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ENVIRONMENTAL MICROBIOLOGY

Construction of *Bacillus thuringiensis* Simulant Strains Suitable for Environmental Release

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ABSTRACT For a surrogate bacterium to be used in outdoor studies, it is important to consider environmental and human safety and ease of detection. Recently, *Bacillus thuringiensis*, a popular bioinsecticide bacterium, has been gaining attention as a surrogate bacterium for use in biodefense. In this study, we constructed simulant strains of *B. thuringiensis* with enhanced characteristics for environmental studies. Through transposon mutagenesis, pigment genes were inserted into the chromosome, producing yellow-colored colonies for easy detection. To prevent persistence of spores in the environment, a genetic circuit was designed to produce a spore without sporulation capability. Two *loxP* sites were inserted, one on each side of the *spo0A* gene, which encodes a sporulation master regulator, and a sporulation-dependent Cre expression cassette was inserted into the chromosome. This genetic circuit successfully deleted *spo0A* during sporulation, producing spores that lacked the *spo0A* gene. In addition, two major α/β-type small acid-soluble spore protein (SASP) genes, predicted by synteny analysis, were deleted. The spores of the mutant strain showed increased UV-C sensitivity and quickly lost viability when tested in a solar simulator. When the spores of the mutant strain were administered to the lungs of BALB/c mice, cells were quickly removed from the body, suggesting enhanced *in vivo* safety. All strains constructed in this study contain no antibiotic resistance markers and all heterologous genes were inserted into the chromosome, which are useful features for simulants to be released into the environment.

IMPORTANCE *B. thuringiensis* has recently been receiving increasing attention as a good spore simulant in biodefense research. However, few studies were done to properly address many important features of *B. thuringiensis* as a simulant in environmental studies. Since spores can persist in the environment for years after release, environmental contamination is a big problem, especially when genetically engineered strains are used. To solve these problems, we report here the development of *B. thuringiensis* simulant strains that are capable of forming yellow colonies for easy detection, incapable of forming spores more than once due to a genetic circuit, and lacking in two major SASP genes. The genetic circuit to produce a spore without sporulation capability, together with the deletion of SASP genes, ensures the environmental and human safety of the simulant strains developed in this study. All of these features will allow wider use of *B. thuringiensis* as a simulant for *Bacillus anthracis* in environmental release studies.

KEYWORDS simulant, biodefense, *Bacillus thuringiensis*, sporulation-dependent *spo0A* knockout, small acid-soluble spore protein, environmentally friendly strain, environmental release

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Surrogate bacteria have played a pivotal role in the history of biodefense research. Since the use of pathogens is highly risky to human health and the environment, requiring special facilities like biosafety level 3 laboratories, surrogate organisms with safe characteristics are attractive alternatives. For *Bacillus anthracis*, known to be one of the major biowarfare or bioterror agents, the spore-forming bacterium *Bacillus atrophaeus* (formerly known as *Bacillus globigii*) has been used as a simulant. *B. atrophaeus* has been popular especially for environmental release studies, as it is a common soil-dwelling bacterium and harmless to humans. *Bacillus cereus*, a close relative of *B. anthracis*, is not suitable for biodefense studies because it is a pathogenic bacterium (biosafety level 2). A nonpathogenic strain of *B. anthracis*, lacking the pXO1 and pXO2 plasmids, would be the best and most ideal simulant strain. In the Republic of Korea (and probably some other countries), however, all *B. anthracis* strains are strictly regulated by law regardless of their pathogenicity. Recently, *Bacillus thuringiensis*, another close relative of *B. anthracis*, was also proposed as a simulant for *B. anthracis* (1, 2), as it has many characteristics similar to those of the latter. It is also a common soil-dwelling bacterium that is generally regarded as safe and has a long history of use as a bioinsecticide worldwide.

Two research groups have worked on the development of *B. thuringiensis* as a simulant for *B. anthracis*. Buckley et al. (3) developed simulant *B. thuringiensis* strains in which genetic barcodes were inserted for easy tracking by real-time PCR assays and successfully tested the strains in the outdoor environment (4). The developed strains were based on the *B. thuringiensis* HD-1 strain, a strain producing the Cry protein during spore formation; Cry protein production is essential for bioinsecticide applications, but it is unnecessary for application as a simulant. For this reason, Bishop and Robinson (5) removed two plasmids carrying the *cry* genes from the HD-1 strain, thereby making it a noninsecticidal simulant (5). Although some progress has been made, several characteristics of *B. thuringiensis* need to be improved to use it as a simulant for outdoor studies.

First, unlike many simulant bacteria, *B. thuringiensis* forms white colonies. When air or soil sampling analyses are performed after environmental release, *B. thuringiensis* cannot be distinguished from other bacteria that often form white colonies. As many *Bacillus* species show similar colony morphologies, morphology alone is not sufficient for easy identification. Second, persistence of spores in the environment upon outdoor use is an important issue. Spores of bacteria belonging to the genera *Bacillus* and *Clostridium* are known to be among the toughest life forms, exhibiting strong resistance against UV light, heat, pressure, and gamma irradiation (6). For example, spores of *B. anthracis* were detected 40 years after a biological warfare experiment on Gruinard Island (7). Spores of *B. thuringiensis* sprayed for monitoring purposes in an urban environment were shown to survive for at least 4 years (8). When sprayed in a cabbage plot, they persisted for at least 7 years (9). Such a persistent property is problematic for a simulant, as many spores would continue to survive in the environment after outdoor experiments. The only solution has been the use of harmless, common soil-dwelling bacteria, such as *Bacillus subtilis* and *B. atrophaeus*. Third, although *B. thuringiensis* has been generally regarded as safe and used as a bioinsecticide for a long time, it is not free from concerns of human safety; rare cases of human infection have been reported (10, 11). Also, there have been concerns of food poisoning, because *B. thuringiensis* possesses enterotoxin genes (12). The *B. thuringiensis* 407 Cry$^-$ strain was shown to be lethal in BALB/c mice when administered via the intranasal route (13). More seriously, persistence of spores for 70 days in the lungs of mice with subchronic inflammation has been observed (14). When large amounts of *B. thuringiensis* spores are released for monitoring purposes, human safety can certainly be a concern.

The aim of this study was to develop advanced simulant strains of *B. thuringiensis* for use in outdoor environmental studies. To address the aforementioned problems, strains having the following characteristics were constructed: pigment production for easy detection, sporulation-dependent knockout of *spo0A* to prevent unwanted environ-

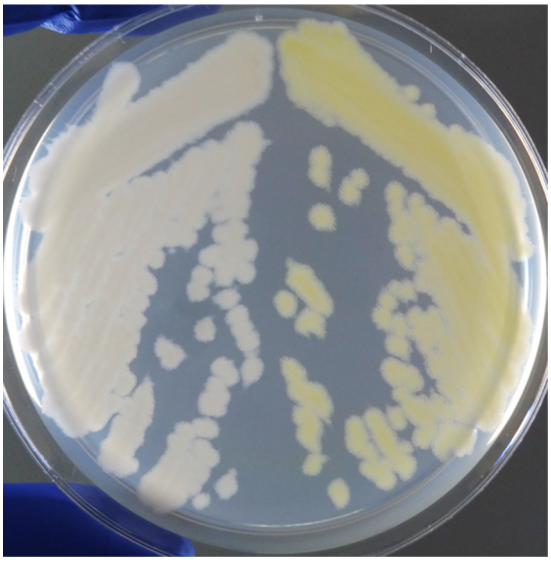

FIG 1 Pigment genes *crtM-crtN* confer a distinctive yellow color to *B. thuringiensis* colonies. Strains were grown on a TSA plate overnight at 30°C. (Left) Wild-type BMB171 strain. (Right) BT-001 strain.

mental persistence, and deletion of two major small acid-soluble spore protein (SASP) genes for environmental friendliness and human safety.

RESULTS

Construction of the yellow-colored strain. After transposon mutagenesis with pAD06, the BT-001 strain was obtained. This strain grew normally and had a distinctive yellow colony color (Fig. 1); only vegetative cells, and not spores, are yellow. Insertion of the *crtM-crtN* genes did not affect sporulation, because no significant difference was observed in spore yield between the BT-001 strain and the wild-type BMB171 strain (data not shown). When grown with other microorganisms sampled from the environment (soil and air), the yellow colony color coupled with the unique colony morphology allowed the strain to be easily identified (see Fig. S1 in the supplemental material).

To map the precise insertion site, the whole genome was sequenced by next-generation sequencing. As a result of *de novo* assembly, 244 contigs were generated from the sequencing files of the MiSeq machine. The *crtM-crtN* genes were found only in contig_87 (total length, 90,839 bp), which originated from the *B. thuringiensis* BMB171 chromosome. A detailed analysis of contig_87 revealed that the *crtM-crtN* genes were inserted into position bp 4524538 in the *B. thuringiensis* BMB171 chromosome. BMB171_C4312 (a hypothetical protein) at that position was knocked out by this insertion. The *crtM-crtN* genes were inserted in reverse orientation to the transcription of BMB171_C4312.

