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Northern, Morphological, and Fermentation Analysis of *spo0A* Inactivation and Overexpression in *Clostridium acetobutylicum* ATCC 824

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The *Clostridium acetobutylicum* ATCC 824 *spo0A* gene was cloned, and two recombinant strains were generated, an *spo0A* inactivation strain (SKO1) and an *spo0A* overexpression strain [824(pMPSOA)]. SKO1 was developed by targeted gene inactivation with a replicative plasmid capable of double-crossover chromosomal integration—a technique never used before with solventogenic clostridia. SKO1 was severely deficient in solvent formation: it produced only 2 mM acetone and 13 mM butanol, compared to the 92 mM acetone and 172 mM butanol produced by the parental strain. After 72 h of growth on solid media, SKO1 formed long filaments of rod-shaped cells that failed to septate. SKO1 cells never achieved the swollen clostridial form typical of the parental strain and did not form endospores. No *spo0A* transcripts were detected in SKO1, while transcription of two solvent formation operons (*aad-ctfA-ctfB* and *adc*; both containing 0A boxes in their promoter regions) was limited. Strain 824(pMSPOA) produced higher butanol concentrations than the control strain [824(pIMP1)] and dramatically elevated *spo0A* transcript levels and displayed a bimodal pattern of *spo0A* transcription similar to that of *B. subtilis*. Microscopic studies indicated that sporulation was both enhanced and accelerated due to *spo0A* overexpression compared to that of both the 824(pIMP1) and parental strains. Consistent with that, expression of the key solvent formation genes (*aad-ctfA-ctfB* and *adc*) and three sporulation-specific genes (*spoIIGA*, *sigE*, and *sigG*) was observed earlier in strain 824(pMSPOA) than in the plasmid control. These data support the hypothesis that Spo0A is a transcriptional regulator that positively controls sporulation and solvent production. Its effect on solvent formation is a balancing act in regulating sporulation versus solvent gene expression: its overexpression apparently tips the balance in favor of accelerated and enhanced sporulation at the expense of overall solvent production.

The series of complex morphological changes that occur during the sporulation process in *Bacillus subtilis* has been extensively studied. Differentiation into endospores involves more than 125 genes, the transcription of which is temporally and spatially controlled by six RNA polymerase sigma factors (σ^A, σ^H, σ^F, σ^E, σ^G, and σ^K), and four DNA binding proteins (Spo0A, AbrB, Hpr, and Sin) (38). Spo0A controls the initiation of sporulation, the development of competence for DNA uptake, and many other stationary-phase-associated processes. In response to environmental, cell cycle, and metabolic signals, the phosphorelay two-component signal transduction system is responsible for phosphorylating Spo0A (Spo0A~P), thus activating its function. Once activated, Spo0A is able to activate or repress transcription at the promoters that it controls (13). The consensus DNA binding site for Spo0A~P is a 7-bp sequence (5′-TGNCGAA-3′, with a preference for N = T) called a 0A box (when the opposite orientation is observed, the sequence is referred to as a reverse 0A box).

Morphological and molecular studies have long suggested that *B. subtilis* and *Clostridium acetobutylicum* employ similar mechanisms for sporulation even though the environmental triggers of sporulation differ in these two organisms (32, 46). To date, four genes encoding homologues to *B. subtilis* sigma factors σ^A, σ^E, σ^G, and σ^K have been cloned and studied in *C. acetobutylicum* (32, 34, 35, 46). The genes *spoIIGA*, *sigE*, and *sigG* are clustered on the *C. acetobutylicum* chromosome (32, 46). The three homologous genes in *B. subtilis*, *spoIIGA*, *spoIIGB* (*sigE*), *and spoIIIG* (*sigG*), form a gene cluster with two different promoters, a σ^A-specific promoter upstream of *spoIIGA* and a σ^H-dependent promoter upstream of *sigG*. Primer extension analysis (32) confirmed that this is also the case in *C. acetobutylicum* DSM 1731 (which is virtually identical [15] to the type strain *C. acetobutylicum* ATCC 824). Despite these similarities between the two organisms, the recently published genome sequence of *C. acetobutylicum* ATCC 824 (25) suggests that many of the identified *B. subtilis* sporulation genes are missing in *C. acetobutylicum*. It is likely, then, that in *C. acetobutylicum* the control of sporulation and other associated stationary-phase events is substantially different from that in *B. subtilis*.

Specific to the transition to the stationary phase in *C. acetobutylicum* is a global response including a metabolic shift to solvent formation, initiation of sporulation, granulose accumulation, and expression of heat shock proteins (1). The molecular nature of the common and separate control mechanisms of these phenomena is still largely unknown (1, 19, 33). Spo0A-mediated regulation of solvent formation has been documented in *C. beijerinckii* NCIMB 8052 (2, 28) with an *spo0A*

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mutant generated by an integration of plasmid pSRW44, which contains an internal fragment (ca. 540 bp long) of the *spo0A* gene (44). This asporogenous mutant (AA243) contains several copies—estimated to be five—of pSRW44 (the sequence of the locus of integration has not been reported). AA243 produces extremely low levels of solvents (<3 mM butanol and no acetone), even under the most favorable conditions (butyrate and acetate addition in controlled-pH fermentations [28]). Ravagnani and coworkers (28) used gel retardation assays to show that the C-terminal domains of both the *C. beijerinckii* and *B. subtilis* Spo0A proteins bind to the *C. beijerinckii ptb* gene promoter (which contains two 0A boxes). *ptb* codes for the phosphotransbutyrylase (PTB) protein, which catalyzes the first step in the conversion of butyryl coenzyme A (CoA) to butyrate. They also examined the *C. acetobutylicum adc* gene, whose promoter region contains three 0A boxes. *adc* codes for acetoacetate decarboxylase, which catalyzes the formation of acetone from acetoacetate. Because DNase I footprinting failed to show protection of the 0A boxes, they carried out in vivo gene expression experiments with the parental *C. beijerinckii* strain with the *gusA* reporter system to show that Spo0A binds to both *ptb* and *adc* promoter 0A boxes, downregulates *ptb* expression, and upregulates *adc* expression (28).

The *spo0A* gene was previously mapped to the *C. acetobutylicum* ATCC 824 chromosome (3) and has been assigned position CAC2071 on the chromosome (25). Here we report the cloning and characterization of this *spo0A* gene by both inactivating it and overexpressing it in this organism. To our knowledge, overexpression of *spo0A* has not been previously reported in any microbial system. We carried out morphological, fermentation, and Northern analyses to examine the role of *spo0A* in the regulation of both solventogenesis and sporulation in *C. acetobutylicum* ATCC 824. *C. beijerinckii* NCIMB 8052 and *C. acetobutylicum* ATCC 824 are two very different organisms with different chromosome sizes (6.5 versus 4 Mb, respectively [25, 43]). They show very little DNA similarity (15) and have different phenotypic characteristics (e.g., *C. beijerinckii* degenerates must faster than *C. acetobutylicum* and displays different degeneration characteristics). The two organisms contain different solvent formation enzymes (e.g., no *aad*/*adhE1* in *C. beijerinckii*) and have different product formation gene arrangements. In fact, in *C. acetobutylicum*, all of the essential solvent formation genes are located on the pSOL1 megaplasmid (whose loss leads to strain degeneration [4]), while there is no such plasmid in *C. beijerinckii*. Thus, control of sporulation and solvent formation may involve different mechanisms in these two organisms.

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 lists the bacterial strains and plasmids used in this study.

Growth and maintenance of strains and fermentation analysis. *Escherichia coli* strains were grown and maintained as previously described (24). All of the recombinant clostridial strains examined in this study were derived from *C. acetobutylicum* ATCC 824. All clostridial strains were grown anaerobically at 37°C either in liquid cultures in 10-ml tubes of clostridium growth medium (CGM [42]) or on agar (15 g/liter) plates of reinforced clostridial medium (Difco Laboratories, Detroit, Mich.). For electroporation, *C. acetobutylicum* was grown under anaerobic conditions at 37°C in liquid 2XYTG (pH 5.2) supplemented with 100 μg of erythromycin (ERM) per ml as needed (22). Transformants were grown on 2XYTG plates (pH 5.8) with the appropriate antibiotics (40 μg of ERM per ml or 25 μg of thiamphenicol [TH] per ml) after electroporation. CBM plates were used to grow cells for morphological studies (26). For long-term storage, *C. acetobutylicum* strains were frozen at −85°C in CGM with 15% glycerol. Controlled pH 5 bioreactor fermentations (2- or 5-liter total volume with clarithromycin, a pH-stable derivative of ERM, used for selection of recombinant strains) and glucose and fermentation product analyses were carried out as previously described (9, 24).

