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Role of Alpha-Toxin in *Clostridium perfringens* Infection Determined by Using Recombinants of *C. perfringens* and *Bacillus subtilis*

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***Clostridium perfringens* type A strains which differed in alpha-toxin (phospholipase C [PLC]) productivity were inoculated intraperitoneally or intravenously into mice, and then their 50% mouse lethal doses (LD_{50}) were determined. Strain NCTC 8237 produced ninefold higher PLC activity than strain 13. The mean LD_{50} for the former was 1 log unit lower than that for the latter. Two isogenic strains were constructed from strain 13: strain 13(pJIR418α) (pJIR418α contains the *plc* gene), which produced ninefold higher PLC activity than strain 13; and strain 13 PLC^-, which showed no PLC productivity at all because of transformation-mediated gene disruption. The mean LD_{50} for strain 13(pJIR418α) was 1 log unit lower than those for strain 13 PLC^- and strain 13. These results indicate that PLC functions as a virulence-determining factor when it is produced in a sufficient amount. Such a difference in LD_{50} was also observed between *Bacillus subtilis* with and without the cloned *plc* gene. Inoculation of *B. subtilis* PLC^+ intravenously into mice caused marked thrombocytopenia and leukocytosis. Mice inoculated with *B. subtilis* at 2 LD_{50} died because of circulatory collapse. Histological examination revealed that intravascular coagulation and vascular congestion occurred most prominently in the lungs. These results suggest that PLC plays a key role in the systemic intoxication of clostridial myonecrosis, probably by affecting the functions of platelets and phagocytes.**

Clostridium perfringens type A is an anaerobic bacterium which is most frequently associated with gas gangrene (myonecrosis). Its histolytic potential is attributed to the numerous toxins and hydrolytic enzymes it produces (8, 32). One of the most important toxins is alpha-toxin (phospholipase C [PLC]), which can degrade phosphatidylcholine and sphingomyelin (16, 44). Various biological activities, such as hemolysis, platelet aggregation (25, 41), contraction of blood vessels (6), vascular permeabilization (42), and cardiovascular dysfunction (39), have been suggested for PLC. Since the substrates of PLC are major constituents of the eukaryotic membrane, it has been speculated that it causes membrane damage leading to cytolysis of many cells and has been regarded as being most responsible for the pathogenesis of myonecrosis (51).

However, some experimental evidence has contradicted this speculation. First, membrane damage by PLC does not lead to cytolysis in human diploid fibroblasts (24, 46) and polymorphonuclear leukocytes (38). Moreover, PLC even exhibits a stimulatory effect on the growth of certain cells (27). Secondly, a correlation between the virulence and the PLC activity of clinically isolated *C. perfringens* was not demonstrated (23). Recent studies on bacterial PLCs, including *C. perfringens* PLC, suggest that their effect is more subtle than cytolysis (47). Furthermore, interpretation of the results of many studies on PLC toxicity are complicated by contamination with other toxins. Thus, the implication of PLC in the pathogenicity of *C. perfringens* is controversial (14, 38).

In order to solve the problem of whether or not PLC contributes to the pathogenicity of *C. perfringens*, we constructed isogenic strains differing only in PLC productivity by two different methods. One method was mutagenization of a PLC-coding gene (*plc*) by transformation-mediated gene disruption in *C. perfringens* type A strain 13, and the other was cloning of the *plc* gene into a nonpathogenic bacterium, *Bacillus subtilis*. Comparison of their degrees of infectivity or lethality toward experimental animals would enable us to draw a definite conclusion as to the problem described above. The advantage of an in vivo study involving the isogenic strains was that the effect of the toxin on tissues infected with an organism could be analyzed in the process of infection. We studied the mechanism underlying PLC toxicity by examining pathological changes induced by infection with *B. subtilis* expressing the *plc* gene.

This paper describes the difference in virulence toward mice between isogenic *C. perfringens* type A strains and also that between *B. subtilis* transformants with and without the *plc* gene. We also describe hematological and histological changes after the intravenous inoculation of PLC-producing *B. subtilis* and present evidence that PLC causes thrombocytopenia, leukocytosis, and pulmonary circulatory failure with intravascular coagulation.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Table 1. Their construction is described below. Plasmid pJIR418 was a gift from J. I. Rood (Department of Microbiology, Monash University, Clayton, Victoria, Australia). Plasmid pHY300PLK (13) was obtained from Takara Shuzo Co., Ltd., Kyoto, Japan.

