JOURNAL OF BACTERIOLOGY, June 2009, p. 3919–3927 Vol. 191, No. 12
0021-9193/09/$08.00+0 doi:10.1128/JB.01455-08

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

Received 16 October 2008/Accepted 30 March 2009

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*. A homologue for *S. aureus agrBD* (*agrBD*$_{Sa}$) was identified in the *C. perfringens* strain 13 genome, and the role of *C. perfringens agrBD* (*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 after the 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*.

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-toxins 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 housekeeping genes are included in the VirRS-VR-RNA-regulon (K. 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 import the resulting amino acids, using them to survive in the host tissue. The VirR/VirS system is therefore very important for the activation of toxin production that results in the degradation of host cells and is critical for the survival of *C. perfringens*, especially within the host. However, it is still unclear what the signal of VirS is and how this signaling system effectively stimulates toxin production.

Many bacteria regulate gene expression in response to cell population density, a phenomenon known as "quorum sensing" (4). Quorum sensing involves the production of extracellular signaling molecules (autoinducers). In general, many known autoinducers of gram-positive bacteria are actively secreted peptides that are processed from larger propeptides. These peptide autoinducers function as ligands for signal receptors such as the two-component sensor histidine kinase (17). In gram-negative bacteria, the *N*-acylhomoserine lactones (AHLs) are well known as autoinducers (14). They diffuse freely in and out of cells and interact directly with intracellular regulatory proteins. AHL accumulates as cells grow, and when it reaches a certain threshold, AHL can efficiently regulate the expression of many genes. In *Vibrio fischeri*, the LuxR protein binds to AHL, and this complex regulates the *lux* operon and many other genes at the transcriptional level (14). Moreover, the *luxS* gene is responsible for the production of another kind of autoinducer, autoinducer 2 (30). Highly conserved *luxS* homologues have been identified in both gram-positive and gram-negative bacteria (2). These quorum-sensing systems play important roles in the regulation of virulence factors and in biofilm formation in various pathogenic bacteria (6, 28, 30).

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 mol-

* Corresponding author. Mailing address: 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: tshimizu@med.kanazawa-u.ac.jp.

▽ Published ahead of print on 10 April 2009.

TABLE 1. *C. perfringens* strains and plasmids used in this study

Strain or plasmid	Description[a]	Source
C. perfringens strains		
Strain 13	Wild-type strain (type A)	
TS133	Strain 13 *virR*::Tet^r	
TS230	Strain 13 Δ*agrBD*::Em^r	This study
TS231	Strain 13 Δ*agrBD*-Δ*virR*::Tet^r Em^r	This study
Plasmids		
pJIR418	*E. coli* -*C. perfringens* shuttle vector, Cm^r Em^r; pJIR418 Ω (PstI 4.3-kb strain 13 genomic library)	
pTS405	*virR*$^+$ *virS*$^+$ complementation vector; Cm^r	T. Shimizu, unpublished data
pTS1301	pJIR418 Ω(Δpromoter CPE1562-CPE1561-*agrD*-CPE1560)	This study
pTS1302	pJIR418 Ω(Δpromoter *agrD*-CPE1560)	This study
pTS1303	pJIR418 Ω(CPE1563-CPE1562-CPE1561-*agrD* -CPE1560) including promoter	This study
pTS1304	pJIR418 Ω(CPE1563-CPE1562-CPE1561-*agrD*) including promoter	This study
pTS1305	pJIR418 Ω(Δpromoter CPE1562-CPE1561-*agrD*)	This study
pTS1306	pJIR418 Ω(Δpromoter CPE1561-*agrD*)	This study
pTS1307	pJIR418 Ω(Δpromoter *agrD*)	This study
pTS1308	pJIR418 Ω(CPE1562-CPE1561-*agrD*)	This study
pTS1309	pJIR418 Ω(CPE1561-*agrD*)	This study
pTS1310	pJIR418 Ω(*agrD*)	This study
pTS1311	pJIR418 Ω(CPE1563-*agrD*)	This study
pTS1312	pJIR418 Ω(CPE1563-CPE1562-*agrD*)	This study
pTS1313	pJIR418 Ω(CPE1562-*agrD*)	This study
pTS1314	pJIR418 Ω(CPE1562-CPE1561-*agrD*)	This study

[a] Cm^r, chloramphenicol resistance; Em^r, erythromycin resistance; Tet^r, tetracycline resistance.

ecule (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 described above (17). In the case of *S. aureus*, the autoinducer propeptide (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 the present 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 the present study (Table 1), were cultured in Gifu anaerobic medium (GAM) or TSF (tryptone, 40 g/liter; soytone, 4 g/liter; fructose, 5 g/liter [pH 5.7]) (9) medium at 37°C under anaerobic conditions as described previously (23). *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 endpoints were confirmed by nucleotide sequencing using reverse or universal primers, a BigDye terminator reaction kit, and an ABI 310 sequencer (Applied Biosystems, Tokyo, Japan).

Northern and Southern hybridization. Total RNA from *C. perfringens* was extracted according to a method described previously (1). Northern hybridization was also carried out as described previously (3, 11) except that DNA fragments were labeled with an AlkPhos-direct kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), and signals were detected by CDPstar chemiluminescence. Southern hybridization was also performed using the same labeling and detection procedures.