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Bacterial strains		
C. acetobutylicum		
ATCC 824		ATCC[b]
SKO1	ATCC 824 *spo0A*::MLS^r	This study
E. coli ER2275	*recA lacZ mcrBC*	NEB[c]
Plasmids		
pAN1	Cm^r; carries the ϕ3TI gene	20
pIMP1	Ap^r	22
pMSPOA	Ap^r MLS^r; carries *spo0A*	This study
pJC4	MLS^r Tc^r	17
pGEM-4Z	Ap^r	Promega[d]
pTHAAD	Ap^r Cm^r; carries *aad*	18
pCLH2	Cm^r	This study
pSCLH2	pCLH2 with 0.58-kb internal *spo0A* fragment	This study
pESCLH2	pSCLH2 with MLS^r	This study
pETSPO	Derivative of pESCLH2	This study
pFNK6	Ap^r MLS^r; carries *adc ctfA ctfB*	21
pHXS5	Ap^r; carries *orf1 aad ctfA ctfB*	23

[a] Abbreviations: Cm^r, chloramphenicol resistant; ϕ3TI, ϕ3TI methyltransferase; Ap^r, ampicillin resistant; Tc^r, tetracycline resistant.
[b] ATCC, American Type Culture Collection, Manassas, Va.
[c] New England Biolabs.
[d] Promega, Madison, Wi.

Amylase activity assay. The presence of the pSOL1 megaplasmid (4) in *C. acetobutylicum* strains was detected by monitoring amylase activity on 2XYTGMA plates (P. Soucaille, personal communication). This was possible due to the presence of two pSOL1-encoded α-amylase genes that are not subject to catabolite repression. The components of 2XYTGMA plates (per liter) were 16 g of Tryptone, 10 g of yeast extract, 2 g of NaCl, 4 g of glucose, 5 g of soluble starch, 41.9 g of morpholinepropanesulfonic acid (MOPS), and 15 g of agar. The pH of 2XYTGMA was adjusted to 6.5 since this pH was optimal for α-amylase activity. After 36 to 48 h of growth on 2XYTGMA plates, 100 μl of a 5% iodine solution was spread over the surface of the plate. Bacterial colonies with amylase activity (thus derived from cells carrying the pSOL1 megaplasmid) degraded the starch surrounding the colonies, resulting in an unstained halo around them following treatment with iodine.

Phase-contrast microscopy. Strains of *C. acetobutylicum* were grown on CBM plates. After 24, 48, and 72 h of growth, cells were suspended in CGM liquid medium and 2 μl of the cell suspension was placed on a poly-L-lysine-coated glass slide. The cells were examined at 1,260-fold magnification in a phase-contrast light microscope (Zeiss Axiophot; Zeiss, Thornwood, N.Y.) equipped with a Zeiss AxioCam camera.

***E. coli* DNA isolation and manipulation.** Isolation of plasmids from *E. coli* by the alkaline lysis method and further manipulation of *E. coli* plasmid DNA were performed with standard protocols (18). Restriction enzymes were purchased from New England Biolabs (Beverly, Mass.) or Pharmacia Biotech Inc. (Piscataway, N.J.) and used under recommended conditions. T4 DNA ligase and alkaline phosphatase were also purchased from Pharmacia. DNA fragments were purified from agarose gels with activated DEAE-cellulose membranes (31).

***C. acetobutylicum* plasmid and genomic DNA isolation.** Plasmid DNA isolation was carried out as previously described (11). Chromosomal DNA was prepared as previously described (22) with minor improvements (9).

Bacterial transformations. DNA was introduced into *E. coli* strains by electroporation with the BTX Electro Cell Manipulator in the high-voltage mode (2.5 kV/Ω) with a resistance of 129 Ω and a charging voltage of 2.44 kV. Electrotransformation of *C. acetobutylicum* was performed as previously de-

TABLE 2. Oligonucleotides used for PCR and sequencing[a]

Primer name	Oligonucleotide sequence (5′-3′)	Use
SPOA5UP	GGAGTTTATATTGAATGgATCCTTAAAAG	PCR *spo0A* gene
3SPOA3DN	TTACTATTCCTTGGTgATCATTAAAGAAA	PCR *spo0A* gene
BSPOFP5′	atggatccGCCTGACCTTGTTGTTCTCGATA	PCR *spo0A* fragment
BSPOFN3′	cgggatccaCGTGACCATGCAACTTCAATAG	PCR *spo0A* fragment
UPERM	cgtggaagcttGTGCTCTACGACCAAAAG	PCR MLS^r gene
DSERM	aggaagcttGTGAATGCGCAAAAGACAT	PCR MLS^r gene
5′NSKO	GGTGGGATAGTTCAAGGAATG	PCR SKO1
3′NSKO	CATCGTAACTCCCTTGGATAC	PCR SKO1
PIMPSEQ	TCCCAGTCACGACGTTGTAA	Sequencing
SPOSEQ2	GACCATGCAACTTCAATAGC	Sequencing
SEQ3SPO	TACATCCATATCAAAAGGTT	Sequencing
SEQDSPO	CGACAAAACTATTATTTATC	Sequencing
PTB-DO	CTATTGGCTTTAAGAGTCCGC	PCR *ptb* probe
PTB-UP	GCTGTGGATGGAGTTAAGTCAG	PCR *ptb* probe
THIOL-3E	CATGATTTTAAGGGGGGTACCATATGCA	PCR *thl* probe
THIOL-5B	GTTATTTTTAAGGATCCTTTATAGCAC	PCR *thl* probe
5′SIGE	GCAATGAGCTTAGAGAGCCTAT	PCR *sigE-sigG* probe
3′SIGG	ACTTCCATGGACTTCATAGCA	PCR *sigE-sigG* probe

[a] Lowercase letters in the oligonucleotide sequences indicate nucleotide substitutions used to generate useful restriction sites near the termini of PCR products. Restriction sites are underlined.

scribed (22). Prior to transformation into *C. acetobutylicum*, plasmids were methylated in *E. coli* ER2275 carrying the methylating plasmid pAN1 (20).

DNA sequencing analysis. DNA sequencing analysis was carried out on an ABI Prism 3100 sequencer by capillary electrophoresis with the ABI Prism dGTP Big Dye Terminator Ready Reaction kit (Applied Biosystems, Foster City, Calif.). Database searches were conducted with the WWW Blast server (www.ncbi.nlm.nih.gov) and the Argonne National Laboratory-WIT site (http://wit.mcs.anl.gov/WIT2/). The University of Wisconsin Genetics Computer Group sequence analysis software package was used to align amino acid sequences of Spo0A homologues.

DNA amplification by PCR. Plasmid DNA was amplified by PCR with 50 ng of DNA in a final volume of 100 μl containing 300 μM each dATP and dTTP, 200 μM each dGTP and dCTP, 100 μg of nonacetylated bovine serum albumin per ml, 0.01 μg of each nucleotide primer per ml, and 0.05 U of Vent DNA polymerase (New England Biolabs) per μl. Amplification reactions were performed in a Perkin-Elmer GeneAmp PCR System 2400 thermocycler programmed for touchdown PCR: 5 min at 94°C, followed by 30 cycles of a 1-min denaturing step at 94°C, 1 min of annealing at a variable temperature (depending on the melting temperature of the PCR primers [Table 2]), and a 72°C extension step for a variable time (ca. 1 min/kb of length). The PCR programs concluded with a 7-min incubation at 72°C before the reaction mixture was cooled to 4°C.

Genomic DNA was amplified with rTth DNA polymerase XL (Applied Biosystems) along with the PCR Optimization Kit (Invitrogen, Carlsbad, Calif.). Typically, 0.5 to 1 μg of chromosomal DNA was used in a 50-μl reaction mixture.

