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Physiological role of carbon dioxide in spore germination of *Clostridium perfringens* S40

Shiro Kato,[1] Atsushi Masayama,[1] Tohru Yoshimura,[1] Hisashi Hemmi,[1] Hidenori Tsunoda,[2] Tomoko Kihara,[2] and Ryuichi Moriyama[2,*]

Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya, Aichi 464-8601, Japan[1] *and Department of Food and Nutritional Sciences, College of Bioscience and Biotechnology, Chubu University, 1200 Matsumoto-cho, Kasugai, Aichi 487-8501, Japan*[2]

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Germination of *Clostridium perfringens* is known to be triggered by nutrients such as L-alanine and inosine, and facilitated by CO_2, however the role of CO_2 has not been fully understood. During the studies of the germination-specific protease GSP, we found that CO_2 could be replaced by bicarbonate or weakly acidic pH (pH 6.0–6.5). We also found that the spores obtained from the *C. perfringens* S40 overproducing GSP could germinate without CO_2. Moreover, the spores could germinate in the absence of nutrients, when the spores were incubated with bicarbonate or under weakly acidic pH. GSP, which might consist of three homologous proteases, CspA, CspB, and CspC, is one of the key enzymes involved in the spore germination, and converts the pre-mature form of the spore cortex-lytic enzyme, SleC, to the mature form. Maturation of SleC in the spores obtained from the mother strain of *C. perfringens* S40 requires nutrients plus bicarbonate or weakly acidic pH. In contrast, mature SleC was found in the spores obtained from the cells overpoducing GSP, when the spores were treated by nutrients, bicarbonate or weakly acidic pH. Each nutrients, bicarbonate and weakly acidic pH can trigger the germination of the spores obtained from *C. perfringens* cells overproducing GSP.

[**Key words:** *Clostridium perfringens*; Bacterial spore; Germination; Carbon dioxide; Anaerobic bacteria]

Carbon dioxide (CO_2) has long been known to have a critical influence on microbiological growth (1). CO_2 and bicarbonate promote the spore germination of clostridia (2–5), while they exhibit an inhibitory effect on the spore germination of bacilli (6, 7). The germination of fungal spores can also be inhibited by high concentrations of CO_2 (8). The effects of CO_2 and bicarbonate on the spore germination of clostridia seem to be the opposite of those on bacilli and fungi (9). These results could be due to a physiological difference between aerobic and anaerobic bacteria, but only several studies to explain these phenomena have been conducted. From Fourier transform infrared analysis, Cheung et al. suggested that sodium bicarbonate probably reacts with protein in the spore, leading to a less orientated structure, and consequently interferes with the germination process of *Bacillus stearothermophilus* (10). Plowman and Peck showed that in the event of amino acid/L-lactate-induced *Clostridium botulinum* spore germination, sodium bicarbonate increased the rate and extent of germination. They proposed that bicarbonate activates the germination receptor; this in turn renders the receptor more responsive to amino acids and L-lactate (11). They noted that the mechanism of germination of *C. botulinum* spores differs from that of the germination of *Bacillus* spores with L-alanine, the AGFK (asparagine, glucose, fructose and K^+) mixture and inosine (11). However, the detailed mode of action of CO_2 and bicarbonate in the germination process remains unclear.

Clostridium perfringens is an endospore-forming, gram-positive bacterium readily found in soil, sediments, and the intestinal contents of humans and animals (12). Bacterial spores are metabolically dormant and are highly resistant to environmental stresses, such as heat, radiation, and chemicals (13, 14). When conditions become favorable, bacterial spores germinate to allow spore outgrowth and the formation of a new vegetative cell (15). Spore germination has an important applied interest, and many attempts to inhibit *C. perfringens* spore germination and outgrowth have been performed (16–18), since *C. perfringens* sometimes causes food poisoning (19). However, spore germination in clostridia is less well studied than in bacilli (20), and therefore further elucidation of the germination mechanism is needed.

In *C. perfringens* S40, there are two enzymes required to initiate cortex degradation during germination: spore cortex-lytic enzyme SleC and germination-specific protease (GSP) (21). SleC is synthesized at an early stage of sporulation, and exists in dormant spore as inactive proenzyme (proSleC) (22–25). The conversion of proSleC to active SleC is achieved via proteolytic cleavage by GSP during germination (23). The GSP fraction contains three serine protease homologues, CspA, CspB, and CspC respectively encoded by the *cspABC* operon (26), which is positioned just upstream of the 5′ end of *sleC* (27). Csp proteins are highly homologous, putative subtilisin-like serine proteases. The proteins are synthesized with an N-

* Corresponding author. Tel./fax: +81 568 51 6084.
E-mail address: moriyama@isc.chubu.ac.jp (R. Moriyama).