Media and culture conditions. *C. perfringens* strains were grown in 100 ml of GAM broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), as described previously (15). *B. subtilis*

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
E. coli		
C600	*supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21*	1
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*)	52
B. subtilis		
ISW1214	Tc^s strain derived from strain 1012; *hsrM1 leuA8 metB5*	13
ISW1214 Spo^-	ISW1214; asporogenous strain	This study
C. perfringens		
NCTC 8237[b]	High alpha-toxin-producing type A strain	Laboratory stock
13	φ9 recipient	18
13 PLC^-	Strain 13; PLC^- strain; *plc*::pJIR418Dα2	This study
Plasmids		
pKMA14	pUC19 with 1.4-kb *Ssp*I-*Hin*dIII fragment of a *plc* gene; Ap^r	48
pTS301	pUC19 with 4.6-kb *Eco*RI-*Nde*I fragment of a *pfoA* gene; Ap^r	35
pTS315	pTS301 digested with exonuclease III; Ap^r	This study
pHY300PLK	*E. coli-B. subtilis* shuttle plasmid containing *ori-177* and *ori-pAMα1*; Ap^r Tc^r	13
pMNα	pHY300PLK with 1.4-kb *Eco*RI-*Hin*dIII fragment of pKMA14 containing a *plc* gene; Ap^r Tc^r	This study
pMNθ	pHY300PLK with 2.0-kb *Hin*dIII-*Eco*RI fragment of pTS315 containing a *pfoA* gene; Ap^r Tc^r	This study
pJIR418	*E. coli-C. perfringens* shuttle plasmid containing *ori-pIP404* and *ori-pUC18*; Cm^r Em^r	36
pJIR418α	pJIR418 with 3.1-kb *Hin*dIII-*Eco*RI fragment of a strain 13 *plc* gene; Cm^r Em^r	This study
pJIR418D	pJIR418; removal of 3.6-kb *Hin*dIII-*Nae*I fragment; Cm^r Em^r	This study
pJIR418Dα2	pJIR418D with 376-bp *Fok*I fragment of a *plc* gene; Cm^r Em^r	This study

[a] Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant; Tc^r, tetracycline resistant; Tc^s, tetracycline sensitive.
[b] *C. perfringens* NCTC 8237 is also known as ATCC 13124 and CN 1491.

strains were precultured overnight in Luria-Bertani (LB) broth at 37°C with shaking at 120 rpm. The precultures were diluted 100-fold with fresh medium and then cultured at 37°C with shaking at 120 rpm. When required, the following were added to the media: egg yolk suspension (5% [vol/vol]), tetracycline (10 μg/ml), and chloramphenicol (10 μg/ml).

Construction of an asporogenous mutant strain of *B. subtilis*. An asporogenous mutant of *B. subtilis* ISW1214 was obtained by ultraviolet irradiation, as described by Takahashi (43). This Spo^- strain exhibited a rate of sporulation of less than 10^{-8} and was used as a host strain for cloning clostridial toxin genes.

Construction of plasmids. Plasmid pKMA14 (48), with a 1.4-kb fragment of the NCTC 8237 *plc* gene, was digested with *Hin*cII and then ligated with the *Eco*RI linker d(pGGAAT TCC). The resulting plasmid was digested with *Eco*RI, and the liberated 1.4-kb *Eco*RI fragment was ligated into the dephosphorylated *Eco*RI site of pHY300PLK. The resultant plasmid with the *plc* gene oriented in the same direction as the *tet* gene was designated pMNα. Plasmid pTS315, with a 2-kb fragment of the *pfoA* gene encoding perfringolysin (theta-toxin), was constructed by nested deletion of pTS301 (35) from an upstream *Eco*RI site. The 2-kb *Eco*RI-*Hin*dIII fragment containing the *pfoA* gene was ligated into the multiple cloning site of pHY300PLK, and the resultant plasmid was designated pMNθ.

Plasmids pJIR418α, pJIR418D, and pJIR418Dα2 were constructed as follows. A 2-kb *Eco*RI-*Hin*dIII fragment containing the *plc* gene was cloned from strain 13 into pUC19 as described previously (15). The fragment was recloned into the multiple cloning site of pJIR418. The resultant plasmid was named pJIR418α. Deletion of a 3.6-kb *Hin*dIII-*Nae*I fragment containing the *C. perfringens* replication origin from pJIR418 was generated by digestion with *Hin*dIII and *Nae*I, filling in, and religation. The resulting 3.8-kb plasmid was named pJIR418D. A 376-bp *Fok*I fragment containing the *plc* coding region (15) was obtained from the 2-kb *Eco*RI-*Hin*dIII fragment of pJIR418α and then inserted into the *Bam*HI site of pJIR418D after filling in and religation. The resulting plasmid was named pJIR418Dα2.

