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Involvement of SpoVG in hemolysis caused by *Bacillus subtilis*

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ABSTRACT

Bacillus subtilis is a facultative anaerobic Gram-positive non-pathogenic bacterium that includes members displaying hemolytic activity. To identify the genes responsible for hemolysis, a random mariner-based transposon insertion mutant library of *B. subtilis* 168 was constructed. More than 20,000 colonies were screened for the hypohemolytic phenotype on blood agar plates. One mutant showed significantly less pronounced hemolytic phenotype than the wild type. DNA sequencing and Southern blot analysis showed this mutant has a single transposable element inserted into the open reading frame (ORF) of the *spoVG* gene; complementation of the *spoVG*-disrupted mutant with a wild-type copy restored its hemolytic phenotype. It was therefore concluded that the *spoVG* gene, which plays a role in regulating asymmetric septation during sporulation in *B. subtilis*, is involved in hemolysis by *B. subtilis*.

1. Introduction

Bacillus subtilis is a well-studied Gram-positive facultative anaerobe that has been used as a source of food-processing and industrial enzymes [1]. In addition, *B. subtilis* has been used in clinical settings, including therapy for chronic gastritis [2] and as a delivery vehicle for oral vaccines [3]. However, its applications are limited due to its hemolysis ability [4].The increasingly widespread use of *B. subtilis* in various fields has raised concern over its risks to human and animals (http://www.epa.gov/oppt/biotech/pubs/fra/fra009.htm).

Strains of *B. subtilis* have the capacity to produce hemolysins that are active against red blood cells [5,6]. Named subtilysin by Bernheimer [7], is identical to surfactin, which belongs to antibacterial cyclic lipoproteins produced by some *B. subtilis* strains [8]. Recent studies have shown that some *B. subtilis* antibiotics, such as surfactins, fengycins and iturins, can cause hemolysis [9–11]. Hemolytic activity is often a virulence determinant [12] and is associated with pathogenicity in many strains, including *Bacillus* cereus, which produces hemolysin BL, and Staphylococci, which produces α-hemolysin and streptolysin-O [13–16]. While *B. subtilis* produces hemolysins with less hemolytic activity than those from pathogenic bacteria, it is necessary to reduce or eliminate the hemolytic activity of *B. subtilis* before *B. subtilis* is used to treat disease in human or other mammals. To date, a few studies have investigated hemolysin production in *B. subtilis* [6,7,17]. Furthermore, mechanisms of hemolysis by *B. subtilis* remain unknown.

Transposon-mediated mutagenesis is often used in bacterial genetics, which facilitates the elucidation of gene function or molecular mechanisms in fundamental research as well as discovery of desirable phenotypes in applied research [18,19]. Compared to the prokaryotic transposons Tn917 and mini-Tn10 [20,21], eukaryotic himar1mariner-based transposons, such as pMarA and pMarB, have been shown no bias toward hotspots and hence are effective for transposon mutagenesis in *B. subtilis* [22,23]. Plasmid pMarB333, a derivative of the pMarB transposon delivery system, which is a remarkably useful tool in *Bacillus thuringiensis* [24], has great potential for further construction of mutant libraries of other *Bacillus* strains. In addition, compared to pMarB, pMarB333 provides an easier and more accurate method of detecting transposon-flanking DNA sequences by means of plasmid rescue. In this report, a pMarB333 transposon-mediated mutagenesis system was used to construct mutant library of *B. subtilis* 168 (BS168) for discovering the genes responsible for hemolysis.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were cultured in Luria–Bertani (LB) medium supplemented with the appropriate antibiotics as needed for *Escherichia coli*: ampicillin (Amp), 100 μg/ml; spectinomycin (Spc), 100 μg / ml; kanamycin (km), 20 μg/ml; or erythromycin (Erm), 400 μg/ml; for *B. subtilis*: Spc, 200 μg/ml; km, 10 μg/ml; or Erm, 10 μg/ml.

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Table 1
Strains and plasmids.

