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An enhanced green fluorescence protein (EGFP)-based reporter assay for quantitative detection of sporulation in *Clostridium perfringens* SM101

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ABSTRACT

Clostridium perfringens type F is a spore-forming anaerobe that causes bacterial food-borne illness in humans. The disease develops when ingested vegetative cells reach the intestinal tract and begin to form spores that produce the diarrheagenic *C. perfringens* enterotoxin (CPE). Given that CPE production is regulated by the master regulator of sporulation (transcription factor Spo0A), the identification of sporulation-inducing factors in the intestine is relevant to better understanding of the disease. To examine these factors, we established assays to quantify *C. perfringens* sporulation stage under microscopy by using two fluorescent reporters, namely, Evoglow-Bs2 and CpEGFP. When the reporter genes were placed under control of the *cpe* promoter, both protein products were expressed specifically during sporulation. However, the intensity of the anaerobic reporter Evoglow-Bs2 was weak and rapidly photobleached during microscopic observation. Alternatively, CpEGFP, a canonical green fluorescence protein with optimized codon usage for *Clostridium* species, was readily detectable in the mother-cell compartment of most bacteria at early stages of sporulation. Additionally, CpEGFP expression predicted final spore yield and was quantifiable in 96-well plates using fluorescence plate reader. These results indicate that CpEGFP can be used to analyze the sporulation of *C. perfringens* and has a potential application in the large-scale screening of sporulation-regulating biomolecules.

1. Introduction

Clostridium perfringens is a Gram positive, spore-forming anaerobe that causes systemic and food-borne diseases in humans and animals. The bacterium produces a variety of toxins that cause tissue disruption and malfunction in the host. Six major toxins (α, β, ε, ι, *C. perfringens* enterotoxin (CPE), and NetB) have been used to classify *C. perfringens* into seven toxinotypes (A-G), and this classification has been used to characterize the epidemiological and pathological behaviors of this bacterium (Hatheway, 1990; Li et al., 2013; Rood et al., 2018). Type F strains, which were formally classified in type A (Rood et al., 2018), produce α toxin and CPE, an important virulence factor for food-borne illness in humans (Sarker et al., 1999). Once type F strains contaminate meat or vegetables, they survive even after adequate cooking because of the formation of resistant spores that grow when the temperature decreases to a permissive level (Paredes-Sabja et al., 2008).

Sporulation is initiated by the activation of the bacterial master sporulation regulator Spo0A (Huang et al., 2004). Activated (phosphorylated) Spo0A induces the expression of dozens of sigma factors and hundreds of downstream genes related to the sporulation process (Yasugi et al., 2016a). The sigma factors *sigE* and *sigK* bind to the promoter regions of *cpe* in the mother-cell compartment and induce the production of CPE at an early sporulation stage (II/III), thus leading to CPE release from lysed bacterial cells into the intestinal tract (Harry et al., 2009). In addition to *sigE* and *sigK*, the importance of *sigF*, as an upstream regulator for CPE production has also been proposed (Li and

Abbreviations: CPE, *C. perfringens* enterotoxin; CpEGFP, *Clostridium*-optimized EGFP; DMEM, Dulbecco's Modified Eagle's Medium; DSSM, Duncan−Strong sporulation medium; EGFP, enhanced green fluorescence protein; PBS, phosphate buffered saline; RFU, relative fluorescence units

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McClane, 2010)

Many bacteria flexibly adapt to different environments and change gene expression patterns to survive or exploit various environmental factors for competitive advantages (Abuaita and Withey, 2009). *C. perfringens* strains that induce food-borne illnesses are believed to sense specific intestinal factors that induce Spo0A activation, which leads to sporulation and CPE production (Al-Hinai et al., 2015; Heredia et al., 1991). We have been examining such environmental sporulation factors and their significance in the pathogenesis of *C. perfringens* infection (Yasugi et al., 2015; Yasugi et al., 2016a, 2016b). Such investigations require the quantification of spore numbers in bacterial culture under different conditions. Conventionally, spores or vegetative cells are enumerated by counting colony-forming units (CFUs) or optically refractile spores under microscopy. However, these methods are laborious and time consuming; therefore, a simple and easily applicable procedure is desired.

Fluorescence proteins have been used extensively as markers or reporters to monitor the physiological and pathological behaviors of bacteria *in vitro* and their interactions with hosts (Kjos et al., 2015; Meyer and Dworkin, 2007). Fusion proteins containing fluorescence proteins have proven particularly useful for monitoring bacterial behavior because their genes are commercially available and easily manipulated. Furthermore, its fluorescence is detectable by conventional fluorescence microscopy. These features enable investigators to analyze the functionality of individual bacterial cells in real time (Fujita and Losick, 2002). In the current study, we compared two fluorescence reporter assays: one employing an anaerobic fluorescent protein (Evoglow-Bs2) (Drepper et al., 2007) and the other using enhanced green fluorescence protein (EGFP) optimized for *Clostridium* (CpEGFP). We speculated that the anaerobic reporter would be more suitable for this anaerobic bacterium, but Evoglow-Bs2 demonstrated unfavorable characteristics, particularly dim basal fluorescence and rapid photobleaching. By contrast, despite the requirement for oxygen to develop fluorescence, CpEGFP proved useful for monitoring sporulation by *C. perfringens* in response to bile acid, a model for the enhanced sporulation in the intestinal tract underlying food-borne illness.