Culture supernatant replacement experiments. *C. perfringens* was cultured in TSF medium to stationary phase (optical density at 600 nm of 5.0) as a primary culture, and then these bacteria were inoculated at 5% concentration. The culture was continued to various growth stages and centrifuged at 15,000 rpm for 5 min, and then the culture supernatant was collected. To prepare recipient cells, *C. perfringens* was cultured in TSF medium for 5 h at 37°C and centrifuged at 15,000 rpm for 5 min, followed by removal of the supernatant from the cells. Recipient cells were suspended with the appropriate culture supernatant and incubated at 37°C for 15 min. Total RNA was then isolated from the incubated cells.

Construction of plasmids for allelic replacements. Specific mutants were constructed by 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 HincII site of pUC118. The fragment downstream of the *agr* region was amplified by PCR using primers 23 and 25 and inserted into the SmaI site of the plasmid containing the upstream region.

TABLE 2. Primers used in this study

Primer	Sequence	Description
1	GAACATATGTTTGCATGGAGG	To make plasmid for allelic replacement
2	CAAGCTCTGGGGCACTAGTT	To make plasmid for allelic replacement
3	ATTGTAAAGAGTGAAGGGAG	To construct pTS1303,1304
4	AAAGTTGGACAATCTATCCTA	To construct pTS1301
5	AGGATAGATTGTCCAACTTT	To construct pTS1308-1310
6	TTTATGGGTAACTATGATGT	To construct pTS1305
7	ACTTGTTCCTATCATATGTA	To make CPE1563 probe
8	ATTCTTCCTCCGCTGTCACT	To construct pTS1314
9	AGTGACAGCGGAGGAAGAAT	To make CPE1562 probe
10	ATGGTATTCATACAATATTG	To construct pTS1306
11	TTTAAACCTTCACATAAA	To make CPE1562 probe
12	TAGGTATTCCATCTACTAT	Sequence primer
13	TTTTTCAGCTATTAACTTCGA	To construct pTS1313,1314
14	TTTACAGCAAGCATACTTA	To construct pTS1310,1311, to make CPE1561 probe
15	TTCTGGAGGAGCACATTCAG	To construct pTS1302
16	TCCTTAGAGTCATACATTGC	To make CPE1561 probe
18	TTGTTAAAAACTATAGATTCTT	To check mutation, *agrD* Northern
19	GGCCGGTTTAAAACCTACCT	To check mutation, agrD Northern
20	TATACTAGATTAGAGAGGGAGAAT	To make CPE1560 probe
21	CTCTTCTCCTCCATATCTAGC	To make CPE1560 probe
22	ACTTCAGCTAAGCTATGCTG	To construct pTS1302
23	AAGGTCATAGGTGTTGTATAGC	To make plasmid for allelic replacement
24	TAACAGTACGTGTTCCAAAC	To construct pTS1301,1303
25	AGATGGGGCGGTAGACGTAG	To make plasmid for allelic replacement

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 of erythromycin/ml and 2.5 μg of tetracycline/ml. The single-crossover 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 HincII 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. The inserted fragments containing various *agr* genomic regions were then subcloned 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 ATCC 13124 genomes (29). However, 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 AIP produced by AgrD protein. Downstream of CPE1561 ($agrB_{Cp}$), there was a small open reading frame (ORF) that was not assigned as an ORF when the *C. perfringens* genome sequence was determined (Fig. 1A). The protein from this ORF (designated CPE1560a) was similar to the AgrD peptide of *S. aureus* (32% identity and 46% similarity in a 43-amino-acid-overlap region), which is a propeptide for AIP. Next to the $agrBD_{Sa}$ gene in *S. aureus*, there are genes for a two-component system (*agrA* and *agrC*) that can act as a receptor for AIP and induce gene expression. However, in the *C. perfringens* strain 13 genome, a similar two-component system could not be found in the vicinity of the *agrBD* gene (Fig. 1A). AIPs in *S. aureus* show a variety of amino acid sequences, but the central cysteine, which is important for the formation of a thiolactone ring with the C-terminal amino acid, is conserved in all of them (15). The amino acid sequence of the *C. perfringens* AgrD ($AgrD_{Cp}$) is completely different from that of AIPs, with the exception that this same central cysteine is conserved (Fig. 1B). However, the predicted peptide sequences are conserved in all three *C. perfringens* whose genome sequences are available (Fig. 1B).

To examine the mRNA corresponding to the $agrBD_{Cp}$ region, Northern analysis was performed using gene probes for $agrD_{Cp}$, CPE1561 ($agrB_{Cp}$), CPE1562, CPE1563, CPE1564, and CPE1560. The mRNA obtained from the CPE1561 region was ~2.5 kb in length (Fig. 1C). This length is consistent with the total length of the CPE1561 operon calculated from genome information. Thus, the CPE1561 operon encodes $agrD_{Cp}$, CPE1562, and CPE1563. These data also suggest that CPE1564 and CPE1560 must be independently transcribed, since mRNAs of different sizes were detected by Northern hybridization using gene probes for CPE1564 and CPE1560 (data 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 de-

