Molecular analyses of genes related to survival and virulence of *Clostridium perfringens* strain 13

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By

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Abbreviations

ATP, adenosine 5’-triphosphate
BHI, brain heart infusion (medium)
BLAST, Basic Local Alignment Search tool
Bp, base pair(s)
CcpA, catabolite control protein A
cDNA, complementary deoxyribonucleic acid
COG, Cluster of Orthologous Groups of protein
CRE, catabolite response element
DAG, diacyl glycerol
dH₂O, distilled water
DNA, deoxyribonucleic acid
ECF, extracytoplasmic function
EDTA, ethylene di-amine tetra-acetic acid
FITC, fluorescein isothiocyanate
GAM, Gifu anaerobic medium
GC, guanine and cytosine
HPLC, high-performance liquid chromatography
IP₃, inositol 1,4,5, triphosphate
IPTG, isopropyl-β-D-thiogalactopyranoside
Kb, kilo base
LB, Luria-Bertani (medium)
mRNA, messenger ribonucleic acid
NAD, nicotinamide adenine dinucleotide
NTCC, National Collection of Type Cultures
OD₆₀₀, optical density at 600

ORF, open reading frame
PAGE, polyacrylamide gel electrophoresis
PBS, phosphate-buffered saline
PCR, polymerase chain reaction
PFO, pefringolysin O
PIP₂, phosphatidylinositol 4,5, biphosphate
PLC, phospholipase C
RBS, ribosome binding site
RBC, red blood cell
RNA, ribonucleic acid
RNase, ribonuclease
rpm, revolutions per minute
RT-PCR, reverse-transcriptase polymerase chain reaction
SD, Shine-Dalgarno (sequence); standard deviation
SDS, sodium dodecyl sulphate
SSC, (buffer) 1X SSC= 0.15 M NaCl and 0.015M sodium citrate
Taq, Thermus aquaticus DNA (polymerase)
TCA, tricarboxylic acid
Tris, tris(hydroxymethyl)aminomethane
tRNA, transfer ribonucleic acid
UV, ultraviolet
VR-RNA, VirR regulated RNA
Chapter 1
General introduction of *Clostridium perfringens*
1.1. *Clostridium perfringens*: An overview

*Clostridium perfringens* was first isolated from a cadaver, and was described completely by Welch and Nuttall in 1892. Although there are a few credible accounts from antiquity of gas-gangrenous infections in man, the disease did not come into any great prominence until the First World War. Hundreds of thousands of soldiers died of gas gangrene due to battlefield injuries during World War I, and *C. perfringens* was widely recognized as being the most important causal organism of the disease.

*C. perfringens* is a Gram-positive, aerotolerant, spore-forming, non-motile bacterium, and known to be the most widely distributed pathogen in nature (McDonel 1980). It forms stout rods, varying considerably in length; 4-8 µm X 0.8-1.0 µm but sometimes shorter and more slender. It forms heat-, oxygen-, and alcohol-resistant endospores that are found especially in soil contaminated with feces. The spores are large, and subterminal but are rarely seen in artificial culture. The sporulation is also strain-specific, and is favored by an alkaline environment (never occur below pH 6.6). Spores are seldom seen in ordinary cultures but may be reliably induced on the bile-bicarbonate-quinoline medium of Phillips (Phillips 1986). Culturally, *C. perfringens* forms two main types of surface colonies. One is round, 2-4 mm in diameter, low convex, amorphous, grayish-yellow, opaque, with smooth surface and entire edge; butyrous and easily emulsifiable. Other is umbonate, and is differentiated into an opaque brownish center and a lighter, more translucent, readily striated periphery with a crenated edge.

*C. perfringens* is commonly found in the environment (in soil and sewage) and in the intestines of animals and humans as a member of normal flora (McDonel 1980; Hatheway 1990). Though considered to be opportunistic, it is frequently encountered in a variety of clinical settings, ranging from simple contamination of a wound to traumatic or
nontraumatic myonecrosis (gas gangrene), gangrenous cholecystitis, post-abortion infection with devastating septicemia and intravascular hemolysis, necrotizing pneumonia, empyema, food-borne and non-food-borne gastroenteritis etc.

*Clostridium perfringens* produces a diverse variety of exoproteins comprising exotoxins and extracellular enzymes, many of which have been implicated as virulence factors, whereas others, despite having identifiable biological activity, have little or no significant effect in the clostridial pathogenesis. Pathogenicity is related to the production of one or more lethal toxins and classification of *C. perfringens* into five groups (A-E) (Table 1.1) is based on the nature of four major toxins (known as alpha-, beta-, epsilon-, and iota-toxins) produced (Petit, Gibert et al. 1999). Of these five types of *C. perfringens*, only type A and C appear to be the significant cause of disease in man. Type A is the main causative agent of gas gangrene and food-borne gastroenteritis, whereas type C is responsible for particularly severe form of food poisoning (enteritis necroticans).

Among the toxigenic clostridial species *Clostridium perfringens* is the paradigm species for genetic studies, because of its oxygen tolerance, faster growth rate (8- to 10-minute generation time in optimal conditions), and ability to genetically manipulate (Rood 1998). Several studies on the genetic regulation of the toxin genes were reported to use the transformable *C. perfringens* strain 13 (Mahony and Moore 1976).

*C. perfringens* mediated gas gangrene is one of the most serious anaerobic infections in human. It is not merely a bacteriological entity, but a rapidly progressive, invasive, clinicopathological condition with liquefactive necrosis of muscle, gas formation and associated clinical sign of toxemia. Because of the large numbers of extracellular factors produced by *C. perfringens*, it has been perplexed to sort out the various contributions by these factors to the development of the disease. In considering the development of
pathogenic processes and their regulation, it is deserved to delve into their biological properties as well as into an area of gene regulation, for the virulent properties (abilities to cause infection) are genetically encoded and the regulation of their expression is just the reaction to cues picked up from the environment.

In order to control microbial infections, it is indispensable to build up a comprehensive knowledge of the virulence strategies by which pathogenic organisms gain access to and mount pathogenic devices in the host. By means of which, it is conceivable to design the drugs that can interrupt the growth and spread of microbes, or that can make the virulent organisms avirulent to its host. It is, therefore, of paramount importance to understand the molecular details of gene control mechanism of an individual virulence factor that has a potential to or is known to be involved in the disease.

1.2. Pathogenicity of *C. perfringens*

*C. perfringens* produces 12 exotoxins and an enterotoxin (Table 1). The major exotoxins are alpha-, beta-, epsilon-, and iota-toxins. Alpha toxin, a lecithinase, is the primary toxin and common to all five types of *C. perfringens*, but is produced in the largest amount by type A. This toxin is known to hydrolyze lecithin and sphingomyelin and the disruption of the cell and mitochondrial membrane phospholipids produces the *in vivo* effects of alpha toxin. All of the major toxins are lethal to laboratory animals. The enterotoxin is produced during sporulation, which inhibits glucose transport, causes protein loss and damages the intestinal epithelium.

To cause skin and soft tissue infections, exogenous contamination of wound may occur from soil, water or sewage, whereas, for endogenous contamination, most infections are secondary to abdominal surgery or trauma, which allow contamination of tissues with
fecal material or genital tract secretion (since *C. perfringens* is part of the normal flora of gastrointestinal and female genital tract). Once tissue is contaminated, several forms of clinical diseases can ultimately ensue: Suppurative infections and abscesses are often seen in the gallbladder, uterus, fallopian tubes, and abdomen. Localized cellulites may occur as in diabetic stump following amputation. The beta toxin of *C. perfringens* type C usually causes a severe form of nectorizing disease, enteritis necroticans, in which an acutely ulcerative process denudes the gut, which is frequently fatal. Gas gangrene (myonecrosis) occurs in contaminated deep wounds and is the most serious type of soft tissue infection, is characterized by necrosis and foul smell. Clostridial food poisoning is usually caused by ingestion of meat dishes (e.g., stews, soups, gravy), which is contaminated by *C. perfringens*, type A spores. The spores resist boiling for variable periods of time but start germinating as the temperature drops below 75°C (e.g., when they reach the gastrointestinal tract). Release of heat-labile enterotoxin upon sporulation occurs primarily in the ileum and large intestine. The disease is usually self-limiting and is characterized by the onset of watery diarrhea within 18 hours of ingestion, and cramps (similar to *E. coli*).

Among all the diseases produced by type A *C. perfringens*, gas gangrene has become the disease of great attention throughout the history of conventional warfare, which cost uncounted lives. Clinically, the incubation period after injury varies from 7 hour to 7 days. Pain in the region of the wound develops early, and steadily increases in intensity along with progressive swelling and edema. Mild to moderate pyrexia is the rule and there is disproportionate tachycardia to fever. Relevant experiments have proved that the disease occurs in two stages: in the first stage the alpha toxin destroys the capillaries; and in the second stage the bacteria invade bloodstream and give rise to general bacteremia. The local lesion provides a reservoir both of toxin and of bacteria (Katitch 1964). As the
disease progresses, bubbles of gas appear in the discharge, the tissues become crepitant, and the skin becomes white and marbled. The patient becomes collapsed, profoundly toxemic, and shocked, but remain mentally alert and anxious. The blood pressure falls rapidly and peripheral venous circulation collapses, and usually terminates with sudden death due to circulatory failure.

1.3. Genomic features of C. perfringens

Whole genome sequence of C. perfringens strain 13 (~3.04 Mb) has been published recently (Shimizu, Ohtani et al. 2002). In the genome, there are 2,660 protein-coding regions that cover 83.1% of the whole chromosomal sequence, with an average size of 946 bp. There are 10 rRNA genes, 96 species of tRNA genes, and low overall GC content (28.6%). The plasmid (named pCP13) has 54310-nucleotide sequence and contains 63 putative open reading frames (ORFs).

In the C. perfringens chromosome, biological roles are assigned to 56.1% (1,492) of the ORFs; 18.9% (502) of the ORFs are similar to hypothetical genes of unknown function and 25% (666) are unique hypothetical genes with no significant similarities to putative or demonstrated genes in other organisms. The majority of the ORFs on pCP13 seem to encode proteins of unknown function; 16 ORFs has similarities to hypothetical proteins from other organisms, and putative functions has been assigned to 17 ORFs that has similarity to proteins of other organisms.

When compared with the genome of Clostridium acetobutylicum (Nolling, Breton et al. 2001) some genes were found to be unique in C. perfringens including those encode myo-Inositol catabolism proteins (myo-Inositol operon), three extracytoplasmic function (ECF)-type sigma factors, α- and β-galactosidases, α-mannosidase, components for V-
type sodium ATP synthase, selenocysteine synthase, and various virulence-associated proteins (Shimizu, Ohtani et al. 2002).

*C. perfringens* has a complete set of enzymes for glycolysis and glycogen metabolism but no genes coding for tricarboxylic (TCA) cycle- or respiratory chain related proteins but all the enzymes are present for anaerobic fermentation to form lactate, alcohol, acetate and butyrate. 61 genes related to sporulation and germination were identified in *C. perfringens*, which were deduced from similarities with genes from the spore-forming bacteria *Bacillus subtilis*, but as many as 80 sporulation- and germination-related genes which are present in *B. subtilis*, are missing in *C. perfringens*. The majority of key genes encoding sporulation-specific sigma factors and other stage-specific sporulation proteins are present but the genes for the major phosphorelay system of the Spo0A protein, required in the initiation process of sporulation, encoding such protein as Spo0F, Spo0B, KinA to KinE, and Rap family phosphatase are missing in *C. perfringens* genome.

When compared with the *C. acetobutylicum* genome (Nolling, Breton et al. 2001), the existence of virulence-related genes in *C. perfringens* is clearly distinct. The important virulence-related genes in *C. perfringens* are presented in Table 1.2. Among them, phospholipase C, perfringolysin O, Collagenase are noteworthy and encoded in the chromosome, while beta 2 toxin, a novel putative virulence factor associated with digestive tract diseases of piglet and horses (Gibert, Jolivet-Reynaud et al. 1997) is located in the plasmid (pCP13). Interestingly, all these virulence-associated genes found in *C. perfringens* do not form any pathogenicity island.

*C. perfringens* has 48 genes that are classified into the bacterial two-component signal transduction system, including 28 sensor histidine kinase and 20 response regulators (Shimizu, Ohtani et al. 2002). A little is known about the two-component system in *C. perfringens* except for that the VirR/VirS system regulates the expression of certain
toxins and proteins at transcriptional level (Lyristis, Bryant et al. 1994; Ba-Thein, Lyristis et al. 1996; Banu, Ohtani et al. 2000). The response regulator protein, VirR has been reported to bind to a repeated DNA sequence, CCAnTT(n=15)CCAGnTT(n=3), located upstream of the promoter of the theta toxin, and search for the same VirR binding sequence revealed another four previously unidentified putative VirR-binding sites in the promoter region of CPE 0957, CPE 0845, CPE 0920 and CPE 0846 (α clostripain).

The cytolytic toxins, including alpha-toxin, theta-toxin and putative enterotoxins, may destroy the host cells to release various materials. The released proteins and host structural proteins are degraded by proteinases such as α clostripain and collagenase. The resulting amino acids and/or peptides are efficiently imported into C. perfringens cells via various transporters, which is essential for the organism to synthesize proteins because of lack of many enzymes for amino acid biosynthesis. Thus, C. perfringens actively degrades and imports various materials from the host tissue to grow and survive in the host, which in turn causes massive destruction of the host tissues and severe myonecrotic lesion (Shimizu, Ohtani et al. 2002). It is reasonable to assume that C. perfringens needs to quickly synthesize the degradative toxins/enzymes to gain nutritional sources efficiently through the degradation of the host material. Hence, the pathogenicity and nutritional acquisition must be highly coupled in C. perfringens infection, and this unique nutritional feature would be a possible target for the inhibition of growth and prevention of C. perfringens infection.

1.4. Regulation of the virulence factors in C. perfringens: Role of two-component system, VirR/VirS

Two-component signal transduction system comprises a regulatory pair, a sensor and a response regulator. The sensor proteins have the conserved C-domains and the response
regulators have their conserved N-domains (Kofoi and Parkinson 1988; Stock, Ninfa et al. 1989). The N-domain of the sensor protein receives environmental signals and transduces the signals to its C-domain. Upon receipt of environmental signals, a sensor, also known as ‘histidine protein kinase’, autophosphorylates by catalyzing transfer of the γ-phosphoryl moiety of Mg-ATP to an imidazole nitrogen of its essential histidine residue. The phosphoryl group from the phosphohistidine is then transferred to a conserved aspartate residue in the response regulator that undergoes phosphorylation and induces global conformational change, which consequently, triggers the appropriate physiological signals, commonly, but not exclusively, the transcriptional activation/inhibition by binding subservient promoters (Figure 1.1). Ultimately, a specific phosphatase activity may reset the system by restoring the response regulator to its unphosphorylated state, and thence, regulator-phosphate levels are controlled in harmony with the environmental conditions. Although this regulatory family consists of many members (Stock, Ninfa et al. 1989), only few have been studied in detail at the molecular and biochemical levels. Very often, the characteristics of the other membranes have simply been inferred from data obtained from amino acid sequence homologies with the best-studied examples.

The histidine kinases (sensors) are mostly associated with cytoplasmic membrane, and have two sub-domains: the stimulus receiver domain and the nucleotide binding domain—usually with its stimulus receiver domain on the outer surface of the membrane, but some are wholly cytoplasmic (e.g., CheA and NtrB)

A two-component system has been described in C. perfringens, which has a sensor protein, VirS and a response regulator, VirR, together VirR/VirS. Though the actual signal that is sensed by the VirS is yet unknown, several toxins and virulence factors
including alpha toxin, theta toxin and kappa toxin, are controlled either positively or negatively, at the transcriptional level by this VirR/VirS system (Lyristis, Bryant et al. 1994; Shimizu, Ba-Thein et al. 1994; Ba-Thein, Lyristis et al. 1996). Later, a secondary RNA molecule, VR-RNA (VirR regulated RNA) was reported to play important regulatory role in the VirR/VirS signal transduction system (Shimizu, Yaguchi et al. 2002). After completion of genome sequencing of C. perfringens, DNA microarray experiments were carried out to understand more about the VirR/VirS regulatory system, and this microarray experiments revealed many more unidentified genes, which are regulated by this two-component systems. On a post-sequencing level, though microarray experiments are desirable, it is crucial to confirm the microarray result either by Northern analysis or by RT-PCR.

Briefly, alpha toxin was found to be directly and positively regulated by the VirR/VirS system, whereas theta- and kappa-toxins are positively regulated but indirectly, through a secondary RNA molecule, VR-RNA. VR-RNA itself is positively and directly regulated by the VirR (Figure 1.2). Microarray experiments revealed several other genes which are regulated by the VirR/VirS system, including myo-Inositol operon, which is absent in C. acetobutylicum and plasmid encoded beta 2-toxin (cpb2) and possible collagen adhesion genes (cna) (Ohtani, Kawsar et al. 2003) and a putative enterotoxin (entA).

1.5. Conclusion

Among several novel revelations by completion of genome sequencing, specifically important candidates, which demand further analysis, were enterotoxin gnes (ent), ECF-type sigma factors and myo-Inositol operon. The reasons for the specific importance for these genes are:
a) Enterotoxin A (entA) is regulated by the VirR/VirS system as seen from microarray experiment. *C. perfringens* is a naturally *spo0A* mutant and several experiments showed that enterotoxin gene (*cpe*) is not expressed in other *C. perfringens* strain when *spo0A* is mutated.

b) ECF-type sigma factors are not present in *C. acetobutylicum*, which is non-pathogenic to human.

c) *myo*-Inositol operon, which is also present in *B. subtilis* and *C. tetani*, is positively regulated by the VirR/VirS system as revealed by microarray experiments.

**AIM**

The aim of present study was to investigate:

1. Whether enterotoxin genes (*ent*) are expressed in naturally *spo0A* mutant, *C. perfringens* strain 13, and if expressed, are they regulated by the VirR/VirS system.

2. Whether the ECF-type sigma factors are expressed in *C. perfringens*, and do they have any role in virulence factor/toxin production. Are they regulated by the VirR/VirS system? Do they have any role in bacterial stress response?

3. Organization and transcriptional regulation of *myo*-Inositol operon and role of VirR/VirS system in the regulation of the operon expression

In general, the common goal was to investigate the transcriptional profile and the role of VirR/VirS system in expression of above genes at their transcriptional level.
1.6. Tables and figures
### Table 1.1. Extracellular toxins produced by *Clostridium perfringens*

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Biological activity</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
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<tr>
<td><strong>Major toxins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α</td>
<td>Lethal, necrotizing, phospholipase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β</td>
<td>Lethal, necrotizing</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>-</td>
</tr>
<tr>
<td>ε</td>
<td>Lethal, necrotizing</td>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ι</td>
<td>Lethal, binary toxin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td><strong>Minor toxins</strong></td>
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<tr>
<td>γ</td>
<td>Lethal</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>δ</td>
<td>Lethal, hemolysin</td>
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<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>η</td>
<td>Lethal toxin (?)</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>θ</td>
<td>Lethal, Perfringolysin O</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<td>κ</td>
<td>Collagenase</td>
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<td>Neuraminidase</td>
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Table 1.2. Important virulence-related genes in *C. perfringens*¹

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Product</th>
<th>Length, Amino acids</th>
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<tr>
<td><em>hlyA</em></td>
<td>Hemolysin-related protein</td>
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<tr>
<td><em>plc</em>²</td>
<td>Phospholipase C (Alpha toxin)</td>
<td>398</td>
</tr>
<tr>
<td><em>pfoA</em>²</td>
<td>Perfringolysin (Theta toxin)</td>
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<tr>
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<td>Collagenase (Kappa toxin)</td>
<td>1104</td>
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<tr>
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1. Shimizu, Ohtani et al, 2002
2. Lyristis, Bryant et al. 1994; Ba-Thein et al. 1994

(2-4) Indicates the genes regulated by the VirR/VirS two-component signal transduction.
Figure 1.1. The "two-component" paradigm for sensory signaling via communication modules
Figure 1.2. VirR/VirS-VR-RNA regulatory cascade in *Clostridium perfringens*
Chapter 2
Transcriptional regulation of enterotoxin genes (ent) by two-component regulatory system, VirR/VirS
2.1. Introduction

*C. perfringens* is well known for its potential to cause various disease, and ranks among the most important of the anaerobic pathogens affecting humans and animals (McDonel 1980; Rood and Cole 1991). One of the most common human diseases caused by *C. perfringens* is *C. perfringens* type A food poisoning, which annually ranks among the leading foodborne diseases in industrialized countries (Czeczulin, Collie et al. 1996). The symptoms (diarrhea and cramping) of this food poisoning are caused by *C. perfringens* enterotoxin (CPE), a single 35-kDa polypeptide with unique amino acid sequence and mechanism of action (McClane 1996). CPE-producing type A isolates are major human gastrointestinal pathogens causing food poisoning (chromosomal-brone *cpe*), and such non-food-borne diseases as antibiotic-associated diarrhea and sporadic diarrhea (plasmid-borne *cpe*). Recent molecular epidemiology surveys suggested that only a low percentage (~5%) of all *C. perfringens* isolates carry the *cpe* gene.