Construction of the sporulation-dependent *spo0A* knockout strain. A strain with two *loxP* sites inserted at both ends of the *spo0A* gene (BT-006) sporulated normally, and its spore yield (96.8%) was not significantly different from that of the wild-type BMB171 strain (100%). Four strains (BT-008, BT-009, BT-010, and BT-011) were constructed based on the BT-006 strain. All of them were leaky at very low frequency ($<$0.1%) in deleting *spo0A* during vegetative cell growth (Table 1). After sporulation, these strains showed various degrees of *spo0A* knockout (70.64% to 99.96%) (Table 1). Compared to the wild-type strain, the spore yields for the BT-008, BT-009, BT-010, and BT-011 strains were 86.6%, 93.7%, 82.3%, and 92.6%, respectively.

Construction of the *sspA/sspB* knockout strain. Alignment of the amino acid sequences of seven SASP homologs of *B. thuringiensis* with those of SspA and SspB of *B. subtilis* showed many conserved amino acids (see Fig. S2 in the supplemental material). By synteny analyses, the regions immediately surrounding the *sspA* and *sspB*

TABLE 1 Efficiency of sporulation-dependent *spo0A* knockout during vegetative growth and during sporulation[a]

Strain	Cre recombinase expression locus	Cre recombinase expression promoter	% knockout efficiency ± SD during:	
			Vegetative growth	Sporulation
BMB171			0.00 ± 0.00	0.00 ± 0.00
BT-008	BMB171_C4815	BMB171_C0536	0.04 ± 0.04	99.77 ± 0.02
BT-009	BMB171_C1559	BMB171_C0536	0.03 ± 0.02	70.64 ± 1.14
BT-010	BMB171_C4815	BMB171_C4286	0.09 ± 0.02	99.96 ± 0.04
BT-011	BMB171_C1559	BMB171_C4286	0.03 ± 0.02	96.43 ± 0.08

[a]For measurement of efficiency during vegetative growth, cells were grown in 10 ml of LB broth until the OD_{600} reached approximately 1.4 to 1.6. One milliliter of the culture was serially diluted in 0.9% (wt/vol) NaCl and spread on DSM agar plates. For measurement of efficiency during sporulation, spores were prepared in GYS medium, and 1 ml of the culture was sampled. After heat treatment at 65°C for 30 min, the spores were serially diluted in PBS solution and spread on DSM agar plates. The plates were incubated overnight at 30°C and then left for 1 day at room temperature. Then, approximately 2,500 colonies were counted. White colonies were counted as wild-type colonies, and gray colonies were counted as *spo0A* knockout colonies. Experiments were performed three times independently.

genes of *B. subtilis* 168 were found to be conserved at the *B. thuringiensis* BMB171_C4286 gene (annotated as *sspB*) and BMB171_C0753 gene (annotated as *sasP1*), respectively (see Fig. S3). Therefore, BMB171_C4286 and BMB171_C0753 were selected as the orthologs of *sspA* and *sspB*, respectively.

Three strains were constructed: the BT-003 strain for *sspA* single knockout, the BT-004 strain for *sspB* single knockout, and the BT-005 strain for *sspA sspB* double knockout. UV-C sensitivity testing showed increased sensitivities of spores of all three mutant strains to UV-C compared to the sensitivity of spores of the wild-type strain (Fig. 2). The *sspA* and *sspB* double mutant exhibited a dramatic increase in UV-C sensitivity compared to those of the single knockout mutants. During 10 weeks of storage at 4°C, the spores of the BT-005 strain were stable until 6 weeks (see Fig. S4A in the supplemental material). After 6 weeks, the viability of the BT-005 strain decreased slightly, whereas the viability of the wild-type strain showed no decrease. At 37°C, the spores of the BT-005 strain showed rapid loss of viability compared to those of the wild-type strain (see Fig. S4B).

Construction of the BT-016 strain. Strain BT-016 produced yellow-colored colonies and had three gene deletions (*sspA*, *sspB*, and *plcR*), with a sporulation-dependent *spo0A* knockout genetic circuit inserted on the chromosome. Due to the deletion of *plcR*, hemolysis capability was lost, which was confirmed by growing the mutant on

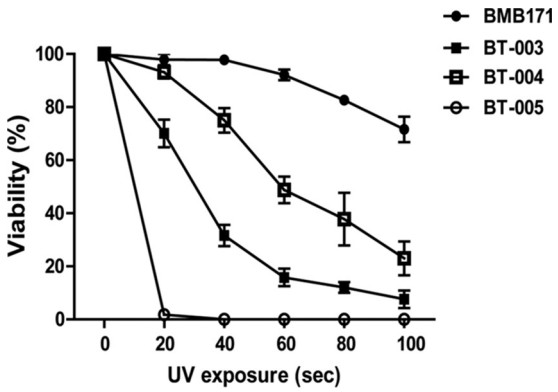

FIG 2 *sspA sspB* gene disruption increases UV-C sensitivity of spores. About 200 to 220 spores were spread onto three TSA plates and immediately irradiated with a UV-C lamp for the indicated times. After incubation overnight at 30°C, the number of colonies was counted. The experiment was repeated independently three times. BMB171, wild type; BT-003, *sspA* mutant; BT-004, *sspB* mutant; BT-005, *sspA sspB* double mutant.

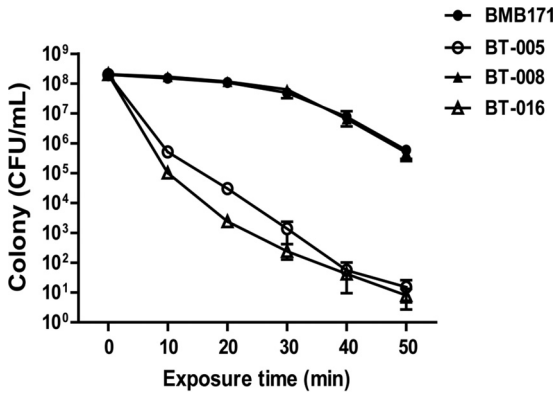

FIG 3 Solar simulator test. Spores of each strain (1.2 ml of 2.0×10^8 CFU ml^{-1} suspension) were placed into wells of 6-well plates and irradiated with artificial sunlight for the indicated times. After exposure, 1 ml of the suspension was serially diluted and plated on TSA plates to determine the number of viable cells. BMB171, wild type; BT-005, *sspA sspB* double mutant; BT-008, sporulation-dependent *spo0A* knockout strain; BT-016, yellow-colored, *sspA sspB plcR* deletion, sporulation-dependent *spo0A* knockout mutant.

sheep blood agar. After 20 s of exposure to UV-C, 1.6% of the spores survived, which was comparable to the survival rate of the BT-005 strain. The efficiency of the *spo0A* knockout was 0.07% during vegetative growth and 99.97% during sporulation. These results indicated that the *crtM-crtN* insertion, *sspA sspB* double gene deletion, *plcR* gene deletion, and *spo0A* knockout circuit insertion could be combined into one strain showing all desirable characteristics.

Solar simulator test. To simulate an outdoor environment, a solar simulator was used. Since UV-C usually does not reach the surface of the earth, the UV-C sensitivity test was inadequate to predict spore decay in a real outdoor environment. In addition, only a small number of spores (200 to 220 CFU per plate) was used for the UV-C sensitivity test. For the solar simulator test, spores at a concentration of 2.4×10^8 CFU per well were used, with 500 W/m^2 of artificial sunlight. The BT-005 and BT-016 strains were chosen to determine the effect of *sspA sspB* double deletion, whereas the BT-008 strain was chosen to determine whether the sporulation-dependent *spo0A* deletion affected normal spore resistance. The viability of spores of the BT-005 and BT-016 strains decreased dramatically, from 2.0×10^8 CFU ml^{-1} to less than 2.0×10^1 CFU ml^{-1} after 50 min of exposure, whereas the viability of spores of the BMB171 wild-type strain decreased from 2.0×10^8 CFU ml^{-1} to 5.9×10^5 CFU ml^{-1} (Fig. 3). A similar decrease was observed in the BT-008 strain as in the wild-type strain, showing normal spore resistance (Fig. 3).

Bacterial clearance in the mouse lung. To compare bacterial clearance *in vivo*, we extracted lung tissue from BMB171-, BT-005-, and BT-016-infected mice and counted the number of remaining bacterial cells (spores and vegetative cells). At 1, 2, and 4 weeks, the number of bacteria in BMB171-infected lungs decreased in a time-dependent manner (Fig. 4A). The average bacterial counts at 1, 2, and 4 weeks were 1.2×10^7 CFU, 9.8×10^5 CFU, and 4.6×10^4 CFU, respectively (expressed as CFU per gram of tissue). In contrast, the average numbers of bacteria for the BT-005 and BT-016 strains at 1 week were 1.6×10^4 CFU and 3.1×10^4 CFU, respectively (Fig. 4B), and the average numbers of bacteria for the BT-005 and BT-016 strains at 2 weeks were 2.9×10^0 CFU and 2.0×10^0 CFU, respectively. At 4 weeks, no spores were detected from the lung homogenates of BT-005- and BT-016-infected mice. The pulmonary bacterial loads were significantly higher in BMB171-infected mice than in BT-005- and BT-016-infected mice at 1 week after instillation (Fig. 4B). These results indicated that the BT-005 and BT-016 strains were much more vulnerable to the *in vivo* environment than the wild-type BMB171 strain.