Strain or plasmid	Characteristics	References
E. coli		
DH5α	F⁻ϕ80lacZΔM15Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 (rK−, mK+) phoA supE44 λ-thi-1 gyrA96 relA1	Invitrogen, Carlsbad, CA
B. subtilis		
168	trpC2	[25]
1A751	his nprR2 nprE18 DaprA3 DeglS102 DbglT bglSRV	[26]
BS224	Wild type	[27]
BS168B333	pMarB333 in B. subtilis 168	Present study
BS168Mu1	Spc^r, 168 spoVG::pUC19-ori-Spc	Present study
MuspoVGkan	Spc^r, 168 spoVG::pUC19-ori-Spc,Km^r	Present study
Plasmids		
pMarB333	Erm^r Spc^r, a transposable element (pUC19-ori-Spc), temperature-sensitive replicon (RepG + ts)	[24]
pWB980	Km^r, Ble^r, sacB signal peptide, P43 promoter	[28]
pAX01	Amp^r, Erm^r, B. subtilis integrative vector, xylose-inducible promoter	[29]
pAX01spoVG	Amp^r, Km^r, P_{spoVG} promoter	Present study

2.2. Assessment of the transposition of pMarB333 in B. subtilis

The mariner plasmid pMarB333 was transformed into strain BS168 competent cells as described by Spizizen et al. [30], and Spc-resistant (Spc^r) colonies were selected for at 30 °C. Transformants containing the mariner plasmid were Spc^r and Erm-resistant (Erm^r) at the permissive temperature for plasmid replication (30 °C). Plasmid DNA was then extracted from transformants, and restriction analysis and DNA sequencing confirmed the presence of the original intact plasmids [31].

To assess the transposition of pMarB333 in *B. subtilis* 168 strain, the original pMarB333-containing strain, designated BS168B333 for *B. subtilis* 168, was cultured overnight in LB at 37 °C, and then portions of the culture were plated on LB, LB plus Spc or LB plus Erm, and incubated at 50 °C, which is a nonpermissive temperature for plasmid replication [22]. The determination of transposition efficiency and plasmid clearance was measured according to the methods described by Li et al. [24].

2.3. Mapping of transposon insertion sites

Genomic sequences flanking transposon insertion sites were captured simultaneously using the plasmid rescue method, as described previously [32]. Chromosomal DNA of transposants displaying Spc^r and Erm^s was extracted and digested with *Eco*RI, *Sal*I, and *Hind*III, none of which are found within the transposon itself. Digested total DNA was purified, ligated and transformed into *E. coli* DH5α. Re-ligated DNA containing pUC19-*ori*-Spc allowed the transformed colonies to grow on LB agar plus Spc. The sequence of transposon-flanking DNA was determined using primer oMarSO [24].

2.4. Construction of transposon mutagenesis library

Strain BS168B333 was incubated overnight in LB broth plus Spc and Erm at 30 °C. The culture was diluted 1:10,000 with fresh LB broth before spotting 250 μl onto LB-plus-Spc Vented QTray with cover (Genetix, U.K.) and incubated for 24 h at 50 °C to screen transposants. Using a Qpix2 automated colony picker (Genetix, U.K.), more than 20,000 transposants were arrayed into 96-well microtiter plates (Greiner Bio-One, Germany) filled with 150 μl of LB broth plus Spc with the use of Genetix QFill3 and grown overnight at 37 °C on an orbital shaker (Multitron II; Infors AG, Bottmingen, Switzerland). All the colonies in 96-well plates (Greiner Bio-One, Germany) were replicated in duplicate with a Qpix 2 automatic replicator (Genetix, U.K.), harvested, and stored at −80 °C.

2.5. High-throughput method of detecting hemolytic phenotype of the mutant strains

A frozen stock transposon mutant library was defrosted and cultured at 37 °C for approximately 18 h. Bacterial cultures with 96 Solid Pin Multi-Blot Replicators (V&P Scientific, U.S.) were stamped from 96-well plates onto 150-mm-diameter LB agar plates plus 5% rabbit blood. The hemolysis zone (i.e. clearance zone) around each spot was examined after 24 h of incubation at 37 °C. Mutants that formed the decrease or disappearance of hemolytic zone around colonies compared to the wild type were picked for further evaluation.

