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Original

Spore-DNA localization and extraction efficiencies of *Bacillus subtilis* for accurate results in quantitative real-time polymerase chain reaction

MIYO NAKANO*

Division of Food Science, Toyo Institute of Food Technology, 23-2, 4-chome, Minami-hanayashiki, Kawanishi, HYOGO 666-0026, Japan

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Abstract: Mechanical bead disruption is an efficient DNA extraction method from spore cells for subsequent quantification of the spore population by quantitative polymerase chain reaction（qPCR）. In this study, to validate spore DNA localization and extraction efficiencies, the fractionated DNA included the total DNA（tDNA）extracted from spore cells and intracellular（iDNA）and extracellular DNA（eDNA）extracted from fractionated spores through chemical decoating and alkaline lysis buffers, each followed by bead disruption. Furthermore, alkaline lysis buffer-treated spore cells were intensively washed three and five times after each centrifugation to determine how the amount of DNA is affected by repeated centrifugation. This process was achieved through fractionated spore pellet and suspension treatments with propidium monoazide xx（PMAxx）before mechanical bead disruption. Three fractionated and extracted DNAs were assessed with qPCR. The amount of eDNA was higher than that of iDNA, and closer to tDNA levels in the qPCR assay. These results indicted the following: 1）amount of eDNA was more than iDNA and responsible for majority of amount of tDNA through the combination method involving alkaline lysis buffer and bead disruption, 2）lysis buffer partially eliminated the eDNA fragments through multiple washing steps, but it was not largely independent of the number of times centrifugation was performed.

Keywords：spore DNA localization / total DNA / intracellular DNA / extracellular DNA / spore DNA extraction / quantitative real-time PCR.

INTRODUCTION

Psychrotolerant *Bacillus* spp. and *Paenibacillus* spp. are essential aerobic spoilage bacteria in the food industry. Their rapid identification and enumeration is essential to said industry. To this end, nucleic acid-based methods such as qPCR-based identification and quantification are a suitable alternative, as they are comparatively easy and fast, with a high sensitivity. The previous study demonstrated that the combining alkaline lysis and mechanical bead disruption improved DNA extraction from spore cells, allowing for the detection of spore DNA at ≧10^2 CFU/mL in food matrices and beverages（Nakano 2023）.

Meanwhile, *Bacillus* spores carry extracellular DNA（eDNA）, which may be bound to the spore surface coat, and which cannot be quickly removed by multiple washing steps, getting amplified during PCR without mechanical lysis（Belgrader 1999; Johns 1994）, leading to overestimating the spore population（Brauge 2018）. Little is known about bacterial spore DNA localization, the amount of intracellular DNA（iDNA）and eDNA in each species and their behavior during DNA extraction procedures with/without the exosporium. The complete recovery of iDNA and eDNA is challenging and could affect downstream qPCR analysis. This study aimed to clarify the DNA localization of *Bacillus subtilis* spore cells and determine the individual amount of total DNA（tDNA）, iDNA, and eDNA through chemical decoating buffer or alkaline lysis buffer treatment combined with mechanical bead disruption. The yielded fractionated individual DNAs were subjected to qPCR. Additionally, we also determined whether the amount of fractionated DNAs obtained were affected by the extraction procedure, particularly through the multiple washing steps with/without PMAxx treatment. In this study, initially we recovered DNA extracted from DNase-treated spore cells to remove free DNA fragments after spore purification.

Fractionated DNAs included tDNA extracted from spore cells and iDNA and eDNA extracted from spore pellets and supernatant by treatment with lysis buffer, followed by centrifugation and bead disruption. Experiment 1 consisted on fractionated tDNA, iDNA, and eDNA extraction using a

*Corresponding author. Tel: +81-72-740-3300, Fax: +81-72-758-6934, E-mail : miyo_nakano@shokuken.or.jp

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chemical decoating buffer with minor modification (Üstok 2014; Bagyan 1998). Comparatively, in Experiment 2 we applied an alkaline lysis buffer (Nakano 2023) to fractionate tDNA, iDNA, and eDNA extractions, incorporating three or five washings between each centrifugation to refine the effects on DNA recovery via repeated centrifugation. Additionally, fractionated spore cells and supernatants were each treated with a propidium monoazide xx (PMA xx) dye to block superficial DNA fragments before mechanical bead disruption. Finally, the individual extracted DNAs were quantified through qPCR, and fractionated spore cells and supernatants were analyzed to verify spore coat removal efficacy.

