation suggests that strain-specific variation could account for the contrasting CT production results obtained in these two studies. Alternatively, the differences in CT production could be due to the methods that were used for CT detection and quantification in each respective study.

Since CT and TCP production are controlled by the ToxR regulon, we assayed to determine if the presence of cFP in AKI broth affected the induction of the ToxR regulon. This was accomplished by using bioluminescence reporters (kindly provided by Jun Zhu, University of Pennsylvania) (22) to quantify *toxR*, *tcpP*, *toxT*, *ctxAB*, and *tcpA* transcription. Strain N16961 containing the indicated reporters was cultured under AKI growth conditions in the presence or absence of 1 mM cFP for 5 h, at which time aliquots of the cultures were transferred to white 96-well microtiter plates for quantification of luminescence production using a BioTek Synergy HT spectrophotometer. Luminescence production was reported as the relative light units divided by the optical density at 630 nm (OD_{630}). We first examined the effect of cFP on *ctxAB* and *tcpA* transcription. We found that the addition of 1 mM cFP resulted in ~ 21 - and 29-fold reductions in *ctxAB* and *tcpA* promoter activities, respectively, suggesting that cFP inhibition of CT and TcpA occurred at the transcription level (Fig. 2A). Because the *ctxAB* and *tcpA* promoters are directly regulated by ToxT, which is itself regulated by ToxR and TcpP, we next assayed to determine if cFP affected the expression of any of these regulatory genes during growth under AKI growth conditions. The results showed that cFP had little, if any, effect on *toxR* transcription (Fig. 2A). As enhanced *ompU* expression in the presence of cFP appears to be *toxR* dependent (20), this result suggests the possibility that cFP could affect the activation state

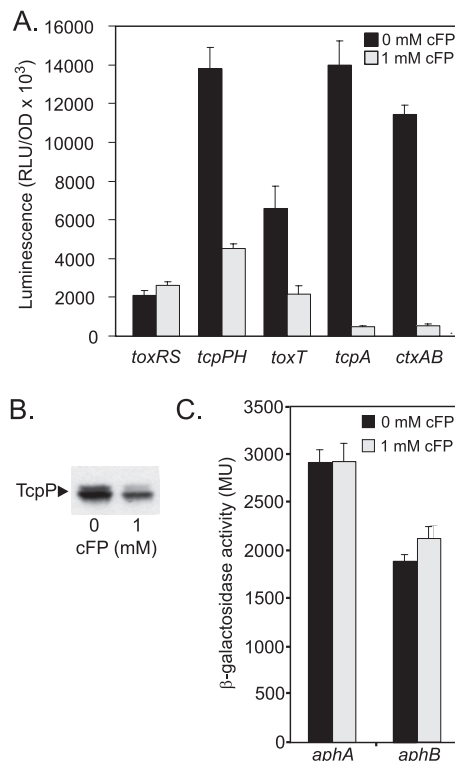


FIG. 2. cFP inhibits *tcpPH*, *toxT*, *tcpA*, and *ctxAB* transcription. *V. cholerae* containing the indicated promoter-*lux* reporters was grown under AKI conditions in the presence or absence of 1 mM cFP for 5 h, at which time luminescence production was quantified. (A) Effects of 1 mM cFP on gene expression. RLU, relative light units. (B) Results of TcpP Western blot analysis of strain N16961 grown in the presence or absence of 1 mM cFP. (C) Effects of 1 mM cFP on *aphA* and *aphB* expression at 5 h. MU, Miller units. The reported results are the averages of the results of three or more experiments \pm standard deviations.

of ToxR and thereby enhance *ompU* expression, but additional work will be required to test this hypothesis. In contrast, *tcpPH* and *toxT* expression were both reduced by ~ 3.5 -fold. As *toxT*, *ctxAB*, and *tcpA* are all downstream of *tcpPH* in the *toxR* regulon, these results suggested that cFP inhibits *tcpPH* expression during growth under AKI conditions.

If cFP inhibits *tcpPH* transcription, we hypothesized that cFP treatment should also result in a corresponding reduction in the amount of TcpP protein. To test this, we performed a TcpP Western blot analysis. Strain N16961 was grown under AKI conditions in the presence or absence of 1 mM cFP for 5 h, at which time aliquots of the cultures were collected and normalized to equivalent OD_{600} . The cells from equal volumes of each culture were then collected by centrifugation, resuspended in equal volumes of SDS-PAGE solubilization buffer, and heated at 100°C for 10 min. Equal volumes of each sample were then resolved by SDS-12.5% PAGE before being transferred to a polyvinylidene fluoride membrane. The membrane was incubated with rabbit polyclonal antisera against TcpP (kind gift of Vic DiRita, University of Michigan) (16), and immunoreactive proteins were visualized as previously described (3). The results of the Western blot analysis showed that the addition of 1 mM cFP inhibited TcpP production (Fig.

2B). This result is consistent with that of the *tcpPH* reporter assay and provides further evidence indicating that cFP is an inhibitor of *tcpPH* transcription.