Complete genome sequencing of *C. perfringens* strain 13 revealed the presence of four putative enterotoxin genes (*ent*) in the chromosome. These genes are different from *cpe* gene, and have similarity with enterotoxin from *B. cereus*. Genome-wide search showed that the enterotoxigenic *cpe* gene is not present in its genome. The Spo0A, a sporulation-specific transcription regulator, tightly regulates transcriptional regulation of the *cpe* gene, and mutation of *spo0A* resulted in inability to produce enterotoxin and sporulation (Huang, Waters et al. 2004). *C. perfringens* strain 13 is a naturally *spo0A* mutant, which is caused by a frame-shift mutation resulting in introduction of a premature termination codon within the ORF. Though *spo0A* negative, *C. perfringens* produces several toxin and virulence genes, which are independent of Spo0A, and some of them are transcriptionally regulated by the VirR/VirS system.
Objectives of this study

Revelation of these newly identified chromosome-borne enterotoxin (ent) genes formed the scaffold to consider experimentation to address the following unanswered questions:

1. Are these putative enterotoxin genes transcribed under normal growth conditions in naturally spo0A negative C. perfringens strain 13?
2. If they are expressed, when the expression reaches to the peak during the growth cycle of bacteria?
3. Are these enterotoxin genes regulated by the VirR/VirS system at transcriptional level?
4. Can these proteins be expressed and purified, and do they have the toxicity to the host cell to justify them as enterotoxin?

2.2. Materials and methods

2.2.1. Bacterial strains, plasmids and materials

C. perfringens type A strains 13 (Mahony and Moore 1976), TS133 (virR mutant) (Shimizu, Ba-Thein et al. 1994), and the virR complement strain, E. coli DH5α (Sambrook, 1989), E.coli Top10 (cloning host), pUC19 (cloning vector) (Yanisch-Perron, Vieira et al. 1985), pTrcHis (Xpress™ system, Invitrogen, CA, USA) and pJIR418 (E.coli-C. perfringens shuttle vector) (Sloan, Warner et al. 1992) were used for this study. The laboratory equipments/reagents were purchased from the following sources: all chemicals (Wako Pure Chemicals, Osaka, Japan), AneroPack (Mitsubishi Gas Chemical(s) Co., Inc.), Restriction and modification enzymes (Toyobo Co. Ltd., Osaka, Japan; Nippon Gene Co. Ltd., Toyama, Japan; TaKaRa Shuzo Co. Ltd., Kyoto, Japan;
NEB, New England Biolabs), Geneclean II Kit (Bio 101, Inc., La Jolla, Calif.), Defibrinated sheep blood (Nippon Bio-Test Laboratories Inc., Tokyo), Antibiotics (Sigma Chemical Co., Ltd., St. Louis, MO.), GeneScreen Plus membrane (Nylon filter membranes) (NEN Research Products, Boston. Mass.), Cellulose Acetate (Syringe sterile filter membrane) (Millipore), DNA ligation kit Ver. 2, TaKaRa BKL Kit, TaKaRa Blunting Kit (TaKaRa Biomedicals, Shuzo, Japan), Gifu Anerobic Media (GAM) (Nissui Pharmaceuticals Co., Japan), Other meida materials (Difco, Sparks, USA), Alk-Phos Direct (northern blot labeling reagent), Northern blot hybridization buffer, Hyperfilm (Amersham Pharmacia Bio-tech, England), CDPStar Detection Reagent (AlkPhos Direct), PCR primers (Hokkaido System Science, Hokkaido, Japan), were used in these experiments.

2.2.2. Media and culture conditions

GAM (Gifu anerobic medium) and LB (Luria-Bertani) medium were used to culture bacteria. LB plate (LB medium with 15 g L⁻¹ Bacto-agar), Blood agar plate (37g L⁻¹ Brain heart infusion with 15g L⁻¹ Bacto-agar and 75 ml L⁻¹ defibrintated sheep blood) containing respective antibiotics was used for bacterial inoculum. E. coli was cultured in LB medium at 37°C with vigorous shaking at 180-190 rpm. Aneropacks were used for C. perfringens plate growth at 37°C and broth culture was performed in GAM at 37°C (water bath/incubator) under anerobic conditions in tube with air-tight cap. Ampicillin (50 µg ml⁻¹), Tetracycline (2.5 µg ml⁻¹), Chloramphenicol (20 µg ml⁻¹) and Erythromycin (25 µg ml⁻¹) were used appropriately in broth or agar medium.

2.2.3. General DNA techniques
The chromosomal DNA and plasmid DNA from *C. perfringens* was prepared as described by Okabe (Okabe, Shimizu et al. 1989) and Roberts (Roberts, Holmes et al. 1986), respectively, by using Sodium Dodecyl Sulfate (SDS) lysis method. The general recombinant DNA techniques, such as restriction and modifications, agarose gel electrophoresis, DNA elution from gel, ligation and transformation, Southern blot analysis, Colony hybridization etc. were done as described by Ausubel et al. (1994) and Sambrook et al. (1989), unless otherwise specified.

### 2.2.4. Plasmid DNA preparation from *E. coli*

*E. coli* DH 5α was cultured overnight at 37°C in 5 ml of LB-broth, from where bacterial cells were harvested by centrifugation at 15000 rpm for 5 minutes at 4°C. The bacterial pellets were re-suspended in 250 µl of SET solution (20% sucrose, 50 mM EDTA, pH 8.0, 50 mM Tris-HCL, pH 8.0) by vortex. 600 µl lysis solution (1% SDS, 0.2 N NaOH) was added and mixed by inversion. After keeping it on ice for 10 minutes, 400 µl of cold 3M sodium acetate (pH 4.8) was added, mixed thoroughly, and kept on ice for 10 minutes. The mixer was centrifuged at 15000 rpm for 10 minutes and 900 µl of the supernatant was transferred into new eppendorf tube. 2 µl of RNase solution (10 µg/ml) was added and then incubated at room temperature for at least 20 minutes. Chloroform extraction was done by adding 900 µl of chloroform-Iso-amyl-alcohol at the ratio of 24:1, mixed hard and spinned for 5 minutes at 15000 rpm. 750 µl of the supernatant was collected and the equal volume of isopropanol was added, mixed by inversion and kept on ice for at least 20 minutes. After centrifugation at 15000 rpm for 10 minutes, the supernatant was discarded and the precipitate was washed with 500 µl of 70% ethanol by
spinning at 15000 rpm for 5 minutes and then dried in vacuum drier and dissolved in 15-30 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or H2O.

2.2.5. Chromosomal DNA preparation from C. perfringens
From overnight culture of C. perfringens in 10 ml of GAM broth, 2 ml was taken and centrifuged for 5 minutes at 15000 rpm, the pellet was suspended in 400 µl of Tris-EDTA-Sucrose buffer (50 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0; 6.7% sucrose) and incubated at 37°C for 5 minutes. 100 µl of lysozyme solution (20 µg/ml in 25 mM Tris) was added, mixed by gentle inversion and incubated at 37°C for 5-10 minutes. 100 µl of 0.25 M EDTA, 50 mM Tris-HCl (pH 8.0) and 60 µl of 20% SDS (in 50 mM Tris-HCl and 20 mM EDTA) was added, mixed by inversion and incubated at 37°C for 10-15 minutes. The mixer was incubated with 2 µl of RNase at 37°C for more than 15 minutes at room temperature. Phenol extraction was done by adding the same volume of phenol-chloroform (1:1), and was mixed by inversion and then spun for 5 minutes. Equal volume of chloroform-isooamylalcohol (24:1) was added into the phenol-extract supernatant, mixed by inversion and centrifuged for 5 minutes at 15000 rpm. 480 µl of the supernatant was taken carefully and equal volume of isopropanol was added. The solution was mixed gently, kept at –80°C for 10 minutes and centrifuged for 5 minutes. After decanting the supernatant, the precipitate was rinsed with 70% ethanol and dried in vacuum drier. The precipitate was dissolved in 20-40 µl of TE buffer.

2.2.6. Ligation of DNA fragments to plasmid vectors
Plasmid vector, pUC19, which contains 1.1 kb erythromycin resistant gene was digested with appropriate restriction enzyme. 50 µl vector was digested and then 10 µl of alkaline phosphatase buffer and 1 µl of CIAP (Calf intestine alkaline phosphatase) (18 units/ml)
were added and incubated at 37°C for 1 hour. The mixer was then incubated with 2 µl of 0.5M EDTA (pH 8.0) at 65°C for 15 minutes. Phenol-chloroform extraction was done and the precipitate formed after centrifugation was rinsed with 70% ethanol and finally dissolved in 20 µl of TE buffer. For the DNA insert-fragment preparation, PCR amplified DNA was digested by appropriate restriction enzyme. After ethanol precipitation, the digested DNA was also dissolved in 20 µl TE buffer. Both the vector and insert were separated by 1% agarose gel-electrophoresis. The desired fragments were cut from the gel and DNA was recovered from gel with glass-milk using GeneClean II kit. The glass-milk pellet was washed with NEW wash solution. The pellet was suspended in 10 µl D/W and 10 µl ligation buffer solution A (Ver. 2) was added and incubated at 16°C for more than 4 hours. The ligated DNA was subjected to transformation.

2.2.7. Preparation of competent cell from *E. coli*

*E. coli* was precultured in 5 ml of LB broth at 37°C overnight on shaker at 180 rpm. 300 µl of preculture was inoculated into 30 ml of LB broth and incubated at 37°C with shaking for 2 hours until the OD (optical density) at 600 nm reached between 0.2 and 0.25. The culture was centrifuged at 3500 rpm for 10 minutes. The supernatant was discarded. The pellet was suspended with 25 ml of 50 mM CaCl₂, kept on ice for 30 minutes and centrifuged for 10 minutes at 3500 rpm. The pellet was resuspended with 3 ml of stock solution (50 mM CaCl₂, 20% glycerol). The competent cell solution was aliquoted as 100 µl in each 1.5 ml tube and stored at –80°C for future use for transformation.
2.2.8. Cloning of enterotoxin genes

All four enterotoxin genes were first cloned into pUC19 to check their expression in *E. coli* DH5α. The whole lengths of the ORF of the respective genes were PCR amplified with appropriate set of primers using the chromosomal DNA as the template. PCR products were checked by agarose gel electrophoresis and cleaned up using GeneCleanII kit. The ends of the amplified products were modified and were cloned into the multiple cloning sites of the vector with appropriate restriction enzyme digestion followed by ligation and were transformed into the competent cells prepared from *E. coli* DH5α. Plasmids were prepared from the transformed cells to examine the accuracy of ligation. Expressions of the cloned genes were checked by SDS-PAGE. Later, all four genes were cloned into His-tagged expression vectors, pTrc-His with appropriate restriction enzymes to align in-frame with the open reading frame of the upstream histidine coding sequences. The resultant plasmids were transformed into *E. coli* Top10 competent cells. Plasmids were prepared from the transformed cells and accuracy of ligation was checked by restriction enzyme digestion and agarose gel electrophoresis. All the genes were ligated in correct orientation. The expressions of the cloned genes were checked by IPTG induction and SDS-PAGE experiments.

2.2.9. Transformation into *E. coli* (Simple method)

The competent cells were thawed by keeping on ice for 30 minutes, 8 µl of 30% PEG (polyethylene glycol) solution, 10 µl of 500 mM MgCl₂-100 mM CaCl₂ were added to 10 µl of ligated DNA solution and messed upto 100 µl with dH₂O. 100 µl of the aliquot of the competent cells were added to 100 µl DNA solution, mixed by pipetting and then
incubated on ice for 20 minutes. Heat shock was applied by incubating the solution at 37°C for 3 minutes. The mixture was then chilled on ice for 1 minute. 500 µl of LB broth was added and incubated at 37°C shaker at 180 rpm for 1 hour. The transformed *E. coli* was spread on LB agar plates containing appropriate antibiotics and incubated at 37°C overnight. The single colonies were checked by plasmid extraction and selected enzyme digestion of plasmid DNA that was then separated in 1% agarose gel electrophoresis. Resulted properly ligated plasmid containing *E. coli* new strains were selected as right clones.

### 2.2.10. Colony hybridization

Colony hybridization experiment required several steps:

**a. Colony transfer and DNA immobilization:** Both culture plates containing transformed colonies and the hybridization membranes were numbered accordingly. The membrane was placed onto the culture plate for ~5 minutes, and then removed from plates. The membrane was then placed on the filter paper which is placed on the saran wrap and already soaked with 10% SDS. The surface of the membrane carrying the transferred bacterial colonies was facing upwards. The membrane was left on filter soaked with 10% SDS for 5 minute to allow lysis of bacterial cells. Then the membrane was placed on the filter papers soaked with denaturing solution (0.5N NaOH, 1.5M NaCl) and left for 5 minutes for denaturing DNA. The membrane was then transferred onto the filter paper soaked with neutralizing solution (1M Tris-HCl, 1.5 M NaCl, pH 7.5) for 5 minutes. Finally, the membranes were placed on a filter paper for drying and then placed under UV lamp for immobilization of DNA. The membrane was washed with 200 ml of washing solution (50 ml 20X SSC, 2 ml 10%
b. **Preparation of digoxigenin labeled DNA probe:** For preparing digoxigenin labeled probe, ~0.5 kb internal fragment of specific gene was amplified by PCR using appropriate primer sets, and the PCR product was purified by GeneClean Kit. 10 µl of purified PCR product was taken in a 1.5 ml eppendorf tube and mixed with 5 µl of dH₂O, and boiled for 5 minutes for denaturing the DNA, and was placed on ice for 2 minutes. 2 µl of hexanucleotide mixture, 2 µl of dNTP mixture and 1 µl of Klenow enzyme was then added to the denatured DNA and was incubated at 37°C overnight. The labeled DNA was precipitated with 2.5 µl of 4M LiCl and was vortexed. 75 µl of cold 100% ethanol was added, vortexed and was incubated at ~80°C for at least 30 minutes. It was then centrifuged at 15000 rpm for 10 minutes, and the pellet was rinsed with 500 µl of 70% of ethanol and centrifuged at 15000 rpm for 10 minutes. The supernatant was decanted and pellet was dried under vacuum. The pellet was finally dissolved with 50 µl of TE buffer.

c. **Hybridization:** The membranes were placed into hybridization bag, 20 ml of Dig-hybridization solution (5X SSC, 0.5% blocking reagent, 0.1% N-Lauroylsarcosine Na-salt, and 0.2% SDS) was poured and 50 µl of the prepared Digoxigenin labeled probe was added, all bubbles were removed and the bag was sealed. The bag was then incubated at 68°C overnight for hybridization.

d. **Washing the membrane:** The membranes were washed with 100 ml of 2X SSC, 0.1% SDS at room temperature on a shaker for 5 minutes. This washing step was repeated twice. The membranes were then washed twice (15 minutes for each washing) with 0.1X SSC and 0.1% SDS at 68°C. The membranes were washed with
100 ml of Buffer 1 (Tris-HCl 100 mmol/L, NaCl 150 mmol/L; pH 7.5) on a shaker at room temperature for 1 minute. Then the membrane was incubated with 100 ml of Buffer 2 (0.5 gm blocking reagent dissolved in 100 ml of Buffer 1; 0.5% (w/v)) and placed on a shaker at room temperature for 30 minutes. The membranes were washed again with Buffer 1 for 1 minute.

e. Detection of positive colonies: The membranes were then incubated with 20 ml of diluted antibody-conjugation solution (4 µl of AP antibody-conjugate mixed with 20 ml of Buffer 1; 5000x dilution), and were placed on a shaker at room temperature for 30 minutes. Unbound antibody-conjugate was removed by washing twice with 100 ml of Buffer 1 for 15 minute (each washing) at room temperature on a shaker. The membranes were then incubated with 10 ml of color solution [(45 µl of NBT-solution and 35 µl of X-phosphate solution into 10 ml of Buffer 3), Buffer 3 (Tris-HCl 1100 mmol/L, NaCl 100 mmol/L, MgCl₂ 50 mmol/L; pH 9.5)] using sealed plastic bag and kept in the dark. When the desired spots were detected, the corresponding colony on the culture plate was picked up and stocked as a positive colony having the plasmid carrying the desired insert.

2.2.11. Electrophoresis and photography

DNA or RNA samples were analyzed by electrophoresis in 0.8% to 1.0% agarose gel at a constant current of 5 V/cm, using a Mupid II electrophoresis apparatus (Cosmo bio Inc., Tokyo, Japan). The gel was visualized under UV 254 and 360 nm after staining for 20-30 minutes with 0.005% ethidium bromide solution. The photograph was taken by an Ultra-lum electrical transilluminator (Toyobo Co. Ltd., Osaka, Japan)
2.2.12. Polymerase chain reaction (PCR) conditions

DNA fragments used as probe in various hybridization analyses were amplified from their respective templates (C. perfringens chromosomal DNA) by PCR using the oligonucleotide primers in a Perkin Elmer DNA thermal cycler (USA) as described below: A reaction mixture of 50 µl containing 3 µl of 10X chromosomal DNA, 1 µl (10 pmol) of each primers, 5 µl of 10X Taq buffer, 4 µl of dNTP mixture and 0.25 µl of Taq polymerase and dH2O to mess up to 50 µl. The PCR condition used was 94°C for 3 minutes followed by 94°C for 1 minute and then with annealing temperature of 47-52°C for 1 minute and 72°C for extension for 1 minute or more (1 minute for each kb length). Amplification was carried out for 30 cycles.

2.2.13. Homology search program

To predict the putative function of the entA, entB, entC and entD, the nucleotide sequence was translated using the appropriate frame by MacMolley software. The translated amino acid sequence was used to search database for the homologue using BLAST-P program (Altschul, Gish et al. 1990). The highly homologous proteins from other bacteria in database were matched for predicted functions.

2.2.14. Total RNA preparation from C. perfringens

Pre-culture of C. perfringens strain 13/pJIR418 and other strains were grown on blood agar plate with appropriate antibiotics under anaerobic condition at 37°C overnight. The preculture was inoculated into 5 ml of GAM or 20 ml of 2X YT medium with antibiotics and was allowed to grow anaerobically at 37°C for 5-6 hours (for GAM) or 10 hours (for 2X YT medium). The culture was transferred to fresh GAM or 2X YT medium at a final concentration of 5% and was allowed to grow anaerobically at 37°C until OD at 600 nm
reached to 0.3~0.8. Two milliliter of culture was taken into eppendorf tube and centrifuged at 15000 rpm for 5 minute at 4°C. The supernatant was discarded and the pellet was suspended with 0.2 ml of solution A (0.5% SDS, 20 mM sodium acetate, 10 mM EDTA of pH 5.5). Then 0.2 ml of phenol solution was added and mixed well by pipetting. It was subjected to vigorous shaking at 65°C water bath for 5 minutes followed by centrifugation at 15000 rpm for 5 minute at 4°C. 100~125 µl of upper aqueous phase was taken into new tube and 2.5 volume of 100% cold ethanol was added and mixed by inversion and centrifuged at 15000 rpm for 5 minute at 4°C. The pellet was rinsed with 500 µl of cold 70% ethanol to remove any salt from the pellet and centrifuged. Finally, the pellet was dissolved in 30~40 µl of RNAse free dH2O. The concentration of RNA was measured at OD of 260 nm. Extreme precaution was taken during the whole procedure to prevent RNA degradation by using RNAse free environment, RNAse free solutions and using hand gloves.

2.2.15. **Northern blotting and hybridization**

The concentration of total RNA from was measured using OD at 260 nm. Requisite volume as 10 µg (in some cases 15 µg)/lane were calculated and made upto 10 µl/lane with RNAse free H2O. Same volume of sample buffer [2X TBE (Tris 10.8g, Boric acid 5.5g and EDTA 8.3g/ L), 13% Ficol pH 8.3, 0.01% Bromophenol blue and 7 M Urea] was added to each sample. It was mixed and incubated at 65°C for 5 minutes and placed on ice. Each 20 µl of denatured RNA solution was loaded in one lane of 0.8% agarose gel in TBE. Electrophoresis was done with a continuous current at 100 V until the dye reached the lower margin of the gel. RNA marker was also used on the same gel to calculate the size of desired bands. The gel was placed as upside down on 4 pieces of Whatmann paper that were immersed under 20X SSC (3M NaCl, 0.3 M Na-citrate) in the
custom made aluminium foil chamber. Nitrocellulose membrane that is same size of gel was placed on the gel and all bubbles in the interface were removed carefully and a piece of Whatmann paper was placed on the membrane. The gel was wrapped from all side by Saran wrap to prevent leakage of buffer from any side except through the gel. 10-15 pieces of paper towel was placed on the gel and light-weight (~300 g) was placed on it and left for 8-10 hours for effective transfer of RNA from gel to the membrane. The membrane was then separated from the gel and RNA was immobilized by UV light of CL-2000 Ultraviolet Crross-linker. The specific probe was prepared as follows: Desired nucleotide sequence of approximately 500 bp internal to specific gene was amplified with appropriate primer directed PCR. The PCR product was cleaned by GeneClean II kit. 2 µl of purified DNA was then denatured at 95°C for 5 minute and kept on ice and mixed with 10 µl of reaction buffer, 2µl of labeling reagent and 10 µl of working cross-linker solution of Alk-Phos direct (Amersham Pharmacia Biotech). It was then incubated at 37°C for 30 minutes and 30 µl of glycerol was added and mixed well by vortex. The probe was ready to use and stored at –20°C for future use. The membrane was then subjected to hybridization with specific probe (12µl) at 55°C in presence of 5 ml of hybridization buffer in Robins’ Science Hybridization shaker for 2-3 hours. After hybridization, the membrane was washed twice with primary wash buffer and twice with secondary wash buffer as was done in case of Southern analyses. The membrane was incubated with 500 µl of CDPStar detection reagent for 5 minutes and then it was wrapped with Saran wrap and was exposed to Hyperfilm. The film was later developed and the band size on the film was calculated by ‘Gel fragment sizer’ program of the Machintosh computer using the RNA marker band size as standard. The picture was
imported to the computer using conventional scanner and edited by Adobe photoshop software.

