The *spo0A-sinI-sinR* Regulatory Circuit Plays an Essential Role in Biofilm Formation, Nematicidal Activities, and Plant Protection in *Bacillus cereus* AR156

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The rhizosphere bacterium *Bacillus cereus* AR156 is capable of forming biofilms, killing nematodes, and protecting plants. However, the underlying molecular mechanisms of these processes are not well understood. In this study, we found that the isogenic mutants $\Delta Bcspo0A$ and $\Delta BcsinI$ have significantly reduced colonization and nematicidal activity in vitro and biological control efficacy on the tomato plant under greenhouse conditions. We further investigated the role of the *spo0A-sinI-sinR* regulatory circuit in biofilm formation, killing against nematodes, and biological control in AR156. Results from mutagenesis of those regulatory genes in AR156 and their heterologous expression in *B. subtilis* suggested that the *spo0A-sinI-sinR* genetic circuit is not only essential for biofilm formation and cell differentiation in AR156 but also able to functionally replace their counterparts in *B. subtilis* in a nearly indistinguishable fashion. Genome-wide transcriptional profiling in the wild type and the $\Delta Bcspo0A$ and $\Delta BcsinI$ mutants further revealed hundreds of differentially expressed genes, likely positively regulated by both Spo0A and SinI (via SinR) in AR156. Among them, 29 genes are predicted to be directly controlled by SinR, whose counterpart in *B. subtilis* is a biofilm master repressor. Collectively, our studies demonstrated the essential role of the *spo0A-sinI-sinR* regulatory circuit in biofilm formation, cell differentiation, and bacteria–host interactions in *B. cereus* AR156.

Southern root-knot nematode (*Meloidogyne incognita*), an obligate, sedentary endoparasite, is one of the most economically important plant-parasitic nematodes of horticultural crops (Hunt and Handoo 2009). It can infect the roots of more than 3,000 different horticultural crops and subsequently form giant cells, causing huge yield loss worldwide (Jones et al. 2013; Sikora and Fernandez 2005). Due to the increase of greenhouse-grown crops and multiple cropping (growing two or more crops consecutively or at the same time on the same field in the same year), this nematode results in considerable average annual vegetable yield losses of 20 to 30% in China (Shi et al. 2010). *M. incognita* is difficult to control, in part due to its short generation

time and a high reproductive rate (Manzanilla-López et al. 2004; Trudgill and Blok 2001). For decades, control of *M. incognita* has heavily relied on the use of chemical nematicides, especially the fumigate nematicides. However, use of some chemical nematicides has been restricted or withdrawn because of their potential negative effects on the environment, nontarget organisms, and human health (Giannakou et al. 2002; Huang et al. 2014; Zasada et al. 2010). Therefore, there is an urgent need for finding novel, environmentally friendly, and more effective alternative solutions. Biological control has shown promise as an ecologically friendly approach for controlling *M. incognita*. Several biological control agents (BCA) and their secondary metabolites with killing activities against *M. incognita* have been developed for the control of root-knot nematode diseases (Kerry 2000). To date, more than 700 species of nematophagous fungi have shown potential nematicidal activities, including *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, and *Zygomycota* spp. (Li et al. 2015). Among them, *Myrothecium verrucaria*, *Paecilomyces lilacinus*, *Pochonia chlamydosporium*, and *Trichoderma* spp. have been commercialized for biological control of root-knot nematodes. In addition to nematophagous fungi, bacterial BCA are also important microorganisms able to reduce the root-knot nematode diseases. Based on their mode of action, bacterial BCA against nematodes can be classified into the following groups: obligate parasitic bacteria, opportunistic parasitic bacteria, rhizobacteria, parasporal Cry protein-forming bacteria, endophytic bacteria, and symbiotic bacteria (Tian et al. 2007). Bacteria belonging to the genera *Bacillus*, *Pseudomonas*, and *Pasteuria* represent the dominant populations of nematophagous bacteria in the soil (Ali et al. 2002; Nielsen-LeRoux et al. 2012; Niu et al. 2010; Preston et al. 2003; Tan et al. 1999).

Biofilms are microbial multicellular communities in which individual cells are held together by a self-produced extracellular matrix (O'Toole et al. 2000). It has been widely recognized that most microbes can colonize abiotic and biotic surfaces in the formation of biofilms in natural, clinical, and industrial settings (Davey and O'Toole 2000). In the last decade, the structure and regulation of the bacterial biofilms have been extensively investigated in model microorganisms such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis* (Branda et al. 2005; Lemon et al. 2008). In *B. subtilis*, the biofilm matrix consists of the hydrophobin protein BslA, exopolysaccharides (EPS), and protein fibers TasA, encoded by the *bslA* gene and the *epsA-O* and *tapA-sipW-tasA* operons, respectively (Fig. 1A) (Branda et al. 2001, 2006; Chu et al. 2006; Hobley et al. 2015; Romero et al. 2010). In *B. subtilis*, biofilm formation can be triggered by

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multiple histidine kinases sensing environmental and physiological signals, leading to protein phosphorylation and activation of the master regulator Spo0A, via a phosphor-relay (Grossman 1991; Jiang et al. 2000; McLoon et al. 2011). Spo0A activates biofilm formation by increasing production of SinI, a small protein antagonist of the biofilm master repressor SinR (Bai et al. 1993). SinR directly represses two matrix component operons, *epsA-O* and *tapA-sipW-tasA* (Branda et al. 2006; Chai et al. 2011; Newman et al. 2013). The gene for BslA was shown to be directly or indirectly regulated by the response regulator DegU and the transcription repressors SinR and AbrB (Verhamme et al. 2009). Two additional small antagonists of SinR, SlrA and SlrR, also play a role in the regulation of biofilm formation by interacting with SinR (Chai et al. 2009, 2010). Evidence indicates that SinR and SlrR constitute a self-reinforcing double-negative loop that locks a population of cells in the matrix-producing state (Fig. 1A) (Chai et al. 2010). SlrA has been shown to directly interact with SinR and relieve SinR-mediated repression of matrix genes (Fig. 1A) (Chai et al.

2009; Newman and Lewis 2013). Like *B. subtilis*, various *B. cereus* strains have been reported to form biofilms under either laboratory or environmental conditions (Gao et al. 2015; Ryu and Beuchat 2005; Shemesh and Chai 2013; Wijman et al. 2007). However, in contrast to *B. subtilis*, few genes involved in biofilm formation have been characterized in *B. cereus* and the regulatory mechanisms that control biofilm formation in that bacterium are poorly understood.

Rhizosphere bacteria are frequently found to form micro-colonies or biofilm-like structures on plant roots (Morris and Monier 2003). Studies have demonstrated that biofilm formation by BCA plays an important role in plant protection and disease control. For instance, mucoid mutants of the biocontrol strain *P. fluorescens* CHA0 showed more robust biofilm formation and increased colonization on carrot roots, which correlated with decreased plant infection (Bianciotto et al. 2001). *Paenibacillus polymyxa* was shown to colonize peanut root tips, forming biofilm-like structures and suppressing the crown rot disease caused by *Aspergillus niger* (Haggag and Timmusk 2008;

Fig. 1. Putative regulatory circuit for biofilm formation in *Bacillus cereus* AR156. **A**, Schematic presentation of the regulatory circuit for the control of biofilm formation in *B. subtilis*. Spo0A is at the center of the regulatory circuit, controlling genes involved in biofilm formation. Multiple histidine kinases directly or indirectly activate Spo0A through phosphorylation. SinR and AbrB are two master repressors of the biofilm matrix genes *tapA-sipW-tasA*, *epsA-O*, and *bslA*. SinR is antagonized by two parallel antirepressors (SinI and SlrA). SlrR is another counteracting protein of SinR. These two proteins constitute a self-reinforcing double-negative loop for the control of matrix genes. YwcC is another biofilm repressor and negatively regulates the *slrA* gene. Gene regulation is indicated in red, while protein-protein interaction is indicated in blue. **B**, Arrangement of genes in the genomic *sinI-sinR* region in *B. cereus* AR156 and the comparable region in *B. subtilis* 168. **C**, Amino acid sequence alignment of SinI and SinR orthologs in *B. cereus* AR156, *B. subtilis* 3610, and *B. anthracis* str. Ames. The DNA-binding domain and the domain interacting with SinI are underlined in blue and red, respectively. **D**, Sequence alignment of the two-helical hook regions of SinR and SinI orthologs in both *B. subtilis* and *B. cereus*. Key residues involved in the interaction between BsSinI and BsSinR, as revealed in the cocystal structure, are highlighted in red. Corresponding residues are also indicated in the BcSinI-BcSinR alignment, with the differentiation residues indicated in green. Alignments were created using ClustalW.