2. Material and methods

2.1. Bacterial strains and plasmids

The bacterial strains, plasmids, and primers used in this study are listed in Supplementary Tables 1 and 2. DNA manipulation was performed by conventional procedures described previously (Ausubel et al., 1987), except that *C. perfringens* SM101 was transformed by electroporation as described (Jirásková et al., 2005). Glycerol stocks of *C. perfringens* SM101 and its derivatives were prepared as described (Yasugi et al., 2015) and stored at −80 °C until use. The stocks were regrown in fluid thioglycolate medium at 37 °C for 16 h and inoculated (1/10, v/v) into either Duncan−Strong sporulation medium (DSSM) (Duncan and Strong, 1968) or glucose-deficient Dulbecco's Modified Eagle's Medium (DMEM(−)) (Thermo Fisher Scientific, Inc., Product No. #1966-025) supplemented with 0.4% starch (Becton, Dickinson, and Company). The bacteria in fluid thioglycolate medium were washed with phosphate buffered saline (PBS) three times before inoculation into DMEM(−) with starch to reduce noncell fluorescence.

2.2. Plasmid creation

2.2.1. Construction of pJIR-cpe-Bs2 and pJIR-Bs2 vectors

The *Evoglow-Bs2* (*Bs2*) gene was excised from pGlow-C-Bs2 using *Eco*RI and *Sal*I, and inserted into pBluescript SK− to obtain pBS-Bs2. The promoter region of the *cpe* gene was amplified from *C. perfringens* SM101 grown on brain heart infusion agar plates by PCR using CPE-F and CPE-R primers. The PCR product was subcloned into the pCR2.1 vector by TA cloning. The resulting plasmid, namely, pCR-cpe pro, was validated by sequencing. The *cpe* promoter region was excised from pCR-cpe pro using *Sac*I and *Nde*I and inserted into the same restriction site of pBS-Bs2, thus yielding pBS-cpe-Bs2 with the *cpe* promoter upstream of the *Bs2* gene. The DNA fragments excised from pBS-cpe-Bs2 and pBS-Bs2 using *Sac*I and *Xba*I were inserted into the same restriction sites of pJIR751, yielding pJIR-cpe-Bs2 and pJIR-Bs2, respectively.

2.2.2. Construction of the pJIR-cpe-Clostridium-optimized enhanced green fluorescent protein (CpEGFP) vector

The pJIR-cpe-CpEGFP reporter plasmid was constructed as follows. A sequence of GFP from *Aequorea victoria* (PubMed accession no. M62653.1) was optimized for *C. perfringens* using published information (Musto et al., 2003) to avoid rare codons and major restriction enzyme recognition sequences. The resultant *CpEGFP* gene (PubMed accession no. LC379923) was obtained by chemical synthesis (Eurofins Genomics, Tokyo, Japan) and cloned between *Avr*II and *Xho*I sites in pFN (Tamai et al., 2008) to create pFN-CpEGFP. The *CpEGFP* gene was amplified from pFN-CpEGFP by PCR using CpEGFP-F and CpEGFP-R primers. The PCR product was digested with *Nde*I and *Xho*I and inserted into the same restriction site of pCR-cpe pro. The new vector was named pCR-cpe-CpEGFP after validation by sequencing. The DNA fragment containing the *cpe* promoter and *CpEGFP* gene was excised from pCR-cpe-CpEGFP using *Sac*I and *Xho*I and inserted between the *Sac*I and *Sal*I restriction sites of pJIR751, yielding pJIR-cpe-CpEGFP.

2.2.3. Construction of pBS-Bs2Δlac and pBS-EGFP vectors

pBS-Bs2Δ*lac*, a plasmid for constitutive expression of Bs2 in *E. coli*, was created by eliminating 29 bases from pBS-Bs2 to match the reading frame length of the lactose operon. After digestion with *Sac*II and *Sma*II, pBS-Bs2 was blunted with T4 DNA polymerase. Blunted DNA was self-ligated, and the new vector named pBS-Bs2Δ*lac*. pBS-EGFP was constructed as follows. The *EGFP* gene was amplified from pEGFP-N1 by PCR using EGFP-F and EGFP-R primers. The resultant PCR product was digested with *Bam*HI and *Eco*RI and inserted into pBluescript SK- digested with the same enzymes, yielding pBS-EGFP.

