THE ROLE OF A NOVEL SECOND MESSENGER, C-DI-GMP IN *PSEUDOMONAS PUTIDA* VIA ENZYMATIC STUDY OF MORA

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Summary

Bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is a global second messenger uniquely found in bacteria that modulates diverse biological processes such as biofilm formation, motility, photosynthesis and virulence. Formation of c-di-GMP is catalyzed by diguanylate cyclase (DGC) from two GTP molecules with the release of two pyrophosphates, while degradation of c-di-GMP to linear dimer pGpG is accomplished by phosphodiesterase A (PDE-A). Studies have revealed that two widespread conserved domains, GGDEF and EAL domains are responsible for the DGC and PDE-A activities, respectively.

Here we study the possibility of the presence of c-di-GMP in *Pseudomonas putida* and its relationship with MorA, a GGDEF-EAL domain containing protein that controls the timing of flagellar development and affects motility, chemotaxis and biofilm formation. A method for extracting and detecting c-di-GMP was established using *Gluconacetobacter xylinus* as a model and c-di-GMP was successfully detected from *P. putida*. The intracellular level of c-di-GMP is growth-dependant with peak levels at middle log phase and dropping drastically at log-to-stationary transition phase.

MorA has been shown to affect c-di-GMP levels via a direct or indirect way. In *morA* knock-out strain of *P. putida*, the intracellular c-di-GMP level is higher than that in wild type, whereas when *morA* is overexpressed, the c-di-GMP concentration reduces below that in the wild type. DGC activity assay showed that MorA does not have apparent in

vitro DGC activity under the conditions tested. Combined with the observation that MorA negatively regulates c-di-GMP levels of *P. putida*, it is highly possible that MorA may function as a PDE-A.

With the interest to investigate factors affecting c-di-GMP metabolism, we identified potential GTP-binding proteins in *P. putida* using GTP-agarose affinity chromatography. Four proteins were identified as being polyribonucleotide nucleotidyltransferase, dihydrolipoamide dehydrogenase, lysine-arginine-ornithine-binding periplasmic protein and phosphoribosyltransferase. As GTP is the substrate for c-di-GMP production, these putative GTP-binding proteins may have an impact on GTP pool and subsequently, affect c-di-GMP metabolism.

Findings from this study have established a protocol for isolation and detection of c-di-GMP and identification of GTP-binding proteins in bacteria and will be helpful in further investigation on the mechanism of c-di-GMP action and MorA signaling pathway.

List of Abbreviations

Bacteria

A. crystallopoietes	*Arthrobacter crystallopoietes*
A. tumefaciens	*Agrobacterium tumefaciens*
A. xylinum	*Acetobacter xylinum*
B. burgdorferi	*Borrelia burgdorferi*
B. mallei	*Burkholderia mallei*
B. pertussis	*Bordetella pertussis*
B. subtilis	*Bacillus subtilis*
C. crescentus	*Caulobacter crescentus*
C. magnum	*Clostridium magnum*
E. coli	*Escherichia coli*
G. europaeus	*Gluconacetobacter europaeus*
G. hansenii	*Gluconacetobacter hansenii*
G. oboediens	*Gluconacetobacter oboediens*
G. xylinus	*Gluconacetobacter xylinus*
M. gallisepticum	*Mycoplasma gallisepticum*
R. leguminosarum	*Rhizobium leguminosarum*
P. aeruginosa	*Pseudomonas aeruginosa*
P. carbinolicus	*Pelobacter carbinolicus*
P. entomophila	*Pseudomonas entomophila*
P. fluorescens	*Pseudomonas fluorescens*
P. putida	*Pseudomonas putida*
P. syringae	*Pseudomonas syringae*
S. antibioticus	*Streptomyces antibioticus*,
S. enterica	*Salmonella enterica*
S. oneidensis	*Shewanella oneidensis*

S. pneumoniae	*Streptococcus pneumoniae*,
S. typhimurium	*Salmonella enterica* serovar typhimurium
V. cholerae	*Vibrio cholerae*
V. vulnificus	*Vibrio vulnificus*
X. axonopodis pv citri	*Xanthomonas axonopodis pv citri*
X. campestris pv. *campestris*	*Xanthomonas campestris* pv. *campestris*

Chemicals and reagents

Amp	ampicillin
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
$CaCl_2$	Calcium chloride
Cm	chloramphenicol
DTT	dithiothretiol
EDTA	ethylene-diamine-tetra-acetate
Gm	gentamycin
GTP	guanosine-5-triphosphate
cGMP	cyclic guanosine 3',5'- monophosphate
HCl	hydrochloric acid
$HClO_4$	perchloric acid
IPTG	isopropyl-β-D-thiogalactopyranoside
K_2CO_3	potassium carbonate
LB	Luria-Bertani
$MgCl_2$	Magnesium chloride
NaCl	sodium chloride
NBT	4-nitro blue tetrazolium chloride
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PVDF	polyvinylidene fluoride
Rf	rifampicin
SDS	sodium dodecyl sulphate
TEAB	triethylammonium bicarbonate
Tet	tetracycline
TFA	trifluoroacetic acid
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol

Units and Measurements

^{o}C	degree Celcius
g	centrifugal force
h	hour
kDa	kilodalton
l	litre
M	moles per litre
mg	miligram
min	minute
ml	millilitre
mM	millimoles per litre
m/z	mass/charge ratio
OD	optical density
rpm	revolutions per minute
UV	ultraviolet
µg	microgram
µl	microlitre
µM	micromoles per litre
µm	micrometre
v/v	volume per volume
w/v	weight per volume

Others

ABC	ATP binding cassette
AcP	acetyl phosphate
ATCC	American Type Culture Collection
BSA	bovine serum albumin
cAMP	cyclic adenosine 3',5'- monophosphate
c-di-GMP	bis-(3',5')-cyclic dimeric guanosine monophosphate
CO	carbon monoxide
CO_2	carbon dioxide
CRP	cAMP-receptor protein
CSPs	cold shock proteins
DG	diacylglycerol
DGC	diguanylate cyclase
DLA	dihydrolipoamide
DLDH	dihydrolipoamide dehydrogenases
DNA	deoxyribonucleic acid
et al.	*et alter* (and others)
GMP	guanosine-5'-monophosphate
GST	glutathione S-transferase
H_2O	water
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
HPLC	high performance liquid chromatography
i. e.	that is
IMP	inosine-5'-monophosphate
ITC	isothermal titration calorimetry
IP_3	Inositol 1,4,5-trisphosphate
KO	knock out
LAO	lysine-arginine-ornithine-binding periplasmic protein
MALDI-TOF	matrix assisted laser desorption/ioniziton time-of-flight
NO	nitric oxide

OE	overexpress
PAGE	polyacrylamide gel electrophoresis
PDE	phosphodiesterase
pGpG	linear dimeric GMP
PGPR	plant growth-promoting rhizobacterial
PKA	cyclic AMP-dependent protein kinase
PNPase	polynucleotide phosphorylase
ppGpp	guanosine-3',5'-(bis) pyrophosphate
pppGpp	guanosine pentaphosphase
PRPP	phosphoribosyl pyrophosphate
PRT	phosphoribosyltransferase
RPH	ribonuclease PH
TME buffer	buffer containing 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, and 1 mM EDTA
TME-DTT buffer	TME buffer containing 1 mM DTT
TME-PEG buffer	TME buffer with the presence of 20% (w/v) PEG-4000
T3SS	type III secretion system
WT	wild type

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Chapter 1. Introduction

1. Introduction

Cyclic nucleotides such as cyclic adenosine 3',5'- monophosphate (cAMP) and cyclic guanosine 3',5'- monophosphate (cGMP) are the best known second messengers involved in numerous intracellular signaling pathways. They regulate a great number of physiological processes in mammalian systems, including neuromuscular transmission, cell proliferation and apoptosis, smooth muscle relaxation, retinal photo transduction, intermediate metabolism, etc. In higher plants, they play a role in ion channel regulation, cell cycle progression, action of phytochrome and plant defence response (Newton and Smith, 2004). While their actions are well characterized in complex eukaryotic cells, cyclic nucleotides are also present in simplest organisms such as fungus, Eubacteria and Archae (Botsford and Harman, 1998). However, their functions in lower organisms seem to be mainly involved in response system to starvation, as in the case of *Escherichia coli* and yeast (Newton and Smith, 2004).

While cAMP is widely found in both prokaryotes and eukaryotes, cGMP seems to function only in the eukaryotic kingdom and its presence in bacteria as a functional molecule is controversial. However, increasing experimental evidence shows that another cyclic guanosine compound, bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is used as a global second messenger uniquely in bacteria, modulating diverse biological processes such as biofilm formation, motility, photosynthesis and virulence. c-di-GMP was first discovered 19 years ago in bacterium *Gluconacetobacter xylinus* (formerly called *Acetobacter xylinum*) as an allosteric activator of cellulose synthase (Ross *et al.*, 1986). Levels of c-di-GMP are controlled by the actions of two opposing enzymatic activities involved in the

formation and turnover, respectively, of this molecule. Formation of c-di-GMP in *G. xylinus* is catalyzed by diguanylate cyclase (DGC) from two GTP molecules with the release of two pyrophosphates, while phosphodiesterase A (PDE-A) is responsible for degrading c-di-GMP to linear dimer pGpG (Ross *et al.*, 1987). DGC and PDE-A proteins in *G. xylinus* share conserved domain structures, GGDEF and EAL domains, which were named after highly conserved sequence motifs, Gly-Gly-Asp-Glu-Phe and Glu-Ala-Leu (Tal *et al.*, 1998).

GGDEF and EAL domains are widespread in diverse bacteria. Recently, the functions of these two domains have been experimentally characterized. GGDEF domain is demonstrated to possess DGC activity whereas EAL domain is responsible for the hydrolysis of c-di-GMP. However, the studies on the biological functions of proteins containing both GGDEF and EAL domains (DGC-PDE proteins) still remain insufficient. Among the few DGC-PDE proteins which have been studied, some exhibit DGC activity while others have a function of PDE-A. No bifunctional DGC-PDE proteins have been reported so far.

Recently, MorA, a novel regulator affecting flagellar development and biofilm formation in diverse *Pseudomonas* species, was discovered in our laboratory. MorA is a transmembrane DGC-PDE protein, the mutation of which leads to constitutive expression of flagellar and reduction of biofilm formation. However, the molecular biochemical function and regulation of MorA is still unknown and needs to be investigated. With such an overall goal, we outlined the following objectives:

(i) To establish method for isolating and detecting c-di-GMP from *Pseudomonas* and *Gluconacetobacter*, and compare its levels between wild type *P. putida* PNL-MK25,

morA knockout and *morA* overexpressed strains, respectively. Our hypothesis is that MorA regulates multiple bacterial phenotypes via intracellular c-di-GMP levels.

(ii) To examine the enzymatic activity of MorA. Although MorA has both GGDEF and EAL conserved domains, it is not currently known whether one or both domains have functional activities. Here, we designed the enzymatic assay to test its DGC activity. The PDE-A activity assay could not be performed at the current time due to lack of sufficiently purified c-di-GMP and a suitable protein control.

(iii) To identify putative GTP-binding proteins in *P. putida.* GTP acts as a substrate for the synthesis of c-di-GMP. In order to test whether GTP is freely available for conversion to c-di-GMP or it is possibly sequestered in bound form, we attempted to identify GTP-binding proteins in *P. putida*. This is an initial step to study how GTP might affect selected pathways in *Pseudomonas*. There is currently no report of GTP-binding proteins involved in c-di-GMP signaling pathway in bacteria.

Chapter 2. Literature Review

2. Literature Review

2.1. Introduction of bacteria used in this study

Two bacterial strains from two different genera were used in this study. One is *Gluconacetobacter xylinus* 1306-21 (ATCC®53524), the other is *Pseudomonas putida* PNL-MK25 (Adaikkalam and Swarup, 2002).

2.1.1. *Gluconacetobacter xylinus*

Gluconacetobacter, under the family Acetobacteraceae, is a genus comprised of 11 species of gram-negative acetate-oxidizing bacteria. These bacteria species are unicellular and have an elongated rod shape typical of the *Gluconacetobacter* strains. This genus was recently elevated from a subgenus *Gluconoacetobacter (sic)*, previously belonging to the genus *Acetobacter*, to the generic level and renamed as *Gluconacetobacter* by Yamada et al. (Yamada *et al.*, 1997). Some species under the sublineage B of *Gluconacetobacter*, such as *G. oboediens*, *G. hansenii, G. europaeus*, can be used for vinegar fermentation (Yamada, 2003).

G. xylinus also belongs to the sublineage B of *Gluconacetobacter*. It has served for decades as a classical model system of investigating cellulose synthesis due to its excellent cellulose-synthesizing capacity. Each bacterial cell can polymerize 200,000 D-glucose units per second. Certain *G. xylinus* strains, such as strain 1306-3 can utilize numerous carbon and nitrogen source for cellulose synthesis. For example, mannitol, sorbitol, sucrose, fructose and glucose can serve as carbon sources whereas

casein hydrolysate, protein hydrolysate, yeast extract, malt extract, ammonium salts, corn steep liquor and other nitrogen-rich substances can be used as a general source of amino acids, nitrogen, minerals and vitamins (Johnson and Neogi, 1989). The colonies of the strain on agar plate are small, circular, smooth, glistening and opaque while in the broth, it forms a thick, rubbery pellicle comprised of mainly cellulose at the surface of the broth. However, the broth itself remains clear.

G. xylinus 1306-21 was used in this study as a model for establishing methods for isolating and detecting c-di-GMP and as a source of DGC from which c-di-GMP can be enzymatically synthesized in vitro. This is mainly because c-di-GMP was discovered and described first in this strain (Ross *et al.*, 1987). The strain 1306-21 was derived from *G. xylinus* 1306-3 by treating the latter with a mutagen. Compared with 1306-3, *G. xylinus* 1306-21 produces less gluconic acid and keto-gluconic acids yet retains a stable productivity of cellulose.

2.1.2. *Pseudomonas putida*

The genus *Pseudomonas* comprises more than 140 species of bacteria, which share the following characteristics: gram-negative, aerobic, rod-shaped, non-spore forming, typically motile with one or more polar flagella, able to grow on a wide range of organic substrates. These bacteria are common inhabitants of water and soil, and most of them are saprophytic. They are present on the surfaces of plants, occasionally on the surfaces of animals. Many species of *Pseudomonas* can cause opportunistic infections in humans. Among them, *P. aeruginosa* is most well-known since it is a

leading cause in hospital-acquired infections such as urinary tract infections, respiratory system infections and so on.

Unlike *P. aeruginosa*, *P. putida* is a non-pathogenic bacterium with multitrichous flagella commonly found colonizing the root area of plants. As a rhizosphere-associated bacterium, it can enhance plant growth by the exclusion of pathogenic microorganisms, release of antifugal compounds, indole acetic acid (IAA) production or phosphate solubilization (Mehnaz and Lazarovits, 2006). In addition, it can resist adverse effects of organic solvent such as aliphatic or aromatic hydrocarbons and has the most genes of any known bacteria species engaged in aromatic hydrocarbon degradation. Therefore, it is one of the most promising bacteria which have the possibility to be applied in environmental biotechnology. For these reasons, its genome has been fully sequenced from *P. putida* KT2440 and the related strains *P. fluorescens* PfO-1. KT2440 genome is 6.1 million base pairs in length with an average GC content of 61.6%. There are a total of 5,420 open reading frames (ORFs), of which putative role assignments could be made for 3,571 ORFs. PfO-1 genome is 6.4 million base pairs in length with an average GC content of 60%. It has 5,833 genes of which 5,736 are protein coding genes. *P. putida* and *P. fluorescens* both belong to the non-phytopathogenic and non-necrogenic type species and they are often responsible for meat and milk degradation (Bossis *et al.*, 2000).

P. putida PNL-MK25, an antibiotic-resistant derivative of the plant growth-promoting rhizobacterial (PGPR) strain ATCC 39169 (Adaikkalam and Swarup, 2002) was taken as a model in this study for the reason that it is the strain where MorA was first discovered and most previous experiments were done. We hypothesize that findings

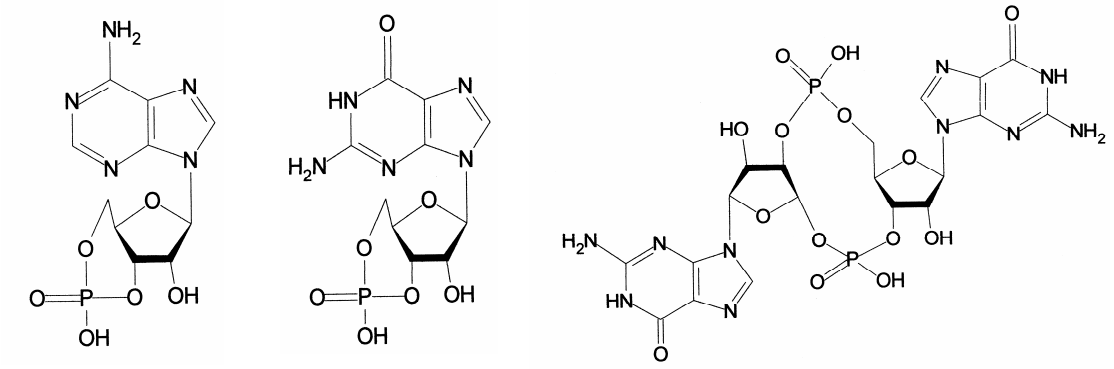
here will be directly relevant to *P. aeruginosa*, where MorA ortholog has been previously described by our laboratory (Choy *et al.*, 2004).

2.2 Cyclic nucleotide messengers

Second messengers are low-molecule weight molecules that are involved in signal transduction pathway to relay signals received by the receptors on the cell surface to target molecules inside the cells. They are usually synthesized or released by specific enzymatic reactions in response to external signals received by surface receptors and subsequently cause massive adaptive changes in the cell.

There are four basic types of second messengers, which include: (1) Inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG), which are generated through the hydrolysis of plasma membrane lipid phosphatidylinositol 4,5-bisphosphate by phospholipase C in animal cells. The water soluble IP_3 diffuses from the plasma membrane to the endoplasmic reticulum to trigger transient increase of cytosolic Ca^{2+} level, which in turn, trigger other responses in the cell, whereas hydrophobic DG stays at the plasma membrane and activates protein kinase C that can phosphorylate many other proteins (Sato *et al.*, 2006). (2) Calcium ions (Ca^{2+}), one of most widely used second messengers in eukaryotic organisms that regulates various intracellular responses, such as exocytosis in neurons and endocrine cells, muscle contraction, plant defence, etc (Lecourieux *et al.*, 2006). (3) Gases such as nitric oxide (NO) and carbon monoxide (CO), which can diffuse both in cytosol and across the cellular membrane in eukaryotic cells. NO functions as a neurotransmitter by simply diffusing from nerve terminals into adjacent cells and forming covalent linkages to the target

molecules. In addition, it has a role in inflammatory responses, blood vessel reactivity, brain functions and so on (Snyder *et al.*, 1998). Similarly, CO is discovered to play a role in neurotransmission, hormone release and regulation of vascular tone (Farrugia *et al.*, 2003). (4) Nucleotides, such as guanosine-3',5'-(bis) pyrophosphate (ppGpp), a major stringent response factor in bacteria; and cyclic nucleotides, such as cAMP, cGMP and c-di-GMP (Fig 2-1).



cAMP cGMP c-di-GMP

Fig 2-1 Chemical structure of cyclic nucleotide messenger molecules.

Cyclic nucleotides such as cAMP and cGMP are among the most widely studied second messengers. In both unicellular and multicellular organisms, they play a role in numerous physiological processes, for example, ion channel regulation, cell cycle progression, neuromuscular transmission, smooth muscle relaxation, to just name a few. Research on their function and mechanism of action has shaped our understanding of this important area of biology and paved the road for further studies. Recently, a novel cyclic nucleotide, c-di-GMP, is emerging as a global second messenger in various bacterial species and studies are undergoing to unravel more about its role and mode of action in signaling transduction pathway (for reviews, see Römling and Amikam, 2006; Römling *et al.*, 2005).

2.2.1 cAMP signaling

The discovery of cAMP in animal tissues can be dated back to 1957 when Rall and Sutherland found that a heat stable factor, produced by certain liver enzyme in response of adrenaline and glucagon, can stimulate liver phosphorylase formation (Berthet *et al.,* 1957). This heat stable factor was later characterized to be cAMP (Sutherland and Rall, 1958). Subsequent studies revealed cAMP was synthesized from ATP by the adenylyl cyclase and was degraded by phosphodiesterase (Butcher and Sutherland, 1962). In 1968, another research group demonstrated that cAMP can mediate cell activity by binding to the regulatory subunit of a specific tetrameric protein complex, namely cyclic AMP-dependent protein kinase (PKA) complex, (Walsh *et al.*, 1968) and causing the dissociation of active PKA, which subsequently phosphorylates various substrate proteins. With this discovery, the concept of cAMP signaling pathway system was established that the activation of adenylyl cyclase with the presence of hormones or neurotransmitters leads to the production of cAMP, which binds to PKA and trigger downstream cellular responses.

cAMP does not only exist in mammalian cells. In fact, it is present both in eukaryotic cells and in prokaryotes. In eukaryotes, it plays a role in the action of various hormones and neurotransmitters, olfactory signal transduction, metabolic processes, cell cycle progression and plant defence response. In bacteria, cAMP is involved in a variety of regulatory networks, ranging from the classical role of cAMP as a regulatory cofactor in control of carbon utilization, amino acid biosynthesis, transport, stress resistance, to the expression of virulence factors by pathogens (Lory *et al*.,

2004). For example, in *E. coli*, cAMP is synthesized in response to reduced glucose levels and induces expression of other nutrient degradative enzymes such as β-galactosidase; in *V. cholerae*, cAMP is involved in regulation of expression of cholera toxin co-regulated pili (Skorupski and Taylor, 1997). Instead of binding to PKA, cAMP in most prokaryotic cells acts as an activator of cAMP-receptor protein (CRP), an allosteric DNA-regulatory protein which mediates the transcription of downstream genes (Fig 2-2).

