

# An *agr* Quorum Sensing System That Regulates Granulose Formation and Sporulation in *Clostridium acetobutylicum*

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**The Gram-positive, anaerobic, endospore-forming bacterium *Clostridium acetobutylicum* has considerable biotechnological potential due to its ability to produce solvents as fermentation products, in particular the biofuel butanol. Its genome contains a putative *agr* locus, *agrBDCA*, known in staphylococci to constitute a cyclic peptide-based quorum sensing system. In staphylococci, *agrBD* is required for the generation of a peptide signal that, upon extracellular accumulation, is sensed by an *agrCA*-encoded two-component system. Using Clostron technology, *agrB*, *agrC*, and *agrA* mutants of *C. acetobutylicum* ATCC 824 were generated and phenotypically characterized. Mutants and wild type displayed similar growth kinetics and no apparent differences in solvent formation under the conditions tested. However, the number of heat-resistant endospores formed by the mutants in liquid culture was reduced by about one order of magnitude. On agar-solidified medium, spore formation was more strongly affected, particularly in *agrA* and *agrC* mutants. Similarly, accumulation of the starch-like storage compound granulose was almost undetectable in colonies of *agrB*, *agrA*, and *agrC* mutants. Importantly, these defects could be genetically complemented, demonstrating that they were directly linked to *agr* inactivation. A diffusible factor produced by *agrBD*-expressing strains was found to restore granulose and spore formation in the *agrB* mutant. Furthermore, a synthetic cyclic peptide, designed on the basis of the *C. acetobutylicum* AgrD sequence, was also capable of complementing the defects of the *agrB* mutant when added exogenously to the culture. Together, these findings support the hypothesis that *agr*-dependent quorum sensing is involved in the regulation of sporulation and granulose formation in *C. acetobutylicum*.**

The Gram-positive, endospore-forming bacterium *Clostridium acetobutylicum* is strictly fermentative and gains energy by converting sugars and starch to organic acids and solvents (5, 17). In the past, it has been exploited for the large-scale production of acetone and butanol, but the classical industrial fermentation process is currently considered uneconomical in most countries, despite increasing oil prices (16). Essential for the engineering of more efficient strains and processes is a more thorough understanding of the organism's physiology and metabolism (22).

The fermentation metabolism of *C. acetobutylicum* is complex. During exponential growth in batch culture, acetic and butyric acid are produced, which allows the cells to gain more than 3 ATPs per molecule of glucose. However, continued acid production poses a problem as the pH of the medium decreases and undissociated acids diffuse back into the cells, thereby affecting intracellular pH and leading to proton gradient dissipation. To avoid acid death, cells shift their metabolism to solvent formation: some of the produced acids (mainly butyric acid) are taken up again and, together with remaining sugars, converted to butanol, acetone, and also some ethanol (5, 17). This switch to solvent production coincides with the initiation of the complex developmental program of sporulation. As part of these processes, a starch-like storage compound termed granulose is transiently formed and accumulates in the cytoplasm (24, 35). Both solventogenesis and sporulation are linked via the transcription factor Spo0A, the master regulator of sporulation: Spo0A is essential for the initiation of sporulation in solventogenic clostridia (as in *Bacillus subtilis*) but also required for high solvent production (8, 34). In contrast to *B. subtilis*, Spo0A in *C. acetobutylicum* is not activated via a complex phosphorelay system. Instead, its phosphorylation state is controlled by multiple orphan histidine kinases through direct interaction (37). The whole cascade of sigma factors that regulates the sporulation process downstream of phosphorylated Spo0A in *B.*

*subtilis* is present in *C. acetobutylicum* (2, 18) although some differences seem to exist (for instance in the activation of  $\sigma^F$  by SpoIIIE). However, while general conditions for solventogenesis and sporulation are known, and progress is being made in unraveling the underlying regulatory networks, the chemical or physical nature of the initiating signals still remains to be elucidated.

There is, however, some evidence to suggest that cell-cell communication (quorum sensing) systems are widely distributed in *Clostridium* spp. (43) and that they may play a role in regulating both solventogenesis and sporulation (3, 20, 21). Quorum sensing is a communication mechanism that relies on small, diffusible signal molecules and allows bacteria to coordinate changes in gene expression with cell population density (42). Most Gram-positive quorum sensing systems are based on secreted peptides which can be linear or cyclic and sometimes contain extensive posttranslational modifications (23, 39). Relatively little is known about the operation of such systems in clostridial species, but for the solventogenic *Clostridium sacharoperbutylaceticum*, an as-yet-unidentified, self-generated signal in the culture supernatant has been reported to induce solvent formation in a "low-solvent" mutant, suggesting that the metabolic shift from acid to solvent production might be subject to quorum sensing control (20). Furthermore, *in silico* analysis suggests that *agr*-type quorum sensing

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systems may exist in many of the sequenced members of the class *Clostridia* (reference 43 and unpublished data from this laboratory) although thus far, experimental evidence has been provided for only three species. In *Clostridium sporogenes*, group I *Clostridium botulinum*, and *Clostridium perfringens*, agr-homologous systems are required for efficient sporulation. In group I *C. botulinum* and *C. perfringens*, the system is also involved in the control of toxin production (3, 21, 30, 41).

The agr system was first discovered in *Staphylococcus aureus*, where it comprises five genes: *agrB*, *agrD*, *agrC* and *agrA* (which make up the actual cell-cell signaling system) and RNAIII (the main effector of target gene regulation) (see reference 28 for an excellent review). A cyclic, so-called autoinducing peptide (AIP) is derived from an internal AgrD fragment, through the action of the membrane-associated protein AgrB, and acts as a signal molecule. Upon extracellular accumulation, the AIP is sensed by a two-component system consisting of the histidine kinase AgrC and the response regulator AgrA. Phosphorylated AgrA then further activates the *agrBDCA* operon and induces RNAIII expression but also controls a subset of target genes independently of RNAIII (33).

Interestingly, *C. acetobutylicum* is one of the members of the genus that appears to possess a complete *agrBDCA* cluster (Fig. 1A), although a regulatory RNA similar to RNAIII has not yet been identified. In contrast to the staphylococcal system, *agrBD* is predicted to be transcribed independently of *agrCA* (31), and this is experimentally supported by a gene array time course series that revealed strong increases in *agrB* and *agrD* transcript levels when the cells entered stationary phase, i.e., just before the initiation of solvent formation and sporulation, whereas *agrCA* expression remained relatively constant (analysis of microarray data provided in the supplemental material of reference 1).

Here we report the mutational analysis of the agr locus in *C. acetobutylicum* ATCC 824.

