Acetic Acid Acts as a Volatile Signal To Stimulate Bacterial Biofilm Formation

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ABSTRACT Volatiles are small air-transmittable chemicals with diverse biological activities. In this study, we showed that volatiles produced by the bacterium _Bacillus subtilis_ had a profound effect on biofilm formation of neighboring _B. subtilis_ cells that grew in proximity but were physically separated. We further demonstrated that one such volatile, acetic acid, is particularly potent in stimulating biofilm formation. Multiple lines of genetic evidence based on _B. subtilis_ mutants that are defective in either acetic acid production or transportation suggest that _B. subtilis_ uses acetic acid as a metabolic signal to coordinate the timing of biofilm formation. Lastly, we investigated how _B. subtilis_ cells sense and respond to acetic acid in regulating biofilm formation. We showed the possible involvement of three sets of genes (ysbAB, epsO, and yxaKC), all encoding putative holin-antiholin-like proteins, in cells responding to acetic acid and stimulating biofilm formation. All three sets of genes were induced by acetate. A mutant with a triple mutation of those genes showed a severe delay in biofilm formation, whereas a strain overexpressing _ywbHG_ showed early and robust biofilm formation. Results of our studies suggest that _B. subtilis_ and possibly other bacteria use acetic acid as a metabolic signal to regulate biofilm formation as well as a quorum-sensing-like airborne signal to coordinate the timing of biofilm formation by physically separated cells in the community.

IMPORTANCE Volatiles are small, air-transmittable molecules produced by all kingdoms of organisms including bacteria. Volatiles possess diverse biological activities and play important roles in bacteria-bacteria and bacteria-host interactions. Although volatiles can be used as a novel and important way of cell-cell communication due to their air-transmittable nature, little is known about how the volatile-mediated signaling mechanism works. In this study, we demonstrate that the bacterium _Bacillus subtilis_ uses one such volatile, acetic acid, as a quorum-sensing-like signal to coordinate the timing of the formation of structurally complex cell communities, also known as biofilms. We further characterized the molecular mechanisms of how _B. subtilis_ responds to acetic acid in stimulating biofilm formation. Our study also suggests that acetic acid may be used as a volatile signal for cross-species communication.
therefore, biofilm formation, the chemical nature of the putative ligand has not been characterized. Next to \( \text{slrA} \) is a two-gene operon consisting of \( \text{ywcB} \) and \( \text{ywcA} \) (\( \text{ywcB} - \text{ywcA} \)) whose function is unknown but which is predicted to encode a small-molecule transporter (Fig. 2A).

Biofilm formation of \( \text{B. subtilis} \) can be triggered by various environmental signals (25, 26). These putative signals have been investigated recently (8, 10, 27–31). In \( \text{B. subtilis} \), there are five histidine kinases (\( \text{KinA}, \text{KinB}, \text{KinC}, \text{KinD}, \text{and} \text{KinE} \)) that can potentially sense those signals and activate directly or indirectly...
the Spo0A master regulator by protein phosphorylation (Fig. 1) (21). In one recent study, KinA and KinB were proposed to sense redox signals in response to oxygen limitation (27). KinC, on the other hand, was shown to sense structurally diverse molecules, including those present in the environment as well as surfactins produced by *B. subtilis* itself (29). The results of several recent studies suggest that another histidine kinase, KinD, may directly or indirectly sense environmental or host signals and activate Spo0A when triggering biofilm formation (8, 10, 30). Those putative signals include small organic acids from the plant host and plant cell wall polysaccharides (8, 10). In addition to environmental signals, bacterial cellular metabolism also plays an important role in biofilm formation. Metabolic molecules constitute an important category of signals in triggering or fine-tuning biofilm formation. As a well-studied example, in some bacteria, accumulation of the ppGpp stress response alarmone during the stringent response was shown to regulate biofilm formation (32–34). Nucleotide-based secondary messengers represent another example of metabolically derived signals that can regulate biofilm formation in a variety of bacteria (35). In a recent study (36), we showed that in *B. subtilis*, intracellular levels of the amino acid serine function as both a proxy for nutrient conditions and a trigger for biofilm formation when the level of serine runs low (36). Serine limitation, which coincides with the entry into biofilm formation, caused lowered protein levels of SinR, the key transcription repressor of the matrix genes, probably by reducing the translation efficiency of the sinR mRNA mediated by ribosome pausing (36).

Holin-antiholin proteins were initially characterized as phage proteins that cause host cell lysis by stimulating the activity of murein hydrolases (37). It was later shown that some bacterial genomes include similar genes that resemble the phage counterparts (38). In *Staphylococcus aureus*, two sets of genes, *cidAB* and *lrgAB*, were shown to encode holin-antiholin-like proteins (Fig. 2B) (38, 39). These proteins were shown to be involved in programmed cell death and biofilm formation (38–41). Expression of *cidAB* is induced by acetic acid (39). The induction is mediated by an acetate-responsive, LysR-type transcription regulator, CidR, whose gene is transcribed divergently from *cidAB* (Fig. 2B) (42). *lrgAB* is regulated by the LysR-LyrT two-component system, which is proposed to sense changes in membrane potential in response to environmental stresses such as those represented by cationic antimicrobial peptides (42). Our search indicated that there are at least three sets of genes, *ywbHG*, *ysbAB*, and *yxaKC*, encoding putative holin-antiholin-like proteins in the genome of *B. subtilis* (Y. Chai, personal observations) (Fig. 2B). Among them, the *ywbHG* genes highly resemble *cidAB* in *S. aureus*, whereas the *ysbAB* genes share strong sequence similarity with *lrgAB*. Finally, the *yxaKC* genes seem to be uniquely present in *Bacillus* species. All three sets of genes are also conserved in *B. cereus* and *B. anthracis* (Y. Chai, personal observations), although a recent report suggested that the *B. anthracis* genome contained a fourth pair of such genes (43). In *B. subtilis*, the function and regulation of these genes are unknown.

