國立交通大學

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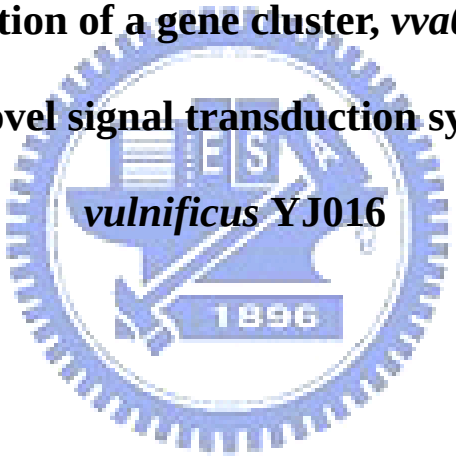
碩士論文

創傷弧菌 YJ016 中一套訊號傳遞系統 *vva0326~vva0329* 的特性分析探討

Characterization of a gene cluster, *vva0326~vva0329*, encoding a novel signal transduction system in *Vibrio vulnificus* YJ016



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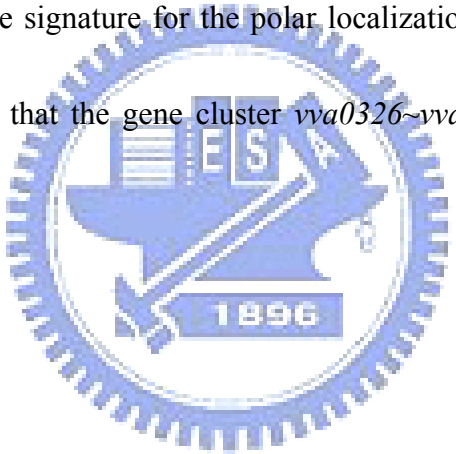
中文摘要

在創傷弧菌 YJ016 的基因體中，我們發現了一組基因群和霍亂弧菌的三分子調控系統 VieSAB 序列相似。而分析這套基因組轉譯後的蛋白序列顯示 VVA0329 為感應蛋白，VVA0328 和 VVA0327 分別和 VieA 的 N 端、C 端的序列相似，而 VVA0326 則為具有 GGDEF 功能區塊的蛋白。許多報導證實：含有 GGDEF 和 EAL 功能區塊的蛋白，分別具有雙鳥苷酸環化酶（Digunylate Cyclase）及磷酸雙脂鍵分解酶（Phosphodiesterase）的活性。在此論文研究中，我們發現將 VVA0328 中的 EAL 區域剔除，會降低創傷弧菌 YJ016 的泳動性，但會增加生物膜的生成。相對的，剔除 VVA0326 的 GGDEF 區塊，會使創傷弧菌的泳動力增加，而減少生物膜的生成。而量測 VVA0326 缺損的創傷弧菌細胞內環狀雙鳥苷酸（cyclic di-GMP）的濃度時，我們發現環狀雙鳥苷酸的含量在 VVA0326 缺損株中明顯降低，而在此缺損株轉形給予 VVA0326 的表現質體時，可以提高環狀雙鳥苷酸的濃度，這個結果暗示 VVA0326 蛋白具有雙鳥苷酸環化酶的活性。同時，我分別構築了攜帶 VVA0326 的 GGDEF 區域及 VVA0328 的 EAL 區域的表現質體，進一步在大腸桿菌中大量表現並純化這兩個重組蛋白。經酵素活性測試，我們發現這兩個僅帶有 GGDEF 及 EAL 區塊的重組蛋白分別具有微弱的雙鳥苷酸環化酶和磷酸雙脂鍵分解酶的活性。由此可推知，VVA0326 和 VVA0328 蛋白可能藉著相對的酵素活性來調控細菌體內環狀雙鳥苷酸的濃度，進而影響細菌的表現型態。我們實驗室先前的實驗發現，分別將 VVA0326 和 VVA0328 和螢光表現蛋白作轉譯融合的重組蛋白，在大腸桿菌中會有極化的表現，顯示這兩個蛋白中可能帶有造成蛋白極化分佈的序列。我將這兩個蛋白分別截短後發現，這種極化分佈的現象便消失了。這項結果指出，VVA0326 及 VVA0328 蛋白極化分佈的現象可能需要完整長度的蛋白；而另一個可能性是我們在作截短構築時破壞了特定接受極化信號的序列。最後，我以反轉錄核酸增幅反應（RT-PCR）確認 *vva0326~vva0329* 基因組同屬一操縱子（operon）。

Abstract

In *Vibrio vulnificus* YJ016 genome, a gene cluster highly homologous to the three-component system VieSAB of *V. cholerae* was identified. This consists of VVA0329, encoding a VieS homolog, VVA0328 and VVA0327, exhibiting homology with the N- and C-terminal parts of VieA, respectively, and VVA0326, encoding a protein with a GGDEF domain. The GGDEF- and EAL-domain containing proteins have been reported to carry enzymatic activity of diguanylate cylase and phosphodisterase, respectively. Deletion of the EAL domain from VVA0328 reduced the swimming motility but enhanced the biofilm formation activity of *V. vulnificus* YJ016. On the other hand, deleting GGDEF domain from VVA0326 protein increased the bacterial swimming activity but diminished the biofilm formation. The reduced level of c-di-GMP could be complemented upon introducing a VVA0326 expression plasmid into the VVA0326 mutant, indicating that VVA0326 containing GGDEF domain likely possesses diguanylate cyclase activity involved in c-di-GMP synthesis. In addition, the GGDEF and EAL domains have been subcloned into expression vector, the recombinant proteins synthesized in *Escherichia coli* and purified. The subsequent enzyme activity analysis revealed that the recombinant proteins respectively carry low level of DGC and PDE activity. This indicates that VVA0326 and VVA0328 regulate intracellular c-di-GMP level inversely to influence the

bacterial behaviors. We have previously reported that the recombinant *E. coli* JM109 carrying either VVA0326-GFP or VVA0328-GFP exhibited green fluorescence at the cell poles. To investigate if a specific sequence is present for the polar localization, several truncated forms of VVA0326 and VVA0328 were generated. The following fluorescent microscopy analysis revealed that all the truncation led to disperse distribution of the fusion proteins. This suggested that the entire protein of VVA0326 or VVA0328 is required for the polar localization. However, the possibility that the truncation interrupted the signature for the polar localization could not be ruled out. Finally, we have shown that the gene cluster *vva0326~vva0329* is an operon using RT-PCR analysis.



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Introduction

Vibrio vulnificus

Vibrio vulnificus, a motile Gram-negative curved rod-shaped bacterium with a single polar flagellum is an opportunistic pathogen causing rare and yet devastating disease. People who drink excess or have hepatitis infected by the bacteria are at high risk of causing death up to 60% (1,2). *V. vulnificus* commonly exists in marine environment is halophile and a naturally occurring, free-living inhabitant of estuarine throughout the world. The infections generally occur through the consumption of raw oysters or contamination of wounds, leading to complications such as invasive septicemia (3). Clinically, tetracycline was the best treatment in the past. The recent reports, however, indicated that cefotaxime and minocyclinex are more efficient in treating patients (4). Except for antibiotic treatment, development of a new antimicrobial approach is also investigated.

V. vulnificus contains two chromosomes, the larger one has 3377 kbp and the other has 1857 kbp (5). Two-chromosome configuration is commonly existed in vibrios demonstrated by pulsed-field gel electrophoresis (6). The wide range of the size of the small chromosome (0.8 to 2.4 Mb) comparing to the narrow range of the large chromosome (3.0 to 3.3 Mb) implies a more flexible feature of the small chromosome during the evolution of vibrios. Up to now, several factors including an

extracellular hemolysin/cytolysin (7), an elastolytic protease (8), the capability of iron acquisition (9), a polysaccharide capsule (10) and an endotoxic lipopolysaccharide (11), and serum resistance activity (12) have been implicated as possible virulence determinants for *V. vulnificus* infection. Homology search and gene annotation using BLAST analysis in NCBI (http://www.ncbi.nlm.nih.gov) and domain search in Pfam database (http://www.sanger.ac.uk/Software/Pfam/) revealed two virulence associated RTX genes VVA0331 and VVA1030 on the small chromosome of *V. vulnificus* YJ016 (5).

RTX toxin in *V. vulnificus*

RTX toxins (repeats in the structural toxin), the pore-forming protein toxins, which produced by many gram-negative bacteria play an important role in the bacterial infections in human or animals by exhibiting cytotoxic and hemolytic activities (13). Commonly, the RTX family toxins possessed a conserved sequence composed of a C-terminal Ca^{2+} binding domain of acidic glycine-rich nonapeptide repeat. After posttranslational modification by acylation via a specific activator protein, they are exported out of the cell by type I secretion system (14). Ca^{2+} binding, an important process for the virulence of RTX toxins occurs following the exportation (15). VcRtxA has common features of RTX family such as posttranslation, C-terminal

Ca2+ binding domain and export via type I secretion system. In *V. vulnificus*, the RtxA encoding gene VVA1030 (VvRtxA) is followed by the gene cluster coding for type I secretion system. In comparing with the amino acid sequences of VcRtxA, 80-90% identity was found throughout most regions of VvRtxA (38).

On the small chromosome, a large protein (4656 aa) encoding gene *vva0331* located next to *vva0332~vva0334* which also encodes a type-I-secretion-like system has previously been identified (5). Accordingly, the large protein encoding gene was named *rtxL* for RTX like protein. Upstream of the *rtxL* gene, a two component signal transduction system (2CS) encoding gene cluster (*vva0326~vva0329*) was identified (Appendix.1). Sequence analysis revealed that VVA0329 is a VieS homolog of His-kinase. VVA0328 and VVA0327 share homology with N- and C-portion of VieA respectively. The last open reading frame in the gene cluster, VVA0326, encodes a GGDEF protein (Appendix. 2).

VieSAB, a three component system, influences the virulence of *V. cholerae*

In *V. cholerae*, the three-component system VieSAB encoded by a tricistronic operon has been shown to regulate the expression of CT (cholera toxin) during infection (16-18). VieS is a sensor kinase that harbors a three cytosolic domains, a transmitter, a medial receiver domain, and a C-terminal transmitter (19). Both VieA

and VieB are response regulators. VieA carries a LuxR-family type of DNA binding motif and an EAL domain. VieB has also a typical N-terminal phosphoreceiver domain of response regulator, but lacking any recognizable DNA binding motif (20). The EAL domain of VieA carries a cyclic diguanylate (c-di-GMP) phosphodiesterase (PDE) activity to control the intracellular concentration of c-di-GMP. The c-di-GMP level determines if an optimal expression of *vps* (*Vibrio* exopolysaccharide synthesis) occurred (21). VPS of rugose variant is responsible for resistance to osmotic, acid, and oxidative stress (22,23). When the bacteria turn to rugose from smooth variants, the exopolysaccharides are increased together with an increased capacity to produce biofilm.

C-di-GMP is a second messenger in bacteria

The molecular structure of bis-3',5'-cyclic dimeric guanosine monophosphate (c-di-GMP) and its presence in bacteria was discovered 17 years ago (24). The intracellular level of c-di-GMP has been implicated to affect many bacteria surface structure such as biofilm information, cellulose, fimbria, and exopolysaccharide. High level c-di-GMP leads bacteria to sessile state, and increasing biofilm formation and cellulose synthesis. In contrast, low level c-di-GMP stimulated bacteria to stay in mobile state and increase of swimming and swarming motility. The c-di-GMP level

also affects virulence of bacteria such as *V. cholera*. Investigation of the regulatory network of *ctxAB* transcription in *V. cholera* showed that the decrease in c-di-GMP level mediated by VieA positively affected transcription of ToxT, a transcriptional regulator that directly activates expression of *ctxAB* to enhance the bacterial virulence (25). The inverse regulation of biofilm formation and intensity of virulence indicated c-di-GMP is an important signal for *V. cholera* to respond for the transition from environment to host (25). The regulatory effects of c-di-GMP were observed not only in prokaryote but also in eukaryote. Recent report indicated that c-di-GMP can inhibit the colon cancer cell growth (26).

GGDEF and EAL proteins have been demonstrated to respectively exert c-di-GMP cyclase and c-di-GMP PDE activity to regulate intracellular c-di-GMP level inversely (27,28). The search using HMMER in the genome of *V. vulnificus* YJ016 revealed 63 GGDEF and 31 EAL proteins, and 18 of them carry both domains (5). With so many proteins involved in modulation of the intracellular c-di-GMP level, complexity of the regulation could be expected in the bacteria.