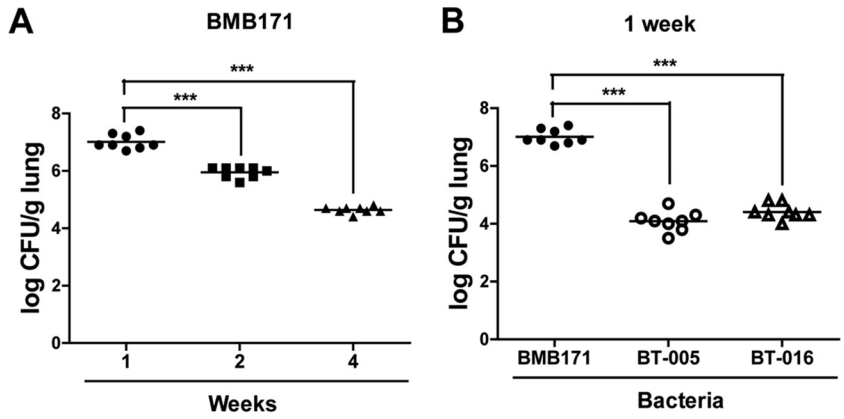

FIG 4 Comparison of bacterial clearance in the lungs of mice infected with spores of the BMB171, BT-005, and BT-016 strains. Groups of mice were intratracheally infected with spores of each strain (1.0×10^7 CFU per mouse). At 1, 2, and 4 weeks after infection, the numbers of bacteria (spores and vegetative cells) in lung homogenates were determined (CFU g^{-1} lung tissue). (A) Numbers of bacteria in the lung homogenates of BMB171-infected mice at 1, 2, and 4 weeks. Because fewer than three colonies (at 2 weeks) or no colonies (at 4 weeks) grew from the lung homogenates of BT-005- and BT-016-infected mice, only the results from BMB171-infected mice are shown. (B) Numbers of bacteria in the lung homogenates of BMB171-, BT-005-, and BT-016-infected mice at 1 week. Horizontal bars show the mean value for each group. ***, $P < 0.001$. BMB171, wild type; BT-005, *sspA sspB* double mutant; BT-016, yellow-colored, *sspA sspB plcR* deletion, sporulation-dependent *spo0A* knockout mutant.

DISCUSSION

Colony morphology, characterized by size, surface, and edge shape, is a unique feature of a bacterial species. This feature alone, however, cannot be used to differentiate between species when many bacteria are grown together. For this reason, many surrogate microorganisms used in biodefense research communities, including *B. atrophaeus* and *Pantoea agglomerans* (formerly known as *Erwinia herbicola*), exhibit distinct colony colors. Colony color, when coupled with colony morphology, makes the bacterial species easier to distinguish from other bacterial species, especially in field experiments such as air and soil sampling analyses. However, *B. thuringiensis* shows a white colony color, which is disadvantageous for a simulant to be used in environmental studies. To circumvent this problem, the use of Brilliance *Bacillus cereus* agar (Oxoid, Basingstoke, UK) was proposed (5). On this selective and differential agar, *B. thuringiensis* grows in blue-green colonies because of special chromogenic substrates. However, this kind of special medium is more expensive than a common medium, and thus, it is more desirable for a simulant to produce its own pigment. To broaden its applicability as a simulant, we thus aimed to make yellow-colored *B. thuringiensis* by inserting *crtM-crtN* genes from *Staphylococcus aureus*. *crtM-crtN* produces 4,4′-diaponeurosporene from farnesyl diphosphate (15). The Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg) predicts that *B. thuringiensis* produces farnesyl diphosphate. When *crtM-crtN* genes were introduced into *B. subtilis* on an expression plasmid, the engineered cells successfully produced 4,4′-diaponeurosporene from farnesyl diphosphate (16). Thus, the introduction of the genes was expected to produce 4,4′-diaponeurosporene in *B. thuringiensis* as well. As pigment-forming genes on expression plasmids can be easily lost without appropriate selective pressure, i.e., antibiotics, these genes were introduced into the chromosome. The pigment-forming genes (*crtM-crtN*) were randomly inserted by transposon insertion, and after screening, the colony with the strongest yellow color was selected as the BT-001 strain (Fig. 1). BT-001 sporulated normally, showing no defect in the sporulation process. Next-generation sequencing analysis showed that the *crtM-crtN* genes were inserted into the middle of a hypothetical protein (BMB171_C4312), thereby disrupting this gene. Since the strain grew normally, the BMB171_C4312 gene was assumed to be nonessential. As the promoterless *crtM-crtN* genes were inserted in reverse orientation to the direction

of BMB171_C4312 transcription, these genes seemed to be transcribed by the promoter of the neighboring BMB171_C4310 gene (encoding virulence factor MviM).

For a simulant that will be released into the environment, the persistence of spores can be a problem. Since this is an inherent characteristic of spores, there has been no solution except for the use of harmless and environmentally friendly strains (e.g., *B. atrophaeus*). We suggested two solutions in this study. The first approach is to insert the sporulation-dependent *spo0A* knockout circuit into the chromosome. With this system, more than 99% of spores cannot form spores again after germination. Although *B. thuringiensis* organisms have been shown to persist as vegetative cells in the soil (17), their survival is severely diminished when *spo0A* is deleted (18, 19). Therefore, *spo0A* deletion as described in our system would prevent not only spore formation but also vegetative cell survival. Although the spore yields are 6.3% to 17.7% lower than that of the wild-type strain, the advantages of this conditional *spo0A* knockout system outweigh the slightly lower spore yields. Between two expression loci, BMB171_C4815 and BMB171_C1559, the former showed better results, with more than 99% *spo0A* knockout efficiency during sporulation (Table 1). Leakage during vegetative growth was negligible (less than 0.1%) when this locus was chosen as the integration site. Contrary to our expectation, the *spo0A* knockout efficiency during sporulation was poorer at the BMB171_C1559 locus, even though the difference between the two loci in the extent of leakage during vegetative growth was negligible (Table 1). Two promoters (BMB171_C0536 and BMB171_C4286) showed similar results when used at the BMB171_C4815 locus (Table 1). Since the BMB171_C0536 promoter showed slightly less leakage and slightly higher spore yields, this promoter was finally chosen for use with the BMB171_C4815 locus for Cre recombinase expression in the BT-016 strain. This result indicates the importance of selecting a proper promoter and an expression locus to construct a sporulation-dependent *spo0A* knockout circuit.

The second approach to the problem of persistence of spores in the environment is the deletion of major α/β-type genes of SASPs. SASPs have been extensively studied in *B. subtilis*. They are DNA-binding proteins that are produced only in the forespore compartment during sporulation and play a crucial role in protecting the spore's DNA from environmental stress (20). Deletion of two major α/β-type SASP genes (*sspA* and *sspB*) has been shown to severely diminish the resistance of spores to UV, heat, H_2O_2, desiccation, and chemicals (20, 21). It was expected that deletion of these genes in *B. thuringiensis* would produce an environmentally friendly simulant. As in *B. subtilis*, the deletion of two major α/β-type SASP genes made *B. thuringiensis* highly sensitive to UV-C (Fig. 2). The solar simulator test also showed that spores became extremely sensitive to artificial sunlight (Fig. 3). With this feature, the engineered *B. thuringiensis* organisms would quickly lose viability once released into the environment. Because they also showed less temperature tolerance (see Fig. S4 in the supplemental material), it was expected that they would persist for only a short period of time after release to the outdoor environment. This feature might be disadvantageous for live-cell detection analyses. However, PCR-based detection is still possible, since its DNA would be still available.

Deletion of the two major α/β-type SASP genes also showed the potential to solve the problem of long-term persistence *in vivo*. Since *B. thuringiensis* has been regarded as a safe organism for a long time, a recent report on spore persistence in the lungs of mice was alarming (14). The present study suggests that SASP gene deletion can make a safer *B. thuringiensis* simulant because the *sspA sspB* mutant quickly disappeared from mouse lungs (Fig. 4). Reduced resistance to heat alone cannot account for the rapid removal from the lungs, because even a 10-week-long incubation in a 37°C incubator (i.e., body temperature) did not decrease the number of BT-005 spores as much as was observed after 1 week in the infected mouse lungs (see Fig. S4 in the supplemental material). Host phagocytes seem to play a role in clearing spores of the *sspA sspB* mutant in the mouse lung. Phagocytes are well known to use reactive oxygen species, including hydrogen peroxide, to kill invading microorganisms (22). Also, *sspA sspB* double deletion has been shown to greatly reduce resistance against

hydrogen peroxide (23). This is the first report on *in vivo* application of SASP gene deletions. This finding can be useful not only for simulant applications but also for bioinsecticide applications for which genetically modified strains are being developed for outdoor use.

