Inactivation of $\sigma^E$ and $\sigma^G$ in Clostridium acetobutylicum Illuminates Their Roles in Clostridial-Cell-Form Biogenesis, Granulose Synthesis, Solventogenesis, and Spore Morphogenesis\textsuperscript{V,}\textsuperscript{†}

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Central to all clostridia is the orchestration of endospore formation (i.e., sporulation) and, specifically, the roles of differentiation-associated sigma factors. Moreover, there is considerable applied interest in understanding the roles of these sigma factors in other stationary-phase phenomena, such as solvent production (i.e., solventogenesis). Here we separately inactivated by gene disruption the major sporulation-specific sigma factors, $\sigma^E$ and $\sigma^G$, and performed an initial analysis to elucidate their roles in sporulation-related morphogenesis and solventogenesis in Clostridium acetobutylicum. The terminal differentiation phenotype for the $\sigma^E$ inactivation mutant stalled in sporulation prior to asymmetric septum formation, appeared vegetative-like often with an accumulation of DNA at both poles, frequently exhibited two longitudinal internal membranes, and did not synthesize granulose. The $\sigma^E$ inactivation mutant did produce the characteristic solvents (i.e., butanol and acetone), but the extent of solventogenesis was dependent on the physiological state of the inoculum. The $\sigma^G$ inactivation mutant stalled in sporulation during endospore maturation, exhibiting engulfment and partial cortex and spore coat formation. Lastly, the $\sigma^G$ inactivation mutant did produce granulose and exhibited wild-type-like solventogenesis.

Clostridium is a diverse genus of obligate anaerobic, endospore-forming, Gram-positive bacteria. Pathogenic species such as C. botulinum, C. difficile, C. perfringens, and C. tetani produce upwards of 18% of all known bacterial toxins, thus making Clostridium the “most toxic” prokaryotic genus (37) and a significant concern to human and animal health. Cellulolytic and solventogenic species, such as C. thermocellum, C. saccharobutylicum, C. cellulolyticum, and C. acetobutylicum are some of the best-studied biomass-degrading bacteria and exhibit significant potential for renewable biofuel and chemical production (22, 25, 26, 33). Additionally, nontoxigenic, proteolytic species, such as C. sporogenes and C. novyi, are being engineered into promising chemotherapeutic vehicles, in what is called clostridial-directed prodrug therapy (31). Despite the increased interest and activity in clostridia research, key fundamental questions regarding differentiation and physiology remain which are not only significant to the general understanding of the bacterium but also necessary for exploring the broad spectrum of clostridia applications.

Arguably one of the most important fundamental questions is the genetic orchestration of clostridial sporulation and its coupling to other stationary-phase phenomena (22, 33, 34). For example, solvent formation (solventogenesis) is the characteristic stationary-phase phenomenon in solventogenic clostridia, and it is tightly if not causally associated with sporulation except in a set of genetically uncharacterized mutants obtained by random mutagenesis or continuous culture (15, 16, 23, 27). Recent comparative genomics approaches and DNA microarray analyses (17, 34) have reinforced the prevailing assumption that clostridial sporulation is similar if not identical to Bacillus subtilis sporulation, but experimentally this has recently been challenged (12).

In B. subtilis, sporulation is initiated by a multicomponent phosphorelay that activates SpoaA in the predivisional cell (3), which then promotes the expression of mother cell-specific and prespore-specific sigma factors $\sigma^E$ and $\sigma^F$ (45). Active $\sigma^E$ regulates the expression of numerous mother cell-specific genes (9) and, with the combined activity of SpoIID, activates the mother cell-specific $\sigma^F$. The combined activities and intercompartamental communication of $\sigma^E$ and $\sigma^F$ lead to activation of the prespore-specific $\sigma^{\text{III}}$ (45), which then regulates the expression of many prespore-specific genes (51). Disruption of $\text{sigF}$ in B. subtilis blocked sporulation at stage II, resulting in normal-looking sporulation septum but also an accumulation of disporic cells (14). The $\text{sigE}$ disruption also blocked sporulation at stage II, resulting in a similar, disporic morphology as the $\text{sigF}$ mutant. Disruption of $\text{sigG}$ blocked sporulation at stage III, exhibiting engulfment but no spore cortex or coat (7). $\sigma^F$ activation is prevented until septation (41), when SpoIIE dephosphorylates the anti-anti-sigma factor SpoIHA, which then binds the anti-sigma factor SpoIAB to release active $\sigma^E$ (41). $\sigma^E$ activity is stalled until SpoIIGA processes the pro-$\sigma^E$, which requires septation and a physical interaction between SpoIIGA and the $\sigma^E$-regulated SpoIIR (20, 24, 36). Regulation of $\sigma^F$ appears to be complex and multilayered (4, 28).
Transcriptional analysis of pH-controlled *C. acetobutylicum* batch cultures suggested that the orchestration of Spo0A and the major sporulation sigma factors (σ^B^, σ^E^, and σ^G^) are similar to those of *B. subtilis* (17). Spo0A activity spikes during the transition from acidogenesis to solventogenesis (−12 h after inoculation) and remains active throughout the duration of the culture. σ^B^ and σ^E^ both exhibited a major spike in activity during mid-stationary phase (24 h after inoculation), while σ^G^ activity spiked 6 h later, at 30 h after inoculation. Regarding the control of solventogenesis, relatively little is known beyond Spo0A activity (11, 38). Spo0A induces the expression of key solventogenic genes in *C. acetobutylicum* (11, 47), namely, the sol locus genes organized in two operons (aad-cfA-cfB and ade) located on the pSOL1 megaplasmid (6, 32). Furthermore, clostridia exhibit the unique clostridial cell form (16), which is an important sporulation-associated morphology. The clostridial cell form is characteristic of all clostridia, is commonly assumed to be the solvent-producing cell type in solventogenic clostridia (16, 49), and is characterized by the accumulation of granules of a glycogen-like (1,4-glucosyl glucan) biopolymer (39).

Here we examined the roles of σ^B^ and σ^G^ in *C. acetobutylicum* by inactivating their genes and assessed their impact on solvent production and morphogenesis of the clostridial cell form. The disruption strains were characterized by Western blot analysis, Southern blot analysis, semiquantitative reverse transcription-PCR (RT-PCR), sporulation assays, phase-contrast microscopy, electron microscopy, flow cytometry (FC), and metabolite analysis. We found significant differences between these asporogenous clostridia disruption mutants and the corresponding *B. subtilis* mutants. Lastly, we examined the necessity of σ^B^ and σ^G^ for granulose formation and the development of the clostridial cell form, as well as for solvent formation.

