278 into the culture supernatant, where it regulates gene expression via a two-component
279 system consisting of AgrA and AgrC (17). To determine whether *agrBD*^{Cp} is related to
280 the signaling component that is secreted from *C. perfringens* cells, we assayed the
281 ability of *agrBD*^{Cp} to modulate toxin expression. Culture supernatant was collected
282 from the wild-type *C. perfringens* strain 13 or the *agrBD*^{Cp} mutant TS230 at early log
283 phase (OD_{600} = 0.5), and was then added to TS230 cells. The cells were incubated at
284 37°C for 15 min and total RNA was prepared and analyzed by Northern analysis. The
285 transcription of toxin genes was significantly increased in the TS230 cells only when
286 the wild-type supernatant was added (Fig. 5A), suggesting that the TS230 cells lacked
287 the ability to produce the signal molecule and release it into the supernatant. To further
288 confirm that the signal molecule in the supernatant of strain 13 was produced from the
289 *agrBD*^{Cp} region, the supernatant was collected from a TS230 mutant strain that had
290 been complemented with an intact *agrBD*^{Cp} (TS230/pTS1304). When this supernatant
291 was tested on TS230 cells, the expression of toxin genes, especially that of *pfoA*, was
292 strongly induced (Fig. 5A). These data clearly indicate that the *agrBD*^{Cp} gene is
293 responsible for production of an extracellular autoinducible signal molecule that
294 controls the expression of toxin genes in *C. perfringens*.

295 In *C. perfringens*, the VirR/VirS-VR-RNA system is known as a global regulator and
296 can regulate the expression of many toxin genes, including *plc*, *pfoA* and *colA*; however,
297 the signal that activates the sensor protein VirS has not been identified. Since the
298 *agrBD*^{Cp} locus controls the expression of a subset of toxin genes similar to that of the
299 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 up-regulated 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 will be 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

322 TS230. At first, RNA was isolated from the wild-type strain (strain 13), TS133, and its
323 complement strain TS133/pTS405, which were cultured for 2 h and 3 h. As in previous
324 experiments, transcription of *pfoA* was absent in TS133 but recovered in TS133/pTS405
325 (Fig. 6A). In contrast, the transcriptional levels of $agrD^{Cp}$ and the 2.5-kb operon in the
326 three strains were almost the same at 2 h under a *virR/virS*-negative background (Fig.
327 6A), although the level of $agrD^{Cp}$ transcript was slightly decreased in TS133/pJIR418 at
328 3 h, which was thought to be not significant.
329 Next, Northern analysis was performed by using strains 13/pJIR418, TS230/pJIR418
330 and TS230/pTS1304 to check the *virR/virS* transcription under $agrBD^{Cp}$-negative
331 conditions. As shown in Fig. 6B, the transcription of the *virR/virS* operon was too faint
332 to confirm its regulation, but the mRNA level was almost the same in all three strains.
333 These results suggested that the *agr* regulatory system involving the $agrBD^{Cp}$ and
334 *virR/virS* operons in *C. perfringens* is not completely analogous to the *agr* regulation
335 system in *S. aureus*.
336
337 **Effect of a stationary culture supernatant on *pfoA* transcription**
338 To further analyze the mechanism by which the extracellular signal in the culture
339 supernatant of *C. perfringens* regulates toxin gene expression, the effect of addition of
340 the *C. perfringens* culture supernatant on *pfoA* expression was examined in more detail
341 by Northern analysis. Although the expression of *plc* and *colA* was also partially
342 regulated by the extracellular signal molecule in the supernatant, we focused on the
343 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, 2h-8h). These cells were used as recipient cells, and were re-suspended in fresh TSF medium. As a control, cells were re-suspended 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 15min after the supernatant was replaced with fresh TSF medium (Fig. 7A, lane TSF). Surprisingly, the transcription of *pfoA* occured even in the 8h-cultured recipient cells following replacement of the supernatant with fresh medium (Fig. 7A). Furthermore, the transcription of *pfoA* in the 3h-8h cultured recipient cells (lane TSF; 3 h-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 2 h 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. And 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.