2.3. Detection, analysis, and quantification of spores and bacterial cells by fluorescence

The numbers of viable vegetative cells in culture were enumerated by colony-forming assays on brain heart infusion agar plates after anaerobic incubation at 37 °C for 24 h. For spore counting, an aliquot of the culture was heated to 70 °C for 20 min before dilution as described. Bacterial cultures were also analyzed under phase-contrast microscopy and/or fluorescence microscopy. For this purpose, cultures were centrifuged at 5000 × *g* for 5 min, and the collected bacterial cells were mounted in a thin agarose cushion between the cover and slide glasses. Cultures labeled with CpEGFP were exposed to air for 20 min prior to collection and mounting (Hartman et al., 2011). Cell stage (Errington, 1993) was determined by the following procedure. Bacterial cells and spores were collected by centrifugation at 400 × *g* for 5 min and suspended in 10 μl of PBS containing 0.002% Hoechst33342 and Rhodamine B for 5 min to stain DNA and cell membranes, respectively. Developmental stages were determined on the basis of cell morphology under fluorescence microscopy as described (Yasugi et al., 2016a). Microscopic images were captured by a digital camera (Nikon ECLIPSE 80i with DS-Fi1) and analyzed using ImageJ software to quantify the means of fluorescence intensity in single cells as relative fluorescence units (RFU). The expression levels of fluorescent reporter proteins in bacterial populations were also quantified using a fluorescence plate reader (2030 Arvo X5 multilabel plate reader, PerkinElmer) with excitation and emission wavelengths at 485 nm and 535 nm, respectively.

2.4. Detection of CPE in the culture supernatant

Culture supernatants were collected by centrifugation at 2350 × *g*

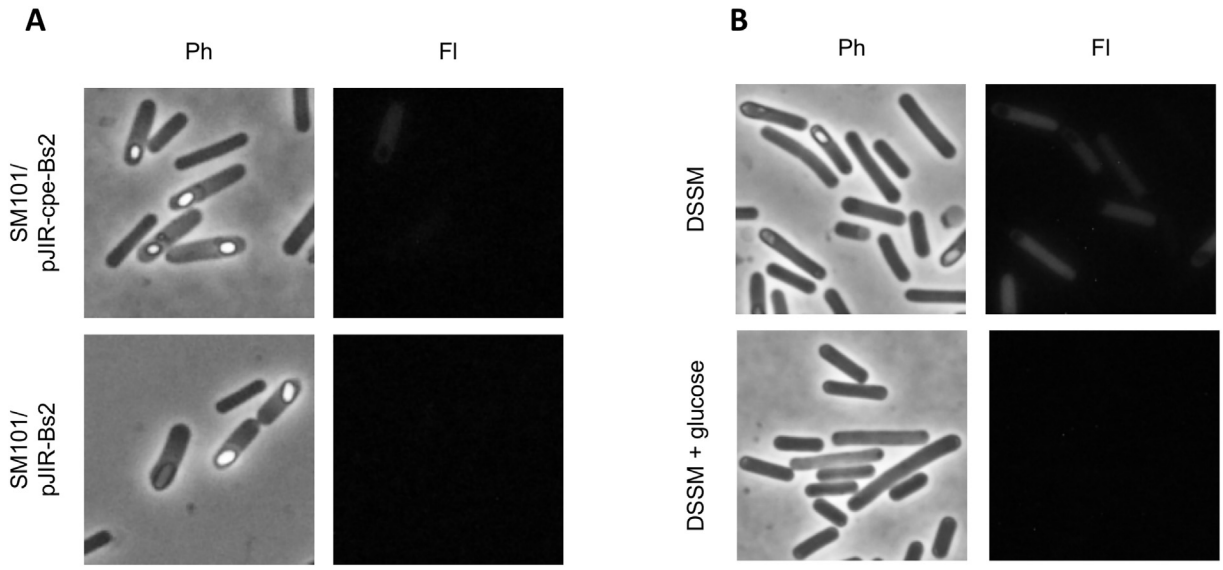

Fig. 1. Microscopic images of fluorescent reporters Evoglow-Bs2 and CpEGFP during sporulation. (A) Phase-contrast (Ph) and fluorescent microscopic (Fl) images of cells transformed with SM101/pJIR-cpe-Bs2 (upper) or SM101/pJIR-Bs2 (lower) and cultivated in DSSM for 12 h. (B) Phase-contrast (Ph) and fluorescent microscopic (Fl) images of cells transformed with SM101/pJIR-cpe-CpEGFP and cultivated in DSSM without and with 0.4% glucose (upper and lower, respectively) for 8 h.

for 10 min and subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Yasugi et al., 2015). The separated supernatant proteins were transferred to polyvinylidene difluoride membranes and then labeled with a rabbit anti-CPE antiserum (a kind gift by Dr. Horiguchi, Research Institute for Microbial Diseases, Osaka University, Osaka) for 1 h at 37 °C. After washing, the membranes were incubated with a horseradish peroxidase-conjugated antirabbit IgG (Jackson ImmunoResearch) for 1 h at 37 °C, and the immunoreactions were visualized using Immobilon Western chemiluminescence reagent (Merck Millipore).

2.5. Statistical analyses

Paired means were compared by Student's *t*-test or Welch's *t*-test, and multiple means were compared by one-way ANOVA followed by Scheffe's F test for pair-wise comparisons. A $p < 0.05$ was considered significant.