MATERIALS AND METHODS

The *B. subtilis* NBRC 13719^T strain (ATCC 6051) was initially cultivated in trypticase soybean broth (TSB), and sporulation was performed in Difco sporulation medium (DSM) plates, as described by Nicholson and Setlow (Nicholson and Setlow 1990). Briefly, the initial overnight culture samples were spread on DSM agar plates and aerobically incubated at 35℃ for 5-7 days until >95% of cells were sporulated, as determined by phase-contrast microscopy. The spore cells were then harvested by scraping the surface and washed twice by centrifugation (8,000×*g*, 15min) at 4℃. In this study, we performed a lysozyme treatment (Tavares 2013); the spores were first washed twice with distilled water by centrifugation (8,000×*g*, 15min) at 4℃; then, the obtained pellet was suspended in 20mM Tris–HCl (pH 7.5) containing 10mg/mL of lysozyme, and the suspension was incubated for 1 h at 37℃ by shaking at 120rpm and washed once with distilled water. Next, the pellets were resuspended in 0.05% SDS solution and incubated at 37℃ for 1 h with shaking at 120rpm. Finally, the spore cells were collected, rinsed thrice with distilled water, and treated with DNase (37℃ for 90 min) (Takara Bio Shiga, Japan); subsequent DNase inactivation to remove unbound DNA fragments was accomplished through heating at 65℃ for 10min. The final spore populations were adjusted to ~10^8 spores/mL following the culture cell count on trypticase soy agar. The spore suspension was stored at −20℃ until further use.

Primers and probes were designed as described previously (Nakano 2023): forward primer BS-rpoF: 5′-GAAAG CTGTATCCGAATGGCG-3′, reverse primer BS-rpoR: 5′-CTA CTCCGTGCAGCAGGTTG-3′, probe BS-rpoP: 5′-[FAM]-GGAGAACAGGTTATCAATGTAATCGGC [TAMRA]-3′. Microbial genomic DNA was extracted using the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The genomic DNA was quantified using a spectrophotometer (µQuant) and diluted to the appropriate concentration before use.

The specificity experiment was performed by qPCR in a total volume of 20 µL containing 10 µL 2×TaqMan Fast Advanced Master Mix (Life Technologies, Foster City, CA), 0.3 µM of each primer, 0.25 µM probe, and 2 µL microbial genomic DNA template (adjusted to a concentration of 2–3 ng/µL) in a QuantStudio 3 system (Life Technologies). The following one-step amplification protocol was used for the qPCR assay: 95℃ for 20 s, followed by 50 cycles of 95℃ for 1 s, and 58℃ for 20 s. All reactions were run in duplicate, and positive and no template control reactions were included in each assay. The experiment was repeated three times on separate plates with triplicate qPCR test per experiment (*n* = 3) to determine reproducibility. Bacterial culture of genomic DNA from *B. subtilis* was quantified for standard curve preparation (Fig. 1). The DNA concentration was converted to the equivalent genomic DNA copy number using the bacterial *tuf* gene quantitative PCR kit according to the manufacturer's instructions (Takara Bio, Shiga, Japan) as previously described (Nakano 2023).

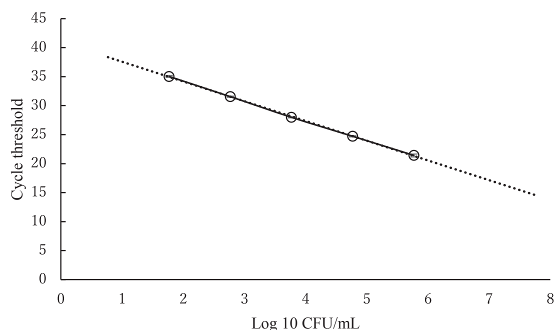

FIG. 1. Standard curve based on the *rpoB* gene copy number for qPCR assay. Values that meet the acceptance criteria from the measured values are shown as solid lines, and values that are extended based on the calibration curve equation are shown as dotted lines.

A spore cell suspension of 100 µL containing 5×10^6 CFU was prepared. The schematic illustration is represented in Fig. 2.