VVA0326-0327-0328-0329, a novel signal transduction system

In analysis of the genome of *V. vulnificus* YJ016 (5), a VieSAB gene cluster VVA0647 to VVA0649 was identified (Appendix. 2). Another set of homolog

VVA0329, VVA0328 and VVA0327 was also found. Both VVA0327 and VVA0328 are typical response regulators with a LuxR-family type of DNA binding motif. In addition to a typical receiver domain at the N-terminus, VVA0328 also carries an EAL domain. Previously, the recombinant proteins VVA0326-GFP and VVA0328-GFP have been shown to express at the cell pole of *E. coli* JM109 (38). The level of c-di-GMP has been shown to act as a messenger to direct the polar localization of GGDEF or EAL protein for the expression of flagella (2). Polar localization is fundamental of many cellular processes including DNA replication, chromosome segregation, protein secretion, chemotaxis, adhesion, motility, cell division, cell shape and virulence (29). Whether the polar localization of VVA0326-GFP and VVA0328-GFP are associated with the modulation of the c-di-GMP level is interesting to be investigated.

Specific aims

The deletion mutants *vva0326*$^-$ and *vva0328*$^-$ have previously been constructed and the deletion of GGDEF domain from VVA0326 was shown to increase the bacterial swimming activity (30). In this study, the complementation plasmids have been constructed and the deletion effects on the intracellular c-di-GMP concentration are measured. In addition, enzymatic activity of the recombinant VVA0326 and

VVA0328 and the role of either GGDEF or EAL domain in determining the cellular localization are investigated. Finally, regulation of the gene cluster is analyzed.

Materials and methods

Bacterial strains, plasmids, and growth conditions. *Vibrio vulnificus* YJ016 is supplied by professor L.I. Hor at the department of Microbiology, National Cheng Kung University. The relevant bacterial strains and plasmids are listed in Table 1 and Table 2. All bacteria were propagated at 37°C in Luria-Bertani (LB, MDbio, Inc) broth or the medium supplemented with suitable antibiotics for 12~16 hours.

Preparation of genomic DNA from bacteria. *V. vulnificus* was grown overnight in rich broth and the bacteria collected by centrifugation. The bacteria was resuspended in 500 μl TE buffer (10 mM Tris-Cl, pH 7.5 1 mM EDTA which was made from 1 M stock of Tris-Cl pH 7.5 and 500 mM stock of EDTA pH 8.0), 30 μl of 10% SDS and 3 μl of 20 mg/ml proteinase K were then added and the mixture incubated 1 h at 37°C. Subsequently, an equal volume of phenol/chloroform was added and the reaction mixed well by inverting the tube but very gently to avoid shearing the DNA until the phases are completely mixed. The DNA/phenol mixture was transferred into a Phase Lock Gel™ tube (green) and was spun at 7,200 *Xg* for 10 min. The upper aqueous phase was moved to a new tube and an equal volume of phenol/chloroform added, and the extraction procedure was repeated. To the aqueous phase, one tenth of pH 5.2 sodium acetate (3 M) and approximately 2X volume of isopropanol were added and the reaction mixed gently until the DNA precipitates. Finally, the DNA was spooled

onto a glass rod (or Pasteur pipet with a heat-sealed end) and washed by dipping end of the rod into 1 ml of 70% ethanol for 30 sec. The DNA was then resuspended in 200 μl TE buffer and was stored at 4°C, or -20 to -80°C for long term storage. Until completely dissolved, DNA concentration is determined by measuring the absorbance at 260 nm.

Construction of VVA0326 and VVA0328 expression plasmids. The primer pairs GP7/GP8 and EP5/EP6 were respectively used for amplifying the fragment G78 1450 bp, and E56 1250 bp then the two fragments cloned into yT&A vector respectively (pG0326.1 and pG0328.1). The *Bam*HI/*Hin*dIII-digested fragments of pG0326.1 and pG0328.1 were then subcloned into pMMB66, and the resulting plasmids pG0326.2 and pG0328.2 transformed into *E. coli S17-1λπ*. Finally, the plasmids were respectively transferred into *V. vulnificus* by conjugation.

Swimming assay. Tryptone swimming plates are composed of 0.3% Bacto Agar, and HI broth. The tested bacteria were inoculated with a sterile tip onto the swimming plate and incubated for 16 h at 30°C.

Biofilm formation analysis. Biofilm formation was assayed by the ability of the cells to adhere to the walls of 96-well microtitre plates made of PVC or glass tube with some modification of the reported protocol (31). The indicator medium (100 μl/well) contained an aliquot of 1:10 diluted overnight bacteria culture. The plate was

incubated at 30℃ with shaking 48 h for biofilm formation. The bacteria were then transferred to a new plate. The unadherent bacteria on the old plate were washed triply with 200 μl saline, the plate dried at room temperature for 15~20 min, and 200 μl of 1% crystal violet (CV) was added to each well. After staining for twenty minutes, each well was washed triply by 200 μl saline. Finally, the CV-stained biofilm was solubilized in 200 μl of 95% ethanol and the absorbance determined at OD_{550nm}.

Analysis of the intracellular c-di-GMP level by reversed-phase HPLC. To prepare for the extracts, 20 ml overnight cultures were inoculated into 500 ml LB and refreshed grown at 37 ºC for 3 h. Then the bacteria collected by centrifugation at 7800 Xg for 10 min and the supernatant discarded. The pellets were then extracted with 0.6 M $HClO_4$, and the denatured protein was removed by centrifugation. Subsequently, the supernatant was neutralized with 5 M K_2CO_3 to pH 6.0, and then subjected to lyophilization (32). The powder was dissolved in 1% NaCl and passed through the 0.22 μm filter. Then the resulting solution were injected into 4.6 X 150 mm ALANTIS DC18.5 μm column (Waters co.) and separated by reversed-phase HPLC. The working buffer A is 0.9% NaCl in water, and the working buffer B is 80% acetonitrile in water. The gradient of A to B ratio started from 100:0 to 40:60 in 60 min, and the detection measured at 254 nm under the flow rate of 1.0 ml/min at 40 ºC.

Construction of pG0326.4 and pG0328.4. The GGDEF domain of *vva0326* was amplified by primers, GP9 and GP2, and the EAL domain of *vva0328* was amplified by primers, EP3 and EP2 listed in Table 3. The PCR products were cloned into yT&A vector and the resulting plasmids are pG0326.3 and pG0328.3. The *Hin*dIII/*Bam*HI fragment of pG0326.3 or pG0328.3 was then subcloned to the expression vector pET30b and the resulting plasmids are pG0326.4 and pG0328.4.

Purification of the recombinant proteins

For purification of the recombinant proteins, G0326, and E0328, the following protocol was used. Briefly, isopropyl-D-thiogalactopyranoside (IPTG) 0.5 mM) was added to exponentially growing *E. coli* BL21 DE3 cells. After 3 h induction, the cells were chilled to 4℃ and collected by centrifugation. The cell pellets were resuspended in buffer containing 200 mM NaCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.6), and 5 % glycerol. The cell suspensions were then subjected to brief sonication and the pellets discarded by centrifugation. Soluble protein fractions were collected and mixed with preequilibrated Ni_2-resin, which was placed into a column and extensively washed with buffer containing 175 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.9). Finally, the recombinant proteins, G0326 and E0328 were

eluted by washing with elute buffer consisted of 200 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.9). The concentration of imidazole was decreased from 200 mM to 0 mM in dialysis buffer, and the protein stored at -80℃ (27). Protein concentration was determined by measuring the absorbance at 260 nm.

Enzyme activity assay. The optimized buffer for DGC assay is composed of 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 0.5 mM EDTA, and 50 mM NaCl (28). The reaction was started by the addition of 100 μl 2 mM GTP and 300 μl protein to the prewarmed reaction mixture (total volume 500 μl) and the reaction was carried out at 28℃ for 1.5 h. The PDE buffer for E0328 is composed of 50 mM Tris-HCl (pH 9.35), 5 mM $MgCl_2$, 0.5 mM EDTA, 50 mM NaCl, and nucleotide substrate at a concentration of 100 mM (27). The reaction was started by the addition of 100 μl 2 mM c-di-GMP supplied by Dr. Yoshihiro Hayakawa (Laboratory of Bioorganic Chemistry, Graduate School of Information Science, Nagoya University Chikusa, Nagoya 464-8601, Japan) and 300 μl protein to the prewarmed reaction mixture and the reaction was carried out at 37℃ for 4 h. Both reactions were stopped in boiling water for 5 min and followed by centrifugation at 7,800 *X*g for 3 min to remove the denatured proteins. Each of the samples of 500 μl was injected into the 4.6 X 150 mm

ALANTIS DC18.5 μm column (Waters co.) and separated by reversed-phase HPLC. The elution buffer A is 0.9% NaCl in water, and buffer B is 80% acetonitrile in water. The gradient of A to B ratio is from 100:0 to 40:60 in 60 min and then the eluted product measured at 254 nm at flow rate of 1.0 ml/min at 40 ºC. Specific activity is a convenient method of communicating theamount of enzyme activity you have in each milligram of protein. The specific activity is (units/ml)/(mg total protein/ml).

Construction of the GFP fusion protein. The primer pairs, GP3/GP2, EP1/EP4 and EP3/EP2 were used respectively to amplify the fragments, G23, E14,and E32 and then cloned into yT&A vector. The resulting plasmids are pVA0326.1yTA, pVA0328.1yTA and pVA0328.2yTA (Table 3). The *Hin*dIII/*Kpn*I fragment of pVA0326.1yTA, pVA0328.1yTA or pVA0328.2yTA was then subcloned in frame fusion to pGFPuv. The resulting plasmids pVA0326.1GFP, pVA0328.1GFP and pVA0328.2GFP were then transformed into JM109 and the location of the GFP fusion protein was examined by fluorescence microscopy. The photographs were taken under a normal light source to reveal the shape of the bacteria and with an excitation light equipped with a green filter to observe the localization of fluorescence in relation to the cell.

Preparation of total RNA from bacteria. Overnight culture of *V. vulnificus* was refreshed grown in LB broth (1:10) at 37℃ for 2 h. The bacteria were then collected by centrifugation, and 1 ml TRI reagent (Sigma) was added and the mixture incubated at 65℃ for 10 min. Then, 200 μl chloroform was added and the mixture vortexed. After centrifugation at 4℃ 7,800 *X*g for 15 min, the supernatant was collected, and 50 μl 3 M sodium acetate (pH 5.2) and equal volume of isopropanol were added. The mixture was frozen at -20℃ for 30 min, and centrifuged at 4℃ 7,800 *X*g for 15 min. The pellet was then washed with 75% ethanol to remove the residual salts. Finally, RNA was dried, dissolved using DEPC H_2O, and appropriate DNAase was added to remove contaminated DNA. The RNA was stored at -80℃ before use. An aliquot of the RNA of was used for first-strand cDNA synthesis and PCR by Maxime RT-PCR PreMix Kit.

Southern blot hybridization assay. The DNA fragments obtained from RT-PCR were resolved on 1% agrose gels by electrophoresis and transferred to nylon membrane (Amersham Pharmacia Inc.). Subsequenctly the membrane was hybridized with either of the probes 1 (for detection of *vva0326*) and 2 (for detection of *vva0328*) to confirm the produsts of RT-PCR.

Construction of the promoter reporter plasmid pGVA0329. The pYC292 (a gift from Dr. Yi-Chyi Lai, Institute of Medcine, Chung Shan Medical University) is a suicide vector carrying ampiciliin resistance gene, and bioluminescence gene. The putative promoter of VVA0329 containing approximately 1832 bp noncoding DNA upstream of *vva0329* was amplified by primer pair VVA03/VVA04 and cloned into TA vector. The restriction enzymes *Bam*HI and *Sac*I were then used to isolate the promoter from the TA vector-derived plasmids and then subcloned into pYC292. The resulting plasmid pPVA0329YC292 was then transferred from *E. coli S17-1λπ* to *V. vulnificus* YJ016 by conjugation. A luxminescence reader (TD-20/20, Turner Designs) was used to measure the luxminescence for the promoter activity.

Results

Deletion of *vva0326* or *vva0328* affected the swimming activity and biofilm formation

The strong mobility activity is an important property of *V. vulnificus* (1,2). As shown in Fig.1, the deletion of either *vva0326* or *vva0328* appeared to affect the swimming activity. Comparing to the wild type, the VVA0326$^-$ has better swimming activity after incubation at 30℃ for 16 hours. The activity was apparently decreased after introducing the plasmid pG0326.2 with an expression of *vva0326* to VVA0326$^-$. On the other hand, VVA0328$^-$ had lower swimming activity than the wild type bacteria. Transformation of VVA0328$^-$ with pG0328.2 with an expression of *vva0328* could increase the swimming activity. It has been reported that the intracellular c-di-GMP level could affect the flagella formation leading to alteration of the swimming activity (2). The deletion effect of VVA0326 and VVA0328 on the swimming activity suggested they both play a role in regulation of the intracellular c-di-GMP level.