## MATERIALS AND METHODS

**Bacterial strains and media.** *C. acetobutylicum* ATCC 824 was grown at 37°C in an anaerobic cabinet (MG1000 Anaerobic Work Station, Don Whitley Scientific) containing an atmosphere of 80% nitrogen, 10% hydrogen, and 10% carbon dioxide. Clostridial strains were cultured either in clostridial basal medium (CBM) (29) or 2× YTG (tryptone, 16 g/liter; yeast extract, 10 g/liter; NaCl, 5g/liter; glucose, 10g/liter; adjusted to pH 5.8 for agar-solidified medium and pH 5.2 for broth). *Escherichia coli* TOP10 (used as a host for ClosTron and complementation plasmids) was grown in Luria-Bertani medium (37) at 37°C. Antibiotics were used at the following concentrations: chloramphenicol, 25 µg/ml; erythromycin, 10 µg/ml (CBM) or 40 µg/ml (2× YTG); thiamphenicol, 15 µg/ml.

**Plasmids, primers, DNA techniques.** Oligonucleotides were synthesized by Eurofins MWG Operon, Germany (see Table S1 in the supplemental material). PCR amplifications were carried out using the FailSafe PCR system (Epicentre) or *Taq* DNA polymerase (New England BioLabs). Electroporation of *C. acetobutylicum* was performed as described previously (38). Plasmid isolation and genomic DNA preparations were carried out using the QIAprep miniprep kit (Qiagen, United Kingdom) and the DNeasy blood and tissue kit (Qiagen), respectively. Restriction enzymes were supplied by New England BioLabs and were used according to the manufacturer's instructions. Standard methods were used for agarose gel electrophoresis and transformation of *E. coli* (37).

**Construction of mutants using ClosTron technology.** Construction of mutants was based on a modified group II intron (the ClosTron) which carries an erythromycin resistance marker and has been retargeted so that it integrates into specific sites of the host's chromosome. Mutants were

constructed in *C. acetobutylicum* ATCC 824 targeting genes *agrB* (*cac0078*), *agrC* (*cac0080*), and *agrA* (*cac0081*), using the standard methodology described by Heap et al. (10, 12). IBS, EBS1d, and EBS2 primers used for retargeting the intron are listed in Table S1 (in the supplemental material). Numbers in the primer names indicate the retargeting site used. PCR-amplified retargeted HindIII/BsrGI fragments were cloned into the pMTL007C-E2 vector. Genomic DNA from putative agr mutants (*agrB*::CTermB, *agrC*::CTermB, and *agrA*::CTermB) was subjected to two PCR screens. First, primers flanking the insertion site were employed to confirm that the intron was present in the desired target gene (primer pairs, CAC0078-F/CAC0078-R, CAC0080-F/CAC0080-R, and CAC0081-F/CAC0081-R for *agrB*, *agrC*, and *agrA*, respectively). To confirm the orientation in which the intron was present, primers amplifying across the intron-exon junction were used (primer pairs, CAC0078-R/EBS universal, CAC0080-F/EBS universal, and CAC0081-R/EBS universal for *agrB*, *agrC*, and *agrA*, respectively).

**Generation of complementation vectors.** Complementation vectors for the mutant strains encompassed the complete coding sequences of *agrA*, *agrBD*, *agrC*, and *agrCA* together with their respective putative promoter regions. For the *agrBD* cluster, the cognate intergenic upstream region was assumed to harbor the promoter. For the *agrCA* cluster, the 224-bp region upstream of the start codon of *agrC* was assumed to contain the necessary regulatory elements. Coding sequences, including their putative promoter regions, were PCR amplified from genomic DNA. The *agrBD* and *agrC* regions were amplified using primer pairs *agrBD*-F/*agrBD*-R and *agrCA*-F/*agrC*-R, respectively. The resulting PCR fragments were digested with XhoI and BamHI and subcloned into the pMTL85141 vector (11) linearized with the same enzymes. The *agrCA* cluster, including the 224-bp upstream region, was amplified as two fragments, using primer pairs *agrCA*-F/*agrC*-R and *agrCA2*-F/*agrA*-R. Taking advantage of a SacI recognition site in the *agrC* gene, the two fragments were digested with XhoI/SacI and SacI/BamHI, respectively, and ligated into the XhoI/BamHI-linearized pMTL85141 vector. For expression of *agrA*, the putative promoter region of the *agrCA* cluster was amplified using primer pair *agrCA*-F/*agrCProm*-R (introducing an NdeI site at the start codon of *agrC*) and the *agrA* gene was amplified using primer pair *agrA*-F/*agrA*-R (introducing an NdeI site at the start codon of *agrA*). The XhoI/NdeI-digested promoter region and the NdeI/BamHI-digested *agrA* fragment were introduced into XhoI/BamHI-linearized pMTL85141 in a three-fragment ligation step. All complementation plasmids were verified by sequencing.

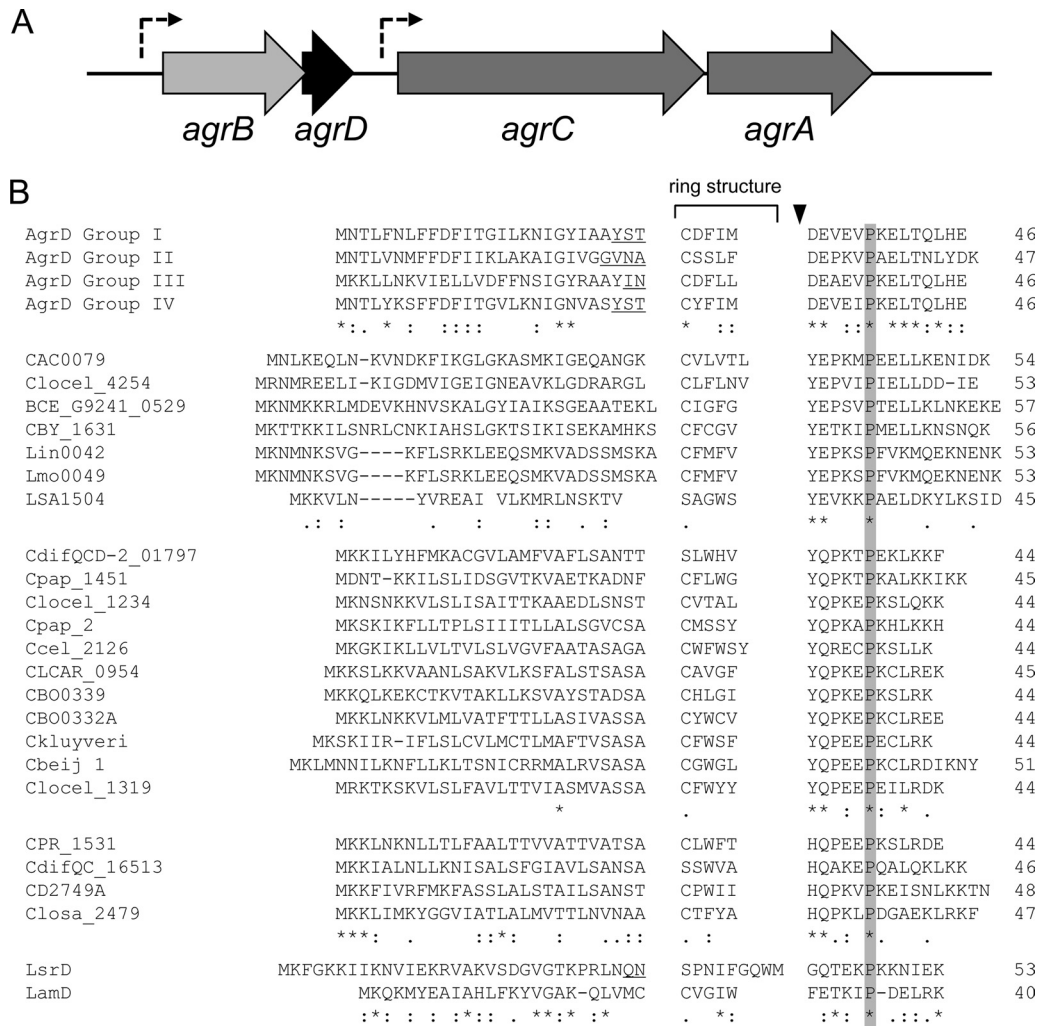
**Spore assays.** *C. acetobutylicum* strains were grown in 5 ml CBM broth containing 0.5% CaCO<sub>3</sub> and 5% glucose (CBM-S) to enable sporulation. After 5 days, a 200-µl sample of culture was heated to 80°C for 10 min. Serial dilutions were carried out and 10-µl aliquots of the heat-treated cell suspension plated onto CBM agar. Colonies were enumerated after 24 h of incubation.