A number of recent studies have shown that bacteria use volatile molecules to communicate with each other and to regulate bacterium-bacterium and bacterium-host interactions (44–47). For example, in one study (44), it was shown that both a *B. subtilis* strain and a *B. amylophiliqueficiens* strain produced a blend of volatile chemicals, including 2,3-butanediol and acetoin, to promote growth of *Arabidopsis thaliana*. Another study demonstrated that a *B. nematocida* strain was able to lure *Caenorhabditis elegans* by emitting a mixture of volatile chemicals, including benzaldehyde, chloromethyl, 2-pentanone, etc. (47). Bacterial volatiles were also shown to significantly alter the physiology of the neighboring cells from different species, as a way to mediate cross-species interactions (46). *B. subtilis* is a strong volatile producer. Several previous studies already identified a collection of volatiles produced by *B. subtilis* cells under different conditions that showed diverse activities in bacterium-bacterium and bacterium-host interactions (44, 47).

In this work, we show that biofilm formation by *B. subtilis* can be stimulated by bacterial volatiles and that acetic acid is a volatile that is potent in triggering early biofilm formation. We also present evidence that three pairs of genes, *ywbHG*, *ysbAB*, and *yxaKC*, encoding holin-antiholin-like proteins, may be involved in cells responding to acetic acid and stimulating biofilm formation. Our investigations on *B. subtilis* as well as our preliminary results from *S. aureus* implicate acetic acid in a broader role as a signal for bacterial multicellularity.

**RESULTS**

**Bacterial volatiles induce early biofilm formation in *B. subtilis***

In our studies of biofilm formation in *B. subtilis*, we occasionally observed that the timing of robust biofilm formation by *B. subtilis* could be influenced by the presence of neighboring *B. subtilis* cells even when the two populations of cells were physically separated from each other. In one such experiment (Fig. 3A), when *B. subtilis* cells were surrounded by physically separated neighboring cells (Fig. 3A, lower right-hand panel, showing 1 well of cells surrounded by 11 wells of cells), biofilm induction was earlier and more robust than when there were no surrounding cells (lower left-hand panel, showing 1 well of cells surrounded by 11 wells of media only). One possible explanation for this phenomenon is that neighboring cells produced unknown chemicals that influenced, via air transmission, the timing of biofilm formation of *B. subtilis* cells that grew in proximity. To further test whether there are such volatiles that can influence biofilm formation by *B. subtilis*, we developed a simple assay as illustrated in Fig. 3B. To describe the assay briefly, in a 6-well polystyrene plate (VWR), well a (top and side views in Fig. 3B) was used to preinoculate *B. subtilis* cells on day 1 to allow growth and volatile production. On day 2, a distant well (well b) was inoculated with fresh wild-type *B. subtilis* cells for pellicle biofilm development. The plate was sealed and incubated at 30°C for one more day. The result of pellicle formation in well b was recorded on day 3. A detailed description of the volatile assay is provided in Materials and Methods.

The results from our volatile assays showed that when well a was filled with medium only as a control, the wild-type *B. subtilis* cells in well b had just started to initiate pellicle formation on day 3 (Fig. 3C, no. 1). Interestingly, when well a was inoculated with *B. subtilis* cells, it clearly stimulated early pellicle formation of the cells in well b on day 3 (Fig. 3C, compare no. 1 and no. 2). A plausible explanation for the potent observation described above is that *B. subtilis* cells in well a produced volatiles that induced early pellicle formation by cells in well b. We point out that under both conditions, cells in well b eventually formed pellicle biofilms that were similarly robust (on day 4; data not shown), suggesting that the key difference between the two different conditions is likely the timing of biofilm induction.
To provide further evidence that the volatile effect can be better described as stimulating earlier and more-robust biofilm induction, and to more quantitatively compare the levels of pellicle biofilm development in the presence and absence of strong volatiles, we measured both the induction of the matrix genes and the accumulation of biofilm biomass at various time points during pellicle formation under the two conditions described above. Induction of the matrix genes was assayed by using cells bearing a promoter reporter for the epsA-O operon (P_{epsA-lacZ}), whereas biofilm biomass was assessed by measuring cell optical density (see Text S1 in the supplemental material). Our results show that the induction of expression of matrix genes in the presence of strong volatiles (conditions similar to those described for Fig. 3C, no. 2) (see diamond symbols in red in Fig. S1A) was stronger than the induction seen in the absence of strong volatiles (conditions similar to those represented in Fig. 3C, no. 1) (see Fig. S1A, square symbols in blue). In parallel, the measurement of cell optical density suggested that the number of cells in the pellicle biofilms modestly increased in the presence of strong volatiles (see Fig. S1B, diamond symbols in red) compared to the level seen in their absence (see Fig. S1B, square symbols in blue) at most given time points.

LBGM medium promotes strong production of biofilm-stimulating volatiles. In performing the volatile assays, we also learned that the types of media used to inoculate B. subtilis cells in well a were important. It appears that LBGM (lysogenic broth [LB] [10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter broth] plus 1% [vol/vol] glycerol and 0.1 mM MnSO₄) medium, previously characterized as a biofilm-inducing medium for B. subtilis (30), is an excellent medium for strong production of biofilm-stimulating volatiles (Fig. 3C, no. 2). In contrast, when LB medium was used, the biofilm-stimulating effect largely disappeared (Fig. 3C, no. 3). In our previous study (30), we showed that supplementation of glycerol and manganese converted LB, a biofilm-inert medium, to a medium that strongly induces biofilm formation for B. subtilis as well as several other Bacillus species. Additional features associated with growing B. subtilis cells in LBGM medium include strong pigment production and (perhaps) strong volatile production (judging by the presence of strong smells) (30). Results of our unpublished studies based on genome-wide transcriptomic analysis suggest that the presence of glycerol caused a major metabolic shift in that several dozens of metabolic genes involved in fermentation and production of small volatile products (such as lactic acid, acetic acid, acetoin, 2,3-butanediol, etc.) were strongly induced in the cells (Y. Chai, unpublished data). We are currently exploring the molecular basis for this “glycerol effect.” Our findings presented here further explain why LBGM medium is a medium that strongly induces biofilm formation, since the yet-to-be-identified volatile(s) may function as a self-stimulating signal for biofilm formation.