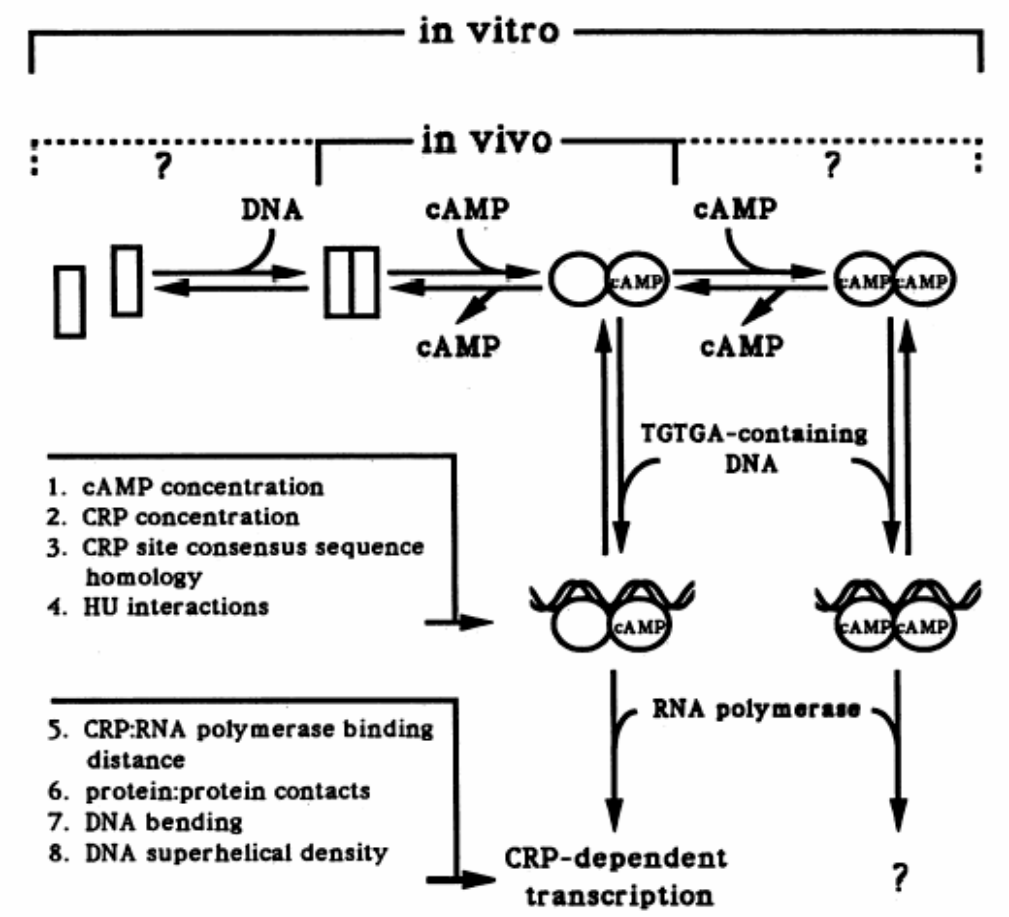


Fig 2-2 Summary of CRP-cAMP-mediated binding and activation of CRP-dependent promoters in *E. coli*.
"In vitro" designates the properties of the two-component system for which data have been obtained from in vitro experiments. "In vivo" represents the properties of the two-component system for which data have been obtained in whole-cell experiments. Rectangles represent either the monomeric or the dimeric form of unliganded CRP. Circles represent the cAMP bound form(s) of CRP whose conformation differs from that of unliganded CRP. DNA refers to nonspecific DNA lacking sequences homologous to the consensus CRP-binding sequence. TGTGA-containing DNA is represented by a double-stranded helical structure and refers to DNA that contains sequences homologous to the consensus CRP-binding sequence. No data exist to indicate that (i) nonspecific DNA sequences cause unliganded CRP to monomerize in vivo, (ii) a stoichiometry of CRP-cAMP complex can be 1:2 in vivo, and (iii) a CRP-cAMP complex having 1:2 stoichiometry can function in activating transcription in vivo or in vitro; these regions are therefore identified by question marks. Adapted from Botsford and Harman (1992).

In contrast to many enterobacteria, the catabolite response of *P. aeruginosa* is not regulated by the levels of cAMP. However, *P. aeruginosa* does express proteins necessary for the synthesis of cAMP and for transcriptional control. Recent studies show that cAMP influences *P. aeruginosa* gene expression by acting as an allosteric regulator of Vfr, a functional homologue of the *E. coli* CRP. Genome microarray analyses revealed that mutants lacking cAMP or *vfr* exhibited reduced expression of nearly 200 genes, including those involved in the type III secretion system (T3SS), type IV pilus biogenesis and type II secretion (Wolfgang *et al.*, 2003). This suggested that T3SS is integrated into a global regulatory network that specifically controls genes related to pathogenesis. Genetic evidence indicated that cAMP and Vfr act upstream of or at the same level as ExsA, a master transcriptional regulator of all T3SS genes, because an *exsA* mutant can not be complemented by overproduction of either cAMP or Vfr. Also, another recent report suggested that the cAMP/Vfr complex does not directly regulate *exsA* expression (Shen *et al.*, 2006). *P. aeruginosa* produces two adenylate cyclases (CyaA and CyaB), but they do not contribute equally to the production of cAMP necessary for the virulence regulator network. Mutants in the *cyaB* gene were more severely attenuated than those in *cyaA*, based on enumeration of bacteria in lungs, liver and spleen, as well as by assessment of mouse lung pathology (Lory *et al.*, 2004). Fig 2-3 showed the environmental signals and regulatory systems controlling the expression of the T3SS, in which the role of cAMP was also explained.

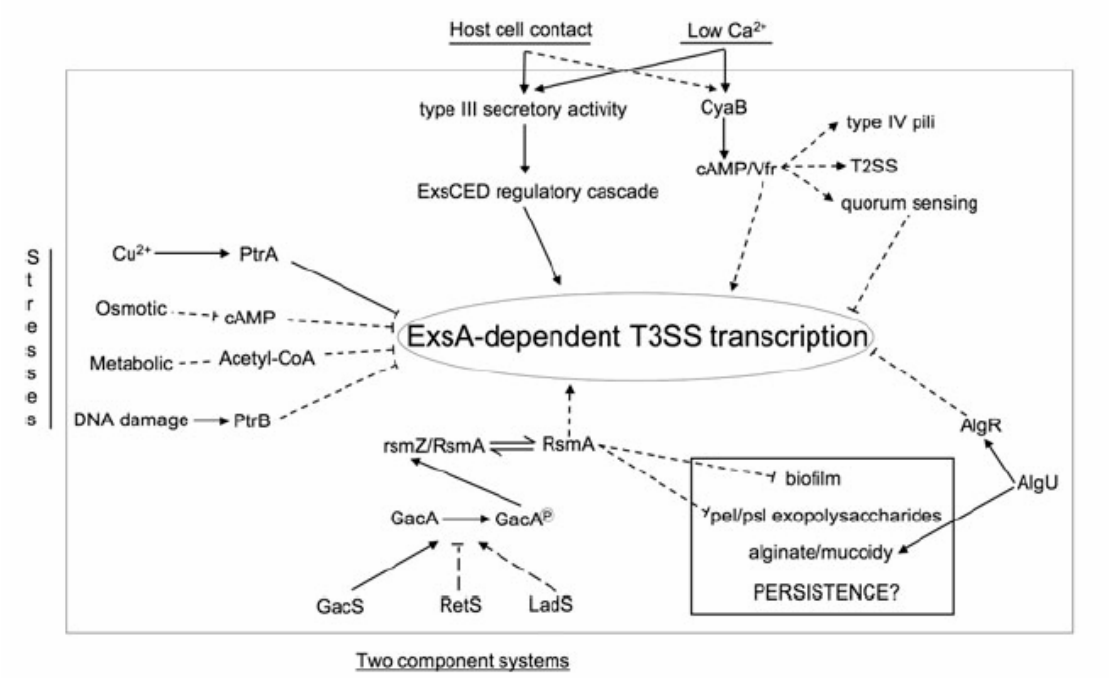


Fig 2-3 Environmental signals and regulatory systems controlling expression of the T3SS.
Solid lines indicate regulatory connections that have been demonstrated experimentally whereas dashed lines indicate hypothetical regulatory connections. Adapted from Yahr and Wolfgang (2006).

2.2.2. cGMP signaling

cGMP, a structural homolog of cAMP, is also an important second messenger, which was first discovered in rat urine (Ashman *et al.*, 1963). In contrast to the ubiquity of cAMP, cGMP is only restrict to eukaryotic kingdom and it regulates a less number of cellular and physiological processes compared with cAMP, including phototransduction, vascular and smooth muscle function, learning and memory and so on. Currently, there are no confirmative reports of cGMP for prokaryotes.

Although cGMP functions differently from cAMP in many aspects, the metabolism of cGMP, which involves guanylyl cyclases for synthesizing cGMP and cGMP-specific PDEs, is similar to that of cAMP. According to their common features, nucleotide cyclases in eukaryotes and prokaryotes can be divided into six different classes that have no sequence similarity with each other. However, all the adenylyl cyclases (ACs) and guanylyl cyclases (GCs) have homologous catalytic domains and they catalyze stereochemically analogous reactions, which proceed with inversion of configuration, presumably by direct in-line attack of the 3' hydroxyl on the α-phosphate (Liu *et al.*, 1997). With respect to cAMP and cGMP specific PDEs, they all contain a conserved catalytic domain of approximately 270 amino acids at the carboxyl terminus (Zhang *et al.*, 2004). High resolution three dimensional crystal structures of cAMP and cGMP specific PDEs reveal a glutamine switch mechanism for the control of PDE selectivity toward cyclic nucleotides. To be more specific, an invariant glutamine residue in the catalytic site can alternatively adopt two different orientations, one is in favor of guanine binding while the other supports adenine binding, leading to selectivity of two cyclic nucleotides, respectively (Zhang *et al.*,

2004).

In mammalians, cGMP plays a role in visual transduction. Upon receiving light stimulus, cGMP-specific retinal PDE is activated. The resultant decreased level of cGMP leads to the hyperpolarization of plasma membrane in rod cells, after which the output signal is sent to visual cortex of the brain to generate vision (Takemoto and Cunnick, 1990).

Apart from light, cGMP signaling pathway is also mediated by natriuretic peptide hormones such as atrial natriuretic peptides and guanylin (Kuhn, 2004). Natriuretic peptides, once released into the blood stream, activate the membrane-bound homodimeric guanylate cyclase and increase the cGMP level. cGMP then exerts its effect on cGMP-gated ion channels, cGMP-specific phosphodiesterase or cGMP-dependent protein kinases to regulate different physiological processes in the cardiovascular and gastrointestinal system, the kidney, bone and other tissues.

The third type of molecule which mediates the cGMP signal transduction is NO (Synder *et al.*, 1998). In the cells, NO is synthesized by isoforms of nitric oxide synthase, which is mostly present in the nervous system. When NO binds to the heme of the soluble isoforms of heterodimeric guanylate cyclase, it triggers the synthesis of cGMP.

2.2.3 c-di-GMP biology

It is widely known that cGMP, unlike cAMP, is absent from bacteria as a second messenger. Therefore, whether any guanosine nucleotide plays a role of second

messenger in the bacteria becomes an open question. As studies going on, there is accumulating evidence that c-di-GMP, the homolog of cGMP, is ubiquitously present in numerous bacteria and functions as a signaling molecule to regulate complicated biological processes from motility, biofilm formation, virulence to photosynthesis (Römling and Amikam, 2006).

2.2.3.1 Conservation of GGDEF and EAL domains in bacterial genomes

The discovery journey of c-di-GMP began 21 years ago when late Moshe Benziman's group from Israel found an unusual guanyl dinucleotide while studying the mechanism of cellulose biogenesis in *G. xylinus*, which could activate the cellulose synthase (Ross *et al*, 1986). One year later, they identified the guanyl oligonucleotide to be c-di-GMP by mass spectrometry and nuclear magnetic resonance analysis (Ross *et al*, 1987). In 1989, c-di-GMP was also found to play the same role in cellulose synthesis in the plant pathogen *Agrobacterium tumefaciens* (Amikam and Benziman, 1989). However, the enzymes responsible for the turnover of c-di-GMP remained obscure until the first evidence came in 1998, when Benziman's group discovered DGCs and PDE-As in *G. xylinus*, which control the synthesis and degradation of c-di-GMP, respectively (Tal *et al*, 1998). The DGC and PDE-A proteins contain two conserved domains, GGDEF and EAL domains, which were named after highly conserved sequence motifs, Gly-Gly-Asp-Glu-Phe and Glu-Ala-Leu. The domain sizes are approximately 180 and 240 amino acid residues, for GGDEF and EAL domains, respectively. Genome analysis showed that these two domains are present in diverse branches of the phylogenetic tree (Fig 2-4) of bacteria but are absent in genomes of any Archaea or Eukarya. Over 2200 proteins with either GGDEF/EAL

domain or both domains are found in public protein databases currently. However, the abundance of the two domains is not equal in diverse bacteria (Römling *et al*, 2005). For example, *Vibrio vulnificus* encodes 66 proteins with GGDEF domain and 33 with EAL domain, which is the number one among bacteria species, whereas *Bacillus subtilis* only has 4 and 3, respectively (Römling *et al*, 2005).

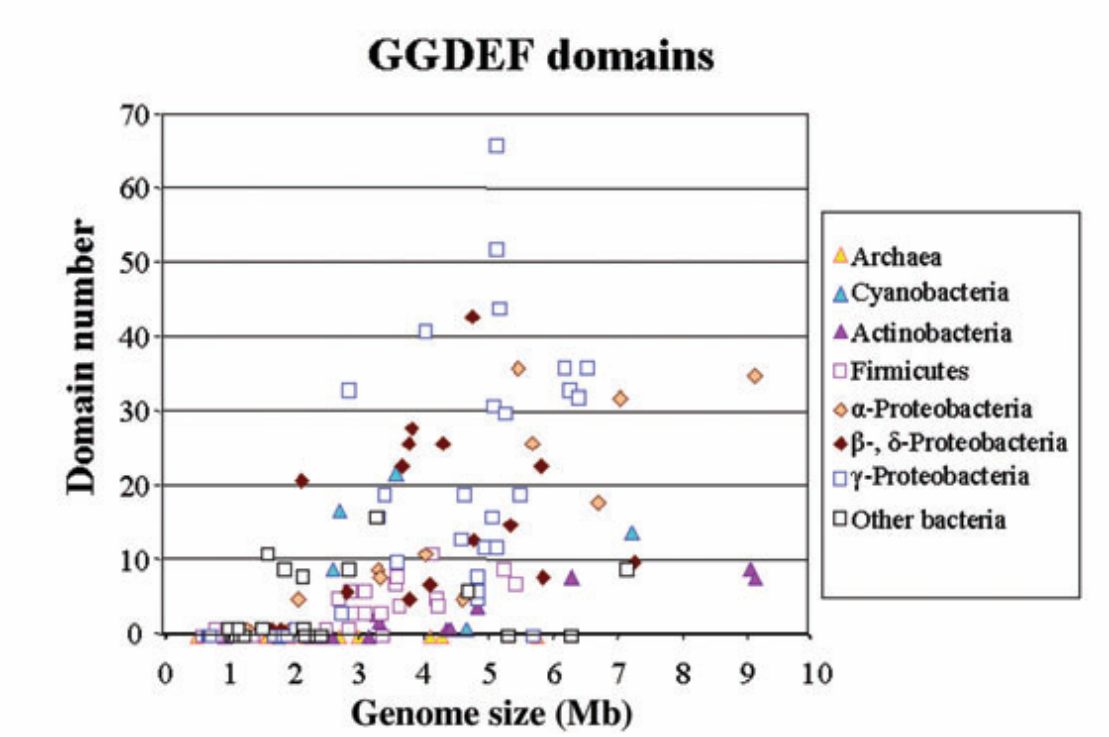


Fig 2-4 Phylogenetic distribution of the GGDEF domains in sequenced prokaryotic genomes. Adapted from Römling *et al* (2005).

2.2.3.2 c-di-GMP cyclase and phosphodiesterase enzymology

Recently, the precise roles of GGDEF and EAL domains were experimentally verified. PleD, a response regulator in *Caulobacter crescentus* polar development has GGDEF domain in its C-terminal serving as an output module (Hecht and Newton, 1995). Purified PleD was shown to have GTP-specific c-di-GMP synthesizing ability and mutations at GGDEF motif abolish the nucleotide cyclase activity (Paul *et al*, 2004). Six GGDEF domain-encoding genes from diverse branches of the bacterial phylogenetic tree were overexpressed and all the purified recombinant proteins were demonstrated to possess DGC activity (Ryjenkov *et al*, 2005). It was shown that GGDEF domain alone is sufficient to encode DGC activity as an oligomeric form, although at a very low level compared with full-length protein. In many proteins, both GGDEF and EAL domains either singly or jointly, can be found in the same molecule together with various types of sensory domains (Fig 2-5). It has been shown that regulations by sensory domains can affect activities of DGC and PDE-A domains. For example, the GGDEF protein Rrp1 from *Borrelia burgdorferi* only displays DGC activity when its input receiver domain, REC domain, is phosphorylated (Ryjenkov *et al*, 2005). In other cases, the presence of GAF domain stimulated DGC activities of the proteins (Ryjenkov *et al*, 2005). It is noteworthy that GAF domains are implicated in cyclic nucleotide signaling with the capability of binding to cAMP/cGMP (Hurley, 2003) and are found together with GGDEF and EAL domain-containing proteins.

While more is known currently of the GGDEF domain, functional studies of EAL domain are also undergoing. The *E. coli* EAL domain-containing protein YahA and

its individual EAL domain were overexpressed, purified and characterized in vitro. Results suggested that both full length YahA and EAL domain have c-di-GMP specific phosphodiesterase activity that leads to hydrolysis of c-di-GMP into linear dimeric GMP (pGpG) (Schmidt *et al*, 2005). Similarly, the *V. cholerae* EAL domain protein was demonstrated in vitro to possess c-di-GMP specific hydrolytic activity (Tamayo *et al*, 2005). Nevertheless, in both cases, the phosphodiesterase activity of EAL domain containing proteins is only responsible for digesting c-di-GMP into pGpG while the subsequent hydrolysis of pGpG into two 5'-GMP are performed by other enzymes in the bacteria. In addition, when tested, the phosphodiesterase activity is dependent on Mg^{2+} and inhibited by Ca^{2+} (Schmidt *et al*, 2005).

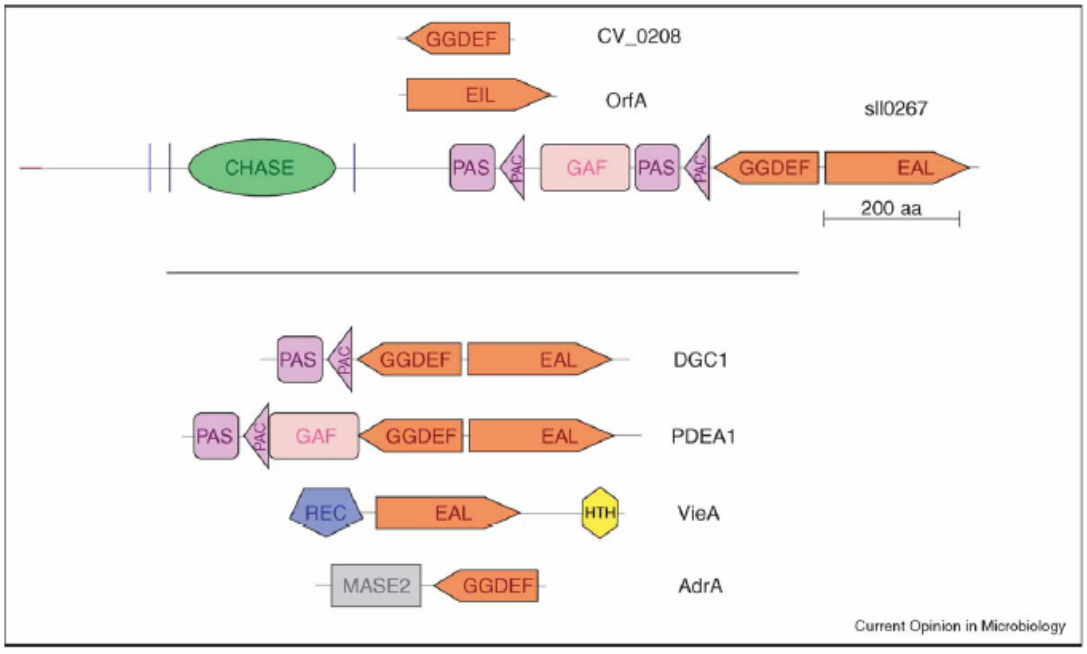


Fig 2-5 Domain structure of GGDEF and EAL domain proteins. Adapted from Römling and Amikam (2006).

Since GGDEF and EAL domains possess opposite functions from each other towards c-di-GMP enzymology, question arises that what the function of proteins containing both domains is. Only a limited number of GGDEF and EAL fusion proteins have been studied so far. In *G. xylinus*, six proteins with both GGDEF and EAL domains were identified. However, three of them were assigned to possess DGC activity while others possess PDE-A activities, respectively. STM 3388, a protein of *Salmonella enterica* serovar typhimurium with conventional GGDEF and EAL domains, only has DGC activity (Kader *et al.*, 2006). CC3396, a GGDEF-EAL protein from *C. crescentus* is a soluble PDE-A with an altered inactive GGDEF domain, served as a GTP-dependent regulatory domain (Christen *et al*., 2005). These observations suggest that one of the domains is enzymatically inactive when both domains are present in a protein. However, the factors affecting the inactivity at the molecular level are currently unknown. It is also likely that under in vitro conditions tested, either GGDEF or EAL domains are not activated and hence hypothesized to be "silent" domain in these proteins.

2.2.3.3 Biological processes regulated by c-di-GMP

Although c-di-GMP is a late comer compared with other cyclic nucleotide messengers, intensive research on its biological functions in bacteria has revealed that c-di-GMP is involved in diverse biological processes including motility, biofilm formation, cell morphology, exopolysaccharide production, cell-cell communication, virulence, etc.

Bacterial cells exist either in planktonic forms or in surface-attached communities called biofilms. In biofilms, bacterial cells are enclosed by extracellular matrix consisting of exopolysaccharide, proteins and other components. Biofilm formation is monitored by a complex regulatory system, in which c-di-GMP was found to play a role via GGDEF and/ or EAL domain containing proteins. The main content of extracellular matrix, expolysaccharides, were shown to be activated by GGDEF domain-containing proteins in *G. xylinus*, *E. coli*, *S. typhimurium*, *Rhizobium leguminosarum* and *P. fluorescens* (Tal *et al*., 1998; Ausmees *et al*., 1999; Zogaj *et al*., 2001; Spiers *et al*., 2002). The expression of GGDEF domain proteins raises the cellular c-di-GMP level, leading to the production of extracellular matrix components, which inhibits the motility of cells and promotes the formation of highly structured biofilm. In contrast, the production of EAL domain proteins decreases the c-di-GMP level and consequently suppresses biofilm formation but stimulates motility. In *V. cholerae*, biofilm formation was reduced threefold by arabinose-induced expression of VieA, an EAL domain protein (Tischler and Camilli, 2004). YhjH, an EAL domain-containing protein of *S. typhimurium*, when overexpressed, enhances all the forms of motility such as swimming, swarming and twitching (Simm et al., 2004). In *E. coli*, the swimming motility defect in an *hns* mutant was overcome by producing an EAL domain protein (Ko and Park, 2000). In *Shewanella oneidensis*, the attachment of cells to and detachment from the biofilm matrix is regulated by c-di-GMP in a concentration-dependent manner (Thormann *et al.*, 2006). In *P. aeruginosa*, the mutant of a GGDEF and EAL domain containing protein, FimX, have strongly reduced levels of extracellular pili, indicating the normal pilus function is impaired, which in turn affects the twitching motility (Huang *et al.*, 2003).

c-di-GMP has been reported to play a role in virulence in many bacterial systems. Investigation showed that VieA, which maintains c-di-GMP at a low level in *V. cholerae*, can positively affect the production of cholera toxin (Tischler and Camilli, 2004). In *Bordetella pertussis*, the expression of EAL domain protein BvgR can suppress virulence-inhibiting genes and consequently enhances virulence in the mouse aerosol challenge model (Merkel *et al.*, 1998). A set of mutants created in the selected GGDEF and EAL encoding genes in the opportunistic pathogen *P. aeruginosa* PA14 strain displayed attenuated virulence in a mouse infection model (Kulasakara *et al*., 2006).

2.2.3.4 c-di-GMP binding domains

Although separate role has been assigned to GGDEF and EAL domains, the mechanisms of c-di-GMP signaling pathway still remain unclear due to the limited information on the target of c-di-GMP action.