**Detection of granulose.** To assess the accumulation of granulose, *C. acetobutylicum* strains were grown on agar-solidified CBM containing 5% glucose (CBM-G) to promote granulose accumulation. After incubation for 24 h to 48 h, plates were exposed to iodine vapor, staining granulose-containing colonies dark brown (36).

**Detection of diffusible signals by cross-streaking.** Wild-type *C. acetobutylicum* and agr mutants were precultured in CBM broth until visible growth was detectable. An inoculation loop was used to streak these cell suspensions on CBM-G plates, generating streaks approximately 7 mm wide and taking care that streaks were physically separated. After incubation for 48 h, plates were subjected to iodine staining as described above.

**Chemical complementation of the agrB mutant and detection of AIPs.** Cyclic AIPs were synthesized and high-performance liquid chromatography (HPLC) purified by Peptide Protein Research Ltd. (Fareham, United Kingdom); the purity was estimated to range from 78 to 90%, depending on the AIP. Lyophilized peptides were dissolved in dimethyl sulfoxide (DMSO) to obtain 1 mM stock solutions.

For complementation studies in broth, the *agrB*::CTermB strain was



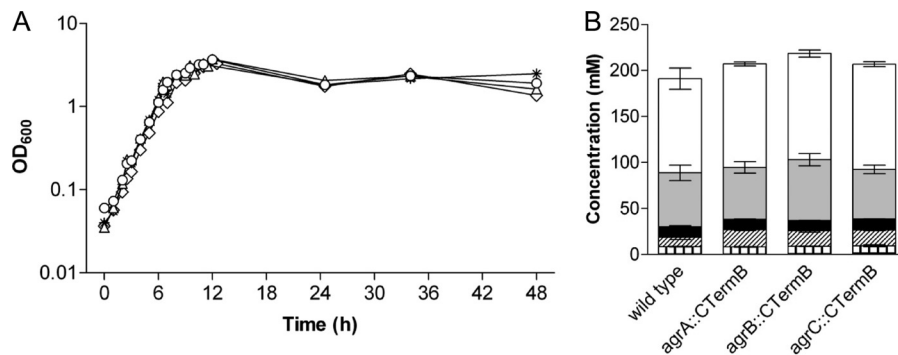
**FIG 1** Schematic representation of the *C. acetobutylicum* *agrBDCA* cluster (A) and alignment of confirmed and putative AgrD sequences (B). (A) The *C. acetobutylicum* *agrBDCA* cluster is predicted to comprise two independent transcriptional units (indicated by the dashed arrows), *agrBD* and *agrCA*. (B) The alignment shows the experimentally confirmed AgrD proteins from *S. aureus* (AgrD group I to IV), *E. faecalis* (LsrD), and *L. plantarum* (LamD), as well as selected putative AgrD sequences from other species. The latter included the following (locus tags are given in parentheses, if available, and identify the respective sequence in the alignment): *C. acetobutylicum* (CAC0079), *Clostridium cellulovorans* 743B (Cloce1\_1234, Cloce1\_1319, and Cloce1\_4254), *Bacillus cereus* G9241 (BCE\_G9241\_0529), *Listeria innocua* Clip11262 (Lin0042), *Listeria monocytogenes* EGD-e (Lmo0049), *Clostridium butyricum* 5521 (CBY\_1631), *Lactobacillus sakei* subsp. *sakei* 23K (LSA1504), *Clostridium difficile* QCD-23m63 (CdifQCD-2\_01797), *Clostridium papyrosolvens* DSM 2782 (Cpap\_1451 and Cpap\_2, the latter not annotated), *Clostridium cellulolyticum* H10 (Ccel\_2126), *Clostridium carboxidivorans* P7 (CLCAR\_0954), *Clostridium botulinum* ATCC 3502 (CBO0332A and CBO0339), *Clostridium kluveri* (Ckluver1; not annotated), *C. beijerinckii* NCIMB 8052 (Cbeij\_1; not annotated), *Clostridium perfringens* SM101 (CPR\_1531), *C. difficile* QCD-66c26 (CdifQC\_16513), *C. difficile* 630 (CD2749A), *Clostridium saccharolyticum* WM1 (Closa\_2479). The black triangle indicates confirmed and proposed processing sites downstream of the ring-encoding part of AgrD. A highly conserved proline, present in all sequences, is shaded grey. Note the sequence conservation immediately downstream of the proposed cleavage site and upstream of the conserved proline. For each AIP group, identities (\*), conserved (:), and semiconserved substitutions (.) are displayed. Amino acids constituting the N-terminal tail of confirmed AIPs are underlined. Numbers indicate the total length of the AgrD sequence.

grown in CBM-S to an optical density at 600 nm (OD<sub>600</sub>) of 0.4 to 0.5. The culture was then divided into 3-ml aliquots and supplemented with an appropriate volume of AIP stock solutions to obtain final AIP concentrations of 2 and 20 μM. DMSO controls were included in all experiments. After 5 days of incubation, spore assays were performed as described above. For plate-based complementation assays, filter discs (6-mm diameter; Whatman) were placed either on the surface of CBM-G plates whose surface had been spread with an appropriate dilution of an early-exponential-phase culture of *agrB::CTermB* or next to the streaks of mutant strains. Samples (5 μl) of the appropriate AIP stock solutions were placed on the filter discs. Plates were incubated for 48 h and subjected to iodine staining to visualize granulose formation as described above.