Experiment 1: Spore cells were suspended in a chemical decoating buffer with minor modification (0.1 M NaCl, 0.1 M NaOH, 0.1 M DTT, and 1% SDS [wt/vol] at 70℃ for 2 h (Üstok 2014; Bagyan 1998; Ghosh 2008). Next, decoated cells were retrieved through three centrifugation washing steps (10,000×*g*, 20 min, 4℃) for iDNA and these supernatants were combined as eDNA fractionated samples, followed by bead disruption. Alternatively, tDNA was extracted from spore cells without fractionation before mechanical bead disruption. All DNA components were included in the qPCR assay. The decoating buffer inhibited PMAxx chemical activity.

Experiment 2: Spore cells were suspended in an alkaline lysis buffer (20 mM Tris-HCL [pH 8.0], 2 mM EDTA, 1.2% Triton X-100, 20 mM NaOH) and proteinase K (Takara Bio), and incubated at 65℃ for 30 min. Concerning iDNA and eDNA, extraction, alkaline lysis buffer-treated spore cells, and supernatant were generated through three or five wash-

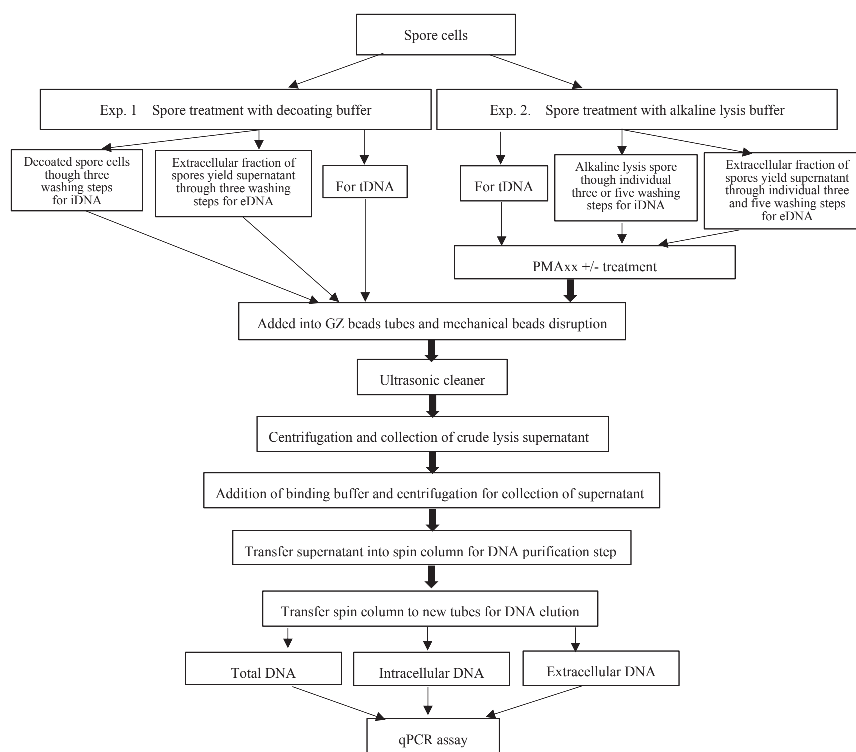

FIG. 2. Sample preparation workflow for fractionated DNA extraction and subsequent DNA quantification through qPCR.

ings to elucidate how multiple washing steps affected retrieved DNA recovery. In addition, individually fractionated spore cells and supernatant were processed with/without PMA treatment as follows: the PMA-based dye PMAxx™ (Biotium Inc., Hayward, CA), is a photoreactive intercalating dye that inhibits PCR amplification of eDNA fragments and DNA from dead or membrane-compromised cells. It has been used for the selective detection of living cells in environmental samples (Nocker 2006; Cawthorn and Witthuhn 2008). Herein, to assess if superficial DNA fragments coexisted on the spore surface in the cell suspension, PMAxx was used to block access to eDNA, and/or if superficial DNA fragments coexisted on the spore surface in the cell suspension, a PMA treatment protocol was carried out according to Rawsthorne et al., (2009) with minor modifications. Briefly, the spores were treated with 20 μM PMAxx for 50 min in the dark at room temperature. Then, the tubes were placed on ice and exposed to halogen light (PH-505N, kandakikou.co.jp, Tokyo) at a distance of 15 cm for 10 min. The spore cells and supernatants were used for mechanical bead disruption and DNA retrieval using spin column purification and subjected to qPCR. tDNA was extracted without fractionation followed by mechanical bead disruption.