**MATERIALS AND METHODS**

**Bacterial strains, media, and growth conditions.** Bacterial strains and their relevant properties are listed in Table S1 of the supplemental material. *Escherichia coli* strains were grown aerobically at 37°C and at 200 rpm in liquid LB medium or solid LB with agar (1.5%) medium supplemented with the appropriate antibiotics (ampicillin at 50 μg/ml, chloramphenicol at 35 μg/ml, or tetracycline at 10 μg/ml). *E. coli* strains were stored at −85°C in 15% glycerol. *C. acetobutylicum* ATCC 824 (here referred to as 824), recombinant strains, and disruption strains were grown anaerobically in liquid clostridial growth medium (CGM) and solid 2% yeast-triptone-glucose (pH 5.8) (2×YTG) at 37°C (52). CGM contained 80 g/liter glucose and was buffered with 30 mM sodium acetate. 2×YTG contained 15 g/liter glucose. The disruption strains were maintained under erythromycin (Em) pressure (100 μg/ml for liquid medium and 40 μg/ml for solid medium) or under chloramphenicol (Th) pressure (5 μg/ml for liquid medium and solid medium). Clostridium strains were stored at −85°C in fresh CGM supplemented with 15% glycerol and revived by anaerobically plating on 2×YTG. For spore-forming strains, colonies were grown for at least a week on 2×YTG and then heat shocked at 80°C for 10 min in CGM. Heat shocking served to kill all vegetative cells, to induce spore germination, and to ensure the presence of the megaplasmid pSOL1, which carries the solventogenic genes (6). Static flask cultures (250 ml) were used for all strain characterization experiments, which were inoculated with a 3% (vol/vol) mid-exponential-phase culture (Log of 0.6 to 1.5). Ten-milliliter static tube cultures were used for all inoculation studies.

**Sporulation assays.** One of the sporulation assays was the heat shocking protocol that was described for starting tube cultures. The second sporulation assay was chloroform treatment, which was performed by combining 0.5 ml of culture and 0.5 ml of 100% chloroform in an Eppendorf tube, incubating anaerobically with mild agitation on a Labquake (Thermo Scientific, Waltham, MA) for 10 min, and then plating 100 μl of serial dilutions of the aqueous phase on 2×YTG plates.

**Cell density, metabolite, and substrate analyses.** Cell growth was monitored by measuring the optical A_600 of a BioMate 3 series spectrophotometer (Thermo Spectronic, Rochester, NY). Culture supernatants were analyzed for glucose, acetate, butyrate, butanol, acetone, ethanol, and acetoin via a Waters (Milford, MA) high-performance liquid chromatograph (48).

**Construction of sigE and sigG gene disruption plasmids.** The plasmids and primers used are listed in supplemental Tables S1 and S2. To specifically target sigE (CAC1695), a 557-bp region of the sigE gene that included the 25th codon through the second nucleotide of the 210th codon was PCR amplified from 824 genomic DNA with Taq polymerase (New England Biolabs [NEB], Ipswich, MA) and the SigE-F/R primer set. The PCR product was cloned into the Invitrogen pcRS-GW-TOPO TA cloning plasmid (Carlsbad, CA) and Invitrogen One Shot TOP10 *E. coli*, resulting in the pcRS-SigE plasmid. pcRS-SigE was linearized by a single NdeI endonuclease digestion site in approximately the middle of the sigE gene fragment, resulting in two regions of homology. The first region of homology started at the first nucleotide of the 25th codon and continued through the second nucleotide of the 109th codon (total of 254 bp), and the second region of homology continued from the third nucleotide of the 109th codon through the second nucleotide of the 210th codon (total of 303 bp). The linearized pcRS-SigE plasmid was blunt ended via NEB Klenow (large fragment) treatment and then dephosphorylated. A thiampenicol resistance cassette (Th' with an optimized Shine-Dalgarno (SD) sequence and under the clostridial phosphotransbutyrylase (pbt) promoter (F_pbt) (44)) was ligated into the linearized pcRS-SigE via NEB Quick Ligase and cloned into TOP10 *E. coli* Quick ligase and cloned into TOP10 *E. coli*. The final replicating, sigE-targeted plasmid was called pKORSIGE.

To target sigG (CAC1696), a 300-bp region of the sigG gene was PCR amplified from 824 genomic DNA with NEB Taq polymerase and the SigG-F/R primer set. The PCR product was cloned into the Invitrogen pcRS-GW-TOPO cloning plasmid and TOP10 *E. coli*, resulting in the pcRS-SigG plasmid. pcRS-SigG was linearized by a single Scal endonuclease digestion site in approximately the middle of the sigG gene fragment, resulting in two regions of homology. The first region of homology started at the first nucleotide of the start codon and continued through the second nucleotide of the 49th codon (149 bp total), and the second region of homology continued from the third nucleotide of the 49th codon through the second nucleotide of the 210th codon (total of 303 bp). The linearized pcRS-SigG plasmid was blunt ended, dephosphorylated, ligated to the 1,610-bp SigG/Cm/pbt gene disruption cassette, and cloned into TOP10 *E. coli*. The final replicating, sigG-targeted plasmid was called pKORSIGO. The final replicating, sigG-targeted plasmid was called pKORSIGO. pKOREC was created by linearizing pRecU (B. Tracy and E. T. Papoutsakis, U.S. patent application 12,437,985) with AvaII and XcmI and gel band purifying the resulting 4,398-bp product. The plasmid backbone was blunt ended, dephosphorylated, ligated to the 1,610-bp SigG/Cm/pbt gene disruption cassette, and cloned into TOP10 *E. coli*. The final replicating, sigG-targeted plasmid was called pKORSIGE.

Prior to transforming into 824, all plasmids were methylated in *E. coli* ER2275(pAN2) to avoid degradation by the endonuclease CAC2241 (29). Plasmids were electrophoresed into 2×YTG as reported previously (30). Th' mutants were outgrown in 10 ml of CGM supplemented with 5 μg/ml Th until the mid-exponential phase of growth, and then 100 μl was spread onto 2×YTG plates supplemented with 5 μg/ml Th and allowed to grow for 24 h. Plates were vegetatively transferred by replica plating every 24 h onto fresh 2×YTG plates with 5 μg/ml Th for four more consecutive days. After the fifth day of Th selection transfers, plates were replica plated onto 2×YTG without antibiotics and vegetatively transferred after another 4 days without antibiotic selection in order to cure cells (i.e., encourage plasmid loss) of the targeted gene disruption plasmid. After the fifth transfer, plates were replica plated onto 2×YTG with 5 μg/ml Th, outgrown for 36 h, and then replica plated onto 2×YTG plates supplemented with 40 μg/ml Em. The backbone of the
replicating gene disruption plasmid contains the macrolide-lincosamide-streptogramin B antibiotic resistance (MLS) gene, which confers Em resistance. Cells that did not lose the plasmid grew rapidly on both Th and Em plates due to the higher copy number of the resistance genes, which was 7 to 10 for this specific plasmid (21). Cells that lost the plasmid and did not integrate the plasmid could not grow at all. Cells that lost the plasmid and underwent a double crossover event were Th resistant and Em sensitive. However, we did not observe any double-crossover mutants. Cells that lost the plasmid and underwent a single crossover contained only a single copy of Th and MLS. Since the Th' cassette has an optimized SD sequence and a P

plasmid expression of Th' on 2×YT plates with 5 μg/ml Th. However, there was a significant delay (~24 h) in growth on 2×YT plates with 40 μg/ml Em for single-copy expression versus plasmid expression. Thus, after 36 h of outgrowth on 2×YT plates with 40 μg/ml Em, Th and Em selection plates were compared for regions of growth/no growth (positive double-crossover events) and growth/delayed growth (putative single-crossover events).