**Statistical analysis.** The data were analyzed in GraphPad Prism using the unpaired *t* test. Significance levels are indicated by asterisks in graphs.

## RESULTS

**Insertional inactivation of *agr* genes using ClosTron technology.** A putative *agr* quorum sensing system is present in the genome of *C. acetobutylicum* ATCC 824 and also in that of the recently sequenced EA 2018 strain (13, 27). It consists of a gene cluster comprising the genes *agrB*, *agrD*, *agrC*, and *agrA* (Fig. 1A). Using ClosTron technology, all *agr* genes apart from *agrD* were insertional inactivated in the ATCC 824 strain, resulting in the



**FIG 2** Growth and solvent formation of *C. acetobutylicum* *agr* mutants. Wild-type *C. acetobutylicum* and *agr* mutants were grown in CBM-S broth. (A) Growth was monitored by following the optical density at 600 nm: wild type (asterisks), *agrA* mutant (triangles), *agrB* mutant (circles), *agrC* mutant (diamonds). (B) After 72 h, culture supernatant samples were taken and analyzed for the produced acids (acetate, checks; butyrate, lines) and solvents (butanol, white; acetone, gray; ethanol, black). The values shown are the means  $\pm$  standard errors of the means of results from six independent experiments. Product concentrations are not significantly different between the wild-type and the mutant strains, except for the difference between butyrate produced by the *agrA* mutant and the wild type ( $P = 0.02$ ).

mutant strains *agrB*::CTermB, *agrC*::CTermB, and *agrA*::CTermB. A defect in the *agrB* gene is expected to abolish production of the postulated AIP signal, whereas *agrA* and *agrC* mutants should be unable to respond to AIP accumulation. For each gene, four independent mutant clones were isolated and further characterized. This was done to avoid accidental isolation of strains carrying undesired second site mutations: the isolation of ClosTron mutants involves multiple restreaking of candidate colonies and this may increase the risk of obtaining spontaneously “degenerated” strains, typically characterized by a reduced or abolished capacity to form solvents and to sporulate (9, 19).

Correct insertion of *ermB*-carrying introns into the target genes was confirmed by several PCR screens using primers flanking the insertion site and primers amplifying across the intron-exon junction (data not shown).

**Phenotypic characterization of *agr* mutants.** A preliminary analysis revealed that one of the obtained *agrA* and one of the obtained *agrC* mutant clones differed phenotypically from the other three and showed signs of degeneration (data not shown). These clones were therefore excluded from further, more detailed analyses.

When cultured in CBM-S broth, *agrA*::CTermB, *agrB*::CTermB, and *agrC*::CTermB strains showed no marked differences in their growth kinetics (Fig. 2A) or their ability to form solvents (Fig. 2B) compared with the wild type. The ability to degrade starch and swimming motility were also not affected (data not shown). However, microscopic examination revealed a noticeable reduction in the number of endospores formed by all three mutant strains. This decrease in spore numbers appeared to be more pronounced in samples taken from colonies than in those from liquid cultures.

To obtain a more quantitative measure for the differences observed, the number of spores present in a given culture volume (liquid cultures) or in similar-sized colonies was determined (more precisely, this procedure quantifies the number of heat-resistant CFU as a measure for spores that can germinate and grow after a heat treatment for 10 min at 80°C). In liquid cultures, an about 10-fold reduction in the number of spores was observed for all *agr* mutants compared to the wild-type strain (Fig. 3A). In contrast, colonies of the mutant strains grown on CBM plates

contained between 1 and 5 orders of magnitude fewer spores than the wild type. Interestingly, the severity of the observed sporulation defect under these conditions was dependent on the sampling time point and the inactivated *agr* gene. After 6 days, spore numbers for the *agrA* and *agrC* mutant were reduced by about 5 orders of magnitude, whereas for the *agrB* mutant, sporulation efficiency was reduced by only about 1 order of magnitude compared to that of the wild type (Fig. 3B).

Granulose accumulation is known to coincide with the initiation of sporulation, and the simple method of staining granulose with iodine vapor—which stains granulose-containing cells dark brown—appears to be a reliable indicator for sporulation on plates. As shown in Fig. 3C and D, all mutants tested negative for granulose after 24 h (Fig. 3C) and produced only very little granulose along the edges of the streak after 48 h (Fig. 3D). This is in stark contrast to results with the wild-type strain, which started granulose accumulation at around 24 h and, at 48 h, stained intensely brown, suggesting that cells had produced considerable amounts of this glycogen-like storage product.

**Genetic complementation of *agr* mutants.** The obtained *agr* mutants were genetically complemented to confirm that the ClosTron insertions rather than second site mutations were responsible for the observed phenotypes. For these complementation studies, the *agr* genes under the control of their native promoters were introduced into the modular vector pMTL85141 (11) and transformed into the respective mutant strains by electroporation. Introduction of the *agrA* complementation vector (pMTL85141-*agrA*) successfully restored spore formation and granulose production in the *agrA*::CTermB strain, whereas the *agrC* complementation vector (pMTL85141-*agrC*) failed to complement the *agrC*::CTermB strain (Fig. 4A and B). Since *agrC* and *agrA* appear to form an operon, it is possible that the ClosTron insertion in *agrC* had also negatively affected expression of the downstream located *agrA*. Therefore, the complete *agrCA* cluster (pMTL85141-*agrCA*) was introduced into the *agrC* mutant and found to restore sporulation and granulose formation to wild-type levels. In view of this result, the complete *agrBD* cluster was cloned (pMTL85141-*agrBD*) and transformed into the *agrB*::CTermB strain. Introduction of pMTL85141-*agrBD* partially restored spore formation of the *agrB* mutant in liquid culture (Fig. 4A) and reestablished