2.6. Growth measurement of wild-type and mutant strains

BioLector, a real-time monitoring technique, was used to compare the growth rate of the parent strain 168 to that of the mutant with hypohemolytic phenotype. This enabled real-time monitoring of the intensity of scattered light in a manner analogous to the use of absorbance for bacteria during the continuous shaking of samples in microtiter plates [33]. A 100 μl aliquot of test strain suspension with an optical density of 0.01 at 600 nm (OD_{600}) (three replicates) was inoculated into 900 μl of LB broth and cultured in the Flowerplate (Art. No. MTP-48-BOH) with a gas-permeable sealing film (Art. No. F-GPS48-10) under defined conditions (37 °C, 1200 rpm shaking frequency, 3 mm shaking diameter) in a BioLector microbioreactor system (m2p-labs GmbH, Aachen, Germany) over 48 h. Biomass was monitored at 20 min intervals via measuring of scattered light at an excitation wave length of 620 nm.

2.7. Determination of the copy numbers of inserted transposons

To determine copy numbers of transposon insertion mutant, Southern blot analysis was performed with Probe 1 and Probe 2 for wild-type and *spoVG* mutant strains. Probe 1 was PCR-amplified with primers P5/P6 using *B. subtilis* 168 genomic DNA as template. Probe 2 was PCR-amplified with primers P7/P8 using plasmid pMarB333 as template. Genomic DNA was extracted and digested overnight with *Eco*RI, and Southern blot assay was performed as previously described [34].

2.8. Construction of spoVG-complementing plasmid

A *spoVG–kan* element that had been obtained by overlapping a 625 bp fragment containing the *spoVG* promoter region [35] amplified from the *B. subtilis* 168 genomic DNA with primers P9/P10 and

a *kan* gene expression cassette amplified from plasmid pWB980 with primers P11/P12 were used to construct the *spoVG*-containing plasmid [28]. The resulting overlap PCR product was gel-purified, digested with *Sma*I, and inserted into the 4801-bp-*Sma*I backbone fragment treated with calf intestine alkaline phosphatase (ClAP) from pAX01 [29]. The integrity of the inserts was verified by sequencing. The resulting plasmid was named pAX01*spoVG*.

3. Results and discussion

3.1. Analysis of transposition activity of pMarB333 in B. subtilis

In pMarB333-containing *B. subtilis* 168 strain, transposition occurred at a frequency of 9×10^{-3} of the viable cells (Section 2). These probably represented transposition events. There was 82% plasmid clearance, indicating that mariner-based transposon was effective in removing the pMarB333 plasmid backbone after transposition. It is reported previously the transposable element of pMarB was randomly integrated into the BS168 genome [22]. To evaluate the randomness of integration of a transposable element (pUC19-*ori*-Spc) of pMarB333 into the BS168 genome during transposition, 10 Spc^r and Erm^s transposants were randomly selected from the pMarB333-inducing transposition collection. We assumed that the mariner has no obvious bias in the genome of *B. subtilis* according to data of insertion sites of randomly chosen mariner transposon mutants (data not shown), which was in accordance with the result described by Le Breton [22]. The high frequency of pMarB333 transposition and the apparent randomness of its insertion sites on the *B. subtilis* genome indicated that pMarB333 should be an efficient tool in the construction of a transposon-integrated library.

3.2. Construction of a transposon-inserted mutant library and screening for hypohemolytic phenotype of mutants

In *B. subtilis* 168, eight genetic loci, *yhdP*, *yhdT*, *yrkA*, *yqxC*, *yplQ*, [36], *yugS, yqhB,* and *ytjA* (http://bacillus.genome.ad.jp/) were predicted to be related to hemolysin or hemolysin-like coding sequences. However, from the available documents, it is difficult to reduce or eliminate hemolysis in *B. subtilis* solely through knock-out single gene [27,37]. In this study, we constructed a random mutant library consisting of 20,000 clones using the mariner transposition system. To accurately determine the mutant hemolysis reflected by clearance zone, assay conditions were optimized in a series of preliminary experiments that were taken into account the growth state of the strain, the level of strain cell inoculation, and the incubation period. Screening for hypohemolytic mutants was performed using blood agar plates. Of these, one designated BS168Mu1, which exhibited reduced hemolytic defect compared to the wild type, was selected and evaluated further.

3.3. Growth analysis of the strain BS168 and its mutant BS168Mu1

To investigate whether the reduced hemolytic activity observed for BS168Mu1was a consequence of a slow growth rate, growth rate parameters of all strains were monitored in real time. Strains BS168 and BS168Mu1 were cultured, and their growth parameters were measured in a BioLector microbioreactor system (Section 2). Aside from a 30 min difference at the start of growth, growth rates and final cell densities for wild-type and mutant strains were similar (Fig. 1). Thus, the difference observed for hemolytic activities between the wild type and mutant strains is not due to differences in growth characteristics.