Timmusk et al. 2005). *B. subtilis* strain ATCC 6051 was able to form biofilm-like structures on the roots of *Arabidopsis* plants and protect *Arabidopsis* from infections by *Pseudomonas syringae* (Bais et al. 2004). In our previous study (Chen et al. 2012), we demonstrated that *B. subtilis* BCA could sense plant-released small signal molecule L-malic acid via a histidine kinase receptor KinD, and subsequently trigger formation of plant-root-associated biofilms. We also showed that the biofilm-forming ability of various *B. subtilis* BCA correlated with their biocontrol efficacy against tomato Ralstonia wilt disease (Chen et al. 2013). In another study, a *B. subtilis* UMAF6614 strain produced surfactin to trigger biofilm formation on melon phylloplane, which ensured long-term persistence and adequate secretion of suppressive lipopeptides against plant pathogens (Zerriouh et al. 2014). Although increasing evidence has suggested that beneficial biofilms by BCA are important for biocontrol activities against plant-pathogenic fungal and bacterial diseases in agriculture in general, few direct data have been presented to demonstrate the role of biofilms by BCA in the biocontrol against root-knot nematodes.

B. cereus AR156 was originally isolated from rhizosphere soil in Nanjing, China. This bacterium exhibited a strong inhibition on egg-hatching and mortality in second-stage juveniles (J2), and good biocontrol efficacy under greenhouse conditions against *Meloidogyne incognita* (Wei et al. 2010). This BCA was later identified as *B. cereus* by whole-genome sequencing (GenBank access number CP015589). Strain AR156 has been registered as a BCA and applied for control of root-knot nematodes in China. However, the biocontrol mechanism of AR156 against nematodes is not well understood. Results from our recent study indicated that *B. cereus* AR156 is capable of forming robust floating pellicles in biofilm-inducing media (Yan et al. 2016). The molecular mechanism governing biofilm formation in this bacterium is largely unknown. Here, we plan to investigate the underlying molecular mechanism that governs biofilm formation in AR156. We are also interested in knowing whether biofilm formation by this bacterium plays a role in the control of root-knot nematodes by this bacterium. Our specific goals include (i) characterizing morphological features of the AR156 biofilms, (ii) investigating the role of the *BcspoA-BcsinI-BcsinR* regulatory circuit in governing AR156 biofilm formation, and (iii) exploring the link between biofilm formation and the nematocidal activity in AR156.

RESULTS

Analyses of candidate genes involved in biofilm formation in AR156.

In *B. subtilis*, the regulatory network that controls biofilm formation has been well studied (Fig. 1A) (Branda et al. 2005;

Lemon et al. 2008; López et al. 2010; Vlamakis et al. 2013). Using the known biofilm genes in *B. subtilis* as queries, we identified several homologous genes in the AR156 genome encoding highly similar proteins. For example, Spo0A of *B. cereus* AR156 (designated as BcSpo0A) and that of *B. subtilis* (designated as BsSpo0A) share 81% identity in amino acid sequence (Supplementary Fig. S1; designation of other *B. cereus* and *B. subtilis* genes and proteins was applied similarly in this study). In the *B. subtilis* NCIB3610 genome, there is a locus containing both the biofilm regulatory genes *sinI-sinR* and the matrix operon *tapA-sipW-tasA* (Fig. 1B). The corresponding region in the *B. cereus* AR156 genome contains several homologous genes. For example, the encoded BcSinI and BcSinR proteins share 32 and 64% identity, respectively, in amino acid sequence with their *B. subtilis* counterparts (Fig. 1C). Meanwhile, the homologous gene cluster in *B. cereus* corresponding to the *tapA-sipW-tasA* operon in *B. subtilis* shows different composition from the latter. There is no *tapA* homolog in the genome of AR156; instead, there is a function-unknown hypothetical gene in that region (ORF3431) (Fig. 1B). In addition, this region harbors two *tasA*-like genes (*calY1* and *calY2*). At the protein level, CalY1 and CalY2, although 57% identical between each other, share only 28 and 32% amino acid sequence identity, respectively, with *B. subtilis* TasA. A recent study showed that both CalY1 and CalY2 were critical for formation of submerged biofilms in *B. cereus* strain CECT148, yet CalY2 was more important than CalY1 in promoting floating pellicles at the air-liquid interface (Caro-Astorga et al. 2015). Our bioinformatics analyses indicated that these two *tasA*-like genes and the entire gene cluster are highly conserved in various species in the *B. cereus* group, including *B. cereus*, *B. anthracis*, and *B. thuringiensis*.

In addition to the TasA protein fibers, the EPS made by the products encoded in the 15-gene *epsA-O* operon is another key component of the *B. subtilis* biofilm matrix (Branda et al. 2001). Previous reports identified similar gene clusters predicted to be involved in capsular polysaccharide biosynthesis (BC5279 to BC5263) in *B. cereus* ATCC14579 (Ivanova et al. 2003) and EPS biosynthesis (BC5445 to BC5465) in *B. cereus* B905 (Gao et al. 2015). The latter EPS biosynthesis gene cluster in *B. cereus* 905 has been shown to not be important for biofilm formation by that bacterium (Gao et al. 2015). Surprisingly, in the *B. cereus* AR156 genome, we could not identify such a homologous gene cluster based on bioinformatics analysis. We did identify a putative polysaccharide biosynthesis operon but with only eight genes (ORF3769 to ORF3776) (Table 1). Among those putative genes, four (ORF3770, ORF3771, ORF3773, and ORF3774) resemble genes in the *epsA-O* operon, encoding the bacterial tyrosine kinase modulator EpsA, the EpsB kinase, the oligosaccharide exporter protein EpsK, and the polymerase EpsG, respectively. Another

Table 1. Putative exopolysaccharide biosynthesis genes in gene cluster in *Bacillus cereus* AR156

<i>B. cereus</i> AR156	Predicted function	Homolog in <i>B. subtilis</i> 168	Amino acid identity between AR156 and 168 (%)	Homolog in <i>B. cereus</i> ATCC14579	Amino acid identity between AR156 and ATCC14579 (%)
ORF3769	Acytransferase	YrhL	26	BC1583	86
ORF3770	Capsule biosynthesis protein CapK	EpsA	27	BC1584	89
ORF3771	Tyrosine protein kinase	EpsB	23	BC1585	87
ORF3772	UDP-galactose phosphate transferase	EpsM	41	BC1586	88
ORF3773	Oligosaccharide exporter protein	EpsK	24	BC1587	94
ORF3774	Polysaccharide polymerase	EpsG	24	BC1588	90
ORF3775	Glycosyl transferase	TuaG	46	BC1589	92
ORF3776	Mannosyl transferase	EpsD	27	BC1590	90

two genes (ORF3776 and ORF3772) resemble glycosyl-transferase genes *epsD* and *epsM*, respectively. This eight-gene operon is also present in other *B. cereus* species, including ATCC14579 and B905. The function of this putative operon has not been reported. Taken together, our analyses suggest that EPS biosynthesis in different *B. cereus* strains is likely quite different from that in *B. subtilis*, and may not be well conserved even within the *B. cereus* group.

***ΔBcspo0A* and *ΔBcsinI* mutants of AR156 showed decreased biological control efficacy toward root-knot nematode diseases.**

Host colonization by biocontrol agents is the first and critical step during biocontrol, and bacterial colonization on biotic and abiotic surfaces has been shown to be promoted by biofilm formation (Bais et al. 2004; Chen et al. 2013; Haggag and Timmusk 2008). To assess whether biofilm formation by AR156 is important for its biocontrol activity against the nematode, we picked two key regulatory genes, *Bcspo0A* and *BcsinI*, whose counterparts play essential roles in biofilm formation in *B. subtilis* (Branda et al. 2001; Chu et al. 2008; Kearns et al. 2005). We constructed *ΔBcspo0A* and *ΔBcsinI* deletion mutants in AR156, and conducted in vitro nematode killing using the wild-type strain and the two mutants. Treatment of *M. incognita* J2 by cell suspension of AR156 resulted in worm mortality with 97.40% efficacy in vitro, in comparison with the water control ($P \leq 0.05$) (Fig. 2A). Both *ΔBcspo0A* and *ΔBcsinI* mutants were dramatically attenuated in the ability to kill *M. incognita* J2, with an efficacy of 13.9 and 42.3%, respectively (Fig. 2A). The efficacy reduction in *ΔBcspo0A* was more severe than that in *ΔBcsinI*.

One possible explanation for the reduced killing activity in the *ΔBcspo0A* and *ΔBcsinI* mutants was that there was decreased attachment of the mutants to the nematode body surfaces. To test this, we performed an assay to evaluate bacterial attachment to the nematode body surfaces (see Materials and Methods). To explain briefly, *B. cereus* cells (wild type and mutants) and J2 nematodes were incubated in Eppendorf tubes for 24 h at room temperature. J2 were picked up by needles under a stereomicroscope and suspended in sterilized water. Body surface-associated bacterial cells were washed off and plated on Luria-Bertani (LB) agar media to count CFU. The number of recovered bacterial cells (normalized to one J2) was

significantly lower in the treatments of the two mutants than that of the wild-type AR156 (an approximately fourfold decrease) (Fig. 2B). Thus, bacterial attachment to the nematodes is impaired in both the *ΔBcspo0A* and *ΔBcsinI* mutants. This is possibly due to their inability to form biofilms because biofilms often possess strong adhesive properties on worm body surfaces (Atkinson et al. 2011; Darby et al. 2002; Drace and Darby 2008; Joshua et al. 2003).