The first available experimental information on c-di-GMP binding protein came from the study of cellulose synthase. c-di-GMP is the allosteric activator of membrane-bound cellulose synthase system in *G. xylinus*. In 1997, Weinhouse and co-workers discovered a c-di-GMP binding protein complex, which is structurally associated with cellulose synthase. This 200kD-protein complex appears to play a major role in modulating the intracellular concentration of free c-di-GMP and may act as an essential factor in regulating cellulose synthase in vivo (Weinhouse *et al.*, 1997). The protein complex was not further characterized; however, it could correspond to a dimer of the α-subunit of cellulose synthase BscA or a fusion protein of BscA and β-

subunit of cellulose synthase BscB (Saxena and Brown, 1995).

Recently, Amikam and Galperin proposed the first c-di-GMP binding domain — PilZ domain, a 118 amino acid long protein which is distributed in a variety of bacteria with a phyletic pattern similar to those of GGDEF and EAL domains (Amikam and Galperin, 2006). In *P. aeruginosa*, PilZ protein, encoded by PA2960 gene, is involved in type IV pili biosynthesis pathways, which is also regulated by c-di-GMP (Simm, *et al*., 2004). Moreover, PSI-BLAST search identified the PilZ domain near the C-terminus of the α-subunit of cellulose synthase BcsA from *G. xylinus*, which is consistent with the discovery of Weinhouse's. Other available experimental data also indicated that PilZ domain is (part of) the long-sought c-di-GMP binding protein.

Early in this year, another protein domain, HD-GYP domain, was shown to be involved in cell-cell signaling of *Xanthomonas campestris* pv. *campestris* (*Xcc*) via functioning in c-di-GMP turnover (Ryan *et al.*, 2006). HD-GYP domain is a subgroup of the HD superfamily of metal dependent phosphohydrolases (Aravind and Koonin, 1998; Galperin *et al*., 1999; 2001). In *Xcc*, RpfG, a HD-GYP domain containing protein, was shown to possess PDE-A activity. The isolated HD-GYP domain was overexpressed and it alone could degrade c-di-GMP. Recent experiment also revealed that the HD-GYP domain of RpfG interacts with a number of GGDEF domain-containing proteins in *Xanthomonas axonopodis pv citri* (Andrade *et al.*, 2006). These observations, therefore, add new information towards a greater understanding of c-di-GMP regulatory system in diverse bacteria.

2.3 Motility Regulatory (Mor) pathway in *Pseudomonas*

2.3.1 Overview

Ability to move, namely, motility provides numerous advantages to a bacterium for better survival, including movement towards favourable conditions, avoidance of detrimental conditions, and successful competition with other micro-organisms (Fenchel, 2002). In pathogenic bacteria, motility is usually considered a virulence factor essential for colonization of host organism or target organ (Ottemann and Miller, 1997; Josenhans and Suerbaum, 2002). There are several forms of bacterial motility, such as swimming, swarming, twitching and gliding, the first two of which require flagella whereas the last two do not. The flagellum (*pl*. flagella), which is the principal locomotory organelle, plays additional crucial roles in cell adhesion, biofilm formation, colonization, and dispersal in the environment. Flagellar biogenesis is highly complex, requiring coordinated expression of over 40 genes. The flagellar regulatory pathway has been well elucidated in several bacteria, such as *E. coli* and *S. typhimurium* and *P. aeruginosa* (Prouty *et al.*, 2001; Givskov *et al.*, 1995; Amsler *et al.*, 1993, Dasgupta *et al.*, 2003).

Recently, a series of motility regulators, MorA, MorB and MorC were identified in *P. putida* in our laboratory. All these regulators can affect bacterial motility and biofilm formation. These proteins have been included in the Mor family based on their domain structures. All of them contain GGDEF domain. MorA and MorC, additionally, contain EAL domain (Fig 2-6) (Ng, 2006; Fu, 2006).

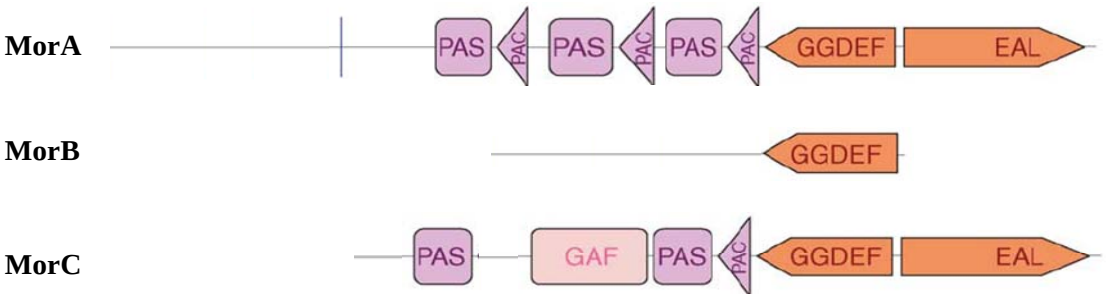


Fig 2-6 Domain structure of MorA, MorB and MorC.
Vertical line represents transmembrane domain.

2.3.2 MorA regulator

MorA is a membrane-localized regulator containing GGDEF and EAL domains, transmembrane domain and PAS-PAC sensory domain (Fig 2-6). It controls the timing of flagellar development and affects motility, chemotaxis, and biofilm formation in *P. putida* (Choy *et al*., 2004). Unlike wild type *P. putida*, where flagellar biogenesis occurs during the log-to-stationary phase, *morA* mutants constitutively expresses flagella in all growth phases. It is noteworthy that flagellar development involves coordinate expression of over twenty proteins and the loss of MorA in *P. putida* affected the expression of *fliC*, a key flagellar biosynthetic gene, in the log-to-stationary transition phase. However, other flagellar pathway transcriptional regulators, such as FleQ and FliA, which stand at a higher hierarchy regulatory level in the flagellar pathway, were not affected. Hence, MorA is perhaps a global regulator of an alternative regulatory system that normally restricts the timing of expression of the flagellar biosynthesis pathway to late phases of growth in *P. putida*. Enhanced motility of MorA mutant leads to secondary phenotype changes such as enhanced chemotaxis and reduced biofilm formation. MorA is highly conserved in various Pseudomonad species such as *P. aeruginosa, P. fluorescens* and others. However, the

morA mutant of *P. aeruginosa* does not show any change in motility, albeit it retains the reduced biofilm phenotype (Choy *et al.*, 2004).

Considering the presence of GGDEF domain in MorA, which implicates the possible DGC activity, isothermal calorimetry (ITC) was performed to investigate influence of GTP on the recombinant MorA proteins. A distinct exothermic change was observed, indicating formation of hydrophobic and electrostatic contacts and hydrogen bonds between the protein residues and GTP substrates. Titration data revealed that the specific binding of GTP to the GGDEF domain is comparatively more favorable than that of the EAL domain and the recombinant MorA could remain active in conformation even when highly expressed in a non-native microorganism such as *E. coli* (Lye, 2006).

In spite of all the above-mentioned information, it is not clear how MorA mediates the flagellar pathway, whether MorA has any enzymatic activity, and whether it affects the c-di-GMP levels in *P. putida*. Therefore, further studies are needed to address the above-mentioned questions.

Chapter 3. Materials and Methods

3. Materials and Methods

3.1 Bacterial strains and media

Bacterial strains used in this study are described in Table 3-1. *G. xylinus* were grown in R-20 medium (Weinhouse, *et al.*, 1997) with the presence of 0.1% cellulase at 30°C. *P. putida* strains were grown in Luria-Bertani (LB) medium at 30°C with suitable antibiotics. *E. coli* strains were grown, unless otherwise specified, in LB medium at 37°C with suitable antibiotics (Table 3-1). Bacterial growth was measured by determining the optical density at 600 nm (OD_{600}).

Table 3-1 Bacterial strains.

Strain	Relevant characteristics[a]	Source or reference
G.xylinus		
1306-21	c-di-GMP production; cellulose production	ATCC® 53524
P. putida		
PNL-MK25	Wild-type *P. putida* strain; Cm^r Rf^r	Adaikkalam and Swarup, 2002
morA knock out strain	PNL-MK25 mutant (*morA*$_{Pp}$::*aacC1*); Cm^r Rf^r Gm^r	Choy *et al*., 2004
morA overexpressed strain	Wile-type PNL-MK25 containing plasmid pGB1 where full-length *morA*$_{Pp}$ gene with its native promoter were cloned into; Cm^r Rf^r Tet^r	Choy *et al*., 2004
E. coli		
morA-BL21	Protein expression strain containing plasmid pGEX where recombinant MorA was cloned into; Amp^r Tet^r	Lye, 2006

[a]Cm, chloramphenicol 15 μg/ml; Rf, rifampicin 20 μg/ml; Gm, gentamycin 20 μg/ml ; Amp, ampicillin 100 μg/ml; Tet, tetracycline 25 μg/ml.

3.2 Extraction and detection of c-di-GMP

3.2.1 c-di-GMP extraction

G. xylinus was used as a model to establish method of extracting and detecting c-di-GMP. Cells of *G. xylinus* were harvested at mid log phase (OD_{600} 1.0) and extracted using an acid method modified from what previously described (Weinhouse *et al.*, 1997). Generally, the cell pellet was extracted with 0.6M perchloric acid. Denatured protein was removed by centrifugation and supernatant was neutralized with 5M K_2CO_3. The precipitate was again removed by centrifugation and resultant supernatant was filtered through 0.2μm filter, lyophilized and resuspended in minimal amount of H_2O for later analysis. *P. putida* wild type (WT) strains were harvested and extracted as above.

3.2.2 HPLC analysis

Reversed-phase high pressure liquid chromatography (RP-HPLC) was performed on a 250×4.6mm Merck LiChrospher® 5 μm RP-18 100A column (Merck KGaA, Germany) at room temperature, detection at 252 nm, on Äkta purifier 10 (Amersham Biosciences, USA). Runs were carried out with isocratic elution (1ml/min) in 50mM triethylammonium biocarbonate (TEAB) buffer pH 7.0 containing 10% (V/V) of methanol. Relevant fractions of 1ml were collected, lyophilized and resuspended in minimal amount of H_2O. HPLC profile of crude c-di-GMP was used as control for the HPLC analysis. Crudely purified c-di-GMP extract is a gift from the late Professor

Moshe Benziman and Dr Haim Wienhouse, Department of Biological Chemistry, Institute of Life Sciences, Hebrew University of Jerusalem, Israel.

3.2.3 Mass spectrometric analysis of c-di-GMP

The resuspended HPLC fractions were mixed 1:1 with saturated a-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% trifluoroacetic acid (TFA). 1 µl was spotted onto a laser-etched stainless sample plate for matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis. The samples were analyzed in negative-ion mode with the Voyager DE™ STR mass spectrometer Biospectrometry™ Workstation (Applied Biosystems, USA).

3.2.4 Relative quantification of c-di-GMP

Relative quantification was carried out by comparing the peak area of c-di-GMP from HPLC profiles. Due to the non-availability of sufficiently purified c-di-GMP, the homolog of c-di-GMP, i. e., cGMP was selected as a HPLC standard. With the presumption that the peak area generated by 1 nmole of c-di-GMP in HPLC analysis is twice the peak area generated by 1 nmole of cGMP, a calibration curve for relative quantification of c-di-GMP levels was established with various concentrations of cGMP in the range of 2µm to 150µm in triplicate.

The nucleotides of *P. putida* WT strain at early log phase (OD_{600} 0.3), middle log phase (OD_{600} 1.0) and log-to-stationary transition phase (OD_{600} 1.7) were extracted and analyzed by HPLC as described above. The relative concentrations of c-di-GMP

were determined according to the calibration curve and expressed as pmole per milligram dry weight of cells. For determination of dry weight, cells were harvested, washed in distilled water, and dried in drying oven until the weight remained consistent.

Comparison of intracellular c-di-GMP levels in *P. putida* WT, *morA* knock out (KO) strain and *morA* overexpressed (OE) strain was carried out. *P. putida* KO strain is a *morA* targeted double cross-over knockout *P. putida* strain in which MorA losses its function while *P. putida* OE strain is wild-type *P. putida* that contains plasmid pGB1 where full-length *morA* gene with its native promoter were cloned into (Choy *et al.*, 2004). For comparison, equal amount of *P. putida* WT, KO and OE strains were harvested at early log phase, middle log phase and log-to-stationary transition phase. Nucleotides were extracted, analyzed and intracellular c-di-GMP levels were determined and compared as described above.

3.3 Enzymatic studies of MorA

To study whether MorA possesses DGC activity, DGC from *G. xylinus* was extracted and used as a positive control. Crude MorA from *P. putida* WT strain was prepared and the presence of MorA was checked by western blotting. Recombinant MorA was extracted and purified, and the resultant protein profile was analysed by SDS-PAGE. DGC activity assay was performed to positive control, crude MorA extract and purified recombinant MorA. The flowchart of experiment is shown in Fig 3-1.

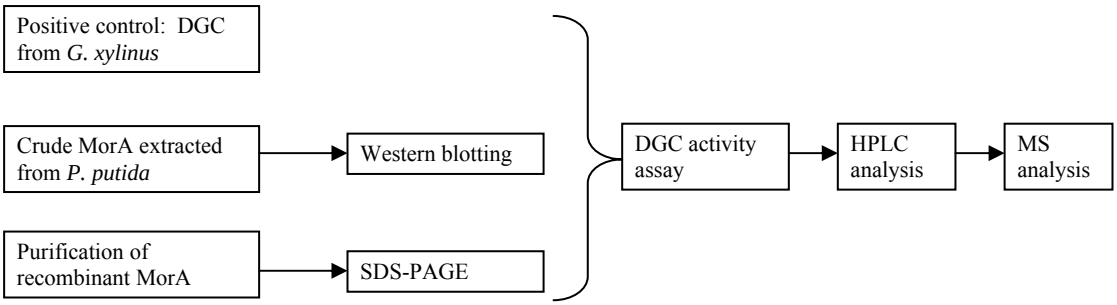


Fig 3-1 Flowchart of experiment to study DGC activity of MorA.

3.3.1 DGC extraction from *G. xylinus*

G. xylinus was grown in R-20 medium supplemented with 0.1% cellulase with constant shaking. Cells were harvested at OD_{600} 1.0 and washed once in buffer containing 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 1 mM EDTA (TME buffer). DGC were extracted with the protocol adapted from Ross *et al*. (1986). Generally, the washed cell pellets were suspended in TME buffer with the presence of 20% (w/v) PEG-4000 (TME-PEG buffer). 1 litre culture of cells was suspended in 30 ml buffer. This suspension was passed through a French press cell. The lysed extract was centrifuged at 12,000 ×g for 10 min and the resultant precipitation prepared in PEG-4000 was homogenized in TME buffer to one fourth the original volume. The resuspended extract was recentrifuged at 1,500 ×g for 3 min to remove large particulate material. The supernatant was recentrifuged at 18,000×g for 20 min at 4^{o}C and the resultant supernatant contained DGC.

3.3.2 Crude MorA extraction from *P. putida*

The protocol for crude MorA extraction was the same as the preparation of DGC from *G. xylinus* described in Section 3.3.1.

3.3.3 Western blotting

The presence of MorA protein was checked by Western blotting as was described in Choy *et al* (2004). The anti-MorA polyclonal antibodies against partial MorA were produced by a previous Ph.D student in our laboratory. Goat anti-rabbit immunoglobulin G, conjugated with alkaline phosphatase (Promega, USA), was used as the secondary antibody for detection of MorA.

Crude MorA extract from *P. putida* WT strain was electrophoresed in 8% polyacrylamide gels and transferred onto Hybond™ ECL™ nitrocellulose membranes (Amersham Biosciences, UK) in Transfer Buffer (25 mM Tris, 0.15 M glycine, 20% (v/v) methanol) at 70 V for 1 h 15 min at 4^{o}C. The membrane blots were first washed three times with phosphate-buffered saline (PBS) after the transfer and then incubated overnight in Blocking Solution (7% (w/v) nonfat day milk, 0.05% Tween 20) at 4^{o}C. The blots were then removed from the Blocking Solution and washed twice for 5 min each in PBS. The blots were incubated in either rabbit anti-MorA antibodies (1500× dilution) prepared in Blocking Solution for 1 h at room temperature. After incubation, the blots were washed four times for 10 min each in Wash Buffer 1 (PBS, 0.1% Tween 20). Next, they were incubated in goat anti-rabbit IgG, conjugated with alkaline phosphatase (2500× dilution, Promega) prepared in Blocking Solution for 1 h at room temperature followed by washing for 4 times at 10 min each in Wash Buffer 2 (50 mM Tris, 0.15 M NaCl, 0.1% Tween 20, pH 7.5). The final washing step involved 2 rounds of rinsing in Wash Buffer 3 (50 mM Tris, 0.15 M NaCl, pH 7.5) before

immersion in NBT/BCIP detection reagent (Promega). The blots were subsequently washed with 20 mM EDTA after the bands had appeared.

3.3.4 Extraction and purification of recombinant MorA

Recombinant MorA without transmembrane domain was previously cloned into a glutathione S-transferase (GST) gene fusion system for expression (Lye, 2006). *E. coli* strain BL21 transformed with recombinant MorA was grown at 37°C in 2× YTA medium to OD_{600}1.0. The cell culture was cooled down to room temperature before induction with 0.1 mM IPTG overnight at 28°C. Bacterial cells were harvested and lysed by sonication in lysis buffer (400 mM NaCl, 20 mM DTT, 5% glycerol, 50 mM Tris-HCl, pH 8.0, and cocktail protease inhibitors without EDTA). Triton X-100 was then added to a final concentration of 1% and subsequently clarified by centrifugation at 6,000g for 40 min at 4°C. The crude protein extract was partially purified by passing through DEAE sepharose Fast Flow Column (Amersham Pharmacia Biotech., USA). Then GST fusion proteins were further purified under non-denaturing conditions by selectively binding to glutathione–Sepharose 4B Beads (Amersham Pharmacia Biotech.). The GST-fusion protein-bound column was washed with two column volumes of 50 mM Tris-HCl buffer. The GST-fusion proteins were then cleaved by PreScission Protease (Amersham Pharmacia Biotech.) at 4°C for 16 h in the cleavage buffer (50 mM Tris–HCl, pH 7.0, 150 mM NaCl, 1 mM DTT, and 1 mM EDTA). Recombinant proteins were then eluted out and the purify of the eluted proteins analyzed on 8% SDS–PAGE and Coomassie-stained. Protein concentration was determined by the Bradford assay (BioRad, USA) and spectrophotometrically at OD_{595}.

3.3.5 DGC activity assay

The enzymatic reaction was performed at 30^{o}C with occasional gentle shaking. The total volume of the reaction mixture is 2 ml. The components of the reaction mixture contained protein to be tested, Tris·HCl (pH 7.5), $MgCl_2$, EDTA, $CaCl_2$ and NaCl. Different concentrations of the components were applied to obtain the optimized conditions (Table 3-2). For DGC activity assay of MorA, another set of experiment was carried out with the presence of 0.1mM, 1mM and 10mM acetyl phosphate (AcP) in the reaction mixture, while other conditions remained the same. The reaction was started by the addition of the substrate GTP to the prewarmed reaction mixture. Aliquots of 350µl were withdrawn at time points of 0, 5, 30, 60 and 120 min and equal volume of 0.5M EDTA was immediately added to the aliquot to stop the reaction. The mixture was then centrifuged at 15,000×g for 5 min to remove the protein and the supernatant was filtered through a 0.2-µm-pore-size filter. The supernatant was then loaded onto the RP-18 column and analyzed with the same buffer and running conditions as described in Section 3.2.2. The corresponding fractions of c-di-GMP were collected, lyophilized, redissolved in H_2O and analyzed by MALDI-TOF mass spectrometer at negative mode.

Table 3-2 Concentrations of reaction components tested in DGC activity assay. The concentrations in gray cells are optimized conditions.

Component	Concentration		
Protein	0.4~0.6mg per reaction		
GTP	100μM	150μM	200μM
Tris·HCl (pH 7.5)	25mM	50mM	75mM
$MgCl_2$	1mM	5mM	10mM
EDTA	0.1mM	0.5mM	1mM
$CaCl_2$	1mM	2mM	4mM
NaCl	50mM	100mM	150mM

3.4 Isolation and purification of GTP-binding proteins in *P. putida*

3.4.1 Protein extraction and purification

Cells from 3 L culture of *P. putida* WT strain at OD_{600} 1.0 were harvested, first washed with TME buffer and resuspended in TME-PEG buffer as described in Section 3.3.1. PEG-4000 was selected as the precipitating agent due to its non-toxicity and inert chemical properties. The percentage of PEG-4000 was determined to be 20% (w/v), the same as described in another experiment of extraction of c-di-GMP related proteins carried out by Ross *et al.* (1986). Cells were then lysed by passing through a French Press cell and the lysed extract was centrifuged at 12,000 ×g for 10 min. The precipitate was suspended in TME buffer and centrifuged at 1,500 ×g for 3

min to remove large particulate material. The resultant supernatant was then passed through 0.45μm-pore-size filter for further clarification before being loaded onto GTP-agarose column.

3.4.2 Affinity chromatography

GTP-agarose beads (Cat No: G9768) were bought from Sigma-Aldrich, USA. The beads were first thoroughly washed with 50 volumes of cold water to remove the storage buffer and then equilibrated with 20 volumes of TME buffer. After loading the samples, the column was first washed with 20 volumes of TME buffer containing 1 mM DTT (TME-DTT buffer), followed by 15 volumes of TME-DTT buffer containing 1 mM ATP, and 10 column volumes of TME-DTT buffer. Eluting buffer contains 50mM tris-HCl, 10mM EDTA, 1mM DTT and 200mM KCl. The eluted proteins were concentrated using Vivaspin® 15R (Cat No: VS02H91) centrifugal concentrators (Vivascience AG, Germany) before SDS-PAGE analysis. All the steps were carried out at 4^{o}C.

3.4.3 Protein identification by mass spectrometric analysis

Eluted proteins were electrophoresed on 8% SDS-PAGE and coomassie-stained. Prominent bands were excised from the gel, washed and performed trypsin digestion. The resultant peptides were analyzed by MALDI-TOF-TOF mass spectrometry.