Cloning of the *spo0A* gene and construction of pMSPOA. The *spo0A* gene (843 nucleotides [nt]), along with 43 nt from the upstream *spoIVB* gene, 364 nt of the upstream intergenic region (which includes the *spo0A* promoter), and 277 nt from the downstream intergenic region (a total of 1.5 kb), was amplified by PCR from the chromosome of *C. acetobutylicum* ATCC 824 with primers SPOA5UP and 3SPOA3DN (Table 2). Single-nucleotide substitutions created a *Bam*HI restriction site in SPOA5UP and a *Bcl*I site restriction site in 3SPOA3DN. After a *Bam*HI-*Bcl*I double digestion, the 1.5-kb *spo0A* fragment was ligated into *Bam*HI-linearized pIMP1. The resulting plasmid was designated pMSPOA (Fig. 1A). The *spo0A* region of pMSPOA was sequenced with primers PIMPSEQ, SPOSEQ2, SEQ3SPO, and SEQDSPO (Table 2).

Construction of pETSPO. *E. coli* vector pGEM-4Z was double digested with *Dra*I and *Pvu*II, generating a 937-bp fragment containing the gram-negative origin of replication (*ori*). The *ori* fragment was then ligated to the 1.5-kb *Eco*RI-*Hin*dIII fragment of pTHAAD (6) carrying the gene for chloramphenicol acetyltransferase expression, which confers resistance to both chloramphenicol and TH. The product of the ligation was plasmid pCLH2 (2.44 kb). A 580-bp internal *spo0A* fragment was PCR amplified from chromosomal DNA with primers BSPOFP5′ and BSPOFN3′ (Table 2). These primers introduced *Bam*HI restriction sites on the ends of this internal *spo0A* fragment in which 147 bp from the 5′ end and 116 bp from the 3′ end of the structural gene were absent. After *Eco*RI linearization of plasmid pCLH2, the ends were filled and this vector was ligated to the internal *spo0A* fragment, yielding plasmid pSCLH2 (3.03 kb). A 1.05-kb fragment containing the macrolide-lincosamide-streptogramin B resistance (MLS^r)-encoding gene was then PCR amplified from pIMP1 with primers UPERM and DSERM (Table 2). These primers introduced *Hin*dIII restriction sites on the ends of the MLS^r-encoding fragment. After digestion of the PCR product with *Hin*dIII, the fragment was ligated into the *Hin*dIII site located in the center of the internal *spo0A* fragment of pSCLH2. The product of the ligation was plasmid pESCLH2 (4.06 kb). A gram-positive origin of replication (*repL*) was obtained on a 1.04-kb DNA fragment after digestion of pIMP1 with *Fsp*I. *Eco*RI linkers were added to the ends of the *repL* fragment, and the product was ligated into pESCLH2 at the unique *Eco*RI site to construct pETSPO (Fig. 1B).

Targeted inactivation of *spo0A*. Methylated pETSPO (Fig. 1B) was introduced into *C. acetobutylicum* ATCC 824 by electroporation. A liquid culture of strain 824(pETSPO) was then used to confluently streak agar (15 g/liter) plates of reinforced clostridial medium containing 40 mg of ERM per liter. The cultures were transferred to fresh plates containing no antibiotics every 24 h for 5 consecutive days with replica plating. Cells that grew on ERM but not on TH were selected by comparing cultures that were replica plated onto medium containing TH (25 mg/liter) and ERM (40 mg/liter). These isolates were grown in liquid medium containing 40 mg of ERM per liter, and the cultures were used for plasmid DNA isolation, chromosomal DNA isolation, and frozen stock preparation. The liquid cultures were also plated onto starch-containing 2XYTGMA plates to confirm amylase activity and thus the presence of pSOL1. The criteria for further screening were absence of a replicating plasmid, presence of pSOL1, sensitivity to TH (chloramphenicol acetyltransferase), and resistance to ERM (MLS^r). Isolates that met these criteria were further analyzed by PCR amplification and sequencing.

***C. acetobutylicum* RNA isolation.** RNA was isolated from 5 to 15 ml of fermentation culture. Following centrifugation (5 min, 4°C, 4,000 × *g*), the supernatant was suspended in 200 μl of SET solution (25% sucrose, 0.05 M Tris-HCl, 0.05 M EDTA) containing 20 mg of lysozyme per ml. After a 5-min incubation at 37°C, 1 ml of RNA STAT-60 (Tel-Test Inc., Friendswood, Tex.) was added to the mixture and the lysed cells were frozen at −85°C. Upon thawing, samples were processed in accordance with the manufacturer's (Tel-Test) instructions. Total RNA was quantified by spectrophotometric analysis at 260 nm. The quality of the RNA preparations was assessed by the spectrophotometric 260-nm/280-nm ratio, which was ≥1.7 for all of the samples used for Northern analysis. Furthermore, RNA integrity and loading were determined by first separating 15 μg of all RNA samples on 1% agarose denaturing gels and then staining by ethidium bromide in order to visualize the rRNA bands (9). Only good-quality RNA was used for Northern analysis.

mRNA probe preparation for Northern analysis. Probes were prepared for detection of the following six mRNA transcripts: *spo0A*, thiolase (*thl*), PTB-butyrate kinase (*ptb-buk*), aldehyde/alcohol dehydrogenase-acetoacetyl-CoA:acetate-butyrate:CoA transferase (*aad-ctfA-ctfB*), acetoacetate decarboxylase (*adc*), *solR* (24), and *sigE-sigG*. The *solR* and *aad-ctfA-ctfB* probes were prepared with plasmid pHXS5. After *Acc*I-*Eco*RI double digestion of pHSX5, one of the

resulting fragments (1.8 kb) contained the entire *solR* gene and a second one (4.9 kb) contained the entire *sol* operon (*aad-ctfA-ctfB*). The *adc* probe was a 1.2-kb fragment resulting from *Acc*I digestion of pFNK6. *Acc*I-*Eco*RI double digestion of pMSPOA (Fig. 1A) resulted in a 1.5-kb fragment containing the *spo0A* gene. PCR amplification from chromosomal DNA was used to obtain probes for the remaining three transcripts, *thl*, *ptb-buk*, and *sigE-sigG*. The thiolase PCR primers were THIOL-5B and THIOL-3E (Table 2), and they were used to amplify a 1.25-kb fragment. Primers PTB-UP and PTB-DO (Table 2) were used to amplify a 1.3-kb fragment containing only the *ptb* gene to be used as a probe for the *ptb-buk* transcript. The *sigE* and *sigG* genes were amplified together as a 2.1-kb fragment with primers 5′SIGE and 3′SIGG (Table 2). Double-stranded DNA probes were purified from agarose gels with activated DEAE-cellulose membranes (31). The probes were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; ICN Biomedicals, Inc., Costa Mesa, Calif.) with the Prime-It II Random Primer Labeling Kit from Stratagene (La Jolla, Calif.). Unincorporated radiolabeled deoxynucleoside triphosphate, [α-^{32}P]dCTP, was removed with Nuc Trap columns (Stratagene).

Northern analysis. Fifteen-microgram RNA samples were used for Northern analysis as previously described (24), with small modifications (9). InstantImager Electronic Autoradiography (Packard Instrument Company, Meriden, Conn.) was used to image all blots. The InstantImager software was used to quantify the hybridization signals, and the values reported are total counts. Since the same membrane was probed for each of the seven messages, it was necessary to strip the blot after each round of hybridization and autoradiography. Membranes were stripped in a 55% formamide solution containing 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) and 1.0% sodium dodecyl sulfate at 65°C for 60 min, followed by washing for 15 min at 65°C in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate. In order to be able to compare Northern blot data for the two pairs of strains [*C. acetobutylicum* ATCC 824 (wild-type or parental strain) versus SKO1 and 824(pMSPOA) versus 824(pIMP1)], the blots for each pair and each probe were probed, imaged, and stripped simultaneously. The probes used to detect each transcript were prepared at the same time and used in the same amounts.