Next, we compared biological control efficacy of the wild-type AR156 and the *ΔBcspo0A* and *ΔBcsinI* mutants on tomato plants. In the biocontrol experiments, the sterilized sandy loam soil treated with AR156 cells effectively suppressed the root-knot development on tomato roots and obtained nearly 65% biocontrol efficacy in comparison with the nonbacterial treatment (Fig. 2C). Application of the *ΔBcspo0A* or the *ΔBcsinI* mutant resulted in a significant reduction in disease control (23 and 38%, respectively) (Fig. 2C). The biocontrol efficacy of *ΔBcspo0A* was even lower than that of *ΔBcsinI*, in line with the results from assays of nematode killing (Fig. 2A).

To test whether the reduction in the biocontrol activity in the mutants was caused by lowered bacterial colonization, the population dynamics of the wild type and the two mutants in the tomato rhizosphere was monitored over a period of 56 days by the plating method. Overall, the cell population of all three strains in the rhizosphere decreased incessantly in the first week and stabilized after 7 days postinoculation (dpi) (Fig. 2D). For instance, the wild-type cell population declined from 1.57×10^8 CFU/g of rhizosphere soil at the beginning of the experiment to 1.55×10^7 CFU/g of soil after 1 week and stabilized afterward to approximately 10^7 CFU/g of soil (at 14, 21, 30, 40, and 56 dpi, the total numbers of cells were 2.04, 1.58, 1.95, 2.04, and 1.26×10^7 CFU/g of rhizosphere soil, respectively) (Fig. 2D). In comparison with the wild type, the *ΔBcspo0A* and *ΔBcsinI* mutants showed a seven- and threefold reduction, respectively, in the total number of root-associated cells after 1 week, which was in the range of 10^6 CFU/g of rhizosphere soil. Noticeably, the cell number of *ΔBcsinI* in the rhizosphere soil was approximately twofold higher than that of *ΔBcspo0A* (Fig. 2D).

Together, our results suggest that *Bcspo0A* and *BcsinI* and, likely, the biofilm formation controlled by them play critical roles in the bacterial colonization of the nematode body surface and in bacterial survival in the rhizosphere, both of which subsequently promote biocontrol activity against root-knot

Fig. 2. Both the *Bcspo0A* and *BcsinI* genes are important for the nematocidal activity, root colonization, and biological control against *Meloidogyne incognita* by *Bacillus cereus* AR156. **A**, Assays of the mortality rate of *M. incognita* in treatments of the wild type and the *ΔBcspo0A* and *ΔBcsinI* mutants. Juveniles were cocultured with cell suspension of the wild type or the two mutants and incubated at room temperature. After 12 h, live and dead worms were counted using stereoscopy. **B**, Colonization capability of the wild type and the two mutants on the body surface of the worms. Juveniles in each treatment were picked and resuspended with sterilized water after coculture. Suspension was serially diluted and cultured on the Luria-Bertani agar medium for CFU counting. CFU was normalized per juvenile. **C**, Biocontrol efficacy of the wild type and two mutants against tomato root-knot nematode diseases under greenhouse conditions. The average $\pm$ standard deviation was calculated from three independent trials and analyzed using Student's *t* test ($P \leq 0.05$). In A, B, and C, bars marked with different letters indicate that there was a significant difference. **D**, Population density of AR156 and the *ΔBcspo0A* and *ΔBcsinI* mutants in the rhizosphere monitored over a period of 56 days in the biocontrol experiment against root-knot nematode diseases. Root-associated bacterial cells were collected periodically and CFU of the samples was determined by a conventional plating method.

nematodes. Therefore, we decided to further investigate the role of *Bcspo0A* and *BcsinI* in biofilm formation in *B. cereus* AR156.

***BcsinI* was required for biofilm formation and swarming motility in AR156.**

Because SinI is a key positive regulator for biofilm formation in *B. subtilis*, we were curious about whether the SinI homolog in *B. cereus* has a similar function. To test this, we compared pellicle biofilm formation by the $\Delta BcsinI$ mutant to that of the wild-type strain. Cells were inoculated in a biofilm-inducing medium, LB supplemented with 1% glycerol and 200 μ M $MnSO_4$ (LBGM) (Shemesh and Chai 2013), and incubated at 25°C. After 72 h of incubation, wild-type cells formed robust pellicle biofilms at the air–liquid interface, whereas the $\Delta BcsinI$ mutant was unable to form a strong pellicle (Fig. 3A, left-hand panels), even after incubation for 10 days (data not shown). The total biomass of the pellicle by the $\Delta BcsinI$ mutant was 14-fold less when compared with that of the wild-type pellicle (Fig. 3B). To further gain insight into the morphological details of the wild-type pellicles (or thin floating films in the case of the $\Delta BcsinI$ mutant), pellicles by both the wild type and the $\Delta BcsinI$ mutant were harvested and imaged using scanning electronic microscopy (SEM). Cells in the wild-type pellicles were tightly stuck together and individual cells were heavily cross-linked by a web-like matrix (Fig. 3A, upper middle and right-hand panels). In contrast, only very short filamentous fibrils were seen on the cell surfaces of the $\Delta BcsinI$ mutant and little web-like matrix was seen in the pellicles prepared from that mutant (Fig. 3A, middle and right-hand panels).

Motility of the bacterial biocontrol agents has been suggested as an important factor in bacterial colonization on plant host surfaces (Andersen et al. 2003; Gao et al. 2016). We briefly tested swarming motility of the wild-type strain and the $\Delta BcsinI$ mutant on 0.7% LB agar plates. The result showed that the $\Delta BcsinI$ mutant had a reduced swarming capability (Fig. 3C and D). Note that no growth defect was seen in the $\Delta BcsinI$ mutant (Supplementary Fig. S2). Thus, we concluded that, in *B. cereus* AR156, SinI is critical for biofilm formation, similar to what was seen in *B. subtilis* (Kearns et al. 2005). In addition, SinI also seems to be important for swarming motility in *B. cereus* AR156, a feature different from that in *B. subtilis*.

***BcsinI* can functionally replace *BssinI* in regulating biofilm formation in *B. subtilis*.**

Previous studies showed that, in *B. subtilis*, disruption of *sinI* caused a severe biofilm defect (Kearns et al. 2005). Our results above indicated that *sinI* is essential in biofilm formation in *B. cereus* (Fig. 3A). Because both SinI proteins share sequence similarity (Fig. 1C), we wondered whether BcSinI could functionally substitute for *B. subtilis* SinI. To test our hypothesis, we performed heterologous expression of the *BcsinI* gene at an ectopic *amyE* locus in a *B. subtilis* $\Delta BssinI$ mutant, and examined the biofilm phenotype of the engineered strain. The *BcsinI* gene was fused with the *gfp* gene for green fluorescent protein (GFP) for easy detection of gene expression and protein production. The expression of *gfp-BcsinI* was driven by a strong and constitutively expressed *hyperspank* promoter (Britton et al. 2002). A similar *gfp-BssinI* fusion (*sinI* from *B. subtilis*) was constructed previously and used as a positive control here (Chai et al. 2010). The wild-type *B. subtilis* 3610 displayed robust colony architecture on the LBGM agar plate and formed wrinkled pellicles in the LBGM liquid medium (Fig. 4A), consistent with the previous study (Shemesh and Chai 2013). As expected, the $\Delta BssinI$ mutant completely lost the ability to form pellicle biofilms and showed flat colony morphology in LBGM (Fig. 4B). Overexpression of the *gfp-BssinI* fusion

completely rescued the biofilm defect caused by $\Delta BssinI$ (Fig. 4C). Interestingly, overexpression of *gfp-BcsinI* also complemented $\Delta BssinI$ in biofilm formation (Fig. 4D), although the colony morphologies by the two overexpression strains somewhat differed from each other (Fig. 4C and D). These results indicate that *BcsinI* can functionally replace *BssinI* in regulating biofilm formation in *B. subtilis*.

In *B. subtilis*, the interactions between the biofilm master repressor BsSinR and the antagonist BsSinI have been well studied biochemically and structurally (Colledge et al. 2011). The full-length BsSinI (amino acids 1 to 57) shares strong sequence similarity with the C-terminal domain of BsSinR (amino acids 60 to 115) (Fig. 1D). In the cocrystal structure of the SinI–SinR complex, amino acid residues 3 to 39 of BsSinI and the residues 74 to 111 of BsSinR form an intermolecular four-helix bundle, in which each chain contributes a pair of helices that hook together. Eight conserved apolar residues are integral to or surrounding the intermolecular hydrophobic core and critical to the interaction (Fig. 1D, highlighted in red in the upper panel) (Colledge et al. 2011). Many of those residues in BsSinI involved in interactions with BsSinR are also conserved in BcSinI (Fig. 1D, highlighted in red in the lower panel). Thus, it is possible that BcSinI can interact with and antagonize *B. subtilis* SinR due to sequence (and possibly structural) similarity to its *B. subtilis* counterpart. Just like the counterparts in *B. subtilis*, BcSinR and BcSinI are also similar to each other, with conserved interaction sites seen in the *B. subtilis* counterparts (Fig. 1D, lower panel). Our protein sequence and domain analyses indicate that the BcSinR–BcSinI interaction in *B. cereus* may be similar to that of their orthologs in *B. subtilis*. We also speculate that BcSinI may cross-interact with BsSinR due to its similarity to BsSinI, which explains why BcSinI can functionally replace BsSinI in the regulation of biofilm formation in *B. subtilis*.