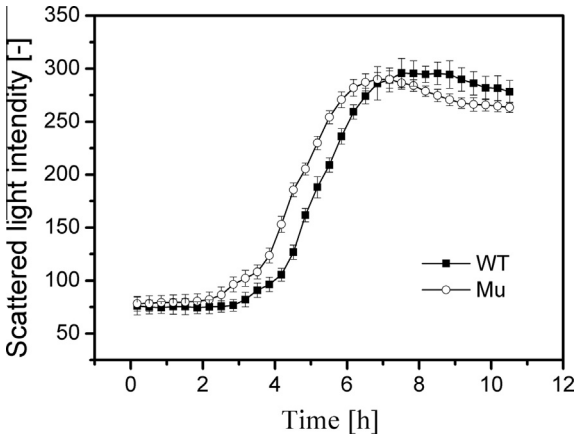

Fig. 1. Growth rate of the wild type (WT) and BS168Mu1 (Mu) as indicated by scattered light curves. Results represent average values from three independent, repeated experiments. BioLector conditions: 48-well Flowerplate, 1 ml filling volume, at 37 °C temperature, 1200 rpm shaking frequency, 3 mm shaking diameter, scattered light (ex: 620 nm/em: -, gain: 20).

3.4. Assessment of the hemolytic activity of wild-type and mutant strains

The hemolytic phenotype of the transposon-inserted mutant was examined using a modified agar-well diffusion method [38]. Two replicates of 20 µl mid-exponential-phase cultures of wild-type and mutant strains were individually inoculated at OD_{600} 0.1 into blood-agar-wells 7 mm in diameter at 37 °C for 24 h, and hemolysis zones were examined. Results showed that the mutant (BS168Mu1) has less hemolytic activity than the wild type (Fig. 3A), with hemolytic zones produced by the wild type and mutants of 13.8 ± 0.2 and 9.6 ± 0.1 mm in diameter (average of three replicates), respectively, reflecting a decrease by ~70% in the hemolytic activity. To further confirm the differences in the hemolytic phenotype of wild-type and mutant strains, the hemolytic zones of single colonies were examined by streaking on blood-agar plate. At 12 h, colonies of wild-type strains exhibited hemolysis on the blood agar, while no zones were observed for the mutant strains. Clear hemolytic zones had formed around wild-type colonies at 24 h, but low levels of hemolysis were observable around mutant colonies (Fig. 3B).

3.5. Characterization of the spoVG gene disrupted in the hypohemolysis mutant

Mutant BS168Mu1 was chosen for further characterization. The site, where the transposable element pUC19-*ori*-Spc was integrated, was determined by sequencing the genomic DNA flanking the site of transposon insertion (Fig. 2A). Nucleotide sequence analysis revealed that the transposon insertion occurred at nucleotide 91 of the *spoVG* gene's coding sequence. The presence of the fragment of pUC19-*ori*-Spc within *spoVG*, was confirmed by PCR using primer pairs P1/P2 and P1/P3 (Fig. 2B, Table 2). Sequencing of the PCR product with the primer pairs P1/P2 showed that a fragment of about 2 kb has been integrated into *spoVG*. The *Eco*RI-digested genomic DNAs of the wild type and mutant were subjected to Southern blot analysis (Fig. 2C). The blotting with probe 1 showed a larger sized DNA fragment for the mutant, which is consistent with the insertion of the transposable element within the *spoVG* locus, and the blotting using probe 2 showed a similar sized DNA fragment, indicating insertion into the genome. The results indicated that the *spoVG* mutant arose as a consequence of a single insertion within the *spoVG* gene coding region, which led to a decrease in hemolytic activity.