Confirming disruption mutants. Putative single, integration disruption mutants, as determined by antibiotic selection, were used as templates in colony PCRs (44) to determine in which region of homology crossover occurred. To confirm integration through the first region of homology, a primer flanking the 3′ end of the targeted gene open reading frame (ORF) (SigE_Conf_F or SigG_Conf_F) and a primer specific to the 5′ end of the ORF (SigE_Conf_R or SigG_Conf_R) were used to amplify a fragment specific to the 3′ end of the ORF. An ~1,500 bp fragment would generate an ~1,500-bp product. The primer flanking is not complementatory to any sequence on the targeted disruption plasmid; therefore, the PCR product can only be generated if integration occurs through the first region of homology. To confirm integration through the second region of homology, a primer flanking the 3′ end of the targeted gene ORF (SigE_Conf_R or SigG_Conf_R) and a primer specific to the 5′ end of the ORF marker would generate a fragment specific to the 3′ end of the ORF marker (Tc_R) that would generate an ~5,000 bp fragment. To confirm integration from both reactions, integration likely occurred through double crossover, but we never obtained such mutants. We also confirmed the absence of an 824 bp fragment when using the flanking PCR primers for the region of integration (SigE_Conf_F/R or SigG_Conf_F/R). An ~1,000-bp product would be amplified from 824 colony PCR, but nothing would be amplified from the knockout strains, since the product would be >6,000 bp. Additional PCR primer sets were designed to amplify and sequence the entire region of integration, and these are listed in Table S2 in the supplemental material.

Construction of complementation and control plasmids. The entire spoIIGA-sigE-sigG operon, including ~150 bp upstream and downstream, was PCR amplified with Nip Vent polymerase from 824 genomic DNA with primers Sig_OP_F/R. The plHLi plasmid (10) was digested with PvuII, and the 4,802-bp fragment was gel band purified. To prepare the plasmid control, pT, the 4,802-bp fragment was ligated into pBluescript KS II (+) to generate a 4,802-bp product. A 1,500-bp product (Thr_F) if a prominent product was generated from both reactions, integration likely occurred through double crossover, but we never obtained such mutants. We also confirmed the absence of an 824 band when using the flanking PCR primers for the region of integration (SigE_Conf_F/R or SigG_Conf_F/R). An ~1,000-bp product would be amplified from 824 colony PCR, but nothing would be amplified from the knockout strains, since the product would be >6,000 bp. Additional PCR primer sets were designed to amplify and sequence the entire region of integration, and these are listed in Table S2 in the supplemental material.

DNA preparation for Southern blot analysis. Agarose plugs of intact genomic DNA were prepared from cultures at an A900 of 1.8 to 2.0. Sixty-millimeter volumes of culture were anaerobically pelleted (3,100 rpm), resuspended in 10 ml ice-cold wash buffer (100 mM EDTA, 50 mM Tris-HCl, 500 mM KCl), pelleted again (3,100 rpm), and resuspended in 2 ml of prewarmed 37°C lysis buffer (100 mM NaCl, 50 mM EDTA, 1 mg/ml lysozyme; pH 7.5). Cells were incubated in the lysis buffer for 15 min at 37°C and then mixed with an equal volume of CleanCut agarose (2%; Bio-Rad, Hercules, CA). The suspension was sucked into the lysis buffer for 15 min at 37°C and then mixed with an equal volume of DNA electrophoresis was used to separate the DNA fragments of the digested DNA slices. Gels were composed of 0.5× Tis-borate EDTA (TBE) buffer and 0.7% (wt/vol) agarose (Bio-Rad, Hercules, CA) and run for 2.5 h at 80 V. After electrophoresis, gels were washed in 0.25 M HCl for 20 min at room temperature with gentle shaking, rinsed with water, and then treated twice with denature solution (1.65 M NaCl, 0.5 M NaOH) for 20 min each with gentle shaking at room temperature. DNA was then transferred to a BrightStar-Plus positively charged nylon membrane (Ambion, Austin, TX) using upward capillary transfer with the transfer solution (1.65 M NaCl, 0.25 M NaOH). Membranes were then washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and dried for 30 min at 80°C.

Membranes were prehybridized in Ambion ULTRAhyb ultrasonic hybridization buffer for 30 min at 42°C. Probes were denatured prior to being added to the hybridization buffer by boiling for 5 min and then incubating for 5 min on ice. Probes were incubated with the membrane for 16 h at 42°C in a Fisher Scientific hybridization oven (Pittsburgh, PA) with moderate rotation. Membranes were washed in 2× SSC, 0.1% SDS, and then twice in 0.1× SSC, 0.1% SDS for 5 min each at 42°C. Membranes were visualized using the NEB Phototope-star detection reagent (Ambion) according to the manufacturer’s instructions. The CDPSM Khorasan Reagent was diluted to 1:200 for visualization.

Western blot protocol. Crude cell lysates were prepared by pelleting to 3 to 10 ml of culture, discarding the supernatant, resuspending in 500 μl of 1× SDS buffer, boiling for 5 min, and collecting supernatant via centrifugation. Protein was quantified by using the Bio-Rad DC protein assay kit. Fifty micrograms of total protein was resolved on 12% Tris-HCl SDS gels. Five microliters each of the Invitrogen MagicMark XP Western standard and Bio-Rad precision plus kaledochrome standard were run on each gel. Affinity-purified, polyclonal antibody against σE, σG, and SpooA were custom made by ProteinTech (Chicago, IL) in rabbits. Blocking was performed overnight in 2% (wt/vol) nonfat milk. Dilutions of 1:1,000 and 1:2,000 of the primary and secondary antibodies, respectively, were used. Secondary antibody was goat anti-rabbit IgG, horseradish peroxidase conjugated, from Cell Signaling Technology (Danvers, MA). Detection was performed with an Amersham ECL Plus Western blotting detection chemiluminescence kit (GE Healthcare, Piscataway, NJ) and X-ray film development.

Western analysis was performed using ImageJ and normalized to SpooA detection. As detailed below in Results, SpooA detection was nearly constant for all samples and thus served as a housekeeping protein to account for variations in protein loading. To compare relative sigma factor protein amounts between samples and strains, the intensity for a specific sigma factor was divided by the SpooA intensity for that same sample. Exposure times for each membrane were varied to achieve optimal detection, so comparisons are only valid between samples on the same membrane.

Morphological analysis using FC, phase-contrast microscopy, and electron microscopy. Flow cytometric light-scattering analysis was performed as previously detailed (49). FC analysis was performed on a BD LSRII apparatus (Becton-Dickinson, Franklin Lakes, NJ), and forward scatter characteristics (FSC) and side scatter characteristics (SSC) were detected using the 488-nm (blue) laser. Microscopy samples and slides were prepared and imaged as previously described (17, 50).