The expression of *tcpPH* is positively regulated by two cytoplasmic regulators, AphA (21) and AphB (13), which bind cooperatively to the *tcpPH* promoter to activate its expression. Thus, one mechanism by which cFP could affect *tcpPH* expression is by altering *aphA* or *aphB* expression. To test this hypothesis, we constructed transcriptional reporters for *aphA* and *aphB* in pTL61T (15), as previously described (3). N16961 cells containing pXB203 (*aphA-lacZ*) or pXB204 (*aphB-lacZ*) were then grown under AKI conditions in the presence or absence of 1 mM cFP for 5 h, at which time the β -galactosidase activity was quantified. The results showed that the expression levels of both *aphA* and *aphB* in cFP-treated cultures were similar to their expression levels in the cFP-negative control cultures (Fig. 2C), suggesting that cFP probably does not affect *tcpPH* transcription via the expression of *aphA* or *aphB*.

We hypothesized that if the negative effects of cFP on CT and TCP production resulted from the inhibition of *tcpPH* expression, then the overexpression of *tcpPH* or the downstream regulatory gene *toxT* should complement for CT and TCP production during growth in the presence of inhibitory concentrations of cFP. To test this, we overexpressed *tcpPH* and *toxT* from the arabinose promoter in pBAD24 (7). N16961 containing pBAD24, pBAD24::*toxT*, or pBAD24::*tcpPH* (8) was cultured in the presence and absence of 1 mM cFP under AKI conditions in AKI broth supplemented with 0.004% arabinose to induce gene expression from the arabinose promoter in pBAD24. The cultures were then assayed for CT and TCP production as previously described (3). The results showed that 1 mM cFP strongly inhibited CT production in the pBAD24 control culture (Fig. 3A). In contrast, the overexpression of *toxT* (gray bars) or *tcpPH* in the cFP-exposed cultures complemented CT production to the levels observed in the cFP-negative control cultures. Corresponding results were observed in the TcpA Western blot analysis, where 1 mM cFP strongly inhibited TCP production in the vector control culture (Fig. 3B) and the overexpression of *toxT* or *tcpPH* alleviated the inhibitory effects of cFP on TcpA production in the cFP-treated cultures. Overall, these results showed that ectopic overexpression of *tcpPH* or *toxT* alleviated the inhibitory effects of cFP on CT and TCP production. Based on these results, we concluded that the negative effects of cFP on CT and TCP production resulted from inhibition of *tcpPH* transcription in the strains assayed.

The collective findings presented here indicate that cFP inhibits the production of CT and TCP during the growth of O1 El Tor strains under virulence gene-inducing conditions by negatively affecting *tcpPH* transcription. There are a number of known mechanisms by which cFP could affect *tcpPH* expression. For example, it is possible that cFP alters AphA or AphB activity. The crystal structure of AphA showed that it had an N-terminal winged-helix DNA-binding domain similar to that of MarR family proteins (5). As MarR activity has been shown to be modulated by effector molecules (1), it is possible that cFP could affect AphA activity at the *tcpPH* promoter. AphB is a LysR family transcriptional regulator that has been hypothesized to activate *tcpPH* expression in response to yet-unknown environmental signals (13). Thus, cFP could function by alter-

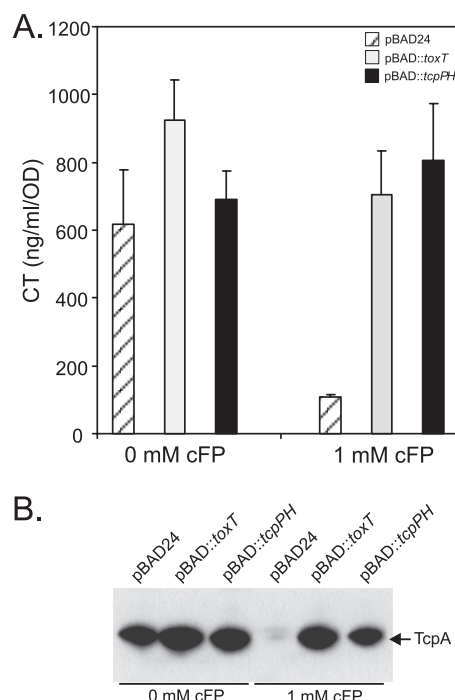


FIG. 3. Overexpression of *tcpPH* and *toxT* alleviates cFP-dependent inhibition of CT and TCP production. *V. cholerae* containing the indicated expression vector was grown under AKI conditions in the presence or absence of 1 mM cFP. Arabinose (0.004%) was added to the AKI broth to induce expression of *toxT* and *tcpPH*. (A) Cholera toxin production. The CT results are the averages of the results of three experiments \pm standard deviations. (B) Results of TcpA Western blot analysis of whole-cell lysates.

ing AphB activity at the *tcpPH* promoter in a similar manner. Alternatively, cFP could affect cyclic AMP (cAMP) production and thereby affect *tcpPH* expression, since cAMP receptor protein-cAMP has been shown to be a negative regulator of *tcpPH* expression (12). Lastly, it is also possible that cFP affects *tcpPH* transcription through a novel mechanism. Work is ongoing in our laboratory to determine the mechanism of cFP action in virulence gene expression and its significance in *V. cholerae* pathogenesis.

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