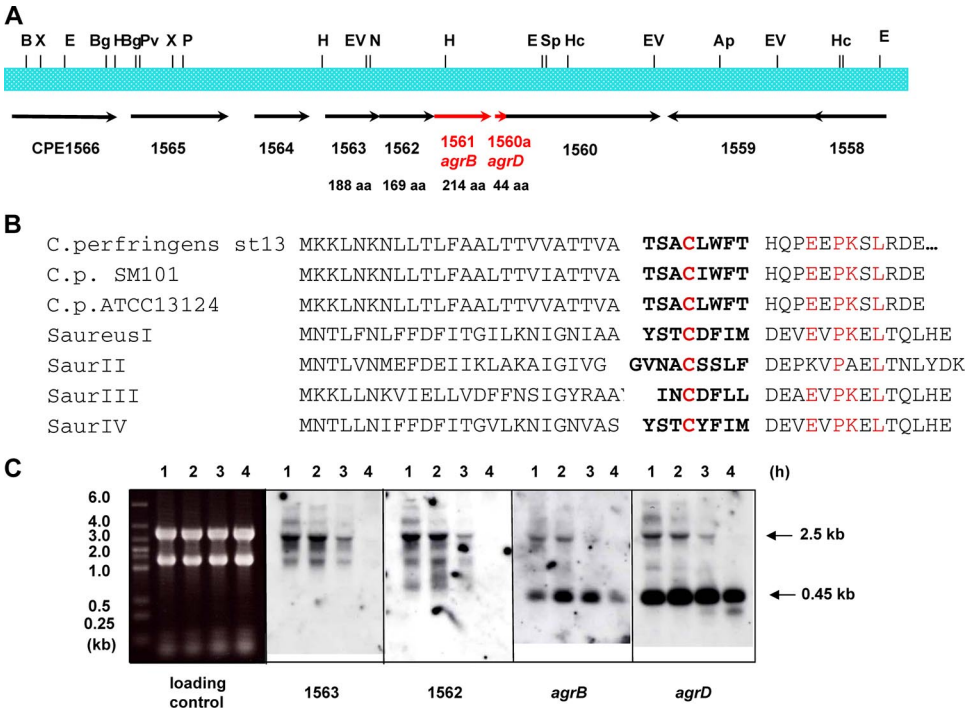

FIG. 1. Analysis of the *agr* region in *C. perfringens*. (*A*) Gene map of the *agr* region in *C. perfringens*. (*B*) Alignment of the deduced amino acid sequence of $AgrD_{Cp}$ in *C. perfringens* and *S. aureus* AIPs. Conserved residues are indicated in red, and the deduced sequence of the mature peptides is in boldface. (C) Northern analysis of the $agrBD_{Cp}$ region. RNA was isolated from strain 13 after culture for 1, 2, 3, and 4 h.

tected 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 Materials and Methods. The resulting mutant strain (TS230) lacked PfoA-hemolytic activity on blood agar plates (see 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 readthrough transcription occurring in the recombinant plasmid (Fig. 2). The transcription of *pfoA* in TS230/pJIR418 was very low, and the *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

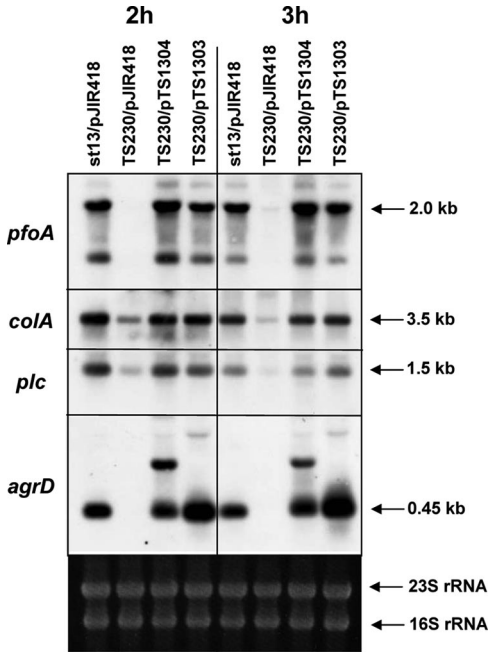

FIG. 2. Northern analysis of the $agrBD_{Cp}$ mutant and complemented strains. An $agrBD_{Cp}$-null mutant (TS230) was constructed by a double-crossing-over method, and the $agrBD_{Cp}$ region was complemented by transformation with pTS1304 and pTS1303. Total RNA was prepared from 2- and 3-h-cultured cells, and 10 μg of total RNA was used for Northern analysis. The internal regions of *pfoA*, *colA*, *plc*, and *agrD* were used as probes.

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, pTS1307, pTS1310, pTS1311, pTS1312, and pTS1313) 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, pTS1302, pTS1305, and pTS1306) 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 into the culture supernatant, where it regulates gene expression via a two-component system consisting of AgrA and AgrC (17). To determine whether $agrBD_{Cp}$ is related to the signaling component that is secreted from *C. perfringens* cells, we assayed the ability of $agrBD_{Cp}$ to modulate toxin expression. The culture supernatant was collected from the wild-type *C. perfringens* strain 13 or the $agrBD_{Cp}$ mutant TS230 at early log phase (optical density at 600 nm of 0.5) and was then added to TS230 cells. The cells were incubated at 37°C for 15 min, and total RNA was prepared and analyzed by Northern analysis. The transcription of toxin genes was significantly increased in the TS230 cells only when the wild-type supernatant was added (Fig. 5A), suggesting that the TS230 cells lacked the ability to produce the signal molecule and release it into the supernatant. To further confirm that the signal molecule in the supernatant of strain 13 was produced from the $agrBD_{Cp}$ region, the supernatant was collected from a TS230 mutant strain that had been complemented with an intact $agrBD_{Cp}$ (TS230/pTS1304). When this supernatant was tested on TS230 cells, the expression of toxin genes, especially that of *pfoA*, was strongly induced (Fig. 5A). These data clearly indicate that the $agrBD_{Cp}$ gene is responsible for the production of an extracellular autoinducible signal molecule that controls the expression of toxin genes in *C. perfringens*.