2.2.16. Computer assisted data analyses

The following software programs were used to analyze the experimental data in this study: Gel fragment sizer v 1.4 for calculating the size of electrophoretically separated DNA or RNA fragments; Genetyx-Mac V 7.1.2 and Mac Molley (Enter & List v 3.5.1, Translate v 3.5.2, Compare v 3.5.2) for analyses of DNA sequences; Oligo 4.01 (Primer analyses software) for designing oligonucleotide primers, Canvas 3.5 and Apple works for illustration, MS Excel for statistical works and Microsoft word 2000 for word processing.

2.3. Results

2.3.1. The putative enterotoxin genes (ent) are located on the chromosome

All the enterotoxigenic C. perfringens carry a single copy of enterotoxin gene (cpe) either in their chromosome or in the plasmid, and are responsible for food-borne and non-food-borne gastroenteritis, respectively. The newly identified four putative enterotoxin genes (ent) are located on the chromosome and their location on the chromosome is shown (Figure 2.1) and did not show any similarity with the well-studied cpe gene of C. perfringens. Initial version of annotation showed that these genes have similarity with the enterotoxin genes of B. cereus. Homology search across the known protein databases using BLAST-P (Altschul, Gish et al. 1990) revealed that these putative enterotoxin genes have similarities with peptidase, surface protein and enterotoxin genes of B. cereus, and the result is presented in Table 2.1. The cpe gene present in enterotoxigenic clostridia was
neither present in the chromosome nor in the plasmid of *C. perfringens* strain 13 as revealed by the complete genome sequencing (Shimizu, Ohtani et al. 2002).

### 2.3.2. Transcription of enterotoxin genes is independent of the *spo0A* in *C. perfringens* strain 13

The transcription of the *cpe* gene that is present in the chromosome or the plasmid of enterotoxigenic type A *C. perfringens* is tightly regulated by the *spo0A* gene and and its expression is linked with sporulation (Czeczulin, Collie et al. 1996; McClane 1996; McClane 1998). *C. perfringens* strain 13 is a natural *spo0A* negative (Huang, Waters et al. 2004) and it was investigated whether its newly identified putative enterotoxin genes (*ent*) are expressed in absence of Spo0A. Total RNA was prepared throughout the log phase and was subjected to Northern analyses. All four enterotoxin genes were expressed, and expression of *entA* was maximal during 2h and 3h, and *entB* and *entC* were mostly expressed during 2h whereas *entD* showed maximum expression during 1h and 2h (Fig. 2.2, 2.3). These results indicate that the expression of putative enterotoxin genes (*ent*) in *C. perfringens* strain 13 is independent of Spo0A.

### 2.3.2. VirR/VirS signal transduction system positively regulates *entA* and *entB* and negatively regulates *entC*

The role of VirR/VirS system on the regulation of these putative enterotoxin genes were investigated using total RNA prepared from wild type strain 13, TS133 (*virR* mutant) and *virR* complemented strains. Prepared RNA was subjected to Northern analyses. *entA* and *entB* showed reduced expression in TS133 (*virR* mutant) and this reduction was recovered in *virR* complemented strain, which was comparable to the wild type. This finding clearly indicates that these two enterotoxin genes are positively regulated by the
VirR/VirS system at transcriptional level (Fig. 2.2). Same experiment was carried out for entC and it showed increased expression in TS133 (virR mutant) compared to both wild type and virR complemented strain, and this finding is indicative of negative control of VirR/VirS system on the transcription of entC (Fig. 2.3). The transcription of entD did not show any visible change among the three strains used in this study, leading to the conclusion that its transcription is independent of the VirR/VirS system.

2.3.3. Cloning of enterotoxin genes in expression vector

Since these enterotoxin genes (nt) of C. perfringens strain 13 did not show any homology with the enterotoxin gene (cpe) present in other type A C. perfringens, it was aimed to clone these enterotoxin genes into expression vector (pTrc-His) under inducible promoters to induce their expression, and to purify the protein which would be used for investigating their toxigenic effects. All four enterotoxin genes were cloned successfully and were transformed into E. coli, but under IPTG induction, none of their protein was expressed as was determined by SDS-PAGE. The reason for their non-expression could not be explained, but it could be partly due to their potential toxicity, which might be too toxic for the host to survive and led to their non-expression in E. coli.

2.4. Discussion

Enterotoxin (cpe) gene, which is 1.6 kb long, is present in almost all types of enterotoxigenic C. perfringens (Table 1.1). The enterotoxin gene (cpe) can be either chromosomal- or plasmid borne (Cornillot, Saint-Joanis et al. 1995), and is responsible for food-borne and non-food-borne gastroenteritis, respectively (Brynestad, Sarker et al. 1995).
Completion of whole genome sequencing of type A *C. perfringens* strain 13 (Shimizu, Ohtani et al. 2002), revealed the presence of four putative enterotoxin genes in their chromosome (*entA*, *entB*, *entC* and *entD*), and homology search for the known protein database showed that these putative enterotoxin gene have similarity with bacterial surface protein, endopeptidase and enterotoxin of *B. cereus* (Table 2.1). Enterotoxigenic *C. perfringens* carry a single copy of the *cpe* gene either on the chromosome or on the plasmid and this gene is somewhat unstable and could be gained or lost suggesting that the gene was on a mobile element (Brynestad, Synstad et al. 1997). An IS 200-like element, IS1469, is almost always upstream of *cpe*, and IS 1470, a member of the IS 30 family is found both up- and downstream of *cpe* gene in the type A strain NCTC 8239 (Brynestad, Synstad et al. 1997). A genome-wide search showed the presence of possible insertion point for *cpe* gene in CPE 0397 but *cpe* gene is not present in the genome of *C. perfringens* strain 13, and thus it is naturally *cpe*-negative. Presence of four putative enterotoxin genes (*ent*) instead of *cpe* gene, which has similarity with enterotoxin of *B. cereus* made it worthy to examine their transcriptional regulation and possible function in *C. perfringens* strain 13.

The four putative enterotoxin genes,*entA* (CPE 1258), *entB* (CPE 1354), *entC* (CPE 0452), and *entD* (CPE 0606) which are 2865 bp, 1647 bp, 1875 bp, and 1905 bp, respectively, are located in the chromosome of *C. perfringens* strain 13. The putative protein products of these four genes did not show any similarity with the enterotoxin produced by other enterotoxigenic *C. perfringens*, but revealed similarity with enterotoxin of *B. cereus*. Presence of signal peptide sequence in the N-terminal of the protein products were revealed using PSORT (Shimizu, Ohtani et al. 2002)

In enterotoxigenic *C. perfringens*, sporulation-specific transcription regulator, *spo0A*, tightly regulates expression of the *cpe* gene, and mutation of *spo0A* results in cessation of
enterotoxin production and inability to form endospore, which can be recovered by spo0A complementation (Huang, Waters et al. 2004). *C. perfringens* strain 13 is naturally spo0A mutant and do not from endospore (Shimizu, Ohtani et al. 2002; Huang, Waters et al. 2004). The expression of enterotoxin (CPE) by *C. perfringens* NCTC 8239 was 1500-fold more in sporulating cultures than that of vegetative cultures (Czeczulin, Collie et al. 1996). It was reported that normal sporulation is required for CPE synthesis but CPE is not necessary for sporulation (Melville, Labbe et al. 1994; Zhao and Melville 1998). Identification of four putative enterotoxin genes in *C. perfringens* strain 13, which is different from cpe gene made it interesting to examine the relation between their expression and Spo0A. spo0A gene showed a premature termination in strain 13 when compared to the CPE (both chromosomal- and plasmid-borne) producing other type A *C. perfringens* including strains SM101, NCTC8239, F4969 and B11 (Huang, Waters et al. 2004). All four newly identified putative enterotoxin genes were expressed (entA was expressed mostly during 2 h, and 3 h, entB and entC, were expressed mostly at 2h and entD was mostly expressed during 1 h and 2 h) under normal growth conditions in naturally spo0A mutant *C. perfringens* strain13, which led to surmise the conclusion that the transcription of these enterotoxin genes are independent of Spo0A.

Many of the toxin and virulence genes have been reported to be regulated by the two-component VirR/VirS system (Lyristis, Bryant et al. 1994; Ba-Thein, Lyristis et al. 1996; Banu, Ohtani et al. 2000). Whether and how this VirR/VirS system regulates the expression of all four putative enterotoxin genes was investigated. Northern analyses showed that entA and entB are positively regulated, and entC is negatively regulated by, whereas entD is independent of VirR/VirS system. entA showed a single transcript which is ~2.9 kb long (ORF length is 2865 kb), mostly expressed during 1h and 2h, and positively regulated by the VirR/VirS system. entB is also expressed as a single transcript
of ~1.7 kb in length, mostly expressed during 1h and 2h, and showed positive regulation by the VirR/VirS system. entC produced a transcript which is ~2.6 kb long, and mostly expressed during 1h and 2h, and appeared to be positively regulated by the VirR/VirS system. A second lower band also appeared in Northern analyses which seems to correspond with the rRNA and different expression pattern, and was concluded as a non-specific band detected with the probe for entC. Though the ORF length of entC is 1875 bp, the presence of ~2.6 kb band detected in Northern analyses required further analyses of both upstream and downstream genes. The downstream gene is completely different and is located on the other strand of the chromosome, so it was excluded from further analysis but upstream gene showed a same band size of ~2.6 kb, but also a second band of ~1.0 kb, the origin of which could not be explained. The 2.6 kb band corresponds with the gene length of CPE 0451 (upstream gene) and CPE 0452 (entC). For entD, it was mostly expressed during 1h and 2h, and two bands (~3.4 kb and ~1.3 kb) were detected by Northern analysis. Upstream gene analysis showed a single band of ~3.4 kb in length. Taken these findings together, it was concluded that the upper band (~3.4 kb) is the product of transcription from CPE 0606 (entD) and CPE 0607 (downstream gene), and the lower band (~1.3 kb) present while probed for entD could be the result of RNA processing. entD was not found to be regulated by the VirR/VirS system.

All these putative enterotoxin genes showed similarity with the enterotoxin of B. cereus (Table 2.1). B. cereus is a Gram-positive, spore forming, motile, aerobic rod, commonly found in soil and water. It is capable of causing food poisoning by the formation of entero- and emetic toxins. So far, one emetic toxin (named cereulide) and three enterotoxins (named hemolysis BL, Enterotoxin-T and non-hemolytic enterotoxin) has been identified (Granum and Lund 1997). The hemolysis BL (HBL) enterotoxin complex consists of three parts (named B, L₁ and L₂) and all these parts are needed for activity.
(Beecher, Schoeni et al. 1995). The enterotoxin-T (becT) is a single component toxin (Agata, Ohta et al. 1995; Ombui, Schmieger et al. 1997), and recently, the third three component non-hemolytic enterotoxin (NHE) has been reported and characterized (Granum, Andersson et al. 1996; Granum and Lund 1997; Granum, O'Sullivan et al. 1999). Both hbl and nhe forms operon, and hbl operon is composed of 4 genes while nhe operon is comprised of 3 genes, and the transcription of the nhe operon is positively regulated by plcR, a gene that also regulates the expression of phospholipase C (PLC) in B. cereus (Granum, O'Sullivan et al. 1999). Much of the studies concerned to B. cereus enterotoxigenicity were focused mainly on the molecular epidemiology and toxin structure instead of mechanism of actions. In this study, it was aimed to investigate the toxicity of the putative enterotoxin genes of C. perfringens, and all the enterotoxin genes were cloned successfully into expression vector, pTrc-His, and was transformed into E. coli Top10 competent cells, but unfortunately, enterotoxin genes were not expressed under IPTG induction, so it could not be purified and the toxicity could not be investigated. The reason of non-expression could not be explained but it was assumed that E. coli did not express these genes to be protected from the potential toxicity to its own.

2.5. Conclusion

In conclusion, these experiments revealed that:

a. All four enterotoxin genes (ent) are present in the chromosome, but not in plasmid of C. perfringens.

b. These ent genes are different from the enterotoxin gene (cpe) of enterotoxigenic C. perfringens.
c. These enterotoxin (ent) genes are expressed under normal growth conditions, and independent of Spo0A and sporulation.

d. entA and entB are positively regulated, whereas, entC is negatively regulated by the VirR/VirS two-component system.
2.6. Tables and figures
Figure 2.1. Location of four putative enterotoxin genes (ent) in the genome of Clostridium perfringens.
Table 2.1. Results of comparison with protein database of the putative enterotoxins using BLAST-P

<table>
<thead>
<tr>
<th>Gene No.</th>
<th>Gene/product name</th>
<th>Homologous protein and organism(s)</th>
<th>% identity for amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPE 0452</td>
<td>entC</td>
<td>Cell wall associated hydrolases (Invasion-associated proteins (C. thermocellum ATCC 27405))</td>
<td>40% (21/52)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell wall endopeptidase (B. cereus ATCC 14579)</td>
<td>35% (26/74)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enterotoxin (B. cereus G9241)</td>
<td>32% (19/58)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enterotoxin (B. cereus ATCC 10987)</td>
<td>30% (28/91)</td>
</tr>
<tr>
<td>CPE 0606</td>
<td>entD</td>
<td>Bacteriolytic enzyme (B. clausii)</td>
<td>29% (115/391)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell wall hydrolase (B. thuringiensis)</td>
<td>27% (108/388)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enterotoxin (B. cereus ATCC 10987)</td>
<td>27% (108/390)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enterotoxin (B. cereus ATCC 10987)</td>
<td>20% (92/445)</td>
</tr>
<tr>
<td>CPE 1258</td>
<td>entA</td>
<td>Peptidase (B. anthracis)</td>
<td>22% (99/441)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell wall endopeptidase (B. cereus G9241)</td>
<td>26% (67/257)</td>
</tr>
<tr>
<td>CPE 1354</td>
<td>entB</td>
<td>Cell wall associated hydrolase (Invasion associated proteins (T. fusca))</td>
<td>38% (59/154)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enterotoxin (B. cereus ATCC 10987)</td>
<td>22% (118/519)</td>
</tr>
</tbody>
</table>
Figure 2.2. Diagram of the gene arrangement (not in scale) and Northern analysis of *entA* and *entB* genes using total RNA prepared from wild type strain 13 (1), *virR* mutant, TS133 (2), and *virR* complementation (3).
Figure 2.3. Diagram of the gene arrangement (not in scale) and Northern analysis of entC and entD genes using total RNA prepared from wild type strain 13 (1), virR mutant, TS133 (2), and virR complementation (3).
Chapter 3
Study of Extracytoplasmic function (ECF)-type sigma factors: approach to investigate their role in survival and virulence in *Clostridium perfringens*
3.1. Introduction

Extracytoplasmic function (ECF)-type sigma factors are a heterogeneous group of alternative sigma factors that regulate gene expression in response to a variety of conditions, including stress. This group was originally defined by conservation of sequence, and in some cases of function, of these proteins among several bacterial species (Lonetto, Brown et al. 1994). Many of these proteins, including those first described for *Escherichia coli* and *Pseudomonas aeruginosa*, have been shown to play a role in regulation of gene expression required for survival following exposure to stress (Erickson and Gross 1989; Deretic, Schurr et al. 1994; Raina, Missiakas et al. 1995). With the rapid expansion of bacterial genome sequence data, it has become apparent that many bacterial species have several genes that encode ECF-type sigma factors, although in most cases the functions of these proteins have not been defined. This has been evident that members of ECF subfamily of sigma factors share at least two properties. The first one consists in the regulation of extracytoplasmic functions: membrane, periplasm and extra-cellular. The second is their ability to respond to specific extra-cytoplasmic stimuli.

Until now, a general mechanism of regulation of ECF type sigma factors has not been proposed. Nevertheless, there seems to be a conserved mechanism of regulation between the ECF sigma factors and a cognate anti-sigma activity. Organizational analyses of these sigma factors in many bacteria showed that the gene encoding ECF-type sigma factor usually constitutes an operon, where the immediate downstream gene of the sigma gene encodes a cognate negative regulator, the anti-sigma factor, with or without more downstream genes encoding positive and negative regulators. Usually, the gene encoding
The ECF factor is the first gene of the operon and its transcription is autoregulated (Missiakas and Raina 1998). The precise interactions between sigma and cognate anti-sigma await further molecular and biochemical analyses, but at present, it is believed that sigma factors are sequestered by the cognate anti-sigma factors, and when the environmental signal is sensed by the membrane bound anti-sigma factors, it undergoes conformational changes and release the sigma factor which in turn causes transcriptional activation of a specific set of genes. It has been reported that anti-sigma factors are inserted in the cytoplasmic membrane in a fashion that positions the N-terminal domain in the cytoplasm, whereas the C-terminal part protrudes into the periplasmic space of Gram-negative bacteria. The C-terminal domain of anti-sigma factor could sense the signal and serve as a ‘signal-transducing’ domain, in a manner reminiscent of histidine kinase of the two-component regulatory system (Missiakas and Raina 1998).

Because the role of some sigma factors in regulating the interaction of bacteria with the extra-cellular environment and in the adaptation of bacteria to stress, these proteins are of special interest as potential regulators of virulence factors in bacterial pathogens (Fernandes, Wu et al. 1999).

In *E. coli*, it has been noted that presence of misfolded protein in the periplasm causes activation of its two-component regulatory system CpxA/CpxR (Raina, Missiakas et al. 1995; Danese and Silhavy 1997). *HtrA*, which encodes a periplasmic protease and degrades the misfolded protein, is under the transcriptional control of both CpxA/CpxR and $\sigma^E$ (an ECF-type sigma factor of *E. coli*) (Missiakas and Raina 1998). In *C. perfringens*, the two-component VirR/VirS system has been shown to regulate several toxins and virulence factors (Shimizu, Ba-Thein et al. 1994; Ba-Thein, Lyristis et al. 1996), and complete genome sequencing showed the presence of three putative ECF-type
sigma factors, designated as $\text{sig}X$, $\text{sig}V$ and $\text{sig}W$, which are not present in non-pathogenic $C. \text{acetobutylicum}$. Based on the facts discovered in other bacteria that ECF-type sigma factors can sense the environmental conditions and cause the necessary changes in cellular machinery to cope with the alteration and also might be involved in modulation of bacterial virulence as well as the finding of the involvement of two-component system in sensing the environmental conditions and virulence-gene regulation, a preliminary aim was set to design experiments to answer the following questions in $C. \text{perfringens}$:

1. Do the putative ECF-type sigma factors have organizational and structural similarity to the proven ECF-type sigma factors in other bacteria?
2. Do these ECF-type sigma factors influence bacterial growth and are they expressed under normal growth conditions?
3. Is there any regulatory interaction between ECF-type sigma factors and two-component VirR/VirS system under normal growth conditions?
4. Do they regulate the important toxin (alpha-, theta-, and kappa toxin) production?
5. Are these ECF-type sigma factors expressed under different environmental stressful conditions?
6. What are the genes regulated (positive/negative) by these ECF-type sigma factors under normal growth conditions (by DNA microarray experiments)?

3.2. Materials and methods

3.2.1. Bacterial strains and plasmids

$C. \text{perfringens}$ type A strains 13 (Mahony and Moore 1976), TS133 ($\text{vir}R$ mutant) (Shimizu, Ba-Thein et al. 1994), HK201 ($\text{sig}X$ mutant) (this study), HK202 ($\text{sig}V$ mutant)
(this study), HK203 (sigW mutant) (this study), *E. coli* DH5α (Sambrook, 1989), pUC19 (cloning vector) (Yanisch-Perron, Vieira et al. 1985), pJIR418 (*E.coli*-*C. perfringens* shuttle vector) (Sloan, Warner et al. 1992) were used for this study.

### 3.2.2. Media and culture conditions

GAM (Gifu anaerobic medium), LB (Luria-Bertani) medium, and BHI (Brain Heart infusion) were used to culture bacteria. LB plate (LB medium with 15 g L⁻¹ Bacto-agar), Blood agar plate (37g L⁻¹ Brain heart infusion with 15g L⁻¹ Bacto-agar and 75 ml L⁻¹ defibrintated sheep blood) containing respective antibiotics was used for bacterial inoculum. *E. coli* was cultured in LB medium at 37°C with vigorous shaking at 180-190 rpm. Aneropacks were used for *C. perfringens* plate growth at 37°C and broth culture was performed in GAM or 2X YT medium at 37°C (water bath/incubator) under anaerobic conditions in tube with air-tight cap. Ampicillin (50 µg ml⁻¹), Tetracycline (2.5 µg ml⁻¹), Chloramphenicol (20 µg ml⁻¹) and Erythromycin (25 µg ml⁻¹) were used appropriately in broth or agar medium.