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

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

384 In *C. perfringens* the functions of $agrBD^{Cp}$, the VirR/VirS system, and VR-RNA seem
385 to be quite similar to those of *S. aureus* $agrBD^{Sa}$, AgrA/AgrC and RNAIII, respectively.
386 Consequently, the two bacteria might have evolved similar regulatory systems to control
387 their pathogenicity towards humans. 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 of note 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 this 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, 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 co-exist 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*.

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FIGURE LEGENDS

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 in red and the deduced sequence of the mature peptides is in bold. C. Northern analysis of the $agrBD^{Cp}$ region. RNA was isolated from strain 13 after culturing for 1-h, 2-h, 3-h and 4-h.

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-h 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.

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 following 2 h 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 the activity is lower than that of pTS1304. The internal regions of *pfoA, colA, plc, agrD* were used as probes.

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 supernatant was

543 collected from the wild-type strain, strain 13/pJIR418 and strain TS230/pTS1304, after
544 culturing for 1.5 h, respectively. Total RNA was prepared 15 min after addition of the
545 supernatant. A. The supernatant (sup) was added onto the *agr* null mutant, TS230. B.
546 The supernatant (sup) was added onto the *agr* null-*virR* mutant, TS231/pJIR418. C. The
547 supernatant (sup) was added onto TS231 that contains an intact *virR/virS*,
548 TS231/pTS405
549
550 Fig. 6. The regulatory relationship between $agrBD^{Cp}$ and *virR/virS*.
551 A. The regulation of $agrBD^{Cp}$ by *virR/virS*. Total RNA was isolated from 2 h and 3
552 h-cultured strain 13/pJIR418, TS133/pJIR418 and TS133/pTS405. B. The regulation
553 of *virR/virS* by $agrBD^{Cp}$. Total RNA was isolated from strain 13/pJIR418,
554 TS230/pJIR418 and TS230/pTS1304. 10 μg of total RNA was used for Northern
555 analysis.
556
557 Fig. 7. Effect of the supernatant on toxin gene expression.
558 A. The supernatant was removed from the various time points of the culture. The cells
559 from each time point were incubated with TSF at 37°C. As a control, the removed
560 supernatant was re-added to the same cells. RNA was isolated after 15 min of
561 incubation. Lane 1, TSF control; lane 2, culture supernatant. B. The supernatant
562 from strain 13 after 6 h of culture was diluted with TSF medium and added to TS230
563 cells. RNA was isolated after a 15-min incubation.
564
565
566 Table 1. Strains and plasmids
567 Table 2. Primers used for PCR analysis.
568

Fig. 1.

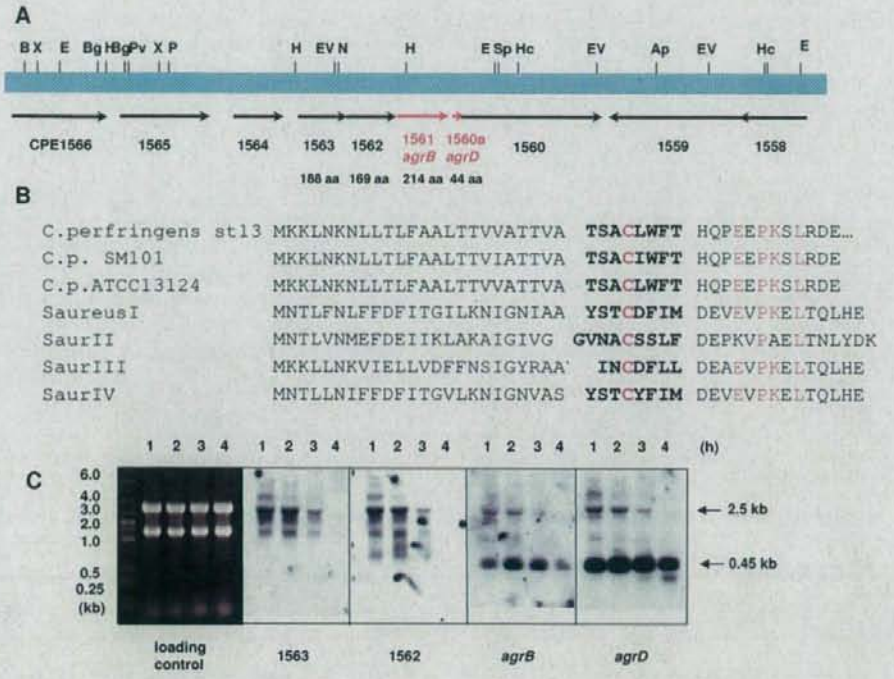


Fig .2.