In *C. perfringens*, the VirR/VirS-VR-RNA system is known as a global regulator and can regulate the expression of many toxin genes, including *plc*, *pfoA*, and *colA*; however, the signal that activates the sensor protein VirS has not been identified. Since the $agrBD_{Cp}$ locus controls the expression of a subset of toxin genes similar to that of the VirR/VirS-VR-RNA system, it seemed highly probable that VirS is a sensor protein for the signal molecule produced from the $agrBD_{Cp}$ region. To examine this hypothesis, an $agrBD_{Cp}$-*virR*/*virS* double-knockout mutant was constructed (designated TS231), and the effect of the wild-type supernatant on toxin transcription in the double mutant was examined. The transcription of *pfoA* in the TS231 mutant was not activated by the wild-type supernatant (Fig. 5B). In contrast, when TS231 was complemented with the plasmid pTS405, which contains the intact *virR*/*virS* genes, the resulting strain (TS231/pTS405) could sense the extracellular signal, and the transcription of toxin genes was significantly induced by the addition of wild-type or TS230/pTS1304 supernatants (Fig. 5C). In addition, the transcription of *plc* and *colA* in TS231/pJIR418 or TS231/pTS1304 was also upregulated by addition of the wild-type supernatant (Fig. 5C). It was suggested from these data that VirR/VirS is important for sensing of the extracellular signal and activation of toxin gene transcription in *C. perfringens*. However, it remains possible that another two-component system or another protein plays a role in the sensing of this signal, and thus further experiments are needed to elucidate the relationship between the signal molecule from $agrBD_{Cp}$ and the VirS sensor protein.

Regulation between *agr* and *virR*/*virS*. In *S. aureus*, the *agr* signaling system results in a positive-feedback loop, and the expression of both $agrBD_{Sa}$ for AIP production and *agrA*/*agrC* for AIP sensing are positively regulated in an operon (15). To examine the regulatory mechanism of the *agr* system in *C. perfringens*, Northern analysis was performed by using TS133 and TS230. At first, RNA was isolated from the wild-type strain (strain 13), TS133, and its complement strain TS133/pTS405, which were cultured for 2 h and 3 h. As in previous experiments, transcription of *pfoA* was absent in TS133 but recovered in TS133/pTS405 (Fig. 6A). In contrast, the transcriptional levels of $agrD_{Cp}$ and the 2.5-kb operon in the three strains were almost the same at 2 h under a *virR*/*virS*-negative background (Fig. 6A), although the level of $agrD_{Cp}$ transcript was slightly decreased in TS133/pJIR418 at 3 h, which was thought to be not significant.

Next, Northern analysis was performed using strains 13/pJIR418, TS230/pJIR418, and TS230/pTS1304 to check the *virR*/*virS* transcription under $agrBD_{Cp}$-negative conditions. As shown in Fig. 6B, the transcription of the *virR*/*virS* operon was