Chapter 4. Results

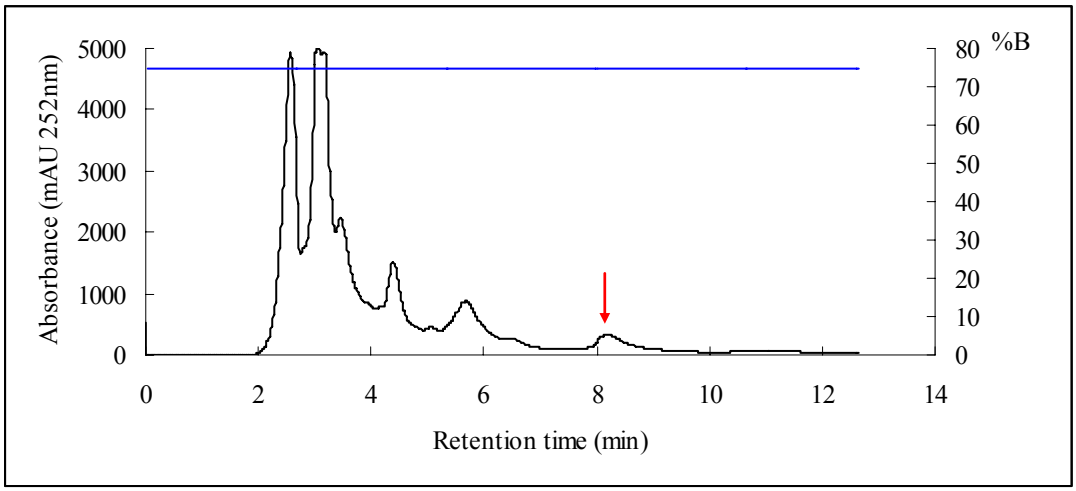
4. Results

4.1. Extraction and detection of c-di-GMP

4.1.1 Detection of c-di-GMP in *G. xylinus* and *P. putida*

In a previous report, c-di-GMP was shown to be produced by DGC in *G. xylinus* (Ross *et al*., 1987). Therefore, *G. xylinus* was used as a model to establish a protocol for extraction and detection of c-di-GMP. Nucleotides were extracted with an acid method and analyzed by HPLC. The HPLC profile of nucleotide extracts of *G. xylinus* is shown in Fig 4-1A. Compared with the control, i. e., the HPLC profile of the crude c-di-GMP sample from G. xylinus (gift by Dr. Weinhouse, Israel) (Fig 4-1B), a peak at retention time of 8-10 min was identified as a putative c-di-GMP peak. The relevant fraction was collected, lyophilized, resuspended in minimal amount of water and further analyzed by MALDI-TOF mass spectrometry in the negative mode. Fig 4-2 shows the mass spectrum of the relevant HPLC fraction. Major ion was detected at m/z of 688.9, which corresponds to $[\text{c-di-GMP-H}]^-$. These results suggested that the extraction and detection method was efficient for *G. xylinus*.

A



B

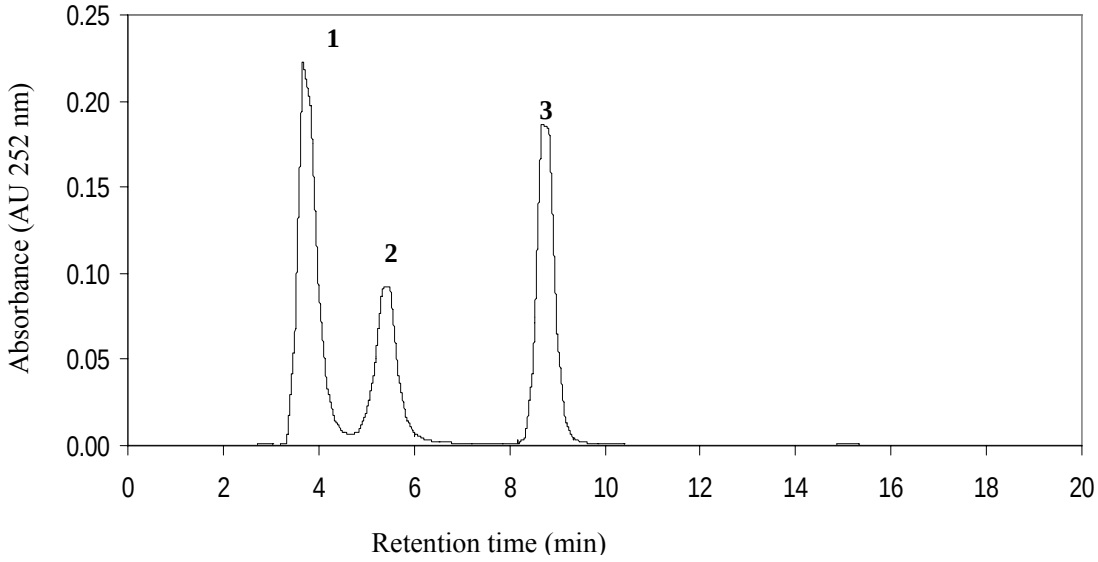


Fig 4-1 HPLC analysis of c-di-GMP in crude nucleotide extracts from *G. xylinus*. (A) HPLC profile of crude nucleotide extracts from *G. xylinus*. The red arrow indicates the peak position of c-di-GMP.

(B) HPLC profile of crude c-di-GMP control sample. Peak 1 contains GTP and its degradation products. Peak 2 and 3 contain c-di-GMP degradation products and c-di-GMP, respectively (adapted from Dr. Choy Weng Keong, Ph.D thesis).

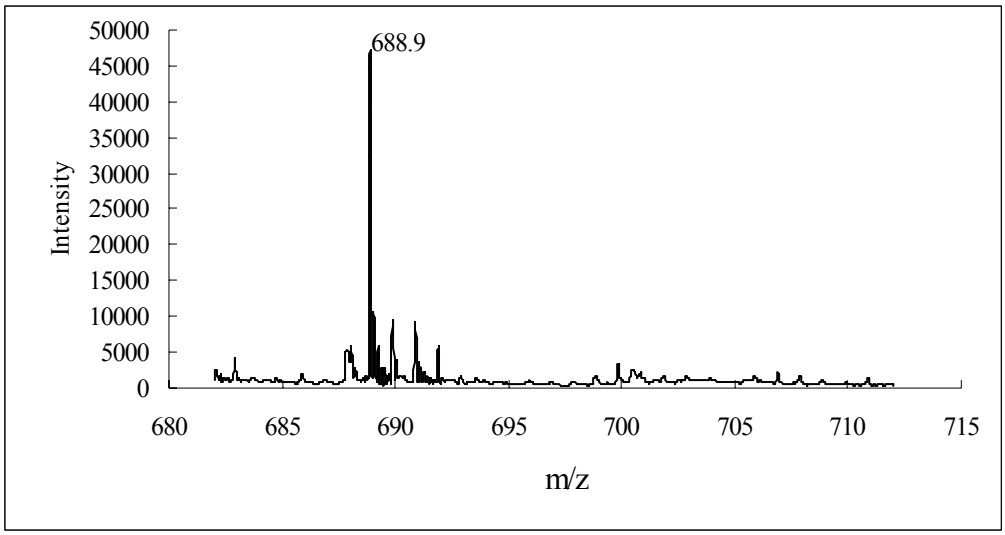


Fig 4-2 MALDI-TOF analysis of c-di-GMP from HPLC fraction of *G. xylinus.*
The HPLC fraction, as shown with red arrow in Fig. 4-1A, was analyzed by MALDI-TOF mass spectrometer in negative mode. Major ion was detected at m/z of 688.9, representing $[c\text{-di-GMP-H}]^-$.

Next, the extraction and detection of c-di-GMP were carried out with *P. putida* WT strain following the same protocol. A c-di-GMP peak was detected from HPLC analysis (Fig 4-3) and confirmed by mass spectrometry (Fig 4-4). Therefore, the method of extracting and detecting c-di-GMP could be applied to *P. putida*.

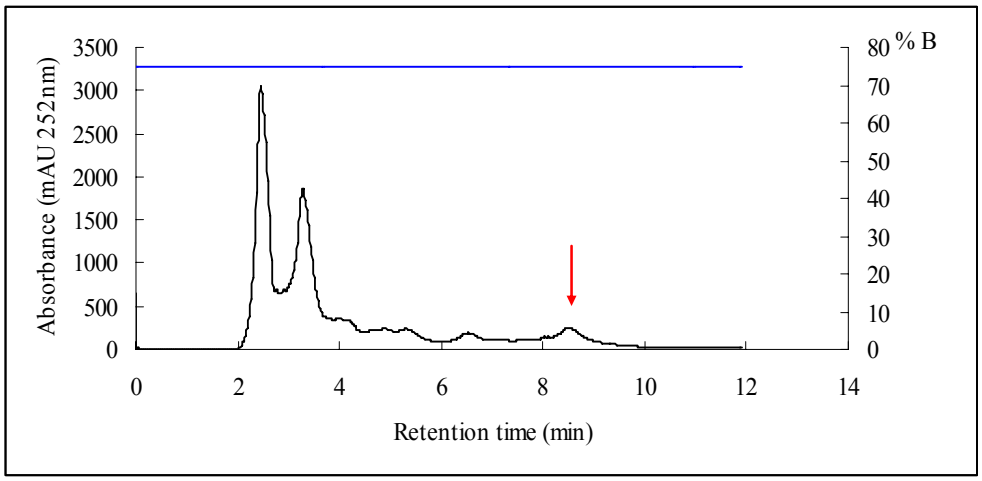


Fig 4-3 HPLC profile of crude nucleotide extracts from *P. putida* WT strain.
Red arrow indicates the peak position of c-di-GMP.

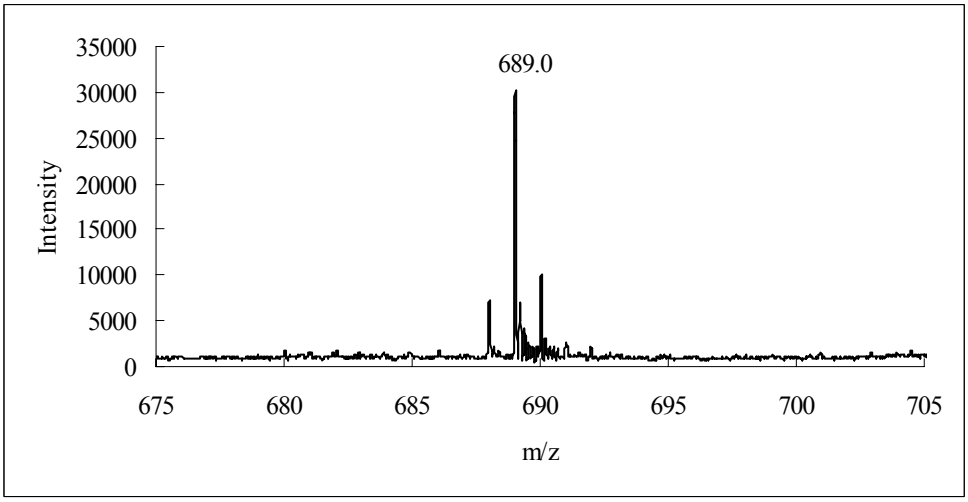


Fig 4-4 MALDI-TOF analysis of c-di-GMP from HPLC fraction of *P. putida* WT strain.
The HPLC fraction, as shown with red arrow in Fig. 4-3, was analyzed by MALDI-TOF in negative mode. Major ion was detected at m/z of 689.0.

4.1.2 Comparison of c-di-GMP levels

To study c-di-GMP levels in *P. putida* WT strain at different growth phases, equal amount of cells were harvested at early log phase, middle log phase and log-to-stationary transition phase. c-di-GMP levels were determined by HPLC analysis from area under the curve of c-di-GMP peak (Fig 4-6). We also investigated whether MorA affects c-di-GMP levels in *P. putida* by comparing intracellular c-di-GMP levels in *P. putida* WT, KO and OE strains at different growth phases (Fig 4-7). Due to the limited availability of c-di-GMP, a c-di-GMP homolog, cGMP was used as a HPLC standard to plot the calibration curve (Fig 4-5). The graph was linear in the range of 2μm to 150μm with a correction coefficient, R^2, >0.999. The relative intracellular concentration of c-di-GMP was calculated according to the HPLC profile and expressed as pmole per milligram (dry weight) of cells.

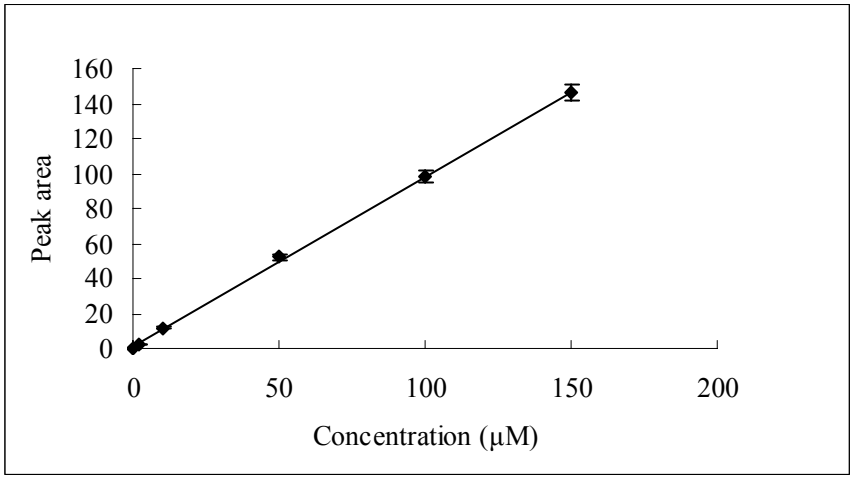


Fig 4-5 Standard curve of cGMP as detected by HPLC analysis.
Peak area was calculated using the software of "UNICORN" (Amersham Biosciences). Experiment was repeated three times.

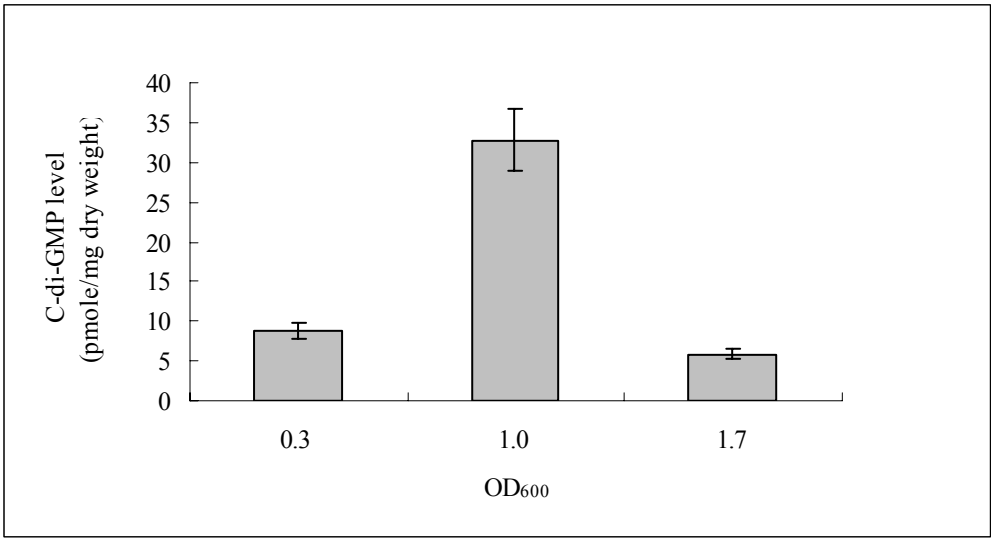


Fig 4-6 Intracellular c-di-GMP levels in *P. putida* WT strain at different growth stages.
Intracellular concentrations of c-di-GMP in *P. putida* WT at early log phase, middle log phase and log-to-stationary transition phase were determined by HPLC analysis and expressed per milligram (dry weight) of cells. Experiment was done in triplicate and error bars represent standard deviation.

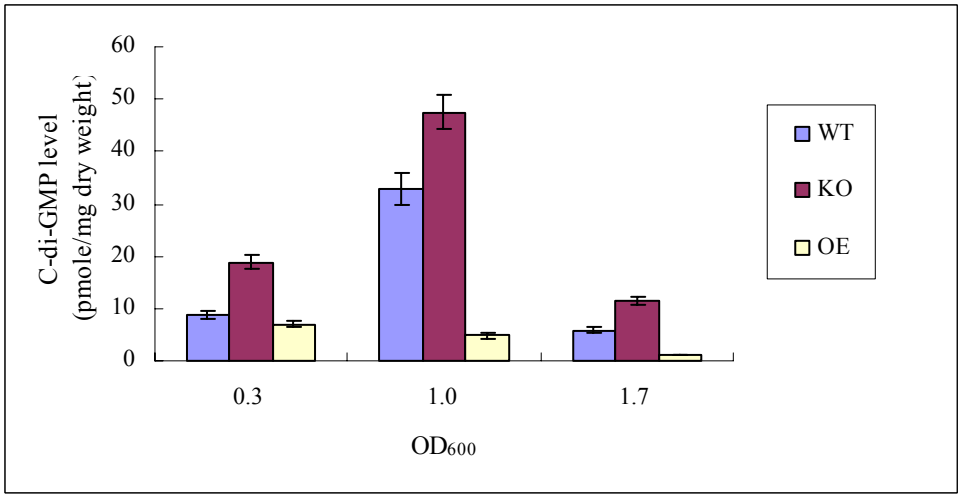


Fig 4-7 Intracellular c-di-GMP levels in *P. putida* WT, KO and OE strains at different growth phases.
Intracellular concentrations of c-di-GMP in *P. putida* WT, KO and OE strains at early log phase, middle log phase and log-to-stationary transition phase were determined by HPLC analysis and expressed per milligram (dry weight) of cells. Experiment was done in triplicate and error bars represent standard deviation.

Relative concentrations of c-di-GMP in *P. putida* were determined in mg of pmole mg^{-1} dry weight cells, which are consistent with the c-di-GMP levels in several bacteria (Christen *et al.*, 2005; Kader *et al.*, 2006; Weinhouse *et al.*, 1997; Simm *et al.*, 2005). Due to the low abundance of c-di-GMP, a large amount of bacterial culture was raised in order to get sufficient starting material for the extraction and detection work. Especially in the case of extracting c-di-GMP from cells at early log phase when cell density was relatively low, the yield c-di-GMP is about 1.7 nmole per liter culture.

The intracellular levels of c-di-GMP in *P. putida* WT strain are growth- dependent. Compared with that of cells at early log phase, the intracellular concentration of c-di-GMP in WT at middle log phase increased by 5-fold. This might be explained that fast growing cells are undergoing vigorous metabolism and the demand for c-di-GMP, which is essential for coordinating several biological pathways, is also higher. When the cells are about to enter the stationary phase, the concentration dropped even below that of cells at early log phase. However, the concentration difference between the early log phase and log-to-stationary transition phase was not significant.

The comparison of c-di-GMP levels of *P. putida* WT, KO and OE strains at different growth phases showed that the level of c-di-GMP increased in KO strain whereas when MorA was overexpressed, the c-di-GMP level was reduced below that of WT. This observation is more significant at middle log phase when 10-fold difference of c-di-GMP level could be seen between KO and OE strains. The variation of c-di-GMP levels in these three strains indicated that MorA affects intracellular c-di-GMP levels, via a direct or indirect way.

4.2 Enzymatic study of MorA

Since MorA contains both GGDEF and EAL domains, which have been implicated in the synthesis and degradation of c-di-GMP, respectively, it is possible that MorA may possess DGC and/or PDE-A activity. Therefore, experiments were designed to test the possible enzymatic activities of MorA. However, only DGC activity assay was performed in this study. PDE-A activity assay cannot be done currently owing to lack of sufficient amount of purified c-di-GMP as a substrate.

4.2.1 DGC extraction and enzymatic assay

To study the DGC activity of MorA, DGC was extracted from *G. xylinus* with the protocol described in "Materials and Methods" to serve as a positive control during the enzymatic assay. The protein content was determined to be 2~3 mg protein per ml extract using Bradford method. The enzymatic activity of DGC was tested by DGC activity assay, as mentioned in Section 3.3.2. Different concentrations of reaction components were used to obtain optimized conditions. Fig 4-8 showed the HPLC profile of the reaction mixture, where the c-di-GMP peak at retention time of 8~9 min was clearly seen. To further confirm the presence of c-di-GMP, the HPLC fraction of the relevant peak was collected, lyophilized, resuspended in minimal amount of water and analyzed by MALDI-TOF mass spectrometry in the negative mode. Fig 4-9 is the mass spectrum of the HPLC fraction, where the m/z value of 689.0 represented $[\text{c-di-GMP-H}]^-$. Hence we could successfully obtain functional DGC from *G. xylinus*.

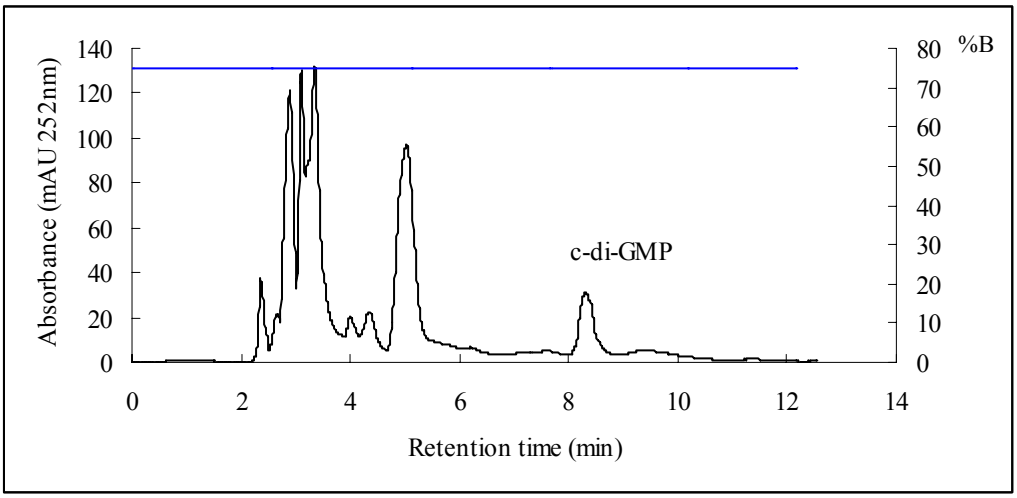


Fig 4-8 HPLC analysis of enzymatic activity of crude DGC extract from *G. xylinus*.
The reaction mixture was analyzed by HPLC using the running buffer and gradient described in Section 3.2.2. The peaks other than c-di-GMP were mainly GTP and its degradation products. After 2-hour incubation, the c-di-GMP concentration in the reaction mixture reached around 4μM.

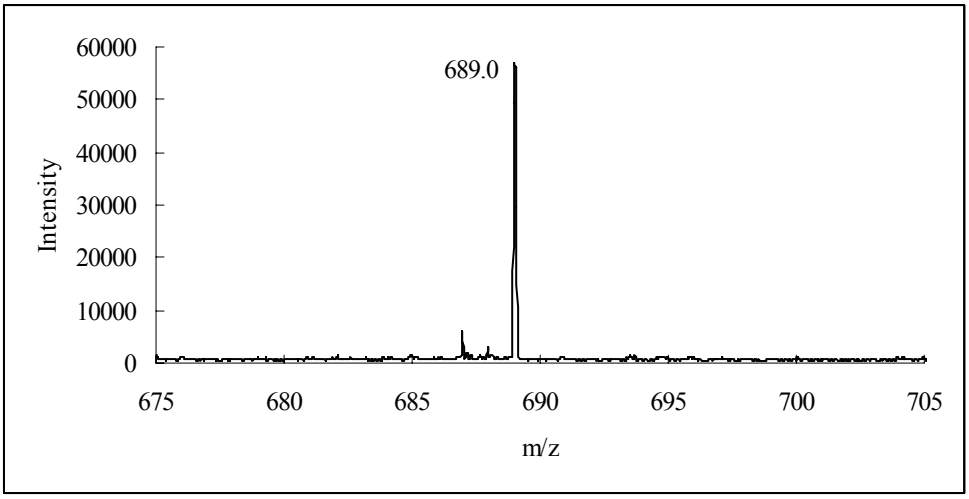


Fig 4-9 MALDI-TOF analysis of c-di-GMP from HPLC fraction of enzymatic mixture.
The HPLC fraction of c-di-GMP, as shown in Fig. 4-7, was analyzed by MALDI-TOF in negative mode. Major ion was detected at m/z of 689.0.