RESULTS

Stable sigE and sigG chromosomal disruptions by single chromosomal crossover. A replicating plasmid approach, similar to a previously reported technique (11), was used for separately disrupting sigE (CAC1695) and sigG (CAC1696) in C. acetobutylicum. Here, we refer to these mutants as EKO1 and GKO1, respectively. Homologous recombination was stimulated by the recombinant expression of the B. subtilis 168 ATCC 23857 resolvase protein, RecU (BSU22310), as described in Materials and Methods. Two regions of homology

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were employed for targeting each of the two genes, but we were only able to isolate single-integration mutants. Regions of homology were 250 to 300 bp in length for \( \text{sigE} \) and 150 bp in length for \( \text{sigG} \). Putative single-integration mutants were identified and confirmed by colony PCR and DNA sequencing. Sequencing the chromosomal region of integration and flanking regions confirmed that \( \text{sigE} \) disruption occurred by a single chromosomal integration through the 254-bp first region of homology (Fig. 1A), and \( \text{sigG} \) disruption occurred by a single integration through the 151-bp second region of homology (Fig. 1B). For both mutants, the \( \text{Th} \) cassette was inserted in the same coding strand as \( \text{sigE} \) or \( \text{sigG} \), which is also shown in Fig. 1A and B. To determine the stability of the integrants, we vegetatively transferred cultures for more than 80 generations under no antibiotic pressure and compared CFU counts on YTG plates with and without 5 \( \mu \)g/ml Th or 40 \( \mu \)g/ml of TH.

FIG. 1. Schematic representation of chromosomal integration events based on sequencing and colony PCR confirmation for EKO1 (A) and GKO1 (B). Abbreviations: RIT, rho-independent terminator; \( \text{P}_{\text{ptb}} \), \( \text{ptb} \) promoter; HR, homologous region; HR(n), homologous region that is native to the chromosome; bb, backbone; TH, thiamphenicol resistance gene (Th\(^{r} \)). (A) The \( \text{sigE} \)-targeted, replicating plasmid (pKORSIGE) with two contiguous regions of homology (top) and the resulting integration events (bottom). Integration occurred through the first region of homology as confirmed by sequencing (bottom) and colony PCR (right). The junction sequences between chromosomal DNA (lowercase letters) and plasmid DNA homologous regions (uppercase letters) or nonhomologous plasmid sequence (uppercase and underlined) are shown (bottom) based on full sequencing data. Colony PCR products shown on ethidium bromide-stained 1% agarose gel (right) were the following: product from the SigE_Conf_F/Th\(^{r}\)_R primer set (EKO1 lane 1); no product from SigE_Conf_R/Th\(^{r}\)_F primer set (EKO1 lane 2); no product from SigE_Conf_F/SigE_Conf_R primer set (EKO1 lane 3); product from Th\(^{r}\)_F/Th\(^{r}\)_R primer set (EKO1 lane 4). We only obtained product from the SigE_Conf_F/SigE_Conf_R primer set for 824 colony PCR (824 lane 3). The ~1,500-bp product amplified from EKO1 with the SigE_Conf_F and Th\(^{r}\)_R primer set suggests that the antibiotic marker was incorporated near the 5\(^{\prime}\) end of the \( \text{sigE} \) ORF. (B) The \( \text{sigG} \)-targeted, replicating plasmid (pKORSIGG), showing the two contiguous regions of homology (top) and the resulting integration events (bottom). Integration occurred through the second region of homology as confirmed by sequencing (bottom) and colony PCR (right). The junction sequences between chromosomal DNA (lowercase letters) and plasmid DNA homologous regions (uppercase letters) or nonhomologous plasmid sequence (uppercase and underlined) are shown (bottom) and were determined based on full sequencing data. Colony PCR products shown on ethidium bromide-stained 1% agarose gel (right) were the following: no product from SigG_Conf_F/Th\(^{r}\)_R primer set (GKO1 lane 1); product from SigG_Conf_R/Th\(^{r}\)_F primer set (GKO1 lane 2); no product from SigG_Conf_F/SigG_Conf_R primer set (GKO1 lane 3); product from Th\(^{r}\)_F/Th\(^{r}\)_R primer set (sigE mutant lane 4). We only obtained product from the SigG_Conf_F/SigG_Conf_R primer set (lane 3) for 824 colony PCR. The ~1,500-bp product amplified from GKO1 with the SigG_Conf_R and Th\(^{r}\)_F primer set suggests that the antibiotic marker was incorporated near the 3\(^{\prime}\) end of the \( \text{sigG} \) ORF.
Em. There was no difference in the CFU between the antibiotic-free plates and the 5 μg/ml Th plates, and all CFU exhibited delayed growth on 40 μg/ml Em, which is consistent with a single-integration mutant. Colony PCR from 20 CFU (i.e., 10 EKO1 and 10 GKO1) confirmed that single integration was still present and that the plasmid did not excise from the chromosome. Cultures inoculated from cells that had been vegetatively transferred for >80 generations still produced solvents, which suggests that sigE and sigG disruptions did not encourage strain degeneration (loss of the pSOL1 megaplasmid [6]).

**Complementation studies.** In order to demonstrate that the resulting phenotypes were due strictly to sigE or sigG disruptions, we first attempted to complement sigma factor function by expression from a low-copy number replicating plasmid (i.e., copy number of 7 to 10 [21]). Specifically, we expressed the entire spoIIGA-sigE-sigG operon in EKO1, GKO1, and the parent strain, 824, from a replicating plasmid with Tc resistance (i.e., pTSEG) and compared the sporulation frequency to the respective plasmid control (i.e., pT), which only contained the Tc resistance marker. None of the complemented or plasmid control EKO1 or GKO1 strains formed heat- or chloroform-resistant CFU when plated onto solid medium. Interestingly, 824(pTSEG) did not form heat- or chloroform-resistant CFU either, but the plasmid control strain 824(pT) exhibited 80 to 90% of the sporulation frequency of 824. The 824(pTSEG) results, especially, suggest that plasmid-borne expression of the spoIIGA-sigE-sigG operon disrupts normal sporulation, even though the spoIIGA-sigE-sigG operon was transcribed from its natural promoter and the plasmid was present at a low copy number. Previously, it was shown in *B. subtilis* that plasmid complementation of sigE mutants greatly reduced sporulation efficiency to 0.2% that of wild-type (WT) *B. subtilis* (2), and similar results were reported for plasmid complementation of a *C. perfringens* sigE mutant (12). Thus, even better-characterized hosts, such as *B. subtilis*, demonstrate a significant deviation in sporulation control when sigma factors are expressed from a replicating plasmid.

Further investigation in *B. subtilis* demonstrated that by incorporating a single copy of the sigE operon into the amyE chromosomal locus of the *B. subtilis* sigE mutant, sporulation efficiency was restored to WT levels (2). Consequently, we suspected that plasmid complementation of the spoIIGA-sigE-sigG operon in EKO1 and GKO1 could not restore 824 sporulation characteristics due to physiologically inappropriate gene copy number, as previously suggested for σ^K complementation in *B. subtilis* (1). We are currently working to develop genetic tools that will allow us to integrate a single copy of the spoIIGA-sigE-sigG operon back into the chromosome of our disruption mutants. Therefore, as described next, we performed Southern blot analysis to confirm that integration only occurred in the suspected sigE or sigG locations. We also generated and examined several, independent, single-integration mutants in order to provide further evidence that the resulting phenotypes were strictly due to sigE or sigG disruption.