By constructing BT-016, we showed that all of the properties studied here could be combined into one strain. Since the gene deletions and insertions did not cross-affect one another, one can choose any of these properties based on the needs in strain construction. For example, the *sspA sspB* knockout strain is not suitable when live cells are needed, such as when air samples are tested on agar plates in culture-based detectors. In that case, all of the properties except *sspA sspB* deletions could be combined into the strain. Since the sporulation-dependent *spo0A* knockout system does not affect the resistance of spores to artificial sunlight (Fig. 3), the strain can be used for an outdoor air sampling analysis with enhanced environmental safety. When a yellow colony color is not needed, other properties can be combined in the wild-type BMB171 strain rather than in the yellow-colored BT-001 strain.

In BT-016, *plcR* was deleted for additional safety because it is a pleiotropic regulator of many virulence genes; *plcR* deletion reduced the virulence of the *B. thuringiensis* 407 Cry$^-$ strain (13). Importantly, *plcR* controls the expression of many enterotoxins and hemolysins (24). In our study, the reduced virulence of the *plcR* mutant could not be measured because the wild-type BMB171 strain did not show any toxicity, even when spores at a high concentration (5.0×10^8 CFU per mouse) were given to BALB/c mice via the intranasal route. The virulence of *B. thuringiensis* (13) seems to be dependent on the strain. Regardless of the actual toxicity of a strain, we suggest that the *plcR* gene should be deleted for simulant application, as its involvement in virulence is well established.

Finally, as all of the strains constructed in this study contain no antibiotic resistance markers on their chromosomes, undesirable environmental contamination with antibiotic resistance genes will not be an issue when the strains are released. In addition, no expensive antibiotics are needed to maintain the plasmids during cultivation because all of the introduced genes and expression cassette are on the chromosome, rather than on plasmids. Furthermore, the genes and genetic circuits will not be lost after environmental release. All these features of the engineered *B. thuringiensis* strains developed in this work will be useful for studying the *Bacillus*-based simulants, including *B. thuringiensis*, for various purposes, including as *B. anthracis* surrogates in outdoor environments.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. The strains and plasmids used in this study are listed in Table 2. *Escherichia coli* DH10β was used for routine cloning and plasmid construction. *E. coli* JM110 was used to prepare unmethylated plasmids, which are necessary for efficient transformation into *B. thuringiensis* (26). The *B. thuringiensis* BMB171 strain lacking *cry* genes (no Cry protein production) was used throughout this study. This strain can be transformed at relatively high efficiency and, thus, allows easy genetic manipulation (27). For routine cultivation of *E. coli*, Luria-Bertani (LB) medium was used with appropriate antibiotics when necessary. For *B. thuringiensis*, LB medium and tryptic soy agar (TSA) were used, with appropriate antibiotics when necessary. Antibiotics were used at the following concentrations: 100 μg ml^{-1} ampicillin and 100 μg ml^{-1} spectinomycin for *E. coli* and 200 μg ml^{-1} spectinomycin and 20 μg ml^{-1} tetracycline for *B. thuringiensis*. For preparation of *B. thuringiensis* spores, GYS medium (28) was used. For measurement of *spo0A* knockout efficiency, Difco sporulation medium (DSM) (29) with 1.5% (wt/vol) agar was used.

Animals. Six-week-old female BALB/c mice were purchased from Orientbio (Sungnam, Republic of Korea). Animal studies were approved and animals maintained according to the regulations of the Institutional Animal Care and Use Committee at the Agency for Defense Development (ADD).

Genome information. Throughout this study, the publicly available full genome sequence of *B. thuringiensis* BMB171 (27) was used (NCBI reference sequences NC_014171.1 [chromosome] and NC_014172.1 [plasmid pBMB171]). Since most genes of *B. thuringiensis* BMB171 in this study were not characterized experimentally, locus tag names were used in this paper instead of gene names when gene names were not available.

Bacterial strain deposition. Strains BT-001 and BT-008 were deposited in the Korean Collection for Type Cultures (KCTC; Jeongeup, Republic of Korea). The deposition numbers for BT-001 and BT-008 are KCTC 18453P and KCTC 18454P, respectively.

TABLE 2 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
E. coli strains		
DH10β	Host for cloning and vector construction	Laboratory stock
JM110	*dam dcm* mutant, host for preparation of the unmethylated plasmid	Laboratory stock
S. aureus KCTC 3881	Source of *crtM-crtN* genes	KCTC[a]
B. thuringiensis strains[b]		
BMB171	Parental strain for all strains constructed in this study	BGSC[c]
YBT-020	Source of the *slpA* promoter	BGSC
BT-001	BMB171 BMB171_C4312::*crtM-crtN*	This study
BT-003	BMB171 Δ*sspA*	This study
BT-004	BMB171 Δ*sspB*	This study
BT-005	BMB171 Δ*sspA* Δ*sspB*	This study
BT-006	BMB171 *spo0A*::*loxP*[d]	This study
BT-007	BMB171 Δ*spo0A*	This study
BT-008	BMB171 *spo0A*::*loxP* ΔBMB171_C4815::P_{C0536}-*cre*[e]	This study
BT-009	BMB171 *spo0A*::*loxP* ΔBMB171_C1559::P_{C0536}-*cre*	This study
BT-010	BMB171 *spo0A*::*loxP* ΔBMB171_C4815::P_{C4286}-*cre*[f]	This study
BT-011	BMB171 *spo0A*::*loxP* ΔBMB171_C1559::P_{C4286}-*cre*	This study
BT-016	BMB171 BMB171_C4312::*crtM-crtN spo0A*::*loxP* ΔBMB171_C4815::P_{C0536}-*cre* Δ*plcR* Δ*sspA* Δ*sspB*	This study
Plasmids		
pUC19	Backbone vector for pAD01	NEB[g]
pDG1662	Source of spectinomycin marker	BGSC
pUCTV2	Source of tetracycline marker and Ts[h] replicon	25
pJW168	Source of Cre recombinase gene	Lucigen[i]
pAD01	Amp^r is replaced by Spc^r; I-SceI recognition site was introduced	This study
pAD02	I-SceI expression vector with *slpA* promoter; Ts replicon	This study
pAD03	FLP expression vector with *slpA* promoter; Ts replicon	This study
pAD04	*plcR* knockout vector	This study
pAD05	*sspA* knockout vector	This study
pAD06	Himar1 transposon delivery vector with *crtM-crtN*; Ts replicon	This study
pAD07	*sspB* knockout vector	This study
pAD08	*loxP* insertion vector at the *spo0A* locus	This study
pAD09	*spo0A* knockout vector	This study
pAD10	Cre recombinase expression cassette (driven by the BMB171_C0536 promoter) insertion vector at the BMB171_C4815 locus	This study
pAD11	Cre recombinase expression cassette (driven by the BMB171_C0536 promoter) insertion vector at the BMB171_C1559 locus	This study
pAD12	Cre recombinase expression cassette (driven by the BMB171_C4286 promoter) insertion vector at the BMB171_C4815 locus	This study
pAD13	Cre recombinase expression cassette (driven by the BMB171_C4286 promoter) insertion vector at the BMB171_C1559 locus	This study

[a]KCTC, Korean Collection for Type Cultures (Jeongeup, Republic of Korea).
[b]Locus tag names were used instead of gene names when gene names were not available (e.g., for hypothetical proteins) or three-letter symbols were not available.
[c]BGSC, Bacillus Genetic Stock Center (Columbus, OH, USA).
[d]Two *loxP* sites were inserted at both ends of the *spo0A* gene.
[e]Cre recombinase expression cassette driven by the BMB171_C0536 promoter.
[f]Cre recombinase expression cassette driven by the BMB171_C4286 promoter.
[g]New England Biolabs (Ipswich, MA, USA).
[h]Ts, temperature sensitive.
[i]Lucigen Corporation (Middleton, WI, USA).

Plasmid construction. All plasmids used in this study are shown in Table 2**.** All primers used for their construction are presented in Table 3. The use of antibiotic resistance markers in this study was approved by Korean Centers for Disease Control and Prevention following regulations of the Republic of Korea (approval number 15-RDM-019).

Plasmid pAD01 is the backbone plasmid for all gene insertion (replacement) vectors in this study. It was constructed by replacing the ampicillin marker in pUC19 with a spectinomycin marker that was PCR amplified from pDG1662 using primers AD01-1 and AD01-2. An I-SceI recognition site was also inserted using the primer AD01-2, which enabled I-SceI-based gene replacement.