3. Results and discussion

The primary goal of this study was to develop a quantitative fluorescence-based assay for monitoring the sporulation of the anaerobic bacterium *C. perfringens*. For this purpose, we initially chose to express the anaerobic fluorescent protein Evoglow-Bs2 under control of the *cpe* promoter because detection does not require an aerobic environment (Drepper et al., 2007). Cells that were transformed with the SM101/pJIR-cpe-Bs2 vector and cultured in spore-promoting DSSM for 12 h exhibited fluorescence labeling that was observable under microscopy (Fig. 1A). Furthermore, the scatter plots of fluorescence emission intensity from individual cells revealed that substantial numbers of transformed cells emitted greater fluorescence than untransformed SM101 cells (Fig. 2A). By contrast, the fluorescence emission intensity scatter plot of cells transformed with the promoterless Evoglow-Bs2 expression vector SM101/pJIR-Bs2 did not differ from that of untransformed cells (Fig. 2A), thus indicating that the signals emitted were mainly due to autofluorescence. These results suggest that the *cpe* promoter-driven fluorescence reporter Evoglow-Bs2 is expressed during sporulation. When the ratio of transformed cells emitting greater intensity fluorescence than untransformed cells (SM101) was calculated, the proportion of fluorescent cells in SM101/pJIR-cpe-Bs2-transformed cultures (2.8% ± 0.37%) was significantly higher than that in SM101/pJIR-Bs2-transformed cultures (0.12% ± 0.094%). However, the actual ratio of heat-resistant spores under these conditions was 35% ± 23% after 24 h of cultivation, as measured by colony-forming assay. Therefore, < 10% of SM101/pJIR-Bs2-transformed cells emitted measurable fluorescence possibly because of fluorescence protein instability or high susceptibility to photobleaching upon exposure to excitation light. The instability of protein fluorescence under illumination was confirmed by additional experiments showing that emission intensity from the *E. coli* derivative DH5α/pBS-Bs2Δ*lac* constitutively expressing Evoglow-Bs2 was drastically decreased by prolonged light exposure (Supplementary Fig. 1A). Therefore, despite the anaerobic nature of Evoglow-Bs2, it appeared unsuitable for the quantitation of *C. perfringens* sporulation.

As an alternative, we examined the feasibility of using EGFP, a canonical fluorescent protein widely employed for cell-specific labeling and lineage tracing when expressed under the control of various endogenous promoters. Both *E. coli* expressing Evoglow-Bs2 and those expressing EGFP demonstrated fluorescence emission under microscopy after exposure to air for 20 min. However, the intensity of EGFP emission was significantly higher than that of Evoglow-Bs2 (data not shown), and emission was detected in a larger proportion of bacterial cells in suspension (Fig. 2B). Moreover, EGFP fluorescence showed greater stability after prolonged light exposure (Supplementary Fig. 1B). Higher baseline fluorescence and greater light stability are advantageous for the serial monitoring of sporulation. Unfortunately, EGFP was not expressed in *C. perfringens* probably because of the codon usage bias between *E. coli* and *Clostridium* species (data not shown). Therefore, we optimized the codon usage of the EGFP gene for *C. perfringens* and examined the expression of the resultant CpEGFP protein in *C. perfringens* under the control of the *cpe* promoter. Bacteria transformed with SM101/pJIR-cpe-CpEGFP (hereinafter referred to as the SM101/pJIR-cpe-CpEGFP line) exhibited brighter fluorescence when cultured in sporulation-promoting DSSM than cells transformed with SM101/pJIR-cpe-Bs2 growing under the same conditions (Fig. 1B) even after 8 h of cultivation in DSSM (Fig. 3A). When the ratio of transformed cells emitting greater intensity fluorescence than untransformed cells was calculated, 35% ± 11% of cells exhibited EGFP emission, indicating that 85% ± 24% of the total sporulating cells emit fluorescence, considering an observation that the ratio of heat resistant spores among total bacterial count after 24 h cultivation was approximately 40%. This high rate of positivity contrasted to the result