**Southern blot analysis and isolation of independent mutants.** We prepared chromosomal DNA from both mutants and the parent 824 strain in DNA agarose plugs without pipetting in order to prevent DNA shearing. The EKO1 and GKO1 DNA plugs were digested with NdeI and HindIII, respectively. There was a single NdeI and HindIII cut site on the backbone of pKORSIGE and pKORSIGG, respectively, and two cut sites for each enzyme flanking the sigE and sigG regions of integration. Thus, for a probe against the first region of homology for each gene, a single band should be visible in the 824 DNA lanes and two bands should be visible in the EKO1 and GKO1 DNA lanes, because the region of homology is duplicated during single-integration disruption. Results from the sigE Southern blot assay revealed a single band at ~2 kb for 824 DNA and two bands at ~6 kb for EKO1 DNA (Fig. 2A). Results from the sigG Southern blot assay revealed a prominent band slightly larger than 3 kb for 824 DNA and two prominent bands at ~6 kb and ~3 kb for GKO1 DNA (Fig. 2B). There was a faint ~1.2-kb band detected for both the 824 and the GKO1 DNA (we increased the exposure of the 824 DNA lane to better visualize the band). Since this band was noticed in the 824 DNA lane, it is likely nonspecific binding. All DNA bands corresponded to the expected sizes, and aside from the ~1.2-kb band that was also detected in the 824 DNA lane, no additional bands were detected, which strongly suggests that integration only occurred through the intended regions of homology.

To further demonstrate that EKO1 and GKO1 were derived from the intended, single-integration events, we independently generated six more sigE (EKO2 to -7) and four more sigG (GKO2 to -4) disruption mutants. EKO2 to -5 strains were generated with the same replicating plasmid used for generating the original disruption mutant, and EKO6 and -7 were generated with a replicating plasmid that only contained a single region of homology. GKO2 to -5 were generated with the original replicating plasmid. All these independently derived sigE and sigG mutants were single-integration mutants through the same regions of homology as the original mutants (i.e., the first and second regions of homology for sigE and sigG mutants, respectively). The resulting morphologies were all identical to each other with regard to the lack of sporulation...
and the ability to generate solvents, as we describe below for EKO1 and GKO1.

Temporal protein expression of $\sigma^F$, $\sigma^E$, and $\sigma^G$ in EKO1 and GKO1. Western blotting against $\sigma^F$, $\sigma^E$, $\sigma^G$, and Spo0A was performed for EKO1 and GKO1, and results were compared to those for the parent 824 strain. Western blot analysis served to verify that single-integration disruptions did eliminate protein expression and to determine the presence, absence, and relative amounts of other major sporulation factors in these mutants over the course of a batch culture. As previously described, DNA microarray analysis of a pH-controlled batch culture suggested that $\sigma^E$ activity is initiated at 24 h and is maintained throughout the duration of the batch culture. $\sigma^G$ activity appeared to lag 6 h behind (starting at ca. 30 h) and then continued throughout the duration of the batch culture (17). We used this knowledge to choose an appropriate sampling schedule to detect all three sigma factors, although the present batch cultures did not employ pH control. Crude cell lysates were prepared at 24, 32, and 40 h for EKO1, at 32 and 40 h for GKO1, and at corresponding time points for 824 batch cultures. We observed that the intensity of the Spo0A protein was nearly the same for all samples and between all biological replicates. Therefore, and in the absence of an alternative, we used Spo0A intensity as a loading control and for quantifying the relative band intensities. Exposure times for each membrane were varied to achieve optimal detection, and thus comparisons are only valid between samples on the same membrane. Western analysis was repeated for three biological replicates, and similar results were observed as those described below.

$\sigma^E$ was not detected in any of the EKO1 cultures, and $\sigma^G$ was not detected in any of the GKO1 cultures (Fig. 3A and B). There was 10 and 5 times more $\sigma^F$ in 824 cultures than with EKO1 (Fig. 3A) at 24 and 40 h, respectively. There was 3.3 times more $\sigma^G$ in EKO1 cultures than with 824 at 40 h. We also noticed a steady increase in $\sigma^G$ amounts from 24 to 40 h in EKO1 cultures but not in the 824 cultures (Fig. 3A). For GKO1 cultures (Fig. 3B), there was 2.2 and 1.9 times more $\sigma^F$ than 824 at 32 and 40 h, respectively. There was also 2.1 times more $\sigma^F$ in GKO1 cultures than with 824 at 40 h.
**sigE disruption abolishes endospore formation, granulose accumulation, and the clostridial cell form.** We performed sporulation assays (chloroform and heat treatment) on samples taken at 52, 76, and 120 h from flask cultures of EKO1 to test for the presence of mature endospores and free spores. No CFU were observed for any EKO1 to -7 sample from either assay performed on three biological replicates, while 10^2 to 10^4 CFU/ml were observed for samples 76 h or older of two biological replicate cultures of the parent 824 strain. Sporulation frequency can vary significantly for 824, from less than 1% to as high as 30% of the cell population. Low spore counts of 10^2 to 10^4 CFU/ml are often observed for 824, while the remainder of the population presumably lyses over the course of the stationary phase. This was suggested by phase-contrast microscopy images that revealed what appeared to be lysed cells (data not shown) and by comparing CFU between chloroform-treated and untreated 824 cells at 48, 60, 76, and 112 h (data not shown). The CFU for chloroform-treated cells increased from 48 to 76 h, plateaued at 76 h, and persisted at this count through 112 h and later. The CFU of untreated cells dramatically decreased from 48 to 76 h, and the CFU counts of treated and untreated cells converged at 76 h, which suggests that the only viable cells were chloroform-resistant endospores and free spores.

In order to characterize the morphological impact from disrupting sigE, we imaged cells by phase-contrast microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). Phase-contrast microscopy of EKO1 samples taken during exponential growth revealed similar morphological development as in 824 samples (Fig. 4A, compared to C, exponential at 18 h). Specifically, we noticed rod-shaped and vegetative or vegetative-like cells in all fields of view. During early stationary phase (i.e., 32 h), rod-shaped morphologies with small, dark “polar bodies” at one or both ends of the cell were the predominant morphology (Fig. 4A, early stationary phase) for EKO1. Cells did not exhibit the characteristic swelling of the clostridial cell form (Fig. 4C, early stationary), nor phase-bright endospore and mature spore morphologies (Fig. 4B, late stationary phase) were witnessed in 824 cultures.
4C, late stationary, 76 h). Instead, the rod-shaped morphologies with small, dark polar bodies remained the predominant morphology throughout the duration of EKO1 batch cultures (Fig. 4A, late stationary), thus appearing to be the terminal differentiation phenotype.

All SEM fields of view for EKO1 samples taken at late stationary phase (i.e., 76 h) confirmed the presence of this rod-shaped morphology, the absence of swollen clostridial cell forms, and the presence of what appeared to be vegetatively dividing cells (Fig. 5A). TEM images from the same late-stationary-phase samples revealed intracellular details of this apparently terminal differentiation state of EKO1 (Fig. 5B and C). The dark, polar bodies seen by phase-contrast microscopy (Fig. 4A) persisted and appeared to be electron-translucent DNA condensing at the poles of the cell (Fig. 5B). TEM also revealed what appeared to be a longitudinal membrane (Fig. 5B and C), which has never been observed in images of the parent 824 strain or an appropriate plasmid control (Fig. 6A to E). The membrane appears to originate from observable “nodes” at the middle of the cell as opposed to the poles (Fig. 5C), and we thus suspect that it represents a malformed asymmetric septum that grew longitudinally as opposed to laterally. Greater than 65% of the cells observed by TEM had the longitudinal membranes, and only one EKO1 cell (i.e., <2%)

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**FIG. 5.** SEM and TEM images of EKO1 cells. All images were captured for batch cultures that were 76 h old (late stationary phase of cultures). (A) SEM image showing the rod-shaped, vegetative-like cells. There were no swollen, clostridial-cell-form morphologies observed. (B) TEM image obtained to interrogate the apparent terminal differentiation morphology. Solid arrows point to what appears to be phase-bright, condensed DNA. Arrows with dotted lines point to what appear to be malformed, longitudinal membranes. (C) TEM image. Arrows with dotted lines point to what also appears to be two malformed, longitudinal membranes with “nodes” at the center of the cell, from which two membranes appear to originate.