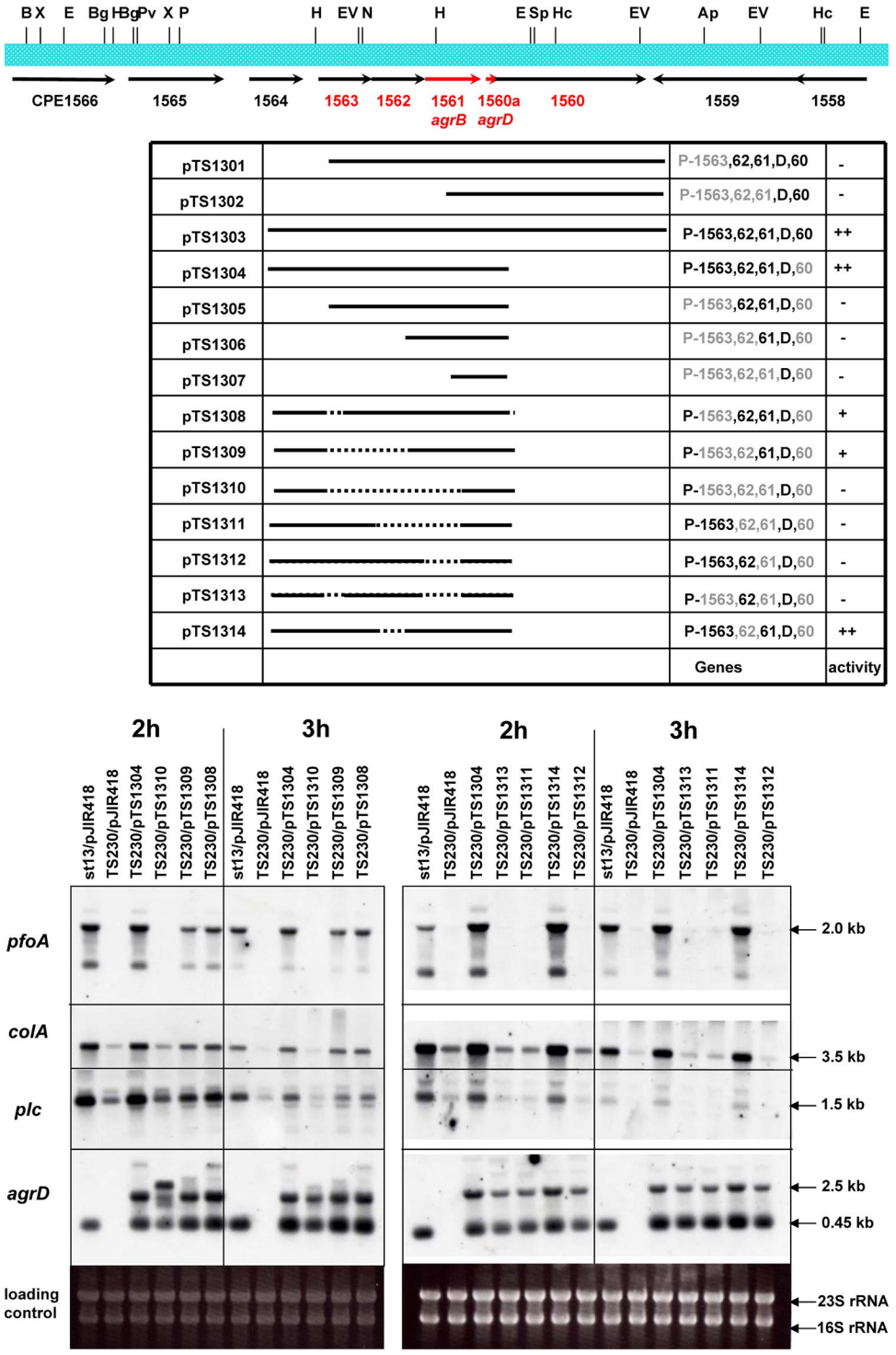

FIG. 3. Deletion analysis of the *agr* region. To determine the role of each gene in the operon, deletion plasmids were constructed and transformed into the $agrBD_{Cp}$-null mutant, TS230. Each strain was cultured, and RNA was isolated after 2 and 3 h of culture. The RNA was used for Northern analysis of the indicated toxin genes. In the deletion table, "−" indicates no activity, "++" indicates the plasmid has activity to induce the expression of toxin genes, and "+" indicates the plasmids have activity but that the activity is lower than that of pTS1304. The internal regions of *pfoA*, *colA*, *plc*, and *agrD* were used as probes.

too faint to confirm its regulation, but the mRNA level was almost the same in all three strains. These results suggested that the *agr* regulatory system involving the $agrBD_{Cp}$ and *virR*/*virS* operons in *C. perfringens* is not completely analogous to the *agr* regulation system in *S. aureus*.

Effect of a stationary culture supernatant on *pfoA* transcription. To further analyze the mechanism by which the extracellular signal in the culture supernatant of *C. perfringens* regulates toxin gene expression, the effect of addition of the *C. perfringens* culture supernatant on *pfoA* expression was exam-

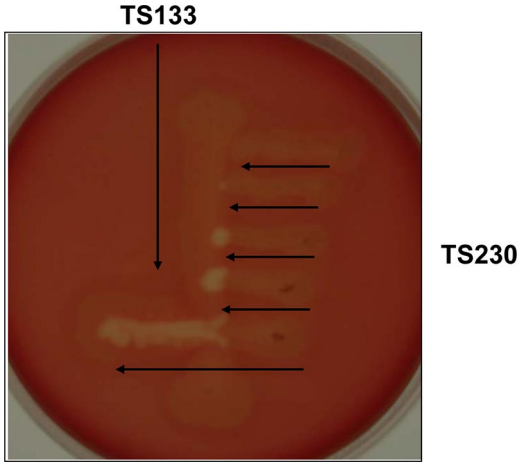

FIG. 4. Cross-streaking of TS230 and TS133. The *virR* mutant strain, TS133, was streaked onto a blood agar plate, and then several streaks of TS230 were made at a right angle to TS133. The distance between the two strains was decreased with each successive streak.

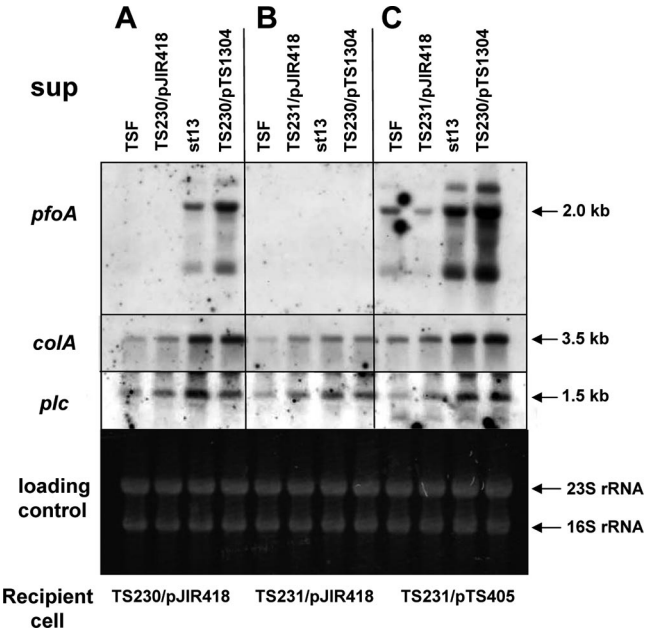

FIG. 5. Effect of the wild-type supernatant on the expression of toxin genes in TS230 and TS231. The culture supernatant was collected from strain 13 and added to the indicated strains to check the effect of the supernatant on sensor protein activity. The supernatants were collected from the wild-type strain, strain 13/pJIR418, and strain TS230/pTS1304 after culture for 1.5 h. Total RNA was prepared 15 min after addition of the supernatant. (A) The supernatant (sup) was added onto the *agr*-null mutant, TS230. (B) The supernatant (sup) was added onto the *agr*-null *virR* mutant, TS231/pJIR418. (C) The supernatant (sup) was added onto TS231 that contains an intact *virR*/*virS*, TS231/pTS405.