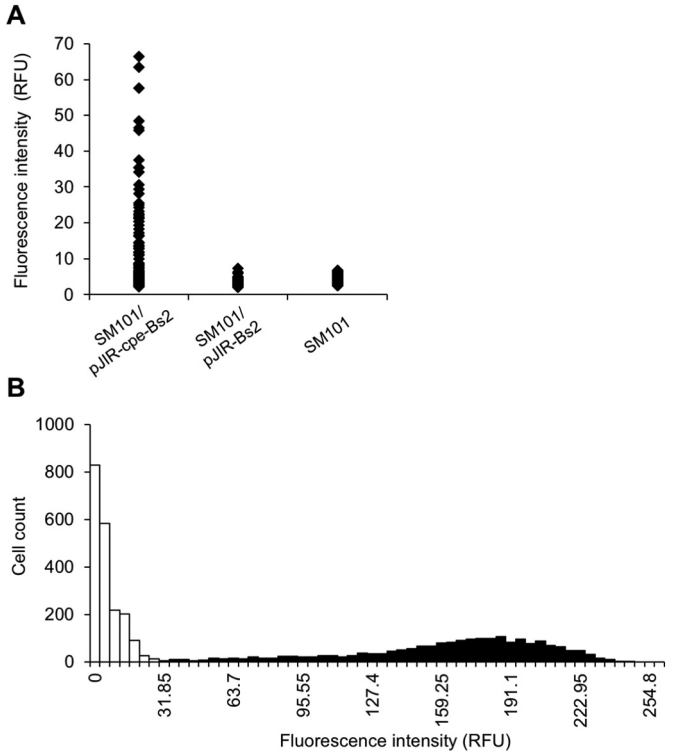

Fig. 2. Fluorescence of Evoglow-Bs2-expressing *C. perfringens* under microscopic observation. (A) Distribution of fluorescence intensities among individual bacterial cells (2059, 1563, and 2027 SM101/pJIR-cpe-Bs2, SM101/pJIR-Bs2, and SM101 cells were analyzed, respectively; individual fluorescence intensities are presented as scatter plots). (B) Histogram of the strength of fluorescence among each *E. coli* cell population expressing either Bs2 (open bar) or EGFP (filled bar). DH5α/pBS-BS2Δ*lac* (open bar) or DH5α/pBS-EGFP (filled bar) were cultivated in LB broth for 16 h, and 1970 and 2001 cells were analyzed for Bs2 and EGFP fluorescence, respectively. Class interval width is 4.55 (RFU).

obtained with Bs2 reporter showing that only 4.0% ± 5.7% of sporulating cells emit fluorescence.

We then examined if the expression of CpEGFP driven by the *cpe* promoter is sporulation-specific. It is known that high glucose in growth medium suppresses sporulation via the CcpA-mediated downregulation of Spo0A (Philippe et al., 2006; Varga et al., 2004); thus, we cultivated SM101/pJIR-cpe-CpEGFP cells in DSSM supplemented with 0.4% glucose. As expected, bacterial sporulation was strongly inhibited by the addition of glucose, and the fluorescence intensity decreased to the level of untransformed cells (Fig. 3B). The proportion of fluorescent-positive cells (with emission RFU greater than that of untransformed SM101 cells), which was 26% ± 1.9% in the absence of glucose, significantly decreased to 0.048% ± 0.049% in the presence of glucose. Similar results were also obtained when DMEM(−) was used as a basal medium (data not shown).

It is possible that the expression level of a reporter gene in a plasmid differs from that of chromosomal genes under control of the same promoter (i.e., CPE). However, Western blot analysis confirmed that CPE production was also inhibited in the presence of glucose (Supplementary Fig. 2), thus suggesting that reporter gene expression in this system is sporulation-specific and parallels the expression of the endogenous chromosomal gene.

We analyzed the kinetics of CpEGFP reporter expression (Fig. 4A). When the SM101/pJIR-cpe-CpEGFP line was cultured in DSSM, the expression of CpEGFP was detected after 4 h of incubation prior to the detection of heat-resistant spores. This indicates that *cpe* promoter-driven gene expression begins at an early stage of sporulation as previously suggested using another reporter (Harry et al., 2009; Melville et al., 1994). The morphological observation of bacterial cells stained with Hoechst33342 and Rhodamine B (Supplementary Fig. 3) revealed that most CpEGFP-positive cells at 4 h exhibited asymmetric septum formation and segregated chromosomes in shorter compartments (data not shown); this observation is consistent with Stage II sporulation (Yasugi et al., 2016a). To our knowledge, this is the first report to directly confirm that the *cpe* gene is upregulated at sporulation Stage II in single cells, although several population analyses have already suggested this notion (Harry et al., 2009; Duncan et al., 1972). The intensity of CpEGFP emission reached a plateau at 8 h and then decreased over the next 16 h of incubation (Fig. 4A). The decreased intensity at 24 h can be explained by the lysis of the mother-cell wall during spore maturation, which causes the release of cytoplasmic contents (including CpEGFP) into the extracellular milieu (Errington, 1993; Yasugi et al.,