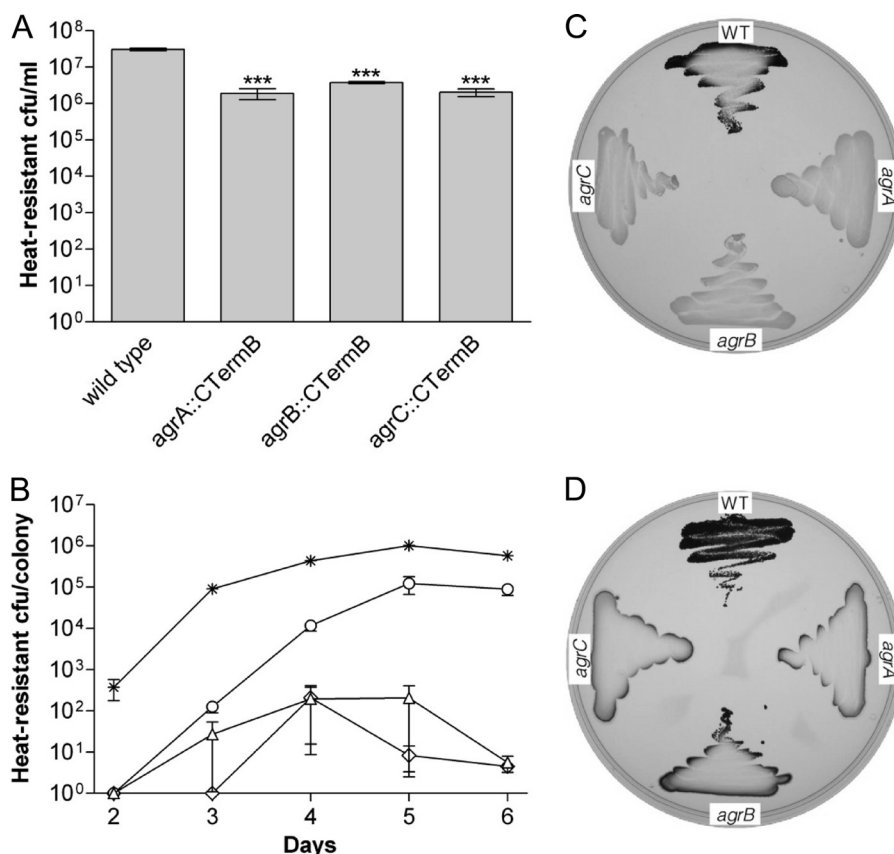


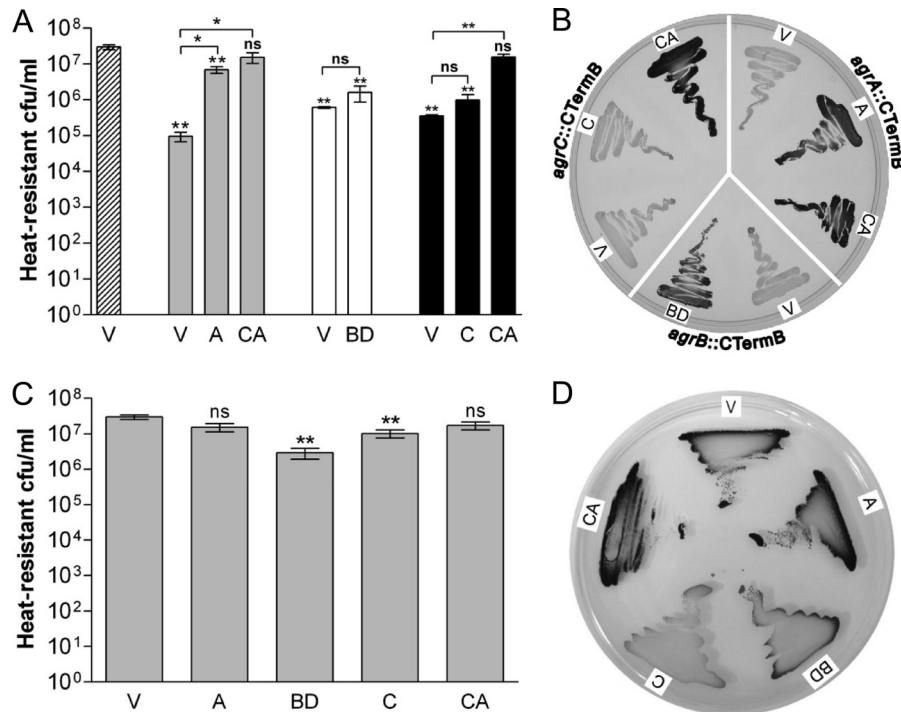
FIG 3 Sporulation and granule accumulation in *C. acetobutylicum* *agr* mutants. (A) Sporulation assays of wild-type *C. acetobutylicum* and *agr* mutants cultured in CBM-S broth for 5 days. The values shown are the means  $\pm$  standard errors of the means (SEM) of results from  $\geq 3$  clones of each strain (each clone representing an independently derived mutant). All values are significantly different from the wild-type strain (\*\*\*,  $P < 0.001$ ). (B) The number of heat-resistant endospores formed per colony was determined for *C. acetobutylicum* (asterisks) and the mutant strains *agrA*::CTermB (triangles), *agrB*::CTermB (circles), and *agrC*::CTermB (diamonds) over a time course of 6 days. The values shown are the means  $\pm$  SEM of results from 10 colonies of each strain and time point. Mutant strains and wild type are significantly different in all given time points ( $P < 0.001$ ). (C and D) Granulose assays. Wild-type *C. acetobutylicum* (WT), *agrA*::CTermB (*agrA*), *agrB*::CTermB (*agrB*), and *agrC*::CTermB (*agrC*) were grown on CBM-G plates and assayed for granulose formation after 24 h (C) and 48 h (D).

granulose formation on CBM-G plates (Fig. 4B). Taken together, these results confirmed that the observed phenotypical changes in the *agr* mutants were directly linked to the inactivation of the respective genes.

The constructed complementation plasmids were also introduced into the ATCC 824 wild-type strain to assess the phenotypic consequences of *agr* gene overexpression. This was motivated by the finding that sporulation was only partially restored in the complemented *agrB* mutant. As can be seen from Fig. 4C, expression of *agrA* and *agrCA* in this background had no marked effect on sporulation in liquid culture compared to the vector control. In contrast, overexpression of *agrBD* reduced the number of spores about 10-fold and a slight, but significant, reduction was also observed for *agrC*. A possible explanation for this could be that expression of the *agrBD* cluster from a multicopy plasmid resulted in too much signal or the wrong timing and thus disturbed the regulatory network in some way. This could also explain why the *agrB* mutant appeared to be only partially complemented in liquid broth. For granulose formation on plates, a different picture emerged. Again, overexpression of *agrA* had no obvious effect, but in comparison to the vector control granulose formation was clearly increased in the *agrCA* overexpressing strain. Furthermore,

granulose formation was reduced in wild-type strains overexpressing *agrBD* or *agrC* (Fig. 4D and data not shown).

**A diffusible factor produced by wild-type *C. acetobutylicum* restores sporulation and granulose formation in the *agrB* mutant.** If the *C. acetobutylicum* *agrBDCA* cluster represents a true quorum sensing system working in the same way as its staphylococcal counterparts, the *agrB* mutant's phenotype should be caused by a lack of extracellular AIP. Therefore, several experiments were performed to test whether the defects in sporulation and granulose production in this mutant could be restored by a quorum sensing signal secreted by the wild type. The *agrB*::CTermB strain was grown in coculture with wild-type *C. acetobutylicum* in a number of different ratios ranging from 0.5 to 5% wild type. After 5 days, these cultures were heat treated to kill off all vegetative cells and to induce germination of the spores that had been formed. Spore counts were performed on CBM plates containing erythromycin to ensure that only germinating spores derived from the *agrB* mutant could grow and form colonies (the Clostron insertion confers erythromycin resistance). Interestingly, the presence of wild-type cells increased spore formation in the *agrB*::CTermB strain by up to one order of magnitude, even when only present at low ratios (Fig. 5A and data not shown), thus