In *B. subtilis*, BsSinR, together with another regulatory protein SlrR, negatively controls genes for matrix production while it positively regulates those for cell separation (Chai et al. 2009; Chu et al. 2008). In other words, antagonizing SinR activities is expected to derepress matrix genes to achieve robust biofilm formation while simultaneously shutting off cell separation genes (e.g., genes encoding various autolysins) for formation of cell chains and bundles, which likely function as a scaffold for biofilm assembly (Chai et al. 2010). Consistent with the above proposed regulation, we observed that overexpression of *gfp-BssinI* in $\Delta BssinI$ not only rescued the biofilm defect but also caused extensive cell chaining, likely due to SinR antagonization and downregulation of cell separation genes (Fig. 4E). The complementation strain by overexpression of *gfp-BcsinI* in $\Delta BssinI$ also showed a chaining phenotype. Intriguingly, it formed much shorter and bent cell chains when compared with that of the *gfp-BssinI*-complemented strain (Fig. 4F). Moreover, the average size of individual cells in the short chains of the *gfp-BcsinI*-complemented strain was significantly smaller than that of cells in the chains of the *gfp-BssinI*-complemented strain (Fig. 4E and F; Supplementary Fig. S3). To better measure the cell size of above strains, cells were prepared and observed with SEM. Consistent with the results from fluorescent microscopy, the complementation strain overexpressing *gfp-BssinI* formed long cell chains, whereas the *gfp-BcsinI* overexpression strain displayed chains that were much shorter (Fig. 5A to D). Furthermore, cells of the *gfp-BssinI* complementation strain ($4.58 \pm 0.36 \mu$ m) were bigger in size than the wild-type 3610 ($2.16 \pm 0.20 \mu$ m), $\Delta BssinI$ ($1.73 \pm 0.28 \mu$ m), and the *gfp-BcsinI* overexpression strain ($1.72 \pm 0.17 \mu$ m) (Fig. 5E). The difference between the two *sinI* overexpression strains was not likely to be due to differential production of the two fusion proteins because cells overexpressing *gfp-BssinI* and

those of *gfp-BcsinI* demonstrated similar fluorescence intensity (Fig. 4E and F). Considering that the amino acid sequence identity between BsSinI and BcSinI is only 32%, we presumed that BcSinI may have another unknown cellular target (other

than BsSinR) in *B. subtilis* that is involved in cell size control. Alternatively, BsSinR controls cell size through a yet-unknown mechanism in *B. subtilis* and the antagonization efficacy of BsSinI against BsSinR is better than that of BcSinI.

Fig. 3. *BcsinI* and *Bcspo0A* mutants are defective in pellicle formation and swarming motility. **A**, The wild type (WT) and the $\Delta BcsinI$ and $\Delta Bcspo0A$ mutants were grown in Luria-Bertani (LB) agar supplemented with 1% glycerol and 200 μ M $MnSO_4$ for 3 days at 30°C in 12-well plates before images were taken (left-hand panels). Morphological details of the pellicles were visualized by scanning electron microscopy at $\times 5,000$ (middle panels) and $\times 15,000$ (right-hand panels) magnifications. **B**, Biomass of the pellicles formed by the WT or the $\Delta BcsinI$ and $\Delta Bcspo0A$ mutants. The 3-day pellicles were harvested from four individual wells for each strain and air dried prior to measurement of dry weight. Experiments were repeated three times. **C**, Swarming motility of the WT and the $\Delta BcsinI$ and $\Delta Bcspo0A$ mutants. Strains were inoculated on the 0.7% LB agar plates and incubated at 30°C for 12 h before images were taken. **D**, Size of the swarming zone on the plates inoculated with either the $\Delta BcsinI$ or the $\Delta Bcspo0A$ mutant was normalized against that of the WT. All values are the average of three replicates. Bars marked with different letters indicate a significant difference according to a least significant difference test at $P = 0.05$.

***BssinR* was also functionally replaceable by *BcsinR* in biofilm regulation in *B. subtilis*.**

BsSinR is a biofilm master repressor in *B. subtilis* (Chu et al. 2006; Kearns et al. 2005). The *B. subtilis* mutant of *sinR* ($\Delta BssinR$) showed a robust biofilm phenotype in LBGM and even in LB, a medium less favorable for biofilm induction (Fig. 6B). Because construction of the knockout mutant of *BcsinR* in AR156 was not successful, we decided to apply heterologous expression of *BcsinR* in *B. subtilis* to see whether *BcsinR* can functionally substitute for *BssinR* in *B. subtilis*, similar to what we did for *BcsinI*. In our previous study, we showed that, in *B. subtilis*, by providing a wild-type copy of *sinR* (*BssinR* with its own native promoter) at the ectopic *amyE* locus, it not only complemented $\Delta sinR$ but further downregulated the expression of SinR-repressed matrix genes, resulting in a severe biofilm defect (Chai et al. 2011). This is likely due to both an increased gene dose of *sinR* when complemented at the replication origin-proximal *amyE* locus (28°) compared with the origin-distal native locus (218°) and ultrasensitivity of SinR-repressed genes to the protein levels

of SinR (Chai et al. 2011). As expected, the complemented strain with *BssinR* at *amyE* in $\Delta BssinR$ displayed smooth, featureless colony morphology and a severe defect in pellicle biofilm formation (Fig. 6C). Interestingly, the complementation strain by *BcsinR* had a nearly identical biofilm phenotype (Fig. 6D), suggesting that *BcsinR* can substitute *BssinR* in biofilm regulation in *B. subtilis*. This was somewhat expected based on high amino acid sequence identity between BcSinR and BsSinR (64%), especially in their N-terminal DNA-binding domains (Fig. 1C). This also suggests that BsSinR and BcSinR may recognize similar DNA motives in transcriptional regulation. Indeed, the consensus DNA sequences recognized by BsSinR (GTTCTCT, SinR box) (Chu et al. 2008) were also seen arranged in tandem repeats in the regulatory regions of the *sipW*, *orf3431*, and *calY2* genes in *B. cereus* AR156, with a few variations (Figs. 1B; Supplementary Fig. S4). Taken together, our results suggested that BsSinR was also functionally replaceable by BcSinR in biofilm regulation in *B. subtilis* and that, possibly, BsSinR and BcSinR

Fig. 4. Heterologous expression of *Bacillus cereus sinI* in the *B. subtilis* $\Delta BssinI$ mutant rescued the biofilm defect. Colony biofilm and pellicle formation by A, the wild type *B. subtilis* 3610; B, $\Delta BssinI$; and C and D, indicated heterologous complementation strains in Luria-Bertani (LB) agar or LB supplemented with 1% glycerol and 200 μ M $MnSO_4$ (LBGM). Images were taken after 3 days of incubation at 25°C. To compare green fluorescence intensity, colonies or pellicles formed by cells bearing either the *gfp-BssinI* or the *gfp-BcsinI* fusion were visualized by fluorescent dissecting microscope. Microscopic analyses of the cell chaining phenotype in E, YCC227 ($\Delta BssinI$, *P_{hyspantk}-gfp-BssinI*) and F, CY617 ($\Delta BssinI$, *P_{hyspantk}-gfp-BssinI*). Shown were phase contrast, green fluorescence, and merged images (from top to bottom). Cells were grown in LBGM medium to an optical density at 600 nm = 1.0, harvested, and observed by confocal microscopy. Scale bars: 5 μ m.

recognize nearly identical operator sequences in the regulatory regions of their controlled genes. Furthermore, BcSinI and BcSinR may cross-interact with their counterpart repressor and antirepressors in *B. subtilis*. Finally, although we do

not have direct evidence yet, we speculate that BcSinI and BcSinR may possess regulatory functions in *B. cereus* AR156 biofilm formation similar to their counterparts in *B. subtilis*.

Fig. 5. Heterologous expression of *Bacillus cereus sinI* in the *B. subtilis* $\Delta BssinI$ mutant reduced the cell size. **A**, The wild type; **B**, FC333 ($\Delta BssinI$); **C**, YC227 ($\Delta BssinI$, *Physpantk-gfp-BssinI*); and **D**, CY617 ($\Delta BssinI$, *Physpantk-gfp-BssinI*) were grown in Luria-Bertani agar supplemented with 1% glycerol and 200 μM MnSO₄ to an optical density at 600 nm = 1.0, and visualized by scanning electron microscopy at ×5,000 (left panels) and ×10,000 (right-hand panels) magnifications. The arrow indicates the shorter chaining. **E**, Average cell size was measured and analyzed. Average ± standard deviation was calculated from three independent trials and analyzed using Student's *t* test (*P* ≤ 0.01). Bars marked with different letters indicate that there was a significant difference.