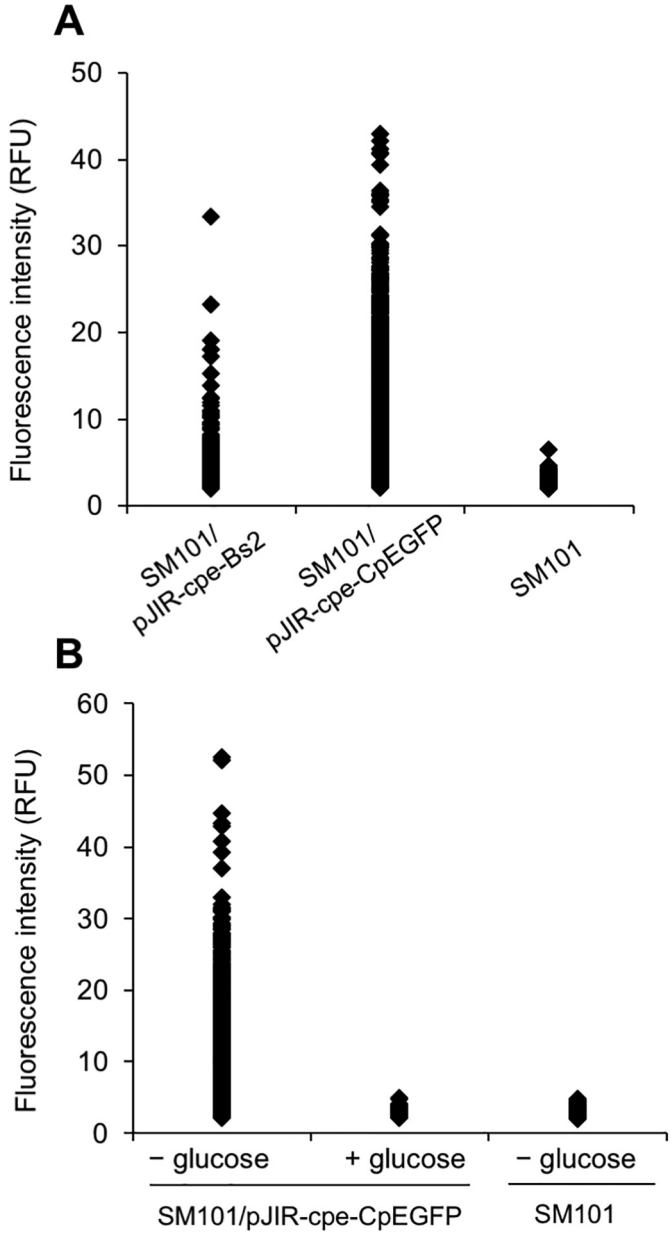

Fig. 3. Fluorescence of SM101/pJIR-cpe-CpEGFP-expressing *C. perfringens* during sporulation. (A) Fluorescence intensity distributions of SM101/pJIR-cpe-Bs2 (n = 2100), SM101/pJIR-cpe-CpEGFP (n = 2126), and SM101 (n = 2136) cells, respectively. (B) Distribution of fluorescence intensity from SM101/pJIR-cpe-CpEGFP cells without glucose (n = 2114), SM101/pJIR-cpe-CpEGFP cells with glucose (n = 2133), and SM101 without glucose (n = 2018).