DNA techniques. Plasmids were prepared from *C. perfringens* by the method of Roberts et al. (31). Transformation of *C. perfringens* was performed by electroporation as described previously (20). Plasmids were prepared from *B. subtilis* by the modified method of Birnboim and Doly (3). Induction of competence and transformation of *B. subtilis* were performed as described by Ishiwa and Shibahara (12). To increase the transformation efficiency of *B. subtilis*, plasmids transferred to *Escherichia coli* C600 $RecA^+$ were used (13). *C. perfringens* chromosomal DNA was prepared, and Southern hybridization was performed as described previously (21) by use of the 2-kb *Eco*RI-*Hin*dIII fragment containing the *plc* gene. Other DNA procedures were carried out by standard methods described by Maniatis et al. (19).

Assaying of PLC and theta-toxin activities. Cultures of the *C. perfringens* and *B. subtilis* strains were chilled on ice at the indicated times and then centrifuged at 5,000 × *g* at 4°C for 10 min. The culture supernatants were stored at −80°C until used. PLC activity was assayed by the method of Ottolenghi (26) with an egg yolk suspension as a substrate. One unit of enzyme activity was defined arbitrarily as the amount of enzyme which caused an increase of 1.0 unit of optical density at 560 nm per minute (48). Hemolytic activity of theta-toxin was assayed by use of sheep blood cells as described previously (35), except that samples were diluted in 0.032 M phosphate buffer (pH 6.8) containing 1 mM EDTA to inhibit hemolysis by the PLC present in the culture supernatant of *C. perfringens*.

Assaying of lethality to mice. Cultures of the *C. perfringens* and *B. subtilis* strains were harvested at the late log phase after 3 and 7 h of incubation, respectively. The cell pellets obtained on centrifugation at 5,000 × *g* at 4°C for 10 min were washed once with and then resuspended in a sterile Dulbecco's phosphate-buffered saline (PBS) solution. The optical density of each cell suspension was measured, and the number of viable cells was determined by use of a calibration curve obtained by

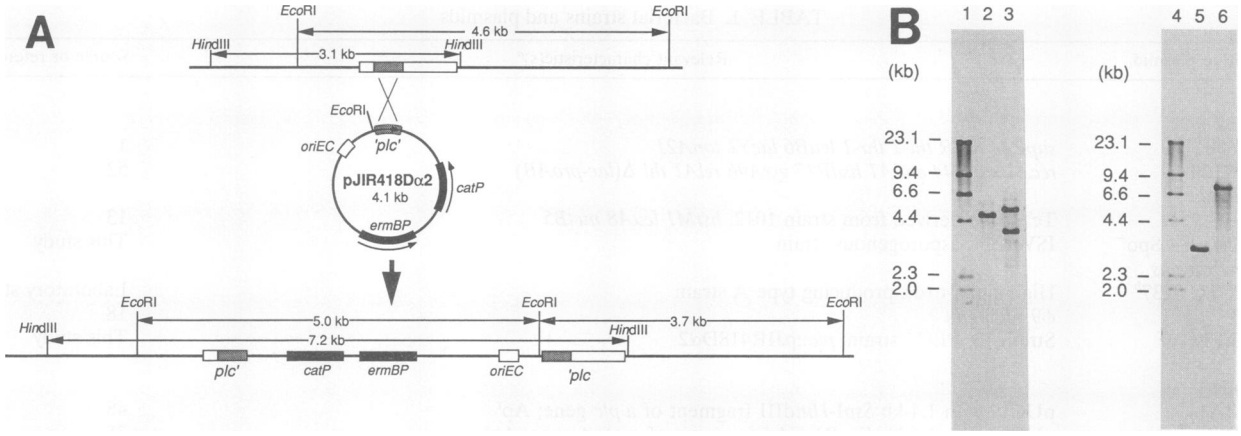

FIG. 1. Disruption of the *plc* gene. (A) Structure of the strain 13 *plc* gene locus before and after recombination with gene disruption vector pJIR418Dα2. The integration of a single copy of pJIR418Dα2 into the *plc* locus by homologous recombination is confirmed by the data shown in panel B. Genetic symbols: *'plc'*, a 376-bp *Fok*I fragment located within the *plc* coding region; *plc'*, a *plc* gene with the downstream coding region deleted; *'plc*, a *plc* gene with the upstream coding region deleted; *catP*, chloramphenicol acetyltransferase gene from *C. perfringens*; *ermBP*, *C. perfringens* erythromycin resistance gene; *oriEC*, the replication origin from pUC18. (B) Southern hybridization of DNAs from *C. perfringens* strains 13 and 13 PLC$^-$ digested with *Eco*RI and *Hin*dIII. Total DNA (about 3 μg) was electrophoresed on a 0.8% agarose gel. Lanes: 1 and 4, size marker; 2, *Eco*RI digests of strain 13 DNA; 3, *Eco*RI digests of strain 13 PLC$^-$; 5, *Hin*dIII digests of strain 13 DNA; 6, *Hin*dIII digests of strain 13 PLC$^-$. The sizes of marker DNA fragments (*Hin*dIII-digested λ DNA) are given in kilobases. The 2-kb *Eco*RI-*Hin*dIII fragment containing the strain 13 *plc* gene was used as a probe after labeling with digoxigenin-dUTP by random priming.