For DNA extraction from the spore cells and supernatants, the lysate was transferred to bead tubes, which were self-prepared GZ beads, 0.5 mm diameter glass (Tomy Seiko), 0.55-0.60 g, and 0.12-0.18 mm diameter zirconia (Shimadzu), 0.20-0.22 g, and disrupted for 1 min at 3000 rpm; this was repeated twice with 1-min intervals. Physical treatment was performed using the bead cell disrupter Micro Smash MS-100R (Tomy Seiko Co., Ltd., Tokyo). The bead tubes were then processed by ultrasonic cleaning for 3 min, and lysate was collected by centrifugation (18,700×*g*, 5 min).

Then, approximately 800 μL of binding buffer (4 M guanidine hydrochloride and 0.5 M potassium acetate, pH 4.2) was added to crude lysates from the total spore lysate, the intra- and extracellular crude lysates, and mixed well. The tubes were gently inverted 10 times and the supernatant was collected by centrifugation (13,800×*g*, 10 min), after which, it was transferred to an EconoSpin column (Ajinomoto Bio-Pharma service) and centrifuged (10,000×*g*, 1 min). The flow-through was discarded and the spin column was washed twice with 500 μL of washing buffer 1 (5 M guanidine hydrochloride, 20 mM Tris-HCL, [pH 6.6] with 38% ethanol), and 500 μL of washing buffer 2 (2 mM Tris-HCL, [pH 7.5] with 80% ethanol) once. The flow-through was then discarded and centrifuged for 2 min to remove the residual ethanol. Finally, the DNA was eluted with 100 μL of DNA elution buffer (10 mM Tris-HCl, pH 8.5) and analyzed

by qPCR. The individual buffer solutions were prepared with minor modification based on the reference material for the EconoSpin column. All DNA components were assessed by qPCR assay.

For transmission electron microscopy (TEM) analysis, the spore cells and supernatants retrieval from two lysis buffers were prepared by the chemical fixation method, and visualized using a JEM 1400 Plus (JEOL Ltd., Tokyo, Japan) at a voltage of 100 kV.

The Tukey-Kramer test was used for statistical analysis. The test statistic T values were calculated by Excel as follows:

$$\mathrm{Tij} = \frac{|\mu i - \mu j|}{\sqrt{\frac{Ve}{n}}}$$

μi, μj are average values between comparative groups, *Ve* is unbiased variance (VAR variance), and *n* is sample size. The sample sizes of Experiment 1 and 2 are three and 10, respectively. The individual T values are referred to in the studentized range table can be found elsewhere (https://real-statistics.com/statistics-tables/studentized-range-q-table/); the obtained T values are exceeded in studentized range table, indicated significant differences $p < 0.05$, or $p < 0.01$, respectively.

RESULTS AND DISCUSSION

The specificity of the primer and probe pair in the present study was previously verified by a qPCR assay using > 4 ng of genomic DNA, including 57 closely related and unrelated strains (Nakano 2023). When testing the specificity of the newly developed qPCR assays, most non-target species had negative C_T values, except of *B. coagulans* (37.1±0.1), in which fluorescence signals were detected at higher C_T values compared with those of the target strain *B. subtilis* NBRC 13719^T (ATCC 6051) (19.4±0.4). When our results were used to generate a standard curve based on the *rpoB* gene of target strain *B. subtilis*, we obtained the mathematical equation, y = -3.41x (±0.1) + 40.98 (±0.2) with an R^2 value of 0.999 (±0.0003) and PCR efficiency of 96.6 (±3.0). The detection range covered > 5 log dynamic units (Fig. 1).