It has been reported that high intracellular c-di-GMP level could enhance bacterial biofilm formation (33). As shown in Fig. 2, biofilm formation was decreased in the mutant, VVA0326$^-$, while the mutant, VVA0328$^-$ had better biofilm formation activity. The complementation assay further confirmed the deletion effect of either *vva0326* or *vva0328* on the biofilm formation capability (Fig.2).

Intracellular level of c-di-GMP

As shown in Table 4, lower level of intracellular c-di-GMP was found in *vva0326*⁻ strain when compared to that of wild type bacteria. After introducing into the plasmid, pG0326.2, the c-di-GMP level appeared to be increased. However, the intracellular c-di-GMP level in *vva0328*⁻ had no apparent change. Additionally the complement of the *vva0328*⁻ has more intracellular c-di-GMP level than wild type. The result is unexpectedly.

Isolation of the recombinant GGDEF- and EAL-domain proteins

The protein containing GGDEF domain commonly expresses diguanylate cylase (DGC) activity and the protein containing EAL domain carries phosphodiesterase (PDE) activity (2). It is hence we speculate that the above phenotypes are probably regulated by the enzymatic activity of VVA0326 and VVA0328. The recombinant plasmid for over-expression of the entire coding region of *vva0328* has been obtained. However, the over-expressed recombinant protein appeared to be in insoluble form. Subsequently, the recombinant plasmids, pG0326.4 and pG0328.4, respectively containing the GGDEF domain of *vva0326* and EAL domain of *vva0328* were generated (Table. 2). As shown in Fig. 3A, the bacteria carrying pG0326.4 synthesized a large amount of the protein with size of approximately 25 kDa which is

comparable to the predicted size of the GGDEF domain of VVA0326. The bacteria carrying pG0328.4 also appeared to overproduce a protein with the expected size of the EAL domain of VVA0328, which is about 37 kDa (Fig. 3B). The recombinant proteins were then purified for enzyme activity measurement.

Enzymatic activity of the recombinant proteins VVA0326-GGDEF and VVA0328-EAL

Fig. 4A shows the peak with elution time 2.79 min for 0.4 mM GTP, the substrate of the reaction. While the peak with elution time 24.62 min in Fig. 4B represents the reaction product of 0.4 mM c-di-GMP. As shown in Fig. 4C, adding the GGDEF domain protein, G0326, into the reaction appeared to transform GTP into c-di-GMP. However, the relatively small peak of c-di-GMP (0.0217 mM) indicated a low level of DGC activity. Nevertheless, not any peak could be observed in Fig. 4D, which containing only the recombinant GGDEF domain protein, supported an authentic DGC activity of the recombinant GGDEF protein. As shown in Fig. 5, the enzyme activity increased with increasing amounts of the purified GGDEF proteins further demonstrated a DGC activity of the protein. The enzyme specific activity of the recombinant protein (GGDEF domain protein) is 1.729 ± 0.2574 unit/mg.

As for the measurement of PDE activity, the elution peak of 0.4 mM GMP with

elution time 2.32 min (Fig. 6A) and 0.4 mM c-di-GMP with elution time 17.9 min (Fig. 6B) were obtained. As shown in Fig. 6C, the recombinant EAL domain protein also appeared to exert a low level PDE activity with the catalytic product of 0.0191 mM GMP. As shown in Fig. 7, the enzyme activity increased with increasing amounts of the purified EAL proteins also supported a PDE activity of the protein. The enzyme specific activity of the recombinant protein (EAL domain protein) is 5.6935 ± 0.2213 unit/mg.

Identification of the sequence responsible for subcellular localization of VVA0326 and VVA0328

To verify which domain of VVA0326 and VVA0328 are responsible for the polar localization that has previously been reported (38), several GFP fusion constructs, pVA26.1GFP, pVA28.1GFP, and pVAE28.2GFP are generated and shown in Fig. 8. The fusion plasmids were then transformed to *E. coli* JM109 and the location of the fusion proteins in bacteria were observed by fluorescence microscopy. As shown in Fig. 9A, the GFP fusion protein containing the entire coding region of VVA0326 appeared to distribute at the cell pole. While truncation of the transmembrane domain of VVA0326 led the VA26.1GFP fusion protein to disperse all over the bacteria (Fig. 9B). The location of VVA0328-GFP protein also appeared at the pole of the bacteria

(Fig. 10A). As shown in Fig.10B and C, either pVA28.1GFP or pVA28.2GFP in *E. coli* JM109 exerted a dispersed fluorescence

The gene cluster *vva0326~ vva0329* is organized as an operon

As shown in Fig.11A, three primer sets were designed to amplify the junctions of the *vva0326~vva0329*, and probe 1 (*vva0328*) and 2 (*vva0326*) were utilized to carry out southern blot hybridization assay. The probe 1 could hybridize with the junctions of *vva0329~vva0328* and *vva0328~vva0327* (Fig.11B-1 and 11B-2) and probe 2 could hybridize with the junction of *vva0327~vva0326* (Fig.11B-3), indicating that the gene cluster *vva0326~vva0329* is an operon. However, there are signals on the negative control (RNA template). Those signals are might RNA contamination, which could be reduced by adding RNase before carrying out PCR.

In order to find the signals, the putative promoter of *vva0329* was fused to the promoterless *luxCDABE* in pYC292 (Fig. 12). The fusion plasmid pPVA0329YC292 was then integrated into the genome of *V. vulnificus* YJ016 by homologous recombination as a real-time reporter for the expression of the promoter. The expression was determined when the bacterial cultures adding with each of the treatments including 0.5~1 mM paraquat, 0.5~1 mM 2-2 dipyridyle, 0.3~0.6 mM NaCl, or grown at 25 or 37℃). However, all the luminescence signals appeared to be

too weak to show a difference of the treatments.

Discussion

Recently reports indicated that c-di-GMP is a second messenger (34). The production of c-di-GMP appears to show temporal and spatial character, which may have variant influence, and have result in different phenotypes. Its correlation with virulence has also been an interesting topic (35). Most proteins containing GGDEF and EAL domain have additional sensory and signal transduction domain, which suggesting that their activities in c-di-GMP turnover are responsive to environmental cues (36).

As shown in Table. 4, different intracellular c-di-GMP levels were obtained in different strains. Unexpectedly, the complement strain of vva0328^{-} has higher c-di-GMP level than wild type indicating that either VVA0328 has no role in regulating the intracellular c-di-GMP level or a cooperative regulation is required from other EAL protein to modulate the intracellular c-di-GMP level. Another possibility is that high copy-number of pG0328.2, activates the expression of the other GGDEF domain proteins which in turn enhances the production of c-di-GMP.

It has been shown the DGC activity is strictly dependent on GGDEF domain but not related to the full length protein (28). However, the neighboring domains including PAS,GAF,HAMP and REC important have also been shown to be the enzyme activity (37). It is hence low level of enzyme activity obtained for both the

recombinant proteins, G0326 and G0328, could be attributed to the missing of their regulatory domains. The possibility remains to be verified with a full length protein of either VVA0326 or VVA0328.

In order to investigate if VVA0329 sensor could relay the phosphoryl group to each of the response regulators in the signal transduction system. I have also tried to generate the recombinant plasmids for the over-expression of VVA0329 and VVA0327. Unfortunately, the recombinant proteins of VVA0329, VVA0328 and VVA0327 were all in insoluble forms. Neither culture at lower temperature (16 or 25℃), nor adding the culture with betain and sorbital could improve their solubility.

Measurement of the RNA level of *vva0326* or *vva0328* under different conditions may help to identify the signal for c-di-GMP production. The experiment could be carried out by adding c-di-GMP in *V. vulnificus* and then to determine the virulence intensity and the bacterial behavior. Whether c-di-GMP level regulates the expression of the neighboring VVA0331 which in turn affect *V. vulnificus* virulence is also interesting to be investigated. Using the reported assay, no apparent change of the promoter activity with either of the treatments may be attributed to a polar effect of the plasmid integration in case that the gene cluster is auto-regulated. It is also possible that the signals to regulate the expression of the promoter have not yet been identified. In stead, real time PCR analysis could be applied to study the expression of

the genes *vva0326~vva0329* in the future.

Polar localization of some proteins is common phenomenon in bacteria, which allows to carry out the activities for diverse cellular processes including protein secretion, chemotaxis, adherence, motility, cell division, cell shape and virulence (29). The polar localization of VVA0326 and VVA0328 proteins suggested that the bacteria utilize these proteins to regulate the c-di-GMP level at the cell pole to alter the phenotype such as flagella-mediated motility. However, the precise information receiver domain for the polar location is still unclear. The fusion proteins, VA26.1GFP, VA28.1GFP, and VA28.2GFP revealed dispersed distribution in bacteria suggested that the polar localization required a full length protein of either VVA0326 or VVA0328. It is also possible that the truncation interrupted the sequence for the focal localization. Nevertheless, measurement of the change of c-di-GMP level at cell pole would be interesting and challenging.

References:

1. Strom, M.S. and Paranjpye, R.N. (2000) Epidemiology and pathogenesis of *Vibrio vulnificus*. *Microbes Infect*, **2**, 177-188.

2. Simm, R., Morr, M., Kader, A., Nimtz, M. and Romling, U. (2004) GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. *Mol Microbiol*, **53**, 1123-1134.

3. Hoi, L., Larsen, J.L., Dalsgaard, I. and Dalsgaard, A. (1998) Occurrence of *Vibrio vulnificus* biotypes in Danish marine environments. *Appl Environ Microbiol*, **64**, 7-13.

4. Chiang, S.R. and Chuang, Y.C. (2003) *Vibrio vulnificus* infection: clinical manifestations, pathogenesis, and antimicrobial therapy. *J Microbiol Immunol Infect*, **36**, 81-88.

5. Chen, C.Y., Wu, K.M., Chang, Y.C., Chang, C.H., Tsai, H.C., Liao, T.L., Liu, Y.M., Chen, H.J., Shen, A.B., Li, J.C., Su, T. L., Shao, C. P., Lee, C. T., Hor, L. I., Tsai, S. F. (2003) Comparative genome analysis of *Vibrio vulnificus*, a marine pathogen. *Genome Res*, **13**, 2577-2587.

6. Okada, K., Iida, T., Kita-Tsukamoto, K. and Honda, T. (2005) Vibrios commonly possess two chromosomes. *J Bacteriol*, **187**, 752-757.

7. Gray, L.D. and Kreger, A.S. (1989) Detection of *Vibrio vulnificus* cytolysin in

V. vulnificus-infected mice. *Toxicon*, **27**, 439-464.

8. Oliver, J.D., Wear, J.E., Thomas, M.B., Warner, M. and Linder, K. (1986) Production of extracellular enzymes and cytotoxicity by *Vibrio vulnificus*. *Diagn Microbiol Infect Dis*, **5**, 99-111.

9. Biosca, E.G., Fouz, B., Alcaide, E. and Amaro, C. (1996) Siderophore-mediated iron acquisition mechanisms in *Vibrio vulnificus* biotype 2. *Appl Environ Microbiol*, **62**, 928-935.

10. Wright, A.C., Miceli, G.A., Landry, W.L., Christy, J.B., Watkins, W.D. and Morris, J.G., Jr. (1993) Rapid identification of *Vibrio vulnificus* on nonselective media with an alkaline phosphatase-labeled oligonucleotide probe. *Appl Environ Microbiol*, **59**, 541-546.

11. McPherson, V.L., Watts, J.A., Simpson, L.M. and Oliver, J.D. (1991) Physiological effects of the lipopolysaccharide of *Vibrio vulnificus* on mice and rats. *Microbios*, **67**, 141-149.

12. Johnson, D.E., Calia, F.M., Musher, D.M. and Goree, A. (1984) Resistance of *Vibrio vulnificus* to serum bactericidal and opsonizing factors: relation to virulence in suckling mice and humans. *J Infect Dis*, **150**, 413-418.

13. Frey, J. and Kuhnert, P. (2002) RTX toxins in *Pasteurellaceae*. *Int J Med Microbiol*, **292**, 149-158.

14. Welch, R.A. (2001) RTX toxin structure and function: a story of numerous anomalies and few analogies in toxin biology. *Curr Top Microbiol Immunol*, **257**, 85-111.

15. Boehm, D.F., Welch, R.A. and Snyder, I.S. (1990) Calcium is required for binding of *Escherichia coli* hemolysin (HlyA) to erythrocyte membranes. *Infect Immun*, **58**, 1951-1958.

16. Tischler, A.D., Lee, S.H. and Camilli, A. (2002) The *Vibrio cholerae vieSAB* locus encodes a pathway contributing to cholera toxin production. *J Bacteriol*, **184**, 4104-4113.