To construct plasmid pAD02 for I-SceI meganuclease expression (see Fig. S5A in the supplemental material), a temperature-sensitive replicon and a tetracycline marker were PCR amplified from pUCTV2 using primers TS-TET-F and TS-TET-R and were inserted into the HindIII site of pUC19. The *slpA* promoter, which was PCR amplified from *B. thuringiensis* YBT-020 genomic DNA with primers AD02-1 and AD02-2, along with the I-SceI open reading frame (ORF), was inserted into SacI and PstI sites of the pUC19. The

TABLE 3 Primers used in this study

Primer	Sequence (5′–3′)[a]	Characteristics
AD01-1	GGGGGGGACGTCGGCGCGCTAGAG (AatII)	Spc^r marker cloning
AD01-2	GGGTAGGGATAACAGGGTAATCATGCAAATGTCACTAA (I-SceI)	Spc^r marker cloning
AD02-1	GGGGGGGAGCTCTGTCGATTAATGTCGTAATATC (SacI)	YBT-020 *slpA* promoter cloning
AD02-2	GGGGGGTCTAGAAATGTAATACTAGTTCCATACTG (XbaI)	YBT-020 *slpA* promoter cloning
AD02-3	GGGGGGTCTAGATAAAAGAGATGGAGGTAACTT**TTG**CATCAAAAAAACCAGGTAATG (XbaI)	I-SceI ORF cloning
AD02-4	GGGGGGCTGCAGTTATTTCAGGAAAGTTTCGGAGG (PstI)	I-SceI ORF cloning
AD03-1	GGGGGGTCTAGATAAAAGAGATGGAGGTAACTT**ATG**CCACAATTTGATATATTATGTAAA (XbaI)	FLP ORF cloning
AD03-2	GGGGGGCTGCAGTTATATGCGTCTATTTATGTAGGATG (PstI)	FLP ORF cloning
AD04-1	GGGGGGGAGCTCCCATAATCCAGTTTCCTTTATC (SacI)	Left arm for pAD04
AD04-2	GGGGGGGGTACCCATACTTACTCACCATCCCA (KpnI)	Left arm for pAD04
AD04-3	GGGGGGGGTACCTAAATATCCTAAAAAATGGGTATG (KpnI)	Right arm for pAD04
AD04-4	GGGGGGTCTAGATTACCAAATCGTCGAAAGTTC (XbaI)	Right arm for pAD04
AD05-1	GGGGGGGAATTCACACGGTGTATATGTGTCAAC (EcoRI)	Left arm for pAD05
AD05-2	GGGGGGGGTACCCATGTTATATCACCTCCTTGT (KpnI)	Left arm for pAD05
AD05-3	GGGGGGGGTACCTAATCCCATATGATGGCTTAG (KpnI)	Right arm for pAD05
AD05-4	GGGGGGGGATCCTACAAGTGCATCCTCTACTTC (BamHI)	Right arm for pAD05
AD06-1	AACTTTATATGAACATAATCAACG (5′ phosphorylated)	pAD06 construction
AD06-2	TCACAATTCCACACAACATAC	pAD06 construction
AD06-3	GGGGGGGAATTCTAAAAGAGATGGAGGTAACTT**ATG**ACAATGATGGATATGAATTT (EcoRI)	*crtM-crtN* cloning
AD06-4	GGGGGGGTCGACTTATACGCCCCGCTCAATATC (SalI)	*crtM-crtN* cloning
AD07-1	GGGGGGGAATTCGTAGAACGGTTTCTTGGCAC (EcoRI)	Left arm for pAD07
AD07-2	GGGGGGGGTACCCATAGGAATATATCCTCCTTAG (KpnI)	Left arm for pAD07
AD07-3	GGGGGGGGTACCTAAATATATATGGCAAAAAGGTC (KpnI)	Right arm for pAD07
AD07-4	GGGGGGGGATCCAGTTTCAGATCGATTGTCTAC (BamHI)	Right arm for pAD07
AD08-1	GGGGGGGAGCTCGACAGCAGAGGCTATCCAG (SacI)	Left arm for pAD08
AD08-2	GGGGGGGGTACCATTTCAACGAATTTTTCAACAGA (KpnI)	Left arm for pAD08
AD08-3	GGGGGGGGTACCGGGGGGGGATCCAGAAAAAGAGATTATTTGATATCA (KpnI, BamHI)	Right arm for pAD08
AD08-4	GCACCGTTTCATACTGTAGTG	Right arm for pAD08
AD08-5	GGGGGGGGTACC*ATAACTTCGTATAGCATACATTATACGAAGTTAT*TTTGTAAAAGCTCCGCAAGAG (KpnI, *loxP*)	*spo0A* cloning with two *loxP*s
AD08-6	GGGGGGGGATCC*ATAACTTCGTATAATGTATGCTATACGAAGTTAT*CAACAAAATAAAAAGACC AACG (BamHI, *loxP*)	*spo0A* cloning with two *loxP*s
AD09-1	GGGGGGGAGCTCTAATAGGTCTTTGTCTCCTTG (SacI)	Left arm for pAD09
AD09-2	GGGGGGGGTACCCATGTGTATCCTTCTAGGCT (KpnI)	Left arm for pAD09
AD09-3	GGGGGGGGTACCTGAGAGTAGAAGTAGCAATGG (KpnI)	Right arm for pAD09
AD09-4	GGGGGGTCTAGACTTGCATCTAACTTGCATAAG (XbaI)	Right arm for pAD09
AD10-1	GGGGGGGACGTCAGGCATGGCTGGGCATTC (AatII)	Left arm for pAD10 and pAD12
AD10-2	GGGGGGGAGCTCGAAACATACTCCTTCTCCGA (SacI)	Left arm for pAD10 and pAD12
AD10-3	GGGGGGGAGCTCGGGGGGAGATCTGATGAAATAAGCTATCCGATTT (SacI, BglII)	Right arm for pAD10 and pAD12
AD10-4	GGGGGGTCTAGACCTTCTGGAATCATATCAGTG (XbaI)	Right arm for pAD10 and pAD12
AD10-5	GGGGGGGAGCTCAATTTGAATATTATGGAAAAAGAC (SacI)	BMB171_C0536 promoter cloning
AD10-6	GGGGGGGGTACC**CAT**ACTACTATCCCTCCTTTTG (KpnI)	BMB171_C0536 promoter cloning
AD11-1	GGGGGGGACGTCGAGTTAGTGAAGGGTGGTC (AatII)	Left arm for pAD11 and pAD13
AD11-2	GGGGGGGAGCTCGGGGGGAGATCTGTAAACAGCCTCCTTTAACAT (SacI, BglII)	Left arm for pAD11 and pAD13
AD11-3	GGGGGGGAGCTCATAAGTAAGGAAGCAGCTTTAG (SacI)	Right arm for pAD11 and pAD13
AD11-4	GGGGGGTCTAGATTTCTCGTGAGAAAGATATAAC (XbaI)	Right arm for pAD11 and pAD13
AD12-1	GGGGGGGAGCTCGAAACAATGGTATTACAAACG (SacI)	BMB171_C4286 promoter cloning
AD12-2	GGGGGGGGTACC**CAT**GTTATATCACCTCCTTGT (KpnI)	BMB171_C4286 promoter cloning
TS-TET-F	GGGGGGAAGCTTCAGAACGGATTGTTGATG (HindIII)	Tet^r marker-Ts replicon cloning
TS-TET-R	GGGGGGAAGCTTTCGAGCGGAGGCCTG (HindIII)	Tet^r marker-Ts replicon cloning
CRE-F	GGGGGGGGTACCATGTCCAATTTACTGACCGTA (KpnI)	Cre recombinase ORF cloning
CRE-R	GGGGGGAGATCTCTAATCGCCATCTTCCAGC (BglII)	Cre recombinase ORF cloning
C4312-F	TGGGATTAACACATGTGGAACGAG	Confirmation of *crtM-crtN* insertion
C4312-R	AGCAGCTGCAATAATTAAGAAATC	Confirmation of *crtM-crtN* insertion

[a]Restriction sites are underlined, *loxP* sites are in italics, and start codons are in boldface. ORF, open reading frame.

I-SceI ORF, which was prepared by gene synthesis (Bioneer, Daejeon, Republic of Korea), was PCR amplified using primers AD02-3 and AD02-4, and the start codon was changed from ATG to TTG to optimize its expression. For the construction of plasmid pAD03 (see Fig. S5B), the same procedure was followed except that the FLP recombinase ORF was used instead of the I-SceI ORF. The FLP recombinase ORF was PCR amplified with primers AD03-1 and AD03-2. The FLP recombinase gene was kindly provided by Chankyu Park at Korea Advanced Institute of Science and Technology (Daejeon, Republic of Korea). Plasmid pAD03 was used to express FLP recombinase for spectinomycin marker removal in the transposon experiment.

For the construction of gene knockout plasmids (pAD04, pAD05, pAD07, and pAD09) (see Fig. S6A in the supplemental material), the left and right homology arms were PCR amplified from *B. thuringiensis*

BMB171 genomic DNA using primers listed in Table 3. Using the restriction sites shown in Table 3, each arm was inserted into pAD01.

To construct the transposon delivery plasmid pAD06 (see Fig. S6B in the supplemental material), the *Himar1* transposase gene (NCBI reference sequence EU146228, nt 1 to 1300) was prepared by gene synthesis (Bioneer). To facilitate plasmid construction, two inverted repeats and two flippase recombination target (FRT) sites were synthesized as well. A temperature-sensitive replicon and a tetracycline marker were PCR amplified from pUCTV2 using primers TS-TET-F and TS-TET-R and ligated to the synthesized DNA. Then, a promoterless spectinomycin marker and a pUC19 replicon were PCR amplified from pAD01 using primers AD06-1 and AD06-2 and ligated with the synthesized DNA. Finally, the *crtM-crtN* genes were PCR amplified from *Staphylococcus aureus* KCTC 3881 genomic DNA using primers AD06-3 and AD06-4 and then inserted into the transposon delivery plasmid. Plasmid pAD06 had a design similar to pAW068 (30).