We decided to further look into the LBGM medium, trying to understand how it promotes strong production of biofilm-
stimulating volatiles. Wild-type *B. subtilis* cells growing in LBGM medium showed little difference in doubling time from the cells growing in LB (Fig. 4A). However, a clear difference was seen in the changes of the pH values of the media over time during growth (Fig. 4B); when *B. subtilis* cells were grown in LBGM medium, the medium became acidic and the pH dropped to as low as 5.2 after 8 h of growth (squares in Fig. 4B), whereas the pH fluctuated only mildly over time when *B. subtilis* cells were grown in LB (diamonds in Fig. 4B). This indicates that more acids were being produced when cells were grown in LBGM medium.

One of the acids predicted to result from overflow metabolism when *B. subtilis* cells are grown in LB plus glycerol is acetic acid (48). We thus compared levels of acetate accumulation in *B. subtilis* cells grown in either LBGM medium or LB (see Materials and Methods). As expected, our results confirmed that acetic acid accumulation was much higher when *B. subtilis* cells were grown in LBGM medium (reaching ~0.55 g/liter after 8 h) (squares in Fig. 4C) than when they were grown in LB (reaching ~0.18 g/liter after 8 h) (diamonds in Fig. 4C). Since more acetic acid was being produced in LBGM medium and since acetic acid is a known volatile, we infer that more acetic acid appears in the volatile mixture when *B. subtilis* cells grow in LBGM medium. Our investigations led us to conclude that LBGM medium promotes strong production of biofilm-stimulating volatiles and, coincidently, strong production of volatile acetic acid. Interestingly, two other biofilm-inducing media, MSgg (5 mM potassium phosphate [pH 7], 100 mM morpholinopropanesulfonic acid [pH 7], 2 mM MgCl₂, 700 μM CaCl₂, 50 μM MnCl₂, 50 μM FeCl₃, 1 μM ZnCl₂, 2 μM thiamine, 0.5% glycerol, 0.5% glutamate, 50 μg ml⁻¹ tryptophan, 50 μg ml⁻¹ phenylalanine, 50 μg ml⁻¹ threonine) and 2× SGG (2× SG [0.05% MgSO₄ · 7H₂O, 0.2% KCl, 1.6% Difco nutrient broth, 0.1% glucose, 0.0001 mM FeSO₄, 0.1 mM MnCl₂ · H₂O, 1 mM Ca(NO₃)₂] medium supplemented with 1% glycerol), frequently used in biofilm studies of *B. subtilis*, also contain significant amounts of glycerol (0.5% [vol/vol]). Volatiles produced from cells growing in those two biofilm media similarly stimulated early pellicle formation (see Fig. S2 in the supplemental material).

**Acetic acid is a potent volatile in stimulating biofilm formation.** On the basis of the results described above, we further speculated that, within the hypothetical volatile mixture, acetic acid could be one such potent molecule stimulating biofilm formation. To explore this idea, we repeated the volatile experiment by testing whether the activity of pure acetic acid is sufficient to mimic the biofilm-stimulating activity of the volatile mixture produced by *B. subtilis*. We added 1% pure acetic acid to well a. As shown in Fig. 3C (no. 5), acetic acid exerted a biofilm-stimulating effect similar to what was seen with the hypothetical volatile mixture (Fig. 3C, compare no. 2 and 5). To test the specificity of this effect, we also applied other volatile acids as controls, including pyruvic acid, lactic acid, propionic acid, and formic acid. The same amounts of pyruvic acid and lactic acid did not show similar stimulating effects (Fig. 3C, no. 6 and 7). Formic acid had a mild biofilm-stimulating affect. Interestingly, propionic acid showed a biofilm-stimulating effect that was at least as strong as that of acetic acid (Fig. 3C, compare no. 5 and 8). We do not yet know whether LBGM medium also promotes production of propionic acid. In *toto*, our results suggest that volatiles produced by *B. subtilis* cells have a stimulating effect on biofilm formation and that acetic acid is one such volatile, being produced at high levels and capable of biofilm stimulation.

To provide further genetic evidence, we constructed a *B. subtilis* ΔackA mutant. The *ackA* gene encodes an enzyme, acetyl kinase, which converts acetyl-phosphate to acetyl and plays a key role in acetic acid production (Fig. 5A) (49). The ΔackA mutant is thought to be largely deficient in acetic acid production. By using an assay to measure acetate accumulation (see Materials and Methods), we were able to confirm that the ΔackA mutant failed to produce and accumulate detectable levels of acetate (Fig. 5B). We next applied the ΔackA mutant to the volatile experiment and found that the volatiles produced by the ΔackA mutant stimulated early pellicle formation only very mildly (Fig. 3C, compare to no. 4). The weak biofilm-stimulating activity of the volatiles produced by the ΔackA mutant could have been due to the presence of other volatile molecules that either had lower stimulating activity or were produced at lower levels. This result further supported the idea that acetic acid functions as a strong volatile signal to stimulate biofilm formation.