17. Lee, S.H., Butler, S.M. and Camilli, A. (2001) Selection for in vivo regulators of bacterial virulence. *Proc Natl Acad Sci U S A*, **98**, 6889-6894.

18. Camilli, A. and Mekalanos, J.J. (1995) Use of recombinase gene fusions to identify *Vibrio cholerae* genes induced during infection. *Mol Microbiol*, **18**, 671-683.

19. Lee, S.H., Angelichio, M.J., Mekalanos, J.J. and Camilli, A. (1998) Nucleotide sequence and spatiotemporal expression of the *Vibrio cholerae* vieSAB genes during infection. *J Bacteriol*, **180**, 2298-2305.

20. Galperin, M.Y., Nikolskaya, A.N. and Koonin, E.V. (2001) Novel domains of the prokaryotic two-component signal transduction systems. *FEMS Microbiol*

Lett, **203**, 11-21.

21. Tischler, A.D. and Camilli, A. (2004) Cyclic diguanylate (c-di-GMP) regulates *Vibrio cholerae* biofilm formation. *Mol Microbiol*, **53**, 857-869.

22. Yildiz, F.H., Liu, X.S., Heydorn, A. and Schoolnik, G.K. (2004) Molecular analysis of rugosity in a *Vibrio cholerae* O1 El Tor phase variant. *Mol Microbiol*, **53**, 497-515.

23. Yildiz, F.H. and Schoolnik, G.K. (1999) *Vibrio cholerae* O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. *Proc Natl Acad Sci U S A*, **96**, 4028-4033.

24. Ross, P., Mayer, R., Weinhouse, H., Amikam, D., Huggirat, Y., Benziman, M., de Vroom, E., Fidder, A., de Paus, P., Sliedregt, L.A.. (1990) The cyclic diguanylic acid regulatory system of cellulose synthesis in *Acetobacter xylinum*. Chemical synthesis and biological activity of cyclic nucleotide dimer, trimer, and phosphothioate derivatives. *J Biol Chem*, **265**, 18933-18943.

25. Tischler, A.D. and Camilli, A. (2005) Cyclic diguanylate regulates *Vibrio cholerae* virulence gene expression. *Infect Immun*, **73**, 5873-5882.

26. Karaolis, D.K., Cheng, K., Lipsky, M., Elnabawi, A., Catalano, J., Hyodo, M., Hayakawa, Y. and Raufman, J.P. (2005) 3',5'-Cyclic diguanylic acid

(c-di-GMP) inhibits basal and growth factor-stimulated human colon cancer cell proliferation. *Biochem Biophys Res Commun*, **329**, 40-45.

27. Schmidt, A.J., Ryjenkov, D.A. and Gomelsky, M. (2005) The ubiquitous protein domain EAL is a cyclic diguanylate-specific phosphodiesterase: enzymatically active and inactive EAL domains. *J Bacteriol*, **187**, 4774-4781.

28. Ryjenkov, D.A., Tarutina, M., Moskvin, O.V. and Gomelsky, M. (2005) Cyclic diguanylate is a ubiquitous signaling molecule in bacteria: insights into biochemistry of the GGDEF protein domain. *J Bacteriol*, **187**, 1792-1798.

29. Janakiraman, A. and Goldberg, M.B. (2004) Recent advances on the development of bacterial poles. *Trends Microbiol*, **12**, 518-525.

30. Romling, U. and Amikam, D. (2006) Cyclic di-GMP as a second messenger. *Curr Opin Microbiol*, **9**, 218-228.

31. O'Toole, G.A. and Kolter, R. (1998) Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol*, **28**, 449-461.

32. Mendez-Ortiz, M.M., Hyodo, M., Hayakawa, Y. and Membrillo-Hernandez, J. (2006) Genome-wide transcriptional profile of *Escherichia coli* in response to high levels of the second messenger 3',5'-cyclic diguanylic acid. *J Biol Chem*, **281**, 8090-8099.

33. Lim, B., Beyhan, S., Meir, J. and Yildiz, F.H. (2006) Cyclic-diGMP signal transduction systems in *Vibrio cholerae*: modulation of rugosity and biofilm formation. *Mol Microbiol*, **60**, 331-348.

34. D'Argenio, D.A. and Miller, S.I. (2004) Cyclic di-GMP as a bacterial second messenger. *Microbiology*, **150**, 2497-2502.

35. Kader, A., Simm, R., Gerstel, U., Morr, M. and Romling, U. (2006) Hierarchical involvement of various GGDEF domain proteins in rdar morphotype development of *Salmonella enterica* serovar Typhimurium. *Mol Microbiol*, **60**, 602-616.

36. Ryan, R.P., Fouhy, Y., Lucey, J.F., Jiang, B.L., He, Y.Q., Feng, J.X., Tang, J.L. and Dow, J.M. (2007) Cyclic di-GMP signalling in the virulence and environmental adaptation of *Xanthomonas campestris*. *Mol Microbiol*, **63**, 429-442.

37. Tamayo, R., Pratt, J.T. and Camilli, A. (2007) Roles of Cyclic Diguanylate in the Regulation of Bacterial Pathogenesis. *Annu Rev Microbiol*.

38. 楊祐俊, (2005) Analysis of a virulence-associated genomic island in *Vibrio vulnificus* YJ016. 交通大學生物科技研究所碩士論文

Table 1. Bacterial strains used and constructed in this study

Strain	Genotype or relevant property	Reference or source
E.coli		
JM109	*RecA1 supE44 endA1 hsdR17 gyrA96 rolA1 thi*Δ *(lac-proAB)*	Laboratory stock
NovaBlue (DE3)	*endA1 hsdR17 (*$rk_{12}^{-}mk_{12}^{+}$*) supE44 thi-1 recA1 gyrA96 relA1 lac*[F *pro AB lac*^{q}*Z*Δ M15::Tn*10*](DE3)*;T*et^{r}	Novagen
S17-1 λ *pir*	TP^{r} Sm^{r} *recA, thi, pro, hsdR*$^{-}$M^{+}[RP4-2-Tc::Mu:Km^{r}Tn*7*] (π)	(de Lorenzo and Timmis, 1994)
BL21 (DE3)	*F*$^{-}$ *ompT hsdS*$_{B}$(r_B-m_B-)*gal dcm*(DE3)	Laboratory stock
G0326	BL21 (pG0326.4)	This study
G0328	BL21 (pG0328.4)	This study
GVA26.1GFP	JM109 (pVA0326.1GFP)	This study
GVA28.1GFP	JM109 (pVA0328.1GFP)	This study
GVA28.2GFP	JM109 (pVA0328.2GFP)	This study
V.vulnificus YJ016		
YJ016	Clincial isolate	(Shao and Hor 2000)
VVA0326^{-}	YJ016△VVA0326	(38)
VVA0328^{-}	YJ016△VVA0328	(38)
VVA0326^{c}	VVA0326^{-} (pG0326.2)	This study
VVA0328^{c}	VVA0328^{-} (pG0328.2)	This study

Table 2. Plasmids used in this study

Plasmid	Discription	Source or reference
yT&A	PCR coloning vector, Ap^r	Yeastern Biotech
pCR2.1-TOPO	PCR coloning vector, Ap^r, Km^r	Invitrogen
pET30a-c	Expression vector, Km^r	Novagen
pGFPuv	GFP expression vector, Ap^r	Laboratory collection
pYC292	A suicide vector containing *luxCDABE* luminescence sequence	Yi-Chyi Lai
pMMB66	A low copy number vector for complement of VVA0326$^-$ and VVA0328$^-$, Ap^r	Laboratory collection
pG0326.1	1,450 bp fragment amplified using primer pairs, GP7 and GP8, and cloned into yT&A vector, Ap^r	This study
pG0326.2	*Bam*HI/*Hin*dIII-digested fragment of pVVa0326-yTA subcloned into pMMB66	This study
pG0328.1	1,250 bp fragment amplified using primer pairs, EP5 and EP6, and coloned into yT&A vector, Ap^r	This study
pG0328.2	*Bam*HI/*Hin*dIII-digested fragment of pVVa0328-yTA subcloned into pMMB66	This study
pG0326.3	453 bp fragment amplified using primer pairs, GP9 and GP2, and coloned into yT&A vector, Ap^r	This study
pG0326.4	*Eco*RI single digested fragment of pVVaGGDEF-yTA subcoloned into pET30b	This study
pG0328.3	768 bp fragment amplified using primer pairs, EP3 and EP2, and cloned into yT&A vector, Ap^r	This study
pG0328.4	*Eco*RI single digested fragment of pVVaEAL-yTA subcloned into pET30b	This study
pVA0326.1yTA	618 bp fragment amplified using primers, GP3 and GP2, and cloned into yT&A vector, Ap^r	This study
pVA0326.1GFP	*Hin*dIII/*Kpn*I digested fragment of pVVaG32-yTA subcloned into GFPuv vector	This study
pVA0328.2yTA	792 bp fragment amplified using primers, EP3 and EP2, and cloned into yT&A vector, Ap^r	This study
pVA0328.2GFP	*Hin*dIII/*Kpn*I digested fragment of pVVaE32-yTA subcloned into GFPuv vector	This study
pVA0328.1yTA	434 bp fragment amplified using primers, EP1 and EP4, and cloned into yT&A vector, Ap^r	This study
pVA0328.1GFP	*Hin*dIII/*Kpn*I digested fragment of pVVaE14-yTA subcoloned into GFPuv vector	This study
pGVA0329	1832 bp fragment amplified using primer pairs, VVA03 and VVA04, and cloned into pYC292, Ap^r	

Table 3. Primers used in this study

Primer	Sequences
Ep3	5'-AAGCTT[a]GGACAACGCCGCCAAT-3'
EP4	5'-GGTACC[a]CATTGGCGGCGTTGTC-3'
GP3	5'-AAGCTT[a]AACCTACATTGCGGATAACAAA-3'
GP4	5'-GGTACC[a]TGGATTGTTCATACAGATTCAAGTA-3'
VVAI	5'-GGATCC[a]CAAGAACTGAATAACAAG-3'
VVAII	5'-AAGCTT[a]TCATCAACAACCAAT-3'
GP5	5'-AAGCTT[a]CACGCTGGAAGATTCT-3'
VVA03	5'-GGATCC[a]GTAGACAGTGAACTCATG-3'
VVA04	5'-GAGCTC[a]TCTAATGTGGGCGTTCAA-3'
EP5	5'-GGATCC[a]ATTCAGCACATTGTC-3'
EP6	5'-AAGCTT[a]GCTCAATTAACGTCT-3'
GP7	5'-GGATCC[a]CAATTCAATGTCGA-3'
GP8	5'-AAGCTT[a]AGGTCGAAATGCAAT-3'
GP9	5'-AAGCTT[a]AGTCTCTTCCGAGGTGATC-3'
VVAIII	5'-GGATCC[a]TCGCAAGCAGTTCGT-3'
VVA05	5'-CCCGGG[a]AACATGGCTAACAGTGC-3'
29RT1	5'-AACGCA[a]ACTGAAACAAGC-3'
28RT2	5'-TTGCTG[a]AATGTCGTTATTGG-3'
28RT1	5'-AACGGC[a]GTCAATGTCTCTA-3'
27RT2	5'-GATCAC[a]TTTGCCTTTATAACC-3'
27RT1	5'-TTATGT[a]GTGCAAATCCGA-3'
26RT2	5'-TGAGTG[a]GATTGAATGATGTT-3'

[a,] the underlined sequences represents designed restriction sites

Table 4. Intracellular level of c-di-GMP

V. vulnificus strains	The amount of c-di-GMP [c-di-GMP/total protein] (nM/μg)	Ratio[a]
YJ016	28.44±2.92	1
0326⁻	24.59±1.84	0.86
0326⁻[p0326]	43.28±4.05	1.52
0328⁻	28.08±0.74	0.99
0328⁻[p0328]	42.91±6.11	1.51