RESULTS

DNA sequence-based bioinformatic analysis. The *B. subtilis* and *C. acetobutylicum* Spo0A proteins show 53% identity and 72% conservation over the entire polypeptide length. The N-terminal domains of the *B. subtilis* and *C. acetobutylicum* Spo0A homologues show 49% identity and 71% conservation over 122 amino acids (aa), with 73% identity and 89% conservation over the 115 aa comprising the Spo0A-specific C-terminal domain. The C-terminal effector or activator domain of Spo0A has three regions (CI, CII, and CIII) (2). Region CII contains the portion of the helix-turn-helix motif (12 aa long: SRVERAIRHAIE) comprising the recognition helix as part of a 22-aa sequence (the putative site of protein-DNA interaction) that is perfectly conserved in *B. subtilis* and *C. acetobutylicum* (9). In view of the very high level of protein homology between the Spo0A proteins from *C. acetobutylicum*, *C. beijerinckii*, and *B. subtilis* (2, 9) and the demonstrated binding of the Spo0A protein on the DNA 0A boxes not only in *B. subtilis* but also in *C. beijerinckii* (28), we assumed that Spo0A binds to such 0A boxes in *C. acetobutylicum* as well.

Two promoters have been identified upstream of the *spo0A* structural gene in *B. subtilis* (39). A vegetative promoter recognized by Eσ^A is responsible for maintaining a low level of Spo0A during exponential growth. A second promoter, recognized by Eσ^H, is responsible for the increased expression of *spo0A* during the transition to the stationary phase. As in *B. subtilis*, the *C. acetobutylicum spo0A* promoter region contain sequences that match promoter consensus sequences recognized by Eσ^A and Eσ^H. There are three 0A boxes upstream of *B. subtilis* and *C. beijerinckii* 8052 *spo0A*. In contrast, only one

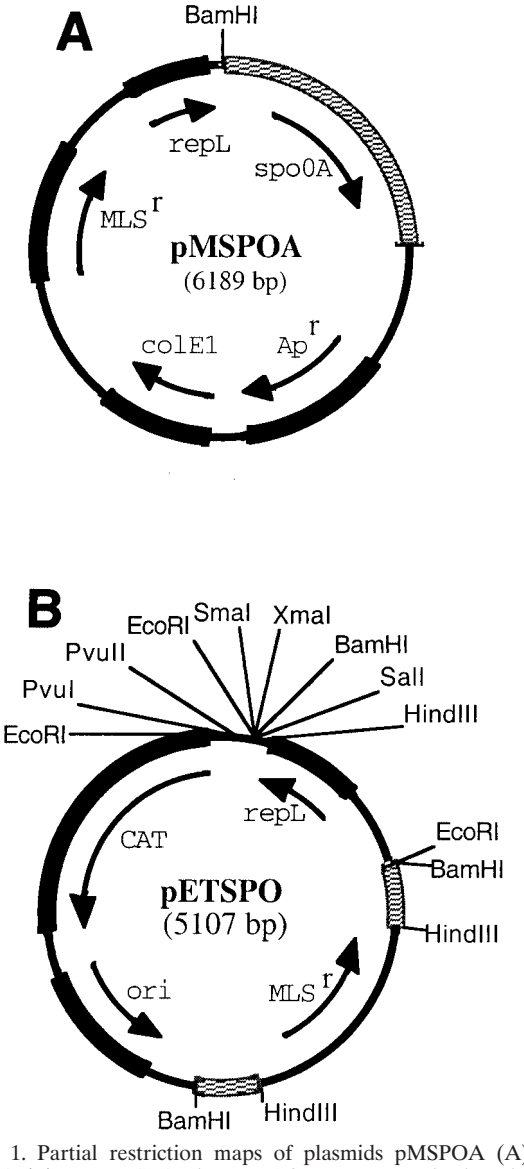

FIG. 1. Partial restriction maps of plasmids pMSPOA (A) and pETSPO (B). In pETSPO, the *spo0A* fragments are checkered.

0A box—it overlaps the −10 element of the Eσ^H promoter—was found in the promoter regions of the *C. acetobutylicum spo0A*. As in *B. subtilis* (37), there are two likely 0A boxes in the promoter region of the *spoIIGA-sigE* operon but none in front of the *sigG* gene. However, all three genes may be transcribed from the *spoIIGA-sigE* promoter (8).

The *sol* operon (5) on the pSOL1 megaplasmid (4), which contains the three genes encoding aldehyde/alcohol dehydrogenase (*aad* [23] or *adhE1* [5]) and the two CoA transferase subunit genes (*ctfA* and *ctfB*), has a single reverse 0A box located 5′ of its distal promoter (28). The monocistronic *adc* gene contains two 0A boxes and one reverse 0A box in its promoter region (28). The chromosomal thiolase (*thl*) gene (27) contains no 0A boxes in its promoter region. Unlike the *ptb* gene in *C. beijerinckii* (28), there are no 0A boxes in the promoter region of the *ptb-buk* operon (responsible for butyrate formation). There are also no 0A boxes in the promoter

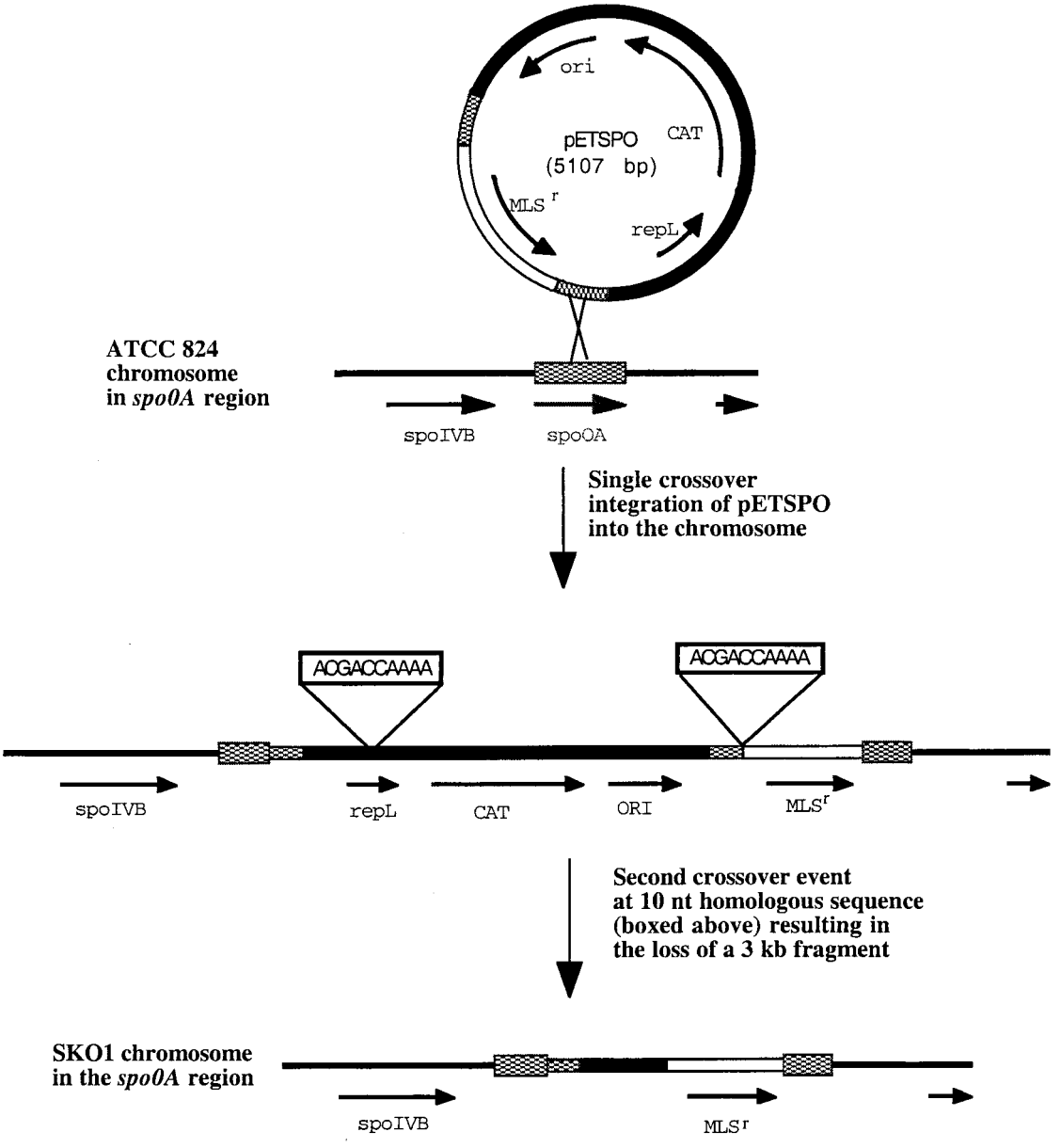

FIG. 2. Putative crossover events during *spo0A* gene inactivation. *spo0A* sequences are checkered, and the MLS^r-encoding gene is represented by an open segment. All other sequences are shown as black lines or filled segments.

region of the *pta-ack* operon (responsible for acetate formation) or in that of the putative regulatory gene *solR* (10, 24).