determining the number of CFU in and the optical density of appropriately diluted suspensions before each set of measurements. The 50% lethal doses (LD_{50}) were determined as follows. A group of 15 to 20 male ddY mice weighing 40 g each were inoculated intraperitoneally (i.p.) with 0.5 ml or intravenously (i.v.) with 0.1 ml of 10-fold serial dilutions of a sample. Death occurring by day 2 was recorded. LD_{50} was calculated by the method of Reed and Muench (30) and expressed as $\log_{10}$ CFU that resulted in 50% mortality within 2 days.

Test for multiplication within macrophages. An in vitro assay for *B. subtilis* strains within macrophages was carried out according to Portnoy et al. (28) with some modifications. The macrophage cell line SV-BP-1 (45), derived from a simian virus 40-transformed BALB/cAnN peritoneal macrophage, was obtained from H. Takayama (Department of Bacteriology, Tottori University School of Medicine, Tottori, Japan). The macrophages were suspended in Dulbecco's modification of Eagle's medium (DMEM; Flow Laboratories, Inc., McLean, Va.) supplemented with 5% newborn calf serum (NCS; Flow Laboratories) to give a cell density of 10^5 cells per ml. Then, 200 μl of this suspension was added to each well of a 96-well microculture plate (Corning Glass Works, Corning, N.Y.) and incubated for 2 days at 37°C in a 5% CO_2 atmosphere with saturated humidity to prepare a monolayer. *B. subtilis* cultures were grown to an A_{600} of 0.5, and then 100 μl of the culture was centrifuged in a microcentrifuge at 15,000 rpm for 30 s. The cell pellet was resuspended in 3 ml of PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ to give a cell density of 2×10^6/ml. Twenty-five microliters of this bacterial suspension was added to each well and incubated for 30 min. After unadhered bacteria had been removed by washing with 5% NCS-containing DMEM, 200 μl of the medium containing 2 μg of gentamicin per ml was added. After the indicated times of incubation, the medium was discarded. Intracellular bacteria were released by lysis of the macrophages with sterile distilled water. The number of viable bacteria was determined by plating of several dilutions of the lysis mix on an LB agar plate.

Hematology. Twenty microliters of blood was drawn from unanesthetized mice into a Unopette (Becton-Dickinson Co., Rutherford, N.J.), and then the mice were anesthetized with ether. About 1 ml of blood was drawn into EDTA-treated tubes. Platelets and leukocytes (WBC) were enumerated by hemacytometer counting of the 20-μl blood sample. With the 1-ml blood sample, the hemoglobin content and erythrocyte count were determined with a microcell counter (CC-130A; Toha Medical Electronics Co., Ltd., Kobe, Japan). Differential WBC counts were performed on Wright-stained smears.

Histological study. Organs (lungs, liver, spleen, and kidneys) of mice inoculated i.v. with 5×10^8 *B. subtilis* organisms were resected at 90 min postinoculation (p.i.) or immediately after they died and fixed with 10% formalin in saline. They were embedded in paraffin wax, sectioned 4 μm thick, and then stained with hematoxylin-eosin by the conventional method for histological examination. Phosphotungstic acid-hematoxylin (PTAH) staining was performed to detect fibrin deposits.

Statistical analysis. Data were expressed as means ± standard deviations or means ± standard errors of the means. Analysis of the difference of means was performed with Student's *t* test. LD_{50} data were analyzed by means of the chi-square test. Probability values of $P < 0.05$ were considered significant.

RESULTS

Mutagenization of the *plc* gene. In order to mutagenize the *plc* gene of strain 13 by homologous recombination, strain 13 was transformed with pJIR418Dα2 containing a 376-bp *Fok*I fragment of the *plc* gene but lacking the *C. perfringens* replication origin (Fig. 1A). A clone selected for chloramphenicol resistance, which was named strain 13 PLC$^-$, did not exhibit PLC activity on egg yolk agar. No plasmid was present in this strain. Therefore, it can be suggested that its *plc* gene was disrupted by an integration event through single-crossover homologous recombination (Fig. 1A). Southern blot analysis with the 2-kb *Eco*RI-*Hin*dIII fragment containing the *plc* gene

TABLE 2. PLC and theta-toxin activities in cultures of various *C. perfringens* and *B. subtilis* strains