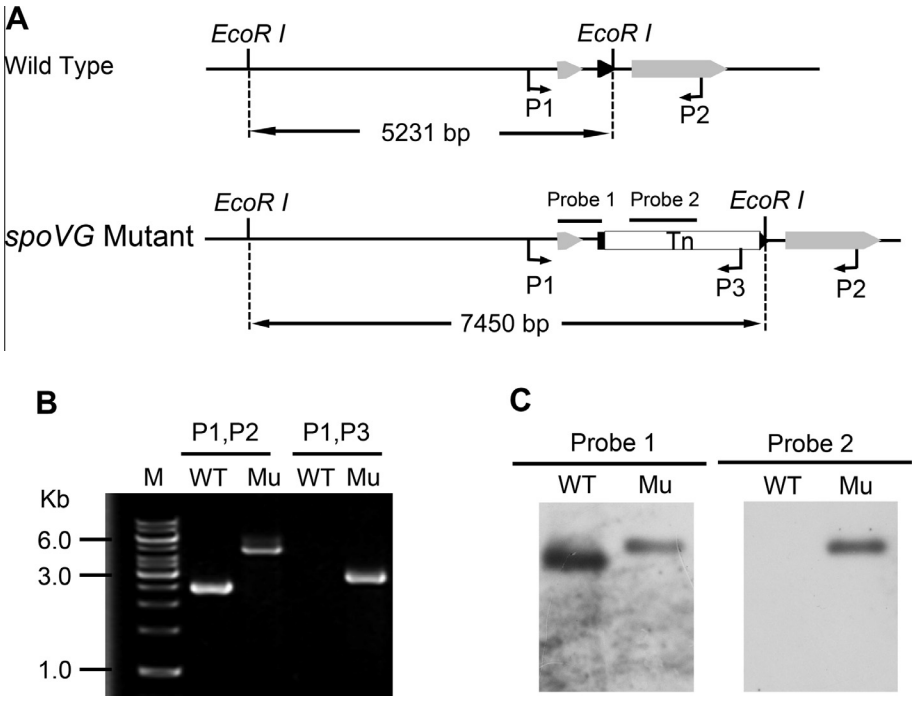

Fig. 2. Identification and analysis of information associated with transposon insertion sites in the mutant BS168Mu1. (A) Schematic representation of *spoVG* in wild-type and mutant strains. Tn: transposable element (pUC19-*ori*-Spc); the *spoVG* gene is indicated by black arrow, and the two genes located in the upstream and downstream regions of *spoVG* are *yabJ* and *gcaD*, respectively (gray arrows). (B) PCR amplification of DNA regions containing transposon insertions. Wild-type and mutant chromosomal DNAs were used as the template. M: GeneRuler 1 kb DNA ladder (Fermentas), WT: the wild type, Mu: BS168Mu1. The positions of primers P1, P2, P3 are indicated in (A). (C) Identification of the *spoVG* mutant (BS168Mu1) by Southern analysis. Hybridization of wild-type and mutant DNA digested with *Eco*RI was performed with probe 1 (left blot) or 2 (right blot). The position of each probe in the mutant sequence is indicated in (A).

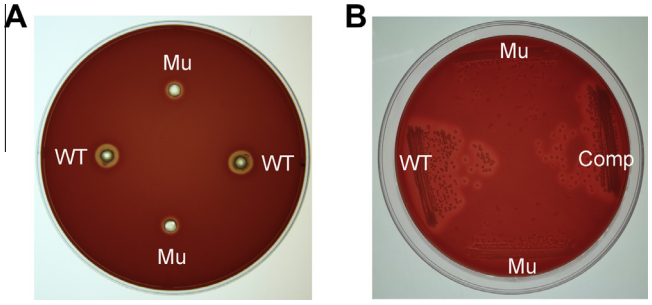

Fig. 3. Hemolytic activities of the wild type, mutant, and *spoVG*-complemented strains grown on rabbit blood agar. (A) Hemolytic activities of BS168Mu1 (Mu) and the wild type (WT), which served as a positive control. Results represent two independent, repeated experiments. (B) Hemolytic zones around single colony. Comp: the *spoVG* mutant (BS168Mu1) complement strain.

3.6. spoVG expression restored the hemolytic ability of mutant BS168Mu1

A complement strain was constructed to test whether the observed hemolytic phenotype of the BS168Mu1 mutant was a consequence of disrupting *spoVG* itself. Because *spoVG* is a developmentally regulated gene involved in *B. subtilis* spore formation [39], expression requires its native promoter [40]. To complement the *spoVG* mutant, plasmid pAX01*spoVG* (Section 2) was transformed into the *spoVG* mutant. This plasmid contains *spoVG* gene under the control of its own promoter, as well as a *kan* gene expression cassette integrated into the non-essential *lacA* locus [41] by homologous recombination. Disruption of *lacA* by the insertion of *spoVG* gene was confirmed by PCR amplification and the strain was designated *MuspoVGkan*. The complement strain displayed hemolytic phenotype similar to that of the wild type

Table 2
Oligonucleotide primers.