[a] Relative to the amount of c-di-GMP in WT

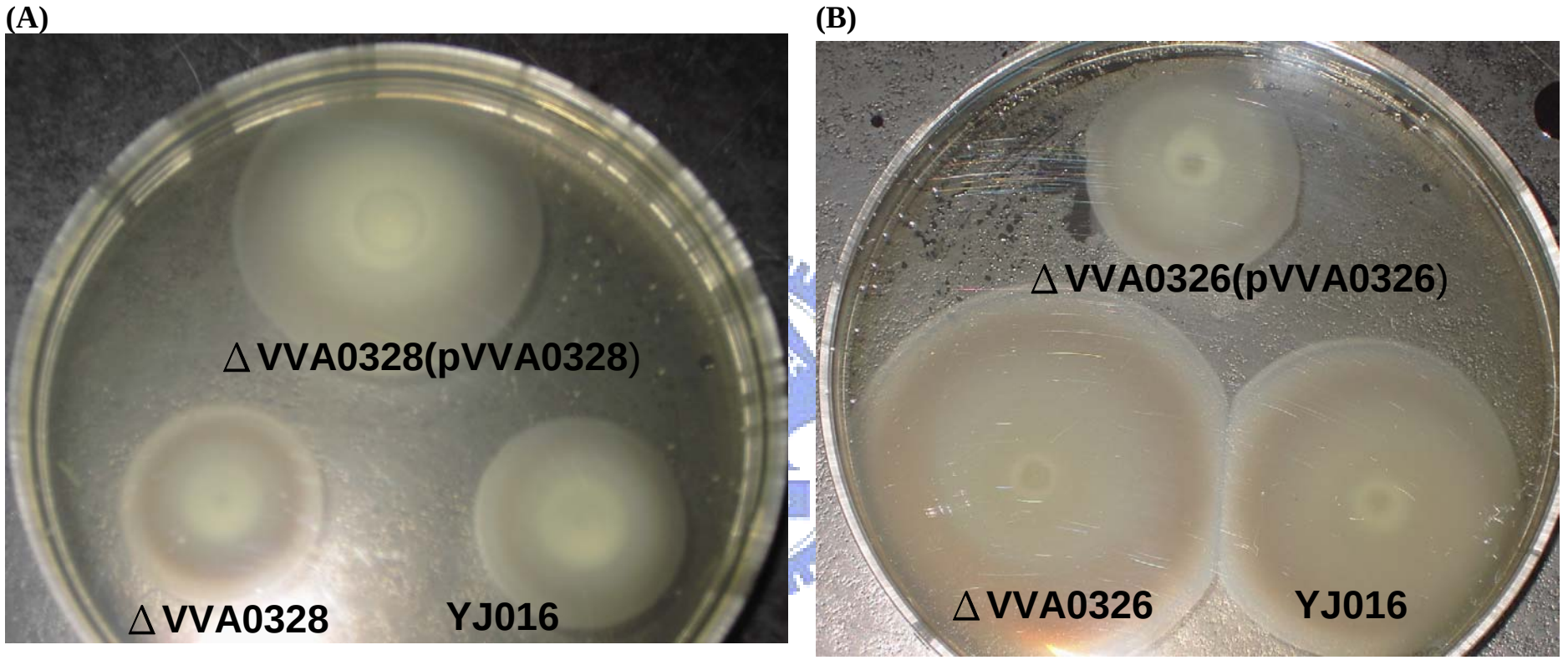


Fig.1. Swimming activity assay. The analyses were performed as described in materials and methods. The photographs were taken after the bacteria have been incubated at 30℃ for 16 h. The mutant of the VVA0328 showed less swimming motility but the complement restored the activity (A), in the contrary VVA0326 has the negative regulation of the swimming motility (B).

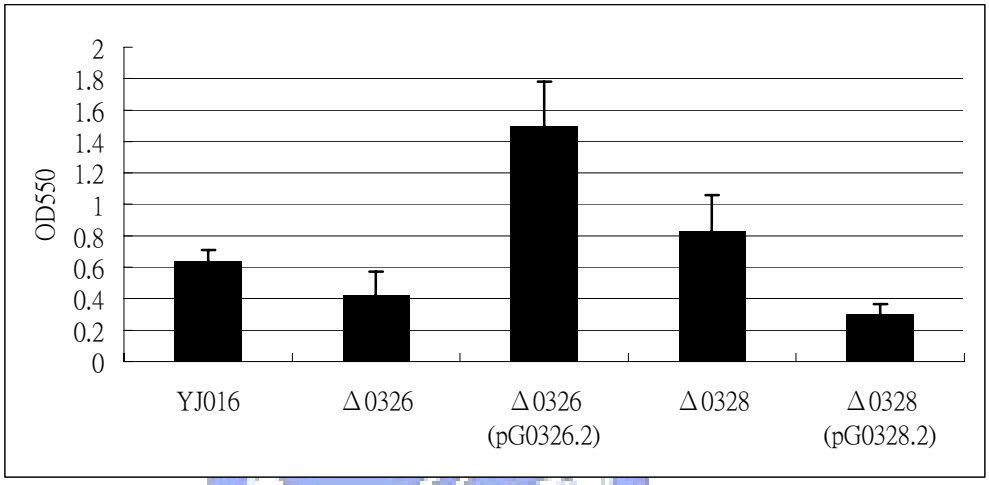


Fig.2. The biofilm formation phenotype. The indicator medium, LB broth (100 μl/well) contained an aliquot of 1:10 diluted overnight bacteria culture and the plate was incubated at 30℃ with shaking(150 rpm) 48 h for biofilm formation. Finally, 1% crystal violet was added to stain for twenty minutes. OD_{550} is expressed for biofilm formation in different strains.

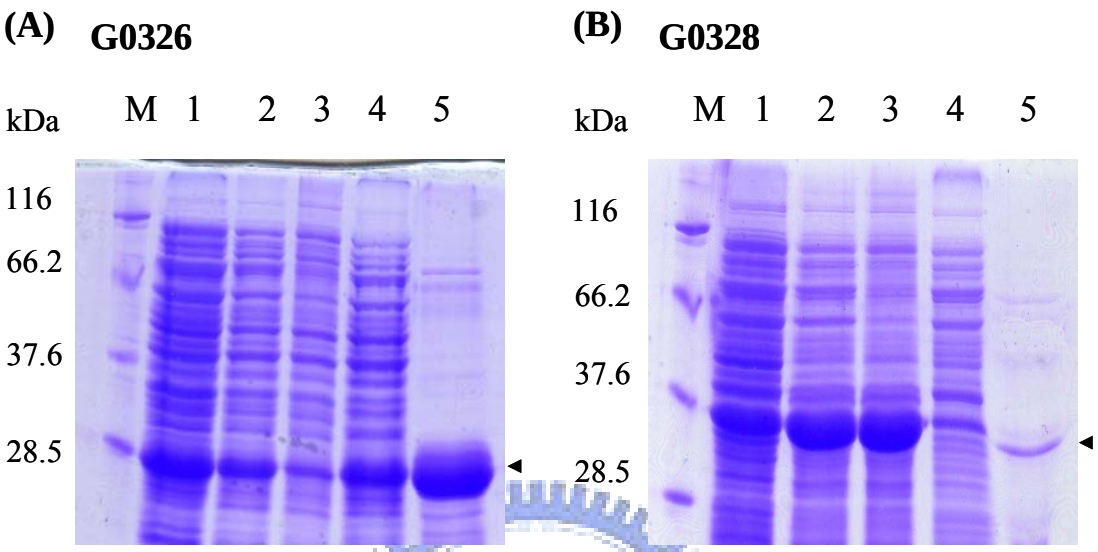
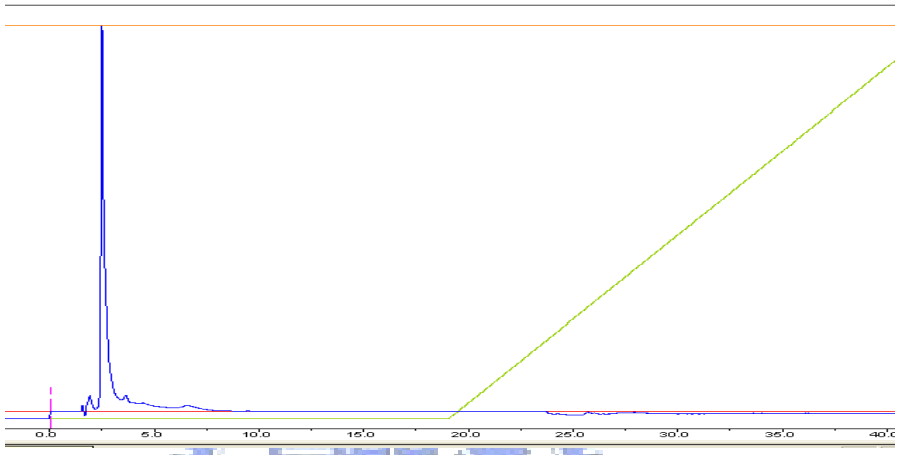
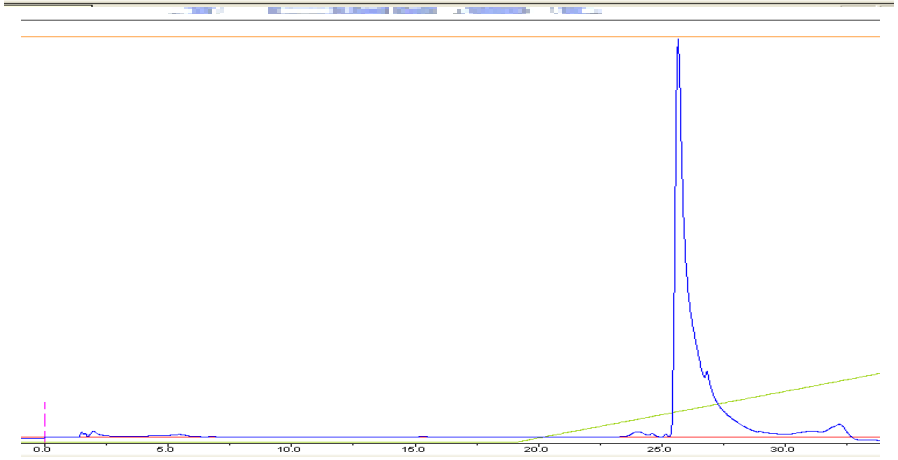


Fig.3. Diagram of the recombinant protein. SDS-PAGE of the purified recombinant protein. Lane M, molecular weight; lane 1, total lysate (unduced); lane 2, total lysate induced by 0.5 mM IPTG; lane 3, induced pellet fraction; lane 4, soluble fraction; lane 5, purified proteins. The arrow head point is the target protein. **(A)** The recombinant GGDEF domain protein, G0326. **(B)** The recombinant EAL **(B)** domain protein, G0328.

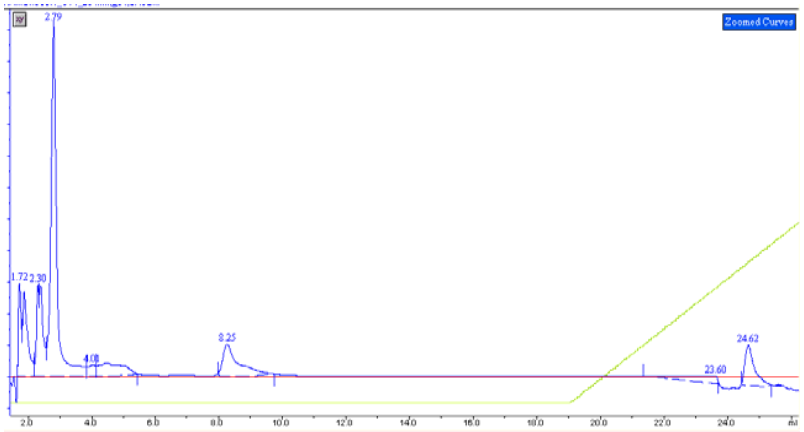
(A)



(B)



(C)



(D)

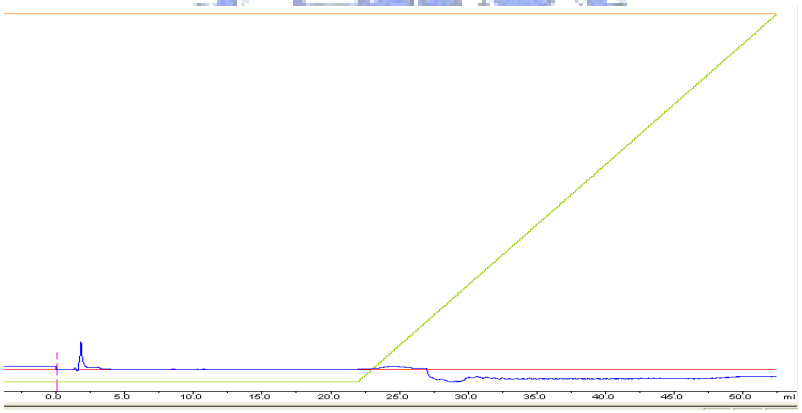


Fig.4. DGC activity assay of the recombinant protein (GGDEF domain protein). (A) Standard**:** GTP for positive control. **(B)** Standard**:** c-di-GMP for positive control. **(C)** The GGDEF domain of VVA0326 (13.31 μg/μl) revealed a low level of DGC activity. **(D)** GGDEF domain protein only (no substrate): negative control.