The *loxP* insertion plasmid pAD08 (see Fig. S7A in the supplemental material) was constructed by first cloning two homology arms flanking the *spo0A* gene into pAD01 with primers AD08-1, AD08-2, AD08-3, and AD08-4. Then, in the middle of the two arms, the *spo0A* gene, prepared using primers AD08-5 and AD08-6, was cloned with the *loxP* site inserted at each end.

Plasmid pAD10 (see Fig. S7B in the supplemental material) for insertion of the Cre recombinase expression cassette was constructed by first cloning two homology arms flanking the BMB171_C4815 gene into pAD01 with primers AD10-1, AD10-2, AD10-3, and AD10-4. Then, the expression cassette, consisting of the BMB171_C0536 (annotated as *sspH*) promoter and Cre recombinase ORF, was inserted into the middle of the two arms. The BMB171_C0536 promoter was PCR amplified from *B. thuringiensis* BMB171 genomic DNA using primers AD10-5 and AD10-6. The Cre recombinase ORF was PCR amplified from pJW168 with primers CRE-F and CRE-R. Plasmid pAD12 was the same as pAD10 except that the BMB171_C4286 (identified as *sspA* in this study) promoter was used instead of the BMB171_C0536 promoter. The BMB171_C0536 promoter in pAD10 was replaced by restriction and ligation at the SacI and KpnI sites to construct pAD12. The BMB171_C4286 promoter was PCR amplified using primers AD12-1 and AD12-2. Plasmid pAD11 was constructed following a method similar to that for pAD10. Two homology arms flanking the BMB171_C1559 gene were cloned into pAD01 with primers AD11-1, AD11-2, AD11-3, and AD11-4. The expression cassette, consisting of the BMB171_C0536 (*sspH*) promoter and Cre recombinase ORF, was inserted into the middle of the two arms using the same primers used for pAD10. The transcriptional orientation of the expression cassette was, however, in the opposite direction of pAD10, because the order of the restriction sites used for expression cassette cloning was reversed (SacI-BglII in pAD10 and BglII-SacI in pAD11). As in pAD12 construction, the BMB171_C0536 promoter was replaced by the BMB171_C4286 promoter to make plasmid pAD13. Three additional amino acids (GTM from the nucleotide sequence GGTACCATG) were added at the N-terminal end of the Cre recombinase for all Cre recombinase cassette insertion plasmids (pAD10, pAD11, pAD12, and pAD13), because the cloned promoters contained their own start codons, as well as KpnI sites.

Electroporation. Transformation of *B. thuringiensis* by electroporation was performed according to the protocol described previously (31). Cells were grown to an optical density at 600 nm (OD_{600}) of 0.18 to 0.20 at 30°C with shaking at 200 rpm in 100 ml of LB broth. In the case of the yellow-colored strain, cells were grown to an OD_{600} of 0.24 to 0.26. Three percent glycine (final concentration) was added, and cells were grown for an additional 1 h. Then, the flask containing cells was held on ice for 5 min. Cells were harvested by centrifugation at 7,000 $\times$ *g* for 5 min at 4°C. Supernatants were removed, and cells were resuspended in 1 ml ice-cold electroporation buffer (272 mM sucrose, 0.5 mM $MgCl_2$, 0.5 mM K_2HPO_4, and 0.5 mM KH_2PO_4, pH 7.2). After washing three times with the ice-cold electroporation buffer, cells were resuspended in 0.3 ml of the electroporation buffer. After incubation with a target plasmid (typically 1 to 3 μg) on ice in a 0.2-cm electroporation cuvette (Bio-Rad Laboratories, Hercules, CA, USA) for 10 min, electroporation was performed using the GenePulser Xcell electroporation system (Bio-Rad Laboratories) under the following conditions: 10 kV cm^{-1}, 200 Ω, and 25 μF. The cuvette was immediately placed on ice. After 10 min, 700 μl of LB broth was added, and cells were transferred to a new tube for a 2-h incubation at 30°C with shaking at 200 rpm. Finally, cells were harvested and spread on LB agar plates containing appropriate antibiotics for an overnight incubation at 30°C.

Procedures for mutant strain construction. For every knockout and insertion (replacement) experiment, the lengths of the left and right homology arms were each about 1.5 kb (about 3 kb in total). Cells were transformed with the gene knockout or gene insertion plasmid and grown on LB agar plates containing spectinomycin at 30°C. After 24 h, the transformants were confirmed by colony PCR using appropriate primers. One correctly identified colony was chosen, transformed with the I-SceI expression plasmid pAD02, and then grown on LB agar plates containing tetracycline at 30°C. After an overnight incubation, 10 transformants were pooled in 1 ml of LB broth containing tetracycline, and 5 μl of this mixture was seeded into 5 ml of LB broth containing tetracycline. This broth was grown for 5 to 6 h with shaking (200 rpm) at 37°C. The culture was appropriately diluted and spread on 20 LB agar plates containing tetracycline. After overnight incubation, colonies on the plates were replica plated onto LB agar plates containing spectinomycin using a replica plater and velveteen squares (SP Scienceware, Wayne, NJ, USA). Colonies that lost the spectinomycin marker were chosen, and colony PCR was performed to search for the desired mutant. A confirmed mutant colony was grown in 5 ml of LB broth containing no antibiotics with shaking at 40°C for 5 to 6 h to remove pAD02. The culture was appropriately diluted and spread on LB agar plates containing no antibiotics. After overnight incubation at 30°C, 10 colonies were chosen and grown on LB agar plates containing tetracycline to confirm curing of pAD02. A colony that lost pAD02 was chosen, and a glycerol stock was prepared.

Construction of the yellow-colored strain. The BMB171 strain was transformed with pAD06 and grown on LB agar plates containing tetracycline. After an overnight incubation at 30°C, 10 transformants were picked and pooled in 1 ml of LB broth. The bacterial suspension was diluted 1:1,000 into 5 ml of LB broth containing tetracycline and grown overnight with shaking (200 rpm) at 30°C. Then, the culture was diluted 1:100 into 5 ml of LB broth containing spectinomycin and grown overnight with shaking (200 rpm) at 30°C. The next day, the culture was appropriately diluted and spread onto 20 TSA plates containing spectinomycin. After overnight incubation at 30°C, yellow colonies were chosen, and after several subcultures, a colony with the strongest yellow color was selected. This strain had a spectinomycin marker with two FRT sequences at both ends on its chromosome. This marker was removed by transforming the FLP recombinase expression plasmid pAD03. After removing pAD03 by culturing at a high temperature (40°C), the final candidate strain, named BT-001, was obtained. Sporulation was tested to see whether the inserted genes affected the sporulation process.

Identification of the insertion site in the yellow-colored strain. Genomic DNA of the BT-001 strain was prepared at a concentration of 200 ng μl^{-1} and was transferred to a microTUBE with an Adaptive Focused Acoustics (AFA) fiber snap-cap (Covaris, Woburn, MA, USA). After shearing DNA using a Covaris M220 instrument (Covaris), a genomic DNA library with 500-bp insert size was constructed using an Illumina TruSeq nano DNA sample preparation kit (Illumina, San Diego, CA, USA), following the manufacturer's protocols. The quality of the library was checked on a DNA 1000 chip with a 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and library quantification was performed with KAPA library quantification kits for Illumina sequencing platforms (KAPA Biosystems, Wilmington, MA, USA) using a 7500 real-time PCR system (Life Technologies, Foster City, CA, USA). The library was normalized to a working concentration of 2 nM using the molarity calculated from the quantitative PCR results. Finally, the library was diluted with HT buffer (in a MiSeq reagent kit, version 2) and PhiX control DNA to a 15 pM concentration to obtain a cluster density of 800 to 1,200 and a passing filter of 70 to 100%. The MiSeq reagent kit, version 2, with 500 cycles was used for sequencing and run with paired-end 251-bp reads on a MiSeq instrument (Illumina). *De novo* assembly was performed on a Linux server with an Intel Xeon processor (16 cores, 2.6 GHz central processing unit, and 256 GB memory). CLC Genomics Workbench and Genomics Server (version 6.5.1; CLC bio, Aarhus, Denmark) were used to carry out *de novo* assembly with the options word size 20, bubble size 30, and minimum contig length 200. The *crtM-crtN* ORF sequences of *S. aureus* (NCTC 8325) were compared with the contigs obtained by BLAST. After the contig (contig_87) containing the *crtM-crtN* genes was identified, it was compared with the *B. thuringiensis* BMB171 sequences using the bl2seq program (32) to investigate whether the contig originated from the chromosome (NCBI reference sequence NC_014171.1) or plasmid pBMB171 (NCBI reference sequence NC_014172.1). Finally, the contig was analyzed in detail to determine the precise insertion site of the *crtM-crtN* genes.