Strain	Activity[a]	
	PLC (U/ml of culture)	Theta-toxin (U/ml of culture)
C. perfringens		
NCTC 8237	1.333 ± 0.032	387.3 ± 35.2
13	0.142 ± 0.006	14.3 ± 0.7
13 PLC$^-$	—[b]	51.9 ± 0.8
13(pJIR418α)	1.287 ± 0.047	16.7 ± 1.5
B. subtilis		
ISW1214 Spo$^-$(pHY300PLK)	—	—
ISW1214 Spo$^-$(pMNα)	10.983 ± 0.340	—
ISW1214 Spo$^-$(pMNθ)	—	1,298.0 ± 20.3

[a] The *C. perfringens* strains were grown in GAM broth. For the cultures of strain 13 PLC$^-$ and 13(pJIR418α), 10 μg of chloramphenicol per ml was added to the broth. The *B. subtilis* strains were grown in LB broth containing 10 μg of tetracycline per ml. At the late log phase, when PLC and theta-toxin activities were highest, the cultures were centrifuged. The culture supernatants were assayed for PLC and hemolytic activities. Activities are expressed as means ± standard deviations for three determinations.

[b] —, not detectable.

TABLE 3. Comparison of virulence among *C. perfringens* and *B. subtilis* strains differing in levels of PLC production

Strain	LD_{50} for mice (log_{10})[a]	
	i.v.	i.p.
C. perfringens		
NCTC 8237	7.5	7.6
13	8.5	8.4
13 PLC$^-$	8.5	8.5
13(pJIR418α)	7.5	7.4
B. subtilis		
ISW1214 Spo$^-$(pHY300PLK)	9.4	9.5
ISW1214 Spo$^-$(pMNα)	8.4	7.5
ISW1214 Spo$^-$(pMNθ)	8.5	8.6

[a] *C. perfringens* and *B. subtilis* cultures were harvested at the late log phase. The cell pellets were washed once with and resuspended in PBS. Tenfold serially diluted suspensions were inoculated i.v. or i.p. into mice. LD_{50} were calculated from the lethality toward five mice for each dilution by the method of Reed and Muench (30).

revealed that the recombination took place correctly on the chromosome of strain 13 PLC$^-$ (Fig. 1B).

Production of PLC by *C. perfringens* strains and *B. subtilis* transformants. The PLC and theta-toxin activities in the culture supernatants of *C. perfringens* strains and *B. subtilis* transformants were determined. In all the cultures, both PLC and theta-toxin activities reached maximal levels at the late-logarithmic growth phase and decreased in the stationary phase, probably because of breakdown by proteases (data not shown). The highest PLC and theta-toxin activities in the late log phase of each culture are summarized in Table 2. No PLC activity was detectable in the strain 13 PLC$^-$ culture, as expected. The level of PLC activity produced by strain 13 was as low as one-ninth of that produced by NCTC 8237. On the contrary, strain 13(pJIR418α) produced almost the same level of PLC activity as the latter. The theta-toxin activity in the strain 13 culture was about 30-fold lower than that in the NCTC 8237 culture. *B. subtilis* ISW1214 Spo$^-$ carrying pHY300PLK did not produce PLC or theta-toxin activity. On the other hand, the same host strain carrying pMNα and pMNθ produced eight times as much PLC activity and three times as much theta-toxin activity as NCTC 8237, respectively. These *B. subtilis* transformants with pHY300PLK, pMNα, and pMNθ are hereafter designated *B. subtilis*, *B. subtilis* PLC$^+$, and *B. subtilis* θ$^+$, respectively.

Lethality to mice of PLC-producing and -nonproducing strains. Two type A strains of *C. perfringens* were inoculated i.v. and i.p. into mice and compared as to lethality to mice. The mean LD_{50} of NCTC 8237 determined with either inoculation were about 1 log unit lower than those of strain 13 (Table 2). The difference in lethality to mice could arise from the difference in PLC and theta-toxin productivity between the two strains. In order to determine the extent of the role of PLC in mouse lethality, the LD_{50} of three isogenic strains, strain 13, strain 13 PLC$^-$, and strain 13(pJIR418α), were determined. As shown in Table 2, there was no significant difference in LD_{50} between strain 13 and strain 13 PLC$^-$. However, strain 13(pJIR418α) was more virulent than the other two strains. Furthermore, the virulence of strain 13(pJIR418α) was almost the same as that of NCTC 8237. These results strongly suggest that PLC is a major virulence determinant of *C. perfringens* when it is produced above a certain critical level. To prove this, we further examined the virulence of *B. subtilis* PLC$^+$, which can produce high levels of PLC activity (Table 3). *B. subtilis* θ$^+$ was also examined for lethality to mice. The mean LD_{50} of both *B. subtilis* PLC$^+$ and *B. subtilis* θ$^+$ were about 1 log unit lower than that of *B. subtilis* on i.v. inoculation into mice. On inoculation i.p. of *B. subtilis* PLC$^+$, the mean LD_{50} decreased a further 1 log unit. This difference in LD_{50} between i.v. and i.p. inoculations was reproduced on reexamination.