(A) **(B)**

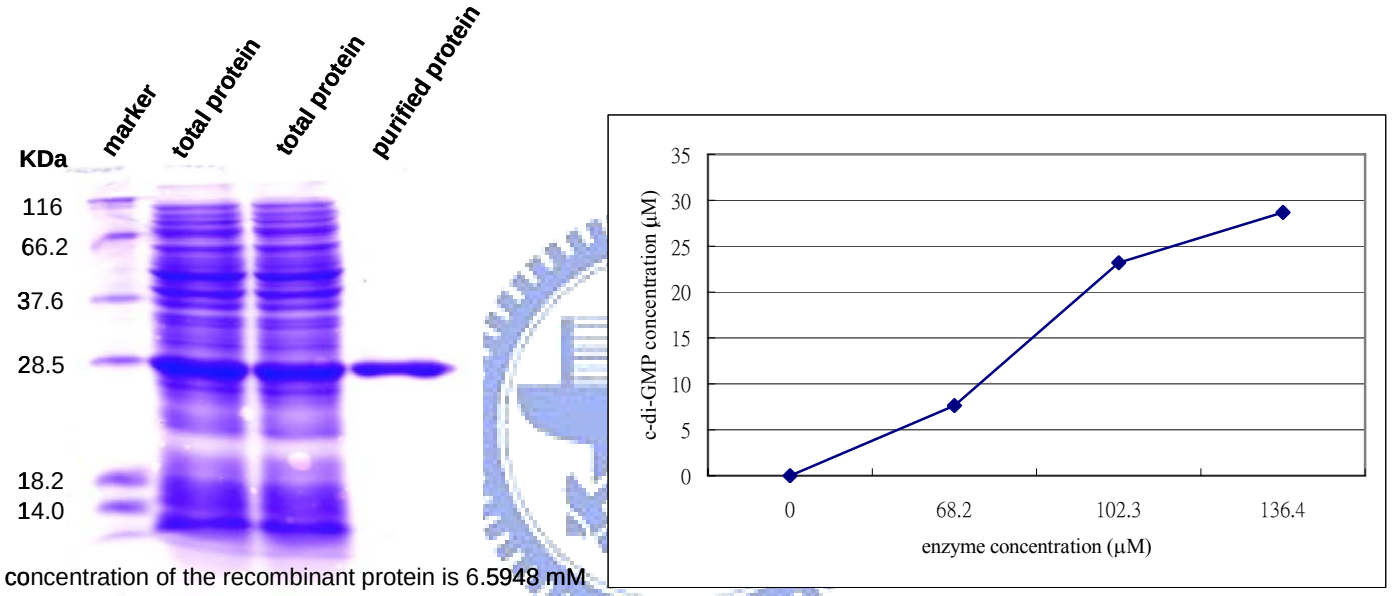
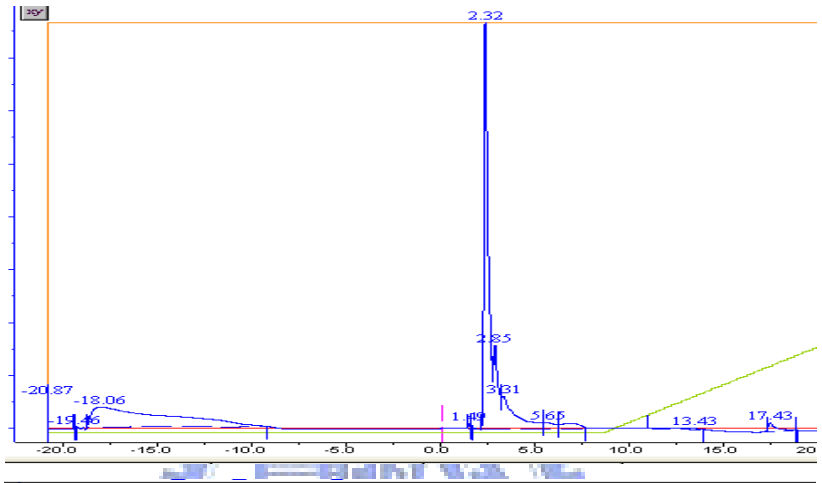
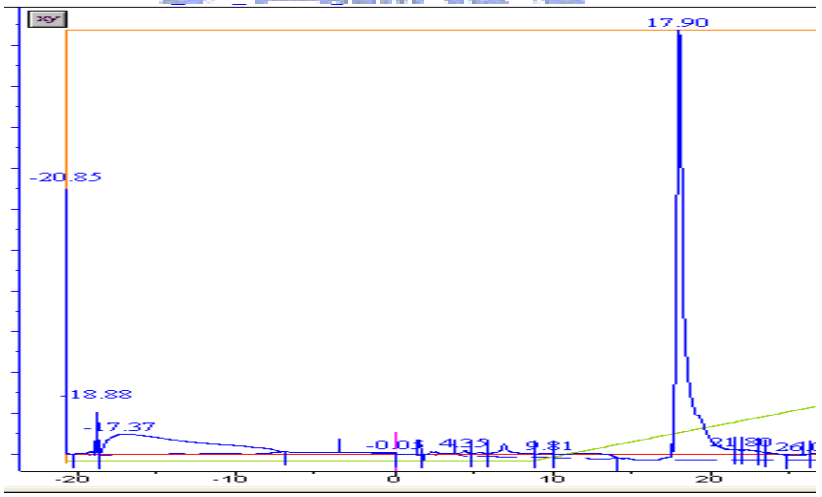


Fig.5. Enzyme specific activity of the recombinant protein (GGDEF domain protein). (A) SDS-PAGE of the purified GGDEF domain purified protein **(B)** More products (c-di-GMP) could be detected by HPLC when adding more recombinant protein (GGDEF domain protein).

(A)


2.32
2.85
3.31
5.65
13.43
17.43
-20.87
-18.06
-20.0
-15.0
-10.0
-5.0
0.0
5.0
10.0
15.0
20

(B)


17.90
-20.85
-18.88
-17.37
-2b
-1b
0
1b
2b

(C)

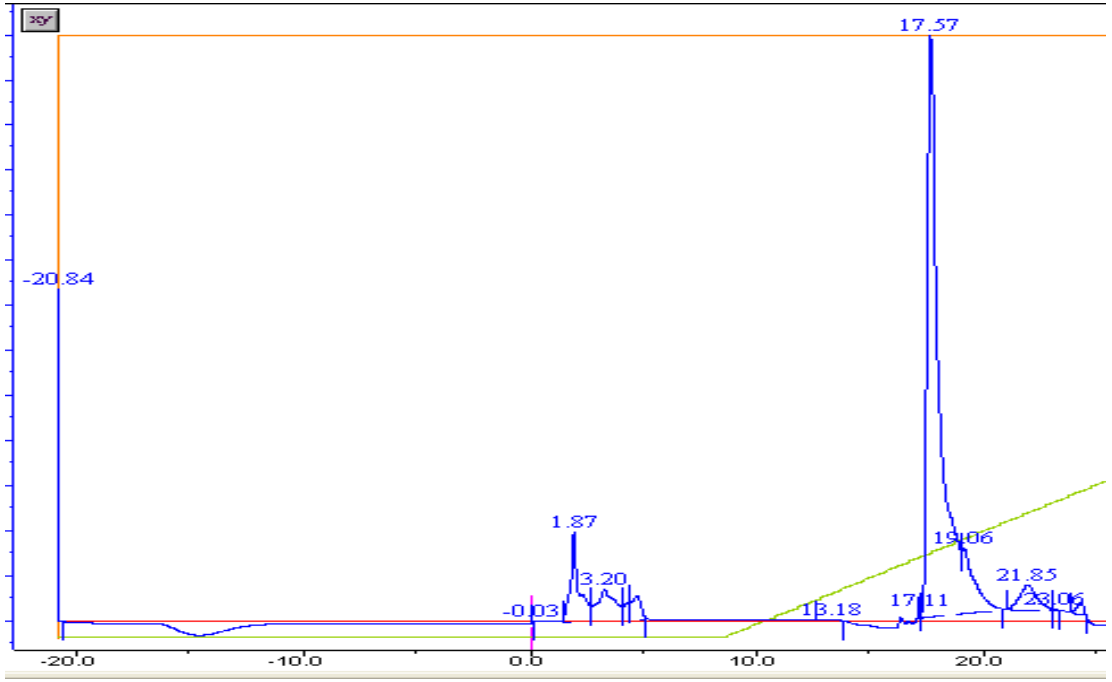


Fig.6. Enzyme activity assay of the recombinant protein (EAL domain protein). (A) Standard: GMP for positive control. **(B)** EAL domain protein free: No EAL protein for negative control. **(C)** C-di-GMP (substrate) and EAL protein (11.248 µg/µl) were incubated in PDE buffer for 4 h. The recombinant protein showed low level PDE activity.

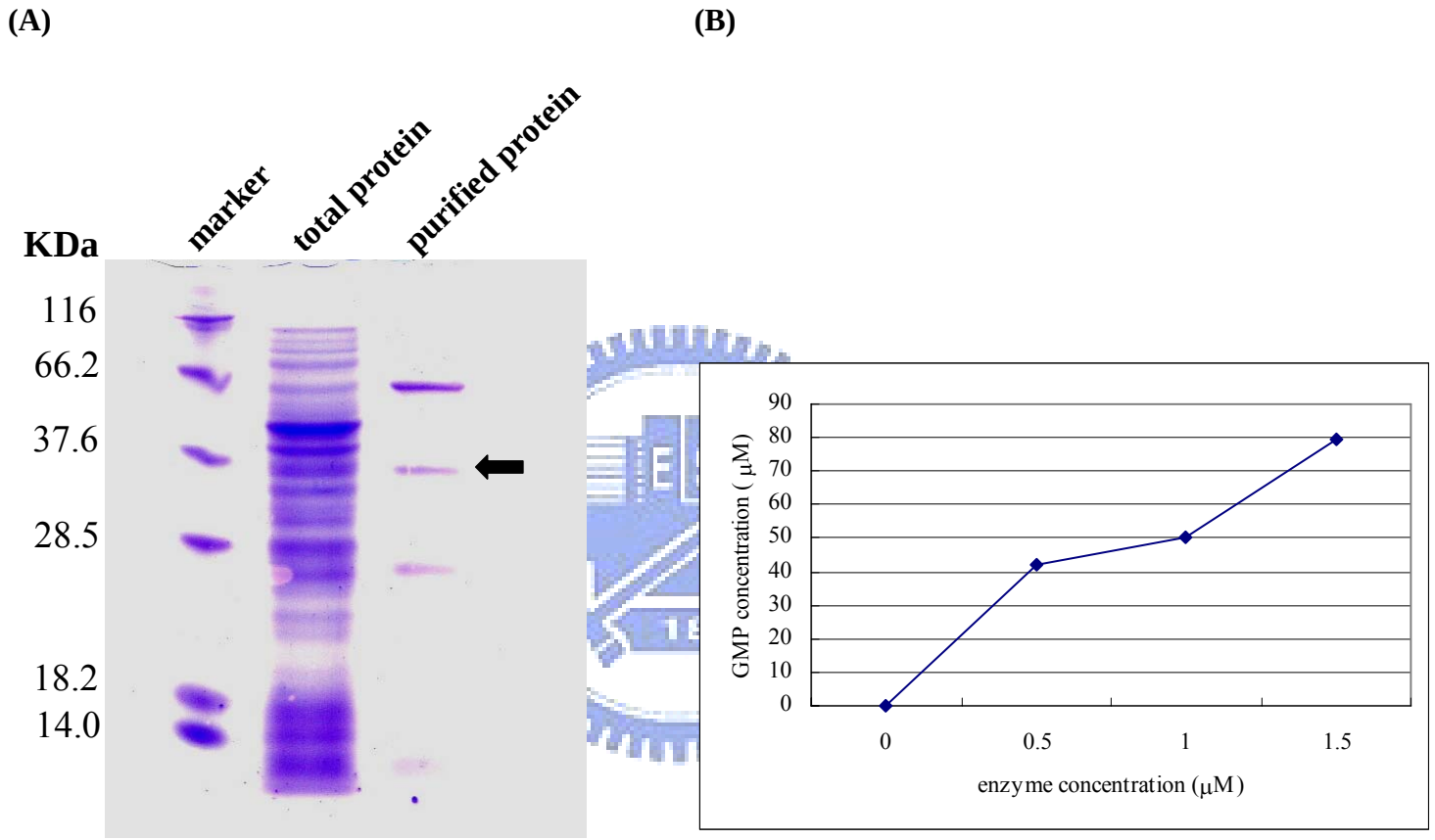


Fig.7. Enzyme specific activity of the recombinant protein (EAL domain protein). (A) SDS-PAGE of the purified recombinant EAL domain protein. The arrow point is the target protein. **(B)** More products (GMP) could be detected by HPLC when adding more recombinant protein (EAL domain protein).