***Bcspo0A* was essential in cell differentiation in *B. cereus* AR156.**

BsSpo0A plays a central role in cell differentiation in *B. subtilis* (Branda et al. 2001; Hoch 1993). A Δ *Spo0A* mutant of *B. subtilis* was completely deficient in both sporulation and biofilm formation (Fig. 7C and E) (Branda et al. 2001, 2006). In contrast, few studies have demonstrated the importance of Spo0A in cell differentiation in *B. cereus* (Gao et al. 2015). Thus, we examined phenotypes in cell differentiation by the Δ *Bcspo0A* deletion mutant of AR156. Similar to the *B. subtilis* Δ *Bsspo0A* mutant, no pellicle biofilm was observed by the Δ *Bcspo0A* mutant of AR156 in LBGM (Fig. 3A). Further examination by SEM revealed that, contrary to the wild type, the pellicles by the Δ *Bcspo0A* mutant completely lacked the web-like matrix holding individual cells together (Fig. 3A). This was similar to what was observed in the Δ *BcsinI* mutant (Fig. 3A). Thus, *Bcspo0A* was also essential for biofilm formation in *B. cereus* AR156. The Δ *Bcspo0A* mutant also showed a defect in swarming motility, though slightly less severe than that of the Δ *BcsinI* mutant (Fig. 3C and D).

We also examined sporulation efficiency for the wild-type AR156 and the Δ *Bcspo0A* mutant. When the Δ *Bcspo0A* cells were grown in Difco sporulation medium for 36 h, only rod-shaped cells but not spores were observed, whereas the wild-type AR156 cells formed mostly phase-bright spores under the

same condition (Fig. 7A). Cells of both the wild type and the Δ *Bcspo0A* mutant were also harvested after 36 h of growth in Difco sporulation (DS) medium and observed by transmission electron microscopy (TEM) to gain more structural details of the cells. The wild-type AR156 spores contained dense inclusions and were dark under TEM (Fig. 7B). In contrast, the Δ *Bcspo0A* cells showed vegetative cell-like rod shape and had less density and darkness in the cytosol (Fig. 7B). The heat kill experiment was further conducted to compare the ratio of heat-resistant spores between the wild type and the Δ *Bcspo0A* mutant. The result was consistent with the observations under a microscope, showing that the wild-type culture contained almost 100% heat-resistant spores after 36 h of incubation in DS medium, whereas virtually no viable cells were recovered from the Δ *Bcspo0A* culture after heat treatment. This confirms that *Bcspo0A* is also essential for sporulation in *B. cereus* AR156.

Heterologous expression of *Bcspo0A* rescued the sporulation and biofilm defects in the *B. subtilis* Δ *Bsspo0A* mutant.

Because the Δ *Bcspo0A* mutant of *B. cereus* AR156 displayed phenotypes in cell differentiation similar to those of the Δ *Bsspo0A* mutant of *B. subtilis* and because the two Spo0A proteins share very high amino acid sequence identity (80%), we speculated that these two proteins might be interchangeable in regulating cell

Fig. 6. *Bacillus cereus sinR* can replace *B. subtilis sinR* in biofilm regulation in *B. subtilis*. Colony biofilm and pellicle formation by **A**, the wild-type *B. subtilis* 3610, **B**, Δ *BssinR*, and complementation strains expressing either **C**, *BssinR* or **D**, *BcsinR* in Luria-Bertani (LB) agar or LB supplemented with 1% glycerol and 200 μ M MnSO_4 (LBGM). Images were taken after 3 days of incubation at 25°C. Scale bar: 1 cm.

differentiation. To test this, we expressed *Bcspo0A* under the control of the *Bsspo0A* native promoter (*P_{Bsspo0A}-Bcspo0A*) in the $\Delta Bsspo0A$ strain of *B. subtilis* and examined whether the heterologous expression of *Bcspo0A* could rescue the defects of sporulation and biofilm formation by $\Delta Bsspo0A$. Interestingly, the heterologous expression strain was completely rescued for the ability to sporulate (Fig. 7C) and to form robust biofilms (Fig. 7E). There was virtually no difference in biofilm formation between the heterologous expression strain with *Bcspo0A* and the complementation strain with *Bsspo0A* (Fig. 7E). Hence, we conclude that Spo0A proteins are not only conserved in amino acid sequence in both *B. cereus* and *B. subtilis* but also structurally similar enough so that BcSpo0A can replace BsSpo0A in interacting with other partner proteins and in regulating cell differentiation in the heterogeneous host *B. subtilis*.

One more interesting observation we had was that the wild-type 3610 and the $\Delta Bsspo0A$ mutant of 3610 grew similarly in

DS medium before reaching late log phase (optical density at 600 nm [OD₆₀₀] = 1.0). However, after reaching late log phase, the culture density of the $\Delta Bsspo0A$ mutant rapidly dropped and eventually reached OD₆₀₀ = 0.1 (a 10-fold decrease) after 36 h of shaking growth, while the wild-type culture reached OD₆₀₀ of approximately 2 (a 2-fold increase) after the same shaking growth (Fig. 7D). There was a strong cell lysis phenotype associated with stationary phase cells of the *B. subtilis* $\Delta spo0A$ mutant. Remarkably, such a strong cell lysis phenotype was not seen in the *B. cereus* *spo0A* mutant, where the cell density of the $\Delta Bcspo0A$ mutant was still retained at OD₆₀₀ = 2.0. Moreover, the cell lysis phenotype of in $\Delta Bsspo0A$ can be rescued by heterologous expression of *Bcspo0A* (Fig. 7D). We hypothesized that the sporulation-defective $\Delta Bcspo0A$ cells experience less autolysis in nutrient-limiting conditions compared with the similar mutant in *B. subtilis*.

Fig. 7. *Bcspo0A* was essential for sporulation and biofilm formation in *Bacillus cereus* AR156. **A**, The *Bcspo0A* deletion mutation completely abolished formation of heat-resistant spores in *B. cereus* AR156. The wild-type strain and the $\Delta Bcspo0A$ mutant were grown in the Difco sporulation (DS) medium for 36 h at 37°C under shaking condition. Cells were observed by phase-bright microscopy. Scale bars: 10 μ m. **B**, Wild-type cells and the $\Delta Bcspo0A$ mutant from **A** were further visualized by transmission electron microscopy for gaining insight into structural details. Scale bars: 1 μ m. **C**, Heterologous expression of *Bcspo0A* in the $\Delta Bsspo0A$ mutant of *B. subtilis* largely rescued the sporulation deficiency of the mutant. Scale bars: 10 μ m. **D**, Cell optical density at 600 nm (OD₆₀₀) of the wild-type strains of both *B. subtilis* (3610) and *B. cereus* (AR156), the corresponding *spo0A* deletion mutants ($\Delta Bsspo0A$ and $\Delta Bcspo0A$), and the $\Delta Bsspo0A$ mutant heterologously expressing *Bsspo0A*. All strains were grown for 36 h under shaking in DS medium before measurement of cell OD. Error bars represent standard deviations from three independent measurements. **E**, Colony and pellicle biofilm formation in Luria-Bertani (LB) agar and LB supplemented with 1% glycerol and 200 μ M MnSO₄ (LBGM) by the wild-type *B. subtilis* 3610, the $\Delta Bsspo0A$ mutant, the $\Delta Bsspo0A$ mutant complemented with *P_{Bsspo0A}-Bsspo0A* at *amyE*, and the $\Delta Bsspo0A$ mutant heterologously expressing *P_{Bsspo0A}-Bcspo0A* at *amyE*. Scale bar: 1 cm.

Genome-wide transcriptional profiling in the wild type and $\Delta Bcspo0A$ and $\Delta BcsinI$ mutants revealed potential biofilm and sporulation genes.

Genes involved in biofilm formation and sporulation are largely unknown in *B. cereus*. To identify putative biofilm genes regulated by either Spo0A or SinI, as well as putative sporulation genes regulated by Spo0A in *B. cereus* AR156, we conducted genome-wide transcriptional profiling in the wild-type strain, the $\Delta Bcspo0A$ mutant, and the $\Delta BcsinI$ mutant. Cells were grown in the biofilm-inducing medium LBGM to $OD_{600} = 2.0$ before harvest (in *B. subtilis*, Spo0A-controlled sporulation and biofilm genes are known to be induced under this condition) (Shemesh and Chai 2013). Genes exhibiting a twofold or greater difference in expression between the wild type and the $\Delta Bcspo0A$ mutant were tentatively designated as BcSpo0A regulon (Supplementary Table S1). In total, 830 genes (or approximately 15% of the encoded genes in the AR156 genome) showed a differential expression between these two strains by at least twofold, among which 259 genes were expressed lower and 571 genes were expressed higher in the $\Delta Bcspo0A$ mutant (Supplementary Fig. S5). Furthermore, of the 830 genes, 558 exhibited at least an eightfold difference in transcription. Those genes were further grouped based on their annotated functions (Fig. 8A). Because BcSpo0A is a transcription factor and based on characterization of Spo0A binding motif (OA box) in *B. subtilis*, we speculated that BcSpo0A likely recognizes a similar conserved DNA-binding motif in the regulatory region of its regulon. To verify the presence of such DNA-binding sites of BcSpo0A, the putative promoter sequences (200 bp upstream of the coding sequence) of these 558 genes were retrieved and analyzed with the MEME-CHIP program (Machanick and Bailey 2011). Results indicated that 161 of the 558 genes contained a consensus binding sequence (5'-TGAAGAA-3') in their corresponding regulatory regions (Fig. 8B). This consensus sequence resembles the Spo0A binding box revealed in *B. subtilis* (5'-TGTCGAA-3') (Molle et al. 2003). Finally, among the 830 genes, 37 have orthologs that have been previously identified as the Spo0A regulon in *B. subtilis* (Fujita et al. 2005; Molle et al. 2003), which includes *spoIIAA*, *spo0J*, *abrB*, *codY*, *glpK*, and so on. (Fig. 8C). Interestingly, there are 165 strongly differentially expressed genes that had no orthologs in *B. subtilis* (E-value $\leq 1e-5$), indicating that these could be Spo0A-regulated cell differentiation genes uniquely present in the *B. cereus* group (Supplementary Table S2).