Inactivation of *spo0A*. After several attempts to generate an *spo0A* knockout with nonreplicative vectors (7) failed, we developed a method with a replicating plasmid—a technique never before used with solventogenic clostridia—in order to inactivate the *spo0A* gene as discussed in Materials and Methods. One isolate, SKO1, that satisfied the criteria for *spo0A* disruption with the MLS^r-encoding gene was selected for further study. To confirm that the MLS^r-encoding gene was inserted by double-crossover recombination events into the *spo0A* gene in SKO1, chromosomal DNA was used as the template for PCR amplification with primers 5′NSKO and 3′NSKO (Table 2). When these primers were used in a PCR with the parent strain chromosomal DNA serving as the template, the product was, as expected, a 2.45-kb fragment. The expected double-crossover event in which only the MLS^r-encoding gene was inserted into *spo0A* would have resulted in the amplification of a 3.47-kb fragment. Instead, when chromosomal DNA from isolate SKO1 was used as the PCR template, a 4.5-kb PCR product was amplified. Sequencing disclosed the precise genetic arrangement in the SKO1 *spo0A* region (9). From this information, the putative crossover events that occurred during *spo0A* inactivation in SKO1 were determined (Fig. 2). First, the entire 5.1-kb pETSPO plasmid was apparently integrated into the chromosome at the *spo0A* gene. This resulted in a strain that was resistant to both TH and ERM. Since the 583-nt internal *spo0A* fragment was duplicated in the chromosome of this single-crossover event strain, a second homologous recombination event at the *spo0A* site would be

expected. If this had occurred in the *spo0A* sequence, the result would have been either reversion to the parental genotype or deletion of plasmid DNA except for the MLS^r marker, an ideal double crossover. This expected second crossover event in the *spo0A* gene apparently did not take place. Instead, a crossover event occurred between two 10-nt homologous sequences (5′-ACGACCAAAA-3′) that were present in the 3′ end of the *repL* structural gene and upstream of the MLS^r marker (Fig. 2). Loss of the 3-kb fragment between these 10-nt-long homologous sequences resulted in the generation of SKO1, whereby the *spo0A* gene was inactivated through insertion of a 2.1-kb fragment containing the MLS^r-encoding gene.

In addition to confirming the presence of the pSOL1 megaplasmid in strain SKO1 by detection of α-amylase activity, PCR was used to amplify the *aad* gene located on the pSOL1 megaplasmid. Results of both of these experiments (9) indicated that SKO1 did, indeed, contain the pSOL1 megaplasmid. After heating of several SKO1 colonies to 70°C for 10 min in liquid culture (a standard procedure used to induce vegetative growth of sporulated *C. acetobutylicum* strains [30]), lack of growth indicated that mature spores were not formed by this recombinant strain. This was in contrast to the behavior of the parental (wild-type) strain, Spo0A-overexpressing strain 824(pMSPOA), and plasmid control strain 824(pIMP1).

Phase-contrast microscopy. In order to investigate possible differences in cell morphology among the parental strain, SKO1, *C. acetobutylicum* ATCC 824(pIMP1), and 824(pMSPOA), cells from cultures grown on CBM plates were examined by phase-contrast light microscopy. Cells from all four strains examined after 24 h of growth had similar shapes and sizes (9). After 48 h, parental-strain cells were swollen in the typical cigar-shaped clostridial form (16) with a small percentage (10 to 20%) of the cells possessing phase-bright endospores. SKO1 cells grown for 48 h were not swollen, and their shape was similar to that observed after 24 h of growth. After 48 h of growth, more than 50% of the 824(pMSPOA) cells contained phase-bright endospores or had released the endospores, resulting in cell debris and phase-bright free spores, and the rest were in the swollen clostridial form. After 48 h, control strain 824(pIMP1) had fewer cells in the swollen clostridial form or containing endospores than did 824(pMSPOA) but more than did the parent strain. After 72 h of growth, debris was prevalent in cell suspensions of all four strain cultures. The intact parental-strain cells were usually in the swollen clostridial form, but some free spores were also present. There was no detectable endospore formation in SKO1 cultures, the cells did not swell, and a large fraction of the cells formed filaments of connected rods. This SKO1 morphology is consistent with that observed when the *spo0A* gene was inactivated in *B. anthracis* and *C. beijerinckii* (2). After 72 h of growth, more than half of the intact 824(pMSPOA) cells photographed contained endospores and there were also a large number of free spores in cell suspensions of the 824(pMSPOA) strain. These numbers were smaller (ca. 40% of the intact cells contained endospores) in plasmid control strain 824(pIMP1).

Effect of *spo0A* inactivation on solvent production. Batch bioreactor SKO1 cultures ($n = 4$) grew slower (by 31% [doubling times were 1.10 ± 0.06 h for the parental strain and 1.44 ± 0.07 h for the SKO1 strain]; all reported differences are based on mean values and are statistically significant [$P < 0.05$] unless otherwise noted) and to a lower density (by 30%) than parental-strain cultures ($n = 4$) (Fig. 3). Fermentations of both strains were complete (no further changes in product concentration) within 60 h. Both acetate and butyrate accumulated to much higher levels (by ca. 50 and 500%, respectively, at the end of the fermentations), and very small amounts were reutilized in the SKO1 cultures compared to the parental-strain fermentations. Accumulation of solvents was very low in SKO1 fermentations (mean values of 2 mM acetone, 13 mM butanol, and 5 mM ethanol) compared to parental-strain fermentations (92 mM acetone, 172 mM butanol, and 21 mM ethanol).

Effect of *spo0A* overexpression on product formation. Batch fermentations (Fig. 3) of strain 824(pMSPOA) ($n = 4$) and plasmid control strain 824(pIMP1) ($n = 2$) showed that neither of these two types of fermentation was complete, even after 70 h, both showing continuing solvent production. Strain 824(pMSPOA) grew slightly faster and to much greater (by ca. 50%) cell densities than strain 824(pIMP1). Maximum and final concentrations of acids were similar in the 824(pMSPOA) and 824(pIMP1) fermentations. Final solvent concentrations in the 824(pMSPOA) fermentations were 20% higher than those in 824(pIMP1) fermentations. Strain 824(pMSPOA) produced 25% less butanol than the parental strain but the same levels of acetone and ethanol. Solvent formation was initiated at about the same time for all of the strains [824, 824(pMSPOA), and 824(pIMP1)], but strain 824 produced solvents at much higher rates during the first 40 to 50 h (Fig. 3). In contrast, strains 824(pMSPOA) and 824(pIMP1) produced solvents at lower rates but for a prolonged time, even after 70 h. It has been documented that the swollen clostridial forms of the cells are the active producers of solvents (spores and forespore-forming cells do not produce solvents) (16, 29). Thus, these fermentation data should be interpreted as mean values from a mixed population of solvent-producing and nonsolvent-producing cells, with the fraction of these two types of cells varying substantially (as revealed by the morphological studies described above) among strains 824, 824(pMSPOA), and 824(pIMP1). Finally, liquid cultures of strain 824(pMSPOA) were quite viscous within 24 h of growth in both tubes and the bioreactor. Culture viscosity decreased when high solvent levels accumulated at the end of the fermentation period.