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terminal extended sequence that is expected to function as an intramolecular chaperone at an early stage of sporulation (26, 27). Previous study indicated that in its 60-kDa mature form, CspC is located outside the cortex in the dormant spore (27).

As described above, Csp proteins are the key enzymes in cortex degradation during *C. perfringens* spore germination. However, since only a small amount of the enzymes could be extracted from germinated spores, biochemical studies for Csp proteins including the relationship with the facilitating effect of CO_2 on germination remain to be performed. We then constructed a strain overproducing Csp proteins, in order to yield a large amount of Csp proteins and elucidate the detailed function of Csp proteins. Interestingly, we found that there are significant differences in germination response between the spores formed from the wild-type cells (WT spores) and the spores formed from the cells overproducing Csp proteins (CSPs spores). In the present study, we compared the germination response of the WT spores and CSPs spores to CO_2 under various conditions, in order to elucidate the physiological role of CO_2 in the germination process of anaerobic bacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions The bacterial strains used in this study are listed in Table 1. *C. perfringens* was grown and sporulated as described previously (28). For the plasmid-carrying strain of *C. perfringens*, chloramphenicol was added to a final concentration of 15 μg/ml.

Construction of Csp proteins overproduced strain The plasmids and the primers used in this study are listed in Tables 1 and 2, respectively. pJCSPs, which harbors the *cspABC* operon, was constructed as follows. The fragment containing the putative *csp* promoter region and the *cspABC* genes was amplified by PCR using the *C. perfringens* chromosomal DNA as the template with primers Csps FW and Csps RV, and ligated into the EcoRV restriction site of the vector pBluescript II KS+(Stratagene). The resultant plasmid was digested with BamHI and KpnI. The BamHI and KpnI fragment, which contains the putative *csp* promoter region and the *cspABC* genes, was ligated into the BamHI and KpnI restriction sites of *Escherichia coli–C. perfringens* shuttle vector pJIR418 (30). The plasmid was transformed into *C. perfringens* S40 by electroporation as described previously (26).

Preparation of spores Spores of both *C. perfringens* WT and the plasmid-carrying strain were prepared as described previously (22, 28).

Spore germination assay All germination experiments shown in this study are the results of duplicate determinations for one experiment. All experiments were also repeated with at least two independent spore preparations.

Germination medium used in this study was 50 mM potassium phosphate containing150 mM potassium chloride, 10 mM L-alanine, and 5 mM inosine, and adjusted at pH 7.0.

Immediately before use, the spores were heat-activated at 75 °C for 20 min. Heat-activated spores were washed with cool distilled water (dH_2O) and suspended in dH_2O at 5% (w/v). The spore suspension was mixed with a 50-fold volume of germination medium that either had or had not been saturated with CO_2, and the suspension was then incubated at 37 °C. Germination was observed by tracking changes in optical density at 600 nm (OD_{600}) of the spore suspension in a germinant solution. The change in OD_{600} was measured using a Ubest-30 UV/VIS Spectrophotometer (Japan Spectroscopic CO.). Germination was also monitored by the conversion of phase-bright (dormant) spore to phase-dark (germinated) spore under phase-contrast microscopy.

Effects of bicarbonate on spore germination Effects of bicarbonate on spore germination were examined with six different final concentrations of potassium bicarbonate in each germination medium; 0% (control), 0.05%, 0.35%, 0.6%, 1%, and 2% (w/v). The pH values were adjusted to 7.0.

Effects of pH on spore germination For the analysis of the effects of pH on spore germination, the following buffers containing 150 mM potassium chloride were used as the germination medium: 50 mM acetate buffer (pH 4.5–5.5), 50 mM citrate buffer (pH 5.5–6.0), or 50 mM phosphate buffer (pH 6.0–7.0).

TABLE 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristics	Source or reference
C. perfringens		
S40	Wild-type strain	Ref. 29
CSPs	*C. perfringens* S40 transformant carrying pJCSPs	This study
Plasmid		
pJIR418	*E. coli–C. perfringens* shuttle vector; Cm^R, Em^R	Ref. 30
pJCSPs	Cloning the *cspABC* operon into pJIR418	This study

Cm^R, Chloramphenicol-resistant gene; Em^R, Erythromycin-resistant gene.

TABLE 2. Oligonucleotide primers used in this study.