**FIG 4** Complementation of *C. acetobutylicum* *agr* mutants and effect of overexpression of *agr* genes. (A and B) For complementation studies, strains *agrA*::CTermB (gray bars), *agrB*::CTermB (white bars), and *agrC*::CTermB (black bars) were transformed with the vector control (V) or the indicated complementation vectors carrying the *agrA* (A), *agrBD* (BD), *agrC* (C), and *agrCA* (CA) genes. As a control, the wild-type strain carrying the vector control (striped bar) was also included. Sporulation assays were performed after 5 days in CBM-S broth (A) and granulose accumulation was assayed on CBM-G plates after 24 h (B). The data obtained for mutant strains carrying either a complementation vector or the empty plasmid were compared to those for the wild-type strain harboring the empty vector; significance levels are indicated immediately above the bars. The significance levels of the differences between complemented mutants and the corresponding mutant strains transformed with empty vector are indicated above the brackets: \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; ns, not significant. (C and D) Wild-type *agr* genes were introduced into the parent *C. acetobutylicum* strain to investigate the effect of overexpression of these genes on sporulation after 5 days (C) and granulose formation after 24 h (D). The values shown are the means  $\pm$  standard errors of the means of results from  $\geq 3$  independent experiments. The data shown in panel C were compared to those for the wild-type strain carrying the empty plasmid, and the significance levels are indicated: \*\*,  $P < 0.01$ ; ns, not significant.

supporting the hypothesis that *agrBD* is required for the production of a diffusible quorum sensing signal.

To exclude the possibility that the wild type exerted this effect via direct cell-to-cell contact, T-streak experiments on agar plates were also performed. The *agrB*::CTermB strain was streaked on CBM-G plates next to wild-type *C. acetobutylicum*, *agrA*::CTermB, and *agrC*::CTermB, incubated for 48 h and then assessed for granulose formation. Close vicinity of the wild type restored granulose formation in the *agrB* mutant but failed to do so in the *agrA* and *agrC* mutants (Fig. 5B). Interestingly, T-streaks of the *agrA* and *agrC* mutant also failed to restore granulose formation in the *agrB* mutant, suggesting that they did not produce the required amounts of diffusible signal. As expected, the complemented *agrB* mutant was capable of restoring granulose formation in the *agrB*::CTermB strain when streaked next to it (Fig. 5C).

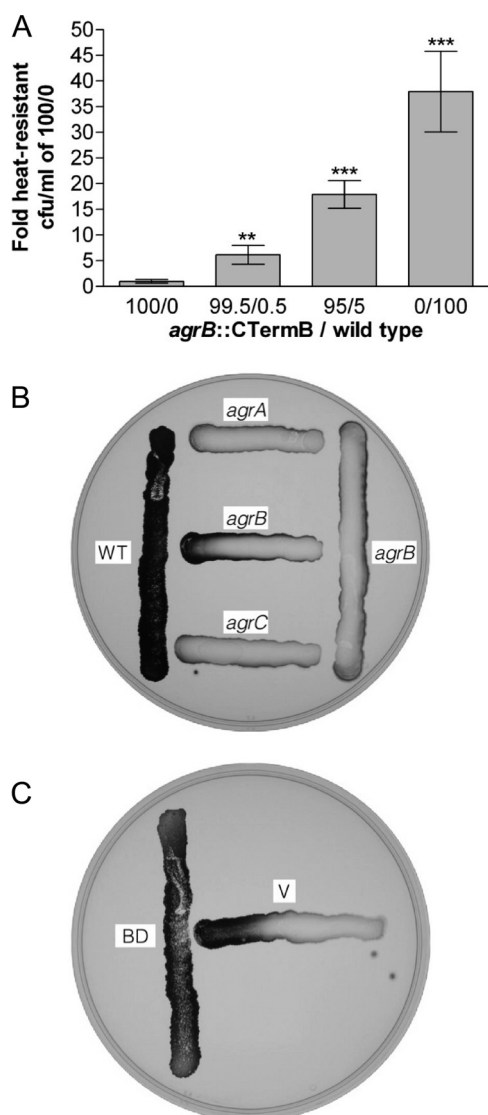
**Chemical complementation of the *agrB* mutant with synthetic peptides.** A number of synthetic, cyclic AIPs (Fig. 6A and B) were assessed for their ability to enhance sporulation of the *agrB* mutant. The sequences for these peptides were derived from the *C. acetobutylicum* AgrD sequence (Fig. 6A), assuming the formation of five-, six-, or seven-amino-acid ring structures, respectively. The length of the N-terminal tail sequence (0 to 4 amino acids) was based on those found in other known AIPs (25, 28, 40). Cultures of *agrB*::CTermB were supplemented with these synthetic AIPs (final concentrations, 2 and 20  $\mu$ M) and assayed for spore

formation after 5 days (Fig. 6C). Interestingly, highest activities in our test system were observed for an AIP consisting of a six-amino-acid ring without an N-terminal tail moiety (termed R6T0). Inclusion of such a tail moiety reduced the activity of the resulting AIP with increasing tail length (R6T1 to R6T4 [Fig. 6C]). As a control, cyclic AIPs consisting of five- and seven-membered rings, but no tail moiety, were used (R5T0 and R7T0, respectively) and showed no significant effect on sporulation.

The synthetic AIPs were also tested for their ability to induce granulose formation in the *agrB* mutant. Filter discs were placed on agar plates freshly inoculated with the *agrB* mutant and impregnated with 5  $\mu$ l AIP stock solution (1 mM). Granulose formation was assessed after 48 h. Under these conditions, the R6T0 AIP restored granulose formation, whereas the other six-membered AIPs containing an N-terminal tail (R6T1-R6T4) had no visible effect. Again, the five- and seven-membered control AIPs without N-terminal tail (R5T0 and R7T0, respectively) showed no activity (Fig. 6D). None of the synthetic AIPs was able to restore granulose formation in the *agrA* and *agrC* mutant (Fig. 6E and data not shown).

## DISCUSSION

In this study, the function of the *C. acetobutylicum* *agrBCDA* gene cluster was investigated. The results suggest that it is a true quorum sensing system involved in the cell density-dependent regu-



**FIG 5** A diffusable factor produced by wild-type *C. acetobutylicum* restores sporulation and granulose formation in the *agrB* mutant. (A) Early-exponential-phase cultures of *agrB*::CTermB were mixed in the indicated ratios with the wild-type strain. Spore assays were performed after 5 days, and heat-treated spores were plated on erythromycin-supplemented CBM plates, which allowed only germinated *agrB* mutant spores to grow. Wild-type controls (0/100) were counted on nonselective medium. Shown is the fold increase in spore numbers compared to the *agrB*::CTermB single culture control (100/0). The values given are the means  $\pm$  standard errors of the means of results from three independent experiments. All values are significantly different from the control 100/0 (\*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ). (B) *agrA*::CTermB (*agrA*), *agrB*::CTermB (*agrB*), and *agrC*::CTermB (*agrC*) were cross-streaked with wild-type *C. acetobutylicum* (WT) on CBM-G plates and assayed for granulose accumulation after 48 h. (C) Shown is the granulose stain of a cross-streak of the complemented *agrB*::CTermB strain (BD) and *agrB*::CTermB carrying the vector control (V) after 48 h.