In addition to being a volatile molecule that acts on cells that are physically separated (but growing in proximity) in the community as we have shown above, acetic acid may also function as a canonical quorum-sensing-like molecule that is produced by cells during growth, is accumulated in the environment, and activates particular targets when its concentration reaches a threshold. If this hypothesis is correct, we should be able to predict not only that genetic mutants deficient in acetic acid production would be unable to stimulate early biofilm formation through volatiles, as shown in Fig. 3C (no. 4, ΔackA cells), but also that those mutants might themselves show a delay in biofilm formation. To test that, we constructed another *B. subtilis* mutant, the Δpta strain, which is mutated for the gene encoding the enzyme phosphotransacetylase (Pta) that converts acetyl-coenzyme A (CoA) to acetyl-phosphate and is therefore blocked in acetic acid production (Fig. 5A) (49). Similarly to the ΔackA mutant results, we were able to confirm that the Δpta mutant is also largely deficient in acetic acid production (Fig. 5B). The residual levels of acetic acid detected in the Δpta mutant could have been due to generation of acetyl-P (and thus acetate) from pyruvate by pyruvate oxidase (YdaP in *B. subtilis* resembles pyruvate oxidase; Y. Chai, personal observation), a reaction that is independent of Pta activity.
We next compared the levels of pellicle biofilm formation by the wild type, the $\Delta^{pta}$ mutant, and the $\Delta^{ackA}$ mutant (Fig. 5C). Our results showed that the $\Delta^{pta}$ mutant and the $\Delta^{ackA}$ mutant showed similar delays in pellicle formation compared to the wild type (upper panels, Fig. 5C). For both the $\Delta^{pta}$ mutant and the $\Delta^{ackA}$ mutant, the delayed pellicle formation was able to be rescued by complementation of the mutation by a wild-type copy of the gene integrated at the ectopic $amyE$ locus (see Fig. S3 in the supplemental material). More interestingly, when 0.2% sodium acetate (NaAc) was added to the medium, it largely rescued the delay of pellicle formation by the two mutants as well (lower panels, Fig. 5C). Rescues were not seen when either 0.2% sodium lactate or 0.2% sodium pyruvate was added to the media as a control (see Fig. S4). These results reinforced the idea that accumulation of acetic acid signals the timing of biofilm induction in $B. \text{subtilis}$. Lastly, another acetic acid metabolic mutant, the $\Delta^{acsA}$ mutant, which is blocked in the conversion from acetate to acetyl-CoA (Fig. 5A), did not show any loss of acetic acid production or any biofilm phenotype (Fig. 5B and data not shown).

### Acetate enhances expression of the matrix genes

We showed that addition of 0.2% NaAc to the medium was largely able to rescue the delay of pellicle formation by the $\Delta^{ackA}$ and $\Delta^{pta}$ mutants (Fig. 5C). In the same experiment, we also noticed that adding 0.2% NaAc to the wild-type $B. \text{subtilis}$ cells in LBGM medium stimulated early and robust biofilm formation (Fig. 5C and 6A). As a control, adding the same amount of sodium chloride (NaCl) did not show any stimulating effect (Fig. 6A), indicating that the stimulating effect was due to acetate. We also tested other types of acetate salts (e.g., potassium acetate). They showed similar biofilm-stimulating activities (data not shown). These results suggest that acetate salts can directly stimulate early and robust biofilm formation.
To investigate whether the biofilm-stimulating effect shown by acetate salts was due to some indirect effect or was a result of acetate promoting expression of biofilm-specific genes, we examined the effect of acetate salts on expression of the matrix genes. To do so, we assayed the activities of the wild-type cells and the ΔywcBA cells harboring either the PtapA-lacZ transcriptional reporter (B) or the PepsA-lacZ transcriptional reporter (C). (D) Assays of β-galactosidase activities of the strains (FY250, FY251, and YC259) bearing one of the three promoter fusions (PtapA-lacZ, PepsA-lacZ, and PywcBA-lacZ, respectively) for the three holin-antiholin operons. (E) Pellicle formation by the wild type, the three single mutants for the holin-antiholin-encoding genes (CY819 [ΔywbHG], CY881 [ΔysbAB], and CY882 [ΔyxakC]), the triple mutant (CY886), and the strain overexpressing ywbHG (CY1250) inoculated in LBGM medium after 24 h of incubation at 30°C.

The ywcB-ywcA operon, predicted to encode a putative acetic acid transporter, is involved in biofilm formation. Since mutations blocking acetic acid production can cause a delay in biofilm formation in B. subtilis (Fig. 5C), one may presume that mutations blocking acetic acid transportation may alter acetate accumulation and therefore the timing of biofilm formation as well. Unfortunately, although acetic acid metabolism has been well studied in some bacteria (49), the dedicated transportation system for acetic acid has not been identified except that Gimenez et al. reported that, in Escherichia coli, the yjcHG genes encode a sodium-dependent acetic acid symporter (Fig. 2A) (52). We performed a BLAST search of the B. subtilis genome, looking for genes homologous to yjcHG, and identified the ywcBA genes in B. subtilis that share strong sequence similarity with yjcHG in E. coli (39% sequence identity and 58% similarity between ywcA and yjcG, Fig. 2A). Our search also revealed that these two genes are highly conserved in different bacteria, including important pathogens such as Pseudomonas aeruginosa and S. aureus (for S. aureus, the homologous genes are present in only a few clinical isolates; Y. Chai, unpublished observations) (Fig. 2A). We hoped to point out that in B. subtilis, the ywbBA genes lie next to slrA and ywcC, whose protein products constitute the YwcC-SlrA pathway for derepression of SinR-controlled matrix genes and thereby biofilm formation (Fig. 1 and 2A) (23).