4.2.2 Crude MorA extraction

Previously, membrane fractions containing MorA were extracted from WT using a method adapted from Minghetti *et al.*, 1992 (Choy *et al.*, 2004). In the present study, the crude MorA was extracted from WT using the same protocol of DGC extraction as designed for *G. xylinus* (Ross *et al*., 1986). To test whether this method is efficient, the protein extracts were checked on 8% SDS-PAGE gel (Fig 4-10). The predicted molecular mass of MorA is 145 kDa. On the SDS-PAGE gel, a band with a molecular mass slightly less than 150 kDa was clearly seen. Next, Western blot was performed to confirm the presence of MorA in the crude protein extracts with anti-MorA polyclonal antibodies (Fig 4-11). Results showed that MorA was present in the crude protein extracts. Hence, the extraction method was applicable to MorA.

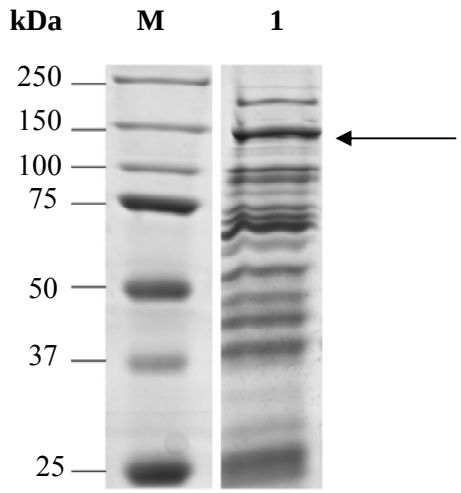


Fig 4-10 SDS-PAGE analysis of crude MorA extracted from wild type *P. putida*. The gel was stained with coomassie blue. Lane M, Bio-Rad Precision Plus Protein standards; Lane 1, crude MorA extract. Arrow indicates the position of MorA band.

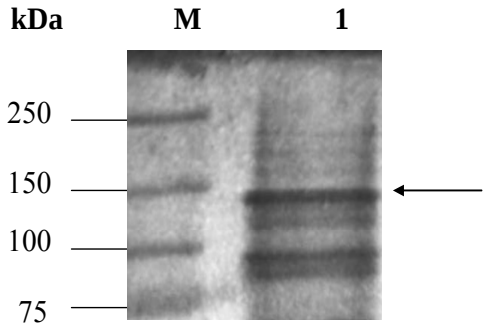


Fig 4-11 Western blot analysis of crude MorA extract from wild type *P. putida*. Membrane was probed with MorA antibody. Lane M, Bio-Rad Precision Plus Protein standards; Lane 1, crude MorA extract.

4.2.3 Extraction and purification of recombinant MorA

In earlier studies, *morA* gene was cloned into GST gene fusion system for expression. The pGEX vector harbouring *morA* gene was introduced into *Escherichia coli* strain BL21. To obtain recombinant MorA, transformed *E. coli* BL21, after induction with IPTG, was harvested and lysed by sonication in lysis buffer. The GST fusion proteins are purified from crude cell lysate under non-denaturing conditions by selective binding to glutathione–Sepharose 4B Beads. The GST-fusion proteins were then cleaved by PreScission Protease and eluted out. Protein purity was assessed on 8% SDS-PAGE gel (Fig 4-12).

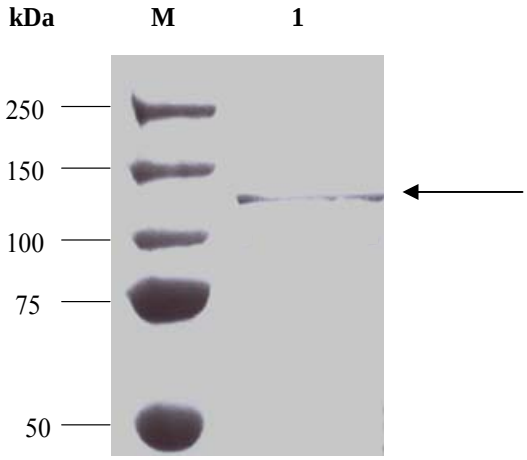


Fig 4-12 SDS-PAGE analysis of recombinant MorA purified from GST gene fusion system.
The gel was stained with coomassie blue. Lane M, Bio-Rad Precision Plus Protein standards; Lane 1, recombinant MorA after the cleavage of GST tag by PreScission Protease. Arrow indicates the position of the band of recombinant MorA.

4.2.4 DGC activity assay of MorA

To investigate whether MorA has DGC activity, the enzymatic assay was carried with the crude MorA extract as well as purified recombinant MorA, following the same conditions as enzymatic assay of DGC from *G. xylinus*. After the reaction was stopped, the reaction mixtures were processed and analyzed by HPLC. However, unlike the positive control of crude DGC (Fig 4-7), the crude MorA extract and purified recombinant MorA had no detectable DGC activity, even after 2-hour incubation (Fig 4-13 A and B).

Taking into account the limit of sensitivity of the HPLC assay, we took the corresponding HPLC fractions of the enzymatic mixture, at which time c-di-GMP was supposed to be eluted out, for mass spectrometric analysis since the mass spectrometer has a much higher sensitivity. However, no c-di-GMP was detected (data not shown). The possibility that no detectable DGC activity for MorA was due to the very low amount of MorA present in the reaction mixture can be ruled out as the rough concentration of MorA was calculated prior to the experiment. The failure of MorA to exhibit DGC activity prompted us to consider further whether the activity of MorA is dependent on the phosphorylation status of the sensory or the catalytic domains. Therefore, in another set of enzymatic assay, MorA was incubated with acetyl phosphate (AcP), a small-molecule phosphate donor at different concentrations. Acp specifically phosphorylates the aspartyl residues of response regulators (Lukat *et al.*, 1992). Nevertheless, no DGC activity was detected in MorA in spite of the addition of AcP (data not shown). As the verification of the phosphorylation of MorA

was not performed, we currently have no clue that whether the undetectable DGC activity was caused by poor efficiency of MorA phosphorylation. However, these results indicated that MorA does not have apparent in vitro DGC activity under the conditions we tested. Further experiments have to be carried out to test whether MorA has PDE-A activity.

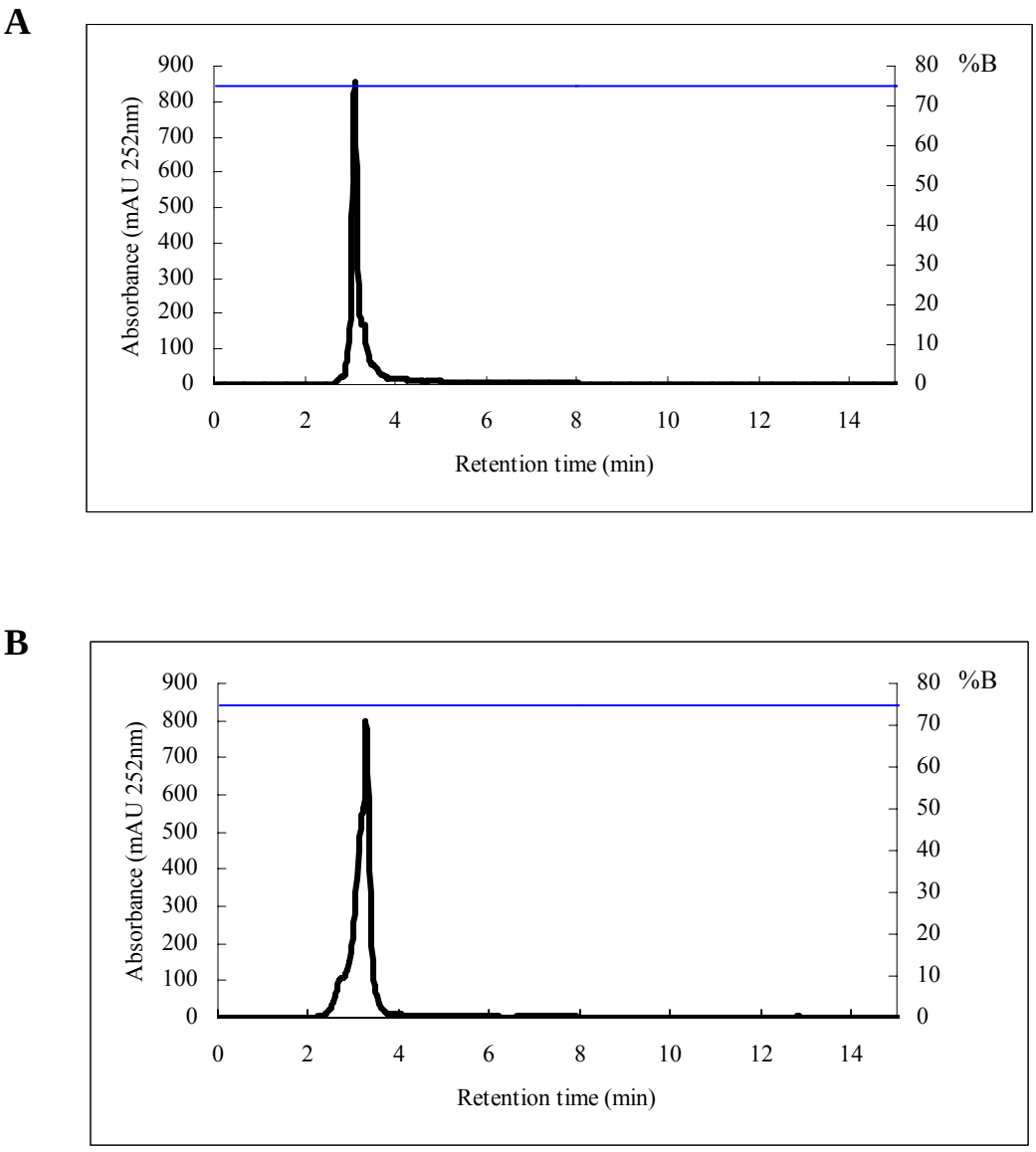


Fig 4-13 HPLC analysis of DGC activity of MorA.
The reaction mixture was incubated up to 2 hours, then stopped and loaded onto HPLC column. Both crude MorA extract (A) and purified recombinant MorA (B) showed no detectable activity. The peak present in (A) and (B) corresponded to that of GTP. Bold black lines were applied to the HPLC profile in order to differentiate it from x-axis, which was shown in fine line.

4.3 Isolation and purification of GTP-binding proteins in *P. putida*

4.3.1 Protein extraction and purification

Involvement of GTP as a substrate for c-di-GMP synthesis prompted us to carry out an identification of GTP-binding proteins in *P. putida* with affinity chromatography. In the first attempt, total proteins from the whole cell lysate were used in GTP-agarose affinity chromatography. However, no bands were detected when checking the eluted proteins on the SDS-PAGE gel. The possible reason could be the limited amount of GTP-binding proteins in the whole cell lysate. Therefore, a method of protein fractionation and enrichment was used next. We then added PEG-4000 for fractional precipitation and purification of proteins due to relatively inert chemical properties of this polymer. The extraction and purification were repeated and the supernatants were combined and loaded onto the affinity column. Since only one specific concentration of PEG-4000 was used in the experiment, we recognize the fact that we could have lost some of the GTP-binding proteins. Hence, these results only provide a partial list of the GTP-binding proteins. Different concentrations of PEG-4000 can be used in future experiment to optimize the protocol and identify more GTP-binding proteins.

4.3.2 Affinity chromatography

Affinity chromatography was carried out as described in Section 3.4.2. Due to the lability of the ligand GTP, all the procedures were carried out at 4^{o}C. The eluted proteins were concentrated using centrifugal concentrators and analyzed by SDS-

PAGE (Fig 4-14). For the convenience of protein sequencing, the gel was Coomassie-stained. Four prominent bands were visualized and given the names of p75, p50, p25 and p20 according to their individual estimated molecular masses. At current stage, we did not perform the verification of the nonspecific binding of these four proteins with GTP-agarose beads, which is one of our future interests.

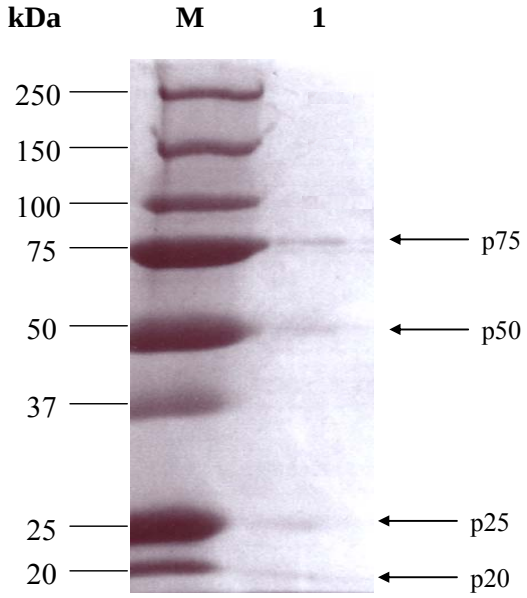


Fig 4-14 SDS-PAGE analysis of putative GTP-binding proteins from *P. putida*.
Lane M, Bio-Rad Precision Plus Protein standards; Lane 1, concentrated putative GTP-binding proteins eluted from affinity chromatography column. Arrows indicate the position of the four bands.

4.3.3 Protein identification by mass spectrometric analysis

The four bands p75, p50, p25, p20 were excised from the gel, washed and their tryptic digestion were done. The digested peptides were analyzed by MALDI-TOF-TOF mass spectrometry. The resulting spectrums were searched against protein sequence database MASCOT and the proteins were identified by peptide mass mapping (Table 4-1; Fig 4-15). Since the complete genome sequence of *P. putida* PNL-MK25 is not available, p75 was identified in *Pseudomonas syringae* pv. tomato str. DC3000 while others were identified in *P. fluorescens* PfO-1. The domain structure of each protein was predicted and the alignment of respective protein with their homologs in *Pseudomonas* species was also performed (Fig 4-16, 4-17, 4-18, 4-19). As they are putative GTP-binding proteins, a comparison of the protein sequence between these four proteins were conducted with the purpose to find any conserved GTP-binding motifs. However, no sequence similarity was found (data not shown). In addition, none of the four proteins possess the classic ATP/GTP-binding site motif A (P-loop), which is a flexible glycine-rich loop that can interact with one of the phosphate groups of the nucleotide (Kjeldgaard *et al.*, 1996). All these results indicate that the four identified proteins may contain GTP-binding motifs that are completely different from that of the P-loop and those motifs may be less characterized or even totally unknown.

Table 4-1 Putative GTP-binding proteins identified by mass spectrometry.
This table shows the accession number, the nominal mass and the name of the proteins identified.

	Locus tag	Nominal mass (Da)	Name
p75	PSPTO_4486	75112	Polyribonucleotide nucleotidyltransferase
p50	Pfl_1616	49999	Dihydrolipoamide dehydrogenase
p25	Pfl_2064	28458	Lysine-arginine-ornithine-binding periplasmic protein
p20	Pfl_4727	20996	Phosphoribosyltransferase

A

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  1 MNPVIKKFQF GQSTVTLETG RIARQASGAV LVTVDDDVSV LVTVVGAKQA
 51 DAGKGFFPLS VHYQEKTYAA GKIPGGFFKR EGRPSEKETL TSRLIDRPIR
101 PLFPEGFMNE VQVVCTVVST SKKIDPDIAA MIGTSAALAI SGIPFDGPVG
151 AARVAFHEST GYLLNPTYEQ LQASSLDMVV AGTSEAVLMV ESEAKELTED
201 QMLGAVLFAH DEFQVVINAI KELAAEAAKP TWDWQPKPEA TALLGAIRAE
251 FGDAISQAYT ITVKADRYAR LGELKDQVVA KLAVEEGSPS AGEVKAAFGE
301 IEYRTVRENI VNGKPRIDGR DTRTVRPLNI EVGVLPKTHG SALFTRGETQ
351 ALVVATLGTA RDAQLLDTLE GEKKDPFMLH YNFPPFSVGE CGRMGGAGRR
401 EIGHGRLARR SVQAMLPGAD VFPYTIRVVS EITESNGSSS MASVCGASLA
451 LMDAGVPMKA PVAGIAMGLV KEGEKFAILT DILGDEDHLG DMDFKVAGTS
501 KGVTALQMDI KIKGITEEIM EIALGQALEA RLNILGQMNQ IIGQSRNELS
551 ANAPTMIAMK IDTDKIRDVI GKGGATIRAI CEETKASIDI EDDGSIKIFG
601 ESKEAAEAAR QRVLGITAEA EIGKIYLGKV ERIVDFGAFV NILPGKDGLV
651 HISMLSDARV EKVTDILKEG EEVEVLVLDV DNRGRIKLSI KDVAAAKASG
701 V
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B

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  1 MSQKFDVVVI GAGPGGYVAA IKAAQLGLST ACIEKYTDAE GKQALGGTCL
 51 NVGCIPSKAL LDSSWKYKEA KESFNVHGIS TGEVKMDVAA MVGRKAGIVK
101 NLTGGVATLF KANGVTSIQG HGKLLAGKKV EVTKPDGSVE VIEAENVILA
151 PGSRPIDIPP APVDQKVIVD STGALEFQSV PKRLGVIGAG VIGLELGSVW
201 SRLGAEVTVL EALDTFLMAA DTAVSKEALK TLTKQGLDIK LGARVTGSKV
251 NGDEVVVNYT DANGEQTITF DKLIVAVGRR PVTTDLLAAD SGVTLDERGF
301 VHVDDHCATT VPGVYAIGDV VRGMMLAHKA SEEGIMVVER IKGHKAQMNY
351 DLIPSVIYTH PEIAWVGKTE QALKAEGVEV NVGTFPFAAS GRAMAANDTG
401 GFVKVIADAK TDRVLGVHVI GPSAAELVQQ GAIGMEFGTS AEDLGMMVFS
451 HPTLSEALHE AALAVNGGAI HIANRKKR
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C

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  1 MKKLVMFGAL ALSMLSLTAV AEDAKPIRIG IEAGYPPFSM KTPDGKLAGF
 51 DVDIGDALCE QMKVKCTWVE QEFDGLIPAL KVKKIDAILS SMTITDDRKK
101 NVDFTIKYYH TPARFVMKAG SGVKDPLTEL KGKKVGVLRA STHDRYATEV
151 LVPAGIELVR YGSQQEANLD MVSGRIDAML ADSVNLSDGF LKTDAGKGFE
201 FVGPTYEDAK YFGGGAGIAV RKGDTELAEK FNTAINEIRA NGKYKQVQDK
251 YFDFDVYGH
```

D

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  1 MSADLEHIRQ IMREADCLYT EAEVEAAIAR VGAQINEQLA DSNPVVFCVM
 51 NGGLIFSGKL LTHLQFPLEA SYLHATRYRN ETSGGDLFWK AKPEVSFIDR
101 DVLIIDDILD EGHTLGAIID FCRHAGARKV HTAVLIDKDH DRKARPDLKA
151 DFVGLPCIDR YIFGYGMDYK GYWRNANGIF AVKGM
```
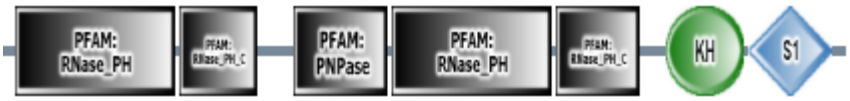

Fig 4-15 Protein sequences of identified GTP-binding proteins.
(A) *P. syringae* pv. tomato str. DC3000 polyribonucleotide nucleotidyltransferase
(B)*P. fluorescens* PfO-1 dihydrolipoamide dehydrogenase
(C) *P. fluorescens* PfO-1 lysine-arginine-ornithine-binding periplasmic protein
(D) *P. fluorescens* PfO-1 phosphoribosyltransferase.
Sequences in red are the peptides identified in mass spectrometry.

A



B

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PSPTO_4486          MNPVIKKFQFGQSTVTLETGRIARQASGAVLVTVDDDVSVLVTVVGAKQADAGKGFFPLS 60
Psyr_4176           MNPVIKKFQFGQSTVTLETGRIARQASGAVLVTVDDDVSVLVTVVGAKQADAGKGFFPLS 60
PSPPH_4185          MNPVIKKFQFGQSTVTLETGRIARQASGAVLVTVDDDVSVLVTVVGAKQADAGKGFFPLS 60
PFL_0848            MNPVIKKFQFGQSTVTLETGRIARQASGAVLVTVDDDVSVLVTVVGAKQADPGKGFFPLS 60
Pfl_0783            MNPVIKKFQFGQSTVTLETGRIARQASGAVLVTVDDDVSVLVTVVGAKQADPGKGFFPLS 60
PSEEN0799           MNPVIKTFQFGQSTVTLETGRIARQATGAVLVTVDNDVTVLVTVVGAKQADPGKGFFPLS 60
PputDRAFT_2361      MNPVIKTFQFGQSTVTLETGRIARQATGAVLVTVDNDVTVLVTVVGAKQADPGKGFFPLS 60
                    ******.******************:********:**:***********.******** 

PSPTO_4486          VHYQEKTYAAGKIPGGFFKREGRPSEKETLTSRLIDRPIRPLFPEGFMNEVQVVCTVVST 120
Psyr_4176           VHYQEKTYAAGKIPGGFFKREGRPSEKETLTSRLIDRPIRPLFPEGFMNEVQVVCTVVST 120
PSPPH_4185          VHYQEKTYAAGKIPGGFFKREGRPSEKETLTSRLIDRPIRPLFPEGFMNEVQVVCTVVST 120
PFL_0848            VHYQEKTYAAGKIPGGFFKREGRPSEKETLTSRLIDRPIRPLFPEGFMNEVQVVCTVVST 120
Pfl_0783            VHYQEKTYAAGKIPGGFFKREGRPSEKETLTSRLIDRPIRPLFPEGFMNEVQVVCTVVST 120
PSEEN0799           VHYQEKTYAAGKIPGGFFKREGRPSEKETLTSRLIDRPIRPLFPEGFMNEVQVVCTVVST 120
PputDRAFT_2361      VHYQEKTYAAGKIPGGFFKREGRPSEKETLTSRLIDRPIRPLFPEGFMNEVQVVCTVVST 120
                    ************************************************************ 

PSPTO_4486          SKKIDPDIAAMIGTSAALAISGIPFDGPVGAARVAFHESTGYLLNPTYEQLQASSLDMVV 180
Psyr_4176           SKKIDPDIAAMIGTSAALAISGIPFDGPVGAARVAFHESTGYLLNPTYEQLQASSLDMVV 180
PSPPH_4185          SKKIDPDIAAMIGTSAALAISGIPFDGPVGAARVAFHESTGYLLNPTYEQLQASSLDMVV 180
PFL_0848            SKKTDPDIAAMIGTSAALAISGIPFDGPIGAARVAFHESTGYLLNPTYEQLAASSLDMVV 180
Pfl_0783            SKKTDPDIAAMIGTSAALAISGIPFDGPIGAARVAFHESTGYLLNPTYEQQAASSLDMVV 180
PSEEN0799           SKKTDPDIAAMIGTSAALAISGIPFEGPIGAARVAFHESTGYLLNPTYEQLAASSLDMVV 180
PputDRAFT_2361      SKKTDPDIAAMIGTSAALAISGIPFEGPIGAARVAFHESTGYLLNPTYEQLAASSLDMVV 180
                    *** ********************:**:**********************  ******** 

PSPTO_4486          AGTSEAVLMVESEAKELTEDQMLGAVLFAHDEFQVVINAIKELAAEAAKPTWDWQ--PKP 238
Psyr_4176           AGTSEAVLMVESEAKELTEDQMLGAVLFAHDEFQVVINAIKELAAEAAKPTWDWQ--PKP 238
PSPPH_4185          AGTSEAVLMVESEAKELTEDQMLGAVLFAHDEFQVVINAIKELAAEAAKPVWDWQ--PKP 238
PFL_0848            AGTEEAVLMVESEAKELTEDQMLGAVLFAHDEFQSVIKAVKELAAEAAKPTWDWDWAAAP 240
Pfl_0783            AGTSDAVLMVESEAKELTEDQMLGAVLFAHDEFQVVINAVKELAAEAAKPTWTWA--PAP 238
PSEEN0799           AGTSDAVLMVESEAQELTEDQMLGAVLFAHDEFQAVIKAVKELAAEAAKPTWDWK--PAD 238
PputDRAFT_2361      AGTSDAVLMVESEAQELTEDQMLGAVLFAHDEFQAVIQAVKELAAEAGKPTWDWK--PAV 238
                    ***.:*********:****************** **:*:*******.**.* *   .    