ined in more detail by Northern analysis. Although the expression of *plc* and *colA* was also partially regulated by the extracellular signal molecule in the supernatant, we focused on the regulation of *pfoA* in this analysis, since *pfoA* appears to be the main target of this system. First, the supernatant was removed from wild-type strain 13 cells that were cultured to various growth stages (Fig. 7A, 2 to 8 h). These cells were used as recipient cells and were resuspended in fresh TSF medium. As a control, cells were resuspended in the supernatant that had been removed. After 15 min of incubation in the added medium or supernatant, total RNA was prepared from the recipient cells. In the control experiment (see the "sup" lane in Fig. 7A), maximum transcription of *pfoA* was observed when the supernatant from a 2-h cell culture was added. However, *pfoA* transcription in the recipient cells was clearly observed within 15 min after the supernatant was replaced with fresh TSF medium (Fig. 7A, lane TSF). Surprisingly, the transcription of *pfoA* occurred even in the 8-h-cultured recipient cells after replacement of the supernatant with fresh medium (Fig. 7A). Furthermore, the transcription of *pfoA* in the 3- to 8-h-cultured recipient cells (lane TSF; 3 to 8 h of culture) was at a much higher level than that observed in the recipient cells cultured for 2 h in the presence of a 2-h culture supernatant (lane 2h sup). These data suggest that there is another signaling molecule in the supernatant that negatively controls *pfoA* expression, especially at the stationary phase, because removal of the culture supernatant and re-addition of fresh medium leads to activation of *pfoA* transcription in the 3-h (mid log)- to 8-h (stationary)-cultured recipient cells. Furthermore, these data presumably suggest that the amount of signal molecule that binds to recipient cells is sufficient to activate *pfoA* transcription. Moreover, through the removal of the stationary-phase supernatant, the concentration of the inhibitory substance might decrease, and the remaining activator bound to cells could stimulate *pfoA* transcription.

To confirm this hypothesis, the supernatant from the stationary phase was diluted with TSF medium and added to TS230 recipient cells. As predicted, diluted supernatant from the stationary phase could activate *pfoA* transcription, with a maximum activation observed at a fourfold dilution (Fig. 7B). These data suggest that there may be an inhibitory molecule in the supernatant from the stationary phase that represses *pfoA* expression but that this inhibition is abrogated when the hypothetical inhibitor is diluted. The proportions of activator concentration and inhibitory molecule might be important for determining the transcriptional level of the *pfoA* gene. Thus, in *C. perfringens*, a gradual accumulation of the inhibitor might occur over the culture period and, when the concentration of the inhibitor reaches a certain threshold, it may completely stop the transcription of *pfoA*. This mechanism could explain the decrease in toxin production at the stationary phase of growth in *C. perfringens*.

In the present study, we examined novel regulatory genes ($agrBD_{Cp}$) for toxin production in *C. perfringens*. These genes are highly similar to the *agr* system in *S. aureus*, and we have shown that the $agrBD_{Cp}$ locus is responsible for the production of an extracellular signal molecule that stimulates the expression of toxin genes in *C. perfringens*. We also found that the two-component VirR/VirS system appears to be required for the regulation by the signaling molecule produced by $agrBD_{Cp}$.

In *C. perfringens* the functions of $agrBD_{Cp}$, the VirR/VirS system, and VR-RNA seem to be quite similar to those of *S. aureus* $agrBD_{Sa}$, AgrA/AgrC, and RNAIII, respectively. Consequently, the two bacteria might have evolved similar regulatory systems to control their pathogenicity toward humans.

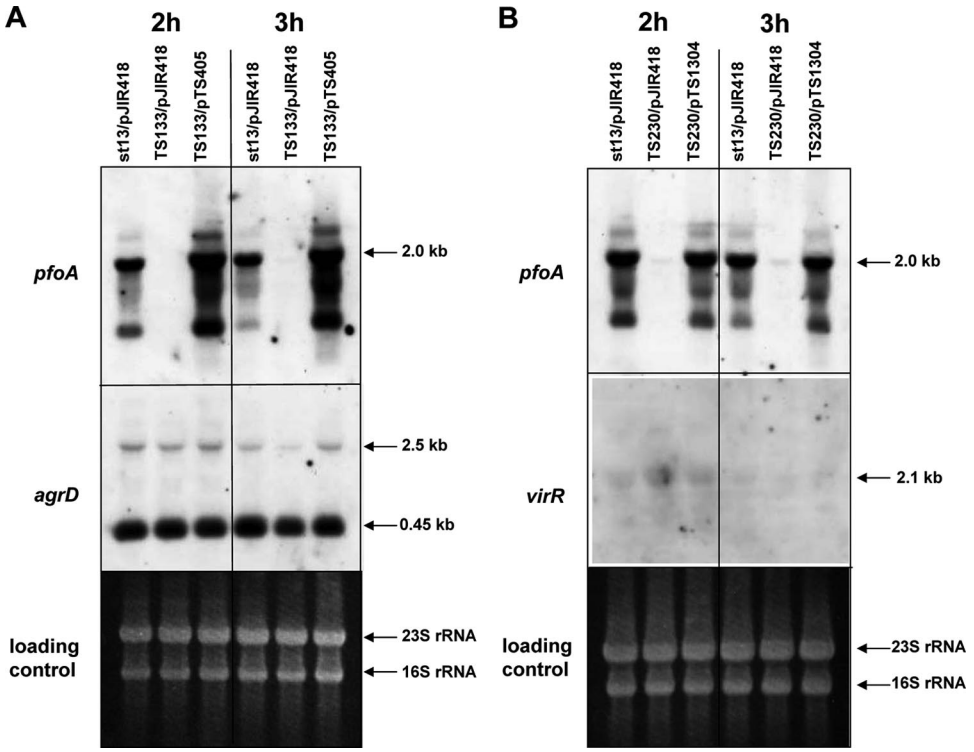

FIG. 6. Regulatory relationship between $agrBD_{Cp}$ and *virR/virS*. (A) Regulation of $agrBD_{Cp}$ by *virR/virS*. Total RNA was isolated from 2- and 3-h-cultured strain 13/pJIR418, TS133/pJIR418, and TS133/pTS405. (B) Regulation of *virR/virS* by $agrBD_{Cp}$. Total RNA was isolated from strain 13/pJIR418, TS230/pJIR418, and TS230/pTS1304. A 10-μg portion of total RNA was used for Northern analysis.