If the ywcBA genes indeed encode an acetic acid transporter, we would predict that mutations in the ywcBA genes might alter acetate acid accumulation in the cells and thus result in an altered biofilm phenotype. To test that, we constructed a B. subtilis mutant with an insertional deletion mutation in the ywcBA genes and compared the levels of pellicle formation by the wild type and the ΔywcBA mutant. Interestingly, the mutant did show early pellicle formation in both LBGM medium and another biofilm-inducing medium, MSgg (Fig. 7A). To test whether early pellicle formation was due to some indirect effect or was due to an alteration in
matrix gene expression, we again introduced the two transcriptional reporters for the matrix operons into the wild type and the ΔywcbA mutant and compared the levels of expression of the matrix genes in the two strains by measuring the activities of the two transcriptional fusions (P_ywcB-lacZ [Fig. 7B] and P_ywcA-lacZ [Fig. 7C]). Our results showed that both reporters were expressed at higher levels in the ΔywcbA mutant than in the wild type, suggesting that early pellicle formation was an outcome of elevated expression of the matrix genes (Fig. 7B and C).

How can the early pellicle formation shown by the ΔywcbA mutant be interpreted? As we speculated above, one possibility could be that there was an early accumulation of intracellular acetic acid due to blocking of acetic acid exportation and therefore that it triggered early biofilm formation. (A second possibility could be the opposite, that is, that after glycerol depletion, import and reutilization of acetate were blocked in the ΔywcbA mutant and lack of intracellular acetate triggered early biofilm formation. The latter possibility seems less likely based on the observation that matrix genes can be induced by exogenously added acetate [Fig. 6].) To test the hypothesis presented above and to explore the putative role of Ywca-Ywcb in acetic acid transportation, we conducted two specific assays. In the first assay, we compared the levels of secretion of acetic acid by the wild type and the ΔywcbA mutant by measuring acetic acid accumulation in the media during growth. Our assumption was that if acetic acid secretion were blocked due to mutations in the ywcbA genes, we would see less accumulation of extracellular acetic acid during growth of the ΔywcbA mutant. Cells of the wild type and the ΔywcbA mutant were grown in LBGM medium, and culture supernatants were collected when the OD600 of the cultures reached 3.0. Concentrations of acetic acid in the supernatants were measured following the method described in Materials and Methods. As shown in Fig. S5A in the supplemental material, we saw a modest (~19%) decrease in acetic acid accumulation in the medium with the ΔywcbA mutant compared to the wild type, indicating that Ywcb-Ywca may act as an acetic acid transporter, but even so, there must be another transporter(s) involved in acetic acid secretion here.

In the second experiment, we compared the levels of importation of acetate by the wild type and the ΔywcbA mutant. We grew the wild type and the ΔywcbA mutant in a minimal medium with a limited amount of glutamate (0.5%) as both the sole carbon source and the sole nitrogen source. We then added 0.1% NaAc to the medium and measured the level of the remaining acetate in the medium periodically for 6 h. Our result showed that both the wild type and the ΔywcbA mutant were able to take up acetate, since the levels of the remaining acetate in the media dropped continuously, but that the ΔywcbA mutant showed a modestly lower uptake rate (see Fig. S5B in the supplemental material). Taken together, our results indicate that the ywcbA operon may encode a putative acetic acid transporter in B. subtilis but may not be the only one that is involved in acetic acid transportation. This finding was not a complete surprise to us, since it was previously shown in E. coli that the yjcHG operon was still able to effectively take up acetic acid, indicating the existence of another transportation system for acetic acid in E. coli (52). Subsequently, a second acetic acid transporter (YaaH) was identified in E. coli. The ability to transport acetic acid was completely abolished for the yaaH yjcGH double mutant, which did not grow on minimal media with acetate as the sole carbon source (53). Unfortunately, no gene homologous to yaaH was found in the B. subtilis genome (Y. Chai, personal observation).

Thus far, results from our investigations have tended to support the idea that YwcbA may be involved in acetic acid transportation in B. subtilis. However, strong biochemical evidence will be needed in the future to demonstrate the direct involvement of the YwcbB-Ywca proteins in acetic acid transportation. In addition, in E. coli, the homologous proteins Yjch-YjcG were investigated previously only for their role in the import of acetic acid (52), whereas in our studies, we showed that YwcbB-Ywca could play a role in both the import and export of acetic acid. Many studies have demonstrated the importance of similar sodium symporters in the import of substrates (often against the substrate concentration gradient and in a manner dependent on a sodium ion gradient as the energy source) (54). The importance of such symporters in the export of substrate remains less clear.

**Putative holin-antiholin-encoding genes are induced by acetic acid and involved in biofilm formation.** The next issues we wanted to test were how B. subtilis cells sense and respond to acetic acid and how acetic acid ultimately stimulates biofilm formation. In S. aureus, two sets of genes shown to be involved in biofilm formation and induced by acetic acid are cidAB and lrAgAB, both encoding putative holin-antiholin-like proteins (Fig. 2B) (39, 41, 42). Expression of cidAB is controlled by a LysR-type transcriptional regulator, CidR, in response to acetic acid (Fig. 2B) (42). As we discussed earlier, the B. subtilis genome contains three sets of genes, ywbHG, yshAB, and yxaKC, encoding putative holin-antiholin-like proteins, and yet their function and regulation are completely unknown (Fig. 2B). In a parallel work designed to characterize genes in B. cereus that are induced under biofilm conditions by using genome-wide transcriptional analyses, we found that the genes that were most highly induced during growth of B. cereus cells in LBGM medium included three genes (B. cereus 5133 [BC5133], BC5438, and BC5439; B. cereus ATCC 14579 was used as a reference genome) that are homologous to lrAgA, lrAgB, and cidB, respectively (Y. Chai, unpublished results).