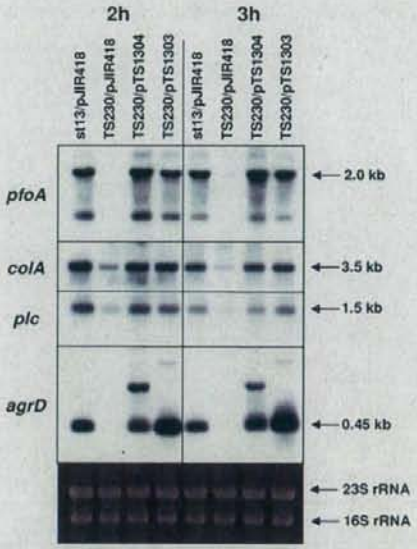
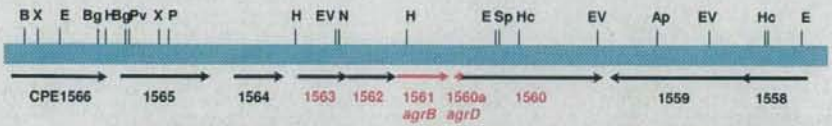


Fig. 3.



pTS1301		P-1563,**62,61,D,60**	-
pTS1302		P-1563,62,61,**D,60**	-
pTS1303		**P-1563,62,61,D,60**	++
pTS1304		**P-1563,62,61,D,**60	++
pTS1305		P-1563,**62,61,D,**60	-
pTS1306		P-1563,62,**61,D,**60	-
pTS1307		P-1563,62,61,**D,**60	-
pTS1308		**P-**1563,**62,61,D,**60	+
pTS1309		**P-**1563,62,**61,D,**60	+
pTS1310		**P-**1563,62,61,**D,**60	-
pTS1311		**P-1563**,62,61,**D,**60	-
pTS1312		**P-1563,62**,61,**D,**60	-
pTS1313		**P-**1563,**62**,61,**D,**60	-
pTS1314		**P-1563**,62,**61,D,**60	++
		Genes	activity

Fig. 3. (continued)

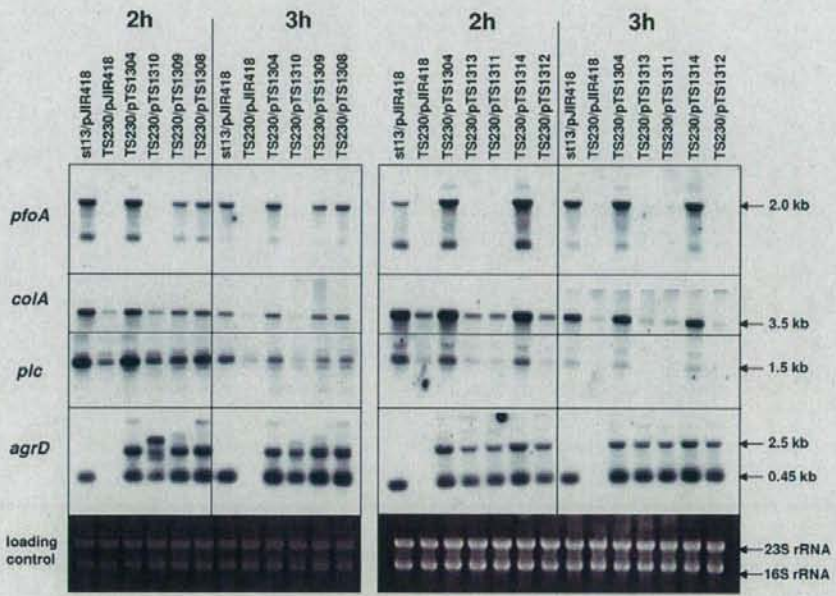


Fig. 4.