Northern analysis. Radiolabeled probes were designed to detect transcripts corresponding to genes with (*spo0A*, *aad-ctfA-ctfB*, *adc*, and *spoIIGA-sigE-sigG*) or without (*thl*, *ptb-buk*, and *solR*) 0A boxes in their promoter regions. In the parental strain, the *spo0A* message was detectable in cells from all stages of the culture examined (samples A through E; an additional late-stationary-phase sample [F] was also analyzed, but the results are not shown here due to excessive RNA degradation) (Fig. 4A). The message was most abundant at the end of the exponential growth phase (sample B), when it was twice as abundant as during the mid-exponential growth phase (sample A) and 50% more abundant than during the stationary phase. That the level of *spo0A* transcripts peaked just prior to the transition to the stationary phase correlates well with its putative role as a transcriptional regulator responsible for controlling changes in gene expression that occur as the organism ceases exponential growth. As expected, no *spo0A* message was detected in strain SKO1 (Fig. 4A).

The *thl* gene encodes a central metabolic pathway enzyme

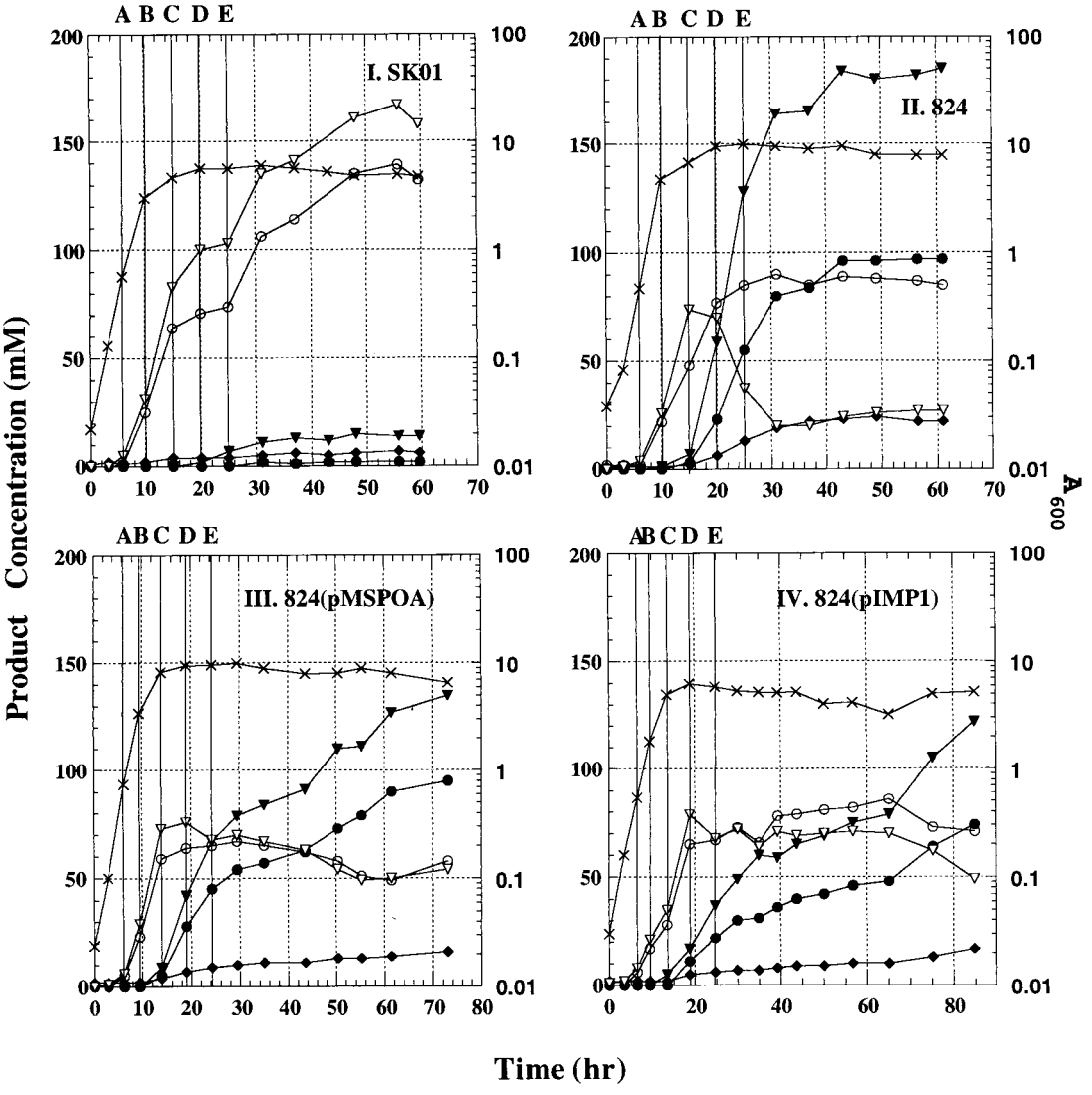

FIG. 3. Representative fermentation kinetics from bioreactor experiments of strain SKO1 (I), the parental strain (II), strain 824(pMSPOA) (III), and strain 824(pIMP1) (IV). Samples from these experiments were used for the Northern analysis data presented in Fig. 4 and 5. The five sampling points (A, B, C, D, and E) for each experiment are indicated by solid vertical lines. Symbols: ×, A_{600}; ▼, butanol concentration; ●, acetone concentration; ◆, ethanol concentration; ▽, butyrate concentration; ○, acetate concentration.

that has significant activity throughout batch fermentations (12, 40). The levels and patterns of thiolase gene expression were similar in the parental and SKO1 strains (Fig. 4A). The highest *thl* transcript level was detected at the end of exponential growth (sample B) in the fermentations of both strains, a pattern that correlates well with previous observations that thiolase activity reaches its peak at the end of the exponential growth phase (12, 40). These results indicate that *spo0A* inactivation does not significantly alter the level of thiolase transcription, and this is consistent with the fact that there are no 0A boxes upstream of the *thl* gene.

For both strains, only background levels of the 4.1-kb *aad-ctfA-ctfB* transcript were detected during exponential cell growth (samples A and B) (Fig. 4B). In the parental strain, the maximum level of *aad-ctfA-ctfB* was detected during the transition from exponential growth to the stationary phase of fermentation (sample C). The levels of the *aad-ctfA-ctfB* transcript detected in SKO1 were much lower than in the parental strain. In fact, the maximum SKO1 *aad-ctfA-ctfB* transcript level during the stationary phase of fermentation (sample D) was about 20 times lower than the maximum parental-strain *aad-ctfA-ctfB* transcript level. A drastic decrease in the abundance of the 0.8-kb *adc* transcript upon inactivation of *spo0A* was also observed (Fig. 4B). The highest levels of SKO1 *adc* transcripts, detected in the transition from exponential growth to stationary phase or in stationary phase (samples C and D), were about 10 times lower than parental-strain levels.

An attempt was made to detect a *sigE-sigG* message in the parental and SKO1 strains, but none was reliably detected in any of the samples analyzed. A substantial amount of a degraded low-molecular-weight message hybridized to the radioactive *sigE-sigG* probe in stationary-phase samples E and F of the parental strain, but none did so in the SKO1 strain (data not shown).