Coculture with natural environmental microorganisms. For isolating soil microorganisms, 10 g of soil was sampled from GapHa Mountain (Daejeon, Republic of Korea) and suspended in 20 ml sterile deionized water. The suspension was filtered twice through Whatman filter paper (qualitative grade 2; GE Healthcare Life Sciences, Pittsburgh, PA, USA) to remove particulate matter. After centrifugation at 1,000 $\times$ *g* for 10 min at 4°C, the pellets were resuspended in 1.5 ml sterile deionized water. The suspension was heat treated (at 70°C for 30 min) to kill vegetative cells. Then, it was mixed with spores of the BT-001 strain and was appropriately diluted in phosphate-buffered saline (PBS) solution and spread on TSA plates. After overnight incubation at 30°C, colonies were observed and photographed. For airborne microorganisms, 4,000 liters of outdoor air (GapHa mountain) was sampled on a TSA plate with a MAS-100 NT air sampler (Merck Millipore, Darmstadt, Germany) for 40 min. Then, spores of the BT-001 strain were appropriately diluted in PBS solution and spread on this plate. After an overnight incubation at 30°C, colonies were observed and photographed. The identity of the BT-001 strain was confirmed by colony PCR using primers C4312-F and C4312-R. The primers were located on both sides of the insertion site of the *crtM-crtN* genes on the chromosome of the BT-001 strain.

Spore preparation. Cells were grown on LB agar plates at 30°C overnight. A single colony was chosen and inoculated into 5 ml of LB broth in a 50-ml conical tube. Cells were grown at 30°C with shaking at 200 rpm for 8 h. Then, 250 μl of this culture was inoculated into 25 ml of GYS medium and grown at 30°C with shaking (200 rpm) for 48 h. For spore purification, the culture was harvested by centrifugation at 5,400 $\times$ *g* for 5 min at 4°C. After decanting the supernatant, the pellets were washed with 25 ml PBS solution with vigorous shaking. After centrifugation at 5,400 $\times$ *g* for 5 min at 4°C, the pellet was washed again. After suspension in 25 ml PBS solution, the spores were heat treated at 65°C for 30 min to kill vegetative cells, followed by two additional washes with PBS solution. Finally, the spores were suspended in PBS solution and stored at 4°C until use. For UV sensitivity, solar simulator, and animal experiments, spores were produced in 100 ml of GYS medium with a 48-h incubation at 30°C. Spores were washed four times with PBS solution without heat treatment because spores of the *sspA sspB* knockout strain were highly sensitive to heat.

Construction of the *sspA sspB* knockout strain. The *B. thuringiensis* BMB171 genome has seven α/β-type SASP genes, six on the chromosome and one on the plasmid (see Fig. S2 in the supplemental material). To identify the *sspA* and *sspB* genes, synteny analysis was performed using the Archaeal and Bacterial Synteny Explorer (Absynte) webserver (http://archaea.u-psud.fr/absynte [33]). Chromosomes of *B. subtilis* 168 and *B. thuringiensis* BMB171 were selected along with a pBMB171 plasmid of *B. thuringiensis* BMB171, and the minimal score threshold was set at 20% of the normalized BLAST score. For the synteny analysis of *sspA*, the amino acid sequence of SspA of *B. subtilis* 168 (NCBI reference sequence NP_390835.1) was used as a query sequence. For *sspB*, the amino acid sequence of SspB (NCBI reference sequence NP_388856.1) was used. After the BMB171_C4286 and BMB171_C0753 genes were selected,

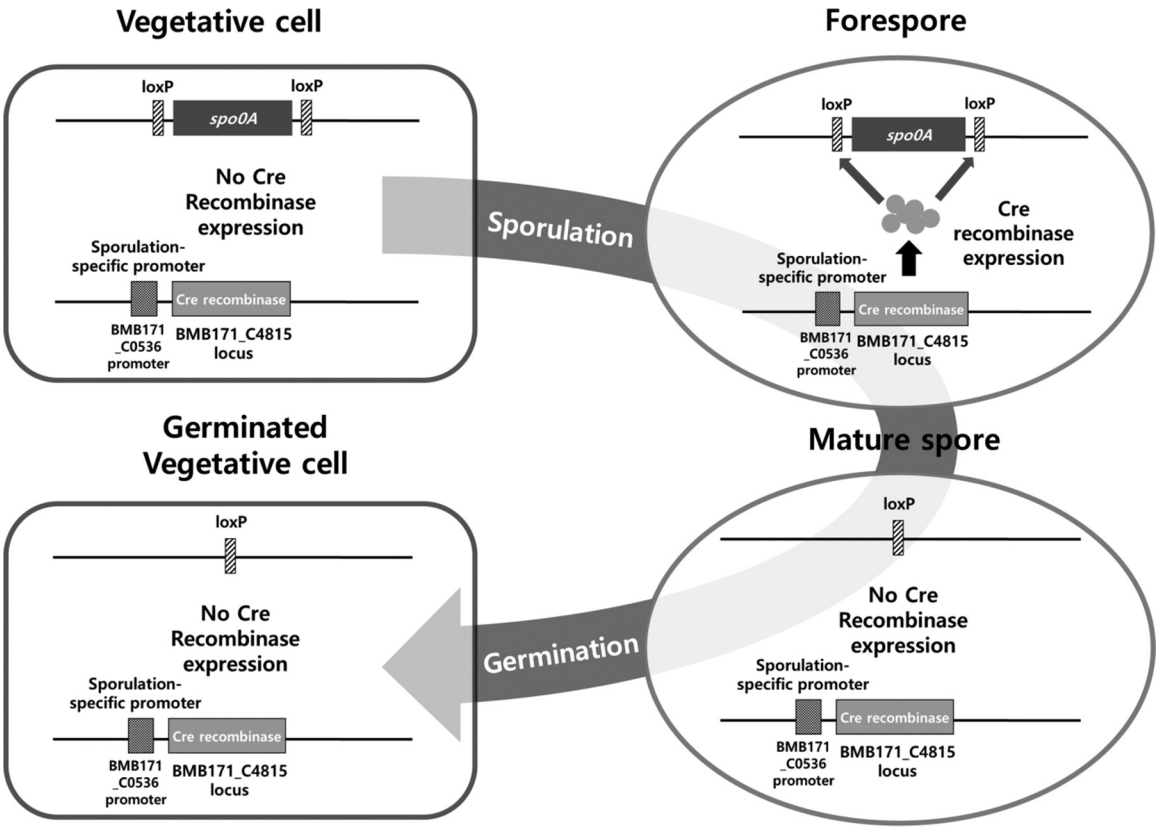

FIG 5 Schematic of sporulation-dependent *spo0A* knockout. A sporulation-specific promoter drives Cre recombinase expression during sporulation, and the Cre recombinase deletes the *spo0A* gene, which is flanked by two *loxP* recognition sites. As *spo0A* is required only for the initial stages of sporulation, the subsequent sporulation process is not affected. Once spores are formed, there is no *spo0A* gene to drive sporulation again.

two gene deletion plasmids, pAD05 for the *sspA* knockout and pAD07 for the *sspB* knockout, were constructed. These plasmids were used to knock out the *sspA* gene (BT-003), the *sspB* gene (BT-004), and both the *sspA* and *sspB* (BT-005) genes for the comparison of the UV-C sensitivities of the respective knockout strains.

Construction of the sporulation-dependent *spo0A* knockout strain. The overall scheme of the sporulation-dependent *spo0A* knockout strain is shown in Fig. 5. To make the sporulation-dependent *spo0A* knockout strain, it was necessary to insert two *loxP* sites at the upstream and downstream regions of the *spo0A* gene. However, *spo0A* transcription in *B. thuringiensis* has not been studied experimentally, and improper selection of the *loxP* insertion sites could affect *spo0A* transcription in an undesirable way. The BMB171_C3832 gene was annotated as the *spo0A* gene of *B. thuringiensis* BMB171 in the reported full genome sequence. The upstream and downstream genes annotated are BMB171_C3833 (annotated as stage IV sporulation protein B) and BMB171_C3831 (annotated as a hypothetical protein), respectively. We performed transcription terminator prediction on BMB171_C3832 (*spo0A*) and BMB171_C3833 using the ARNold webserver (http://rna.igmors.u-psud.fr/toolbox/arnold [34]). The upstream *loxP* insertion site was selected to be 26 bp downstream from the predicted BMB171_C3833 transcription terminator. The downstream *loxP* insertion site was selected to be 14 bp downstream from the predicted *spo0A* transcription terminator (see Fig. S8 in the supplemental material). By this design, it was expected that the two *loxP* insertions would not significantly interfere with the *spo0A* transcription process. The two *loxP* sites were inserted at both ends of the *spo0A* locus using pAD08. The resulting strain was named BT-006. All of the following strains were constructed from the BT-006 strain.