Experiment 1: A qPCR assay assessed three individual fractionated DNAs retrieved through three washing steps before bead disruption (Fig. 3). The C_T values of eDNA and tDNA were 20.2 (±0.2) and 19.6 (±0.2), meanwhile, iDNA had a 22.5 (± 0.2) C_T value, less than that of eDNA. Moreover, as shown in TEM image (Fig. 5D), it was speculated that the supernatant obtained from the decoating buffer treatment may recover most of the eDNA, which stripped off the broken coat surface, whereas, it retrieved less of iDNA due to mechanical disruption efficiency. The Tukey-Kramer test mentioned previously indicated significant differences between tDNA and iDNA, iDNA and eDNA ($p < 0.01$), and tDNA and eDNA ($p < 0.05$) content (T values

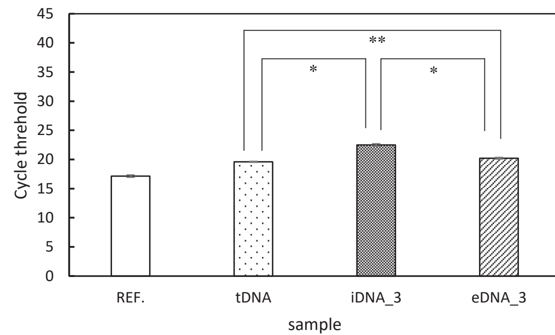

FIG. 3. Fractionated total spore (total), intracellular, and extracellular DNA abundance were extracted with/without decoating treatment, followed by bead disruption and qPCR assay evaluation. The Tukey–Kramer test was used for statistical analysis; all values indicate significant differences between groups, *, $p < 0.01$, **, $p < 0.05$.

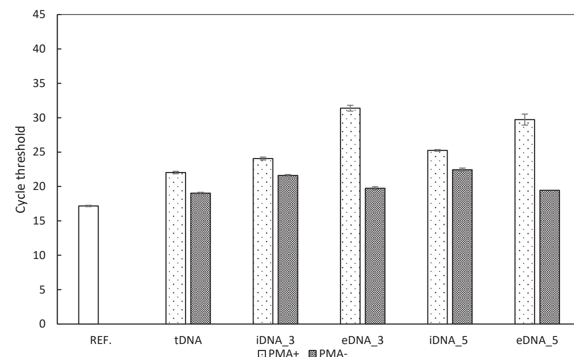

FIG. 4. tDNA, iDNA, and eDNA abundance extracted with/without PMAxx treatment as determined by quantitative PCR. "_3" and "_5" indicate "three" and "five washing steps", respectively.

were not shown).

Experiment 2: A qPCR assay evaluated three individual fractionated DNAs generated through three or five washing steps with/without PMAxx treatment before bead disruption (Fig. 4). Similar C_T values were obtained based on DNA copy numbers from tDNA and eDNA samples without PMA treatment: 19.0 (±0.1) and 19.8 (±0.1) (three washing steps) and 19.5 (±0.2) (five washing steps), respectively. Concurrently, C_T values for the iDNA sample were 21.6 (±0.1) (three washing steps) and 22.4 (±0.2) (five washing steps). Regardless of washing times, C_T values of the eDNA was closer to that of tDNA, and lower than that of iDNA, similar to Experiment 1's results. The supernatant might contain most of the eDNA obtained via alkaline lysis buffer treatment, suggested that is allowed eDNA to detach from the associated coat surface regardless of the multiple washing times.

PMA-treated tDNA and eDNA C_T values were 22.0 (±

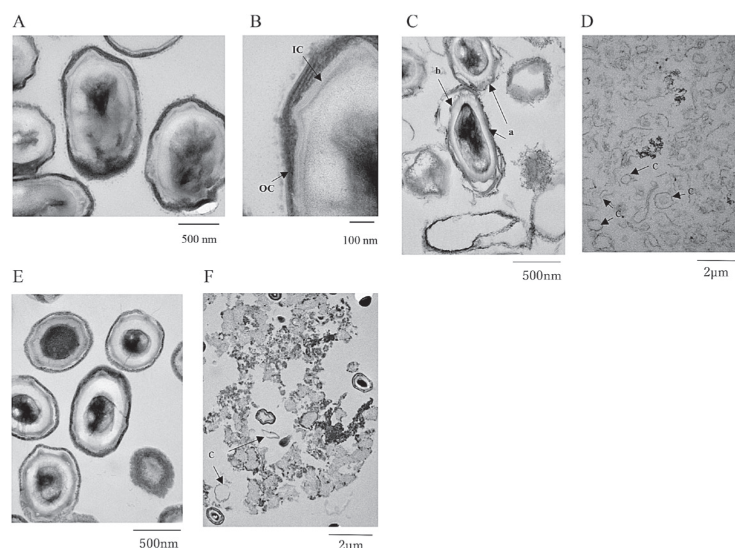

FIG. 5. TEM images obtained before and after decoating and alkaline lysis buffer treatments. (A) Intact spore (B) Magnified image of an intact spore. IC, inner coat; OC, outer coat. (C-D) Cell pellets and supernatant with decoating treatment. (E-F) Cell pellets and supernatant with alkaline lysis buffer treatment. Both the outer (arrow a) and inner lamellar coats (arrow b) were largely removed; several "rind" structures (arrow c in panels D and F) can be observed.