Primer	Sequence (5′ to 3′)
Csps	FW 5′-CTTCTCGAG[a]TCGACAAGCTTTATAGAATAGAATTTG-3′
Csps	RV 5′-CAGGATCC[b]TCTAGGTTCTTAAACTATTAAA-3′
AF	5′-TATGGATCC[b]GGAAAGTCCTGTAGAAGATTCAAAG-3′
AR	5′-ACGCTCGAG[a]CTTATTCGTTAAACTCTTAAATAT-3′
BF	5′-TACCATATG[c]GCTTATGATAGTAATAGAGCAT-3′
BR	5′-TACCTCGAG[a]TCTCCTATTAATTAATAATTCCAT-3′

[a] The XhoI restriction sites are underlined.
[b] The BamHI restriction site is underlined.
[c] The NdeI restriction site is underlined.

Preparation of anti-CspA and anti-CspB antisera To prepare a polyclonal antibody against CspA, a recombinant CspA–His fusion protein was obtained with the plasmid constructed as follows. A part of the *cspA* gene encoding Glu-78 to Lys-578 was amplified by PCR using the *C. perfringens* chromosomal DNA as the template with primers AF and AR. The resultant PCR product digested with BamHI and XhoI was ligated into the BamHI and XhoI restriction sites of the vector pET-22b(+) to generate the plasmid pE-AMH. The plasmid pE-AMH encoding a part of CspA with a C-terminal six-histidine tag was introduced into *E. coli* BL21(DE3). The production of the recombinant protein ($r\Delta_{1-77}$CspA) was induced with 1 mM isopropyl-1-thio-β-galactopyranoside (IPTG). The resulting fusion protein obtained from an inclusion body was dissolved in 6 M urea and purified with His Trap HP (GE Healthcare) according to the recommended protocol by the supplier.

A rabbit was subcutaneously injected with 1 mg of the purified $r\Delta_{1-77}$CspA emulsified in Freund's complete adjuvant. After 2 weeks, the rabbit was injected with 0.5 mg of the same protein. A second boost was administered one week later, and the rabbit was bled one week after the injection. Blood containing 0.1% (w/v) NaN_3 was stored at 4 °C for 20 h. After centrifugation (3 krpm, 3 min, 4 °C), the antiserum was stored at −20 °C.

To prepare a polyclonal antibody against CspB, a recombinant CspB–His fusion protein was obtained with the plasmid constructed as follows. A part of the *cspB* gene encoding Ser-97 to Arg-565 was amplified by PCR using the *C. perfringens* chromosomal DNA as the template with primers BF and BR. The resultant PCR product digested with NdeI and XhoI was ligated into the NdeI and XhoI restriction sites of the vector pET-22b (+) to generate the plasmid pE-BMH. The plasmid pE-BMH encoding a part of CspB with a C-terminal six-histidine tag was introduced into *E. coli* BL21(DE3). The production of the recombinant protein ($r\Delta_{1-96}$CspB) was induced with 1 mM IPTG. The resulting fusion protein obtained from an inclusion body was dissolved in 6 M urea and purified with His Trap HP (GE Healthcare) according to the recommended protocol by the supplier. An antiserum against CspB ($r\Delta_{1-96}$CspB) was also prepared as well as that against CspA.

These antisera did not crossreact with other Csp proteins. However, the sensitivities of the anti-CspA and anti-CspB antisera were lower than that of the anti-CspC antiserum (27).

Preparation of the coat fraction The coat fraction was extracted with 0.2 M H_3BO_3, 5% (w/v) SDS, pH 10.0, containing 2% (w/v) 2-mercaptoethanol at 95 °C for 5 min. Protein in the SDS/alkaline extracts was then precipitated with trichloroacetic

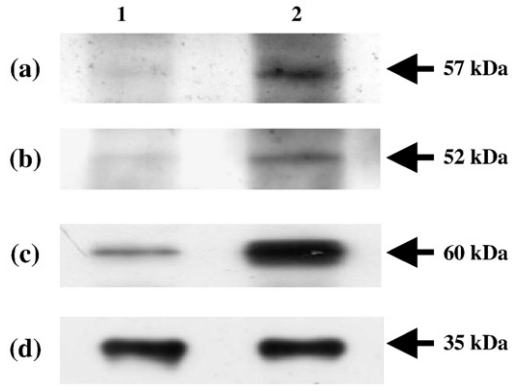

FIG. 1. Immunological detection of overproduced Csp proteins in dormant spores. The spore coat fraction was separated from decoated spores as described in Materials and methods, and subjected to 0.1% SDS-12% PAGE followed by immunoblot analysis. CspA, CspB, CspC and SleC were detected with anti-$r\Delta_{1-77}$CspA (A), anti-$r\Delta_{1-96}$CspB (B), anti-$r\Delta_{1-78}$CspC (C), and anti-C_{31} antisera (D), respectively. Detection of proSleC with the anti-C_{31} antibody was used as a protein loading control. Lanes 1, WT strain (~100 μg protein); 2, strain CSPs (~100 μg protein).