### 3.2.3. Preparation of competent cell from *C. perfringens*

100 ml GAM, two 50 ml centrifuge tubes, and 15% (w/v) glycerol were autoclaved. Pre-culture solution (>5h) of *C. perfringens* strain 13 was made. 5 ml bacterial pre-culture was added to 100 ml of GAM. After 5h and 6h growth at 37°C, each 20 ml culture was centrifuged at 5000 rpm for 15 minutes. Pellet was dissolved in 2 ml of ice-cold 15% glycerol and transferred to 2 ml eppendorf tube. It was then centrifuged for 5 minutes at 15000 rpm and washing step of pellet by 15% glycerol was repeated twice. Finally pellet was dissolved in 1 ml of 15% ice-cold glycerol. Each 80 µl aliquots were distributed in 1.5 ml tube and stored at –80 °C for future use.
3.2.4. Electroporation to *C. perfringens* competent cells

Internal fragment of specific gene was amplified by PCR and was cloned into pUC118 and a marker for selection of clones (erythromycin resistant gene) was also cloned at appropriate location of the vector and was transformed into *E. coli* DH5α competent cell. The clone that contained the correct orientation was stocked and plasmid was extracted. The prepared plasmid was purified by ethanol precipitation. *C. perfringens* competent cell (80 µl) was placed on ice for 30 minutes. 1-3 µl of purified plasmid (1-5 µg DNA) containing the insert of interest was transformed to *C. perfringens* competent cell and was mixed well by pipetting. The mixture was transferred to pre-chilled 0.2 cm gap-cuvette (Bio-Rad) and kept on ice for 10 minutes. Then the cuvette was carefully wiped out to remove moisture on the outside of the tube and placed on the Bio-Rad Gene pulser electroporator. The voltage used was 2.5 Kv and resistance was 200 ohm. A brief electric pulse for a few seconds was applied and then the cuvette was immediately placed on ice for another 10 minutes. Electroporated cells were mixed with 500 µl GAM and incubated at 37°C incubator for 2-3 hours under anaerobic condition. 200 µl aliquot was spread on BHI-blood plates containing erythromycin (25 µg ml⁻¹). The plates were incubated at 37°C with aneropack for more than 12 hours. The single colonies that appeared on the plate are the requisite modified strain, which was subjected to further analysis for confirmation.

3.2.5. Construction of *sigX*, *sigV* and *sigW* mutant

Internal ~0.4-0.5 kb fragments from *sigX* (CPE 0359), *sigV* (CPE 0559) and *sigW* (CPE 0716) were amplified by PCR with appropriate primer sets using *C. perfringens* chromosomal DNA as template and was cloned into *Hinc*II or *Sma*I site of pUC19 or
pUC118 (TaKaRa, Japan) and resultant plasmids, were transformed into *E. coli* DH5α competent cells. Erythromycin resistant determinant (*erm*BP) was introduced into the plasmid and the orientation of the fragment was confirmed by restriction enzyme digestion and electrophoresis. The resultant plasmid, pHIK201 (for *sigX*), pHIK202 (for *sigV*) and pHIK203 (for *sigW*) were introduced into *C. perfringens* strain 13 by electroporation and single-crossing-over sigma mutants were selected on BHI-sheep blood agar plate containing erythromycin (50 µg ml⁻¹) and the insertion mutation was confirmed by Southern blot analysis. The resultant mutant strain was designated as HK201 (*sigX* mutant), HK202 (*sigV* mutant) and HK203 (*sigW* mutant).

**3.2.6. Southern blot hybridization**

Chromosomal DNA was prepared from both wild-type *C. perfringens* and the gene-specific mutant strain of *C. perfringens*, which was subjected to appropriate restriction enzyme digestion and was separated by 1% agarose gel electrophoresis and incubated in EtBr for DNA visualization, and a photo was taken with scale under UV light. The gel was then washed successively at room temperature with 0.1 M HCl for 20 minutes, 0.5 M Tris-HCl (pH 7.5), 3 M NaCl for 20 minutes and 0.5 M NaOH, 1.5 M NaCl for 20 minutes and then the gel was blotted with nylon membrane to transfer DNA from gel to the membrane and the DNA in the membrane was immobilized by UV cross-linker. The membrane was hybridized with AlkPhos labeled gene-specific probe (10 µl) at 55°C for more than 2 hours. The membrane was then washed with primary wash buffer (2M urea, 0.1% SDS, 50 mM Na-phosphate pH 7.0, 150 mM NaCl, 1 mM MgCl₂ and 0.2% blocking reagent) at 55°C twice for 10 minutes each. Then the membrane was washed twice at room temperature for 5 minutes each with secondary wash buffer (121 g Tris, 112 g NaCl, 2 ml/L 1 M MgCl₂ pH 10.0). The membrane was then incubated with 500 µl
of CDPStar detection reagent (Amersham Pharmacia Biotech) for 5 minutes. Finally, the membrane was wrapped by saran wrap and placed under Hyperfilm (Amersham pharmacia) and later the film was developed and the bands of DNA were analyzed by “Gel fragment sizer” software.

3.2.7. Bacterial growth curve

For checking bacterial growth pattern GAM was used. From primary culture, 5% was of the culture was transferred to the new medium with appropriate antibiotic. The initial reading (0h) was taken at optical density (OD) 600 nm. In the same way, reading was taken at every hour until the bacteria entered into the stationary phase. The growth curve was plotted using Microsoft Excel program.

3.2.8. Homology search analysis

To predict the putative function of the \textit{sigX}, \textit{sigV} and \textit{sigW}, the nucleotide sequence was translated using the appropriate frame by MacMolley software. The translated amino acid sequence was used to search database for the homologue using BLAST-P program (Altschul, Gish et al. 1990). The highly homologous proteins from other bacteria in database were matched for predicted functions.

3.2.9. DNA microarray experiments

This experiment requires several steps of preparation.

A. Preparation of probe (labeled DNA):

For microarray experiment, total RNA was prepared from the bacteria at different specific time point. Forty microgram of total RNA was used for each sample preparation. In each sample RNA, calculated volume of dH$_2$O was added to make the
final volume of 20 µl and then, 2 µl of random primer was added. For each sample, two of 1.5 ml eppendorf tubes were taken and labeled as Cy 5 (Blue) and Cy 3 (Red). Prepared RNA samples were distributed to the eppendorf tubes and were mixed well, and then subjected to denaturation at 68°C for 5 minutes followed by annealing at 42°C for 5 minutes. In a separate tube, 5X buffer: 8 µl/sample, 0.1 DTT: 4 µl/sample, 10X dNTP: 4 µl/sample and RNAsin (ribonuclease inhibitor): 0.1 µl/sample were taken on ice and mixed well. Just before use, Power script (Reverse transcriptase) (Clontech) was added as 2 µl/sample and mixed by pipette. 18 µl of this prepared reaction mixture was added to each RNA sample, mixed by pipette and was incubated at 42°C for 1 hour for strand extension reaction to produce DNA from RNA. 5 µl of 0.5 M EDTA was added to stop reaction, mixed well and 10 µl of 1 M NaOH was added and incubated at 60°C for 30 minutes to remove/digest extra RNA from the solution. Then, 25 µl of 1M Tris-Hcl (pH: 7.5) was added, mixed well and was filtered through Microcon YM-30 (Centifugal filter device) to separate molecules of > 300 KD M' and centrifuged at 20°C at 14000 rpm for 5 minutes. 100 µl of dH₂O was added in each column and centrifuged at 20°C at 14000 rpm for 5 minutes and this step was total for four times. Finally, 30 µl of dH₂O was added to each column and mixed very carefully and the column was put upside-down onto new Microcon-30 tubes and centrifuged at 20°C for 1 minute at 5000 rpm and transferred into a new eppendorf tube and was completely dried under vacuum for 40-50 minutes. After complete drying, 10 µl of 50 mM NaHCO₃ (pH: 8.2-8.3) was added and kept at room temperature for 3 minute. Then, 40 µl of Dimethyl sulfoxide was added to each Cy 5 monofunctional reactive dye and Cy 3 monofunctional reactive dye, and was mixed very well. 10 µl of Cy 5 was added in each blue sample tabue and 10 µl of Cy 3 was
added in each red sample tube, and was mixed well. The tubes were kept in a dark place for 60 minutes with periodic mixing at every 15 minutes. Then, 250 µl of dH$_2$O was added to 72 mg of hydroxylamine hydrochloride, mixed well and 10 µl of this solution was added to each sample and the sample was kept at room temperature for 15 minutes in the dark place. Same sample containing Cy 5 and Cy 3 in different tubes were mixed in one tube and 400 µl of Buffer PB (binding buffer) (Qiagen) and was mixed well until the color became homogenous. The mixture was then filtered through the columns of QIAquick PCR purification kit (250) and was centrifuged at 20°C for 1 min at 13000 rpm, the flow through was discarded and the column was placed on fresh tubes. 500 µl of Buffer PE (Wash buffer) was added to each column, and centrifuged at 20°C for 1 minute at 13000 rpm. The wash step was repeated again. In the next step, only the column was centrifuged (no buffer was added) to remove the ethanol in buffer PE, and then the column was kept at room temperature for 5 minute to dry up ethanol. Then, 25 µl of buffer EB (elution buffer) was added to the column, waited 1 minute, and centrifuged at 13000 rpm at 20°C for 1 minute, and the elution step was repeated again and the flow-through was collected for use. This is the probe (labeled DNA) that will be used for hybridization of the target nucleotides printed on the glass-slides.

**B. Preparation of slides:**

The special microscopic glass-slides were used for printing the target nucleotide sequences. All 2660 ORFs of the complete genome of C. perfringens were cloned into suitable cloning vector (TOPO 10) and the plasmids carrying the target sequencing were PCR amplified and correct insertion was confirmed by agarose gel-electrophoresis. The inserts (each ORF) was printed onto the specially treated glass-
slided by custom-made robots. The glass slides were preserved in strictly maintained environment. These slides that carried the target sequences were used for microarray experiment. One slide was used for one sample of RNA. The slides were first dried under UV light (UV crosslinker) to immobilize the printed DNA. The slides were then taken on a slide hanger, keeping the slides vertically. A solution to treat the slides was made as follows: 5.4 gm of succinate anhydride was dissolved into 340 ml of N-methyl-pyrolidone in a VIDTEC glass beaker, and was put on a stirrer (5-10 minutes) to be completely dissolved. The slide hanger carrying slides was placed into the beaker containing that solution and 38 ml off borate buffer was poured into that beaker. The hanger was shake several times vertically to increase the washing efficiency of the slides and was left in that solution for 30 minutes. The hanger was taken out of the buffer, and buffer was soaked out from hanger and slides by slowly shaking on the Kim paper. The hanger containing slides were washed in water by shaking into a water-containing beaker and were soaked again by Kim paper. Washing step was repeated twice. Then, the slide-hanger carrying slides was then placed into boiling water for 5 minutes, and then, soaked by Kim paper. Then the hanger was shake in 100% ethanol for 1-2 minutes and soaked on Kim paper. The slides are now prepared for use and were transferred to a slide-stand and were kept at room temperature for 5-10 minutes to dry.

C. Making probe solution:

To make probe solution, 20X SSC: 10 µl/sample, 10% SDS: 2 µl/sample, and water: 3 µl/sample were taken into a 1.5 ml tube and it will form precipitate. This solution was then incubated at 65°C to dissolve the precipitate that was formed in the solution. 25 µl of respective probe (labeled DNA) was taken in separate tubes and 15 µl of
probe solution was added to each probe and mixed well by pipette, and was placed at 65°C for 2-5 minutes.

D. Hybridization:
This step is to hybridize the probe with specific target DNA sequences that is printed on the glass-slides. ~38 µl of probe was poured on the one end of the glass-slide (same side where DNA targets are printed). A slide-cover was placed, and the probe spread homogenously on the printed area of the glass-slides and extreme caution was adopted to avoid formation of any bubble in the interphase of the slide and cover slip. Each slides were numbered corresponding to the sample number (probe number). 7 µl of water was placed inside the hybridization cassette to keep it humid. The slide was then placed on the hybridization cassette and the cassette was locked up by the cassette-cover. The hybridization cassette was then placed inside a plastic box, which is already covered with wet Kim paper, the plastic box was closed with its lid to make airtight and was incubated at 60°C overnight for hybridization.

E. Washing the slides:
This step required many washing steps to read the signal effectively from the slides. Briefly, the slides were taken out of the hybridization cassette and were hold by a forceps in 200 ml of 2X SSC+ 0.2% SDS wash buffer. The slide covers will automatically slip-down from the glass-slides. The slides were then placed in a slide-hanger, which was suspended from the time-regulated rotator to shake the slides inside the buffer-containing beaker. The slide was left to wash in the same buffer for 7 minutes. This same washing step was repeated in same buffer for 4 times (each step7 minutes) in different beakers. Then, the slides were transferred to a new hanger
and was washed in the same way in 0.2X SSC+ 0.2% SDS wash buffer for 7 minutes. This washing step was repeated for 3 times. Another three beakers, each containing 200 ml of the above wash buffer were placed in 60°C water bath. The slides were washed in that buffer in each beaker for 15 minutes. Again, four beakers were taken, each filled with 200 ml of 0.2% SSC. The slides were washed slowly in each beaker. Now, two beakers each containing 200 ml of 100% ethanol were taken. The slides were washed in both beakers. Then, the slides were centrifuged quickly at 800 rpm for 3 minutes at 20°C. The slides were checked for any kind of cloudiness. In presence of cloudiness, some washing steps were repeated until the slides became very clear since this cloudiness interferes in the images while the slides are scanned. The washed slides were kept in a box to avoid sunlight.

F. Scanning of the slides and data analysis:

The slides were scanned immediately and the images were transferred to the computer as TIFF images. The mean Cy 3 and Cy 5 fluorescence intensities for each spot were calculated with the background taken as the average of the intensities of negative control DNA spots. After subtracting the background from from all the intensities of the C. perfringens gene spots, and their normalization using total Cy 3 and Cy 5 intensities, the expression ratios were calculated using Gene Spring software. MS Excel was used to analyze the data. Three-fold deviation from the mean value was considered significant for these studies.

3.3. Results

3.3.1. Location and organization of ECF-type sigma factors in C. perfringens genome.
The three ECF-type sigma factors present in the genome of *C. perfringens* are *sigX* (CPE 0359), *sigV* (CPE 0559) and *sigW* (CPE 0716), and 533 bp, 572 bp, and 515 bp in length, respectively. Their location in the genome is shown in figure 3.1. To know the putative function of the protein products of these ECF-type sigma factors, BLAST-P (Altschul, Gish et al. 1990) program was used to search the protein database and the proteins in other bacteria showing significant homology is shown in Table 3.1. It has been reported that anti-sigma factor genes are usually present immediately downstream of the ECF-type sigma genes (Missiakas and Raina 1998; Helmann 1999). To investigate that possibility in *C. perfringens*, showed that all three ECF-type sigma factors have downstream gene which encode for putative/hypothetical protein but their structural analysis and protein products’ homology (Table 3.1) showed that they have homology with other known anti-sigma proteins. Diagrammatic presentations of these sigma factors with possible anti-sigma genes are shown (Fig. 3.2).

### 3.3.2. Mutation of ECF-type sigma factor does not affect bacterial growth

The investigation to elucidate the precise role of ECF type sigma factors in bacteria is still at its infancy. To know if there is any role of these sigma factors on bacterial growth kinetics under normal growth conditions, the growth curves were plotted using wild type, respective sigma mutants and their complemented strains (Fig. 3.3). This showed apparently no effect of ECF sigma factors on the bacterial growth under growth conditions used in this study (i.e., using GAM at 37°C in anaerobic condition). This finding led to conclude that ECF type sigma factors do not play any visible role in bacterial growth in normal conditions which is in well concordance with the
presupposition based on the available scientific informations from other published reports.

3.3.3. **ECF type sigma factors are not expressed and are not regulated by the VirR/VirS system**

To investigate the expression of ECF type sigma factors, normal growth conditions for *C. perfringens* were used. Total RNA was prepared during log phase and was subjected to Northern analyses and was hybridized separately with specific probes for three different sigma factors. This experiment did not detect any bands specific to the ECF-type sigma factors (data not shown). Since these sigma factors were not expressed under normal growth conditions, investigation of the role of VirR/VirS on their expression under normal growth conditions, using wild type and *virR/virS* mutant strain (TS133) also failed to detect any transcript for both wild type and *virR/virS* mutant strains (data not shown). This finding indicates that the under normal growth conditions, ECF-type sigma factors are neither expressed nor regulated by the VirR/VirS system.

3.3.4. **Important clostridial virulence genes (*plc*, *pfoA* and *colA*) are not regulated by the ECF-type sigma factors.**

As mentioned earlier, ECF-type sigma factors have not been reported in human non-pathogenic *C. acetobutylicum* (Nolling, Breton et al. 2001), which also does not produce these virulence factors and toxins (alpha-, theta-, and kappa toxin), the preliminary interest was to investigate any relation between these virulence genes and ECF-type sigma factors. To do this, total RNA was prepared at 1h, 2h and 3h (the expression of these toxin genes peak during 1h and 2h) using wild type and respective ECF-type sigma mutants. Total RNA was used for Northern analysis and was hybridized with the probes
for those virulence genes. All three genes for alpha toxin (Fig. 3.4), theta toxin (Fig. 3.5) and kappa toxin (Fig. 3.6) showed no difference in their expression in wild type and ECF type sigma mutants. This finding apparently indicates that these three clostridial toxin genes are not regulated by the ECF-type sigma factors under the conditions used in this study, but it was not possible to draw the conclusions from this finding due to some perplex interaction among the sigma factors themselves as well as for some intricate, overlapping promoter sequence specificity for the sigma factors (described in ‘discussion’ section)

3.3.5. Role of various environmental stresses on ECF-type sigma factor expression.

Since ECF-type sigma factors were not expressed under normal growth conditions, several altered environment, that is, stress stimuli were applied to investigate the condition that lead to the expression of these three sigma factors. For these experiments, the bacteria was grown at 37°C in GAM under anerobic condition at 37°C until the OD at 600 nm reached ~0.5 (log-phase) and then was expoed to several stresses including heat shock (50°C), cold shock (16°C), acid shock (pH: 4.0), alkaline shock (pH: 9.0), ethanol shock (9% v/v), osmotic shock (1.2 M NaCl) and oxidative shock (50 µM H₂O₂). Total RNA was prepared after 10 minute and 30 minute of exposure to shock, and was subjected to Northern anlyses, which was probed for all three ECF-type sigma factors (data not shown). None of the above stresses induced the expression of these sigma factors.

3.3.6. DNA microarray experiments for ECF-type sigma factor mutants

Neither normal growth condition, nor selected seven stresses induced expression of three ECF-type sigma factors in C. perfringens. In a Gram positive, aerobic, spore
forming soil bacteria, \textit{B. subtilis}, much experimentation has been reported to understand the promoter region and promoter specificity determinants for ECF-type sigma factors (Huang, Decatur et al. 1997; Huang, Fredrick et al. 1998; Huang, Gaballa et al. 1999; Huang, Gaballa et al. 1999; Huang, Gaballa et al. 1999; Huang, Gaballa et al. 1999; Qiu and Helmann 2001; Cao and Helmann 2002; Cao, Kobel et al. 2002; Cao, Salzberg et al. 2003), but very little is known about the conditions which induces their expression. To investigate the global regulation and which genes are regulated by these three ECF-type sigma factors under normal growth conditions in \textit{C. perfringens}, DNA microarray experiments were carried out at whole genome scale using total RNA (prepared at 2h) from both wild type and ECF-type sigma mutants and the important genes appeared to be regulated by them are presented (Table 3.2).

\section*{3.4. Discussion}

The extracytoplasmic function (ECF)-sigma factors are member of a subfamily of the $\sigma^{70}$ family of sigma factors, and are structurally distinct group of proteins that control a great variety of functions often involving the secretion or uptake of macromolecules or ions and responses to a variety of extracellular stress signals (Lonetto, Brown et al. 1994; Missiakas and Raina 1998). This new subfamily of sigma factors were first designated and identified in \textit{Streptomyces coelicolor} (Lonetto, Brown et al. 1994) based on the sequence alignment with the representative members of $\sigma^{70}$ family, and this subfamily showed significant divergence from other $\sigma^{70}$ family members, most noticeably in the regions 2.4 and 3. Bacterial genome sequencing has revealed numerous new members of this class of $\sigma$ factors including seven in \textit{Bacillus subtilis} (Kunst, Ogasawara et al. 1997) and ten in \textit{Mycobacterium tuberculosis} (Cole, Brosch et al. 1998). Determining the roles
of these numerous regulatory proteins is a formidable challenge. None of the ECF-type sigma factors in *B. subtilis* corresponds to known regulatory loci, despite extensive genetic analysis of this organism, which suggests that the functions controlled by these proteins are not relevant in standard laboratory culture conditions, that the sigma factors are redundant in function or both (Huang, Gaballa et al. 1999).