When comparing transcription profiling between the wild-type strain and the $\Delta BcsinI$ mutant, we found differential expression of 955 genes above the twofold threshold. Among them, 315 genes were downregulated and 640 showed an upregulation in the $\Delta BcsinI$ mutant (Supplementary Table S3). Gene ontology analysis suggested that functions of many of those genes are related to amino acid catabolism and oligopeptide transport (Supplementary Fig. S6).

Given that both $\Delta Bcspo0A$ and $\Delta BcsinI$ mutants showed a defect in biofilm formation, we speculated that a subset of genes regulated by both Spo0A and SinI might contribute to biofilm formation, especially those downregulated in both mutants. Thus, those downregulated genes were further analyzed. In all, 256 genes (62.6% of the total downregulated genes) (Supplementary Table S4) were expressed at a lower level in both mutants when compared with the wild type. These 256 genes were predicted to be involved in approximately 10 different biological processes and were further grouped into seven categories based on the annotated molecular functions. Those annotated functions include hydrolase activities, transferase activities, and ion binding function, many of which are similar to the BaSinR regulon characterized in *B. anthracis* (Pflughoeft et al. 2011).

In *B. subtilis*, SinI acts as an antagonist of the biofilm master repressor SinR and the *sinI* gene is activated by Spo0A (Bai et al. 1993; Branda et al. 2006; Chai et al. 2011; Newman et al. 2013). Therefore, genes such as the *epsA-O* and *tapA-sipW-tasA* operons repressed by SinR are under indirect and positive regulation by both SinI and Spo0A. By taking advantage of the known SinR regulon characterized in both *B. subtilis* and *B. anthracis* (Chu et al. 2006; Pflughoeft et al. 2011), we predicted that, among the 256 downregulated genes in both the $\Delta Bcspo0A$ and $\Delta BcsinI$ mutants identified in our transcription profiling, 29 genes might be directly repressed by SinR in *B. cereus* (Table 2). Of these 29 genes, 18 harbored a consensus 7-bp binding sequence (5'-GTCTTGC-3') in their regulatory regions, similar to the consensus SinR binding sequence characterized in *B. subtilis* (5'-GTTCTCT-3'). Ten genes have orthologs that were shown to be involved in biofilm formation in *B. subtilis*, including *ywqD*, *galE*, *guaA*, *sodA*, *kataA*, and so on (Table 2, highlighted in bold). In summary, our results suggest that BcSpo0A, BcSinI, and BcSinR in *B. cereus* may have biological functions in *B. cereus* similar to their counterparts in *B. subtilis* in regulating cell differentiation and biofilm.

DISCUSSION

The *B. cereus* group is widely distributed in natural environments. These bacterial species are found to be associated with soils, plants, foods, equipment, or humans as opportunistic pathogens (Bottone 2010; Elhariry 2011; Kotiranta et al. 2000). Survival of these bacteria in versatile ecological niches is highly dependent on their abilities to form biofilms or become dormant spores under various stress conditions (Auger et al. 2009; Elhariry 2011). Previous studies showed that both spores and biofilms of this genus had a higher resistance to heat, antibiotics, UV, and other environmental stresses. Biofilms also serve to increase adhesion to biotic and abiotic surfaces (Ball et al. 2008; Shaheen et al. 2010). *B. cereus* species form biofilms on solids, at air-liquid interfaces, or as submerged surface-associated biofilms, even though the ability to form biofilms and the morphological robustness of the biofilms vary greatly among the environmental isolates of *B. cereus* (Caro-Astorga et al. 2015; Elhariry 2011; Gao et al. 2015; Peng et al. 2001; Wijman et al. 2007; Yan et al. 2016). On the other hand, structural components of the biofilm matrix and regulatory mechanisms controlling biofilm formation are still very poorly understood in *B. cereus*, in contrast to the well-characterized regulatory networks for biofilm formation in the closely related *B. subtilis*.

Unlike the previously studied *B. cereus* strains, the biocontrol root-knot nematode strain AR156 cannot form submerged biofilms (Yan et al. 2016). Rather, this bacterium forms floating pellicles in the biofilm-inducing medium LBGM with robust surface features (Fig. 3A). In this study, we explored morphological details of the AR156 pellicle biofilms. The pellicles observed by SEM contained fiber-like structures associated with cell surfaces. These fiber-like structures reached out and formed a web-like network connecting individual cells within the biofilm. To some extent, this resembles the TasA fibers seen in the *B. subtilis* biofilms (Branda et al. 2001; Romero et al. 2011). These extracellular structures were largely missing in either $\Delta Bcspo0A$ or $\Delta BcsinI$ mutants (Fig. 3), indicating that Spo0A and SinI may control production of these extracellular components.

A main goal of this study was to characterize the genetic circuit that governs biofilm formation in *B. cereus* AR156. Due to the close relationship between *B. cereus* and *B. subtilis*, we first searched and analyzed orthologs of the major biofilm

genes of *B. subtilis* in AR156. Consistent with the findings in previous studies (Caro-Astorga et al. 2015; Gao et al. 2015), there are two TasA-like proteins (CalY1 and CalY2) and the Spo0A-SinI-SinR module in AR156, similar to their orthologs in *B. subtilis* (Fig. 1). These *tasA*-like genes have been shown to be important in formation of the submerged biofilms in

B. cereus CECT148. On the other hand, no orthologs of the *epsA-O* operon of *B. subtilis* or the capsular polysaccharide operon of *B. cereus* 14579 was found in the *B. cereus* AR156 genome. Instead, only an eight-gene cluster with predicted products resembling the proteins encoded in the *epsA-O* operon was identified in the AR156 genome (Table 1). This eight-gene

Fig. 8. Genome-wide transcriptome analyses of the BcSpo0A regulon in *Bacillus cereus* AR156. **A**, Gene ontology analyses predicted genes regulated by BcSpo0A in AR156 from transcriptional profiling experiments. **B**, Consensus DNA-binding motif of BcSpo0A predicted based on analyses of the differentially expressed genes in ΔBcspo0A using the MEME-CHIP program. **C**, Expression patterns of 37 genes under the Spo0A control in both *B. cereus* and *B. subtilis*.

cluster is highly conserved in *B. cereus* yet its function is completely unknown. Unfortunately, results from the transcriptome profiling indicate that the expression of these eight genes was not significantly altered in either the $\Delta Bcspo0A$ or the $\Delta BcsinI$ mutant under the tested conditions. However, it is still possible that this putative eight-gene EPS cluster plays a role in pellicle biofilm development, although expression of the EPS gene cluster is not controlled by either Spo0A or SinI in AR156. In previous studies, a 21-gene cluster with predicted products bearing many of the hallmarks of EPS biosynthetic machinery was identified in both *B. cereus* 905 and ATCC14579 (Gao et al. 2016). However, the mutant with a deletion of this gene cluster in *B. cereus* 905 was not affected in biofilm formation. Thus far, no published report has suggested an important role of any secreted polysaccharide in biofilm formation in *B. cereus*.

In *B. subtilis*, the genetic circuit of *spo0A-sinI-sinR* plays a central role in regulating biofilm formation (Branda et al. 2001; Chu et al. 2006; Kearns et al. 2005). Our evidence, based on mutagenesis, heterologous expression of gene orthologs, and transcription profiling, suggests that the similar genetic circuit (*Bcspo0A-BcsinI-BcsinR*) also plays a central role in biofilm formation and cell differentiation in *B. cereus* AR156. Those homolog proteins in both *B. subtilis* and *B. cereus* are functionally very similar to each other. For instance, we showed that BcSinI may cross-interact with BsSinR, and that BcSinR, when heterologously produced in *B. subtilis*, was able to regulate the biofilm genes controlled by BsSinR. Obviously, one should still expect major differences in the control mechanism for biofilm formation between *B. subtilis* and *B. cereus*. For example, some of the important regulatory proteins such as YwC, SlrA, and SlrR in *B. subtilis* have no orthologs in the *B. cereus* strains (Chai et al. 2009, 2010).

Hundreds of genes putatively regulated by BcSpo0A and BcSinI in *B. cereus* AR156 were first revealed in this study

based on the transcriptome analyses. In addition, based on the knowledge of biofilm regulation in other *Bacillus* spp., 29 genes under the regulation of BcSpo0A-SinI-SinR were speculated to be under the direct control of SinR in AR156 (Table 2). Interestingly, some of the 29 genes showing differential expression between the wild type and the $\Delta Bcspo0A$ and $\Delta BcsinI$ mutants do not have orthologs in *B. subtilis* 3610. This indicated that some of the BcSpo0A-SinI-SinR-controlled genes in *B. cereus* were unique to the *B. cereus* group. These genes could provide unique features to the *B. cereus* biofilms.