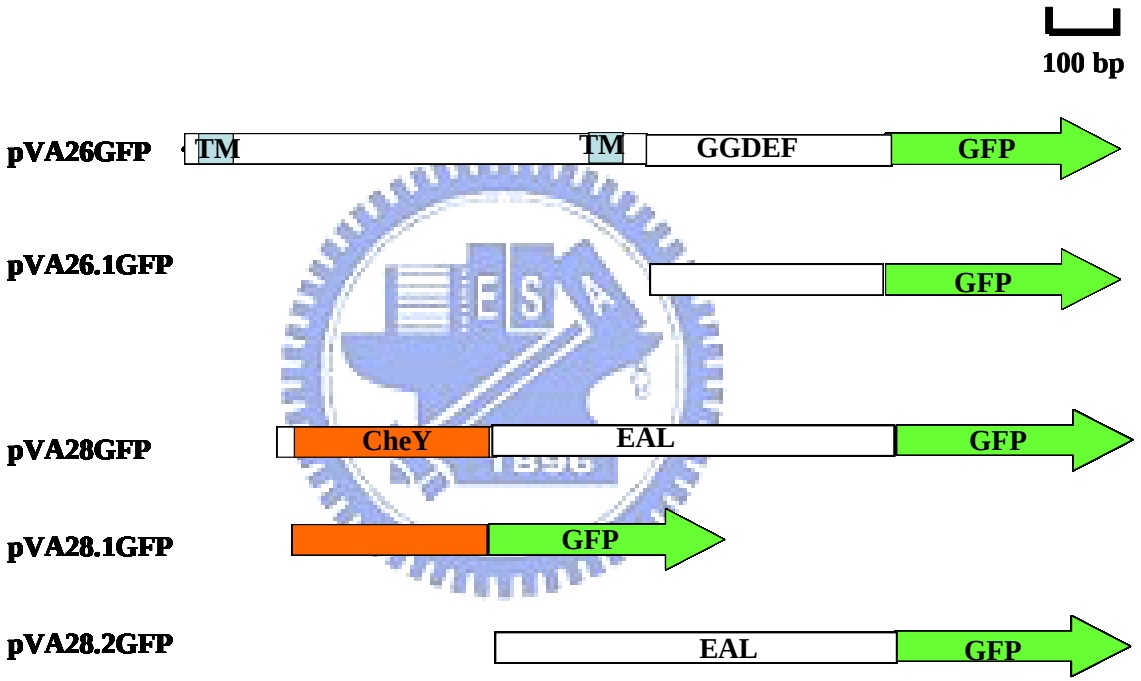


Fig.8. Diagram of the GFP fusion plasmids. Constructs of the pVA26GFP, pVA26.1GFP, pVA28GFP, pVA28.1, and pVA28.2GFP.

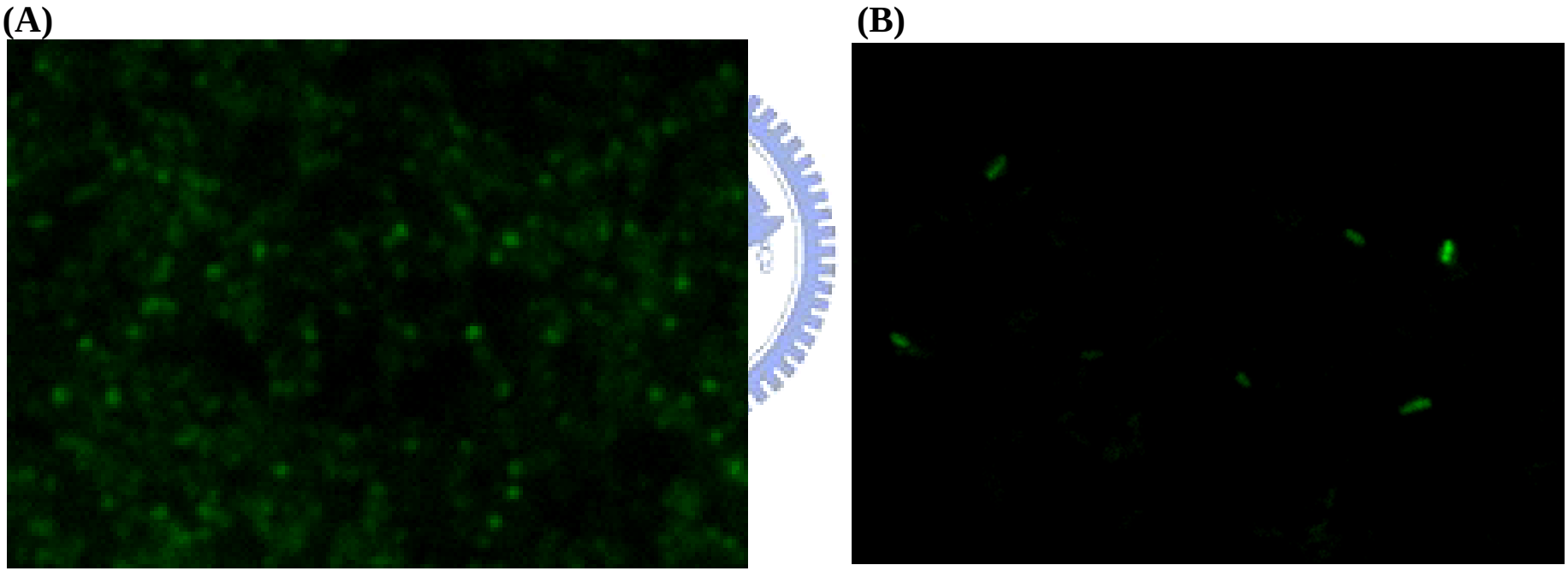


Fig.9. Analyze polar localization by fluorescence microscope. (A) The pVA26GFP is the full length of VVA0326::GFP which showed polar localization. **(B)**The pVA26.1GFP is a GGDEF domain fragment cutting from *vva0326* and cloning into GFP which showed no polar localization.

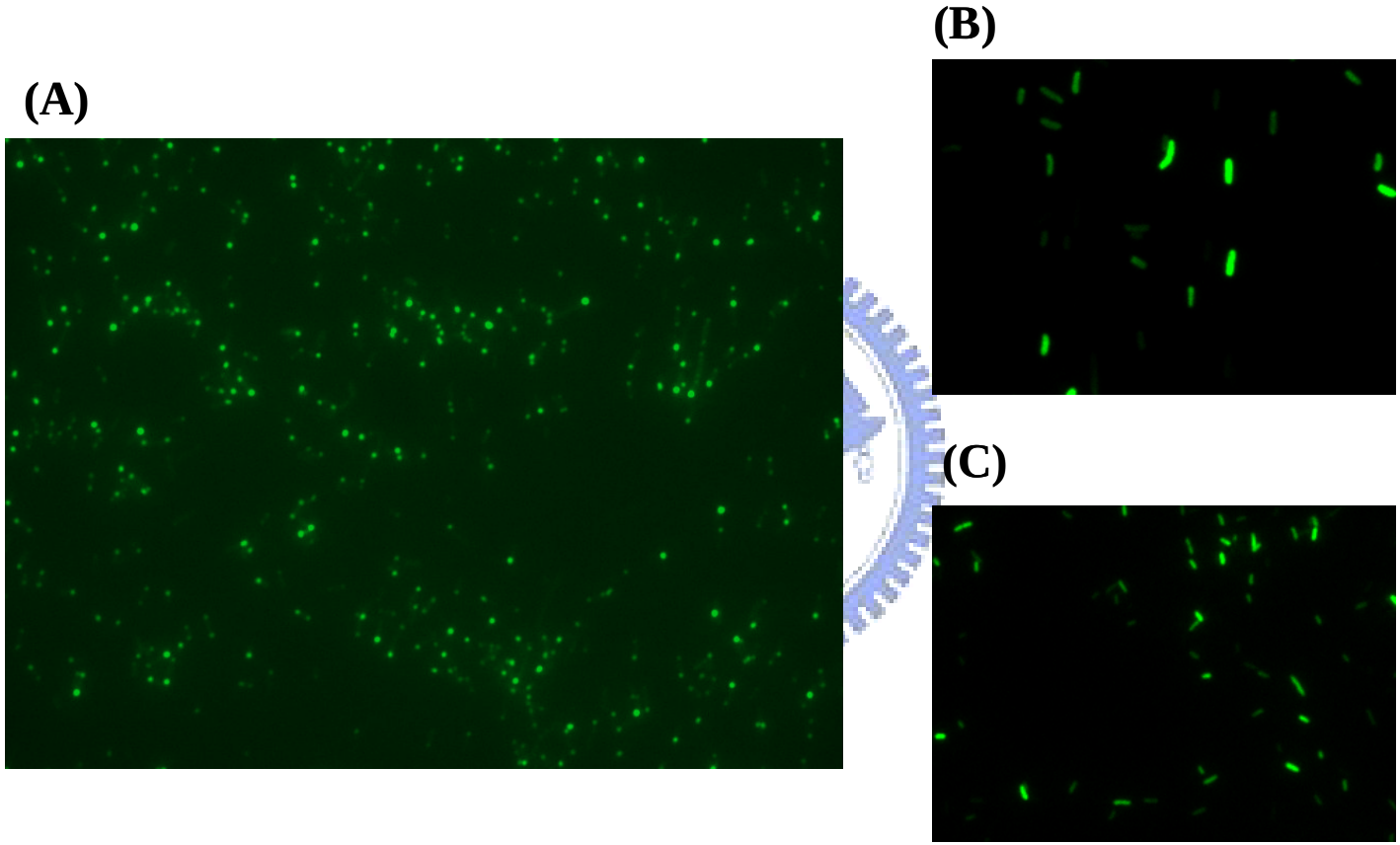


Fig.10. Analyze polar localization by fluorescence microscope. **(A)** The pVA28GFP is the full length of VVA0328::GFP which showed polar localization. **(B)** The pVA28.1GFP is a CheY domain fragment cutting from *vva0328* and cloning into GFP and **(C)** pVA28.2GFP is an EAL domain fragment cutting from *vva0328* and cloning into GFP both of them showed no polar localization.

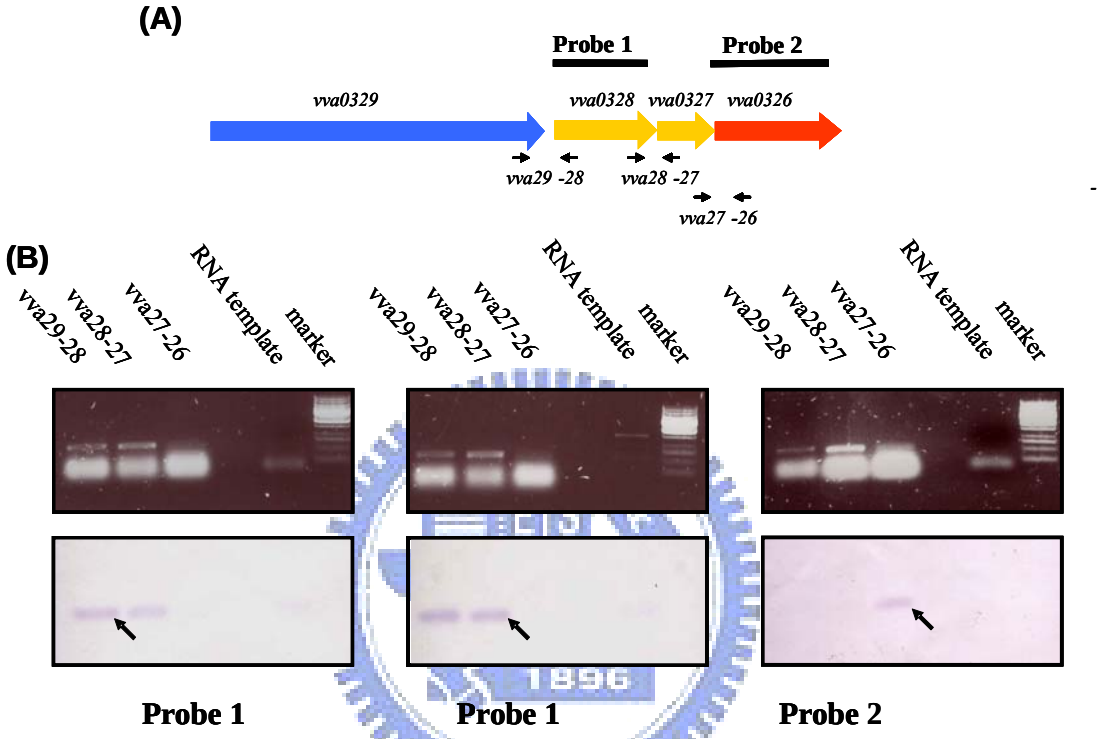


Fig.11. RT-PCR and southern blot analysis. (A) Probe 1 is *vva0328* and Probe 2 is *vva0326*. Three primer pairs 29RT1/28RT2, 28RT1/27RT2 and 27RT1/26RT2 were used to generate the connected sequence of the operon. **(B)** Primer pairs, 29RT1/28RT2, 28RT1/27RT2 and 27RT1/26RT2 were used to prove whether the *vva0326* to *vva0329* is an operon or not. Up panel are the electrophoresis of the RT-PCR products and low panel are the southern blot to confirm the result of RT-PCR. The hybridization using probe 1 to (1), (2) and probe 2 to (3) are shown.