Organizationally these sigma factors usually form an operon where the first gene codes for the sigma factor, followed by downstream gene, which usually codes for the anti-sigma factors with or without presence of other positive and negative regulators and they are co-transcribed (Missiakas and Raina 1998). In most cases, these sigma factors are positively autoregulated (Lonetto, Brown et al. 1994; Missiakas and Raina 1998). In the absence of an external signal, the sigma factor is held in an inactive, stoichiometric complex with an anti-sigma, often located in the cytoplasmic membrane. By virtue of its trans-membrane disposition, the anti-sigma is poised to activate a transcriptional response signaled by the presence of molecules external to the cytoplasmic membrane (Helmann 1999). The organization of putative ECF-type sigma factors in *C. perfringens* were analysed and is presented schematically in figure 3.2. *sigX* is present on the negative strand in the chromosome, whereas both *sigV* and *sigW* are present on the positive strand. Putative anti-sigma factors were present just downstream of the sigma genes in all three cases. Mutation analysis of these sigma factors revealed that these sigma factors plays not role on bacterial growth profile under normal conditions.

In *E. coli*, it has been noted that presence of misfolded protein in the periplasm (e.g., due to heat shock) causes activation of its two-component regulatory system CpxA/CpxR (Raina, Missiakas et al. 1995; Danese and Silhavy 1997). *HtrA*, which encodes a periplasmic protease and degrades the misfolded protein, is under the transcriptional
control of both CpxA/CpxR and $\sigma^E$ (an ECF-type sigma factor of *E. coli*) (Missiakas and Raina 1998). The reason for such redundancy is not known, but it is conceivable that the two signaling mechanisms are devoted to monitoring the folding state of distinct subsets of proteins. It is interesting that both ECF factors and two-component signal transduction system respond to extracytoplasmic stimuli in general, but have developed sensing devices that are very different. Both the two-component system and ECF-type sigma factors were activated while the bacteria was under stressful environment. Whether and how these systems work in *C. perfringens*, the relation between two-component VirR/VirS system with any of three ECF-type sigma factors were investigated by Northern analysis under normal growth conditions, but as mentioned earlier, the ECF-type sigma factors are not usually expressed under normal growth conditions, and no transcripts were detected by Northern analysis, leading to conclusion that there is no regulatory interaction between VirR/VirS with any of the ECF-type sigma factors, at least under the conditions used in this study. In *B. subtilis*, it has been reported that *sigX* is expressed in late-log phase, whereas *sigW* is expressed during late-log to early-stationary phase, and the expression of *sigW* is regulated by both growth phase and medium composition (Huang, Fredrick et al. 1998; Huang, Gaballa et al. 1999)

It has been speculated that ECF-type sigma factors might be potential regulators of virulence factors in bacterial pathogens (Fernandes, Wu et al. 1999), and since these sigma factors are absent in non-pathogenic *C. acetobutylicum* (Nolling, Breton et al. 2001), it was a preliminary interest to investigate any relation between these sigma factors and virulence genes in *C. perfringens*. To examine that assumption, Northern analyses were done with total RNA prepared from both wild type and all ECF-type sigma mutants, and were hybridized with probes specific for three toxins, alpha-, theta-, and
kappa toxin. The result did not show any obvious regulation on those three toxins production at transcriptional level under normal growth conditions. These negative findings do not automatically exclude the other possible subtle molecular interactions among the sigma factors and their target genes. The ECF-sigma factors, \textit{sigX} and \textit{sigW}, in \textit{B. subtilis} share partially overlapping promoter regions of their own regulons, and mutation of either \textit{sigX} or \textit{sigW} neither affected viability nor resulted into any obvious phenotypes and the mutants showed normal behaviour for sporulation, competence and most other post-exponential-phase functions (Huang, Fredrick et al. 1998; Huang, Gaballa et al. 1999; Qiu and Helmann 2001). In \textit{B. subtilis}, it was also shown that promoters recognized by \textit{sigX} and \textit{sigW} have similar $-35$ elements but are distinguished by different base preferences at the two key positions within $-10$ region. Changes in the $-10$ element are sufficient to switch a promoter from \textit{sigX} to the \textit{sigW} regulon and vice versa, but context effects clearly play an important role in determining promoter strength (Qiu and Helmann 2001). It is also reported that \textit{sigX} and \textit{sigW} regulons are mutually antagonistic in \textit{B. subtilis}: a decrease in \textit{sigX} activity leads to increased expression of \textit{sigW}-dependent genes, conversely, an increase in \textit{sigX} activity decreases the \textit{sigW}-dependent expression (Huang, Gaballa et al. 1999). If that is true for \textit{C. perfringens}, mutation of a sigma factor will not produce any obvious phenotype and the effect of mutation will be, at least partially, compensated by the other sigma factors. So, the findings that the mutation of an ECF-type sigma factor apparently did not affect the expression of clostridial virulence genes do not directly exclude the possibility of their regulation. If the sigma factors share overlapping promoter specificity of the target genes of their regulons, the effects of mutation of one sigma factor will be taken over by the increased expression of another sigma factor, and the effect of the mutation will not be obvious by Northern analyses. Though it could not be concluded at this point that what is
true for *C. perfringens*, more extensive studies are required including use of double- or multiple sigma mutant to unravel the existing interaction and regulation.

ECF-type sigma factors has been reported to be expressed under various stresses in other bacteria and mutation of respective sigma factors resulted in reduced survival (Craig, Nobbs et al. 2002) and virulence (Testerman, Vazquez-Torres et al. 2002) under those stressful conditions. In *B. subtilis* alkaline shock (pH:8.9) induced ECF-type sigma W (Wiegert, Homuth et al. 2001), and high salt concentration (0.7M NaCl) induced ECF-type sigma M (Horsburgh and Moir 1999), and in *M. tuberculosis*, ECF-type sigma H is induced by heat shock and oxidative stress (Manganelli, Voskuil et al. 2002). Recently, it was reported that ECF-type sigma X regulates the modification of cell envelope in *B. subtilis* in presence of cationic animicrobila peptides (Cao, Helmann et al. 2004). Since there was no expression of ECF-type sigma factors under normal growth conditions, several stresses (i.e., heat-, cold-, acid-, alkaline, ethanol-, and oxidative shock) were applied to study which of the stimulus can cause expression of these ECF-type sigma factors in *C. perfringens*. Contrarary to the anticipation, none of the stresses used in this study showed induction of any of these three ECF-type sigma factors.

Though it is speculated, and in several bacteria it was reported that ECF-type sigma factors are expressed under stress, and failure to detect their expression in *C. perfringens* using variety of stresses, DNA microarray experiments were planned to examine the role of these sigma factors on the genes at whole-genomic scale. Total RNA was prepared at 2h (early-mid log phase) from both wild type and respective ECF sigma mutants, which were cultured in rich medium (GAM) under normal growth conditions. Each experiment was repeated twice and the genes regulated by each of these sigma factors are presented in Table 3.2. For all three ECF-type sigma factors, numbers of positively regulated genes were at least twice more than that of the negatively regulated genes. In general, most of
the positively regulated genes are presumed to be involved in carbohydrate transport and metabolism, energy production and conversion, whereas, negatively regulated genes are mainly involved in cell envelope/outer membrane biogenesis. Majority of the genes commonly regulated by all three ECF-type sigma factors, though the strength of regulation was different. This finding suggests that these three ECF-type sigma factors may share common genes in their regulons, but the molecular mechanism of this shared regulon was neither addressed nor determined here. The microarray experiments were carried out under normal growth conditions, but not under stresses, so it is important to recall that ECF-type sigma factors are usually expressed under conditions other than normal growth environment. So, the findings from microarray may not be very specific or this regulation may not be dire important for bacterial survival and growth. As an example, *myo*-Inositol operon was positively regulated by all three ECF-type sigma factors, more prominently by SigX and SigW, but analysis of this operon (Chapter 4) showed that the operon is not expressed under normal growth conditions (in presence of sufficient glucose) and only expressed under glucose deprivation. Microarray data showed that the *myo*-Inositol operon is regulated by these sigma factors under normal growth conditions. Though the reason for these discrepancies could not be explained in details, but one possibility is that the amount of that is insignificant for Northern analyses might be significant for microarray experiment. Another possibility is the result obtained from microarray experiments for *myo*-Inositol operon could be non-specific, since the promoter region analyses for the *myo*-Inositol operon is not convincing enough to consider that the operon, in general, is positively regulated by these three sigma factors under normal growth conditions (Chapter 4). To make a conclusion from this microarray result will require more supportive experiments.
3.5. Conclusion

Due to the absence of three ECF-type sigma factors in non-pathogenic \textit{C. acetobutylicum}, it was speculated that they might play some role in the virulence of \textit{C. perfringens}. Through above-mentioned experiments, it was found and concluded that:

1. ECF-type sigma factors do not affect normal growth and are not expressed under normal laboratory growth conditions, at least during log-phase.
2. VirR/VirS system does not regulate the expression of these three sigma factors under the conditions used in this experiment.
3. Apparently clostridial virulence genes are not under control of these three ECF-type sigma factors, because all the virulence genes are expressed through early-to-mid-log phase but these sigma factors are not expressed during that period.
4. Stresses including heat-, cold-, acid-, alkaline-, osmotic-, and oxidative shocks do not cause induction of these sigma factors.
5. Under normal growth environment, these sigma factors might have roles in energy conversation and cell wall biogenesis; confirmation of microarray finding requires additional experiments including Northern analyses.
3.6. Figures and Tables
Figure 3.1. Location of the three ECF-type sigma factors in the genome of *Clostridium perfringens*
Table 3.1. Results of comparison with protein database of the ECF-type sigma factors and putative cognate anti-sigma factors using BLAST-P

<table>
<thead>
<tr>
<th>Gene No.</th>
<th>Product name</th>
<th>Homologous protein and organism(s)</th>
<th>% identity for amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPE 0359</td>
<td>Probable ECF-type sigma factors</td>
<td>RNA polymerase ECF-type sigma factor (B. cereus ATCC 14579)</td>
<td>49% (78/157)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RNA polymerase, ECF subfamily (B. anthracis)</td>
<td>48% (76/157)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RNA polymerase sigma factor (C. tetani E88)</td>
<td>41% (63/151)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ECF-type sigma factor negative effector</td>
<td>21% (57/269)</td>
</tr>
<tr>
<td>CPE 0358</td>
<td>Hypothetical protein</td>
<td>Possible ECF-type sigma factor negative effector (B. thuringiensis)</td>
<td>21% (58/269)</td>
</tr>
<tr>
<td>CPE 0559</td>
<td>sigV</td>
<td>Possible RNA polymerase sigma factor, ECF subfamily (B. thuringiensis)</td>
<td>42% (73/171)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RNA polymerase sigma factor, SigV (C. tetani E88)</td>
<td>45% (77/170)</td>
</tr>
<tr>
<td>CPE 0560</td>
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<td>Conserved protein (C. tetani E88)</td>
<td>22% (58/255)</td>
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<td></td>
<td></td>
<td>ECF-type sigma factor negative effector (B. thuringiensis)</td>
<td>30% (22/71)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ECF-type sigma factor negative effector (B. cereus ATCC 14579)</td>
<td>30% (22/71)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ECF-type sigma factor (B. thuringiensis)</td>
<td>50% (78/155)</td>
</tr>
<tr>
<td>CPE 0716</td>
<td>sigW</td>
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<td></td>
<td></td>
<td>RNA polymerase sigma factor (C. tetani E88)</td>
<td>43% (69/158)</td>
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<tr>
<td>CPE 0717</td>
<td>Hypothetical protein</td>
<td>ECF-type sigma factor negative effector (B. cereus ATCC 14579)</td>
<td>26% (75/282)</td>
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<td></td>
<td></td>
<td>Conserved protein (C. tetani E88)</td>
<td>23% (89/373)</td>
</tr>
</tbody>
</table>
Figure 3.2. Schematic presentation of three ECF-type sigma factors and their putative cognate anti-sigma factors in the genome of Clostridium perfringens (figures not in scale)
Figure 3.3. Comparison of bacterial growth pattern in ECF-type sigma mutants and respective mutated sigma complemented strains
Figure 3.4. Northern analysis to investigate the role of ECF-type sigma factor X on the expression of three important toxin genes (alpha, theta and kappa) in *C. perfringens* (Lane 1: Wild type, strain 13, Lane 2: sigX mutant, Lane 3: sigX complementation).
Figure 3.5. Northern analysis to investigate the role of ECF-type sigma factor V on the expression of three important toxin genes (alpha, theta and kappa) in *C. perfringens* (Lane 1: Wild type, strain 13, Lane 2: *sigV* mutant, Lane 3: *sigV* complementation).
Figure 3.6. Northern analysis to investigate the role of ECF-type sigma factor W on the expression of three important toxin genes (alpha, theta and kappa) in *C. perfringens*. (Lane 1: Wild type, strain 13, Lane 2: *sigW* mutant, Lane 3: *sigW* complementation).
Figure 3.7. DNA microarray analysis of $\text{sigX}$ mutant at 2 hour
Table 3.2.(a). Genes positively regulated by \textit{sigX}

<table>
<thead>
<tr>
<th>Gene No</th>
<th>Gene name</th>
<th>Putative gene product</th>
<th>COG*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPE 0085</td>
<td>\textit{mdh}</td>
<td>Probable alcohol dehydrogenase</td>
<td>C</td>
</tr>
<tr>
<td>CPE 0088</td>
<td>\textit{iolB}</td>
<td>Myo-Inositol catabolism protein, IolB</td>
<td>-</td>
</tr>
<tr>
<td>CPE 0096</td>
<td>-</td>
<td>Propionate CoA-transferase</td>
<td>I</td>
</tr>
<tr>
<td>CPE 0097</td>
<td>\textit{acdS}</td>
<td>Acyl-CoA dehydrogenase</td>
<td>I</td>
</tr>
<tr>
<td>CPE 0310</td>
<td>-</td>
<td>Probable lactate permease</td>
<td>C</td>
</tr>
<tr>
<td>CPE 0312</td>
<td>\textit{etfA}</td>
<td>Electron-transfer flavoprotein alpha subunit</td>
<td>C</td>
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<td>CPE 0313</td>
<td>-</td>
<td>Probable glycolatte oxidase subunit</td>
<td>C</td>
</tr>
<tr>
<td>CPE 0317</td>
<td>\textit{fucK}</td>
<td>Probable fucokinase</td>
<td>G</td>
</tr>
<tr>
<td>CPE 0318</td>
<td>\textit{fucI}</td>
<td>L-fucose isomerase</td>
<td>G</td>
</tr>
<tr>
<td>CPE 0319</td>
<td>\textit{fucA}</td>
<td>L-fuculose phosphate aldolase</td>
<td>G</td>
</tr>
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<td>CPE 0321</td>
<td>-</td>
<td>Mannose specific component IIAB</td>
<td>-</td>
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<td>Mannose specific component IIC</td>
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<td>Mannose specific component IID</td>
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<td>CPE 0324</td>
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<td>Probable glycosyl hydrolase</td>
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<td>CPE 0325</td>
<td>-</td>
<td>Alpha-N-acetylgalactoseaminidase</td>
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<td>CPE 0326</td>
<td>\textit{lacA}</td>
<td>Galactose 6-phosphate isomerase</td>
<td>G</td>
</tr>
<tr>
<td>CPE 0327</td>
<td>\textit{lacB}</td>
<td>Galactose 6-phosphate isomerase</td>
<td>G</td>
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<td>CPE 0328</td>
<td>\textit{lacC}</td>
<td>Tagatose 6-phosphate kinase</td>
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<td>CPE 0372</td>
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<td>Probable ABC transporter (Permease)</td>
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<td>Probable sugar ABC transporter (Permease)</td>
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<td>CPE 0374</td>
<td>\textit{aga}</td>
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<td>CPE 0553</td>
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<tr>
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<td>CPE 1604</td>
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<td>GEPR</td>
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<td>CPE 2058</td>
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<td>Glutamate decarboxylase</td>
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Table 3.2.(b). Genes negatively regulated by sigX

<table>
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<tr>
<td>CPE 0495</td>
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<td>PST-fructose specific enzyme component IIBC</td>
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<td>fruB</td>
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Table 3.2.(c). Genes positively regulated by sigV

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<td>ioIB</td>
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<td>Conserved hypothetical protein</td>
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<td>CPE 0095</td>
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<td>Crotonase</td>
<td>I</td>
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<td>CPE 0096</td>
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<td>Propionate CoA-transferase</td>
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<td>CPE 0097</td>
<td>acdS</td>
<td>Acyl-CoA dehydrogenase</td>
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<td>Rubrerythrin</td>
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<td>CPE 0170</td>
<td>arcD</td>
<td>Arginine ornithine antiporter</td>
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<td>Carbamate kinase</td>
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<td>fucI</td>
<td>L-fucose isomerase</td>
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<td>Mannose specific component IID</td>
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<td>CPE 0324</td>
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<td>Probable glycosyl hydrolase</td>
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<td>Galactose 6-phosphate isomerase</td>
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<td>CPE 0578</td>
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<tr>
<td>CPE 0678</td>
<td><em>dut</em></td>
<td>Probable dUTP nucleotidohydrolase</td>
<td>F</td>
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<tr>
<td>CPE 0818</td>
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<td>Endo-beta-N-acetylglucosaminidase</td>
<td>-</td>
</tr>
<tr>
<td>CPE 0866</td>
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<td>Probable alpha-N-acetylglucosaminidase</td>
<td>D</td>
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<td>CPE 0897</td>
<td><em>eutA</em></td>
<td>Ethanolamine utilization protein</td>
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<tr>
<td>CPE 1150</td>
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<td>Probable CitG protein</td>
<td>HI</td>
</tr>
<tr>
<td>CPE 1152</td>
<td><em>citN</em></td>
<td>Probable citrate/sodium symporter</td>
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<td>CPE 1234</td>
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<td>Beta-galactoidase</td>
<td>G</td>
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<td>-</td>
<td>Probable galactoside ABC transporter</td>
<td>G</td>
</tr>
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<td>CPE 1523</td>
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<td>GEPR</td>
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<td>Stage V sporulation protein AE</td>
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<td>RNA polymerase sigma F factor</td>
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<td>CPE 2080</td>
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<td><em>spoIIIE</em></td>
<td>Stage II sporulation protein E</td>
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<td>-</td>
<td>Probable tagatose-6-phosphate alolodase/ketose isomerase</td>
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Table. 3.2.(d). Genes negatively regulated by *sigV*
Table 3.2 (e). Genes positively regulated by sigW

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<td><em>rubY</em></td>
<td>Rubrerythrin</td>
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</tr>
<tr>
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<td>Probable Na(^+)/H(^+) antiporter</td>
<td>P</td>
</tr>
<tr>
<td>CPE 0322</td>
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<tr>
<td>CPE 0324</td>
<td>-</td>
<td>Probable glycosyl hydrolase</td>
<td>G</td>
</tr>
<tr>
<td>CPE 0325</td>
<td>-</td>
<td>Alpha-N-acetylgalactoseaminidase</td>
<td>-</td>
</tr>
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<td>CPE 0326</td>
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<td>Galactose 6-phosphate isomerase</td>
<td>G</td>
</tr>
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<td>CPE 0327</td>
<td><em>lacB</em></td>
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</tr>
<tr>
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<td>HI</td>
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<td>CPE 1151</td>
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<td>G</td>
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<td>CPE 1343</td>
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<td>CPE 1523</td>
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<td>GEPR</td>
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Table 3.2.(f). Genes negatively regulated by the \textit{sigW} gene.

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<td>ManNAc-6-phosphate epimerase</td>
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<td>nanA</td>
<td>Acylneuraminyl lyase</td>
<td>EM</td>
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<td>CPE 0500</td>
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<td>Probable hexosyltransferase</td>
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<td>CPE 0501</td>
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<td>Capsular polysaccharide biosynthesis protein</td>
<td>M</td>
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<td>Probable liposaccharide biosynthesis protein</td>
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* COG = Cluster of Orthologus Genes

J = Translation, ribosomal structure and biogenesis; K = Transcription; L = DNA replication, recombination and repair; D = Cell division and chromosome portioning; O = Posttranslational modification, protein turnover, chaperones; M = Cell envelope biogenesis, outer membrane; N = Cell motility and secretion; P = Inorganic ion transport and metabolism; T = Signal transduction mechanisms; C = Energy production and conversion; G = Carbohydrate transport and metabolism; E = Amino acid transport and metabolism; F = Nucleotide transport and metabolism; H = Coenzyme metabolism; I = Lipid metabolism; Q = Secondary metabolites biosynthesis, transport and catabolism; R = General function prediction only; S = Function unknown.

Chapter 4
Organization and transcriptional regulation of the myo-Inositol operon in Clostridium perfringens strain 13
4.1. Introduction

_Clostridium perfringens_, an anerobic, Gram-positive organism, is known to produce a number of toxins, which play important roles in its pathogeneicity (gas gangrene or clostirdial myonecrosis) (Hatheway 1990; Petit, Gibert et al. 1999). The roles of these toxins in pathogenicity have been substantially studied (Rood and Cole 1991). A two component regulatory system, VirR/VirS, has been reported to be involved in the global regulation of the production of clostridial toxins (Lyristis, Bryant et al. 1994; Shimizu, Ba-Thein et al. 1994).