The *Caenorhabditis elegans*-bacteria interaction model has been widely used to identify factors in both the bacteria and the host that are involved in biofilm-mediated interactions between bacteria and invertebrates (Begun et al. 2007; Darby et al. 2002; DePas et al. 2014; Drace and Darby 2008; Joshua et al. 2003; Tan et al. 1999). *Yersinia pseudotuberculosis*, *Y. pestis*, and *Xenorhabdus nematophila* were shown to kill *C. elegans* by forming biofilms around the head of the nematodes, which prevented food intake and subsequently caused death of *C. elegans* due to starvation (Darby et al. 2002; Drace and Darby 2008; Joshua et al. 2003). Mutations in the EPS operon *hmsHFRS* significantly reduced biofilm formation and colonization of the above three bacterial species, and attenuated the pathogenicity of the bacteria against *C. elegans* (Drace and Darby 2008). *Streptococcus epidermidis* and *Staphylococcus aureus* are also pathogenic bacteria and form biofilms during the infection of *C. elegans* (Begun et al. 2007). The mutants of the *icaADBC* operon encoding a biofilm-associated polysaccharide adhesin (PIA) lost the nematocidal activity, which led to decreased accumulation of bacteria in the *C. elegans* intestine (Begun et al. 2007). On the other hand, overexpression of the *icaADBC* operon in *S. aureus* increased virulence toward nematodes. Furthermore, biofilm formation is critical for bacterial survival inside the gut of nematodes. PIA-producing *Streptococcus epidermidis* has a significant survival advantage

Table 2. Predicted BcSinR-regulated biofilm genes in *Bacillus cereus* AR156^a

Gene ID	Log ₂ ($\Delta Bcspo0A$ /WT)	Log ₂ ($\Delta BcsinI$ /WT)	Annotation	Gene	Note ^b
ORF0296	-1.63	-1.67	GMP synthase	<i>guaA</i>	Yan et al. 2016
ORF0707	-5.11	-5.96	Ferrous iron transport protein	NA	Yan et al. 2016
ORF0708	-3.89	-3.84	Ferrous iron transport protein	NA	Yan et al. 2016
ORF1047	-4.96	-3.36	Transcriptional regulator Hpr	<i>hpr</i>	Madec et al. 2002
ORF1155	-2.02	-2.97	Catalase	<i>kata</i>	Ostress
ORF5445	-4.03	-3.41	Superoxide dismutase (Mn)	<i>sodA</i>	Ostress
ORF1638	-2.13	-3.15	Flagellar capping protein	<i>filD</i>	Chai et al. 2010
ORF1651	-5.98	-5.21	Flagellar hook protein FlgE	<i>filG</i>	Chai et al. 2010
ORF5263	-4.11	-4.54	UDP-glucose 4-epimerase	<i>galE</i>	Chai et al. 2012
ORF0344	-2.89	-3.50	Pyrroline-5-carboxylate dehydrogenase	<i>ycgN</i>	Chu et al. 2006
ORF0666	-3.02	-4.43	Immune inhibitor A precursor	<i>inhA</i>	Pflughoeft et al. 2011
ORF3882	-6.27	-5.71	Orotatephosphoribosyltransferase	<i>pyrE</i>	Yan et al. 2016
ORF3883	-6.49	-6.39	Orotidine 5'-phosphate decarboxylase	<i>pyrF</i>	Yan et al. 2016
ORF3884	-6.01	-5.71	Dihydroorotate dehydrogenase 1B	<i>pyrD</i>	Yan et al. 2016
ORF3885	-7.21	-6.66	Dihydroorotate dehydrogenase	<i>pyrK</i>	Yan et al. 2016
ORF3886	-7.07	-7.33	Carbamoyl phosphate synthase	<i>carB</i>	Yan et al. 2016
ORF3887	-8.48	-8.14	Carbamoyl phosphate synthase	<i>pyrAA</i>	Yan et al. 2016
ORF3888	-8.53	-7.30	Dihydroorotate	<i>pyrC</i>	Yan et al. 2016
ORF3889	-6.09	-6.02	Aspartatecarbamoyltransferase	<i>pyrB</i>	Yan et al. 2016
ORF3942	-3.80	-4.40	Cytochrome c oxidase polypeptide III	<i>catE</i>	OR
ORF3943	-5.87	-6.30	Cytochrome c oxidase polypeptide I	<i>catD</i>	OR
ORF3944	-4.00	-3.96	Cytochrome c oxidase polypeptide II	<i>catC</i>	OR
ORF3945	-2.97	-2.68	Protoheme IX farnesyltransferase	<i>catB</i>	OR
ORF4126	-7.33	-6.66	Ornithinecarbamoyltransferase	<i>argF</i>	AM
ORF4148	-8.79	-5.92	Arginine ATP-binding protein	<i>artR</i>	AM
ORF4149	-10.67	-7.10	Arginine ABC transporter permease	<i>artQ</i>	AM
ORF4150	-11.85	-8.20	Arginine-binding protein	<i>artP</i>	AM
ORF4629	-6.06	-5.75	Argininosuccinatelyase	<i>argH</i>	AsucM
ORF4630	-8.32	-6.43	Argininosuccinate synthase	<i>argG</i>	AsucM

^a WT = wild type and NA = not available.

^b Ostress = oxidative stress response, OR = oxidative respiration, AM = arginine metabolism, and AsucM = argininosuccinate metabolism.

over the *ica*-deficient mutant within the intestinal tract of *C. elegans* (Begun et al. 2007). Biofilm matrix components curli and cellulose could protect *E. coli* against killing by *C. elegans*. Matrix-encased bacteria at the air–biofilm interface exhibited a 40-fold increase in survival after *C. elegans* killing compared with the non-matrix-encased cells (DePas et al. 2014). Consistent with the above studies, our data in the present study suggested that biofilm formation by the biocontrol agent *B. cereus* AR156 was also very important for killing against the plant-parasitic nematode *M. incognita*. Colonization ability, mortality against J2 of *M. incognita*, and biocontrol efficacy were all significantly declined in the treatments by the biofilm-defective mutants compared with the wild-type strain.

It is worth noting that the killing activity was not completely lost in the $\Delta Bcspo0A$ and $\Delta BcsinI$ mutants of AR156 (Fig. 2A), indicating that a biofilm-independent mechanism may also function in nematode killing. In *Y. pestis* KIM5, four proteins—a protease OmpT, a metalloprotease Y3857, an adhesion protein Yaph, and a putative exported protein Y0340—were found to be associated with virulence on *C. elegans* in an independent process on biofilms (Styer et al. 2005). Hemolysin II, immune inhibitor A, and enhancin have been suggested as virulence factors toward the larvae of Lepidoptera and nematodes in *B. thuringiensis* and *Vibrio cholerae* (Cinar et al. 2010; Wang et al. 2016). Bioinformatics analyses indicated that there were several genes encoding the above factors in the genome of AR156. Genes encoding these factors were significantly downregulated in both the $\Delta Bcspo0A$ and $\Delta BcsinI$ mutants. This indicates that those virulence-related genes may also be regulated by the *spo0A/sinR/sinI* circuitry and contribute to nematode killing and biocontrol efficacy in addition to biofilm formation. Furthermore, the $\Delta Bcspo0A$ and $\Delta BcsinI$ mutants have a similar biofilm defect but differ significantly in mortality rate of the nematodes. This reinforces the idea that, in addition to biofilm formation, other unknown factors controlled by Spo0A also contribute to the nematocidal activity by AR156.

MATERIALS AND METHODS

Strains and media.

B. subtilis wild-type strain 3610, *B. cereus* wild-type strain AR156, and other derivatives were routinely grown in LB broth (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter of broth) at 37°C. Approximately 1.5% agar was included when making solid agar medium. LBGM medium was used for assays of biofilm formation. Sporulation assays were performed in DS medium (Schaeffer et al. 1965). Antibiotics were added at the following concentrations: tetracycline at 10 $\mu\text{g ml}^{-1}$, spectinomycin at 100 $\mu\text{g ml}^{-1}$, kanamycin at 20 $\mu\text{g ml}^{-1}$, chloramphenicol at 5 $\mu\text{g ml}^{-1}$, and erythromycin at 1 $\mu\text{g ml}^{-1}$. Chemicals were purchased from Sigma. Restriction enzymes and other enzymes for molecular cloning were obtained from New England Biolabs. All primers were ordered from IDT DNA technology. DNA sequencing was performed at Genewiz. All strains used in this study are described in Supplementary Table S5. All primers used in this study are listed in Supplementary Table S6.

Strain construction.

