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ウェルシュ菌芽胞形成調節ネットワークの解析と、調節遺伝子をターゲットとした食中毒予防法の開発に関する研究

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研究代表者　大谷　郁

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研究代表者　　大谷　郁　　金沢大学医薬保健研究域医学系　講師

研究要旨
ウェルシュ菌食中毒は、ウェルシュ菌があらゆる環境に耐えうるための芽胞を形成する時に惹起されると考えられている。しかし、その詳細なメカニズムは明らかになっておらず、ウェルシュ菌の食中毒を予防することは困難である。我々は、食中毒予防法の手がかりをつかむため、腸管毒素産生と芽胞形成メカニズムを遺伝子レベルで明らかとすることを試みた。マイクロアレイの解析により、芽胞調節に関係していることが示唆される遺伝子を抽出し、ウェルシュ菌腸管毒素産生株を用いて、遺伝子変異株を作製し解析した。その結果、この調節遺伝子変異株は、野生株と比べ、形態、芽胞形成効率、ならびに腸管産生に変化が見られた。これらの結果は、芽胞形成調節ネットワークを解き明かす手がかりとなり得ると考えられ、ウェルシュ菌においては、食中毒予防法の開発の大きな手がかりとなり得る。

A. 研究目的

グラム陽性嫌気性桿菌ウェルシュ菌は、日本において食中毒の主要原因菌の１つとしてあげられる。この食中毒は、本菌が加熱不十分な食べ物とともに人体に入り、胃酸の刺激により芽胞を形成し、芽胞形成中に下痢を引き起こす腸管毒素（エンテロトキシン）を産生することが原因であると報告されている。しかし、その詳細なメカニズム、特に芽胞形成と腸管毒素産生の制御機構については未知のままである。本菌の食中毒予防法としては食物をよく加熱することしかないのが現状であり、大量に食べ物を調理する学校給食や仕出し弁当等での食中毒は未だに回避することが難しい状態である。そこで、遺伝子レベルで芽胞をコントロールし、食中毒予防につなげることを本研究の目的とした。

B. 研究方法

マイクロアレイデータを網羅的に解析し、様々な調節ネットワークを抽出後、そのデータをノザン解析ならびにリアルタイムPCRを用いて検証した。得られたデータをもとに芽胞形成に関わる調節遺伝子を抽出し、変異株を作製して、芽胞形成、腸管毒素産生性などの検討を行った。

C. 研究結果

マイクロアレイデータの解析により、芽胞調節に関与すると考えられる遺伝子が明らかとなった。この遺伝子変異株を、腸管毒素産生株を用いて作製したところ、野生株と比較して、形態が明らかに異なり、DNA の局在の経時的変化や、芽胞形成時の細胞膜の形成過程が大きく異なっていることが明らかとなった。また変異株においては野生株と比べて、芽胞形成効率が明らかに低下することが明らかとなった。腸管毒素に関しては、変異株において毒素遺伝子の転写がみられなくなり、毒素の産生もみられなくなることが明らかとなった。また、この遺伝子変異株においては、芽胞形成に深く関わる遺伝子群の転写も変化しており、遺伝子がこれらの遺伝子群を転写レベルで調節していることが明らかとなってきた。

D. 考察

これらの結果は、新規調節遺伝子が、芽胞形成調節に関与し、さらには、腸管毒素産生を調節している可能性を強く示唆している。この調節遺伝子を含む芽胞形成調節新規ネットワークをさらに詳細に解析することは、ウェルシュ菌食中毒予防法の開発の大きな手がかりとなり得ると考えられる。

E. 結論

新規調節遺伝子は芽胞形成に深く関与していることが強く示唆されたこと、また、本菌の食中毒は食品に混入した菌が体内に入る時に芽胞になり、腸管毒素を産生することで食中毒が発生することを考えると、この新規調節ネットワークをさらに解析することは、食中毒予防法開発に新たな側面を与える可能性が考えられる。