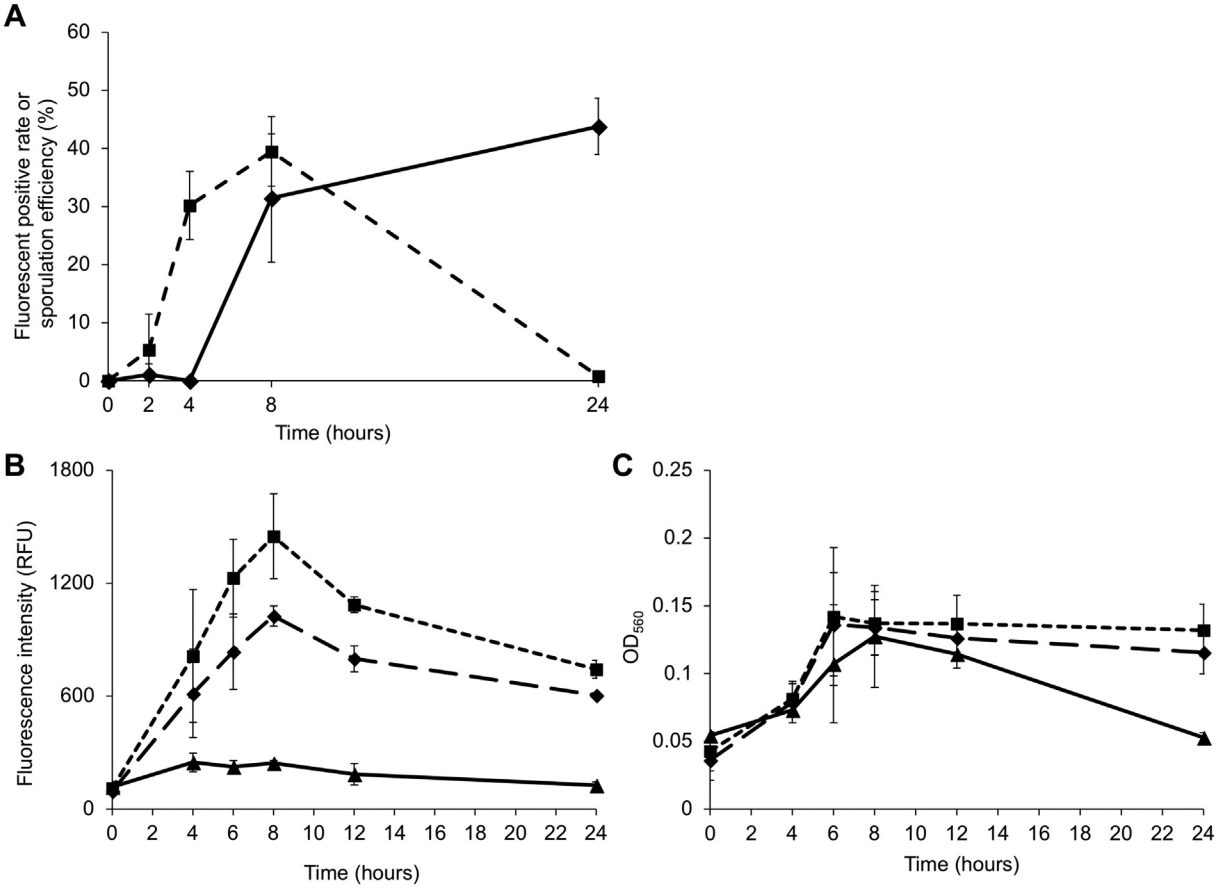

Fig. 4. The time required for assessment of sporulation by the CpEGFP reporter method. (A) Time course of the change in ratio of fluorescent cells to sporulation efficiency calculated after 0, 2, 4, 8, and 24 h of incubation. Data expressed as mean ± standard deviation of three independent experiments. (B) (C) Detection of fluorescence by fluorescence plate reader. SM101/pJIR-cpe-CpEGFP cells were cultivated in DMEM with (squares) and without (diamonds) deoxycholic acid, and SM101 cells were cultivated in DMEM with deoxycholic acid (triangles). Fluorescence intensity (B) and optical density at 560 nm (C) were measured as the means of three wells for each strain by fluorescence plate reader. Data expressed as mean ± standard deviation of three independent experiments. $*p < 0.05$ and $**p < 0.01$ between absence and presence of deoxycholic acid.

2016a). Moreover, the proportion of fluorescent cells at 8 h was approximately equivalent to the proportion of heat-resistant spores at 24 h, thus indicating that most of the fluorescent bacterial cells observed in early stages ultimately formed spores.

We examined whether this reporter can be quantified using fluorescence plate reader. We cultured the bacteria in a defined medium (DMEM(−)−0.4% starch) with a sporulation inducer deoxycholic acid (Yasugi et al., 2015; Yasugi et al., 2016a) after air exposure for 20 min. A significant enhancement of fluorescence intensity was observed after 8 h incubation only in cells treated with deoxycholic acid (Fig. 4B). This result is consistent with previous observations that deoxycholic acid potentiated sporulation via the activation of Spo0A, which in turn causes elevated expressions of *sigE* and *sigK* and the ensuing *cpe* promoter-driven gene expression (Yasugi et al., 2015; Yasugi et al., 2016a). Total bacterial growth from 0 to 12 h was not altered by any condition employed in these experiments (Fig. 4C), thus suggesting that the observed differences in fluorescence intensity reflect the expression of the reporter gene rather than the cell number. Collectively, these results suggest that the CpEGFP reporter is expressed specifically in spore-inducing environments and that the expression level can be easily monitored using fluorescence plate reader.

Finally, we evaluated the correlation between the level of reporter expression as measured by fluorescence intensity and the level of bacterial sporulation. The IH101 strain is an SM101 isogenic mutant that lacks the *spo0A* gene; therefore, it does not sporulate even under sporulation conditions (Huang et al., 2004). The SM101/pJIR-cpe-CpEGFP and IH101/pJIR-cpe-CpEGFP lines were mixed at different ratios from 8:0 to 0:8 and cultured in a defined spore-inducible medium for up to 24 h. Reporter expression was monitored at 8 h by fluorescence plate reader, and the number of spores was counted at 24 h. As shown in Fig. 5, the highest level of spore formation was obtained from the 8:0 culture containing only SM101/pJIR-cpe-CpEGFP cells. By contrast, no spores were detected when the starter culture did not contain sporulation-competent SM101/pJIR-cpe-CpEGFP cells (0:8). A lower proportion of sporulation-competent SM101/pJIR-cpe-CpEGFP cells in the initial mixture leads to a lower number of spores at 24 h (Fig. 5A). Furthermore, there was a strong positive correlation between fluorescence intensity at 8 h and the resultant formation of heat-resistant spores at 24 h (Fig. 5B, Pearson correlation coefficient test, $n = 9$, $p = 0.001$, $r = 0.942$), thus suggesting that our reporter system can be used to estimate the final number of spores at the end of cultivation.

Evoglow-Bs2 originates from *Bacillus* species and has been optimized for *Clostridium* species by the manufacturer. However, this protein was not expressed sufficiently in *C. perfringens* under our conditions (Fig. 2). This insufficient expression may have resulted from the incompatibility of the gene with the promoter and/or the ribosome binding sequence employed in this study. Considering the substantial advantages of anaerobic proteins as markers for anaerobic bacteria, further investigation is warranted to identify the ideal upstream sequences that maximize Evoglow-Bs2 expression in *C. perfringens*. The utilization of newly developed stable derivatives of flavin