The *Bcspo0A* mutant strain was constructed by the double-crossover recombination following a procedure described in a previous study (Gao et al. 2015). To explain briefly, homologous regions of the upstream and downstream regions of the *Bcspo0A* gene were amplified from *B. cereus* AR156 genome using the primers *spo0A-Up-F/R* and *spo0A-Down-F/R*, respectively. These two DNA fragments were digested with

XbaI/BamHI and *PstI/XhoI*, respectively, and sequentially cloned into the pEBSK plasmid to create pEBSK ($\Delta Bcspo0A$). The long flanking DNA fragment (designated as Up-Km-Down) was amplified by polymerase chain reaction (PCR) using pEBSK ($\Delta Bcspo0A$) as the template and primers *spo0A-F* and *spo0A-R*. The fragment was then digested with *BglII* and *MluI*, and cloned into a temperature-sensitive *E. coli* and *Bacillus* shuttle vector pMAD (Arnaud et al. 2004) to generate pMAD ($\Delta Bcspo0A$). The pMAD ($\Delta Bcspo0A$) plasmid was mobilized into *B. cereus* AR156 by electroporation. Transformants were obtained and screened as described in the previously published protocol (Gao et al. 2015). All mutants were subsequently confirmed by PCR amplification and DNA sequencing to verify the presence of the *Bcspo0A::Km* allele on the chromosome. Replacement of *BcsinI* in the *B. cereus* AR156 chromosome with the kanamycin resistance cassette was carried out similarly to that of $\Delta Bcspo0A::Km$, except that different primers were used to amplify the upstream and downstream regions of the *BcsinI* gene.

The heterologous expression strains were generated as follows. For heterologous expression of the *BcsinI* gene at the ectopic *amyE* locus in the $\Delta BssinI$ mutant of *B. subtilis*, the *BcsinI* gene was amplified by PCR using AR156 genome DNA as the template and primers *BcsinI-F* and *BcsinI-R*. The PCR products were digested with *NotI* and *SacII* and inserted into the pYC151 plasmid (Chai et al. 2010), resulting in the recombinant plasmid pCY560. The pCY560 plasmid contains the *gfp-BcsinI* fusion under the control of a constitutively expressed promoter *hyperspank*. To express the *BcsinR* at the *amyE* locus in the $\Delta BssinR$ mutant of *B. subtilis*, the promoter and coding region of *BcsinR* was amplified from *B. cereus* AR156 using the primers *BcsinR-F* and *BcsinR*. The PCR fragment was digested with *EcoRI* and *BamHI* and inserted into the pDG1730 plasmid (Britton et al. 2002), resulting in the recombinant plasmid pCY558. To express the *Bcspo0A* gene under the *Bsspo0A* promoter at the *amyE* locus in the $\Delta Bsspo0A$ mutant of *B. subtilis*, the promoter region of *Bsspo0A* was fused with the *Bcspo0A* coding region using the overlapping PCR, and a fused fragment was inserted into the pDG1730 plasmid to generate the recombinant plasmid pCY559. All of the above recombinant plasmids were then introduced into PY79 by transformation (Gryczan et al. 1978). A double-crossover recombination at the *amyE* locus on the chromosome of PY79 was confirmed. The *amyE* fragments were then introduced into different strain backgrounds by SSP1 phage-mediated transduction.

Analysis of biofilm formation and pellicle biomass.

To analyze pellicle biofilm formation, cells were first grown in 3 ml of LB broth to $\text{OD}_{600} = 1$. Culture (3 μl) was added to 3 ml of LBGM medium in 12-well polyvinyl plates (VWR). The plates were incubated statically at 30°C for 24 to 48 h. For colony biofilm formation, 2 μl of the cells was spotted onto LBGM medium solidified with 1.5% agar. Plates were incubated at 30°C for 48 to 72 h prior to analysis. Images were taken by a Nikon CoolPix camera. The biomass of pellicle was analyzed following in a protocol that we previously described (Yan et al. 2016).

Assays of the swarm and sporulation efficiency.

For the swarm assay, a 5- μl cell suspension ($\text{OD}_{600} = 0.5$) of wild-type AR156 or mutants was inoculated on the center of 0.7% LB agar plates and incubated at 30°C for 12 h. Six plates were used for each individual strain. The experiments were repeated three times.

Heat-killing experiments were performed to compare the sporulation efficiency among the wild type, the mutants, and

the complementation strains of both *B. cereus* and *B. subtilis*. After growing in DS medium for 24 h (for *B. subtilis*) or 36 h (for *B. cereus*), cells were serially diluted and plated on LB agar media to determine the number of total cells by CFU counting. Diluted samples were then incubated in the water bath at 80°C for 20 min, briefly cooled, and plated on the LB agar media again. The next day, CFU were counted for cells before and after heat kill. Sporulation efficiency was calculated as the percentage of heat-resistant spores versus total cells. The spores generated in different strains were visualized by Leica AF6000 Modular microsystems.

Microscopic analysis.

Pellicle biofilms were visualized by SEM. Pellicles were harvested after 3 days of incubation in LBGM medium and fixed in a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.15 M phosphate buffer, pH 7.4, overnight. After fixation, tissues were rinsed three times (15 min each) with 0.1 M phosphate-buffered saline and dehydrated in graded ethanol solutions before critical point drying with CO₂. Examination of samples was performed in a Hitachi S-4700 field emission scanning electron microscope, and secondary electron images were collected as TIFF files. The structure of cells or spores of mutants after incubation in DS medium was examined by a transmission electronic microscope.

Transcriptional profiling.

B. cereus wild-type strain AR156, $\Delta BcsinI$, and $\Delta Bcspo0A$ were grown in LBGM to OD₆₀₀ = 2.0 and harvested for total RNA extraction. After the total RNA extraction and DNase I treatment, magnetic beads with oligo (dT) were used to remove ribosomal RNA from the total RNA. Samples were mixed with the fragmentation buffer, and the mRNA was fragmented into short fragments. The cDNA was then synthesized using the mRNA fragments as templates. Short fragments were purified and resolved in elution buffer for end reparation and single nucleotide A (adenine) addition. After that, the short fragments were linked with adapters. The modified cDNA library was sequenced using Illumina HiSeq 2000. Differential expression statistics reflecting both effective size (fold-change) and statistical significance (*P* value adjusted based on the method of Benjamini and Hochberg) were calculated using DESeq. Duplicate reads were excluded from the analyses. Differentially expressed genes were identified based on a minimum fold-change and maximum *P* value. The consensus DNA-binding sequences of BcSpo0A and BcSinR were analyzed by the MEME-CHIP program (Machanick and Bailey 2011).

Nematicidal activity

against tomato root-knot nematode disease.

To evaluate the nematicidal activity of *B. cereus* AR156 and its mutants against *M. incognita* in vitro, the J2 were cocultured with different strains for an indicated period of time, and the mortality rate of the worms was then calculated. Briefly, the *Bacillus* strains were grown in LB broth at 28°C for 24 h with shaking at 200 rpm, and cells were harvested and resuspended with sterilized water. The concentration of cell suspension was adjusted to 1×10^8 CFU/ml prior to use. J2 were prepared following Fusali's method (Fudali et al. 2013) and the number of J2 was adjusted to 4,000 J2/ml. Bacterial cell suspension (250 μ l) and J2 (250 μ l) were then mixed well in a 1.5-ml Eppendorf tube and incubated at 28°C for 24 h. After incubation, the number of live and dead worms in each treatment was counted by the stereomicroscope. The mortality rate was calculated according to the following formula: mortality rate = (number of dead worms/number of total worms) $\times$ 100%.

To quantify the number of bacterial cells on the worm body surface in coculture treatment, worms in each treatment were picked up with a needle after 24 h of incubation, soaked in sterile water, and vortexed for 5 min. Serial dilutions of a cell suspension were spread on LB medium supplemented with appropriate antibiotics. The number of bacterial cells per worm was analyzed by plating and CFU counting. Experiments were repeated three times.

Biocontrol efficacy

against tomato root-knot nematode disease.

Biocontrol efficacy of *B. cereus* AR156 and derivatives against *M. incognita* was conducted to control tomato root-knot nematode diseases under greenhouse conditions following the protocol that we published previously (Wei et al. 2010). Briefly, tomato seed ('Shanghai Cooperation 903', provided by the Chinese Academy of Agricultural Sciences) were surface sterilized, planted in sterilized soil in pots, and maintained in a greenhouse at 26°C for 4 weeks. Seedlings were transplanted to new pots (10 cm in diameter) containing fertilizer and then maintained at 26°C in a greenhouse with relative humidity of 40% and a photoperiod of 12 and 12 h for 2 days. The individual seedlings in each pot were first inoculated with AR156 or mutants by carefully pouring 40 ml of bacterial suspension (10^9 CFU/ml) into the rhizosphere of each plant. The inoculated pots were maintained under the same conditions as described above for 2 more days. Each pot was inoculated with 10 ml of a nematode suspension containing 600 freshly hatched J2. As a control, an equal amount of sterilized water instead of an antagonist suspension was added to the pots. Each treatment consisted of three replicates and each replicate had 24 plants. All pots were arranged in a randomized block design. The number of galls per root system was counted 56 days after the inoculation with nematodes. The gall indices were confirmed by using the 0-to-11 scale of Bridge and Page (1980) and biocontrol efficacy were calculated as described by Xue et al. (2009). The survival of the wild-type strain and the mutants in the rhizosphere soil was monitored over a period of time by following a previously published protocol (Chen et al. 2013).

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