0.2) and 31.4 (± 0.4) (three washing steps) and 29.7 (± 0.8) (five washing steps), respectively. Meanwhile, PMA-untreated tDNA and eDNA C_T values were 19.0 (±0.1) and 19.8 (±0.1) (three washing steps) and 19.5 (±0.2) (five washing steps), closer to that of tDNA Compared with the results obtained with PMA treatment, it was speculated that eDNA contents recovered less than 0.1% after three and five washing steps. These results suggest that eDNA associated with the spore coat surface may be stripped off and lost over multiple washing steps, followed by covalent PMA xx chemicals and inhibition of qPCR amplification. However, it is largely independent of the number of times centrifugation is performed. C_T values of iDNA with PMA xx treatment were 24.1 (±0.2) (three washing steps) and 25.3 (±0.1) (five washing steps), meanwhile, C_T values of without PMA xx treatment were 21.6 (±0.1) (three washing steps) and 22.4 (±0.2) (five washing steps), obtained approximately three signals of C_T values higher than those obtained without PMA xx treatment, respectively, suggesting reduced amount of DNA washed off compared with the results from eDNA fraction. For tDNA fraction, C_T values with and without PMA treatment were 22.0 (±0.2) and 19.0 (±0.1), observed three signals higher than those obtained without PMA xx treatment. Furthermore, the Tukey-Kramer test (table 1) performed based on the C_T values indicated no significant differences between tDNA and individual iDNA or eDNA content after three or five washings without PMA xx treatment. Similarly, the following combinations showed no significant differences ($p > 0.01$); iDNA with/without PMA xx treatment (three or five washings each), eDNA without PMA xx treatment (three of five washings), and iDNA and eDNA without PMA xx treatment (five washings each) (Table 1).

A previous study indicated eDNA from *B. subtilis* spore cells by PCR even after the spores were extensively washed with water and high salt solutions (Belgrader 1999). More recently, another study suggested that eDNA is primarily bound to the spore coat (for example in *B. cereus* strains) even in the presence of an additional external membrane, the exosporium, which could protect any superficial DNA from detachment, especially during the washing steps (Brauge 2018). According to that study, the amount of spore DNA differed according to the DNA extraction protocols used. Mechanical disruption using bead beating yielded the highest amounts of DNA from *B. cereus* spores while the superficial DNA closely associated with the spore coat could be lost during extraction through column purification (Brauge 2018).

In this study, the extracted eDNA amount was closer to that of tDNA, whereas iDNA exhibited C_T values 2-3 signals higher, reflecting the difficulty of accessing the spore core to retrieve the iDNA. These results suggest that the high salt concentrations of the alkaline lysis buffer potentially assist with eDNA removal. Furthermore, the eDNA fragments initially associated with spore coat layers are removed during the intensive washing steps, potentially covalently bound to PMAxx chemicals, inhibiting their amplification by qPCR.

The TEM analysis revealed intact spore structures (Fig. 5A–B), the decoating treatment released the outer and inner

TABLE 1. Statistical results from Tukey–Kramer test.