In line with the evidence described above, we decided to investigate the possible involvement of these putative genes in responding to acetic acid and stimulating biofilm formation in B. subtilis. We conducted two experiments. In the first experiment, we wanted to test whether any of these genes might affect biofilm formation. We constructed several mutants that were able to take up acetate, since the levels of the remaining acetate in the media dropped continuously, but that the ΔywcbA mutant showed a modestly lower uptake rate (see Fig. S5B in the supplemental material). Taken together, our results indicate that the ywcbA operon may encode a putative acetic acid transporter in B. subtilis but may not be the only one that is involved in acetic acid transportation. This finding was not a complete surprise to us, since it was previously shown in E. coli that the yjcHG operon was still able to effectively take up acetic acid, indicating the existence of another transportation system for acetic acid in E. coli (52). Subsequently, a second acetic acid transporter (YaaH) was identified in E. coli. The ability to transport acetic acid was completely abolished for the yaaH yjcGH double mutant, which did not grow on minimal media with acetate as the sole carbon source (53). Unfortunately, no gene homologous to yaaH was found in the B. subtilis genome (Y. Chai, personal observation).

Thus far, results from our investigations have tended to support the idea that YwcbA may be involved in acetic acid transportation in B. subtilis. However, strong biochemical evidence will be needed in the future to demonstrate the direct involvement of the YwcbB-Ywca proteins in acetic acid transportation. In addition, in E. coli, the homologous proteins Yjch-YjcG were investigated previously only for their role in the import of acetic acid (52), whereas in our studies, we showed that YwcbB-Ywca could play a role in both the import and export of acetic acid. Many studies have demonstrated the importance of similar sodium symporters in the import of substrates (often against the substrate concentration gradient and in a manner dependent on a sodium ion gradient as the energy source) (54). The importance of such symporters in the export of substrate remains less clear.
genes encoding holin-antiholin-like proteins may be collectively involved in regulation of biofilm formation, possibly by the control of the timing for biofilm induction.

**Overexpression of ywbH operon stimulates early and robust biofilm formation.** In *S. aureus*, strong induction of the *cidAB* genes was shown to stimulate biofilm formation (38, 41). The *ywbH* genes of *B. subtilis* are homologous to *cidAB* (Fig. 2B). The *ywbH* genes sit next to *ywbI*, a gene homologous to *cidR* encoding a putative acetate-responsive, LysR-type transcription regulator (Fig. 2B and 8A). *ywbHG-ywbI* are also located five genes away from *ywcBA* (Y. Chai, personal observation) (55). We were thus interested in understanding the exact role of ywbHG in responding to acetic acid and stimulating biofilm formation. We decided to first test whether the induction of *ywbH* genes is mediated by the YwbI LysR-type regulator, as was shown in *S. aureus*. To test this, the transcriptional reporter for the *ywbHG* genes (*P*ywbH-lacZ*) was also introduced into a strain deleted for the *ywbI* gene. NaAc or KAc (0.2%) was added to the shaking LB inoculated with the reporter strains (wild type and Δ*ywbI*), and the β-galactosidase activities of the cells were measured. As shown in Fig. 8B, both NaAc and KAc significantly induced expression of the *P*ywbH-lacZ fusion (white bars, wild type). Induction of the *P*ywbH-lacZ reporter by acetate salts was also dependent on the presence of the YwbI regulatory protein, since no induction was observed in the *ywbI* mutant (gray bars, Δ*ywbI*). These results suggest that the regulation of the *ywbHG-ywbI* genes in *B. subtilis* and that of the *cidAB-cidR* genes in *S. aureus* are similar to each other.

The activity of the reporter-bearing wild-type cells was also tested on LB agar plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in the absence or presence of 1% glycerol. The result seemed to show quite clearly that glycerol also strongly induced *P*ywbH-lacZ expression, as colonies of the reporter cells turned dark blue on the X-Gal plates in the presence of glycerol (Fig. 8C). This result suggests that when *B. subtilis* cells are grown in the biofilm-inducing LBGM medium, the *ywbH* genes are strongly induced. Finally, to test whether induction of *ywbHG* by acetate is the reason that acetate can stimulate biofilm formation, we constructed a *B. subtilis* strain that overexpresses *ywbHG* under the control of an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter. The resulting strain, CY1250, showed early and very robust biofilm formation upon addition of IPTG (Fig. 7E), suggesting that induction of *ywbHG* by acetate can at least partly explain the acetate-mediated biofilm stimulation seen in this study.

**Acetate stimulates biofilm formation in *S. aureus***. Finally, an issue that may have potentially broad interest is whether other bacteria also use acetic acid as a biofilm-inducing signal. We reasoned that it is possible because the putative genes involved in acetic acid metabolism and transportation and the genes encoding holin-antiholin-like proteins are highly conserved in other bacteria (Fig. 2). Furthermore, it was already shown in *S. aureus* that the *cidAB* and *lrgAB* genes are induced by acetic acid and are involved in programmed cell death and biofilm formation (39, 41, 42). Our unpublished results from the genome-wide transcriptome analyses (Y. Chai, unpublished results) also suggest that some of those genes in *B. cereus* are among the ones most highly induced under biofilm-inducing conditions (LBGM medium).