F. 健康危険情報

特になし

G. 研究発表

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Genes Regulated Directly by the VirR/VirS System in *Clostridium perfringens*. J. Bacteriol. 190: 7719-7727

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Virulence gene regulation by the *agr* system in *Clostridium perfringens*

Kaori Ohtani, Yonghui Yuan, Sufi Hassan, Ruoyu Wang, Yun Wang and Tohru Shimizu

Department of Bacteriology, Graduate School of Medical Science, University of Kanazawa, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8203, Japan

Corresponding Author : Tohru Shimizu
Department of Bacteriology, Graduate School of Medical Science
Kanazawa University, 1-13, Takara-machi Kanazawa,
Ishikawa 3920-8640, Japan
Phone : (+) 81-76-265-2200
Fax : (+) 81-76-234-4230
E-mail address: tshimizu@med.kanazawa-u.ac.jp

ABSTRACT

A Gram-positive anaerobic pathogen, *Clostridium perfringens*, causes clostridial myonecrosis or gas gangrene in humans by producing numerous extracellular toxins and enzymes that act in concert to degrade host tissue. The *agr* system is known to be important for the regulation of virulence genes in a quorum-sensing manner in *Staphylococcus aureus*. An $agrBD^{Sa}$ homologue was identified in the *C. perfringens* strain 13 genome, and the role of $agrBD^{Cp}$ was examined. The $agrBD^{Cp}$ knockout mutant did not express the theta-toxin gene and transcription of the alpha- and kappa-toxin genes was also significantly decreased in the mutant strain. The mutant strain showed a recovery of toxin production following addition of the culture supernatant of the wild-type strain, indicating that the $agrBD^{Cp}$ mutant lacks a signal molecule in the culture supernatant. An *agr-virR* double-knockout mutant was constructed to examine the role of the VirR/VirS two-component regulatory system, a key virulence regulator, in $agrBD^{Cp}$-mediated regulation of toxin production. The double-mutant strain could not be stimulated for toxin production with the wild-type culture supernatant. These results indicate that the $agrBD^{Cp}$ system plays an important role in virulence regulation and also suggest that VirR/VirS is required for sensing of the extracellular signal and activation of toxin gene transcription in *C. perfringens*.

INTRODUCTION

Clostridium perfringens is a Gram-positive, spore-forming, anaerobic bacterium. *C. perfringens* is the causative agent of several human and animal diseases, including clostridial myonecrosis, or gas gangrene (7). *C. perfringens* produces various extracellular enzymes and toxins, including alpha-, theta- and kappa-toxin encoded by *plc*, *pfoA* and *colA*, respectively (21). These toxin genes are positively regulated by the two-component VirR/VirS system (25) that is a major regulator of virulence in *C. perfringens*. The VirS is a sensor histidine kinase and VirR is a response regulator. When VirS senses specific stimuli in the environment, VirS autophosphorylates at a histidine residue and then transfers the phosphate to VirR. Once VirR is activated by phosphorylation, it regulates gene expression. The genomic sequence of *C. perfringens* strain 13 was determined in 2002 and it was found that the genome contains only five genes, including *pfoA* and VR-RNA, that have VirR-binding sites on their promoter regions (24). VR-RNA is known to be a small regulatory RNA and positively regulates *colA* and *plc* transcription (26). Recent microarray analysis suggested that many other genes are regulated by the VirRS-VR-RNA cascade. Thus a number of virulence-related genes and also some house-keeping genes are included in the VirRS-VR-RNA-regulon (Ohtani *et al.*, unpublished data). The *C. perfringens* genome contains many genes for toxins or for enzymes that can degrade host tissue, while the genome lacks many genes related to the synthesis of amino acids. Under infectious conditions, *C. perfringens* might secrete these toxins and enzymes in order to degrade the host tissue. It may then