PSPTO_4486          EATALLGAIRAEFGDAISQAYTITVKADRYARLGELKDQVVAKLAVEEGSPSAGEVKAAF 298
Psyr_4176           EATALLGAIRAEFGDAISQAYTITVKADRYARLGELKDQVVAKLAVEDGSPSASEVKAAF 298
PSPPH_4185          EATALLGAIRAEFGDAISQAYTITVKADRYARLGELKDQVVAKLAVEEGSPSASEVKAAF 298
PFL_0848            EATELLGAIRAEFGEAISQAYTITVKADRYARLGELKDQVVAKLSGEEGQPSASDVKAAF 300
Pfl_0783            EATELLAAIRSEFGEAISQAYTITIKADRYARLGELRDQVVAKLSGEEGQPSAADVKAAF 298
PSEEN0799           KNSALFDAIRAEFGEAVSQGYTITVKADRYARLGELRDQAVAKFSGEEGQPSAGEVKDIF 298
PputDRAFT_2361      ANTELFNAIRAEFGEAVSQGYTITVKADRYARLGELRDQAVAKFSGEEGQPSASEVKDIF 298
                      : *: ***:***:*:**.****:**********:**.***:: *:*.***.:**  *  

PSPTO_4486          GEIEYRTVRENIVNGKPRIDGRDTRTVRPLNIEVGVLPKTHGSALFTRGETQALVVATLG 358
Psyr_4176           GEIEYRTVRENIVNGKPRIDGRDTRTVRPLNIEVGVLPKTHGSALFTRGETQALVVATLG 358
PSPPH_4185          GEIEYRTVRENIVNGKPRIDGRDTRTVRPLNIEVGVLPKTHGSALFTRGETQALVVATLG 358
PFL_0848            GEIEYRTVRENIVNGKPRIDGRDTRTVRPLNIEVGVLPKTHGSALFTRGETQALVVATLG 360
Pfl_0783            GEIEYRTVRENIVNGKPRIDGRDTRTVRPLNIEVGVLPKTHGSALFTRGETQALVVATLG 358
PSEEN0799           GEIEYRTVRENIVNGKPRIDGRDTKTVRPLNIEVGVLPKTHGSALFTRGETQALVVATLG 358
PputDRAFT_2361      GEIEYRTVRENIVNGKPRIDGRDTKTVRPLNIEVGVLPKTHGSALFTRGETQALVVATLG 358
                    ************************:********************************** 
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PSPTO_4486          TARDAQLLDTLEGEKKDPFMLHYNFPPFSVGECGRMGGAGRREIGHGRLARRSVQAMLPG 418
Psyr_4176           TARDAQLLDTLEGEKKDPFMLHYNFPPFSVGECGRMGGAGRREIGHGRLARRSVQAMLPG 418
PSPPH_4185          TARDAQLLDTLEGEKKDPFMLHYNFPPFSVGECGRMGGAGRREIGHGRLARRSVQAMLPG 418
PFL_0848            TARDAQLLDTLEGEKKDPFMLHYNFPPFSVGECGRMGGAGRREIGHGRLARRSVQAMLPA 420
Pfl_0783            TARDAQLLDTLEGEKKDPFMLHYNFPPFSVGECGRMGGAGRREIGHGRLARRSVSAMLPA 418
PSEEN0799           TARDAQLLDTLEGEKKDPFMLHYNFPPFSVGECGRMGGAGRREIGHGRLARRSVQAMLPA 418
PputDRAFT_2361      TARDAQLLDTLEGEKKDPFMLHYNFPPFSVGECGRMGGAGRREIGHGRLARRSVQAMLPA 418
                    **************************************************.****.

PSPTO_4486          ADVFPYTIRVVSEITESNGSSSMASVCGASLALMDAGVPMKAPVAGIAMGLVKEGEKFAI 478
Psyr_4176           ADVFPYTIRVVSEITESNGSSSMASVCGASLALMDAGVPMKAPVAGIAMGLVKEGEKFAI 478
PSPPH_4185          ADVFPYTIRVVSEITESNGSSSMASVCGASLALMDAGVPMKAPVAGIAMGLVKEGEKFAI 478
PFL_0848            ADVFPYTIRVVSEITESNGSSSMASVCGASLALMDAGVPMKAPVAGIAMGLVKEGEKFAV 480
Pfl_0783            ADVFPYTIRVVSEITESNGSSSMASVCGASLALMDAGVPMKAPVAGIAMGLVKEGEKFAV 478
PSEEN0799           ADVFPYTIRVVSEITESNGSSSMASVCGASLALMDAGVPMKAPVAGIAMGLVKEGEKFAV 478
PputDRAFT_2361      ADVFPYTIRVVSEITESNGSSSMASVCGASLALMDAGVPMKAPVAGIAMGLVKEGDKFAV 478
                    **************************************************:***:

PSPTO_4486          LTDILGDEDHLGDMDFKVAGTSKGVTALQMDIKIKGITEEIMEIALGQALEARLNILGQM 538
Psyr_4176           LTDILGDEDHLGDMDFKVAGTSKGVTALQMDIKIKGITEEIMEIALGQALEARLNILGQM 538
PSPPH_4185          LTDILGDEDHLGDMDFKVAGTSKGVTALQMDIKIKGITEEIMEIALGQALEARLNILGQM 538
PFL_0848            LTDILGDEDHLGDMDFKVAGTAKGVTALQMDIKIKGITEEIMEIALGQALEARLNILGQM 540
Pfl_0783            LTDILGDEDHLGDMDFKVAGTAKGVTALQMDIKIKGITEEIMEIALGQALEARLNILGQM 538
PSEEN0799           LTDILGDEDHLGDMDFKVAGTAKGVTALQMDIKINGITEEIMEIALGQALEARLNILGQM 538
PputDRAFT_2361      LTDILGDEDHLGDMDFKVAGTAKGVTALQMDIKINGITEEIMEIALGQALEARLNILGQM 538
                    ********************:************:************************

PSPTO_4486          NQIIGQSRNELSANAPTMIAMKIDTDKIRDVIGKGGATIRAICEETKASIDIEDDGSIKI 598
Psyr_4176           NQIIGQSRNELSANAPTMIAMKIDTDKIRDVIGKGGATIRAICEETKASIDIEDDGSIKI 598
PSPPH_4185          NQIIGQSRNELSANAPTMIAMKIDTDKIRDVIGKGGATIRAICEETKASIDIEDDGSIKI 598
PFL_0848            NQIIGQSRTELSANAPTMIAMKIDTDKIRDVIGKGGATIRAICEETKASIDIEDDGSIKI 600
Pfl_0783            NQIIGQSRTELSANAPTMIAMKIDTDKIRDVIGKGGATIRAICEETKASIDIEDDGSIKI 598
PSEEN0799           NQIIGESRTELSANAPTMIAMKIDTDKIRDVIGKGGATIRAICEETKASIDIEDDGSIKI 598
PputDRAFT_2361      NQVIGQSRTELSANAPTMIAMKIDTDKIRDVIGKGGATIRAICEETKASIDIEDDGSIKI 598
                    **:**:**.**************************************************

PSPTO_4486          FGESKEAAEAARQRVLGITAEAEIGKIYLGKVERIVDFGAFVNILPGKDGLVHISMLSDA 658
Psyr_4176           FGESKEAAEAARQRVLGITAEAEIGKIYIGKVERIVDFGAFVNILPGKDGLVHISMLSDA 658
PSPPH_4185          FGESKEAAEAARQRVLGITAEAEIGKIYVGKVERIVDFGAFVNILPGKDGLVHISMLSDA 658
PFL_0848            FGETKEAAEAARQRVLGITAEAEIGKIYVGKVERIVDFGAFVNILPGKDGLVHISMLSDA 660
Pfl_0783            FGETKEAAEAARQRVLGITAEAEIGKIYVGKVERIVDFGAFVNILPGKDGLVHISMLSDA 658
PSEEN0799           FGETKEAADAAKQRILGITAEAEIGKIYVGKVERIVDFGAFVNILPGKDGLVHISMLSDA 658
PputDRAFT_2361      FGETKEAADAAKQRILGITAEAEIGKIYVGKVERIVDFGAFVNILPGKDGLVHISMLSDA 658
                    ***:****:**:**:*************:********************************

PSPTO_4486          RVEKVTDILKEGEEVEVLVLDVDNRGRIKLSIKDVAAAKASGV 701
Psyr_4176           RVEKVTDILKEGQEVEVLVLDVDNRGRIKLSIKDVAAAKASGV 701
PSPPH_4185          RVEKVTDILKEGQEVEVLVLDVDNRGRIKLSIKDVAAAKASGV 701
PFL_0848            RVEKVTDILKEGQEVEVLVLDVDNRGRIKLSIKDVAAAKASGV 703
Pfl_0783            RVEKVTDILKEGQEVEVLVLDVDNRGRIKLSIKDVAAAKASGV 701
PSEEN0799           RVEKVTDVLKEGQEVEVLVLDVDNRGRIKLSIKDVAAAKASGV 701
PputDRAFT_2361      RVEKVTDILKEGQEVEVLVLDVDNRGRIKLSIKDVAAAKASGV 701
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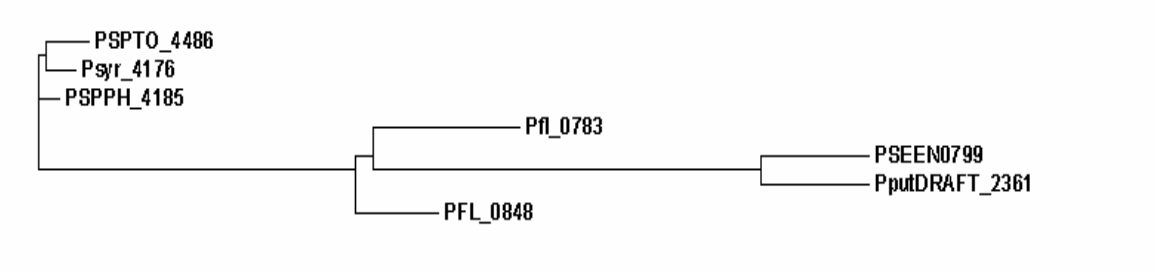
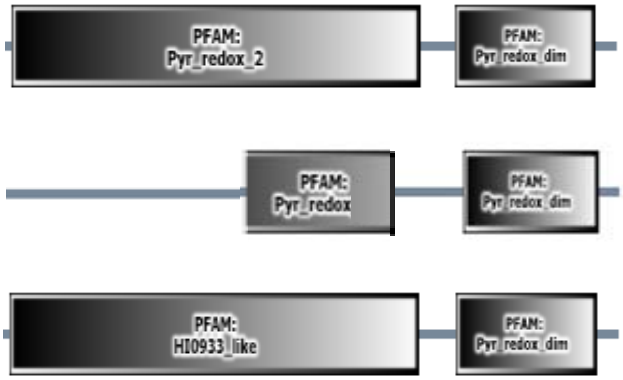


Fig 4-16 Domain structure and alignment of polyribonucleotide nucleotidyltransferase in *Pseudomonas* species.
(A) Domain architecture of polyribonucleotide nucleotidyltransferase of *P. syringae* pv. tomato str. DC3000. Domains are predicted using Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de).

(B) Alignment of *P. syringae* pv. tomato str. DC3000 polyribonucleotide nucleotidyltransferase (PSPTO_4486), *P. syringae* pv. syringae B728a Psyr_4176, *P. syringae* pv.phaseolicola 1448A PSPPH_4185, *P. fluorescens* PfO-1 Pfl_0783, *P. fluorescens* Pf-5 PFL_0848, *Pseudomonas entomophila* L48 PSEEN0799 and *P. putida* F1 PputDRAFT_2361 was performed using ClustalW (http:// www.ebi.ac.uk/clustalw).

(C) Phylogram tree of polyribonucleotide nucleotidyltransferase and its homologues was generated by ClustalW (http://www.ebi.ac.uk/clustalw).

A



B

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Pfl_1616            MSQKFDVVVIGAGPGGYVAAIKAAQLGLSTACIEKYTDAEGKQALGGTCLNVGCIPSKAL 60
PFL_1720            MTQKFDVVVIGAGPGGYVAAIKAAQLGFTTACIEKYTDKEGKLALGGTCLNVGCIPSKAL 60
PP_4187             MTQKFDVVVIGAGPGGYVAAIKAAQLGLKTACIEKYTDAEGKLALGGTCLNVGCIPSKAL 60
PputDRAFT_3136      MTQKFDVVVIGAGPGGYVAAIKAAQLGLKTACIEKYTDAEGKLALGGTCLNVGCIPSKAL 60
PSEEN3638           MTQKFDVVVIGAGPGGYVAAIKAAQLGFSTACIEKYTDAEGKLALGGTCLNVGCIPSKAL 60
PSPPH_1982          MSQKFDVVVIGAGPGGYVAAIKAAQLGLKTACIEKYQDKEGKLALGGTCLNVGCIPSKAL 60
PSPTO_2201          MSQKFDVVVIGAGPGGYVAAIKAAQLGLKTACIEKYQDKEGKLALGGTCLNVGCIPSKAL 60
                    *:*************************:.******* * *** *****************

Pfl_1616            LDSSWKYKEAKESFNVHGISTGEVKMDVAAMVGRKAGIVKNLTGGVATLFKANGVTSIQG 120
PFL_1720            LDSSWKFHEAQDGFAIHGINHAGVTMDVPAMVGRKANIVKGLTSGVATLFKANGVTSIQG 120
PP_4187             LDSSWKYKEAKESFNVHGISTGEVKMDVAAMVGRKAGIVKNLTGGVATLFKANGVTSIQG 120
PputDRAFT_3136      LDSSWKYKEAKESFNVHGISTGEVKMDVAAMVGRKAGIVKNLTGGVATLFKANGVTSIQG 120
PSEEN3638           LDSSWKYKEAKESFNVHGISTGEVKMDVAAMVGRKAGIVKNLTGGVATLFKANGVTSIQG 120
PSPPH_1982          LDSSWKFYEAKNGFSVHGISTSEVNIDVPAMIGRKSTIVKGLTGGVASLFKANGVTTLQG 120
PSPTO_2201          LDSSWKFYEAKNGFAVHGISTSEVAIDVPAMIGRKSTIVKGLTGGVASLFKANGVTTLQG 120
                    ******: **::.* :***. . * :**.**:***: ***.**.***:*********::**

Pfl_1616            HGKLLAGKKVEVTKPDGSVEVIEAENVILAPGSRPIDIPPAPVDQKVIVDSTGALEFQSV 180
PFL_1720            HGKLLAGKKVEVTKPDGSVEVIEAENVILAPGSRPIDIPPAPVDQNVIVDSTGALEFQSV 180
PP_4187             HGKLLAGKKVEVTKADGTTEVIEAENVILASGSRPIDIPPAPVDQNVIVDSTGALEFQAV 180
PputDRAFT_3136      HGKLLAGKKVEVTKADGTTEIIETENVILASGSRPIDIPPAPVDQNVIVDSTGALEFQAV 180
PSEEN3638           HGKLLAGKKVEVTKADGTTEIIEAENVILASGSRPIDIPPAPVDQNVIVDSTGALEFQSV 180
PSPPH_1982          HGKLLAGKKVELTAADGTVEIIEADHVILASGSRPIDIPPAPVDQKVIVDSTGALEFQQV 180
PSPTO_2201          HGKLLAGKKVELTAADGTVEIIEADHVILASGSRPIDIPPAPVDQKVIVDSTGALEFQQV 180
                    ***********:* .**:.*:**:::****.**************:************ *

Pfl_1616            PKRLGVIGAGVIGLELGSVWSRLGAEVTVLEALDTFLMAADTAVSKEALKTLTKQGLDIK 240
PFL_1720            PKRLGVIGAGVIGLELGSVWSRLGAQVTVLEALDTFLMAADTAVSKEALKTLTKQGLDIK 240
PP_4187             PKRLGVIGAGVIGLELGSVWARLGAEVTVLEALDTFLMAADTAVSKEAQKTLTKQGLDIK 240
PputDRAFT_3136      PKRLGVIGAGVIGLELGSVWARLGAEVTVLEALDTFLMAADTAVSKEAQKTLTKQGLDIK 240
PSEEN3638           PKRLGVIGAGVIGLELGSVWARLGAEVTVLEALDTFLMAADTAVSKEAQKTLTKQGLDIK 240
PSPPH_1982          PQRLGVIGAGVIGLELGSVWARLGAQVTVLEALDKFIPAADEAVSKEALKTFTKQGLDIK 240
PSPTO_2201          PQRLGVIGAGVIGLELGSVWARLGAQVTVLEALDKFIPAADEAVSKEALKTFTKQGLDIK 240
                    *:*******************:****:********.*: *** ****** **:********

Pfl_1616            LGARVTGSKVNGDEVVVNYTDANGEQTITFDKLIVAVGRRPVTTDLLAADSGVTLDERGF 300
PFL_1720            LGARVTGSKVNGEEVVVTYTDANGEQTITFDKLIVAVGRRPVTTDLLAADCGVTLDERGF 300
PP_4187             LGARVTGSKVNGNEVEVTYTNAEGEQKITFDKLIVAVGRRPVTTDLLASDSGVTIDERGY 300
PputDRAFT_3136      LGARVTGSKVNGNEVEVTYTNAEGEQKITFDKLIVAVGRRPVTTDLLASDSGVTIDERGY 300
PSEEN3638           LGARVTGSKVNGNEVEVTYTNAEGEQKITFDKLIVAVGRRPVTTDLLAADSGVTIDERGY 300
PSPPH_1982          LGARVTGSKVEGEEVVVSYTDAAGEQSITFDRLIVAVGRRPVTTDLLASDSGVDLDERGF 300
PSPTO_2201          LGARVTGSKVNGEEVVVSYTDAAGEQSITFDRLIVAVGRRPVTTDLLASDSGVDLDERGF 300
                    **********:*:** *.**:* ***.****:****************:*.** :****:
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Pfl_1616            VHVDDHCATTVPGVYAIGDVVRGMMLAHKASEEGIMVVERIKGHKAQMNYDLIPSVIYTH 360
PFL_1720            VHVDDHCATTVPGVYAIGDVVRGMMLAHKASEEGIMVAERIKGHKAQMNYDLIPSVIYTH 360
PP_4187             IFVDDYCATSVPGVYAIGDVVRGMMLAHKASEEGIMVVERIKGHKAQMNYDLIPSVIYTH 360
PputDRAFT_3136      IFVDDYCATSVPGVYAIGDVVRGMMLAHKASEEGIMVVERIKGHKAQMNYDLIPSVIYTH 360
PSEEN3638           IFVDDHCATSVPGVYAIGDVVRGMMLAHKASEEGIMVVERIKGHKAQMNYDLIPSVIYTH 360
PSPPH_1982          IYVDDYCTTSVPGVYAIGDVVRGLMLAHKASEEGIMVVERIKGHKAQMNYNLIPSVIYTH 360
PSPTO_2201          IYVDDYCTTSVPGVYAIGDVVRGLMLAHKASEEGIMVVERIKGHKAQMNYNLIPSVIYTH 360
                    :.***:*:*:************:************.************:*********

Pfl_1616            PEIAWVGKTEQALKAEGVEVNVGTFPFAASGRAMAANDTGGFVKVIADAKTDRVLGVHVI 420
PFL_1720            PEIAWVGKTEQALKAEGVEVNVGTFPFAASGRAMAANDTGGFVKVIADAKTDRVLGVHVI 420
PP_4187             PEIAWVGKTEQALKAEGVEVNVGTFPFAASGRAMAANDTGGFVKVIADAKTDRVLGVHVI 420
PputDRAFT_3136      PEIAWVGKTEQALKAEGVEVNVGTFPFAASGRAMAANDTGGFVKVIADAKTDRVLGVHVI 420
PSEEN3638           PEIAWVGKTEQALKAEGVEVNVGTFPFAASGRAMAANDTGGFVKVIADAKTDRVLGVHVI 420
PSPPH_1982          PEIAWVGKTEQTLKAEGVEVNVGTFPFAASGRAMAANDTGGFVKIIADAKTDRVLGVHVI 420
PSPTO_2201          PEIAWVGKTEQTLKAEGVEVNVGTFPFAASGRAMAANDTGGFVKIIADAKTDRVLGVHVI 420
                    **********:**********************************:***************

Pfl_1616            GPSAAELVQQGAIGMEFGTSAEDLGMMVFSHPTLSEALHEAALAVNGGAIHIANRKKR 478
PFL_1720            GPSAAELVQQGAIGMEFGTSAEDLGMMVFSHPTLSEALHEAALAVNGTAIHIANRKKR 478
PP_4187             GPSAAELVQQGAIAMEFGTSAEDLGMMVFSHPTLSEALHEAALAVNGGAIHVANRKKR 478
PputDRAFT_3136      GPSAAELVQQGAIAMEFGTSAEDLGMMVFSHPTLSEALHEAALAVNGGAIHVANRKKR 478
PSEEN3638           GPSAAELVQQGAIAMEFGTSAEDLGMMVFSHPTLSEALHEAALAVNGGAIHVANRKKR 478
PSPPH_1982          GPSAAELVQQGAIAMEFGSSAEDIGMMVFSHPTLSEALHEAALAVNGGAIHIQNRKKR 478
PSPTO_2201          GPSAAELVQQGAIAMEFGSSAEDIGMMVFSHPTLSEALHEAALAVNGGAIHIQNRKKR 478
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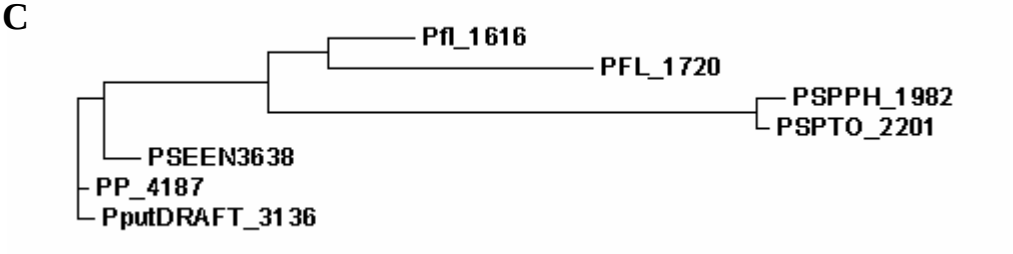

Fig 4-17 Domain structure and alignment of dihydrolipoamide dehydrogenase in *Pseudomonas* species.

(A) Domain architecture of dihydrolipoamide dehydrogenase of *P. fluorescens* PfO-1. Due to overlapping domains, there are 3 representations of the protein**.** Domains are predicted using Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de).

(B) Alignment of *P. fluorescens* PfO-1 dihydrolipoamide dehydrogenase (Pfl_1616), *P. entomophila* L48 PSEEN3638, *P. fluorescens* Pf-5 PFL_1720, *P. putida* KT2440 PP_4187, *P. putida* F1 PputDRAFT_3136, *P. syringae* pv.phaseolicola 1448A PSPPH_1982, *P. syringae* pv. tomato str. DC3000 PSPTO_2201 was performed using ClustalW (http:// www.ebi.ac.uk/clustalw).