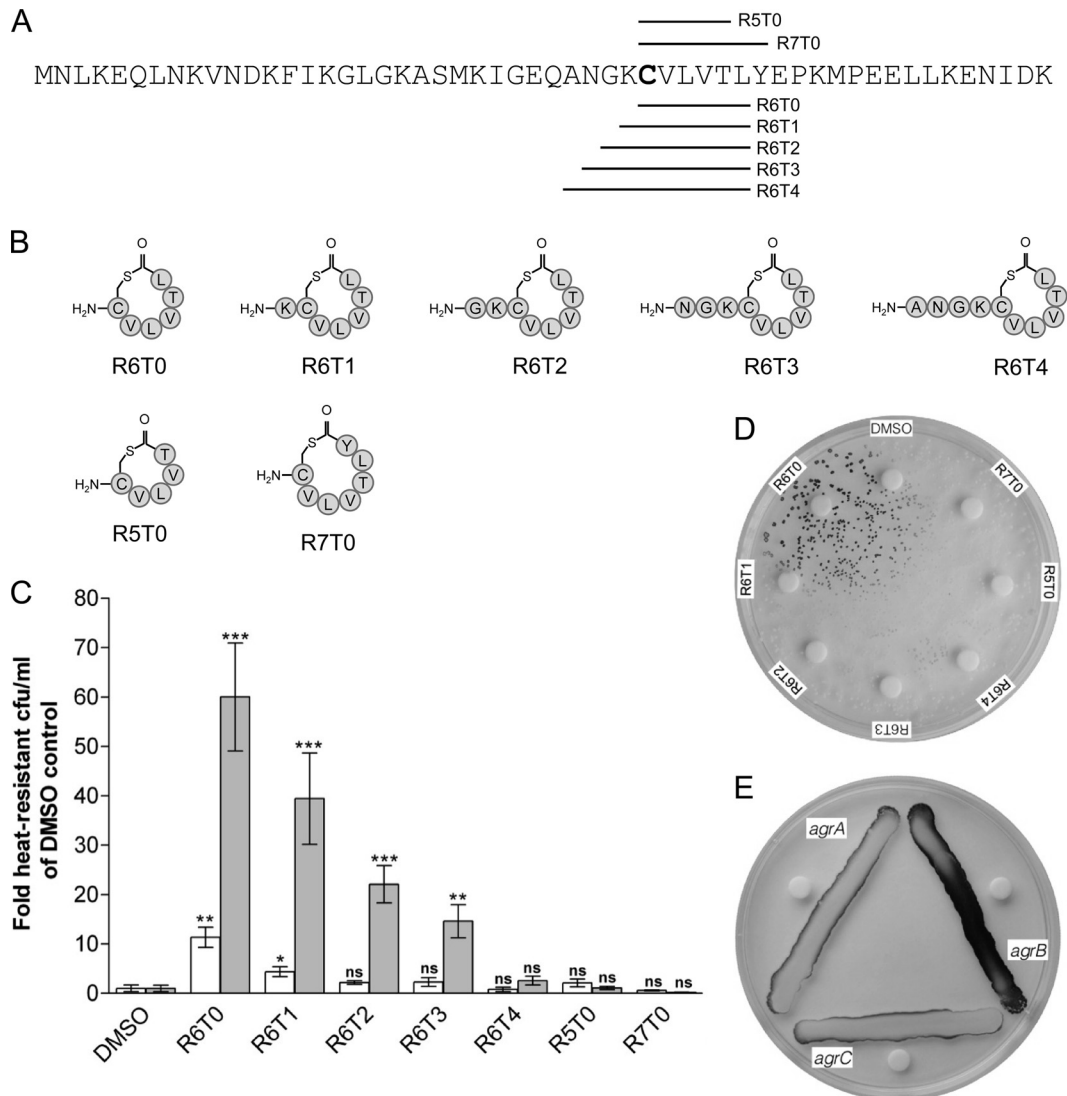
lation of granulose and endospore formation. All genes of the cluster could be inactivated, apart from *agrD*, for which, due to its small size, no suitable ClosTron insertion site could be identified. However, *agrB* and *agrD* inactivation are predicted to have the same consequence: a lack of AIP production. Thus, mutants were constructed that failed to either produce (*agrB*::CTermB) or re-

spond to (*agrC*::CTermB and *agrA*::CTermB) the presumed AIP quorum sensing signal.

One of the problems encountered during *agr* mutant construction was that the organism is inherently prone to degeneration, characterized by a loss of several key features, in particular the ability to sporulate and to produce acetone and butanol (9, 19). The ClosTron mutagenesis procedure employed (10) involved frequent restreaking of colonies and thus carried a risk of enriching and isolating degenerate mutant strains. It was therefore absolutely essential to ensure that the phenotypes of isolated mutants were caused by the ClosTron insertion rather than second site mutations. This was achieved by (i) screening several independent mutant clones for each gene and eliminating potential outliers and (ii) subsequent genetic complementation.

So what is the evidence that the *C. acetobutylicum* *agr* cluster acts as a functional quorum sensing unit responsible for production and detection of an extracellular AIP? First, inactivation of the putative AIP signal-generating (*AgrB*) and -receiving (*AgrCA*) parts of the cluster resulted in almost identical phenotypes, suggesting that they are part of the same regulatory circuit. Second, wild-type characteristics could be restored when growing the signal-deficient *agrB* mutant in close vicinity to the wild type, which suggests *AgrB*-dependent production of a diffusible signaling compound by the latter. Third, this wild-type-produced diffusible signal could not complement *agrC* and *agrA* mutants, suggesting that the *agrCA*-encoded two-component system is required for signal reception and transmission. Fourth, restoration of both granulose formation and sporulation by the *agrB* mutant was also achieved with a synthetic AIP, whose sequence was based on a relevant section of the putative signal precursor polypeptide, *AgrD*. Again, the *AgrCA* system was required for the perception of this signal. Taken together, the results provide strong support for the hypothesis that the *C. acetobutylicum* *agrBD* cluster is required for the production of a cyclic AIP, which accumulates extracellularly and is sensed by the *AgrCA* two-component system. Although *agrCA* are predicted to form a separate transcriptional unit (31) and, in contrast to *agrBD*, are not drastically upregulated during entry into stationary phase (analysis of microarray data provided in the supplemental material of reference 1), they are required for upregulation of AIP production, as *agrA* and *agrC* mutants both failed to chemically complement the *agrB* mutant in the cross-streaking experiments.