Completion of the whole genome sequencing of 3.04 Mb of _C. perfringens_ (Shimizu, Ohtani et al. 2002) revealed the presence of a myo-Inositol operon in its genome. Later, whole genome DNA microarray analysis showed that this operon is positively regulated by the VirR/VirS system (our unpublished data). myo-Inositol operon has been reported in many other bacteria, including two Gram-positive, soil bacteria, *Bacillus subtilis* (Yoshida, Aoyama et al. 1997), and *Clostridium tetani* E88 (Bruggemann, Baumer et al. 2003).

myo-Inositol that belongs to the group ‘Cyclitols’, is abundant in nature, especially in soil. Bacteria can use myo-Inositol as a sole carbon source in absense of other readily metabolizable carbon source, such as glucose. Catabolism of myo-Inositol in bacterial cell requires multiple steps with specific enzymes in each step to yield energy. This catabolic pathway has been well studied in *Aerobacter aerogenes* (Berman and Magasanik 1966; Berman, Magasanik et al. 1966; Anderson and Magasanik 1971; Anderson and Magasanik 1971; Magasanik 1971; Yoshida, Shibayama et al. 1999; Magasanik 2000) and is under investigation in _B. subtilis_ (Yoshida, Fujimura et al. 1995; Yoshida, Aoyama et al. 1997).
This study was aimed at understanding the following unaddressed issues in C. perfringens:

1. Does myo-Inositol catabolism genes constitute an operon, and if it forms the operon, how the genes are organized in the operon? What is the function of the upstream divergent gene?

2. How and when the operon is transcribed? Does the operon transcribe as a single transcript or more?

3. Which nutritional condition turns on the transcription of the operon? Is there any role of glucose on its expression?

4. Is the transcription regulated by the VirR/VirS two-component system?

4.2. Materials and methods

4.2.1. Bacterial strains and plasmids

C. perfringens strain 13 (Mahony and Moore 1976), TS133 (virR mutant) (Shimizu, Ba-Thein et al. 1994), HK301 (iolR mutant) (This study), TS140 (VR-RNA mutant) (Shimizu, Yaguchi et al. 2002), E. coli DH5α (Sambrook, 1989), pUC118 (cloning vector, derivative of pUC19) (Yanisch-Perron, Vieira et al. 1985), pJIR418 (E. coli-C. perfringens shuttle vector) (Sloan, Warner et al. 1992), pHK301 (pUC118 carrying 0.45 kb internal fragment of iolR) (This study) were used for this study.

4.2.2. Media and culture conditions

GAM (Gifu anaerobic medium), LB (Luria-Bertani) medium, BHI (Brain Heart infusion), and 2X YT media were used to culture bacteria. LB plate (LB medium with 15 g L\(^{-1}\) Bacto-agar), Blood agar plate (37g L\(^{-1}\) Brain heart infusion with 15g L\(^{-1}\) Bacto-agar and
75 ml L\(^{-1}\) defibrinated sheep blood) containing respective antibiotics was used for bacterial inoculum. \textit{E. coli} was cultured in LB medium at 37°C with vigorous shaking at 180-190 rpm. Aneropacks were used for \textit{C. perfringens} plate growth at 37°C and broth culture was performed in GAM or 2X YT medium at 37°C (water bath/incubator) under anaerobic conditions in tube with air-tight cap. Ampicillin (50 µg ml\(^{-1}\)), Tetracycline (2.5 µg ml\(^{-1}\)), Chloramphenicol (20 µg ml\(^{-1}\)) and Erythromycin (25 µg ml\(^{-1}\)) were used appropriately in broth or agar medium. myo-Inositol was added in the culture when specified and purchased from Wako pure chemicals, Japan. All the primers used in this study are listed in Table 4.1.

### 4.2.3. Construction of \textit{iolR} mutant

An internal 0.45 kb fragment from \textit{iolR} (CPE 0084) was amplified by PCR with appropriate primer set using \textit{C. perfringens} chromosomal DNA as template and was cloned into \textit{HincII} site of pUC118 (TaKaRa, Japan) and resultant plasmid was transformed into \textit{E. coli} DH5α competent cells. Erythromycin resistant determinant (\textit{erm}BP) was introduced into the plasmid and the orientation of the fragment was confirmed by restriction enzyme digestion and electrophoresis. The resultant plasmid, pHIK301 was introduced into \textit{C. perfringens} strain 13 by electroporation and single-crossing-over \textit{iolR} mutants were selected on BHI-sheep blood agar plate containing erythromycin (50 µg ml\(^{-1}\)) and the insertion mutation was confirmed by Southern blot analysis. The resultant \textit{iolR} mutant strain was designated as HK 301.

### 4.2.4. Bacterial growth curve

For checking bacterial growth pattern, either GAM or 2X YT medium was used. From primary culture, 5% was of the culture was transferred to the new medium with
appropriate antibiotic. The initial reading (0h) was taken at optical density (OD) 600 nm. In the same way, reading was taken at every hour until the bacteria entered into the stationary phase. To see the effect of presence of glucose and myo-Inositol, 10 mM (final concentration) was added to the medium and finally the growth curve was plotted using Microsoft Excel program.

4.2.5. Preparation of RBC from preserved sheep blood

Five ml of preserved sheep blood was taken into 15 ml cornig and centrifuged for 10 minutes at 2000 rpm. The upper phase (serum) was discarded and the cells were washed with 15 ml of filter sterilized 0.9% NaCl solution and centrifuged for 10 minutes at 2000 rpm. The wash step was repeated twice and the purpose of this wash is to remove serum and other preservatives, so that only blood cells can be used. Finally 1.5 ml of RBC was prepared from 6 ml of blood and this 1.5 ml of RBS was added directly to the bacterial culture at specified time point.

4.2.6. Primer extension experiments

There were several steps of preparation for primer extension experiments:

a) **Primer end labeling**: End Tag Nucleic acid labeling system kit (Vector Labo) was used for primer labeling using manufacturer’s protocol. Briefly, 5 µl of primer extension specific primer (100 pmol/µl) was taken in a 1.5 ml tube. 10 µl of dH2O, 2 µl of universal reaction buffer, 1 µl of ATP-(Gamma) S and 2 µl of T4 kinase were added to make final volume of 20 µl. The mixture was incubated at 37°C for 30 minutes and 10 µl of Thio-reactive label (Fluorescin maleimide) was added and kept at 65°C for 30 minutes and was covered with aluminium foli to avoid light. The mixture was then transferred to the middle of the gel-filtration
column (MicroSpin™ G-25 column, Amersham Biosciences) and centrifuged at 3000 rpm for 3 minute at 4°C to filter and purify the mixture. The flow-through was taken in a new tube and 20µl of DW was added to make final volume of 50 µl. This FITC end-labelled primer was stored at –20°C freeze for further use.

b) **PCR cycle sequencing:** Four PCR tubes were taken. In each tube, 2 µl of FITC labeled primer, 5 µl of purified PCR product of template DNA and 2 µl of A/T/G/C was added to make final volume of 9 µl. It was mixed well by pipetting and PCR was done under following cycling condition: 95°C for 5 minutes and then 95°C for 30 second, 60°C for 30 second for 25 cycle followed by 4°C for α. The PCR product was purified as following: In each tube, 1.0 µl of 3M sodium acetate and 23.0 µl of 100 ethanol was added, mixed well and chibitan was done and kept at room temperature for 5 minutes. It was then centrifuged at 15000 rpm for 10 minutes, supernatant was discarded and precipitate was rinsed with 100 µl of 70% ethanol, centrifuged at 15000 rpm for 2 minutes and the supernatant was removed by bent tip. It was then dried under vacuum drier and 6µl of loading dye was added in each tube. It was mixed well by vortex and boiled for 3 minutes and taken on ice. Finally, it was stored at –20°C for future use.

c) **Primer extension:** Total RNA that was prepared from c. perfringens at specific time point under specific condition. 5 µl of total RNA (15 µg/5 µl) was taken in 1.5 ml tube. 5 µl of 2X buffer and 1 µl of FITC labeled primer were added and mixed well and incubated at 58°C for 20 minutes and then kept at room temperature for 10 minutes to allow annealing. For the extension step, 5 µl of 2X buffer, 1.4 µl of sodium pyrophosphate (40 mM), 1.0 µl of AMV reverse transcriptase and 1.6 µl of nuclease free water were added to make a final volume
of 9 µl for each sample and was added to the tube containing RNA sample which was kept for annealing. The solution was mixed well and incubated at 42°C for 30 minutes to allow reverse transcription and extension of RNA. It was subjected to ethanol precipitation for purification by adding 30 µl of dH2O, 1.5 µl of 3 M sodium acetate, 0.5 µl of ethachinmate and 125 µl of 100 ethanol. It was mixed by vortex and centrifuged at 15000 rpm for 10 minutes. The supernatant was discarded and 125 µl of 70% ethanol was added and mixed by tapping and centrifuged at 15000 rpm for 5 minutes and supernatant was discarded. The pellet was dried under vacuum drier and pellet was dissolved with 5 µl of loading buffer, vortex, and denatured by boiling for 3 minutes and taken on ice. It was stored at –20°C for future use.

d) **Cleaning glass plates, and setting apparatus:** Electrophoresis glass plates were cleaned on its both sides by regular detergent, rinsed thoroughly and washed under flowing water, dried in air, wiped by Kim wipe and finally cleaned with 70% ethanol. Spacers were placed in between glass-plates, fixed with clips and adhesive tapes were applied on both sides of the plates.

e) **Making sequencing gel:** In a 50 ml beaker, 18 g of Urea, 7.5 ml of 40% acrylamide, 3.75 ml of freshly prepared 10X TBE and 11.25 ml of dH2O were added to make a final volume of 37.5 ml and put with stirrer for 30 minutes until all dissolves completely. Then 230 µl of freshly prepared 10% ammonium peroxodisulfate (APS) and 10 µl of PEMET were added, mixed well and this solution was poured into the space between the glass-plates. Extreme precaution was taken to avoid formation of bubble in the gel. The comb was placed at the
upper part of the gel, which will form the well for loading the sample. It was allowed to solidify.

f) **Setting of the apparatus and electrophoresis:** Tape was removed from the plates and plate was cleaned again with wet towel and ethanol. The gel was fixed with the apparatus and sufficient 1x TBE buffer was poured into the buffer chambers. The electrophoresis was carried out with the following specification: Final 2025 volt, 22 mA, 44 Watt, Constant = POW. 45 W. Time 1:40. V*H=3305. using Bio Rad electrophoresis machine. The gel was pre-run for 20 minutes and then sample was loaded and run for 80 minutes. The gel was immediately scanned using FluorImager image analyzer (Amersham Bioscience) and imported to the computer through Adobe Photoshop software.

### 4.3. Results

#### 4.3.1. Organization of the myo-Inositol operon

Complete genome sequencing of *C. perfringens* strain 13 revealed the presence of myo-Inositol operon, which is composed of 13 genes (~15.6 kb) with an upstream divergent gene, *iolR* (Figure 4.1). Initial version of annotation of these genes indicates that the protein products of some of these genes might be involved in the myo-Inositol uptake and catabolism in bacterial cells. To know more clearly the putative function of the protein products of this operon, the protein database was searched for the known homologous protein using BLAST-P (Altschul, Gish et al. 1990), and the result is presented in Table 4.2. The presence of 13 genes in the operon suggests that many specific steps are required in inositol catabolism before the end product enters into the common pathway.
The protein product of the upstream gene, iolR, showed significant similarity with the transcriptional repressor of myo-inositol operon, IolR (belongs to DeoR family) of B. subtilis and also with the DNA binding protein, IolR in C. tetani E88. This might suggest that IolR in C. perfringens also has the same function, i.e., repression of transcription of the downstream myo-Inositol operon. If that is true, IolR is the negative regulator of the operon.

An important characteristic of myo-Inositol operon of C. perfringens is the presence of two genes (CPE 0090 and CPE 0093), which are predicted to encode myo-Inositol 2-dehydrogenase, the enzyme required for the first step of myo-Inositol catabolism where myo-Inositol is dehydrogenated to produce 2-keto-myoinositol. Sequence analyses of these two genes, CPE 0090 and CPE 0093, having 335 and 349 amino acids respectively, revealed that both proteins possess an NAD (H)-binding motif (GXGXXG) at their N terminal, which has been proposed to form a βαβ fold involved in binding the ADP moiety of NAD(H) in dehydrogenase. This motif has also been reported to be present in myo-Inositol 2-dehydrogenase in other bacteria (Galbraith, Feng et al. 1998). Except for C. tetani, myo-Inositol operon in all other bacteria has only one gene encoding this enzyme. The presence of two genes for this enzyme could be explained in two ways: first, myo-Inositol is converted to 2-keto myo-Inositol by this enzyme. So, the presence of two genes for this enzyme could essentially accelerate the rate of conversion of myo-Inositol. Secondly, one gene could be a pseudogene, and is not translated into functional protein.

Another unique feature of this operon in C. perfringens is the presence of last four genes (CPE 0094, CPE 0095, CPE 0096 and CPE 0097) in the operon that are absent in myo-Inositol operon of other bacteria including B. subtilis and C. tetani E88. In C.
perfringens, the putative protein product of these four genes may not be directly involved in myo-Inositol catabolism but are thought to be involved in fermentation, presumably coupling the entry and metabolism of myo-Inositol catabolic end product through fermentation to yield energy.

4.3.2. Transcription of the myo-inostiol operon

To confirm that all these 13 genes belong to the operon, total RNA was prepared from C. perfringens and Northern analyses was performed separately using gene-specific probe for all 13 genes. As expected, a full-length transcript of 13 genes, which is 15.6 kb long was detected in all the probes (Fig. 4.2), confirming the speculation that these 13 genes constitute the myo-Inositol operon in C. perfringens. The same kind of full-length transcript of 10 genes of myo-Inositol operon has been reported in B. subtilis. Interestingly and to our surprise, myo-Inositol operon produced more transcripts beside the full-length transcript as detected by Northern analyses, and is unique to C. perfringens, since this kind of additional transcripts has not been reported in other bacteria possessing myo-Inositol operon. One of those transcripts was detected while hybridized with probe for CPE 0090 and CPE 0091 and the calculated length of that transcript is 2.0 kb which perfectly corresponds with the gene length of CPE 0090 and CPE 0091. Based on this finding, it was concluded that this is an additional and separate transcript which would translate to form two enzymes: myo-Inositol 2-dehydrogenase and myo-Inositol catabolism protein, IolE, respectively. Another additional transcript was detected while hybridized with probe for CPE 0094, CPE 0095, CPE 0096 and CPE 0097. The length of this transcript was calculated to be 4.6 kb that match with the length of the genes from CPE 0094-CPE 0097. As mentioned, the product of this transcript is
not thought to be involved in myo-Inositol catabolism directly and was not present in the operon of any of the myo-Inositol operon possessing bacteria. Interestingly, the expression of this transcript was strongest among all the transcripts. Another transcript was detected while hybridized with the probe for CPE 0097 and the size was calculated to be 1.1 kb which corresponds with the gene length for CPE 0097. The last transcript appeared in Northern analysis where probe for CPE 0094 was used for hybridization, which also seems to correspond to the gene size of CPE 0094 (~0.7 kb). The origin of some unclear, faint bands, which appeared as smear in Northern analyses, could not be resolved but thought to be the result of degraded products of the very long (15.6kb) transcript.

4.3.3. IolR is the negative regulator of the myo-Inositol operon

_IolR_, which was divergently oriented, and 482 bp upstream of the operon was identified. The deduced IolR protein has significant homology to IolR of _B. subtilis_ and _C. tetani_ (Table 4.2). To elucidate the role of _IolR_, an _IolR_ mutant (HK301) was constructed by a single cross over event. The _IolR_ mutant, HK301, had constitutive expression of the myo-Inositol operon both in presence and in absence of myo-Inositol in the culture medium, whereas in the wild type, the operon was expressed after 1 hour of addition of myo-Inositol (Fig. 4.3) This result indicates that the IolR is a negative regulator of the myo-Inositol operon at transcriptional level as has been reported in _B. subtilis_ (Yoshida, Aoyama et al. 1997).

4.3.4. myo-Inositol is the inducer of the myo-Inositol operon

To investigate the effect of _myo_-Inositol (Fig. 4.4) on the expression of the operon, various concentrations of _myo_-Inositol was added to the culture medium and total RNA
was prepared, which was subjected to Northern analyses. The rate of transcription of the myo-Inositol operon increased with time and with the increasing concentration of myo-Inositol in the medium (Fig. 4.5). This finding indicates that the expression of this operon is both dose- and time-dependent. Since only myo-Inositol was used to check the induction of the operon, it could not be concluded whether myo-Inositol itself or any of its metabolic products are directly responsible for the induction of myo-Inositol operon. This question remains to be answered also in other bacteria possessing myo-Inositol operon (Yoshida, Aoyama et al. 1997)

However, contrary to our assumption, transcription of iolR was also induced by myo-Inositol (Fig. 4.2), which has also been reported in B. subtilis (Yoshida, Aoyama et al. 1997). It could not be explained why the myo-Inositol operon is induced under the condition in which its negative regulator gene is also induced. Probably, complex repressor-substrate interactions may be involved in the regulation.

**4.3.5. Growth pattern of C. perfringens in presence of different carbon sources**

To understand the difference in growth pattern of *C. perfringens*, myo-Inositol (10 mM) and glucose (10 mM) was used separately in 2X YT medium. *C. perfringens* was cultured in 2X YT only, 2X YT plus 10 mM myo-Inositol and 2X YT plus 10 mM glucose in presence of appropriate antibiotic. The optical density (OD) was count at 600 nm beginning form the inoculation time (0 h) until bacteria entered the stationary phase. The purpose of this experiment was only to see the difference in growth pattern in presence of different carbon source, but it may not reflect the differential usage (and thence differential growth pattern) of different carbon sources. The growth curve was plotted (Fig. 4.6). As expected, *C. perfringens* showed standard growth pattern in presence of
glucose, as always seen in GAM but the growth in only 2X YT medium was significantly slow during the log phase, convincibly due to absence of any carbon source. Presence of myo-Inositol in 2X YT medium did not make any significant difference except for the later stage of the log phase (late-log). Though this finding defies easy conclusion, it paved the way to investigate the role of glucose on myo-Inositol operon in C. perfringens.

4.3.6. Glucose represses the myo-Inositol operon in C. perfringens

Bacteria use myo-Inositol as an alternative carbon source in the absence of glucose in the environment. To unravel the role of glucose on the expression of myo-Inositol operon, first, 10 mM myo-Inositol was added in the medium to induce the expression of the operon for one hour, and then glucose was added to the culture at different concentrations (5 mM, 10 mM and 20 mM) and total RNA was prepared after 30 minutes and 60 minutes. Prepared RNA was subjected to Northern analyses (Fig. 4.7). Glucose caused complete repression of the myo-Inositol operon (catabolite repression) within 30 minutes when its concentration was either equal or more than that of myo-Inositol in the medium (i.e., only 10 mM and 20 mM, but not 5 mM glucose showed repression of the operon). But with increasing time, i.e., 60 minutes, the strength of glucose repression was decreasing, as evident from this figure, which shows that even 10 mM glucose could not repress myo-Inositol operon completely. It signifies that glucose repression was also dependent on the duration of the exposure to glucose; the repression diminished with time. This can be explained in a straightforward way. Since growing bacteria will consume glucose preferentially to myo-Inositol in culture medium, the amount of glucose added in the medium initially will be used up by the bacteria and gradually less glucose will be available to counter the effect of myo-Inositol, which in turn will lead to the induction of the operon. So, as long as sufficient glucose will be available in the
environment, the transcription of the myo-Inositol operon in *C. perfringens* is inhibited even in the presence of myo-Inositol, most probably by catabolite repression.

### 4.3.7. Transcriptional regulation of myo-Inositol operon by VirR/VirS system

The two-component VirR/VirS signal transduction system of *C. perfringens* has been reported to regulate many toxin genes including alpha toxin (*plc*), kappa toxin (*colA*) and theta toxin (*pfoA*) genes (Rood 1998). DNA Microarray analyses also showed that the myo-Inositol operon is positively regulated by the VirR/VirS system (Fig. 4.8). To confirm that result, Northern analysis was done with total RNA prepared from wild-type strain 13 with pJIR418 vector (Sloan, Warner et al. 1992), the *virR* mutant strain TS133 with pJIR418 (Shimizu, Ba-Thein et al. 1994) and TS133 with pBT405 (*virR/virS*'). In the presence of 10 mM myo-Inositol in the medium, the transcription of myo-Inositol operon significantly decreased in TS133 (pJIR418) in comparison to both wild-type and the complemented strains (Fig. 4.9). This finding indicates that the myo-Inositol operon is positively regulated by the VirR/VirS system at transcriptional level. However, myo-Inositol had no effect on the production of toxins, suggesting that myo-Inositol is not a direct signal for the VirS sensor protein.