We selected *S. aureus* HG003, a strain capable of forming biofilms, to test whether *S. aureus* also uses acetate as a biofilm-inducing signal. Note that the biofilms formed by *S. aureus* are submerged, surface-attached biofilms (see Materials and Methods and Fig. 9) that are different from the floating pellicles formed by *B. subtilis*. We first tested whether acetate salts can stimulate biofilm formation in *S. aureus*. To do so, HG003 cells were inoculated in tryptic soy broth (TSB) supplemented with 1% glucose in 24-well polystyrene plates (VWR) to allow formation of surface-attached biofilms (see Materials and Methods). In parallel, NaAc at different concentrations (0.2%, 0.5%, and 1%) was added to the media in order to test whether acetate stimulates biofilm formation. As shown in Fig. 9A, *S. aureus* cells seemed to form more surface-attached biofilms at the bottom of the wells in the presence of increasing amounts of NaAc. A semiquantitative analysis showed approximately 1.4-, 4.5-, and 10.7-fold increases in the biofilm biomass in the presence of 0.2%, 0.5%, and 1% NaAc, respectively, compared to that seen in the absence of NaAc (Fig. 9B). We concluded that acetic acid has stimulatory activity with respect to biofilm formation in *S. aureus* as well. This result is also consistent with findings from a recent study that suggested that production of acetic acid from overflow metabolism and weak-acid properties of acetic acid play important roles in *S. aureus* programmed cell death and biofilm development (51).

**DISCUSSION**

In this study, we showed that *B. subtilis* employs self-produced volatiles as a way to coordinate multicellular development for cells that are physically separated in the community. Our results suggest that acetic acid is one such potent volatile stimulating biofilm formation in *B. subtilis*. We are currently investigating the amount of acetic acid in the volatile mixture and what other volatile chemicals are present in the volatile mixture under our biofilm-
Acetic acid has a variety of known biological functions. In bacteria, intracellular acetic acid has also been implicated as a metabolic signal to coordinate the timing of biofilm formation in the population (Fig. 1). In some ways, this is similar to the behavior seen with quorum-sensing molecules. The really interesting difference appears to be that the signal can be airborne. This also makes very good sense for bacteria during biofilm development since volatiles can provide an efficient method of cell-cell communication among discrete colonies of cells sharing the same community and since cells in natural environments do not always grow in a continuously aqueous environment that permits efficient diffusion of signal molecules.

Bacteria are known to produce and secrete acetic acid (56). Acetic acid is a common byproduct of overflow metabolism when cells grow in media supplemented with glucose or with other favorable carbon sources such as glycerol (Fig. 1) (48, 49). Acetic acid has a variety of known biological functions. In bacteria, intracellular acetic acid has also been implicated as a metabolic signal (56). However, characterizing the cellular targets of acetic acid is often challenging. In this study, we showed that acetic acid strongly induced expression of ywbHG, the genes encoding putative holin-antiholin-like proteins, in B. subtilis and that overexpression of ywbHG stimulated early biofilm formation. We suggest that enhanced expression of ywbHG in response to acetic acid may be one of the main reasons for acetic acid-induced early biofilm formation (Fig. 1). Other holin-antiholin-like genes (ysbAB and yxaKC) may also be involved in biofilm formation since the triple mutant had the most severe delay in biofilm formation in the comparisons with the individual single mutants. Our findings are also consistent with the observations from previous studies in S. aureus showing that some of the bacterial holin-antiholin-like genes (cidAB) can be induced by the metabolic signal acetic acid.

Holin-antiholin proteins were originally characterized as phage proteins that are involved in host cell lysis in the lytic life cycle of phage (37). Previous studies showed that some bacteria also include holin-antiholin-like genes in their genomes. These bacterial proteins are thought to regulate the activity of murein hydrolases in a similar fashion. The biological function and regulation of these bacterial proteins are relatively well studied in S. aureus (39, 41, 42). There is genetic and molecular evidence suggesting that these proteins are involved in programmed cell death and biofilm formation in S. aureus (39, 41, 42). A link was also proposed between programmed cell death and biofilm formation in S. aureus (41, 51) in that programmed cell death leads to cell lysis in some localized regions within the biofilm and to release of extracellular DNA (eDNA) and likely proteins as well. eDNA and proteins released from cell lysis become part of the biofilm matrix and thus contribute to biofilm formation (41, 57). It seems that contributions to programmed cell death and release of eDNA and/or proteins are the main reasons that those holin-antiholin-like genes are involved in biofilm formation in S. aureus. How these putative genes contribute to biofilm formation in B. subtilis is not known. It is also not known whether localized cell death and eDNA release occur in a significant fashion during B. subtilis biofilm formation. In this study, we briefly attempted to address some of these issues by examining the presence of eDNA during B. subtilis biofilm formation. We performed a simple assay to detect and compare levels of eDNA release during biofilm development in the wild type and the triple mutant by reverse transcription-quantitative PCR (RT-qPCR) (see Text S1 in the supplemental material). Our results showed that although there were biofilm-associated eDNAs present in the samples, no significant difference in eDNA abundance (<30%) was observed between samples of the wild type and the triple mutant (see Fig. S6 in the supplemental material). Thus, it is unclear whether eDNA release is a significant feature in B. subtilis biofilm development. Further studies will be needed to address these issues.