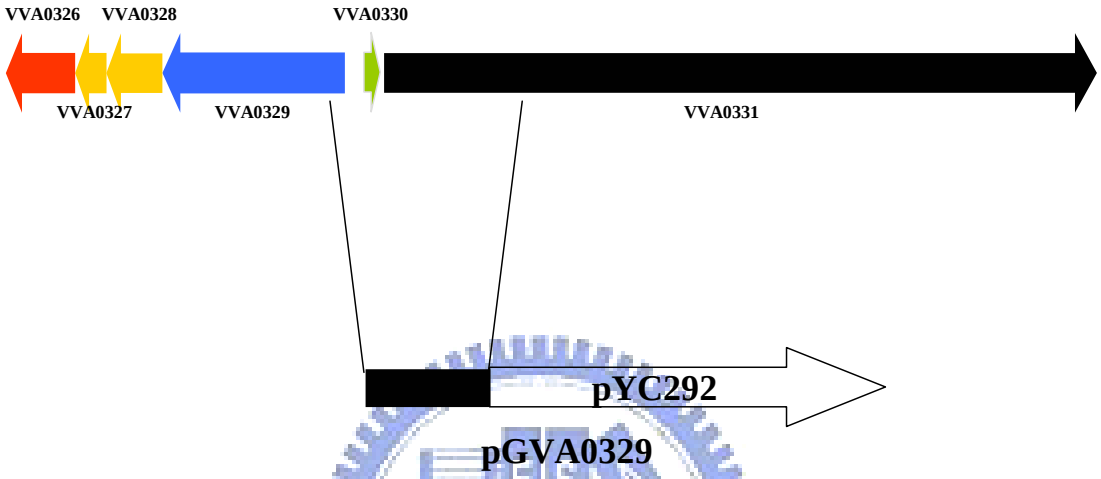
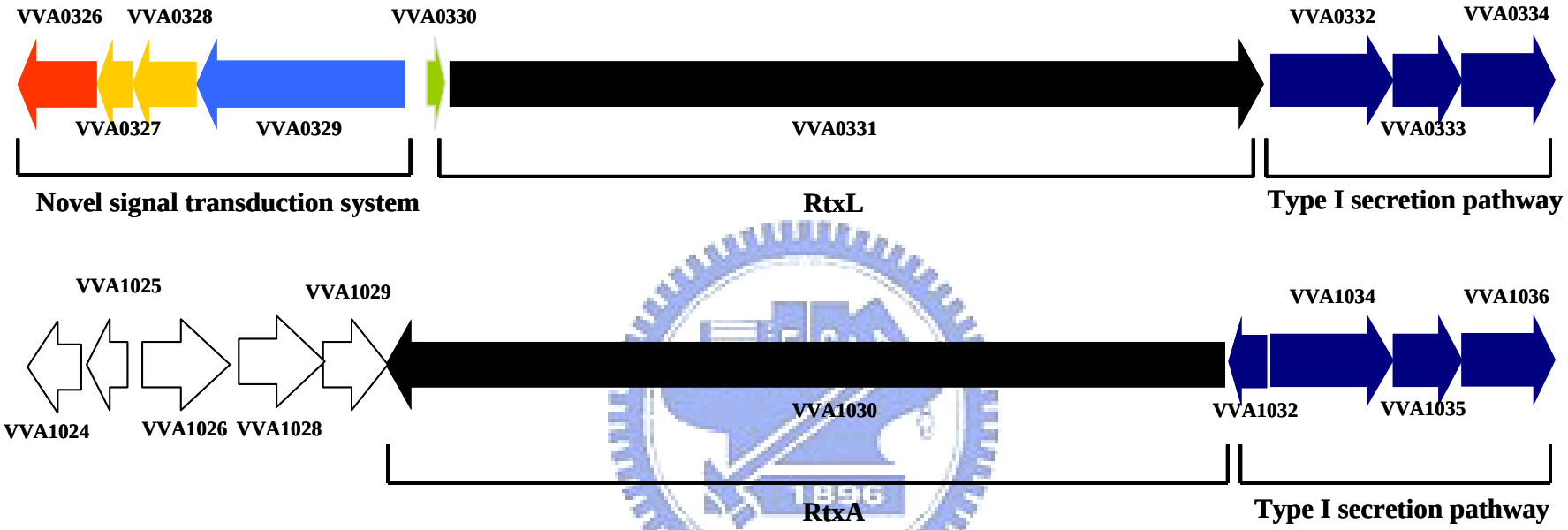
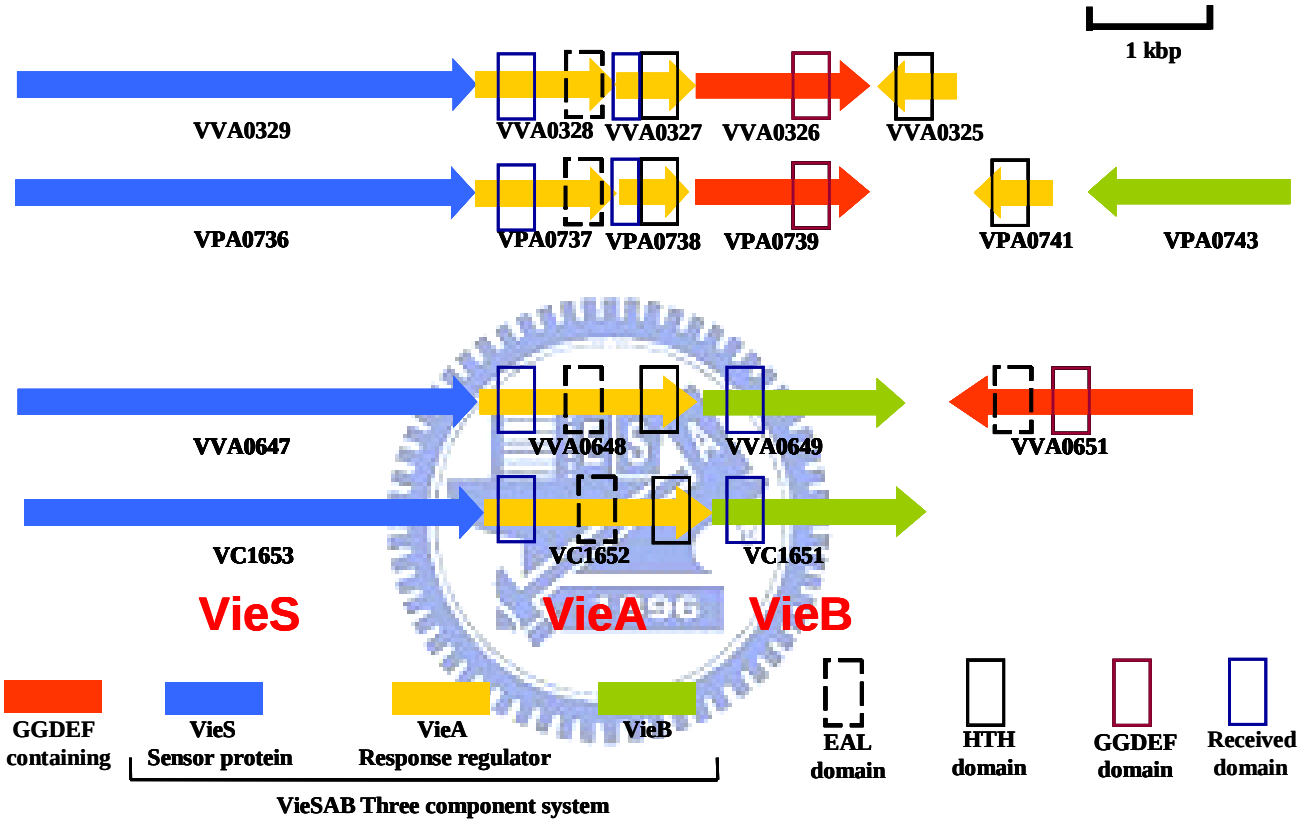


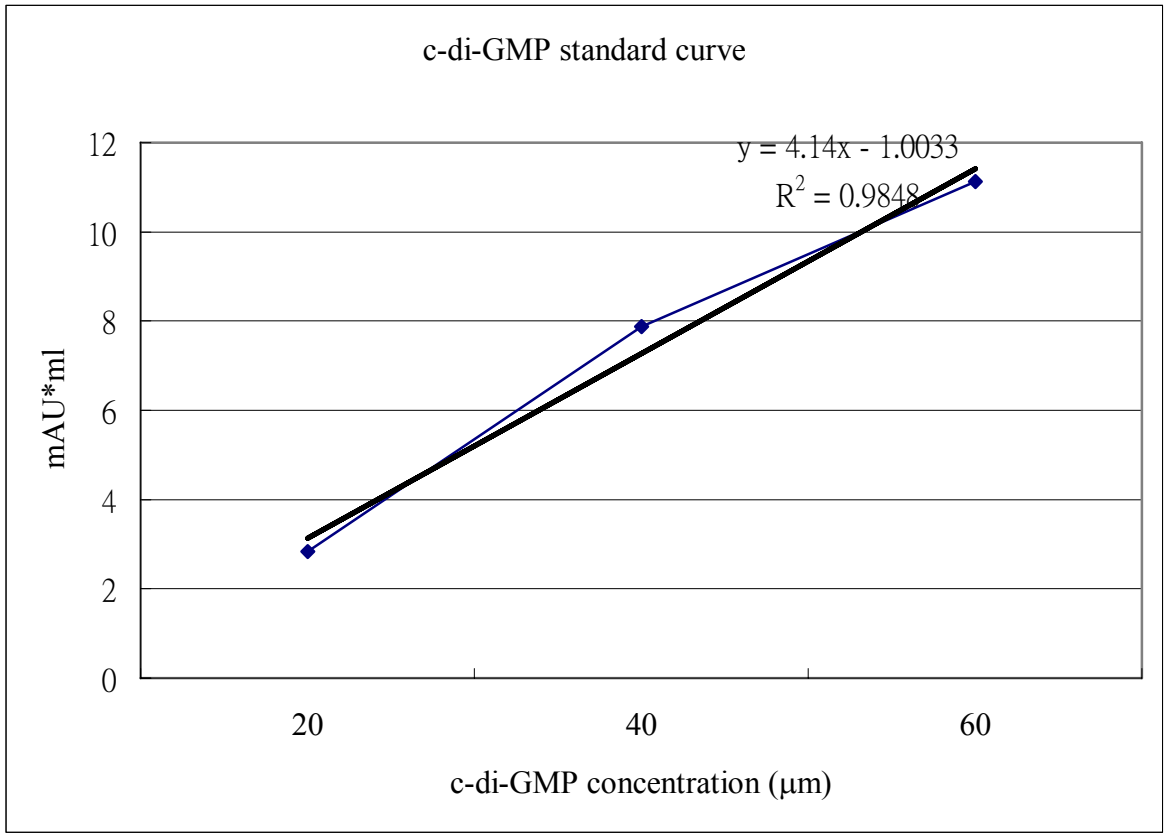
Fig. 12. Schematic diagram of the promoter reporter construct. Primer set VVA05 and VVA04 were used to amplify a fragment (1832 bp) then cloned into a reporter pYC292 containing bioluminescence.



Appendix 1. Schematic diagram of the RTX family in *Vibrio vulnificus*. The VVA0331 is an RTX like protein following a typeI secretion system and having a novel signal transduction system. VVA1030 is a typical of RTX protein. (5)



Appendix 2. Comparative analysis of vieSAB-like gene cluster. The *vva0647* to *vva0649* and vva0326 to vva0329 are homologous of an three component system VieSAB. But *vva0326* to *vva0329* lack VieB but following an RTX like protein, which suggested this gene cluster might regulate RTX expression. (38)



Appendix 3. C-di-GMP standard curve. The sample was supplied by Dr. Yoshihiro Hayakawa (Laboratory of Bioorganic Chemistry, Graduate School of Information Science, Nagoya University Chikusa, Nagoya 464-8601, Japan).