### 4.3.8. Identification of the start sites of the transcripts of myo-Inositol operon

In order to identify the start sites of all the transcripts of the myo-Inositol operon, primer extension experiments were carried out. Since myo-Inositol operon is not expressed under normal growth conditions, total RNA used for primer extension experiments were prepared from cultures both in absence (-) and in presence (+) of 10 mM myo-Inositol in the medium. This experiments revealed start sites for total four transcripts including one
for the iolR (Fig. 4.10). The identified transcriptional start sites were for (a) CPE 0084, iolR, (b) CPE 0085, (C) CPE 0090, and (d) CPE 0094, and the length of these transcripts were 767 bp, 15.6 kb, 2.0 kb and 4.6 kb, respectively. A single start site was detected for all (G for CPE 0084, A for CPE 0090 and T for CPE 0094) but two consecutive nucleotides were detected as the mRNA start site for CPE 0085 (C and A) and it was reproducible. The presence of two start sites for CPE 0085 could not be explained but the second start site showed stronger signal in all primer extension experiments. All these transcripts were also detected in Northern analyses. Repeated experiments failed to show clear ladder sequences for CPE 0094 but it did show clear start site, so the ladder sequence of CPE 0090 was used as an adapter to calculate and locate the start site of the mRNA transcript of CPE 0094. Several attempts were made to identify the start sites of other transcripts, which were detected in Northern analyses but no transcriptional start sites could be detected by primer extension analyses. So, it was concluded that the bands detected only with the probe for CPE 0097 (~1.1 kb) and CPE 0094 (~ 0.7 kb) could be formed by the RNA processing of the 4.6 kb transcript (Fig. 4.2). This kind of bacterial RNA processing has also been reported in other bacteria (Cao, Helmann et al. 2004). The faint, smear like bands neither correspond with any of the calculated size of the genes, nor their start sites could be detected by the primer extension analyses, suggested to assume that those bands are non-specific and might be the products of the degradation of other transcripts.

4.3.9. Promoter region analyses of the transcripts of the myo-Inositol operon

Putative promoter sequences (-35 and –10) in the upstream regions of mRNA start sites were identified. The mRNA start sites of the iolR (CPE 0084), CPE 0085, CPE 0090 and
CPE 0094 were located 25, 55, 75, and 110 bases upstream respectively, from the putative start codon (Fig. 4.11), and this kind of long distance between putative start codon and mRNA start sites has been reported for many other genes in *C. perfringens* (Ba-Thein, Lyristis et al. 1996; Ohtani, Kawsar et al. 2003). The deduced promoter region of CPE 0084, CPE 0085 and CPE 0094 showed resemblance with the consensus promote sequence which is recognized by major sigma factor, $\sigma^{70}$ of *E. coli* and $\sigma^A$ of *B. subtilis* (Amaya, Khvorova et al. 2001). However, the predicted promoter region of the CPE 0090 (GAGAAC and GCCGCT, -35 and –10 respectively) showed GC rich sequences, indicating that this promoter is most probably recognized by alternative sigma factors as has been reported in *B. subtilis* (Huang, Decatur et al. 1997; Amaya, Khvorova et al. 2001; Britton, Eichenberger et al. 2002; Cao and Helmann 2002), though a universal consensus sequence for all alternative sigma factors has not been reported yet. Careful examination surrounding the promoter region did not reveal any similarity across the sequences of these four transcripts. A search for the consensus sequence for binding VirR and VR-RNA in the upstream regions of these transcripts turned out to be negative.

4.3.10. *myo*-Inositol operon is induced in presence of sheep RBC in the culture medium

An extensive search to understand inositol cycle (Fig. 4.12) and signal transduction system in eukaryotic cells led us to postulate the hypothesis that clostridial toxin might activate a cascade in host cells resulting in formation if *myo*-Inositol which can be used up by bacteria for its carbon source when required. Aiming at validation of the hypothesis, this experiment was carried out. Sheep RBC was prepared as described in Materials and Methods section. Bacteria were cultured in 2X YT medium (glucose free), and at specified time point, *myo*-Inositol, 0.9% NaCl, and sheep RBC was added to
identical but separate cultures. Total RNA was prepared as described and Northern blot analysis was performed and hybridized with the probe for any of the gene of myo-Inositol operon. When nothing was added to 2X YT medium, the operon was not expressed under this experimental condition, whereas the culture containing myo-Inositol showed high expression of the operon, which was in line with the expectation. The culture, which contained 0.9% NaCl was used as a control, since NaCl is not responsible for myo-Inositol operon induction and was used to compare the myo-Inositol expression level in the sample containing sheep RBC. As evident from (Fig. 4.13) presence of RBC in the medium caused the myo-Inositol operon to be expressed and it was concluded that something from RBC, and most probably myo-Inositol formed from RBC was the causal factor for this induction, though the presence of myo-Inositol in the medium expected to be formed form RBC could not be confirmed by High pressure liquid chromatography (HPLC) or HPLC-Mass spectrometry. Logical reasoning based on the scientific evidence and published data (Macfarlane 1950; Sakurai, Ochi et al. 1994; Naylor, Eaton et al. 1998; Naylor, Jepson et al. 1999), hinted to consider the potential role of alpha toxin, a major virulent exotoxin of C. perfringens in the cascade that might result in the production of myo-Inositol from sheep RBC (or eukaryotic cells). A pathway relating the alpha toxin to myo-Inositol production in RBC has been postulated.

4.4. Discussion

4.4.1. myo-Inositol, an alternative carbon source for bacteria

myo-Inositol, one of the nine isomers of the group inositols, belongs to ‘Cyclitols’, and is abundant in nature, especially soil, but also common and essential in plants. The necessity of inositol and related compounds as a second-messenger and involvement in
signal transduction in human has been intensively studied and well documented. Several microorganisms, including *B. subtilis* (Yoshida, Aoyama et al. 1997), *Cryptococcus melibiosum* (Vidal-Leiria and van Uden 1973), *Aerobacter aerogenes* (reclassified as *Enterobacter aerogenes/Klebsiella mobilis*) (Berman and Magasanik 1966; Berman and Magasanik 1966; Berman, Magasanik et al. 1966), *Sinorhizobium meliloti* (Galbraith, Feng et al. 1998) and *Sinorhizobium fredii* (Jiang, Krishnan et al. 2001) can grow on inositol as carbon source. It was thought that bacterial inositol catabolism is only required for efficient utilization of this compound. However, the inositol dehydrogenase of *S. fredii* not only catalyses the initial reaction step of inositol catabolism but is also involved in nitrogen fixation and competitiveness to nodulate soybeans (Jiang, Krishnan et al. 2001). *Cryptococcus neoformans*, the causative organism of meningitis, also uses myo-Insitol (Vincent and Klig 1995; Molina, Ramos et al. 1999). myo-Insitol level in cerebrospinal fluid (CSF) reduces gradually after the infection by *C. neoformans* (MacKenzie, EA, California State University, LA; unpublished data, personal communication). Recently, presence of myo-Insitol operon has reported in the genome of *C. tetani* E88 (Bruggemann, Baumer et al. 2003), though functional characterization awaits relevant studies.

**4.4.2. myo-Insitol catabolism in *C. perfringens***

The completion of whole genome sequencing revealed presence of myo-Insitol operon in an anaerobic, Gram-positive bacteria, *C. perfringens* (Shimizu, Ohtani et al. 2002). This operon consists of 13 genes with an upstream divergent gene, iolR. To know the putative functions of the gene products of the operon, BLAST-P program (Altschul, Gish et al. 1990) was used to search the protein database for homology to known protein (Table 3.1). myo-Insitol catabolic pathway has been well studied only in *A. aerogenes* (Berman
and Magasanik 1966; Berman and Magasanik 1966; Berman, Magasanik et al. 1966; Anderson and Magasanik 1971; Anderson and Magasanik 1971). In *B. subtilis*, a Gram positive, spore-forming, soil bacteria, *myo*-Inositol catabolic pathway is under extensive study (Yoshida, Aoyama et al. 1997; Yoshida, Shibayama et al. 1999). *myo*-Inositol catabolic pathway has been shown in *A. aerogenes* and has also been proposed in case of *B. subtilis* based on the information revealed from *A. aerogenes*. Considering the study results from *A. aerogenes* and *B. subtilis* and based on the homology search result, a putative pathway for *myo*-Inositol catabolic pathway for *C. perfringens* has also been proposed here (Fig. 4.14) assigning the probable gene products as enzymes at specific reaction steps. In details, *myo*-Inositol from the environment is transported into the bacteria by the protein product (*Na*⁺/*myo*-Inositol cotransporter) of CPE 0092. The same gene is present in *myo*-Inositol operon of *C. tetani* E88 (Bruggemann, Baumer et al. 2003). A hydropathy plot of CPE 0092 using Kyte and Doolittle algorithm (Kyte and Doolittle 1982) showed the putative membrane spanning regions, an indispensable characteristic of a cell- membrane transporter. Once inside the cell, *myo*-Inositol is converted to 2-keto-*myo*-Inositol (Inosose) by the protein product of CPE 0090 and/or CPE 0093, both of which encode *myo*-Inositol 2-dehydrogenase, and their presence has also been reported in *myo*-Inositol operon of *B. subtilis* (Yoshida, Aoyama et al. 1997) and *C. tetani* (Bruggemann, Baumer et al. 2003). This is a NAD-linked dehydrogenation reaction where hydrogen from carbon atom 2 of inositol is transferred. *Myo*-Inositol 2-dehydrogenase, encoded by CPE 0090 and CPE 0093, possesses an NAD(H) binding motif, GXGXXG at its N terminal, which has been proposed to form a βαβ fold involved in binding the ADP moiety of NAD(H) in dehydrogenase (Thompson and Donkersloot 1992). The presence of two genes encoding same enzyme could
essentially accelerate the rate of conversion of \textit{myo}-Inositol in the first step of catabolic pathway. 2-keto-\textit{myo}-Inositol is then dehydrated to D-2,3, diketo-4-deoxy-\textit{epi}-inositol by 2-keto-\textit{myo}-Inositol dehydratase (\textit{myo}-Inositol catablosim protein, IolE) encoded by CPE 0091. IolE is also present in \textit{myo}-Inositol operon of \textit{B. subtilis} and \textit{C. tetani}, and recently, the catalytic role of IolE in this dehydration reaction has been bio-chemically proved in \textit{B. subtilis} (Yoshida, Yamaguchi et al. 2004). D-2, 3, diketo-4-deoxy-\textit{epi}-inositol is then hydrated to 2-deoxy-5 keto-D-glucoronic acid, the first open chain intermediate, although the specific enzyme involved in this reaction has not been bio-chemically studied in any bacteria, but careful examination of the protein products of the operon will reveal that the role of IolB (CPE 0088) has neither been assigned in the catabolic pathway, nor the function has been reported anywhere. Taken these two points together, IolB is the most potential candidate to be involved in this step of \textit{myo}-Inositol catabolic pathway, which surely demands the bio-chemical evidence to confirm the proposition. 2-deoxy-5 keto-D-glucoronic acid is then phosphorylated to form 2-deoxy-5 keto-D-glucoronic acid phosphate, most probably by the IolC protein (CPE 0087), because this protein showed meaningful homology to fructokinase and 2-dehydro-3-deoxyglucokinase. IolC protein is also present in \textit{myo}-Inositol operon of \textit{B. subtilis} and \textit{C. tetani}. The resulting 2-deoxy-5 keto-D-glucoronic acid phosphate is then probably cleaved by fructose 1,6, biphosphate aldolase (CPE 0086), to yield dihydroxyacetone phosphate and malonic semialdehyde. Dihydroxyacetone phosphate then enters into the glycolytic pathway to yield energy. Remaining malonic semialdehyde is converted to Acetyl CoA and CO$_2$ probably by \textit{myo}-Inositol catabolism protein IolD (CPE 0089), which has similarity with methylmalonate-semialdehyde dehydrogenases. Acetyl CoA produced at this step can be converted to butyrate, ethanol or acetate through multiple steps. Thus, the end products of \textit{myo-}
Inositol catabolism, dihydroxyacetone phosphate and acetyl CoA enter into energy yielding cycles.

Curiously, the protein products of last four genes of myo-Inositol operon, CPE 0094-CPE 0097 has not been found to be involved in any step of this catabolic pathway, and note-worthily, these four genes are not included in the myo-Inositol operon of any of the myo-Inositol operon possessing bacteria, including B. subtilis and C. tetani. Search for putative protein functions revealed that these proteins code enzymes, which are strong candidates to be involved in the anaerobic glycolysis/fermentation pathways, and some of them have already been assigned of their functions in fermentation (our lab’s unpublished data) to yield energy.

Unique in C. perfringens, a myo-Inositol operon protein product not only catabolizes myo-Inositol, but also couples these catabolic end products to the more downstream glycolytic/fermentation pathway, making sure of effective and rapid energy production by keeping the downstream cellular machineries receptive.

**4.4.3. Expression of myo-Inositol operon in C. perfringens**

Northern blot analyses of myo-Inositol operon showed that the operon produced three transcripts, which were 15.6 kb (whole length), 4.6 kb and 2.0 kb in length. iolR produced a separate, ~0.7 kb long transcript. In B. subtilis, myo-Inositol operon produced a single, whole-length transcript (Yoshida, Aoyama et al. 1997). The subtle purpose of three transcripts in C. perfringens could not be clearly reasoned but the presence of a 2.0 kb transcripts composed of CPE 0090 and CPE 0091 is of particular interest. Careful visual scanning will reveal that these two genes encode myo-Inositol 2-dehydrogenase and myo-Inositol catabolism protein, IolE, which are involved in the first and second
steps of myo-Inositol catabolic pathway, respectively. Beside the full length (15.6 kb) transcript, the presence of separate and strongly expressed, 2.0 kb transcript of those two genes might have meaningful involvement in the acceleration of initial catalysis of myo-Inositol, once it is inside bacterial cell. Another 4.6 kb long transcript, composed of CPE 0094-CPE 0097 (not present in other myo-Inositol operon possessing bacteria, described above), which is additional to the whole-length transcript, codes for the enzymes believed to be involved in glycolysis/fermentation draws special consideration in clostridial metabolic pathways. The other two bands detected in Northern analyses while hybridized with probe for CPE 0094 (~0.7 kb) and CPE 0097 (~1.1 kb) were considered to be the product of RNA processing of 4.6 kb transcript since their transcriptional start sites could not be detected by repeated primer extension experiments.

myo-Inositol operon is not expressed under normal growth conditions until the glucose concentration falls critically below the level to maintain the availability of carbon source for the growing bacteria (data not shown). When glucose is exhausted and become the limiting factor, myo-Inositol operon transcription is turned on, though at a very low level, to prepare the bacteria for alternative carbon source and to use that effectively without prolonged time lag. To investigate the role of myo-Inositol on the operon expression, myo-Inositol was added to the glucose free culture medium (2X YT) or glucose containing medium (GAM) at various concentrations, beginning from 0.05 mM to 10 mM and RNA was prepared. Northern analyses showed that as low as 0.2 mM myo-Inositol in the medium can cause induction of the operon and this induction was increased in course of time and with increasing dose of myo-Inositol. Since only myo-Inositol was used in this study, whether myo-Inositol itself or any of its metabolic product, is the actual inducer at molecular level, could not be resolved. The same question remains to be answered in other bacteria.
4.4.4. IolR-mediated transcriptional inhibition of myo-Inositol operon and bacterial catabolite repression

*iolR*, the divergent, upstream gene of myo-Inositol operon is 767 bp long. Homology search across the protein database revealed that the protein product has significant identity with the transcriptional repressor of myo-Inositol operon, IolR of *B. subtilis* (Table 4.2), which belongs to DeoR family of DNA binding protein. To investigate the role of IolR, *iolR* mutant strain, HK301, was constructed. Total RNA was prepared from both wild type strain 13 and *iolR* mutant, HK301 at specified time course, both in presence and absence of myo-Inositol in the culture medium and was subjected to Northern analyses. The result showed that the expression of myo-Inositol operon became constitutive in HK301 (*iolR*), both in presence and absence of myo-Inositol in the medium, whereas in the wild type strain 13, the operon was expressed after 1 hour of addition of myo-Inositol. This clearly indicates the repressive action of the IolR on the operon, which has also been reported in *B. subtilis*. Contrary to presupposition, but alike in *B. subtilis*, transcription of *iolR* itself was also induced by myo-Inositol. We could not conclude why the myo-Inositol operon is induced under the condition in which its negative regulator gene is also induced. Probably, complex repressor-substrate interactions may be involved in the regulation.

To investigate the role of glucose on myo-Inositol operon expression, glucose was added in presence of myo-Inositol in the medium and the result showed (Fig. 4.7) that glucose completely ceases the expression of the operon if the concentration of glucose is equal or more than that of myo-Inositol. This repression (catabolite repression) is thus dose dependent but it also showed that the magnitude of the repression diminished in course of time, as evidenced.
Catabolite repression (originally referred to as ‘glucose effect’) is a regulatory mechanism whereby the expression of many catabolite genes, and some others, is repressed in the presence of a readily metabolizable carbon source such as glucose, fructose or mannitol (Hueck and Hillen 1995). Catabolite repression can be mediated by *cis*-acting catabolite responsive element (CRE), which is present in many genes encoding carbon catabolic enzymes in various species of the Gram-positive bacteria. Catabolite repression of most genes regulated via CRE is also affected by the *trans*-acting factors, carbon catabolite protein A (CcpA) and histidine protein (HPr). This catabolite repression in microorganism has two facets: inhibition of uptake of second substrate (inducer exclusion), and inhibition of gene transcription (Saier 1989). The high repression factors of inositol dehydrogenase might be due to inducer exclusion functioning in addition to catabolite repression as has been demonstrated for xylose isomerase expression in *B. subtilis* (Kraus, Hueck et al. 1994). ‘Inducer exclusion’ summarizes all effects of catabolite repression promoting carbon sources on substrate-specific induction. The mechanism of action of CcpA in catabolite repression is not understood. It might involve direct interaction with the CRE of target genes or may exert its effect on a central pathway of carbon metabolism like glycolysis, which may, in turn, lead to catabolite repression (Hueck and Hillen 1995). Like other bacteria, clostridia exhibits catabolite repression and several studies showed that synthesis of amylolytic enzymes is repressed in the presence of a readily metabolizable sugar such as glucose (Hyun, Shen et al. 1985; Hyun and Zeikus 1985). Catabolite repression has also been reported and a consensus sequence of CRE has also been proposed for *myo*-Inositol operon in *B. subtilis* (Miwa, Saikawa et al. 1994; Miwa and Fujita 2001), but using that consensus sequence to search for CRE for *myo*-Inositol operon in *C. perfringens* was not conclusive.
Very recently, it has been reported that in *C. perfringens*, CcpA is necessary for efficient sporulation and enterotoxin gene (*cpe*) regulation, and maximum synthesis of collagenase (kappa toxin) but CcpA does not regulate the synthesis of phospholipase C (alpha toxin) (Varga, Stirewalt et al. 2004). Since *C. perfringens* possesses CcpA (CPE 2539), and CRE could not be detected (due to unavailability of a proven CRE consensus sequence), catabolite repression might occur through central pathway, as described above, but further studies at molecular level is a prerequisite before an inference can be deduced.

4.4.5. Transcriptional regulation of *myo*-Inositol operon by the VirR/VirS system

The two-component signal transduction system, VirR/VirS, is a global regulatory mechanism and regulates many of the clostridial toxins and virulence factors. Our unpublished VirR/VirS mutant microarray data revealed that the *myo*-Inositol operon is positively regulated by VirR/VirS system. To confirm that microarray result, Northern analysis was performed, and it showed that the operon is clearly positively regulated at transcriptional level by VirR/VirS system both in presence and in absence of *myo*-Inositol (Fig. 4.9). So far, all the genes regulated by VirR/VirS system are either directly or indirectly involved in clostridial virulence, but this study showed the first non-virulent genes to be regulated by the VirR/VirS system. VirR/VirS system regulates the genes in its regulon by two ways: either by binding directly to the promoter region of the target genes or through the secondary regulator, VR-RNA (Shimizu, Yaguchi et al. 2002).

VR-RNA is a putative RNA binding protein, which binds to the target genes to exert its regulation. Our unpublished microarray experiment also showed that VR-RNA also exerts positive regulation on the *myo*-Inositol operon, but whether this regulation is direct
or indirect, could not be concluded. To confirm the role of VR-RNA on the \textit{myo}-Inositol, Northern analyses were performed using total RNA from wild type strain 13, TS 140 (VR-RNA mutant) and VR-RNA complementation (VR-RNA\(^{+}\)) (Fig. 4.15). VR-RNA mutant showed complete cessation of \textit{myo}-Inositol operon compared to wild type, which is in concordance with the microarray result, but surprisingly, VR-RNA complemented strain, failed to complement the expression of the operon. The complete genome-wise search for the consensus sequence for VirR binding (CCCAGTTNTNCAC) (Cheung and Rood 2000), and for VR-RNA (TGTAAGGTT) did not show presence of those consensus sequences in the \textit{myo}-Inositol operon, so, it could not be concluded where and how the VR-RNA is involved in the \textit{myo}-Inositol operon expression, and left more questions than the answers, i.e., is other regulatory molecules involved upstream/downstream of VR-RNA regulatory pathway or is there any other molecule involved downstream of VirR/VirS pathway which is independent of VR-RNA.