However, the genes involved in the regulation of toxin genes are scattered around the genome of *C. perfringens*, whereas the genes involved in the *agr* system are located in a cluster on the *S. aureus* chromosome (17).

It is noteworthy that toxin gene expression in *C. perfringens* reaches a maximum during the log phase of growth and completely stops at the stationary phase, whereas in many other pathogenic bacteria, toxin gene expression commonly starts at the stationary phase. Induction of toxin gene expression at the stationary phase is mainly mediated by a quorum-sensing mechanism. In contrast, the $agrBD_{Cp}$ system of *C. perfringens* induces the expression of toxin genes in the early stages of cell growth. For this expression pattern, there may be other unique systems that ensure the specific expression of toxin genes at the early stages of cell growth. From the data in the present study, we predict that there might exist in *C. perfringens* a system whereby inhibitory molecules are secreted into the medium. However, these molecules would stop toxin gene expression only upon reaching a critical level at the stationary phase. The balance between the $agrBD_{Cp}$ activator system and a second,

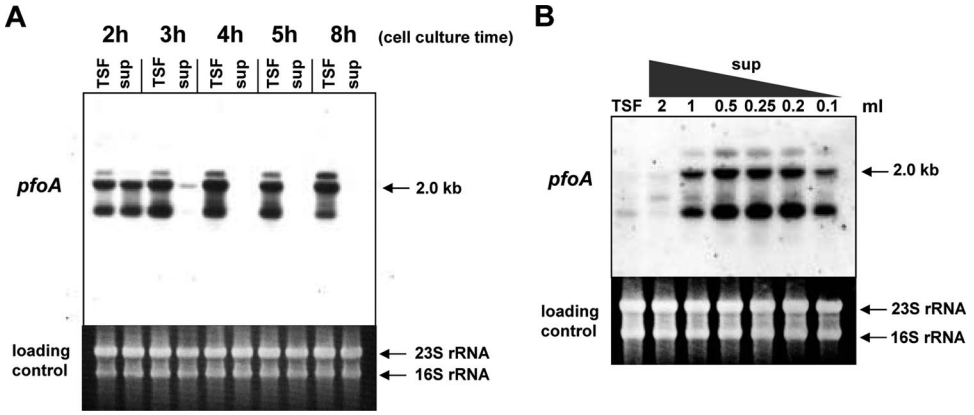

FIG. 7. Effect of the supernatant on toxin gene expression. (A) The supernatant was removed from the various time points of the culture. The cells from each time point were incubated with TSF at 37°C. As a control, the removed supernatant was added again to the same cells. RNA was isolated after 15 min of incubation. Lanes: TSF, TSF control; sup, culture supernatant. (B) The supernatant from strain 13 after 6 h of culture was diluted with TSF medium and added to TS230 cells. RNA was isolated after a 15-min incubation.

as-yet-undefined inhibitory system may be important for the proper control of gene expression in *C. perfringens*.

The unique regulation of toxin expression in *C. perfringens* is consistent with the requirement of *C. perfringens* to secrete various tissue-degrading toxins and enzymes at an early stage of infection. These secreted products enable the organism to acquire essential nutrients from the host (resulting in gas gangrene) that are required for the survival and growth of the bacteria. Genomic analysis has shown that *C. perfringens* lacks many genes related to amino acid biosynthesis, with the exception of genes for the three amino acids cysteine, serine, and glycine. Thus, in order to survive, especially in a host environment, *C. perfringens* may require a well-coordinated system to secrete numerous toxins and enzymes for the degradation of host cells and for the effective import of nutrients from the environment. Therefore, it is very important to precisely elucidate how these extracellular regulatory systems control the virulence of *C. perfringens*. Elucidation of these regulatory systems may lead to an understanding of the relationship between *C. perfringens* and other bacteria that coexist in the intestine or in wounds and, furthermore, to the identification of new therapeutic targets for the treatment of life-threatening diseases caused by *C. perfringens*.

ACKNOWLEDGMENTS

This study was supported by a KAKENHI (Grant-in-Aid for Scientific Research) on the Priority Area "Applied Genomics" from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