Primer name	Sequence (5′–3′)[a]	Source or reference
P1	GACAATAAGGTAACAGAGGGCTCCACAGTC	Present study
P2	CCCGATGACAGAATCAGGTCTGATGTGAGC	Present study
P3	CTCATAGCTCACGCTGTAGGTATCTCAG	Present study
P4 (oMarSO)	AAAGCGTCCTCTTGTGAAAT	[24]
P5	CAACAAATTCGTGATCCAGCGTGATGGATGC	Present study
P6	AGTTGAAGCCGAAGGAGTAGATGAACGTC	Present study
P7	GTCCTTCCCACTTATCATCACACTCTC	Present study
P8	CTACGGGGTCTGACGCTCAGTGGAACGAA	Present study
P9	CCTGATCCCCCGGGGTCGAGATCGAAGTTATTGCACTGGTG	Present study
P10	GCCCGTTTGTTGAACTACTCTTTAAAGTTTGAAAAATCAGAATTAAAAAAGGAC	Present study
P11	GTCCTTTTTTAATTCTGATTTTTCAAACTTTAAAGAGTAGTTCAACAAACGGGC	Present study
P12	CCTGATCCCCCGGGCCTTTATTCCGTTAATGCGCCATGACAG	Present study

[a] Restriction sites are underlined.

(Fig. 3B). The *spoVG* mutants of several *B. subtilis* strains included *B. subtilis* strain 224 and 1A751, were also constructed, and have hemolytic phenotypes similar to that of the strain 168 *spoVG* mutant. Complementation of *spoVG* mutants by wild-type *spoVG* allele were found to restore the hemolytic phenotypes of the mutants, further showed that SpoVG plays some role in hemolysis activity in *B. subtilis*. Furthermore, we observed the phenotype of sporulation in the *spoVG* mutants in line with that described by Matsuno et al. [40]. These effects could be complemented with integration of a copy of wild-type *spoVG* allele. These results indicated that reduced hemolysis level of *spoVG* null mutant are truly due to *spoVG* disruption. It was first demonstrated that SpoVG is responsible for hemolysis in *B. subtilis*.

SpoVG is a negative regulator of the pathway leading to asymmetric septation during sporulation [40]. Interestingly, we found that SpoVG is involved in hemolysis by *B. subtilis*. SpoVG, the ubiquitous proteins, belonging to the highly conserved SpoVG family [42], is found in Gram-positive bacteria including *Staphylococcus aureus* and Gram-negative bacteria [43]. It has been reported that SpoVG, a site-specific DNA-binding protein, counteracts the 5′-non-coding regions of virulence-related loci including *cap5*, *fmtB*, *esxA*, and *lukED* [43], and plays a role in the regulatory cascade modulating virulence factors in *S. aureus* [44]. In *Borrelia burgdorferi*, SpoVG specifically binds a DNA site within the *vlsE* ORF and regulates the VlsE antigenic variation system, which is the causative agent of Lyme disease [45]. However, a new study suggested that SpoVG homologs of different bacterial species may preferentially bind to a different DNA sequence and exert different effects on physiology [43]. It is possible that the effect of SpoVG on hemolysis is accomplished through binding the hemolysis-related genes in *B. subtilis*.

We reported that SpoVG is somehow involved in hemolysis. It is certain that the *spoVG* gene is more dominant in hemolysis. Since this mutant still exist hemolysis, other genes besides *spoVG* are possibly responsible for remaining activity. To discover hemolysis-related genes besides *spoVG*, we tried to purify hemolysis-related productions from the culture filtrates of wild-type and mutant strains (BS168Mu1). However, the yield and activity were low in the mutant strain, as wild-type strain displays a weak hemolytic activity [46,47]. Further work is required to determine how SpoVG affects hemolysis. Moreover, it is also required to identify other players in hemolysis in order to elucidate the complicated mechanism of hemolysis in *B. subtilis*.

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