(C) Phylogram tree of dihydrolipoamide dehydrogenase and its homologues was generated by ClustalW (http://www.ebi.ac.uk/clustalw).

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B

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PFL_4521          MKKLVLLGALALSVLSLPTFADE-KPLKIGIEAAYPPFASKAPDGSIVGFDYDIGNALCE 59
Pfl_4292          MKKLVLLGALALSVLSLPTFADE-KPLKIGIEAAYPPFASKAPDGSIVGFDYDIGNALCE 59
PSEEN3887         -------------MFSLVSHADE-KPLKIGIEAAYPPFAFKQPDGSIAGFDYDIGNALCE 46
Pfl_2064          MKKLVMFGALALSMLSLTAVAEDAKPIRIGIEAGYPPFSMKTPDGKLAGFDVDIGDALCE 60
PFL_2252          MNKFALFGALALSLFSFTASADEAKPIRIGIEAGYPPFSMKTPDGKLTGFDVDLGNALCE 60
PA0888            MKKLALLGALALSVLSLPTFAAD-KPVRIGIEAAYPPFSLKTPDGQLAGFDVDIGNALCE 59
PaerP_01000159    MKKLALLGALALSVLSLPTFAAD-KPVRIGIEAAYPPFSLKTPDGQLAGFDVDIGNALCE 59
                              ::*: : * : **::*****.****: * ***.:.*** *:*:****

PFL_4521          EMKVKCVWVEQEFDGLIPALKVRKIDAILSSMSITDDRKKSVDFTNKYYNTPARLVMKAG 119
Pfl_4292          EMKVKCQWVEQEFDGLIPALKVRKIDAILSSMSITDDRKKSVDFTNKYYNTPARLVMKEG 119
PSEEN3887         EMKAKCTWVEQEFDGLIPALKVRKIDAILSSMSITDDRKKSVDFSKRYYLTPARLVMKEG 106
Pfl_2064          QMKVKCTWVEQEFDGLIPALKVKKIDAILSSMTITDDRKKNVDFTIKYYHTPARFVMKAG 120
PFL_2252          QMQAKCTWVEQEFDGLIPALKVKKIDAILSSMTITDDRKKNVDFTIKYYHTPARFVMKAG 120
PA0888            EMKVQCKWVEQEFDGLIPALKVRKIDAILSSMTITDERKRSVDFTNKYYNTPARFVMKEG 119
PaerP_01000159    EMKVQCKWVEQEFDGLIPALKVRKIDAILSSMTITDERKRSVDFTNKYYNTPARFVMKEG 119
                  :*:.:* ***************:*********:***:**:.***: :** ****:*** *

PFL_4521          TQISDGLAELKGKNIGVQRGSIHERFAREVLAPLGAQIKPYGSQNEIYLDVAAGRLDGTV 179
Pfl_4292          TQVSEGLAELKGKNIGVQRGSIHERFAREVLAPLGAEIKPYGSQNEIYLDVAAGRLDGTV 179
PSEEN3887         TTVSESLDELKGKKIGVQRGSIHDRFAKEVLAPKGATVVPYGTQNEIYLDVAAGRLDGTV 166
Pfl_2064          SGVKDPLTELKGKKVGVLRASTHDRYATEVLVPAGIELVRYGSQQEANLDMVSGRIDAML 180
PFL_2252          TSIKDPLTELKGKKVGVLRASTHDRFATEVLVPAGIDLVRYGSQQEANLDMVSGRVDALL 180
PA0888            ASLNDPKADLKGKKAGVLRGSTADRYASAELTPAGVEVVRYNSQQEANMDLVAGRLDAVV 179
PaerP_01000159    ASLNDPKADLKGRKAGVLRGSTADRYASGELTPAGVEVVRYNSQQEANMDLVAGRLDAVV 179
                  : :.:   :***:: ** *.*  :*:*   *.* *  :  *.:*:*  :*:.:**:*. :

PFL_4521          ADATLLNDGFLKTDAGKGFAFVGPAFTDVKYFGDGVGIAVRKGDKADLDKINAAIAAIRE 239
Pfl_4292          ADATLLNDGFLKTDAGKGFAFVGPAFTDVKYFGDGVGIAVRKGD-ALKDKINTAIAAIRE 238
PSEEN3887         ADATLLEDGFLKTDAGKGFAFVGPSFTDVKYFGDGVGIAVRKGDKENADRINAAIDAIRA 226
Pfl_2064          ADSVNLSDGFLKTDAGKGFEFVGPTYEDAKYFGGGAGIAVRKGDTELAEKFNTAINEIRA 240
PFL_2252          ADSVNLDDGFLKTDAGKGFAFVGPEYNDPKYFGGGAGIAVRKGDQELAGKFNKAITEIRA 240
PA0888            ADSVNLEDGFLKTDAGKGYAFVGPQLTDAKYFGEGVGIAVRKGDSELAGKFNAAIDALRA 239
PaerP_01000159    ADSVNLEDGFLKTDAGKGYAFVGPQLNDVKYFGEGVGIAVRKGDSELAGKFNAAIDALRA 239
                  **:. *.***********: ****   * **** *.********     ::* **  :*

PFL_4521          NGKYKQIQDKYFDFDIYGK--- 258
Pfl_4292          NGKYKAIQDKYFDFDIYGK--- 257
PSEEN3887         NGKYKQIEAKYFNFDIYGPDSK 248
Pfl_2064          NGKYKQVQDKYFDFDVYGH--- 259
PFL_2252          NGKYKQVQDKYFDFDVYGE--- 259
PA0888            NGKYKQIQDKYFSFDVYGSN-- 259
PaerP_01000159    NGKYKQIQDKYFSFDVYGAN-- 259
                  ***** :: ***.**:**
```

C

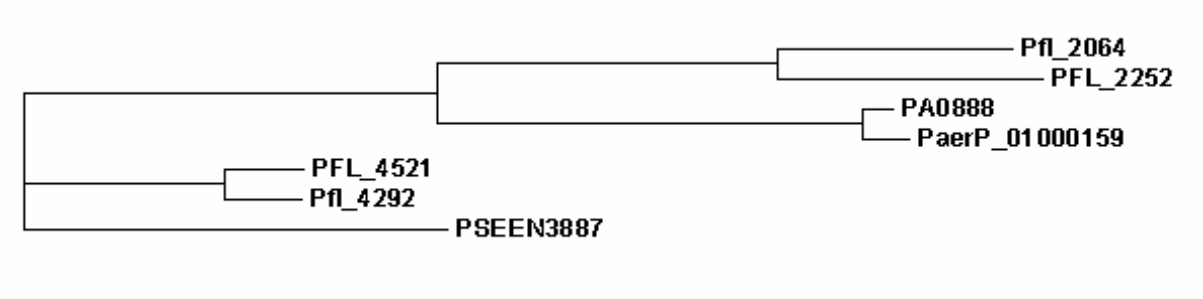

Fig 4-18 Domain structure and alignment of lysine-arginine-ornithine-binding periplasmic protein in *Pseudomonas* species.
(A) Domain architecture of lysine-arginine-ornithine-binding periplasmic protein of *P. fluorescens* PfO-1 (Pfl_2604). Red bar represents signal peptide. Domains are predicted using Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de).

(B) Alignment of *P. fluorescens* PfO-1 lysine-arginine-ornithine-binding periplasmic protein (Pfl_2064), *P. fluorescens* Pf-5 PFL_2252, *P aeruginosa* PAO1 PA0888, *P. aeruginosa* PA7 PaerP_01000159, *P. fluorescens* Pf-5 PFL_4521, *P. entomophila* L48 PSEEN3638, *P. fluorescens* Pf-5 PFL_1720, *P. fluorescens* PfO-1 Pfl_4292, *P.* entomophila L48 PSEEN3887 was performed using ClustalW (http:// www.ebi.ac.uk/clustalw).

(C) Phylogram tree of lysine-arginine-ornithine-binding periplasmic protein and its homologues was generated by ClustalW (http://www.ebi.ac.uk/clustalw).

A

B

```
PSPPH_1019      MSADLEHIRQIMREADCLYTEAEVDAAIARVGAQINAELAERNPVVFCVMNGGLIFSGKL 60
Psyr_0970       MSADLEHIRQVMREADCLYTEAEVDAAIARVGAQINAELAERNPVVFCVMNGGLIFSGKL 60
PSPTO_1131      MSADIEHIRQIMREADCLYTEAEVDAAIARVGAQINAELADRNPVVFCVMNGGLIFSGKL 60
PFL_5138        MSADLEHIRQIMREADCLYTEAEVEAAIARVGAQITEELAERNPVVFCVMNGGLIFAGKL 60
Pfl_4727        MSADLEHIRQIMREADCLYTEAEVEAAIARVGAQINEQLADSNPVVFCVMNGGLIFSGKL 60
PP_0747         MSADLEHIRQVMHEADCLYTEAEVEAAIAKVGEQICKDLHDKNPVVFCVMNGGLIFSGKL 60
PSEEN0889       MSADLEHIRQVMREADCLYNEAEVEAAIAEVGKQICQDLHDKNPVVFCVMNGGLIFSGKL 60
                ****:*****:*:******.****:****.** **  :* : **************:***

PSPPH_1019      LTHLNFPLEASYLHATRYRNETTGGDLFWKAKPEVSFIDRDVLIIDDILDEGHTLGAIID 120
Psyr_0970       LTHLNFPLEASYLHATRYRNETTGGDLFWKAKPEVSFMDRDVLIIDDILDEGHTLGAIID 120
PSPTO_1131      LTHLNFPLEASYLHATRYRNETTGGDLFWKAKPEVSFIDRDVLIIDDILDEGHTLGAIID 120
PFL_5138        LTHLRFPLEASYLHATRYRNETSGGELFWKSKPEVSFIDRDVLIIDDILDEGHTLGAIID 120
Pfl_4727        LTHLQFPLEASYLHATRYRNETSGGDLFWKAKPEVSFIDRDVLIIDDILDEGHTLGAIID 120
PP_0747         LTHLQFPLEASYLHATRYRNQTSGGELFWKAKPEVSFIDRDVLIVDDILDEGHTLSAIIE 120
PSEEN0889       LTHLQFPLEASYLHATRYRNTTSGGELFWKAKPEVSFIDRDVLIVDDILDEGHTLSAIIE 120
                ****.*************** *:**:****:******:******:**********.***:

PSPPH_1019      FCKHAGARAVHTAVLIDKDHDRKARPDLKADYVGLPCIDRYIFGFGMDYKGYWRNAAGIY 180
Psyr_0970       FCKHAGARAVHTAVLIDKDHDRKARPDLKADYVGLPCIDRYIFGFGMDYKGYWRNAAGIY 180
PSPTO_1131      FCKHAGARAVHTAVLIDKDHDRKARPDLKADYVGLPCIDRYIFGFGMDYKGYWRNAAGIY 180
PFL_5138        FCKHAGARAVHTAVLIDKDHDRKARPDLKADYMGLPCVDRYVFGYGMDYKGYWRNAAGIY 180
Pfl_4727        FCRHAGARKVHTAVLIDKDHDRKARPDLKADFVGLPCIDRYIFGYGMDYKGYWRNANGIF 180
PP_0747         FCKHAGARSVYTAVLIDKDHDRKASPDLKANYVGLPCVDRYIFGYGMDYKGYWRNANGIF 180
PSEEN0889       FCKHAGARAVHTAVLIDKDHDRKASPDLKATYTGLPCVDRYIFGYGMDYKGYWRNANGIF 180
                **:***** *:************* ***** : ****:***:**:*********** **:

PSPPH_1019      AVKGM 185
Psyr_0970       AVKGM 185
PSPTO_1131      AVKGM 185
PFL_5138        AVKGM 185
Pfl_4727        AVKGM 185
PP_0747         AVKGL 185
PSEEN0889       AVKGL 185
                ****:
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C

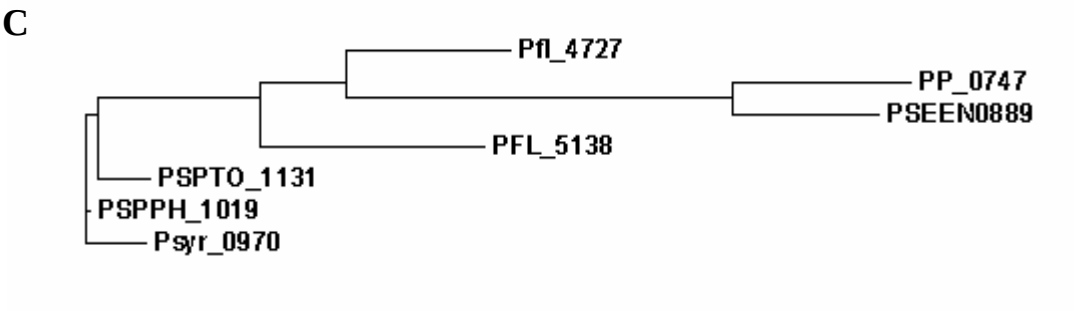

Fig 4-19 Domain structure and alignment of phosphoribosyltransferase in *Pseudomonas* species.
(A) Domain architecture of phosphoribosyltransferase of *P. fluorescens* PfO-1 (Pfl_4727). Domains are predicted using Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de).

(B) Alignment of *P. fluorescens* PfO-1 phosphoribosyltransferase (Pfl_4727), *P. syringae* pv. tomato str. DC3000 PSPTO_1131, *P. syringae* pv. phaseolicola 1448A PSPPH_1019, *P. syringae* pv. syringae B728a Psyr_0970, *P. putida* KT2440 PP_0747, *P. fluorescens* Pf-5 PFL_5138, *P. entomophila* L48 PSEEN0889 was performed using ClustalW (http:// www.ebi.ac.uk/clustalw).

(C) Phylogram tree of phosphoribosyltransferase and its homologues was generated by ClustalW (http://www.ebi.ac.uk/clustalw).

Chapter 5. Discussion

5. Discussion

5. 1 Presence of c-di-GMP in *P. putida*

Since GGDEF and EAL domains have been found in various branches of phylogenetic tree of bacteria, both have been assigned with DGC and PDE-A activities, respectively, and roles of many proteins with these domains are assigned to various pathways, the involvement of c-di-GMP in bacterial signal transduction is well-established now. However, direct experimental data of c-di-GMP detection have for a long time been restricted to a few bacterial species, most of which are pathogenic bacteria. *G. xylinus*, the first bacterium in which c-di-GMP was first discovered, is non-pathogenic, however, it can cause fruit decay. The pathogenic bacteria in which the presence of c-di-GMP has been experimentally confirmed include human pathogens *S. typhimurium* and *V. cholerae*, opportunistic human pathogen *P. aeruginosa*, and plant pathogen *A. tumefaciens* (Simm *et al*., 2004; Tischer and Camilli, 2004; Kulesekara *et al*., 2006; Amikam and Benziman, 1989). The restriction of data to pathogenic bacteria may reflect people's concern about the mechanism of diseases caused by various pathogens.

Earlier this year, c-di-GMP was detected in non-pathogenic mineral-reducing soil bacterium *Shewanella oneidensis* (Thormann *et al*., 2006). Here, we report that c-di-GMP is also present in *P. putida*, a non-pathogenic soil bacterium, adding new information to support the notion that c-di-GMP is widespread in bacterial species. The intracellular c-di-GMP concentrations of *P. putida* are determined to be at pmole per mg dry weight level, consistent with those in *G. xylinus* and *S. oneidensis*

(Weinhouse *et al.*, 1997; Thormann *et al.*, 2006). It is noteworthy that the cAMP concentration of *Arthrobacter crystallopoietes* also falls within this range (Hamilton and Kolenbrander, 1977), indicating this may be the normal physiological level of cyclic nucleotide messengers in bacteria. However, unlike the intracellular level of cAMP, which peaks at the onset of starvation or stationary phase in *Haemophilus influenzae* (Macfadyen *et al.*, 1998), the intracellular c-di-GMP concentration of *P. putida* reaches the highest level during exponential growth phase and decreases when cells enter log-to-stationary transition phase. This suggests that c-di-GMP is actively involved in biological processes during exponential phase, in contrast to cAMP which mainly exerts its function when cells undergo living stresses. It is interesting to note that one of the phenotypes affected by c-di-GMP signaling is the flagellar biogenesis that is also initiated during this transition phase (Amsler *et al.*, 1993). We have previously shown that mutational loss *P. putida* of the GGDEF and EAL-domain containing MorA leads to constitutive flagellar formation during all growth stages (Choy *et al.*, 2004)

5.2 MorA affects c-di-GMP levels in *P. putida*

To study whether MorA affects c-di-GMP levels in *P. putida*, we compared intracellular c-di-GMP levels in *P. putida* WT, *morA* KO and OE strains at early log phase, middle log phase and log-to-stationary transition phase. At all three growth phases, the level of c-di-GMP of KO was higher that of WT and in OE, the c-di-GMP level was slightly below that of WT. In addition, the c-di-GMP concentrations in OE always remained low throughout all the growth stages. This observation implies that MorA has a negative influence on the intracellular concentration of c-di-GMP in *P.*

putida, via a direct or indirect way. It is possible that MorA functions as a c-di-GMP specific PDE, hence the loss of functional MorA leads to the increase of c-di-GMP in the cell while overexpression of MorA leads to a drop of c-di-GMP level. It is also possible that MorA regulates c-di-GMP level indirectly by binding to DGC or other proteins which finally results in the inhibition of certain DGC. When MorA is mutated, DGC is released from inhibition and therefore, the production of c-di-GMP is on the rise. On the contrary, when MorA is overexpressed, the inhibition of DGC is enhanced and consequently the level of c-di-GMP is reduced. These speculations will soon be tested with the availability of synthetic c-di-GMP. It is noteworthy that another cytosolic GGDEF protein MorC has been identified to affect the MorA pathway. It is currently not known whether MorA and MorC interact directly.

5.3 MorA does not have observable in vitro DGC activity

As mentioned above, comparison of c-di-GMP levels in *P. putida* implies that MorA might be a PDE-A. Despite this possibility, the presence of GGDEF domain in MorA suggests that the possibility of DGC activity of MorA cannot be ruled out. Therefore, we performed enzymatic assay to test MorA DGC activity in vitro. Both crude MorA and purified recombinant MorA were tested and no DGC activity was detected, which is contrary to the positive control, DGC extracted from *G. xylinus*. We then hypothesized that the phosphorylation status of the PAS-PAC sensory domains or the catalytic domains may affect MorA activity. Therefore, another set of enzymatic assays with the addition of acetyl phosphate was carried out. Acetyl phosphate is a small-molecule phosphate donor that specifically phosphorylates the acceptor aspartyl residues of response regulators which are phosphorylated in vivo by histine protein

kinases (Lukat *et al*, 1992). However, we still could not detect any DGC activity. Considering the sensitivity limit of HPLC, it was possible that c-di-GMP was synthesized in such low amount that it could not be detected by HPLC. Therefore, we took HPLC fraction of the reaction mixture at the retention time when c-di-GMP was supposed to be eluted out and sent it for MALDI-TOF mass spectrometric analysis. Absence of positive signals indicated that under the conditions tested, mo cyclase activity was detected for MorA.

The fact that MorA does not have apparent in vitro DGC activity under the conditions tested can be explained by the hypothesis that MorA is a likely c-di-GMP specific PDE. The comparison of intracellular c-di-GMP levels in *P. putida* WT, KO and OE strains also supported this hypothesis. If this hypothesis is correct, it is then interesting to know the role of GGDEF domain in MorA. Previous binding experiment showed that GTP could bind specifically to the GGDEF domain of MorA. Does this GTP-binding capacity contribute to the enzymatic activity of MorA? In *C. crescentus*, the GGDEF-EAL protein, CC3396, is a soluble PDE-A, the activity of which is confined to EAL domain. The associated GGDEF domain of CC3396 is inactive. Instead, it is able to bind GTP and activate the PDE-A activity of neighboring EAL domain (Christen *et al.*, 2005). The in vitro PDE-A activity of CC3396 is increased about 40-fold with the addition of GTP and this activation only occurs with the presence of GGDEF domain. However, the GGDEF domain of CC3396 is not a conserved one. It has one of the highly conserved Gly residues of the active site (A-site) motif replaced by Glu (GEDEF). A defined mutation in the A-site motif of the GGDEF domain (GQNEF) abolished allosteric activation of PDE-A activity. Therefore, It is proposed that the missing of key catalytic residues may

contribute to the loss of DGC activity of the GGDEF domain and the mutation may be the strategy of proteins which harbour GGDEF domain as a sensory domain for GTP. But in the case of MorA, the A-site motif is conserved. Previously, site-directed mutagenesis was used to mutate GGDEF motif of MorA to Ala-Ala-Ala-Ala-Ala (Yang, 2004). *P. putida* WT strain containing this mutant construct shows a significant increase in motility over *P. putida* WT and OE strain, suggesting the important role of GGDEF domain in MorA function. It is our hypothesis that the observed dominant negative effect may due to the formation of heterodimers of MorA, which competitively interfere with the function of wild type MorA homodimers.

Regarding EAL domain, sequence alignment of eleven enzymatically active EAL domains led to identification of several highly conserved key residues (Schmidt *et al*., 2005). Among five tested GGDEF-EAL proteins which possess DGC activity, the EAL domain of four proteins lack at least one of these conserved motifs. Although the basic requirement for PDE-A activity is unknown, these motifs may, to some extent, help differentiate active EAL domains from inactive ones. We analyzed the amino acid sequence of MorA and found that the EAL domain of MorA contains all the conserved motifs identified by Schmidt's group, which may indicate the possibility of MorA to be a PDE-A.

If MorA is indeed a PDE-A, based on our experiment that overexpression of MorA lead to an increase in biofilm formation, it implies that PDE-A promotes biofilm formation in *P. putida*. This is contradictory to the observations in *V. cholerae* and *S. typhimurium*, where the overexpression of PDE-A has been linked to a reduction in

biofilm formation (Simm *et al*., 2004; Tischler and Camilli, 2004). Biofilm formation is a complicated process that needs the participation and corporation of various factors so the relationship between c-di-GMP and biofilm formation may not be that direct and simple. For example, *P. aeruginosa* expressing PA2870 and PA3343, two DGCs that produce high levels of c-di-GMP in enzymatic assay do not cause any alteration in the biofilm phenotype (Kulesekara *et al.*, 2006). Another example is Arr, an inner-membrane PDE-A in *P. aeruginosa*, the activity of which can be enhanced by tobramycin, yet leading to augmented rather than decreased biofilm formation (Hoffman *et al*., 2005). Hence, it is also possible that MorA may function as a PDE-A which enhances biofilm formation in *Pseudomonas* species.

5.4 Putative GTP-binding proteins in *P. putida*

Since the synthesis of c-di-GMP needs the substrate GTP, we were interested to gain some knowledge on the potential factors that can influence the GTP pool in *P. putida* and to study whether these factors have an effect on c-di-GMP metabolism. Therefore, GTP-agarose affinity chromatography was performed to identify candidates of GTP-binding proteins. The SDS-PAGE analysis of eluted proteins showed four prominent bands, which were then excised from the gel and sent for protein sequencing analysis. These four proteins were identified to be polyribonucleotide nucleotidyltransferase, dihydrolipoamide dehydrogenase, lysine-arginine-ornithine-binding periplasmic protein and phosphoribosyltransferase, respectively.