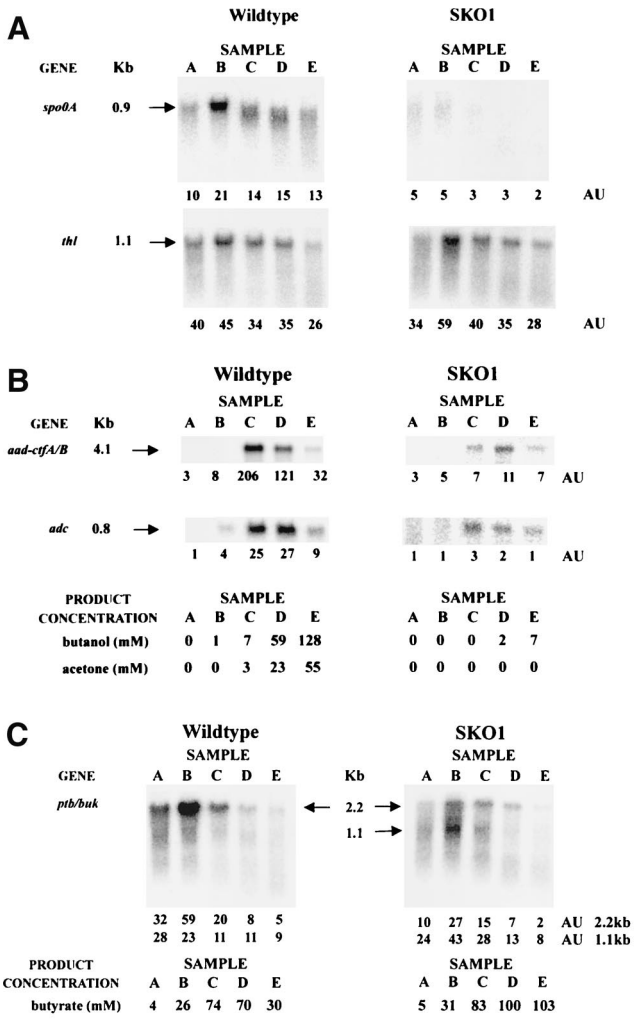

FIG. 4. Parental (wild-type) and SKO1 strain Northern analyses with the *spo0A* and *thl* probes (A), the *aad-ctfA/B* and *adc* probes (B), and the *ptb-buk* probe (C). RNA samples collected during the exponential growth phase (samples A [h 6 and 6.3; A_{600} of 0.57 and 0.47 for the SKO1 and parental strains, respectively] and B [h 10 and 9.5; A_{600} of 3 and 4.7 for the SKO1 and parental strains, respectively]), the transition state (samples C [h 15; A_{600} of 4.6 and 6.7 for the SKO1 and parental strains, respectively]), and the stationary phase (samples D [h 20; A_{600} of 5.6 and 9.5 for the SKO1 and parental strains, respectively] and E [h 25; A_{600} of 5.6 and 9.9 for the SKO1 and parental strains, respectively]) of fermentations (Fig. 3) were electrophoretically resolved and probed with ^{32}P-labeled probes. The blots were electronically imaged with InstantImager Electronic Autoradiography, and the total radioactive counts were measured after 10 min of imaging for *spo0A*, *aad-ctfA-ctfB*, and *adc* and 20 min of imaging for *thl* and *ptb-buk*. The mRNA levels are reported in arbitrary units (AU), which are the total counts divided by 1,000.

The *ptb* gene is the first gene in a bicistronic message also containing the *buk* gene. Together, these genes encode the enzymes responsible for the conversion of butyryl-CoA to butyrate. In the parental strain, this 2.2-kb *ptb-buk* signal was strongest during the exponential growth phase (samples A and B) and was significantly decreased in stationary phase (samples D and E) (Fig. 4C). This pattern of the 2.2-kb *ptb-buk* signals was similar in strain SKO1 (Fig. 4C), but the transcript levels were lower (by 50 to 300% at points A and B). A second, shorter message is quite apparent in the SKO1 samples, so we analyzed the data for the full (2.2-kb) and short (1.1-kb) transcripts separately. For consistency, we did so also for the parental strain, although the short message was not as clearly identifiable in this strain. The probe we used for Northern analysis of the *ptb-buk* operon is made up of a large fragment from the *ptb* gene, the intergenic region and a 40-bp fragment from the *buk* gene. This 40-bp fragment shows a high percentage of homology (90%) with a second *buk* gene (*bukII*), a monocistronic chromosomal gene with a high level of homology to the *buk* gene of the *ptb-buk* operon (14). Thus, our Northern probe would likely also capture any *bukII* transcript that has a calculated size of about 1.2 kb. Therefore, the smaller 1.1-kb transcript could represent this *bukII* transcript or a truncated *ptb-buk* transcript. We noted that Huang et al. (14) reported very low BKII expression levels in *C. acetobutylicum*. We also noted that *bukII* contains a 0A box in its promoter region and a reverse 0A box just inside its coding region. The *ptb-buk* transcript levels and butyrate concentrations corresponded well to the PTB activity previously reported in parental-strain batch fermentations (12) and to β-galactosidase activity during fermentations of a strain with a β-galactosidase reporter gene under the control of the *ptb* promoter (40). We also noted that the SKO1 strain produced higher levels of butyrate despite the lower *ptb-buk* transcript levels.

Collectively, these data show that *spo0A* inactivation decreased the transcription of solvent formation genes more than 10-fold but did not significantly alter the transcription of the thiolase gene. In fact, the *thl* gene appears to behave like a housekeeping gene within the time points examined in this study, and this is consistent with the results from our reporter system study (40). The uncertainty about the two transcripts (Fig. 4C) makes it impossible to draw any conclusions regarding the effect of Spo0A on *ptb-buk* operon expression.

Levels of *spo0A* mRNA in the *spo0A*-overexpressing strain *C. acetobutylicum* ATCC 824(pMSPOA) were up to 40 times greater than those in strain 824(pIMP1) throughout the fermentation period (Fig. 5A). The maximum *spo0A* transcript level detected was observed during the transition from exponential to stationary phase (sample C). The *spo0A* message appeared to be shorter in samples B, C, and D of strain 824(pMSPOA), but this was not the case for strain 824(pIMP1). The parental strain Northern data also show shorter transcripts in samples C, D, and E (Fig. 4A). These shorter messages may correspond to transcripts from the two different promoters (recognized by $E\sigma^A$ and $E\sigma^H$) upstream of *spo0A*, which are 90 nt apart. These data suggest for the parental and 824(pMSPOA) strains a bimodal expression of *spo0A*, similar to what was observed in *B. subtilis*, whereby *spo0A* is transcribed first from the weaker vegetative promoter and later from the sporulation-specific promoter (39). These results show that the presence of plasmid-encoded *spo0A* results in a significant increase in the number of *spo0A* transcripts. The *thl* and *ptb-buk* transcript levels were similar in strains 824(pMSPOA) and 824(pIMP1) (Fig. 5A and B) and thus were apparently not affected by *spo0A* overexpression.

Signals for the *aad-ctfA-ctfB* probe were detected during the transition to stationary phase and in the early stationary-phase samples (C and D, respectively) of both strains 824(pIMP1)