58 import the resulting amino acids, using them to survive in the host tissue. The VirR/VirS
59 system is therefore very important for the activation of toxin production that results in
60 the degradation of host cells, and is critical for the survival of *C. perfringens*, especially
61 within the host. However, it is still unclear what the signal of VirS is and how this
62 signaling system effectively stimulates toxin production.
63 Many bacteria regulate gene expression in response to cell population density, a
64 phenomenon known as "quorum sensing" (4). Quorum sensing involves the production
65 of extracellular signaling molecules (autoinducers). In general, many known
66 autoinducers of Gram-positive bacteria are actively secreted peptides that are processed
67 from larger propeptides. These peptide autoinducers function as ligands for signal
68 receptors such as the two-component sensor histidine kinase (17). In Gram-negative
69 bacteria, the N-acylhomoserine lactones (AHLs) are well known as an autoinducer (14).
70 They diffuse freely in and out of cells and interacts directly with intracellular regulatory
71 proteins. The AHL accumulates as cells grow, and when it reaches a certain threshold,
72 AHL can efficiently regulate the expression of many genes. In *Vibrio fischeri*, the
73 LuxR protein binds to AHL, and this complex regulates the *lux* operon and many other
74 genes at the transcriptional level (14). Moreover, the *luxS* gene is responsible for the
75 production of another kind of autoinducer, autoinducer 2 (AI-2) (30). Highly conserved
76 *luxS* homologues have been identified in both Gram-positive and Gram-negative
77 bacteria (3). These quorum-sensing systems play important roles in the regulation of
78 virulence factors and in biofilm formation in various pathogenic bacteria (6, 28, 30).
79 In *C. perfringens*, the possibility that cell-cell signaling exists has been suggested (8).

In a previous report, two types of toxin-negative strains were cross-streaked on a blood agar plate, and one toxin-negative strain recovered its toxin production just after the crossing point of the two strains on the plate (8, 10). This experiment suggested that there is a signal molecule (called substance A) (9) that stimulates toxin production from outside of the cell. In 2002, cell-cell signaling mediated by *luxS* was reported and it was concluded that the signal produced actually regulated the transcription of toxin genes (18). However, the mutant strain of *luxS* still retained toxin production; therefore, it was concluded that the *luxS* signaling system might be different from that mediated by substance A and thus that there may be a different cell-cell signaling system in *C. perfringens*.

In Gram-positive bacteria, a secreted peptide regulates gene expression in the quorum-sensing manner as described above (17). In the case of *S. aureus*, the autoinducer peptide (AIP) acts as a signal to stimulate gene expression. This peptide contains an intramolecular thiolactone ring. The *agrD* gene is a structural gene for AIP, and AgrB is a protein that is required for modification of the AgrD propeptide. In the genome of *S. aureus*, the genes of a two-component system, *agrA* and *agrC*, lie next to the *agrBD* genes. The AgrA protein is a response regulator and AgrC is a sensor histidine kinase. The AIP, synthesized from the AgrD protein, is secreted and accumulates in the supernatant. Once AIP reaches a certain threshold level it activates its receptor, AgrC sensor histidine kinase, which then activates AgrA by phosphotransfer. Finally, AgrA activates the transcription of the regulatory RNA, RNAIII, that regulates the expression of various virulence genes of *S. aureus* (5, 15, 17). This signaling

system is highly conserved among many Gram-positive bacteria (12, 16, 20).

In this study we identified an *agrBD* gene in *C. perfringens* ($agrBD^{Cp}$) that is homologous to the *agrBD* gene of *S. aureus* ($agrBD^{Sa}$). Functional genetic analysis revealed that $agrBD^{Cp}$ is involved in the positive regulation of alpha-, kappa-, and theta-toxin genes through a cell-cell signaling mechanism that involves a two-component VirR/VirS system.