### 4.4.6. \textit{myo}-Inositol, a recycleable terminal product of G-protein coupled downstream cascade in eukaryotic cells

The well-established fact that inositol plays very important role in human body, especially in signal transduction system and in neurotrasmission and neuro-modulation in the brain. Any deficiency of inositol or any in-milieu homeostatic derangement causes psychological disarrays including psychiatric disorders (Agam, Shamir et al. 2002; Harvey, Brink et al. 2002; Harwood 2003). Careful search of published articles showed the well-established ‘G-protein coupled’ signal transduction cascade where G-protein in the cell membrane is activated by specific ligands, and lead to formation of Inositol 1,4,5 triphosphate (IP\(_3\)) and Diacyl glycerol (DAG), from Phosphatidyl 4,5 bi-phosphate (PIP\(_2\)). IP\(_3\) and DAG activate the catalytic cascade, which finally causes increase in
intracellular Ca\textsuperscript{2+} concentration and degradation of the cell membrane through different steps. IP\textsubscript{3} is recycled in this process and is converted to Inositol 1,3,4,5 tetra phosphate (IP\textsubscript{4}) but part of IP\textsubscript{3} is spontaneously converted to myo-Inositol (Fig. 4.12 and 4.16), which is then converted to Phospahtidyl Inositol to be recycled in cell membrane biosynthesis.

4.4.7. Eukaryotic cellular myo-Inositol and prokaryotic toxins: Implication of clostridial alpha toxin in coupling its virulence to survival

Clostridial alpha toxin (encoded by\textit{plc} gene) has extensive homology with phospholipase C of \textit{B.cereus} which is known to be a PC-PLC (Phosphatidyl choline-PLC) (Guillouard, Garnier et al. 1996), but not PI-PLC (Phosphatidyl Inositol-PLC), which means that clostridial PLC can not cause hydrolysis of PIP\textsubscript{2} directly but it can hydrolyse PIP\textsubscript{2} by activating the eukaryotic intacellular PLC through the G–protein coupled pathway (Sakurai, Ochi et al. 1994). Once the whole cascade is activated, DAG and IP\textsubscript{3} will be formed which in turn will cause the cell membrane to lyse as well as formation of myo-Inositol (though not in significant amount). This myo-Inositol will not be required for recycling since the membrane itself is damaged and the cell lyses.

Assuming that this myo-Inositol can be taken up by the spreading clostridia during pathogenesis (myonecrosis and hemolysis) in host cell, an experiment was designed where sheep RBC was added in the growing bacterial culture (the growing cell will be producing alpha toxin, and since alpha toxin is an exotoxin, it will be released in the environment, and that is a proven fact). Alpha toxin present in the culture is assumed to interact with RBC through G-protein to cause hemolysis, and myo-Inositol will be formed which will be taken up by the bacteria, and will cause induction of the operon. To
investigate that hypothesis, total RNA was prepared from bacteria cultured in presence and in absence of RBC and was subjected to Northern analysis (Fig. 4.13). The result indicates that the myo-Inositol operon is expressed in presence of RBC but not in absence of RBC and this induction was maximal after 2 hour of addition of RBC. This finding led us to postulate a cascade to show the possible relation between PLC and myo-Inositol operon induction (Fig. 4.17), but confirmation of this hypothesis requires precise, quantitative biochemical analysis. If that can be proved, it will show that bacterial virulence is not merely to produce disease in the host, rather to survive by getting its nutrition from damaged host tissues, including red blood cells, white blood cells and endothelium, and thus bacterial virulence will be purposefully coupled to its own survival.

4.5. Conclusion

1. myo-Inositol operon in C. perfringens strain 13 is composed of 13 genes with an upstream, divergent, gene, iolR.

2. The myo-Inositol operon is transcribed to three separate transcripts of 15.6 kb (full-length), 4.6 kb and 2.0 kb in length. iolR produces a separate transcript of ~0.7 kb long.

3. IolR is a negative regulator of the downstream operon.

4. Both myo-Inositol operon and iolR, were induced by the myo-Inositol, in a dose- and time-dependent manner.

5. Glucose causes repression of the myo-Inositol operon, even in presence of myo-Inositol.
6. This operon is positively regulated by the two-component, VirR/VirS system.

7. Primer extension experiments identified the transcriptional start sites of all four transcripts, but a consensus sequence in the promoter region across the four genes could not be identified.

8. The \textit{myo-Inositol} operon was also induced by the presence of sheep RBC in the culture medium, which led to formulate a possible interrelation between alpha toxin and \textit{myo-Inositol} production from RBC.
4.6. Tables and figures
Table 4.1. Primers used in this study

To construct *iolR* mutation:

F primer: 5- GGAAAGGCTGCTGCTGAATT-3
R primer: 5-CATCACTAGGTTTTTGCATCT-3

For primer extension experiments:

**IolR (CPE0084):**

F primer: 5- CTACTAGGTCTATTCTTTTT-3
R primer: 5-CCTGAAATAATAACGTTTTTGTC-3
Extension primer: 5-TTCTACTAGGTCTATTCTTTTTGAACCGCAT-3

**CPE 0085:**

F primer: 5-CTACTAGGTGTATTCTTTTT-3
R primer: 5-CCTGAAATAATAACGTTTTTGTC-3
Extension primer: 5-ACGTTTTGGCATAATAATTTGATTCGCCAT-3

**CPE 0090:**

F primer: 5-ACTGTGGATTTGGATGTATA-3
R primer: 5-CTTCCTATTTCTTCTGCTCC-3
Extension primer: 5-TTCTCCATTTCTTCTGCTCCAATAACACC-3

**CPE 0094:**

F primer: 5-GTTGGATACCGCTTGTAAAGA-3
R primer: 5-CCATTATTCCTTGCCATCTCT-3
Extension primer: 5-GCATTTATTCTCCTTTATATTATATTATATTATGT-3
Table 4.2. Results of comparison with protein database of the genes of myo-Inositol operon using BLAST-P

<table>
<thead>
<tr>
<th>Gene No.</th>
<th>Gene/product name</th>
<th>Homologous protein and organism(s)</th>
<th>% identity for amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPE 0084</td>
<td>ioIR</td>
<td>DNA binding protein, IolR, C. tetani E 88</td>
<td>47% (121/254)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transcriptional repressor of myo-Inositol operon, IolR (DeoR family), B. subtilis</td>
<td>44% (111/250)</td>
</tr>
<tr>
<td>CPE 0085</td>
<td>Probable alcohol dehydrogenase</td>
<td>1, 3,3-propanediol dehydrogenase, C. tetani E 88</td>
<td>57% (220/338)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alcohol dehydrogenase, B. anthracis Str Ames</td>
<td>46% (173/375)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alcohol dehydrogenase, B. subtilis</td>
<td>36% (133/360)</td>
</tr>
<tr>
<td>CPE 0086</td>
<td>Fructose 1,6 biphosphate aldolase (iolJ?)</td>
<td>F-bp-aldolase, Class II, B. anthracis</td>
<td>60% (170/281)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F-bp-aldolase, C. tetani E 88</td>
<td>55% (155/281)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F-bp-aldolase (iolJ), B. subtilis</td>
<td>37% (109/291)</td>
</tr>
<tr>
<td>CPE 0087</td>
<td>Myo-Inositol catabolism protein, iolC</td>
<td>Myo-Inositol catabolism protein, IolC, C. tetani E 88</td>
<td>68% (238/338)</td>
</tr>
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<td></td>
<td>Myo-Inositol catabolism protein, IolC, B. subtilis</td>
<td>46% (148/318)</td>
</tr>
<tr>
<td>CPE 0088</td>
<td>Myo-Inositol catabolism protein, iolB</td>
<td>Myo-Inositol catabolism protein, IolB, C. tetani E 88</td>
<td>50% (128/253)</td>
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<td></td>
<td>Myo-Inositol catabolism protein, IolB, B. subtilis</td>
<td>22% (60/263)</td>
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<td>CPE 0089</td>
<td>Myo-Inositol catabolism protein, iolD</td>
<td>Myo-Inositol catabolism protein, IolD, B. anthracis Str Ames</td>
<td>64% (411/641)</td>
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<td></td>
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<td>Malonic semialdehyde oxidative decarboxylase, B. halodurans</td>
<td>54% (351/642)</td>
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<td></td>
<td></td>
<td>Myo-Inositol catabolism protein, IolD, B. subtilis</td>
<td>53% (315/588)</td>
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<tr>
<td>CPE 0090</td>
<td>Probable dehydrogenase</td>
<td>Myo-Inositol 2-dehydrogenase, C. tetani E 88</td>
<td>59% (190/317)</td>
</tr>
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<td></td>
<td></td>
<td>Opine catabolism homology yrbE (Myo-Inositol 2-dehydrogenase), B. subtilis</td>
<td>49% (156/318)</td>
</tr>
<tr>
<td>CPE 0091</td>
<td>Myo-Inositol catabolism protein, iolE</td>
<td>Myo-Inositol catabolism protein, IolE, C. tetani E 88</td>
<td>75% (223/295)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Myo-Inositol catabolism protein, IolE, B. halodurans</td>
<td>48% (143/294)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Myo-Inositol catabolism protein, IolE, B. subtilis</td>
<td>44% (131/294)</td>
</tr>
<tr>
<td>CPE 0092</td>
<td>Na⁺/myo-Inositol cotransporter</td>
<td>Na⁺/myo-Inositol cotransporter, B. halodurans</td>
<td>50% (256/512)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Na⁺/myo-Inositol cotransporter, C. tetani E 88</td>
<td>49% (254/511)</td>
</tr>
<tr>
<td>CPE 0093</td>
<td>Probable dehydrogenase</td>
<td>Myo-Inositol 2-dehydrogenase, C. tetani E 88</td>
<td>70% (240/340)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Opine catabolism homology yrbE (Myo-Inositol 2-dehydrogenase), B. subtilis</td>
<td>31% (107/340)</td>
</tr>
<tr>
<td>CPE 0094</td>
<td>Conserved hypothetical protein</td>
<td>Probable nitrate transporter, C. tetani E 88</td>
<td>39% (99/252)</td>
</tr>
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<td></td>
<td></td>
<td>Nitrate transporter, B. subtilis</td>
<td>34% (85/245)</td>
</tr>
<tr>
<td>CPE 0095</td>
<td>Crotonase</td>
<td>3-hydroxybutyryl CoA dehydratase, C. tetani E 88</td>
<td>75% (194/257)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3-hydroxybutyryl CoA dehydratase, C. acetobutylicum</td>
<td>57% (148/259)</td>
</tr>
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<td>CPE 0096</td>
<td>Propionate CoA transferase</td>
<td>Acetyl CoA:aceotoacetyl CoA transferase, C. tetani E 88</td>
<td>72% (370/513)</td>
</tr>
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<td></td>
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<td>Propionyl CoA transferase, C. propionicum</td>
<td>56% (292/520)</td>
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<tr>
<td>CPE 0097</td>
<td>Acyl CoA dehydrogenase</td>
<td>Acyl CoA/Butyryl CoA dehydrogenase, C. tetani E 88</td>
<td>71% (272/379)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Butyryl CoA dehydrogenase, C. acetobutylicum</td>
<td>58% (221/379)</td>
</tr>
</tbody>
</table>
Figure 4.1. Schematic presentation of the gene organization of the myo-Inositol operon in *Clostridium perfringens*
Figure 4.2. Transcriptional profile of the myo-Inositol operon in *Clostridium perfringens*
Figure 4.3. Northern experiments showing that IolR is the negative regulator of the myo-Inositol operon in *Clostridium perfringens* (W, wild type strain 13; iolR-, iolR mutant strain). Bacteria were cultured in 2X YT medium, and total RNA was prepared at indicated time both in presence (+) and absence (-) of myo-Inositol (10 mM) in the culture. Ten microgram of total RNA was used for Northern analyses using 0.8% agarose gel.
Figure 4.4. Structure of myo-Inositol. Carbon atoms are numbered.
Figure 4.5. Northern analysis showing that myo-Inositol induces myo-Inositol operon in a dose- and time-dependent manner in Clostridium perfringens. Total RNA was prepared after 1h and 2h of addition of different concentrations of myo-Inositol in the medium (2X YT). Ten microgram of total RNA was used for 0.8% agarose gel electrophoresis, which was subjected to Northern analyses.
Figure 4.6. Growth pattern of *Clostridium perfringens* in presence of glucose (10 mM) or *myo*-Inositol (10 mM) in the culture medium (2X YT, a glucose free medium).
Figure 4.7. Northern analysis showed glucose repression of myo-Inositol operon in a dose- and time-dependent manner. Wild type strain 13 was cultured in 2X YT medium for 3 h, and then 10 mM myo-Inositol was added to induce the expression of myo-Inositol operon. 1 h after addition of myo-Inositol, glucose was added at different concentrations in the culture. Total RNA was prepared after 30 min and 60 min, and was subjected to Northern blotting.
Figure 4.8. DNA microarray results showing the location of *myo*-Inositol operon in the genome and the genes either positively (red) or negatively (blue) regulated by the two-component VirR/VirS system. Yellow color indicates the genes independent of VirR/VirS regulation.
Figure 4.9. Northern analyses result showing positive regulation of the VirR/VirS system on the myo-Inositol operon expression. Wild type strain 13 (lane a), TS133 (virR mutant) (lane b) and virR complemented strain (lane c) were cultured in 2X YT for 3h, then 10 mM myo-Inositol was added, and total RNA was prepared at 1h and 2h, and was subjected to Northern blot analyses.
Figure 4.10. Primer extension studies of the transcripts of the myo-Inositol operon and its negative regulator, iolR in C. perfringens. The primer extension products were electrophoresed on the sequencing gels together with sequencing reactions using the same primers against appropriate PCR templates. The extended products are shown arrows, the mRNA start sites are indicated by bold face letters. Studies were carried out using total RNA prepared after 2h culture in GAM (-) and 1h after addition of 10 mM myo-Inositol (+). Repeated primer extension experiments failed to show clear ladder sequences for CPE 0094-0097, so ladder sequences of CPE 0090-0091 was used as an adapter (I) to locate the start site of mRNA transcript of CPE 0094-0097 (II).
AATACCTTTAGTATAAACTCTATATATTATTTAAATAGATTTTATTTACTCACTAATTAACTAACTTTAATATTATTTGATAAG.(n25) **ATG: PiolR**

TTTTTCGTAATTATGATTATATTGTGTAATAATATGAAAAAGTTATTGACATGTATGTTAAGTTGGTATTATTAAAAC...CA..(n55) **ATG : 84–85**

TTGGAATAGCTGGAGAATCAAGAAATTGATGCTAAATAAGGCATTTTGGAAACAAAGAGAAAAATTTAAAAGCCGCTAGAAGA..(n75) **ATG : 89–90**

TAGCATGGTAAACTTGAGTTGTCTAAAGTTACATCTACATTATTTAAACTCAAAAATCTAATAACTTTAATTTAAAGAT..(n110) **ATG : 93–94**

Figure 4.11. Deduced promoter regions of the transcripts of myo-Inositol operon and its regulator, *iolR* in *C. perfringens*. The -35 and -10 promoter sequences are underlined.
Figure 4.12. Inositol cycle. Formation of \textit{myo}-Inositol from Phosphatidyl inositol 4,5-biphosphate and Inositol 1,4,5 triphosphate is shown in bold face.
Figure 4.13. Northern analyses experiment to investigate the possible role of sheep RBC on induction of myo-Inositol operon. *Clostridium perfringens* wild type strain 13 was cultured in 2X YT medium. myo-Inositol (3 mM), 0.9% NaCl, and sheep RBC (prepared as described in materials and methods) was added to the culture and total RNA was prepared after 2h. Ten microgram of RNA for each sample was used for Northern blot analysis. Lane a, Strain 13 in 2X YT medium; Lane b, 3mM myo-Inositol added into 2X YT medium; Lane c, 1.5 ml 0.9% NaCl added into 2X YT medium (used as control for sheep RBC); Lane d, 1.5 ml (7.5%, v/v) sheep RBC was added into 2X YT medium.
Figure 4.14. Postulated pathway for myo-Inositol catabolism in Clostridium perfringens. Based on the homology of the protein products of the genes of the myo-Inositol operon, specific gene product thought to involve in the catabolic pathway has been mentioned. The final product of this pathway, dihydroxyacetone phosphate enters into the glycolytic pathway to yield energy.
Figure 4.15. Northern experiment to investigate the role of VR-RNA, the secondary regulator of the VirR/VirS system, on the expression of myo-Inositol operon. *Clostridium perfringens* wild type strain 13, VR-RNA mutant (*vrr* mutant, TS140) and VR-RNA complemented strains were cultured 2XYT medium. 10 mM *myo*-Inositol was added to culture and total RNA was prepared just before addition of *myo*-Inositol (0h), and after 1h and 2 h of addition of *myo*-Inositol. Prepared RNA was subjected to Northern blot analyses.
Figure 4.16. Presumed mechanism of action of alpha toxin (PLC) of C. perfringens in host cell membrane disruption. Clostridial PLC is a PC-PLC (Phosphatidyl choline-PLC), and it does not act directly on the membrane, as is the case for PI-PLC (Phosphatidylinositol-PLC). PC-PLC acts on the G-protein and membrane phospholipids, which in turns activate the host cells’ PLC to initiate the cascade resulting in the degradation of cell membrane (lysis).
Figure 4.17. Proposed hypothesis to illustrate the relation between production of alpha toxin by *C. perfringens* and production of *myo*-inositol in the host cells by the action of alpha toxin. *myo*-Inositol produced from host cell is speculated to be taken up by the growing bacteria for its nutritional demand, and effective catabolism of imported *myo*-inositol is ensured by the induction of *myo*-inositol operon.
Summary

Clostridial diseases, though costed uncounted lives throughout the history, the pathogenesis of these diseased remain largely raveled. Rapid progression and completion of whole-genome sequencing of many bacteria, and accumulation of genetic information extracted from these discoveries are enriching the understanding of many basic as well as intricated phenomena of bacterial survival and virulence. Entering into the so-called ‘post-genomic era’ implied the formidable challenges to work out and to ‘put-all-the-pieces-together’ in a logical manner to make meaningful conclusion from the four nucleotides.

Careful examination and comparative analysis of the genome of *C. perfringens* revealed several unique characteristics including four putative enterotoxin genes (*ent*) that is not present in other enterotoxigenic clostridia and also different from well-studied enterotoxin gene (*cpe*), presence of three ECF-type sigma factors that is absent in non-pathogenic *C. acetobutylicum*, and myo-Inositol operon that is present in several soil bacteria including *B. subtilis* and *C. tetani*. Experiments were designed logically based on the available information to focus on the understanding the importance of these unique features in clostridial virulence and survival.

All the putative enterotoxin genes were expressed under normal growth condition, and importantly, during the early-to-mid log phase, which is a special feature of all the major toxins produced by *C. perfringens*. Though the function of these enterotoxins could not be studied, alike several other toxins, it was found that these putative enterotoxin genes are also regulated by the VirR/VirS system. Presence of three ECF-type sigma factors is another important feature of *C. perfringens*. Though these sigma factors did not show any obvious regulation on the expression of three important exotoxins, their role in survival
under stress could not be concluded. Application of several stresses did not show the
expression of these sigma factors under normal growth conditions during the mid-log
phase. DNA microarray experiments using total RNA from bacteria under normal growth
condition showed several genes are regulated by these sigma factors, including those
involved in carbohydrate transport and energy conservation as well as outer membrane
biogenesis. Microarray experiments were also done using total RNA from bacteria
exposed to several selected stressful conditions but the result was not available for
analyses due to some problem.

*myo*-Inositol operon was studied in details and it was found that the operon functions
when concentration of glucose as carbon source declines in the environment. Glucose
was found to be effective to repress the expression of this operon, perhaps through
catabolite repression. Interestingly, this operon is also positively regulated by the
VirR/VirS system along with phospholipase C (Alpha toxin). This finding fueled the
speculation, and led us to propose a hypothesis that clostridia use its virulence, at least
partially to gain its nutrition from the host.

These studies indentified several new genes, which are regulated by the VirR/VirS
system, and also pointed a link between the genes related to virulence with that of
survival. Further precise experiments will be required to answer some key questions for a
complete understanding of the pathogenesis.
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Presentations

Part of this study was presented during the following conferences and meetings:

1. ECF-type sigma factors in *Clostridium perfringens* strain 13.

   **Hameem I. Kawsar**, Kaori Ohtani, Hideo Hayashi, Tohru Shimizu,
   Annual meeting of bacteriology, April 2002, Yokohama, Japan

2. Sigma factors of *Clostridium perfringens* strain 13.

   **Hameem I. Kawsar**, Kaori Ohtani, Tohru Shimizu
   4th International conference on molecular biology and pathogenesis of clostridia.
   April 26-30, 2003, Boston, USA.
   Abstract published in: Anaerobe 9, 2003, 211

3. Transcriptional regulation of *myo*-Inositol operon in *Clostridium perfringens*

   Strain 13

   **Hameem I. Kawsar**, Tohru Shimizu
   104th General meeting of American Society for Microbiology (ASM)
   May 23-28, 2004, New Orleans, LA, USA
Publications

(1). VirR/VirS regulatory cascade affects transcription of plasmid-encoded putative virulence genes in *Clostridium perfringens* strain 13.

Kaori Ohtani, **Hameem I. Kawsar**, Okumura K, Hayashi H, and Shimizu T.


(2). Organization and transcriptional regulation of *myo*-Inositol operon in *Clostridium perfringens*.

**Hameem I. Kawsar**, Ohtani K, Okumura K, Hayashi H, and Shimizu T.


(3). Identification and characterization of a cell-wall anchored Dnase gene in *Clostridium perfringens*

Kayo Okumura, **Hameem I. Kawsar**, Takeshi Shimizu, Toshiko Ohta, Hideo Hayashi and Tohru Shimizu

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