**FIG. 6.** SEM and TEM images of 824(pSOS95del) cells. (A) SEM image at 24 h (i.e., transition phase of growth). The solid arrow points to vegetative, dividing cells, and the arrow with the dotted line points to what appears to be asymmetric septum formation. (B) SEM image at 76 h (i.e., late stationary phase). The solid arrow points to what appears to be an endospore in a mother cell, and the arrow with a dotted line points to a swollen, clostridial cell form. The solid arrow points to what appears to be an asymmetric septum, and the arrow with the dotted line points to a symmetric septum between two cells just before division. (D) TEM image at 36 h (i.e., early stationary phase). The solid arrow points to the engulfed endospore, and the arrow with the dotted line points to granulose accumulation. (E) TEM image at 76 h (i.e., late stationary phase). The solid arrow points to spore coat formation, and the arrow with the dotted line points to the developing spore cortex as the endospore matures, prior to being released from the mother cell.
had what appeared to be a correctly formed asymmetric septum. We compared SEM and TEM images to the plasmid control strain, 824(pSOS95del) (50), because this allowed for comparison to growth under antibiotic pressure (Fig. 6A to E), and the control strain exhibited all the expected sporulation-associated, differentiation morphologies. The 824(pSOS95del) strain formed a correctly positioned polar septum during the transition phase (i.e., 24 h) of growth (Fig. 6A and C). During early stationary phase (i.e., 36 h), the forespore became engulfed (Fig. 6D), and the mother cell accumulated granulose. In late stationary phase (i.e., 76 h), the endospore developed a thick cortex and spore coat (Fig. 6B and E) before being shed from the mother cell.

In contrast, the B. subtilis sigE disruption mutant formed a proper asymmetric septum (14). However, the B. subtilis ftsA inactivation mutant rendered a phenotype that was blocked at asymmetric septum formation (18), which is similar to EKO1. FtsA is an actin-like protein that acts in concert with the cytokinetic protein FtsZ to direct septum formation (18). Interestingly both genes are adjacent to the spoIIGA-sigE-sigG operon [i.e., ftsA/Z (CAC1692/3) and spoIIGA-sigE-sigG (CAC1694/5/6)]. Thus, we wanted to ascertain that the ftsA gene locus had not been affected by the sigE disruption and that polar effects were not occurring. Therefore, we compared the chromosomal sequence and transcription of ftsA/Z (CAC1692/3) in EKO1 to 824. We PCR amplified the ftsA/Z locus (CAC1692/3), including 250 bp upstream and downstream, and sequenced the resulting PCR product. Sequencing data suggest that higher levels of expression were not profoundly different. Additionally, there was no antisense RNA (asRNA) detected for the ftsA/Z region (CAC1692/3) in EKO1 to 824, (50), which is similar to EKO1. FtsA is an actin-like protein that acts in concert with the cytokinetic protein FtsZ to direct septum formation (18). Interestingly both genes are adjacent to the spoIIGA-sigE-sigG operon [i.e., ftsA/Z (CAC1692/3) and spoIIGA-sigE-sigG (CAC1694/5/6)]. Thus, we wanted to ascertain that the ftsA gene locus had not been affected by the sigE disruption and that polar effects were not occurring. Therefore, we compared the chromosomal sequence and transcription of ftsA/Z (CAC1692/3) in EKO1 to 824. We PCR amplified the ftsA/Z locus (CAC1692/3), including 250 bp upstream and downstream, and sequenced the resulting PCR product. Sequencing confirmed that there were no mutations or differences between EKO1 and 824 (see Fig. S1 in the supplemental material). We also performed semiquantitative RT-PCR on RNA samples taken at 6, 16, and 32 h for the sigE and ftsA genes (Fig. 6A and C). During early stationary phase (i.e., 36 h), the forespore became engulfed (Fig. 6D), and the mother cell accumulated granulose. In late stationary phase (i.e., 76 h), the endospore developed a thick cortex and spore coat (Fig. 6B and E) before being shed from the mother cell.

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Significantly, there was no granulose accumulation observed in any field of view of EKO1 (Fig. 4A, and 5B and C). These data suggest that sigE disruption prevents the synthesis of granulose and, thus, plays a significant role in the morphogenesis of the clostridial cell form.

We also sought to capture the dynamics of sporulation (or lack thereof) for a larger population of cells than could be examined by phase-contrast or electron microscopy. We recently showed that FC could accurately capture and quantify the morphologies and kinetics of sporulation by light-scattering (LS) analysis alone (49). Therefore, we used FC-LS analysis to capture the bulk, temporal morphology changes of 50,000 individual EKO1 cells, with the aim to relate these large population assays to the microscopy images of Fig. 4 and 5. In typical 824 batch cultures (Fig. 7C), exponential-phase cells (i.e., 18 h) condense into a low-FSC population that represents rod-shaped cells. During transition to mid-stationary phase (i.e., 44 h), a mid-FSC population evolved out of the low-FSC population; this population represents clostridial cell forms and forespore-containing cells (Fig. 7C). During late stationary phase (i.e., 76 h), a distinct and condensed, high-FSC population evolved out of the mid-FSC population; this population represented endospore-containing cells and mature spores. The same LS phenomena have been noted in the plasmid control, 824(pSOS95del) (49). The EKO1 cultures only exhibited similar LS characteristics as 824 cultures during exponential growth, with the majority of the population condensing into a low-FSC population (Fig. 7A). During all phases of growth, we observed at most 0.3% of the light scattering events in the mid- or high-FSC gates, which strongly suggests that sporulation was stalled prior to clostridial-cell-form formation. Furthermore, this confirmed the microscopy results for the bulk population.

sigG disruption severely impacts endospore architecture and aborts sporulation before mature spores are formed. The morphological characteristics of GKO1 were examined by sporulation assays, FC-LS analysis (Fig. 7B), phase-contrast microscopy (Fig. 4B), and SEM and TEM (Fig. 8). Samples for the sporulation assay were collected at 52, 76, and 120 h. No CFU were observed from either spore assay for any GKO1-5 sample. FC-LS analysis of GKO1 samples taken during exponential, mid, and late stationary growth phases demonstrated similar LS characteristics as 824 cultures through the mid-stationary growth phase (Fig. 7B and C). In contrast to 824, late-stationary-phase GKO1 cells did not exhibit the separate, condensed, high-FSC population that is indicative of free spores (Fig. 7B and C, late stationary). The absence of free spores was consistent with the sporulation assay results.