Amino acid sequence alignment of these four proteins revealed no sequence similarity between each other, indicating that they may each belong to a different GTP-binding

protein family. Furthermore, the comparison of protein sequence of these four proteins with the classic ATP/GTP-binding site motif A (P-loop) showed that these four proteins do not harbour P-loop. P-loop, i. e., (A/G)XXXXGK(S/T), is the first identified motif that can bind purine nucleotide triphosphate (Kjeldgaard *et al.*, 1996). It typically forms a flexible loop between a β-strand and an α-helix and interacts with one of the phosphate groups of the nucleotide. Numerous proteins that bind to ATP/GTP contain P-loop. Nevertheless, not all the ATP/GTP binding proteins are picked up by this motif because their structure of nucleotide binding site may be different from that of P-loop, and this may be the possible reason that the four proteins we identified do not have P-loop.

5.4.1 Polyribonucleotide nucleotidyltransferase

Polyribonucleotide nucleotidyltransferase, which is also called polynucleotide phosphorylase (PNPase) belongs to an expanding family of exoribonucleases with homologues widespread in eubacteria, *Drosophila melanogaster*, plants, mice and humans (Ygberg *et al.*, 2006). It contains 2 RNase PH domains (PNPase 1 and PNPase 2) which are closely related functionally and in sequence similarity to ribonuclease PH (RPH) protein, and one KH domain and one S1 domain, both of which are oligonucleotide-binding motifs (Leszczyniecka *et al*., 2004). PNPase degrades RNA phosphorolytically and processively in a 3' to 5' direction and plays a central role in bacterial RNA degradation. It also assists bacterial adaptation to growth at low temperature(Yamanaka an M, 2001) in *E.coli, Bacillus subtilis, and Yersinia enterocolitica* (Favaro and Dehò, 2005) by specifically degrading mRNAs that code for cold shock proteins (CSPs) so as to allow bacteria to restart replication after

shifting to decreased temperature (Ygberg *et al.*, 2006). In *S. enterica*, apart from being a regulator of the cold shock response, PNPase also functions in turning the expression of virulence genes and bacterial fitness during infection (Ygberg *et al*., 2006). PNPase mutated strain expressed increased levels of mRNAs coded for by genes from Samonella pathogenecity islands (SPIs) and Samonella plasmid virulence (spv) genes clusters, which are essential for bacteria to remain virulent and viable in host cells. It is noteworthy that in *Streptomyces antibioticus*, a bifunctional enzyme, GPSI, was identified to possess both PNPase activity and guanosine pentaphosphate (pppGpp) synthetase activity (Jones and Bibb, 1996). Since pppGpp is known as the biosynthetic precursor of ppGpp, a nucleotide second messenger, GPSI seems to establish a connection between RNA degradation and signal transduction pathway in *S. antibioticus*. Similarly, it is possible that the PNPase we identified from *P. putida* may have some connection with the metabolism of nucleotide second messengers, such as ppGpp or c-di-GMP.

5.4.2 Dihydrolipoamide dehydrogenase

Dihydrolipoamide dehydrogenases (DLDH) are homodimeric flavoproteins that catalyse the NAD^+- dependent reoxidation of dihydrolipoamide (DLA) according to a ping-pong mechanism (de Kok *et al.*, 1998). It is the E3 component of several multienzyme complexes such as pyruvate dehydrogenase, 2-oxo glutarate dehydrogenase and branched chain keto acid dehydrogenase complexes which generally function in the conversion of 2-oxo acids to acyl-CoA derivatives (De Kok *et al*., 1998). Being a subunit of 2-oxo acid dehydrogenase, DLDH in prokaryotes is involved in the cytosolic main stream of energy metabolism. It also plays a role in the

glycine cleavage multienzyme complex and the acetoin dehydrogenase complex in *B. subtilis*, *Clostridium magnum* and *Pelobacter carbinolicus* (Smith *et al.,* 2002).

Although traditionally DLDH would be considered as a metabolic factor, its role in pathogenecity is being investigated. In *Mycoplasma gallisepticum*, a primary etiologic agent of the chronic respiratory disease complex in chickens and infectious sinusitis in turkeys, one gene encoding dihydrolipoamide dehydrogenase has been identified to be a virulence-associated determinant (Hudson *et al.*, 2006). A transposon insertion in the coding sequence resulted in diminished biological function and reduced virulence of the mutant. It is proposed that mutation of DLDH lowers the activity of ATP binding cassette transporters upon which bacteria rely to acquire various precursors necessary for viability, and persistence and adaptation in host cells (Hudson *et al*., 2006). In *Streptococcus pneumoniae*, DLDH-negative bacteria can grow normally in vitro but remain avirulent in sepsis and lung infection models in mice, suggesting the necessity of active DLDH for the survival of the bacteria in the host. In addition, DLDH-negative bacteria produced only 50% of normal capsular polysaccharide and according to the experiment, this reduction is probably not directly linked to the impairment of carbohydrate metabolism (Smith *et al.,* 2002). Currently we have no knowledge on the downstream changes occurred in DLDH mutants that lead to the reduction of capsular polysaccharide. Although so far there is no report on the presence of c-di-GMP in *M. gallisepticum* and *S. pneumoniae*, it is interesting to know that c-di-GMP also regulates virulence and exopolysaccharide production in various bacteria. Therefore, we cannot rule out the probability that there is crosstalk between DLDH and c-di-GMP signaling pathway.

5.4.3 Lysine-arginine-ornithine-binding periplasmic protein

Lysine-arginine-ornithine-binding periplasmic protein (LAO) belongs to the family of periplasmic substrate binding proteins (PBPs), which are involved in ABC (ATP binding cassette) transporter system to transport a variety of substrates such as amino acids, peptides, sugars, vitamins, inorganic ions, etc (Oh *et al.*, 1993). ABC transporter system is also known as the periplasmic binding protein-dependent transport system in Gram-negative bacteria which is made up of three types of components, including one or two integral membrane proteins (permeases) each having six transmembrane segments, two peripheral membrane proteins that bind and hydrolyze ATP, and a high affinity PBP. PBP is thought to bind the substrate in the vicinity of the inner membrane, and to transfer it to a complex of inner membrane proteins for concentration into the cytoplasm.

LAO has high affinity for L-lysine, L-arginine, and L-ornithine *(* Nikaido and Ames, 1993). L-histidine also binds to LAO, but less tightly than the other three amino acids. BLAST analysis of LAO with other *Pseudomonas* species showed that it shares high homology with arginine/ornithine binding protein AotJ in *P. aeruginosa* PAO1 and *P. entomophila* L48. LAO can also be found in *E.coli* and *S. typhimurium*. The three-dimensional structure of LAO from *S. typhimurium* with and without a ligand was determined (Oh *et al.*, 1993). It was proposed that the unliganded protein undergoes a dynamic change between an “open” and a “closed” conformation and the role of the ligand is to stabilize the closed conformation rather than to induce conformational change directly.

The reason that LAO bound to GTP in our experiment is unknown. However, it is noteworthy that in protein SLR2077 from *Synechocystis* sp. PCC.6803, PBP domain is located upstream of GGDEF and EAL domain (Fig 5-1). This suggests that PBP may be involved in c-di-GMP signaling pathway as a sensory domain. It is our hypothesis that upon receiving signals, PBP may induce conformational changes of the protein, affecting the activity of GGDEF or EAL domain. As for LAO homologs in *P. flurescens* Pf-5 and PfO-1, their neighboring genes are mainly ABC transporter genes and those involved in carbohydrate and nucleotide metabolism such as acetyl-coenzyme A synthetase and ribonucleotide reductase (Table 5-1). Hence, it is also possible that PBP plays a role in GTP metabolism.

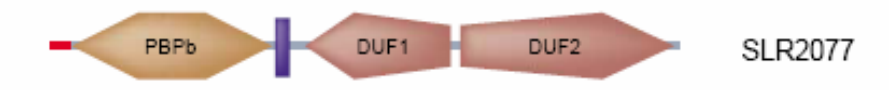

Fig 5-1 Domain structure of protein SLR2077 from *Synechocystis* sp. PCC.6803. Adapted from Jenal, 2004.
GGDEF and EAL domains were previously named as DUF1 (domain with unknown function 1) and DUF2, respectively.

Table 5-1 LAO homologs and their upstream and downstream genes in *P. flurescens* Pf-5 and PfO-1.
Gene names in bold are LAO homologs.

Locus tag	Genes
PFL_4518	conserved hypothetical protein
PFL_4519	arginine/ornithine transport system permease protein AotM
PFL_4520	arginine/ornithine ABC transporter, permease protein
PFL_4521	**arginine/ornithine ABC transporter, periplasmic arginine/ornithine-binding protein**
PFL_4522	acetyl-coenzyme A synthetase
PFL_4523	hypothetical protein
PFL_4524	ribonucleoside-diphosphate reductase, beta subunit, putative
Pfl_4289	succinylglutamate desuccinylase/aspartoacylase
Pfl_4290	amino acid ABC transporter, permease protein, 3-TM region, His/Glu/Gln/Arg/opine
Pfl_4291	amino acid ABC transporter, permease protein, 3-TM region, His/Glu/Gln/Arg/opine
Pfl_4292	**lysine-arginine-ornithine-binding periplasmic protein**
Pfl_4293	acetate--CoA ligase
Pfl_4294	hypothetical protein
Pfl_4295	ribonucleotide reductase

5.4.4 Phosphoribosyltransferase

The family of phosphoribosyltransferase (PRT) includes a range of diverse phosphoribosyl transferase enzymes such as adenine PRT, ribose-phosphate pyrophosphokinase, xanthine-guanine PRT, etc. The PRT identified in our experiment is a hypoxanthine-guanine phosphoribosyltransferase (HPRT, often renamed as HGPRT). It catalyzes the synthesis of inosine-5'-monophosphate (IMP) and guanosine-5'-monophosphate (GMP) from the purine bases hypoxanthine and guanine, respectively. Certain HGPRTs can also convert xanthine to xanthine monophosphate. The enzyme primarily functions to salvage purines from degraded DNA to renewed purine synthesis by acting as a catalyst in the reaction between guanine and phosphoribosyl pyrophosphate (PRPP) to form GMP. In patients with Lesch-Nyhan syndrome, HGPRT activity is strikingly reduced, leading to hyperuricemia and hyperuricaciduria (Arnold *et al.*, 1972). Since HGPRT is essential for purine nucleotide metabolism, it may affect c-di-GMP signaling pathway indirectly by regulating the GTP pool inside bacteria cells.

Chapter 6. Conclusions and Future Work

6. Conclusion and Future Work

Using *G. xylinus* as a model, we have established a method for extracting and detecting c-di-GMP from bacteria. In this method, crude nucleotides are extracted with perchloric acid, analyzed through HPLC and further confirmed by MALDI-TOF mass spectrometry. We have successfully detected c-di-GMP in *P. putida* with this method and this is the first report that c-di-GMP is present in *P. putida*. Although the combination of characteristic HPLC peak and specific m/z value in the mass spectrum can ensure the detected molecule is c-di-GMP, our conclusion will be more affirmative if more characterization work is carried out. For example, extracted c-di-GMP could be analyzed by MALDI-TOF mass spectrometry at different pH so that different m/z values specific to c-di-GMP will be obtained; or tandem mass spectrometry could be used to break down extracted c-di-GMP into fragment ions, the m/z value of which are the characteristics of c-di-GMP.

Comparison of intracellular c-di-GMP levels of wild type *P. putida* at different growth phases revealed that the c-di-GMP level in *P. putida* is growth-dependant. It is low at early log phase, rises to the peak at middle log phase and drops dramatically at log-to-stationary transition phase. MorA has been shown to affect intracellular c-di-GMP levels according to the observation that c-di-GMP level in KO strain is higher than that in WT and in OE strain, c-di-GMP content is lower than that in WT. However, we have no evidence for whether the influence of MorA to c-di-GMP is direct or not.

DGC activity assay suggested that MorA does not have apparent in vitro DGC activity under the conditions tested. Combined with the result of comparison of c-di-GMP levels in *P. putida* WT, KO and OE strains, it seems that MorA may function as a PDE-A. Therefore, PDE activity assay would be important for elucidating the enzymatic activity of MorA. For preparation of PDE activity assay, sufficient high purity c-di-GMP should be provided as the substrate. Since currently c-di-GMP is not commercially available, chemical synthesis and enzymatic conversion are the only two ways for c-di-GMP production. On one hand, we have collaborated with scientists from Department of Chemistry, National University of Singapore for c-di-GMP synthesis; on the other hand, we have started the initial step of enzymatic synthesis of c-di-GMP, i. e., cloning the DGC of *G. xylinus* into expression vector to produce recombinant DGC.

Since GTP is the substrate for c-di-GMP synthesis, with the interest to investigate factors affecting c-di-GMP metabolism, we carried out GTP-agarose affinity chromatography to identify potential GTP-binding proteins in *P. putida*. Four proteins have been identified. They are polyribonucleotide nucleotidyltransferase (PNPase), dihydrolipoamide dehydrogenase (DLDH), lysine-arginine-ornithine-binding periplasmic protein (LAO) and phosphoribosyltransferase (PRT). PNPase mainly functions in bacterial RNA degradation, but it can also regulate virulence and in some case, biosynthesis of nucleotide second messenger, ppGpp. DLDH is an important component of several metabolic multienzyme complexes such as pyruvate dehydrogenase and it also plays a role in pathogenecity in some bacterial species. LAO belongs to the family of periplasmic substrate binding proteins (PBPs), which are involved in ABC transporter system. PRT identified in this study is essential for

purine nucleotide metabolism. Although these four proteins were all eluted out from GTP-agarose column and are supposed to be GTP-binding proteins, they do not share any protein sequence similarity with each other, nor do they have the classic ATP/GTP binding motif, P-loop, indicating that they may have different types of GTP binding sites. Future work such as isothermal titration calorimetry could be carried out to confirm their GTP binding capacity. Furthermore, mutations of these four proteins could be generated to see whether they have any impact on c-di-GMP levels inside the cells. At the same time, c-di-GMP related changes in phenotypes such as motility, chemotaxis and biofilm formation could also be examined.

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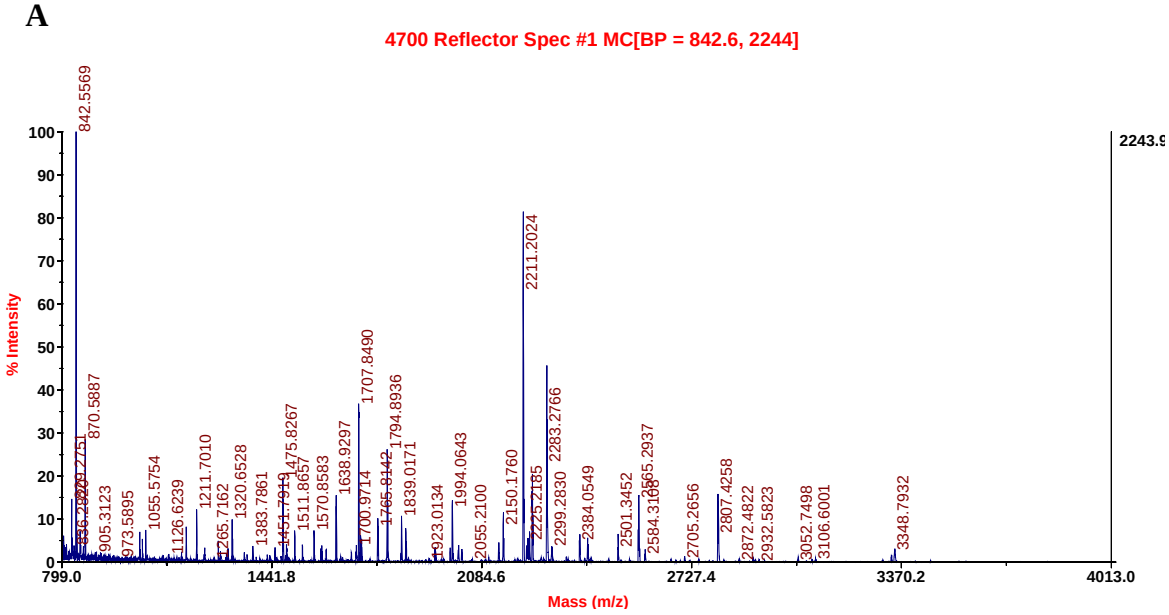
Appendix

A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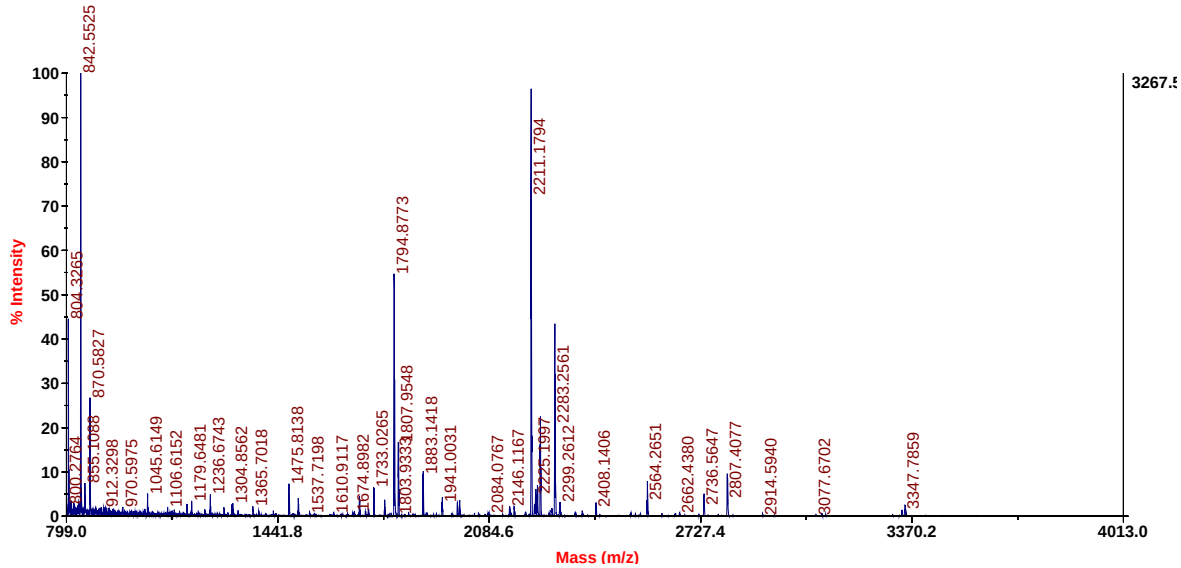
B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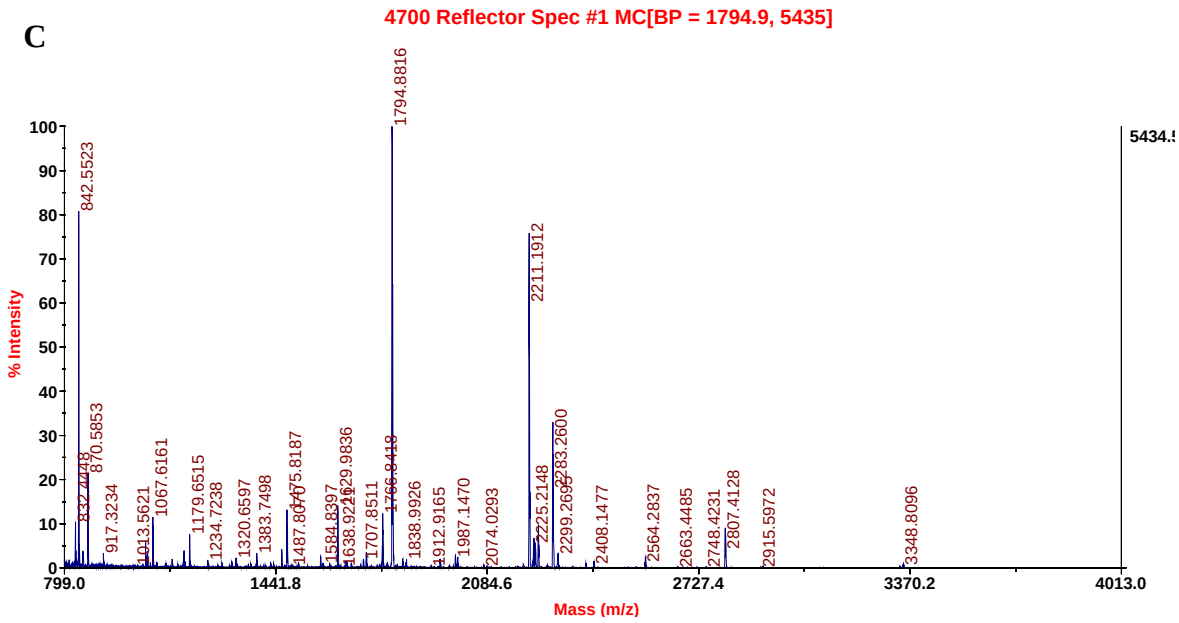

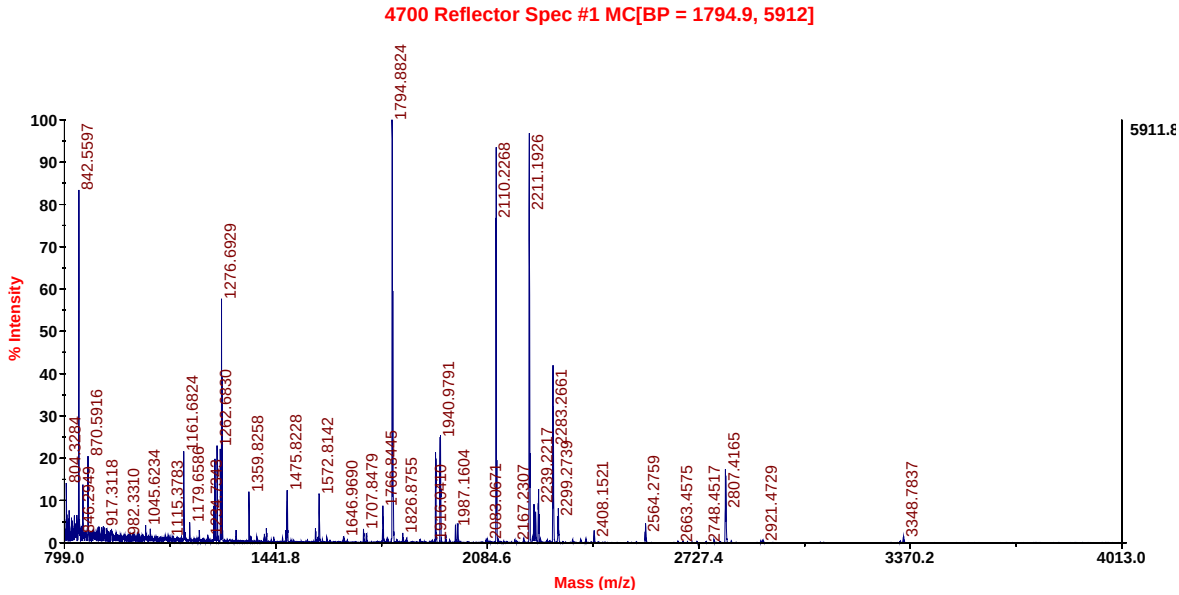

Mass spectrums of protein sequencing analysis of four putative GTP-binding proteins.

(A) Polyribonucleotide nucleotidyltransferase
(B) Dihydrolipoamide dehydrogenase
(C) Lysine-arginine-ornithine-binding periplasmic protein
(D) Phosphoribosyltransferase.