MATERIALS AND METHODS

Strains, media, and culture conditions

The *C. perfringens* strains 13 (13) and TS133 (23), as well as the other strains used in this study (Table 1), were cultured in GAM or TSF (tryptone 40 g, soytone 4 g, fructose 5 g /L, pH 5.7) (9) medium at 37°C under anaerobic conditions as described previously (23). The *Escherichia coli* strain DH5α was cultured under standard conditions (22). The plasmid pUC19 was used for general cloning in *E. coli*, and pJIR418 (27) was used as an *E. coli-C. perfringens* shuttle vector. The plasmid pTS405 was used as a complementation vector for *virR/virS* genes (19).

DNA manipulation

General recombinant DNA techniques were performed as described in Sambrook *et al.* (22) unless otherwise noted. *C. perfringens* strains were transformed by an electroporation-mediated transformation as previously described (23). Deletion

124 endpoints were confirmed by nucleotide sequencing using reverse or universal primers,
125 a Big-Dye terminator reaction kit and the ABI 310 sequencer (Applied Biosystems
126 Japan, Tokyo, Japan).
127
128 **Northern and Southern hybridization**
129 Total RNA from *C. perfringens* was extracted according to a method described
130 previously (1). Northern hybridization was also carried out as described previously (2,
131 11) except that DNA fragments were labeled with an AlkPhos-direct kit (Amersham
132 Pharmacia Biotech, Buckinghamshire, UK) and signals were detected by CDPstar
133 chemiluminescence. Southern hybridization was also performed using the same labeling
134 and detection procedures.
135
136 **Culture supernatant-replacement experiments**
137 *C. perfringens* was cultured in TSF medium to stationary phase (OD_{600}=5.0) as a
138 primary culture, and then these bacteria were inoculated at 5% concentration. The
139 culture was continued to various growth stages and centrifuged at 15,000 rpm for 5 min,
140 and then the culture supernatant was collected. To prepare recipient cells, *C. perfringens*
141 was cultured in TSF medium for 5 h at 37°C and centrifuged at 15,000 rpm for 5 min,
142 followed by removal of the supernatant from the cells. Recipient cells were suspended
143 with the appropriate culture supernatant and incubated at 37°C for 15 min. Total RNA
144 was then isolated from the incubated cells.
145

Construction of plasmids for allelic replacements

Specific mutants were constructed using PCR, and the sequences of all primers used for PCR are shown in Table 2. The fragment upstream of the *agr* region was amplified by PCR using primers 1 and 2 and inserted into the *Hinc*II site of pUC118. The fragment downstream of the *agr* region was amplified by PCR using primers 23 and 25 and inserted into the *Sma*I site of the plasmid containing the upstream region. Reverse PCR was performed using primers 2 and 23 and the erythromycin-resistance gene was cloned into the region deleted by reverse PCR.

Construction of deletion strains.

The resulting plasmid for allelic replacement of *agr* operon was transformed into wild-type strain 13. Transformants were screened on a blood agar plate containing erythromycin (25 μg/ml). A hemolysis-negative colony was picked up and Southern analysis was performed to confirm the null-mutation of the *agr* region in TS230.

To construct double-knockout mutants, an internal PCR fragment of the *virR* gene was inserted into pUC18 containing the *tetA* gene. The resulting plasmid was transformed into TS230 and screened on an agar plate containing 25 μg/ml of erythromycin and 2.5 μg/ml of tetracycline. The single-cross over mutation of *virR* in TS231 was confirmed by PCR using the appropriate primer set.

Construction of deletion mutants

To construct the pTS1304 deletion mutant containing the genomic fragment stretching from CPE1563 to $agrD^{Cp}$, PCR was performed using the primers listed in Table 2. This PCR fragment was inserted into the *Hinc*II site of pUC118 and the resulting plasmid was used as a template for further PCR. Each fragment amplified by PCR was self-ligated and transformed into *E. coli* DH5α. Then the inserted fragments containing various *agr* genomic regions were sub-cloned into pJIR418. To construct the pTS1313 deletion mutant, PCR was performed using pTS1312 as a template.