Phase-contrast microscopy of samples taken between 12 and 120 h revealed similar morphology development to that of 824 batch cultures through exponential growth (Fig. 4B, compared to C). GKO1 cultures generated swollen, phase-bright cells similar to clostridial-form cells during early and mid-stationary phases (Fig. 4B). In late stationary phase, GKO1 cultures did not exhibit well-defined, phase-bright endospores, as observed in 824 cultures (Fig. 4B versus C). Instead, the clostridial-cell-form morphology persisted, and phase-dark morphologies with less defined endospores that were not as phase bright were observed (Fig. 4B). Phase-bright free spores were never observed in GKO1 cultures. SEM analysis of late-stationary-phase (i.e., 76 h) GKO1 cultures confirmed the development of swollen, clostridial-cellform morphologies (Fig. 8A). TEM analysis revealed that GKO1 advanced to forespore and endospore formation; however, the architecture of the developing endospore was severely compromised, and mature spore structures never developed. This was especially apparent compared to images for 824(pSOS95del) (Fig. 6) and images from previous publications (17, 49). In many cases, GKO1 endospores were abnormally elongated (Fig. 8B), and the spore cortex was much thinner or not present (Fig. 8B, C, and D). The spore coat was either incomplete or fragmented (Fig. 8B, C, and D). There was granulose formation in over 90% of endospore-containing cells (Fig. 8C and D).

Overall, sigG disruption appeared to have a large impact on the architecture of the developing endospore, which suggests that sigG activity is localized to the endospore. To investigate sigG localization, we performed intracellular immunofluorescence (ICIF) confocal microscopy against sigG in 824(pSOS95del). As
shown in Fig. S3 of the supplemental material, ICIF revealed that $\sigma^G$ was strongly localized to endospores.

**sigE and sigG disruptions preserve solvent formation, but EKO1 exhibits variable metabolism that depends upon the inoculum.** Typical 824 batch cultures exhibit two phases of metabolism, acidogenesis and solventogenesis. During acidogenesis, cells grow exponentially, consume glucose, and produce acetic and butyric acids to final concentrations of $\sim 20$ mM and $50$ mM, respectively. For reasons not fully understood (43), cells enter the stationary phase of growth and switch into solventogenic metabolism, which is characterized by continued glucose consumption and acid reassimilation into the solvents acetone, butanol, and ethanol. Final solvent concentrations are typically $140$ to $170$ mM butanol, $70$ to $90$ mM acetone, and $10$ to $30$ mM ethanol, and acids are largely reassimilated such that final organic acid concentrations are less than $20$ mM. When inoculated with typical mid-expontial-growth inocula, the EKO1 exhibited normal exponential growth and acidogenesis with maximum acetic and butyric acid concentrations of $21 \pm 2$ mM (mean $\pm$ standard error) and $51 \pm 3$ mM, respectively. However, solventogenesis was only sustained for a brief period of time, resulting in final solvent concentrations of $52 \pm 15$ mM butanol, $30 \pm 6$ mM acetone, and $8 \pm 2$ mM ethanol. Acid reassimilation was also impaired, with final concentrations of $21 \pm 3$ mM acetic acid and $39 \pm 1$ mM butyric acid. The GKO1 mutant exhibited normal exponential growth and acidogenesis,

**FIG. 7. Time course FC analysis of EKO1 (A), GKO1 (B), and 824 (C) batch cultures.** Gates: 1, low FSC (rod-shaped cells); 2, mid-FSC (clostridial-cell-form and forespore-containing cells); 3, high FSC (endospore-containing cells and free spores) (49). Exponential, mid-stationary, and late stationary phases refer to 18, 44, and 76 h, respectively. (A) FC analysis of EKO1 demonstrated that only vegetative morphologies were present throughout the culture time course. No significant gate 2 or 3 cell populations were observed. (B) FC analysis of GKO1 demonstrated that differentiation was stalled at endospore-containing cells. No distinct, condensed cell population in gate 3 was observed, which we did observe in 824 cultures (shown in panel C). Such a population represents free spores, which were not observed in GKO1 cultures. (C) FC analysis of a typical 824 time course.
with maximum acetic and butyric acid concentrations of 20 ± 1 mM and 48 ± 2 mM, respectively. Normal solventogenesis then proceeded, resulting in final solvent concentrations of 160 ± 5 mM butanol, 82 ± 4 mM acetone, and 26 ± 2 mM ethanol.

Since the differentiation program of EKO1 was severely affected, we decided to analyze the impact of the physiological state of the inoculating population on solventogenesis. We did this by withdrawing inocula for fresh 10-ml tube cultures from 250-ml static flask batch cultures at different stages of growth. Specifically, cells were withdrawn at 4 h (pre-exponential stage), 12 h (mid/late exponential), 24 h (early stationary), 32 h (mid-stationary), and 60 h (late stationary). A typical inoculum consisted of mid/late-exponential-phase cells, which is what we used for the original static flask batch culture analyses. The 10-ml tube cultures were grown for 100 h, and then we measured the metabolite concentrations. All 824 and GKO1 cultures produced normal concentrations of solvents and reassimilated acids, independent of the growth phase of the inoculum. Interestingly, EKO1 tube cultures started with pre-exponential-, early-, stationary-, and mid-stationary-phase inocula resulted in normal solvent formation and acid reassimilation. The final butanol concentrations for the respective inocula were 157 ± 7 mM, 159 ± 14 mM, and 162 ± 4 mM. In contrast, the mid/late-exponential-phase inoculum produced only 21 ± 7 mM butanol, which is consistent with the poor solventogenesis we originally observed. EKO1 cultures inoculated with late-stationary-phase inocula did not grow.

DISCUSSION

This study aimed to examine the impact of the two major sporulation-specific sigma factors, σE and σF, on the sporulation and metabolism of C. acetobutylicum and to assess if their role is similar to their role in the well-established B. subtilis model. Based on the sequenced genomes, clostridia appear to have a well-preserved set of homologous sporulation-specific sigma factors that are similar to those in bacilli, despite the large differences in the machinery responsible for the initiation of sporulation, that is, phosphorylation-induced Spo0A activation (34). It was recently reported that the B. subtilis developmental sequence of sporulation-specific sigma factor-controlled events are not preserved in C. perfringens (12). In that study, major differences in the sequence and timing of major sigma factor expression were observed, most notably the expression of annotated σE prior to σF (12). Additionally, the B. subtilis regulons of these sigma factors are not readily recognizable in clostridia (17, 34). Since developmental morphogenesis and function rest largely with genes under the control of these sigma factors, it is important to experimentally interrogate their roles in both sporulation and solventogenesis. Additionally, the metabolism of solventogenic clostridia has great applied potential (22, 33) and is known to be intimately related to sporulation (17). Thus, we also aimed to examine if we could decouple sporulation from solventogenesis by manipulating the expression of these major sigma factors.

sigE disruption arrests sporulation prior to asymmetric cell division. EKO1 did not form an asymmetric septum; rather, it generated what appeared to be a longitudinally directed septum that did not form polar compartments at either pole of the cell (Fig. 4 and 5). There was only one single cell with a correctly formed polar septum observed in all TEM fields of view, which was less than 2% of all cells observed. This suggests that the C. acetobutylicum sigE disruption is stalled in sporulation prior to stage II. In contrast, the B. subtilis sigE disruption mutant (14) and the recently reported C. perfringens sigE disruption mutant (12) stalled in sporulation at early stage II. Specifically, those sigE disruptions were shown to generate disporic cells that had properly formed polar septum and multiple septa at one pole in the case of the C. perfringens sigE disruption. As shown in Fig. 4A, approximately 20% of the sigE mutant cells displayed two dark polar bodies, which is perhaps indicative of an aborted program to morph a disporic cell. Moreover, TEM confirmed that these polar bodies were not enclosed in a normal, asymmetric septum (Fig. 5B compared to 6C).