To drive Cre recombinase in a sporulation-dependent manner, promoters with the following characteristics were necessary. First, transcription should begin after sporulation initiation. Because the *spo0A* gene is necessary for the initial stages of sporulation, in addition to other roles, it should be deleted only after its mission is completed. Second, Cre expression should be minimal when not in the sporulation phase because otherwise, *spo0A* would be deleted during vegetative growth. Third, the transcription of the *cre* gene should occur in the forespore compartment, rather than in the mother cell, during sporulation. The promoters of SASP genes fall into this category. Among many SASP genes, BMB171_C0536 (annotated as *sspH*) and BMB171_C4286 (identified as *sspA* in this study) were chosen. For the chromosomal integration of the *cre* gene, the following two loci were chosen. First, BMB171_C4815, annotated as a phage infection protein, was chosen as it was expected to be nonessential for the normal physiology of *B. thuringiensis*. In addition, this gene was shown to be inactive,

based on high-throughput cDNA sequencing (RNA-seq) data in a previous study (35). Second, BMB171_C1559 was chosen as it was shown to be very active during vegetative growth while being weakly active during sporulation, based on RNA-seq data (35). This BMB171_C1559 locus was chosen just in case SASP promoters were somewhat active during vegetative growth. The Cre recombinase expression cassette was inserted in the opposite direction of transcription of the BMB171_C1559 locus, and thus, strong transcription of this locus during vegetative growth was expected to suppress Cre recombinase expression because of transcriptional interference (see Fig. S9 in the supplemental material). Overall, four sporulation-dependent *spo0A* knockout strains (two promoters and two loci) were constructed (Table 2). The ORFs of each expression locus (the BMB171_C4815 locus and the BMB171_C1559 locus) were replaced by the Cre recombinase expression cassette during strain construction (see Fig. S9).

Measurement of sporulation-dependent *spo0A* knockout efficiency. The knockout of *spo0A* can be easily checked by growing the strains on a DSM agar plate. When grown on DSM agar plates for 1 day at 30°C, the *spo0A* knockout strain BT-007, which was constructed using pAD09, grew as gray colonies. On the other hand, the wild-type cells grew as white colonies (see Fig. S10 in the supplemental material). Since prolonged incubation made the color contrast more apparent, the plates were left one more day at room temperature for knockout efficiency measurements. The cause of this color difference is the different composition of colonies after the incubation. As DSM is a sporulation medium, colonies of the wild-type strain sporulate on the DSM agar plate, whereas colonies of the *spo0A* knockout strain cannot sporulate on it. Phase-contrast microscopy showed that colonies of the wild type (white colonies) were composed of sporulating cells and vegetative cells, whereas colonies of the *spo0A* knockout strain (gray colonies) were composed of vegetative cells only (see Fig. S11). After growing approximately 4,000 colonies each of the wild-type strain and the *spo0A* knockout strain (BT-007) on DSM plates, it was confirmed that all of the colonies of the wild-type strain grew as white colonies and all of the *spo0A* knockout strain grew as gray colonies, proving that this method was robust to distinguish between wild-type colonies and *spo0A* knockout colonies.

For the measurement of *spo0A* knockout efficiency during vegetative growth (a measurement of leakage), strains were grown on LB agar plates at 30°C for 18 h. A single colony was inoculated into 10 ml of LB broth in a 50-ml conical tube, vortexed briefly, and incubated at 30°C with shaking (200 rpm) until the OD_{600} reached 1.4 to 1.6. One milliliter of the culture was sampled and stored on ice. Then, the culture was serially diluted in 0.9% (wt/vol) NaCl and spread on DSM agar plates. After an overnight incubation at 30°C, the plates were left at room temperature for one additional day. Among approximately 2,500 colonies counted, gray colonies were counted as the *spo0A* knockout strain. When a part of a colony appeared white, it was counted as the *spo0A* wild-type strain.

For the measurement of *spo0A* knockout efficiency during sporulation, spores were prepared in GYS medium, and then 1 ml of the culture was sampled. After heat treatment at 65°C for 30 min to kill vegetative cells, the spores were serially diluted in PBS solution and spread on DSM agar plates. After an overnight incubation at 30°C, the plates were left at room temperature for one additional day. As described above, among approximately 2,500 colonies counted, gray colonies were counted as the *spo0A* knockout strain (see Fig. S12 in the supplemental material). When a part of a colony appeared white, it was counted as the *spo0A* wild-type strain (see Fig. S12).

UV sensitivity test. The UV sensitivity test was performed in a clean bench (KUKJE Scientific Instrument, Goyang, Republic of Korea) equipped with a UV-C lamp installed perpendicular to the working surface. The intensity of UV-C was 78 $\mu W/cm^2$ when measured with an HD2102.2 photoradiometer and LP471 UV-C probe (Delta OHM, Padua, Italy). Spores were appropriately diluted in PBS solution at a concentration of 200 to 220 CFU per plate and spread on TSA plates. After spreading spores on three plates, the open plates were immediately placed on the clean bench and irradiated for a given time (0 to 100 s in 20-s intervals) using the UV-C lamp. After irradiation, the plates were wrapped in aluminum foil and incubated at 30°C overnight. This procedure was repeated for every measurement. Three plates were irradiated at each time point, and the average number of surviving spores was counted the next day. The number of colonies at 0 s (no exposure) was regarded as 100%, and the percentage of surviving cells at each point was calculated. The test was performed three times independently.

Solar simulator test. To compare the resistance of spores to artificial sunlight, a Suntest CPS plus solar simulator (Atlas Material Testing Solutions, Mount Prospect, IL, USA) was used with a SunCool cooling assembly and a daylight reduced infrared (IR) filter. The black standard temperature and irradiation level were set at 35°C and 500 W/m^2, respectively. During the test, the chamber temperature and relative humidity were approximately 20 to 22°C and 57 to 60%, respectively. Spores (1.2 ml at 2.0×10^8 CFU ml^{-1}) of the strains to be tested, suspended in PBS solution, were dropped into the wells of 6-well plates (Nunc, Roskilde, Denmark), and the plates were covered with a 2-mm-thick quartz plate. After irradiation for a given time (0 to 50 min in 10-min intervals), 1 ml of the suspension was drawn and serially diluted in PBS solution to count cells on TSA plates. After an overnight incubation at 30°C, the number of colonies was counted and calculated.

Construction of the BT-016 strain. The BT-016 strain was constructed from the BT-001 strain. First, a *plcR* knockout was performed on the BT-001 strain using pAD04. After the deletion, the absence of hemolysis was checked by incubation on sheep blood agar (Hanil Komed, Sungnam, Republic of Korea). Then, two *loxP* sites were inserted at both ends of the *spo0A* gene using pAD08, and the Cre recombinase expression cassette was inserted using pAD10. Finally, the *sspA* and *sspB* genes were deleted sequentially using pAD05 and pAD07. The final strain, BT-016, was checked for UV-C sensitivity, resistance to artificial sunlight, *spo0A* knockout efficiency, and bacterial clearance in the lungs of mice.

Study on bacterial clearance in the mouse lung. The mice were anesthetized by intraperitoneal injection of 80 mg/kg of body weight ketamine (Yuhan Co Ltd., Seoul, Republic of Korea) and 10 mg/kg

xylazine (Rompun; Bayer Korea, Seoul, Republic of Korea) before instillation. Then, the mice were laid in a supine position on a slant board and hung by the incisor teeth. After making a small incision (1 to 2 cm) in the skin around the neck, the trachea was exposed by separating the surrounding muscles and tissues. Fifty microliters of the spore inoculum (2.0×10^8 CFU ml^{-1} for 1.0×10^7 CFU per mouse) was instilled intratracheally in each mouse using a flexible tube attached to a syringe. Correct insertion of the tube into the trachea was ensured by examining the inserted tube through the exposed trachea. After instillation, the skin was closed with a 3-0 silk suture and sterilized with povidone iodide. For each *B. thuringiensis* strain constructed (BT-005 and BT-016) and the control strain (BMB171), 24 mice were exposed by intratracheal instillation with spores of these strains. At 1, 2, and 4 weeks after instillation, eight mice were euthanized and used for the pulmonary bacterial count. After deep anesthesia, the thoracic cavity was opened, and lungs were collected in sterile PBS solution. The extracted lungs were weighed, homogenized, and serially diluted, and then 100 μl of each homogenate was spread onto TSA plates to count the total number of bacteria (including spores and vegetative cells). Heat treatment was not done because the spores of the *sspA sspB* mutant strain (BT-005 and BT-016) were highly sensitive to heat. After an overnight incubation at 30°C, the number of colonies was counted and the number of bacteria (CFU per gram of lung tissue) was calculated. The differences in mean values among different groups were analyzed, and the values were expressed as means ± standard deviations (SD). All statistical calculations were carried out using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). *P* values of <0.05 were considered significant.

Viability check during long-term storage. Spores were produced in GYS medium, washed four times with PBS solution, and then diluted in PBS solution to prepare the spore concentration of 1.0×10^7 CFU ml^{-1}. Spores were stored at 4°C and 37°C in a 25-ml Pyrex 1395 bottle (Corning; Corning, NY, USA) with a parafilm seal. Every week, 1 ml was drawn from the bottle, and cells were counted by the serial dilution method. The experiment was performed in triplicate.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00126-17.

SUPPLEMENTAL FILE 1, PDF file, 1.8 MB.

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The authors declare that the knockout strains and strategies described in this paper are of commercial interest.

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