No AIP structures have been published for any of the clostridial *Agr* systems investigated so far. However, the results obtained here for a set of synthetic AIPs suggest that the native *C. acetobutylicum* AIP has a thiolactone ring structure that, unlike its staphylococcal counterparts, consists of six instead of five amino acids (CVLVTL; cyclized by thiolactone bond formation between the SH group of cysteine and the carboxy group of the N-terminal leucine) (Fig. 6B). This finding was not entirely unexpected, given the sequence conservation at the *C. acetobutylicum* *AgrD* C-terminal end (Fig. 1B). It was reasonable to assume that the N-terminal end of the AIP ring structure is represented by the conserved cysteine. The C-terminal end was predicted to be a leucine, located five amino acids downstream of the conserved cysteine, for several reasons. First, this leucine is located immediately adjacent to a tyrosine which is conserved in a large subgroup of *AgrD* proteins (Fig. 1B) and therefore likely to belong to the C-terminal part of *AgrD* which is not involved in ring formation. Second, the proposed cleavage site between this leucine and the adjacent tyrosine is in



**FIG 6** Effect of synthetic cyclic peptides on sporulation and granulose formation. (A) *C. acetobutylicum* AgrD sequence displaying the position of peptide stretches (horizontal lines) that were used to design the synthetic, cyclic AIPs shown schematically in panel B. The bold C indicates the conserved cysteine required for thiolactone bond formation. (C) The number of heat-resistant endospores formed by the *C. acetobutylicum* *agrB*::CTermB strain was analyzed after 5 days of growth in CBM-S broth in the presence of 2  $\mu$ M (white bars) and 20  $\mu$ M (gray bars) concentrations of the indicated peptides and compared to a DMSO control. The values shown are the means  $\pm$  standard errors of the means of results from three independent experiments. Significance levels of differences between AIP-supplemented cultures and corresponding DMSO controls are indicated above the bars: \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; ns, not significant. (D) Granulose stain of *agrB*::CTermB grown on CBM-G plates supplemented with the indicated AIPs on filter discs and assayed after 48 h. (E) Granulose stain of *agrA*::CTermB (*agrA*), *agrB*::CTermB (*agrB*), and *agrC*::CTermB (*agrC*) grown next to filter discs impregnated with R6T0 AIP stock solution.

the same position, relative to a highly conserved proline, as the respective cleavage sites in all other AgrD homologues for which corresponding AIPs have been identified, i.e., staphylococcal AgrD, *Lactobacillus plantarum* LamD, and *Enterococcus faecalis* FsrD (14, 15, 25, 26, 40). Whether the R6T0 peptide, which showed the strongest effect in the sporulation assays and was the only one to restore granulose formation on plates, represents the true structure of the native signaling peptide remains to be seen. However, given the lack of activity for the related five- and seven-amino acid peptide rings (R5T0 and R7T0), it seems likely that the native AIP will contain a six-membered amino acid ring structure, identical or very similar to that of R6T0. Although the AgrD-derived AIPs produced by staphylococci and *Lactobacillus planta-*

*rum* all contain pentapeptide ring structures (14, 15, 40), larger rings are not without precedent: the *E. faecalis* FsrD-derived AIP contains a lacton ring structure consisting of nine amino acids (25). In this context it is also of interest that the LamD-derived AIP from *L. plantarum* consists only of a cyclic thiolactone pentapeptide without N-terminal tail moiety (40).

For many years, the factors that trigger sporulation in clostridial species have remained elusive. There is now an increasing body of evidence to suggest that *agr*-dependent quorum sensing, and thus presumably cell density, plays an important role in this process. Earlier work from this laboratory has shown that *agrBD* systems are required for efficient sporulation in *Clostridium sporogenes* and group I *Clostridium botulinum* (3), and a recent study on



*Clostridium perfringens* also established drastically reduced endospore numbers in *agrB* mutant cultures (21). Furthermore, almost all currently sequenced members of the genus *Clostridium* possess at least one set of *agrBD* homologous genes (reference 43 and unpublished data from this laboratory). An interesting question is, therefore, what advantage *Clostridium* spp. might gain by linking cell-cell communication (and thus information on cell density) with endospore formation. At least for *C. acetobutylicum* it seems that activation of *agr* quorum sensing at high cell densities is not an absolute prerequisite for sporulation to take place, as spores are still formed when the system is rendered nonfunctional, particularly under conditions of liquid culture. This is similar to the situation in *B. subtilis*, where the role of quorum sensing in the regulation of sporulation is very well established (32). Nutrient limitation is an essential trigger for sporulation in this organism, and cell density is thought to play a modulatory role because *B. subtilis* quorum sensing mutants still sporulate, albeit with much reduced efficiency. Information on high cell density or crowding could be valuable in this context, as it might indicate the degree of competition for scarce nutrients (7) and allow the population to adjust sporulation levels accordingly. For *C. acetobutylicum*, however, this explanation is unlikely to apply, as nutrient limitation does not appear to be a major factor in clostridial sporulation (4). It is also not clear whether granule formation is directly regulated by quorum sensing or indirectly as part of the ongoing sporulation process. Direct regulation in response to cell density might allow cells to increase sugar uptake and initiate granule formation before external resources run out. This would not only take the sugar away from potential competitors but also ensure that *C. acetobutylicum* cells can complete the sporulation process with only limited dependence on external carbon and energy sources. The last point could be important given that high butanol levels are known to affect membrane-associated functions, including transport processes.

How the *C. acetobutylicum* Agr system integrates with the regulatory cascade(s) that controls endospore formation will be an interesting aspect of future studies. An attractive hypothesis could be that AgrA influences the phosphorylation status of Spo0A, either directly or indirectly, by controlling the expression of the recently described Spo0A-phosphorylating/dephosphorylating orphan histidine kinases (38). Alternatively, the system could control *spo0A* expression levels, as has been suggested for *C. perfringens* (21). However, given that Spo0A is important for both spore formation and solvent production (*spo0A* inactivation drastically reduces butanol and acetone production [8]), it is difficult to explain with these hypotheses why the latter remained unaffected, at least in liquid culture, for all *C. acetobutylicum agr* mutants. It is possible that differing levels of phosphorylated Spo0A are needed for the initiation of solventogenesis and sporulation, respectively (similar to the situation in *B. subtilis*, where increased levels appear to be required for the initiation of sporulation [6]). Another explanation could be that the *C. acetobutylicum* Agr system acts by modulating the expression of key sporulation factors downstream of phosphorylated Spo0A. For instance, Jones et al. (18) recently found that inactivation of  $\sigma^F$  in *C. acetobutylicum* abolishes sporulation before cells form granules and undergo asymmetric cell division but does not block solvent formation. Thus, Agr-dependent regulation of an early key sporulation factor such as  $\sigma^F$  may allow the organism to produce solvents without committing to the sporulation pathway. Clearly, Agr-dependent quorum sens-

ing in *C. acetobutylicum* is part of a complex regulatory network that, depending on other environmental and internal stimuli, will place more or less emphasis on cell density-dependent inputs, as illustrated by the differences observed for *agr*-independent sporulation on solid and in liquid medium.

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