RESULTS AND DISCUSSION

Identification of an *agrBD* homologue in *C. perfringens*

The *agr* operon of *S. aureus* is known to mediate a quorum-sensing system (17). It has been reported that there is a homologue of this *agr* system in *C. perfringens* SM101 and ATCC13124 genomes (29). But the function of the *agr* system in *C. perfringens* has not been determined. To investigate the function of the *agr* system in *C. perfringens*, we searched for homologues of the *agr* operon in the genome of *C. perfringens* strain 13. We found that the amino acid sequence deduced from CPE1561 showed a 29% identity and 50% similarity with the $AgrB^{Sa}$ protein of *S. aureus*. The *agrB* gene encodes an integral membrane protein that modifies the auto-inducer pro-peptide (AIP) produced by AgrD protein. Downstream of CPE1561 ($agrB^{Cp}$), there was a small ORF that was not assigned as an ORF when the *C. perfringens* genome sequence was determined (Fig.

190 1A). The protein from this ORF (designated CPE1560a) was similar to the AgrD
191 peptide of *S. aureus* (32% identity and 46% similarity in a 43-amino-acid-overlap
192 region), which is a propeptide for AIP. Next to the $agrBD^{Sa}$ gene in *S. aureus*, there are
193 genes for a two-component system (*agrA* and *agrC*) that can act as a receptor for AIP
194 and induce gene expression. However, in the *C. perfringens* strain 13 genome, a similar
195 two-component system could not be found in the vicinity of the *agrBD* gene (Fig. 1A).
196 AIPs in *S. aureus* show a variety of amino acid sequences, but the central cysteine,
197 which is important for the formation of a thiolactone ring with the C-terminal amino
198 acid, is conserved in all of them (15). The amino acid sequence of the *C. perfringens*
199 AgrD ($AgrD^{Cp}$) is completely different from that of AIPs, with the exception that this
200 same central cysteine is conserved (Fig. 1B). However, the predicted peptide sequences
201 are conserved in all three *C. perfringens* whose genome sequences are available (Fig.
202 1B).

203 To examine the mRNA corresponding to the $agrBD^{Cp}$ region, Northern analysis was
204 performed using gene probes for $agrD^{Cp}$, CPE1561 ($agrB^{Cp}$), CPE1562, CPE1563,
205 CPE1564 and CPE1560. The mRNA obtained from the CPE1561 region was
206 approximately 2.5 kb in length (Fig. 1C). This length is consistent with the total length
207 of the CPE1561 operon calculated from genome information. Thus the CPE1561 operon
208 encodes $agrD^{Cp}$, CPE 1562 and CPE1563. These data also suggest that CPE1564 and
209 CPE1560 must be independently transcribed, since mRNAs of different size were
210 detected by Northern hybridization using gene probes for CPE1564 and CPE1560 (data
211 not shown). The $agrD^{Cp}$ gene is included in the operon, but a second, small independent

mRNA was also identified that corresponds to the *agrD*^{Cp} gene (Fig. 1C). This mRNA is transcribed at a high level up to the stationary phase of growth (data not shown). The length of the *agrD*^{Cp} mRNA was calculated as 0.45 kb (Fig. 1C). This 0.45-kb mRNA was also detected by using the CPE1561(*agrB*^{Cp}) probe, probably because the transcription start site of this mRNA exists in the coding region of CPE1561.