Comparative combination of sample with PMA+ and - treatment		Calculated analytical T values	Significant differences indicated*	
			>9.46 with 5 % level	>16.69 with 1 % level
tDNA+	tDNA-	13.12		
tDNA+	iDNA_3+	8.86		
tDNA+	iDNA_3-	1.81		
tDNA+	eDNA_3+	40.75		
tDNA+	eDNA_3-	9.92		
tDNA+	iDNA_5+	14.01		
tDNA+	iDNA_5-	1.74		
tDNA+	eDNA_5+	33.52		
tDNA+	eDNA_5-	11.23		
tDNA-	iDNA_3+	21.98		
tDNA-	iDNA_3-	11.31		
tDNA-	eDNA_3+	53.87		
tDNA-	eDNA_3-	3.20		
tDNA-	iDNA_5+	27.13		
tDNA-	iDNA_5-	14.86		
tDNA-	eDNA_5+	46.65		
tDNA-	eDNA_5-	2.47		
iDNA_3+	iDNA_3-	10.67		
iDNA_3+	eDNA_3+	31.89		
iDNA_3+	eDNA_3-	18.78		
iDNA_3+	iDNA_5+	5.15		
iDNA_3+	iDNA_5-	7.12		
iDNA_3+	eDNA_5+	24.67		
iDNA_3+	eDNA_5-	20.08		
iDNA_3-	eDNA_3+	42.56		
iDNA_3-	eDNA_3-	8.11		
iDNA_3-	iDNA_5+	15.82		
iDNA_3-	iDNA_5-	3.55		
iDNA_3-	eDNA_5+	35.34		
iDNA_3-	eDNA_5-	9.41		
eDNA_3+	eDNA_3-	50.67		
eDNA_3+	iDNA_5+	26.74		
eDNA_3+	iDNA_5-	39.01		
eDNA_3+	eDNA_5+	7.22		
eDNA_3+	eDNA_5-	51.97		
eDNA_3-	iDNA_5+	23.93		
eDNA_3-	iDNA_5-	11.66		
eDNA_3-	eDNA_5+	43.44		
eDNA_3-	eDNA_5-	1.31		
iDNA_5+	iDNA_5-	12.27		
iDNA_5+	eDNA_5+	19.52		
iDNA_5+	eDNA_5-	25.24		
iDNA_5-	eDNA_5+	31.79		
iDNA_5-	eDNA_5-	5.02		

*Statistical analysis was performed by obtaining the T values using the Tukey–Kramer test, and refereed studentized range, empty column indicates no significant difference.

The individual T values are referred to in the studentized range table can be found elsewhere（https://real-statistics.com/statistics-tables/studentized-range-q-table/）; the obtained T values are exceeded in studentized range table, indicated significant differences $p < 0.05$, or $p < 0.01$, respectively.

lamellar coats (Fig. 5C), and alkaline lysis treatment exhibited no decoating effects in spore cells (Fig. 5E). Moreover, there were no detectable cells in the supernatant in the decoated treatment sample (Fig. 5D), as evidenced by the insoluble "rind" fraction and retained residues. A similar effect was obtained in the alkaline lysis buffer-treated supernatant (Fig. 5F). An intact spore structure has been previously observed (Driks 1999; Goldman and Tipper 1978). The inner coat has a fine lamellar region composed of several lightly stained layers, and the outer coat is often thicker and appears coarser, compared with the inner coat (Driks 1999; Fitz-James 1971). Furthermore, Ghosh (2008) characterized *cotE* and *gerE* mutants that lack most spore coat layers by TEM, finding that the insoluble "rind" fraction was largely retained not only in intact spores but also in mutant spores. Although the components essential for spore resistance to mechanical disruption have not been identified, the highly insoluble rind structure might be involved on the effect (Jones 2005). This study revealed similar rinds in decoated intact spores without deformation (Fig. 5D and 5F). In addition, the coat proteins from a highly crosslinked structure potentially have a role in the rigidity of the spore coat (Driks 1999; Henriques and Moran 2000).

In conclusion, we determined the relationship between individually extracted amount of DNA as tDNA > eDNA > iDNA, where eDNA was notably closer to tDNA than iDNA. These results indicted the following: 1) the eDNA amount is more than the iDNA content and similar to the tDNA amount using the combination method of alkaline lysis buffer and bead disruption, 2) lysis buffer partially eliminated the eDNA fragments through multiple washing steps, but it was not largely independent of the number of times centrifugation was performed. Although we utilized decoating or alkaline lysis buffers to eliminate spore surface coat-associated eDNA and bead disruption to access the bacterial spore core, retrieving iDNA remains challenge. However, overestimating the extracted spore DNA amount is unlikely with these methods as spore eDNA was notably eliminated due to the high salt concentration, lysis buffer treatment, multiple centrifugation steps and purifications in this methodological approach.

1) Author contributions: Author made contributions to the study concept, the data analysis, interpretation, drafted the manuscript and revised it critically for important intellectual content, approved the final version of the manuscript to be published.
2) Conflicts of interest: The authors have no conflicts of interest directly relevant to the content of this article.
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4) Ethical approval: This work does not involve any ethical issues.
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