Viability of *B. subtilis* PLC$^+$ within a macrophage. From the result that *B. subtilis* PLC$^+$ displayed high virulence when inoculated i.p. into mice, we speculated that a peritoneal macrophage might be highly sensitive to PLC toxicity or might sequester the organism. The effect of *B. subtilis* PLC$^+$ on the viability of a peritoneal macrophage cell line was examined by nigrosine staining. However, no lethal effect was observed (data not shown). *B. subtilis* expressing the *pfoA* gene can escape from a phagosome and multiply within a macrophage (29). To determine if PLC had a similar effect on a peritoneal macrophage, we examined the survival rate of *B. subtilis* PLC$^+$ within the macrophage cell line. The number of viable *B. subtilis* PLC$^+$ cells within a macrophage decreased with incubation time, and its survival rate was almost the same as that of *B. subtilis* (Fig. 2). In contrast, *B. subtilis* θ$^+$ increased about 10-fold during a 90-min incubation, coinciding with the result reported by Portnoy et al. (29). Therefore, PLC should not cause damage to the phagosomal membrane by itself.

Hematological examination of mice inoculated with *B. subtilis* PLC$^+$. The PLC prepared from *C. perfringens* culture has been shown to exhibit hemolytic and platelet-aggregating activities. *B. subtilis* PLC$^+$ produces PLC but no other clostridial toxins. To analyze the specific effect of PLC on blood cells, mice were inoculated i.v. with *B. subtilis* PLC$^+$ at the dose of 5×10^7 cells, and then hematological examination was performed at various intervals for 120 min p.i. (data not shown). Erythrocyte counts did not change significantly. On the contrary, platelet counts decreased markedly. Peripheral WBC counts decreased at 60 min and then increased at 120 min p.i. This leukocytosis was shown by counting of differential WBC to be due to granulocytosis (data not shown). Both thrombocytopenia and leukocytopenia followed by leukocytosis, though less prominent, were also observed in mice inoculated with *B. subtilis* at the same dose. To further examine such effects, mice were inoculated with *B. subtilis* PLC$^+$ and *B.*

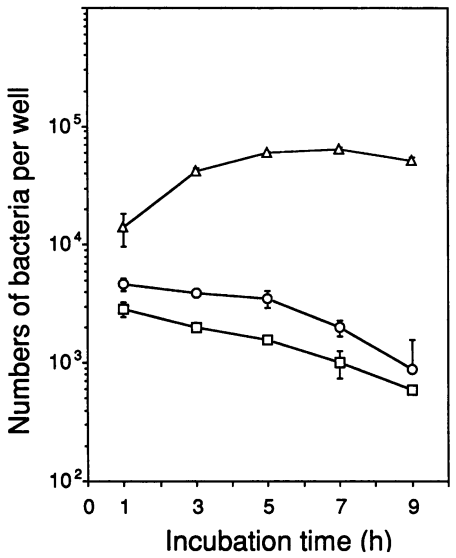

FIG. 2. Replication of *B. subtilis* θ^+ and PLC^+ strains in macrophages. The macrophage cell line SV-BP-1 (2×10^4 cells per well) was incubated on a microculture plate for 2 days. The macrophages were infected with the bacteria (5×10^4 bacteria per well). After incubation for 30 min, extracellular bacteria were removed by washing. Gentamicin-containing culture medium was added to kill residual extracellular bacteria (time 0), and then incubation was continued. At each point, the numbers of living *B. subtilis* (○), *B. subtilis* PLC^+ (□), and *B. subtilis* θ^+ (△) cells were determined by lysis of the macrophages and plating. The results are expressed as means ± standard deviations ($n = 3$).

subtilis at various doses, and platelet and WBC counts were measured at 30 min p.i. Differences in platelet and WBC counts between them were found to be statistically significant when mice were inoculated with 1.25×10^8 and 5×10^8 bacteria (Fig. 3). While inoculation with 5×10^7 cells of *B. subtilis* PLC^+ caused leukocytopenia at 30 min p.i., leukocytosis occurred 30 min after inoculation with the strain at the doses of 1.25×10^8 and 5×10^8 cells. These results indicate that the thrombocytopenia and leukocytosis caused by i.v. inoculation with *B. subtilis* PLC^+ are partly due to septicemia, but mainly due to the effect of PLC.