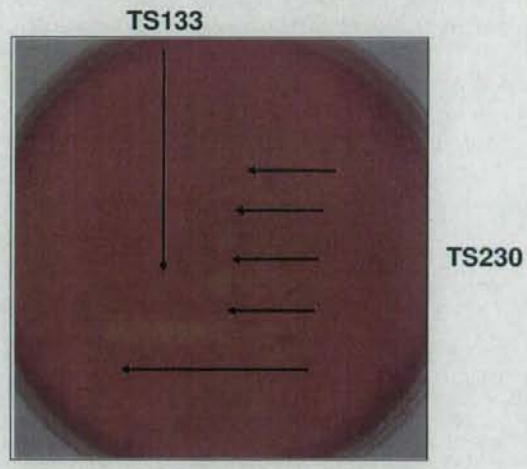


Fig. 5.

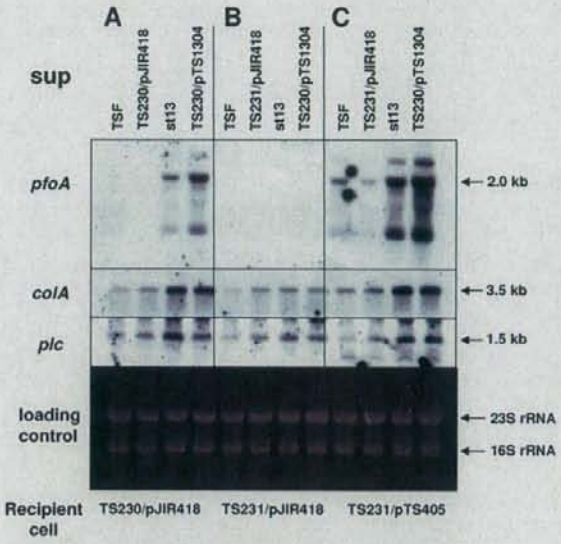


Fig. 6.

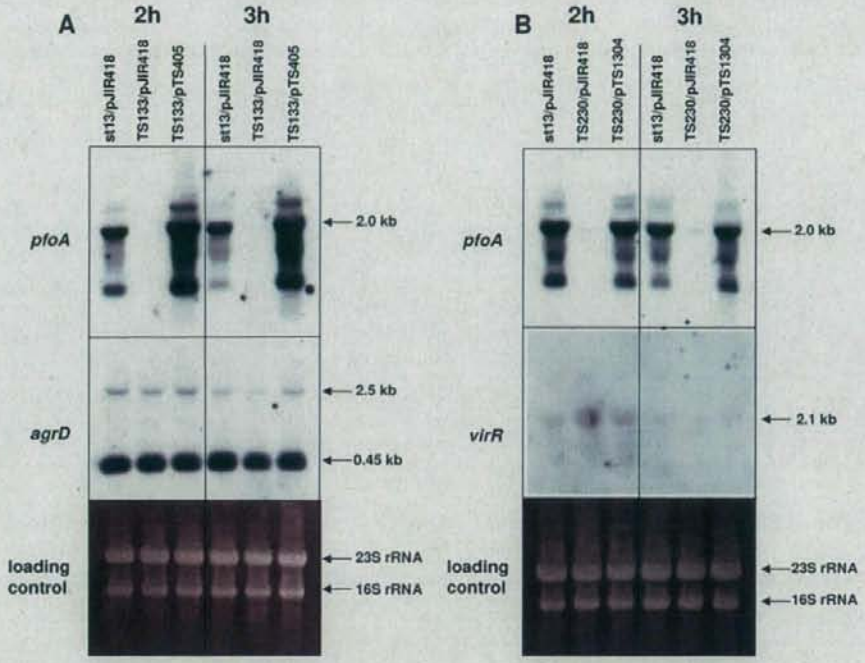


Fig. 7.