One recent study further investigated the link between acetate-mediated programmed cell death and biofilm development in S. aureus (51). In that study, the authors showed that acetic acid production from overflow metabolism played a very important role in regulating programmed cell death and biofilm formation. The authors attempted to explicate the molecular basis of the acetate-mediated effects and came to the conclusion that the trigger of programmed cell death depends on synergistic activities of the weak-acid properties of acetate and increasing levels of reactive oxygen species (ROS). This study suggests a broader link between cell overflow metabolism and programmed cell death and biofilm formation. The importance of the weak-acid properties of acetic acid was also implied in several previous studies (57–59). In our biofilm studies, we primarily used LBGM medium, a medium with excess (1%) glycerol. Our observation of media acidification between decreasing media pH and increasing numbers of cells experiencing oxidative stress (measured by the use of a cellular reporter for detection of DNA damage) (Y. Chai, unpublished results). All these results suggest the presence of such a link be-
between cell overflow metabolism and formation of multicellular communities in bacteria. The metabolic pathway of acetic acid also involves intermediate products such as acetyl-CoA and acetyl-phosphate (Fig. 1 and 5A). The putative biological functions of acetyl-phosphate have been investigated recently (60–62). It was proposed previously that in some bacteria such as _E. coli_, acetyl-phosphate acts as an important global signal and as a phosphor/acyetyl donor for protein modifications and other important regulations (60, 61). Multiple studies showed that acetyl-phosphate acts to directly modify key regulatory proteins whose activities are controlled by either protein acetylation or phosphorylation (60, 61, 63). In present report, we cannot rule out the possibility that the intermediate products of acetate metabolism, especially acetyl-phosphate, may be involved in regulating biofilm formation since activation of key regulators such as Spo0A and ComA by protein phosphorylation is critical to biofilm induction in _B. subtilis_ (5). There was at least one study showing that acetyl-phosphate can directly regulate the activity of ComA by acting as a phosphatase donor for ComA protein phosphorylation (64). ComA is the responsive regulator of the ComA-ComP two-component system, which is involved in regulating surfactin production (62). Surfactin in turn functions as a quorum-sensing molecule for biofilm formation in _B. subtilis_ (29). In future studies, it will be interesting to investigate the possible role of acetyl-phosphate in regulating biofilm formation in _B. subtilis_.

MATERIALS AND METHODS

Strains and media. _Bacillus subtilis_ strain 3610 and its derivatives were routinely cultured in tryptic soy broth (LB) (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter broth) at 37°C. For assays of biofilm formation, three different biofilm-inducing media, LBGM medium, MSgg, and 2× SGG, were used. LBGM medium is composed of LB supplemented with 1% glyceral (vol/vol) and 100 μg/ml MnSO 4·H 2O. The recipes for MSgg and 2× SGG were described in previous publications (6, 23). _Staphylococcus aureus_ was grown in brain heart infusion medium (BHI; Difco) for colony proliferation. For biofilm formation of _S. aureus_, tryptic soy broth (TSB; Difco) was used with the supplementation of 1% glucose. Lists of strains, plasmids, and oligonucleotides used in this work are presented in Table S1 in the supplemental material. Chemicals were purchased from Sigma. All ingredients of media were purchased from Difco. An acetate assay kit (catalog no. 10148261035) was purchased from Biopharm (a Roche company), United Kingdom.

Strain and plasmid construction. We followed published protocols for general methods of molecular cloning (65). SSP1 phage-mediated general transduction was used to transfer antibiotic-marked DNA fragments among different strains of _B. subtilis_ (12, 66). Long-flanking PCR mutagenesis was applied to generate insertional deletion mutations (67). The primers used to generate insertional deletion mutations of ywcBA, ywHBa, yshAB, yxakC, and ywbl genes and used to supplement the Δyna and ΔhekA mutations are described in Table S1 in the supplemental material. Details of strain constructions are described in Text S1.

The volatile assay. In the 6-well polyvinyl plates (VWR), well a (shown in the top and side views in Fig. 3B) was used to preincubate 10⁶ cells of wild-type or mutant _B. subtilis_ per ml on day 1 to allow growth. On day 2, a distant well b was freshly inoculated with 10⁶ cells of wild-type _B. subtilis_ per ml for pellicle development. Meanwhile, well a had already started to show robust cell growth and volatile production. Four glass beads (3 mm in diameter) were added between the lid and the bottom part of the plate (one at each corner of the plate) with the purpose of creating a headspace shared among the wells. The plate was then sealed with Parafilm (Bemis, WI) and cultured at 30°C for another day. On day 3, the plate was unsealed and pellicle formation of the wild-type _B. subtilis_ cells in well b was recorded. For addition of the pure acids, they were directly diluted into appropriate media at a final concentration of 1% (vol/vol). In some experiments, when the defined MSgg medium was used to inoculate volatile-producing cells into well a, plates were incubated at 30°C for 2 days (instead of being incubated for 1 day as when LBGM medium, LB, or 2× SGG was used) before wild-type _B. subtilis_ cells were inoculated into well b with the purpose of allowing robust growth and strong volatile production by cells in well a since _B. subtilis_ tends to grow slower in MSgg. In some experiments (as shown in Fig. 3A), a slightly different assay was performed to assess the influence of volatiles from neighboring cells on pellicle formation. The 12-well polystyrene plates (VWR) were used to inoculate _B. subtilis_ cells into 1 or 4 or 12 wells in the plate, while the rest of the wells were filled with medium only. The plates were sealed and incubated at 30°C for 1 to 2 days. Images of the pellicle biofilms (in the corner well) were similarly taken.

Assays of pellicle formation. Cells were grown in LB with shaking at 37°C to mid-log phase. For pellicle formation, 9 μl of the cells was mixed with 9 ml of LBGM broth and the mixture was added to the 6-well plates (VWR). Plates were incubated at 30°C for an indicated period of time. Images were taken using a Nikon CoolPix S9200 digital camera.

Biofilm assays for _S. aureus_. Details of the biofilm assay for _S. aureus_ HG003 are provided in Text S1 in the supplemental material.

Assays of β-galactosidase activities. Details for the assay of the β-galactosidase activities are provided in Text S1 in the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at mbio.asm.org/lookup/suppl; doi:10.1128/mBio.00392-15/-/DCSupplemental.

Text S1, PDF file, 2.5 MB.
Table S1, PDF file, 1.8 MB.
Figure S1, PDF file, 0.5 MB.
Figure S2, PDF file, 0.4 MB.
Figure S3, PDF file, 0.5 MB.
Figure S4, PDF file, 0.7 MB.
Figure S5, PDF file, 0.3 MB.
Figure S6, PDF file, 0.2 MB.

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