Histological examination of mice inoculated with *B. subtilis* PLC^+. All the mice inoculated i.v. with *B. subtilis* PLC^+ at the dose of 5×10^8 (2 LD_{50}) died between 60 and 90 min p.i. Immediately after the mice had died, their lungs, kidneys, spleens, and livers were dissected out and examined histologically. Mice inoculated with *B. subtilis* at the same dose were sacrificed at 90 min p.i., and then histological examination was performed. The most prominent change was observed in the lungs of the mice inoculated with *B. subtilis* PLC^+, in which extensive intravascular coagulation occurred and the interalveolar septa were thickened as a result of capillary congestion (data not shown). The presence of typical fibrin deposits in aggregates was confirmed by PTAH staining (data not shown). Intravascular coagulation was observed only in mice inoculated with *B. subtilis* PLC^+, while pulmonary capillary congestion, though less prominent, was also observed in the mice inoculated with *B. subtilis*. Compared with these changes, cellular infiltration and hemorrhage were not so prominent. Intravascular hemolysis was observed in mice inoculated with *B. subtilis* PLC^+, but not in those inoculated with *B. subtilis*. The hemolytic activity of PLC depends on the temperature shift down (hot-cold hemolysis). However, not all hemolysis observed in sections seemed to occur as a postmortem change, since hemoglobinuria was noticed as a characteristic manifestation before death and renal tubules were shown histologically to be filled with hemoglobin.

DISCUSSION

The finding that both *C. perfringens* 13 and *B. subtilis* showed increased virulence upon acquisition of an extrachromosomal *plc* gene clearly indicates that PLC is essentially a virulence determinant of *C. perfringens*. Another important finding is that no significant difference in lethality to mice between strain 13, a moderate PLC producer, and its PLC^- mutant was observed. This indicates that sufficient levels of PLC activity must be produced for PLC-producing clostridia to exhibit their lethal toxicity. This may reflect the possible existence of a fine balance between membrane damage and repair in the PLC-induced membrane change, as was suggested by Titball (47). Although the virulence of *C. perfringens* is not determined solely by PLC, a producer of high levels of PLC seems to be a candidate for serious gas gangrene. Mutation of the *plc* gene resulted in a slight but significant increase in theta-toxin productivity (Table 2). This may imply that a change in PLC productivity affects the production of other toxins regulated by the VirS/VirR system (17, 34).

Various toxicities of PLC have been suggested on the basis of results obtained by use of PLC preparations of various degrees of purity. Therefore, some toxicities might be due to other contaminating toxins. The *B. subtilis* PLC^+ does not produce any clostridial toxin. In this study, the systemic toxicity of PLC has been investigated by pathological examination of mice infected with the *B. subtilis* PLC^+. The result presented here proves that platelet aggregation reported by others (25, 41) is attributable to PLC but not to other contaminants. We also showed that *B. subtilis* PLC^+ induced leukocytopenia followed by leukocytosis more markedly than did *B. subtilis* PLC^-. The histological examination revealed that intravascular coagulation and vascular congestion were marked in the lungs of the mice inoculated with *B. subtilis* PLC^+. This pulmonary circulatory failure seems to lead to cardiac failure and hence to circulatory collapse, which is a characteristic manifestation often encountered in serious myonecrosis (51). However, it should be noted that *B. subtilis* differs largely in many biological properties from *C. perfringens*. The two organisms may differ in tissue tropism. Furthermore, murine and human tissues may also differ in susceptibility to PLC. Therefore, not all the pathological features of the mice injected with *B. subtilis* PLC^+ may reflect the systemic intoxication in myonecrosis, and more-detailed study is necessary to draw a definite conclusion.

The molecular mechanism(s) responsible for the systemic toxicity displayed by PLC may be complex. The action of PLC on membrane phospholipids has been considered to trigger a chain of signal transduction in various cells, e.g., activation of enzymes such as PLC, protein kinase C, and phospholipases A_2 and D, an increase in the intracellular Ca^{2+} concentration, and stimulation of arachidonic acid metabolism (5, 33, 47). Agents which are capable of inducing transient granulocytopenia followed by rebound granulocytosis include interleukin-8 (IL-8) (4), IL-1, and tumor necrosis factor alpha (7). Leukocytosis induced by the inoculation of *B. subtilis* PLC^+ is similar to that induced by IL-8 in that it occurs as early as 1 h p.i., suggesting the possible involvement of IL-8 in the observed leukocytosis. The production of IL-8 by neutrophils is stimulated on a respiratory burst (10), and PLC can induce neutro-