REFERENCES

1. **Aiba, H., S. Adhya, and B. de Crombrugghe.** 1981. Evidence for two functional *gal* promoters in intact *Escherichia coli* cells. J. Biol. Chem. **256:**11905–11910.
2. **Bassler, B. L.** 1999. How bacteria talk to each other: regulation of gene expression by quorum sensing. Curr. Opin. Microbiol. **2:**582–587.
3. **Ba-Thein, W., M. Lyristis, K. Ohtani, I. T. Nisbet, H. Hayashi, J. I. Rood, and T. Shimizu.** 1996. The *virR/virS* locus regulates the transcription of genes encoding extracellular toxin production in *Clostridium perfringens*. J. Bacteriol. **178:**2514–2520.
4. **Fuqua, W. C., S. C. Winans, and E. P. Greenberg.** 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J. Bacteriol. **176:**269–275.
5. **George, E. A., and T. W. Muir.** 2007. Molecular mechanisms of *agr* quorum sensing in virulent *staphylococci*. Chembiochem. **8:**847–855.
6. **Gobbetti, M., M. De Angelis, R. Di Cagno, F. Minervini, and A. Limitone.** 2007. Cell-cell communication in food related bacteria. Int. J. Food Microbiol. **120:**34–45.
7. **Hatheway, C. L.** 1990. Toxigenic clostridia. Clin. Microbiol. Rev. **3:**66–98.
8. **Higashi, Y., M. Chazono, K. Inoue, Y. Yanagase, T. Amano, and K. Shimada.** 1973. Complementation of theta toxinogenecity between mutants of two groups of *Clostridium perfringens*. Biken J. **16:**1–9.
9. **Imagawa, T., and Y. Higashi.** 1992. An activity which restores theta toxin activity in some theta toxin-deficient mutants of *Clostridium perfringens*. Microbiol. Immunol. **36:**523–527.
10. **Imagawa, T., T. Tatsuki, Y. Higashi, and T. Amano.** 1981. Complementation characteristics of newly isolated mutants from two groups of strains of *Clostridium perfringens*. Biken J. **24:**13–21.
11. **Kobayashi, T., T. Shimizu, and H. Hayashi.** 1995. Transcriptional analysis of the β-galactosidase gene (*pbg*) in *Clostridium perfringens*. FEMS Microbiol. Lett. **133:**65–69.
12. **Lyon, G. J., and R. P. Novick.** 2004. Peptide signaling in *Staphylococcus aureus* and other gram-positive bacteria. Peptides **25:**1389–1403.
13. **Mahony, D. E., and T. J. Moore.** 1976. Stable L-forms of *Clostridium perfringens* and their growth on glass surfaces. Can. J. Microbiol. **22:**953–959.
14. **Miller, M. B., and B. L. Bassler.** 2001. Quorum sensing in bacteria. Annu. Rev. Microbiol. **55:**165–199.
15. **Muir, T. W.** 2003. Turning virulence on and off in *staphylococci*. J. Pept. Sci. **9:**612–619.
16. **Nakayama, J., A. D. Akkermans, and W. M. De Vos.** 2003. High-throughput PCR screening of genes for three-component regulatory system putatively involved in quorum sensing from low-G+C gram-positive bacteria. Biosci. Biotechnol. Biochem. **67:**480–489.
17. **Novick, R. P., and T. W. Muir.** 1999. Virulence gene regulation by peptides in staphylococci and other gram-positive bacteria. Curr. Opin. Microbiol. **2:**40–45.
18. **Ohtani, K., H. Hayashi, and T. Shimizu.** 2002. The *luxS* gene is involved in cell-cell signaling for toxin production in *Clostridium perfringens*. Mol. Microbiol. **44:**171–179.
19. **Okumura, K., K. Ohtani, H. Hayashi, and T. Shimizu.** 2008. Characterization of genes regulated directly by the VirR/VirS system in *Clostridium perfringens*. J. Bacteriol. **190:**7719–7727.
20. **Rieu, A., S. Weidmann, D. Garmyn, P. Piveteau, and J. Guzzo.** 2007. Agr system of *Listeria monocytogenes* EGD-e: role in adherence and differential expression pattern. Appl. Environ. Microbiol. **73:**6125–6133.
21. **Rood, J. I.** 1998. Virulence genes of *Clostridium perfringens*. Annu. Rev. Microbiol. **52:**333–360.
22. **Sambrook, J., E. F. Fritch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
23. **Shimizu, T., W. Ba-Thein, M. Tamaki, and H. Hayashi.** 1994. The *virR* gene, a member of a class of two-component response regulators, regulates the production of the perfringolysin O, collagenase, and hemagglutinin in *Clostridium perfringens*. J. Bacteriol. **176:**1616–1623.
24. **Shimizu, T., K. Ohtani, H. Hirakawa, K. Ohshima, A. Yamashita, T. Shiba, N. Ogasawara, M. Hattori, S. Kuhara, and H. Hayashi.** 2002. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. Proc. Natl. Acad. Sci. USA **99:**996–1001.
25. **Shimizu, T., A. Okabe, and J. I. Rood.** 1997. Regulation of toxin production in *Clostridium perfringens*, p. 451–470. *In* J. I. Rood, G. Songer, B. A. McClane, and R. W. Titball (ed.), The clostridia: molecular biology and pathogenesis. Academic Press, London, England.
26. **Shimizu, T., H. Yaguchi, K. Ohtani, S. Banu, and H. Hayashi.** 2002. Clostridial VirR/VirS regulon involves a regulatory RNA molecule for expression of toxins. Mol. Microbiol. **43:**257–265.
27. **Sloan, J., T. A. Warner, P. T. Scott, T. L. Bannam, D. I. Berryman, and J. I. Rood.** 1992. Construction of a sequenced *Clostridium perfringens-Escherichia coli* shuttle plasmid. Plasmid **27:**207–219.
28. **Waters, C. M., and B. L. Bassler.** 2005. Quorum sensing: cell-to-cell communication in bacteria. Annu. Rev. Cell Dev. Biol. **21:**319–346.
29. **Wuster, A., and M. M. Babu.** 2008. Conservation and evolutionary dynamics of the *agr* cell-to-cell communication system across firmicutes. J. Bacteriol. **190:**743–746.
30. **Xavier, K. B., and B. L. Bassler.** 2003. LuxS quorum sensing: more than just a numbers game. Curr. Opin. Microbiol. **6:**191–197.