Effect of *agrBD*^{Cp} on toxin gene expression

To examine the role of the *agrBD*^{cp} region in detail, an *agrBD*^{Cp}-null mutant strain and its complement strain were constructed as described in the Materials and Methods section. The resulting mutant strain (TS230) lacked PfoA-hemolytic activity on blood agar plates (Fig. 4). Transcription of *agrD*^{Cp} was completely absent in TS230 but was recovered in the strains that carry pTS1303 and pTS1304 (Fig. 2). In the TS230/pTS1304, an extra band was detected above the *agrD*^{Cp} mRNA; this band presumably originated from a read-through transcription occurring in the recombinant plasmid (Fig. 2). The transcription of *pfoA* in TS230/pJIR418 was very low and *plc* and *colA* mRNA levels were significantly decreased (Fig. 2). In the TS230 strain that was complemented with a plasmid containing the intact 2.5-kb *agrBD*^{Cp} operon (TS230/pTS1304), transcription of the toxin genes increased to almost the same level as that in the wild-type strain (Fig. 2). Since the level of toxin gene transcription was practically the same between the TS230/pTS1304 strain complemented with the 2.5-kb operon and the TS230/pTS1303 strain complemented with the 2.5-kb operon and the downstream CPE1560 (Fig. 2), it was concluded that CPE1560 does not have a

significant effect on toxin gene expression. From these data it was concluded that the *agrBD*^{Cp} operon is responsible for the transcriptional activation of toxin genes in *C. perfringens*.

Function of each gene in the operon

In *S. aureus*, *agrBD*^{Sa} and a two-component regulatory system are all included in a single operon. However, in the case of *C. perfringens* there is no apparent two-component system in the vicinity of *agrBD*^{Cp} in the genome. Instead, two other hypothetical genes exist upstream of CPE1561 (*agrB*^{Cp}) and compose a 2.5-kb operon together with *agrBD*^{Cp} (Fig. 1A). It was therefore considered a possibility that these genes might also have a regulatory effect on toxin gene expression. To analyze the effect of these genes on toxin transcription, plasmids encoding various deletions in these genes were constructed and transformed into the *agrBD*^{Cp} mutant TS230 (Fig. 3). Deletion plasmids containing both an intact *agrD*^{Cp} and the CPE1561 gene (pTS1303, pTS1308, pTS1309 and pTS1314) could restore transcription of the toxin genes, whereas plasmids that do not contain the CPE1561 gene (pTS1302, 1307, 1310, 1311, 1312 and 1313) could not recover toxin gene transcription even when the plasmids contained an intact *agrD*^{Cp} gene (Fig. 3). Plasmids that contain both the *agrD*^{Cp} and CPE1561 genes but that do not contain a potential promoter region located upstream of CPE1563 (pTS1301, 1302, 1305 and 1306) also could not activate transcription of the toxin genes (Northern blot data not shown). These experiments suggest that at least CPE1561 (*agrB*^{Cp}) and *agrD*^{Cp} appear to be essential to the regulatory function of this

operon and that transcription is started from a position upstream of CPE1563. Interestingly, in TS230/pTS1308 and TS230/pTS1309 (the plasmids that contain CPE1561 and $agrD^{Cp}$ but not CPE1563) toxin genes are transcribed but the level of transcription is much weaker than that in TS230/pTS1304 (the plasmid containing all of the genes). However, transcription of the toxin genes in the mutant strain with pTS1314 (ΔCPE1562) was at almost the same level as that in the TS230/pTS1304 strain. These data indicated that CPE1561 ($agrB^{Cp}$) and $agrD^{Cp}$ are essential genes for toxin gene activation but that CPE1563 is required for complete activation.

Activation of toxin production by the toxin-negative strain TS133

We examined whether TS230 can recover its hemolytic activity by exposure to a signal molecule produced from another toxin-negative *virR* mutant strain, TS133. First, TS133 was streaked on a blood agar plate and then TS230 was streaked at a right angle to TS133 at various distances (Fig. 4). As the two strains became closer, hemolysis from TS230 became stronger. This finding suggested that TS133 secreted a signal molecule that activated toxin production and that TS230 recovered its toxin production by absorbing this molecule from TS133. However, this signal molecule did not appear to diffuse widely in the agar medium because hemolysis of TS230 only occurred when the distance between TS230 and TS133 was quite short (Fig. 4).

Production of the signal molecule and its putative sensor protein

In *S. aureus*, AIP is produced from the $agrBD^{Sa}$ region and is secreted from the cell