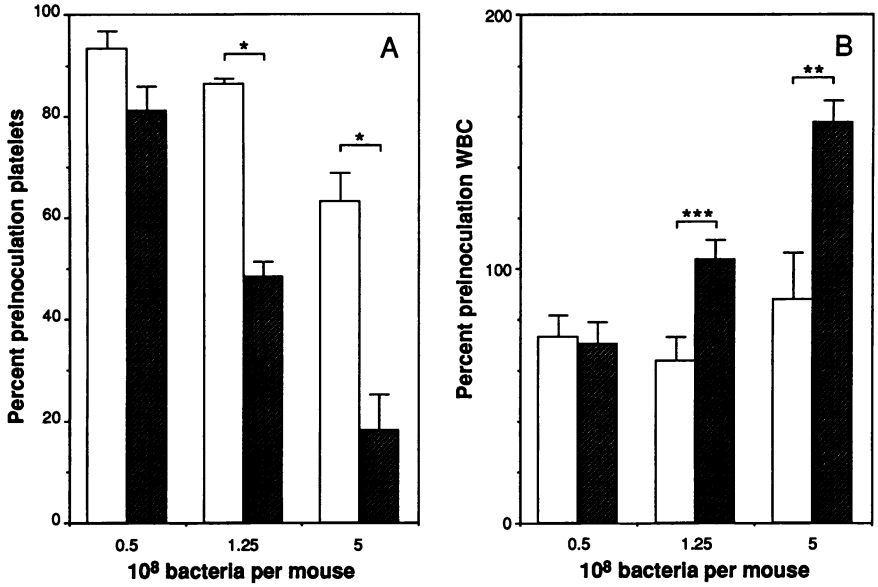

FIG. 3. Changes in platelet and white blood cell counts after inoculation of different doses of *B. subtilis* strains into mice. Mice were inoculated i.v. with *B. subtilis* PLC$^+$ or *B. subtilis* at the indicated dose. Platelet and WBC counts were determined 30 min p.i. The results, three determinations for three mice per group, are expressed as the mean + standard error of the mean (vertical bar) percentages of blood cell counts in mice without inoculation of bacteria. (A) platelet counts. (B) WBC counts. *, **, and ***, $P \leq 0.008$, $P = 0.026$, and $P = 0.034$, respectively, for *B. subtilis* PLC$^+$ versus *B. subtilis* at each dose. Open bars, *B. subtilis*; hatched bars, *B. subtilis* PLC$^+$.

phils to produce O_2^- (40). Additionally, the superoxide-generating NADPH oxidase system is activated by protein kinase C as well as activated small G protein (22). Taking these facts into account, we speculate that PLC activates protein kinase C in neutrophils, triggering a respiratory burst and IL-8 production. An oxidative burst is induced on phagocytosis of bacteria (2), which explains well why WBC counts initially decreased and then gradually increased in mice inoculated with *B. subtilis*.

One likely explanation for the thrombocytopenia and intravascular coagulation caused by *B. subtilis* PLC$^+$ is that PLC activates phospholipase A_2 and thereby stimulates thromboxane A_2 synthesis in platelets. It is uncertain whether or not PLC exhibits cytolytic activity toward platelets like *Staphylococcus aureus* PLC (49, 50). If this is the case, coagulating factors released from platelets may also stimulate platelet aggregation and thrombocytopenia. Activation of complements, which was suggested to cause disseminated intravascular coagulation in infections with gram-positive bacteria (9, 37), may be responsible for the thrombocytopenia caused by *B. subtilis*.

Although the role of PLC in systemic intoxication has been suggested by the animal model used in this study, its role in the early stages of wound infection remains to be determined. Platelet aggregation by PLC may contribute to reduction of the blood supply, thereby creating conditions favorable for the multiplication of *C. perfringens*. PLC may induce a premature respiratory burst in phagocytic cells, helping overcome the host defense system (47). Local responses of tissues infected with *C. perfringens* to PLC must be examined to understand the role of PLC in early events of myonecrosis. Purified PLC is not well suited for this purpose. Atraumatic inoculation of experimental animals with anaerobes does not produce infection unless the redox potential of healthy tissues is reduced sufficiently (51). Another obstacle is that strain 13, which is an atypical type A strain in terms of productivity of the toxins, is the only strain permitting the efficient transformation required for gene disruption. The *B. subtilis* transformant can be expected to overcome these problems. Recently, pneumolysin, a hemolysin similar to theta-toxin, was shown to stimulate the production of tumor necrosis factor alpha and IL-1β (11). The concerted actions of PLC and theta-toxin can be expected to increase the inflammatory response. *B. subtilis* expressing the *plc* and *pfoA* genes would be very useful for analyzing the concerted actions of PLC and theta-toxin. Thus, *B. subtilis* transformants should be a useful system for approaches towards roles of clostridial toxins in the pathogenesis of myonecrosis. However, they also have a disadvantage and limitation in their application. The LD_{50} of *B. subtilis* PLC$^+$ determined for intravenous and intraperitoneal inoculations were different, unlike those of *C. perfringens*. This discrepancy may arise from differences in the properties of the bacteria, such as aerotolerance and growth rate. Furthermore, *B. subtilis* gave a background of the effects of *B. subtilis* PLC$^+$ on blood cells. In this context, precaution against such differences and effects is required for interpretation of the results, which should be assessed by a different approach using isogenic strains of *C. perfringens* or purified toxins.

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