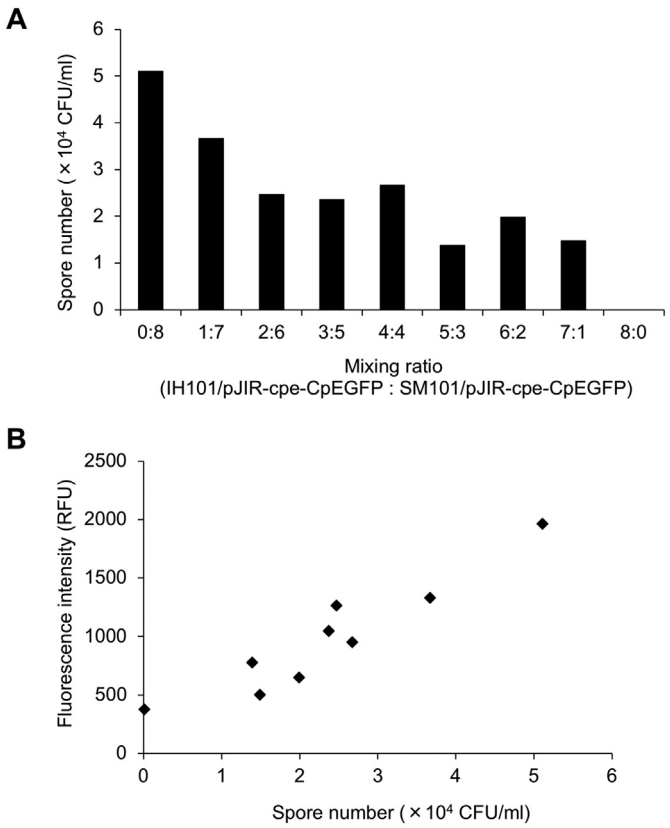

Fig. 5. Correlation between CpEGFP fluorescence intensity and level of bacterial sporulation. (A) The numbers of heat-resistant spores in bacterial cultures at different initial ratios of SM101/pJIR-cpe-CpEGFP to IH101/pJIR-cpe-EGFP cells (8:0 to 0:8). (B) Correlation between the fluorescence intensity of the 8 h cultures (vertical) and the number of heat-resistance spores in 24 h cultures (horizontal). A strong positive correlation was confirmed by Pearson correlation coefficient test ($n = 9$, $p = 0.001$, $r = 0.942$).

mononucleotide-based fluorescent proteins may be another choice for this purpose (Chapman et al., 2008).

In this study, CpEGFP proved to be a suitable reporter for high-throughput detection of *C. perfringens* sporulation because its fluorescence intensity was strong enough to be detected and quantified by conventional fluorescence microscopy and plate reader. Ideally, reporter expression should be directly measurable in bacterial culture without additional treatment. However, in our system, resuspension in PBS was required because some of the media constituents emit fluorescence, thus interfering with de novo measurements. The development of an alternative medium that induces sporulation without fluorescent emission is necessary for further study.

In the current study, we used the *cpe* promoter to monitor sporulation and expressed it in the SM101 strain as its host. This combination is considered advantageous for screening sporulation-related elements by genetic procedures because SM101 is competent for genetic manipulation. After the elements are determined with SM101, application of the results to other food-borne strains of *C. perfringens* is necessary to generalize their biological significance in bacteria. Even though most *C. perfringens* strains are refractory to genetic manipulation, using several protocols may solve this technical challenge (Scott and Rood, 1989; Jirásková et al., 2005). It is reportedly known that several *C. perfringens* strains have the *cpe* gene on the plasmid, although most clinical isolates that cause food-borne illness (including SM101 strain) possess this gene on the chromosome (Lahti et al., 2012; Li et al., 2013). The promoter regions of *cpe* genes have been mapped from 58 to 143 bp upstream of the initiation codon, which serve as binding sites for sigma factors, SigE and SigK (Harry et al., 2009). Regardless of their genetic locations, these regions show very similar sequence identity (Harry et al., 2009). Thus, in theory, *cpe*-promoter-driven CpEGFP can be expressed and visualized in a sporulation-specific manner in most food-borne isolates if the construct is successfully introduced into the bacterial cells. Non-food-borne strains universally utilize *sigE*, *sigF*, and *sigK* for controlling sporulation although they do not possess the *cpe* gene (Harry et al., 2009; Myers et al., 2006). It is likely that our reporter system would be expressed in these strains under sporulation-inducing conditions because the *cpe*-promoter is under the control of these sigma factors. The observations that the *cpe* gene introduced into *cpe*-negative strains resulted in normal CPE production (Czeczulin et al., 1996) supports this hypothesis; however, in some strains, this may not be the case, as some of the sporulation-associated genes (especially upstream genes) had been mutated (Myers et al., 2006; Shimizu et al., 2002). Regardless, maximal achievement and timing for the start and peak reporter expression, would vary among strains depending on their origin of isolation and etiological and epidemiological characteristics, all of which should influence the regulatory network of sporulation cascades.

The expression levels of specific genes in *Clostridium* species have been assessed mainly by using β-glucuronidase (*gusA*) or chloramphenicol acetyl transferase (*catP*) as the reporter (Matsushita et al., 1994; Raju et al., 2006). However, these reporters are not easily applicable to single cell analysis because they require substrate conversion for visualization. Phillips-Jones (1993) reported an alternative photoluminescence reporter, namely, the *lux* operon from *Aliivibrio fischeri*. However, the nucleotide sequence of the operon is relatively large (7 kb), thus making gene manipulation difficult. Recently, Stanton et al. (2015) reported a procedure to visualize single anaerobic bacteria using a fluorescent probe activated by bacterial nitroreductase. This procedure may prove to be particularly powerful but requires additional modification for use in the expression analysis of target genes. By contrast, CpEGFP shows several advantages: the gene is small and easy to manipulate (700 bp), it is available from commercial sources, and it does not require a substrate for visualization. Furthermore, the demonstrated correlation with the extent of sporulation (Fig. 5) means that it is possible to predict sporogenesis potential before the actual detection of heat-resistant spores. Applicability for high-throughput screening will be an important issue to be investigated in the future.

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Conflict of interests

The authors declare no conflict of interest.

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