Western blot analysis of EKO1 showed a downregulation of σF.
at the protein level (Fig. 3A), which we believe can be explained by the B. subtilis model of $\sigma^F$ activity regulation. In B. subtilis, $\sigma^F$ protein levels are partly due to positive autoregulation, but such self-stimulation is dependent on activation of $\sigma^F$ by a $\sigma^E$-dependent pathway, which also requires a correctly formed asymmetric septum (8, 13). Since EKO1 cannot activate $\sigma^F$-dependent pathways and did not correctly form an asymmetric septum, $\sigma^F$ is likely not activated. Additionally, it was shown in B. subtilis that $\sigma^F$ activity promotes its own expression from the dacF-spoIIA operon (dacF-spoIIA-spoIAB-sigF) later in sporulation (42). The C. acetobutylicum spoIIA operon [spoIIA-spoIAB-sigF (CAC2308-6)] has a predicted $\sigma^F/\sigma^G$ promoter (35), which, coupled with the predicted bimodal activity of $\sigma^F$ and expression data for sigF and spoIIE (17), suggests that $\sigma^F$ can also autoregulate its expression in C. acetobutylicum.

Detection of $\sigma^G$ protein in EKO1 supports the presence of a monocistronic sigG transcript (Fig. 3C, T3). Computational (35) and DNA microarray analyses (17) suggest that sigG is expressed from the tricistronic mRNA, spoIIGA-sigE-sigG (CAC1694 to -1696). Yet, Northern analysis (11) and primer extension analysis (40) also suggest the presence of a dicistronic spoIIGA-sigE (CAC1694 to -1695) and a monocistronic sigG (CAC1696) transcript. Northern analysis further demonstrated that the mono- and dicistronic transcripts were more prevalent during early to mid-stationary phases of growth than the tricistronic spoIIGA-sigE-sigG transcript (11). If $\sigma^G$ were only translated from the tricistronic transcript in EKO1, transcription should be aborted by the rho-independent terminator positioned at the 3’ end of the disrupting Th’ cassette (Fig. 1A). Thus, a monocistronic sigG transcript could explain the presence of $\sigma^G$ in EKO1. For B. subtilis, there is a known $\sigma^F/\sigma^G$ promoter upstream of the sigG gene, and it has been shown that $\sigma^G$ can augment its own expression following engulfment (5, 46). In C. acetobutylicum, a stem-loop structure has been predicted immediately downstream of the spoIIGA-sigE operon (40), but a promoter has yet to be determined in front of the sigG gene (35), and engulfment does not occur in EKO1. The increased detection of $\sigma^G$ could be due to polar effects. The Th’ cassette does have its own promoter, and it is in the same coding strand as sigG (Fig. 1A). However, this would require read-through through a rho-independent terminator and ~4 kbp of additional inserted plasmid DNA. Overall, the presence of $\sigma^G$ in EKO1 is not necessarily surprising, but we cannot conclude at this time why there was an increased amount of $\sigma^G$ compared to 824 (Fig. 3A).

(sigG) disruption arrests sporulation at the engulfment stage. GKO1 differentiation appeared to be blocked at stage V of sporulation. Forespores and engulfed endospores were formed (Fig. 8), but spore cortex and coat development were severely impaired, which was further demonstrated by the inability to survive spore assays. Significantly, and in contrast to EKO1, GKO1 produced granulose and clostridial cell forms (Fig. 8). The presence of spore cortex and spore coat fragments suggests that GKO1 advances further in sporulation than the B. subtilis sigG disruption mutant. The B. subtilis strain did not produce spore cortex or coat proteins (7). The increased amounts of $\sigma^F$ and $\sigma^E$ detected in GKO1 compared to 824 were possibly due to sporulation stalling at $\sigma^G$ activation. Thus, cells actively expressing high levels of $\sigma^F$ and $\sigma^E$ could accumulate over time and become a larger fraction of the population, compared to equivalent time points in 824 cultures.

$\sigma^E$ plays a regulatory role in granulose synthesis and the clostridial cell morphogenesis. EKO1 did not produce granulose or generate clostridial cell forms. Therefore, it appears that $\sigma^E$ activity is required for granulose production and the development of the clostridial cell morphology in C. acetobutylicum. The abolishment of granulose biosynthesis upon $\sigma^E$ inactivation suggests that the genes or proteins that control granulose biosynthesis are directly or indirectly controlled by $\sigma^E$. The two genes that were previously suggested to be responsible for granulose formation, CAC2237 and CA2240, have a proposed $\sigma^F/\sigma^G$ binding site (35). Thus, if these promoter predictions are correct, this supports our previous hypothesis that $\sigma^E$ is not activated in EKO1. Additionally, a recent DNA microarray analysis (17) showed that the CAC1664 gene was highly expressed during the transition phase of growth and just prior to the expression of the CAC2237 and CA2240 genes. CAC1664 encodes a putative glycogen phosphorylase and has a predicted $\sigma^E$ binding motif in its promoter (35). Thus, it too may play a role or the major role in granulose biosynthesis (17).

In B. subtilis, glycogen (likely similar to granulose) is synthesized only under special conditions and apparently by a $\sigma^F$-regulated promoter (19). In C. perfringens, $\sigma^E$ inactivation did not abolish granulose formation, but $\sigma^E$ inactivation did. In the C. perfringens analysis it was hypothesized that $\sigma^E$ activity is initiated earlier than $\sigma^F$ activity (12). Thus, it appears that there is a lot of diversity in the role of these sporulation-specific sigma factors in clostridia, and studies such as this will greatly advance our fundamental understanding of this class of bacteria.

$\sigma^G$ is not necessary for solventogenesis, while $\sigma^E$ may play a role in certain cell states. GKO1 produced solvent titers that were comparable to the parent 824 strain, which strongly suggests it does not play a regulatory role in solventogenesis. EKO1 could also produce solvent titers that were comparable to 824, albeit using a modified inoculation. Solvent formation for EKO1 cultures depended upon the physiological state of the EKO1 inoculum, which might be explained by an epigenetic inheritance event or the prevalence of a rigorous solvent-forming and proliferative cell state in certain inocula. For example, if $\sigma^E$ disruption stalled sporulation very early and prevented cells from reverting back into a vegetative cell state, then cells that did not undergo endospore differentiation would be the only cells that can vegetatively grow when inoculated into a fresh culture. Subsequently, this might suggest that the solvent-forming cell type does not actually go into endospore differentiation, which is in accordance with our recent suggestion (49) that the clostridial cell form is not the primary solvent-producing cell, but rather detracts from solventogenesis by storing carbohydrate sources in anticipation of energetic and carbon needs for sporulation. Significantly, the $\sigma^E$ disruption does demonstrate that solvent formation and endospore differentiation can be decoupled from each other at a very early stage of endospore differentiation.

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