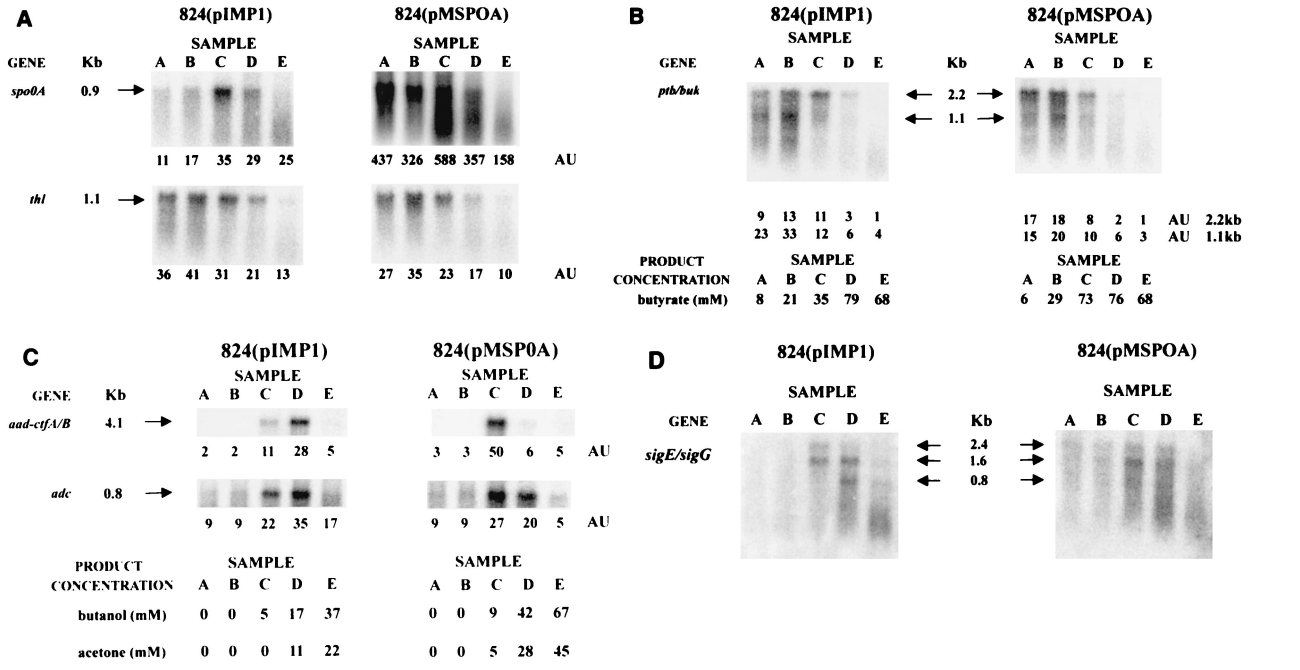

FIG. 5. Northern analysis of strains 824(pIMP1) and 824(pMSPOA) with the *spo0A* and *thl* probes (A), the *ptb-buk* probe (B), the *aad-ctfA-ctfB* and *adc* probes (C), and the *sigE-sigG* probe (D). RNA samples collected during the exponential growth phase {samples A [h 6.3 and 6.5; A_{600} of 0.74 and 0.54 for the 824(pMSPOA) and 824(pIMP1) strains, respectively] and B [h 9.5 and 9.5; A_{600} of 3.4 and 1.8 for the 824(pMSPOA) and 824(pIMP1) strains, respectively]}, the transition state {samples C [h 14 and 13.5; A_{600} of 8.2 and 4.9 for the 824(pMSPOA) and 824(pIMP1) strains, respectively]}, and the stationary phase {samples D [h 19 and 18.8; A_{600} of 9.4 and 6.2 for the 824(pMSPOA) and 824(pIMP1) strains, respectively] and E [h 24.3 and 24.8; A_{600} of 9.6 and 5.8, for the 824(pMSPOA) and 824(pIMP1) strains, respectively]} of fermentations (Fig. 3) were electrophoretically resolved and probed with ^{32}P-labeled probes. The blots were electronically imaged with InstantImager Electronic Autoradiography, and the total radioactive counts were measured after 10 min of imaging for *spo0A*, *aad-ctfA-ctfB*, and *adc* and 20 min of imaging for *thl*, *ptb-buk*, and *sigE-sigG*. The mRNA levels are reported in arbitrary units (AU), which are the total counts divided by 1,000.

and 824(pMSPOA) (Fig. 5C). In both strains, and in contrast to the parental strain (Fig. 4B), the *aad-ctfA-ctfB* signals show an abrupt downregulation. This is also in contrast to the slower downregulation of the *adc* signals in both strains 824(pIMP1) and 824(pMSPOA) (Fig. 5C) and suggests a differential regulation of these *sol* locus genes. Similar levels of *adc* transcripts were detected after the transition to stationary phase (samples C, D, and E) in both strains 824(pIMP1) and 824(pMSPOA), although, as in the case of the *aad-ctfA-ctfB* transcripts, the points of maximal transcript levels were different. Higher levels of both *aad-ctfA-ctfB* and *adc* transcripts appear to be expressed earlier in strain 824(pMSPOA) than in strain 824(pIMP1), and this is consistent with the earlier expression of *spo0A* in 824(pMSPOA) (Fig. 5A) and the faster rate of solvent formation early in the fermentation of strain 824(pMSPOA) than in that of strain 824(pIMP1) (Fig. 3). The ratio of maximal (*aad-ctfA-ctfB*)/*adc* signals in strain 824(pMSPOA) was approximately twice that in strain 824(pIMP1) but fourfold lower than that in the parental strain (Fig. 4B). This again suggests a differential regulation of the *aad-ctfA-ctfB* operon versus the *adc* gene.

A 2.1-kb radioactive probe containing both the *sigE* and *sigG* genes was used to examine the expression of these two sigma factor genes. Three different transcripts were detected in RNA samples from both 824(pIMP1) and 824(pMSPOA) strains with the radioactive *sigE-sigG* probe (Fig. 5D). These transcripts (2.4, 1.6, and 0.8 kb) correspond to messages containing *spoIIGA-sigE-sigG*, *spoIIGA-sigE*, and *sigG*, respectively. In strain 824(pIMP1), no *sigE-sigG* message was detected during exponential growth (samples A and B), the 2.4- and 1.6-kb transcripts were detected in the transition to stationary phase (sample C), and all three transcripts were detected in early stationary phase (sample D). In strain 824(pMSPOA), two faint transcripts (2.4 and 1.6 kb) were detected in samples A and B and all three transcripts were detected in samples C and D. Degradation made detection of bands difficult in samples E from both 824(pIMP1) and 824(pMSPOA). However, in samples E (Fig. 5D) and F (data not shown) from both of these strains, a substantial amount of a low-molecular-weight message hybridized to the *sigE-sigG* probe, similar to that observed in the parental strain Northern analysis, as discussed above. The *sigE-sigG* data of Fig. 5D should be contrasted to our inability to detect any *sigE-sigG* messages in the SKO1 strain and only a very low-level and degraded message in the parental strain. These results indicate that overexpression of the plasmid-encoded *spo0A* gene results in overexpression of a functional Spo0A protein, which stimulates earlier and greater expression of the *spoIIGA*, *sigE*, and *sigG* genes.

We also probed for differential expression of *solR*. The signals were very weak, and no differences could be detected. Thus, we are not able to conclude that *solR* is or is not under Spo0A control. We should note, however, that there are no 0A boxes in the *solR* promoter region or inside the coding region.

DISCUSSION

The morphological and fermentation (Fig. 3) data suggest that *spo0A* inactivation leads to an asporogenous phenotype without complete abolition of solvent formation. In contrast, *spo0A* inactivation in *C. beijerinckii* completely abolished acetone formation and allowed only very low levels of butanol formation (<3 mM) (28). Strain SKO1 also produces much higher concentrations of both butyrate (by ca. 5-fold) and acetate (by ca. 1.5-fold) compared to the parental strain. In *C. beijerinckii*, *spo0A* inactivation results in ca. 20% less acetate formation but ca. 35% higher butyrate levels (28). We believe that the reduced cell densities of strain SKO1 (Fig. 3) is not a direct effect of the *spo0A* inactivation but rather an indirect one due to the accumulation of the two acids as a result of the inability of the cells to take up butyrate and acetate. We base this interpretation on the fact that two other degenerate mutants (M5 and DG1) resulting from loss of the pSLO1 plasmid show almost identical growth and acid formation characteristics (reference 4 and data not shown). These two mutants produce no solvents, except for some ethanol, but apparently still express the chromosomal *spo0A* gene. The inability of these two mutants to sporulate and produce solvents is due to the loss of pSOL1 genes. *C. beijerinckii* contains no such plasmid. These findings suggest that *spo0A* expression has different effects on product formation by *C. acetobutylicum* and *C. beijerinckii*.

Overexpression of *spo0A* in strain 824(pMSPOA) appears to accelerate both the sporulation process and solvent formation compared to those in plasmid control strain 824(pIMP1). Strain 824(pMSPOA) [and, to a lesser extent, strain 824(pIMP1)] show dramatically accelerated and increased sporulation compared to that of the parental strain. Fermentations of both strains 824(pMSPOA) and 824(pIMP1) show a slower rate of solvent formation than the parental strain, and this is consistent with the lower levels of the *aad-ctfA-ctfB* transcripts (Fig. 5C). However, these results should be reinterpreted on the basis of the actual fraction of cells producing solvents, i.e., cells in the clostridial form versus nonproducing forespore-forming cells and spores (16, 29). In view of the fact that 824(pMSPOA) [and, to a lesser extent, 824(pIMP1)] contain a much larger fraction [by at least 50% for strain 824(pMSPOA)] of forespore-forming cells and spores than does the parental strain, the fermentation data suggest that the ability of clostridial-form cells of strain 824(pMSPOA) to produce solvents is as great as and likely greater than that of those of the parental strain. Similarly, if the Northern analysis data could be presented on the basis of the cells that contain the solvent formation gene transcripts, they might show that such transcripts are indeed more abundant and present at higher concentrations in clostridial-form cells of the 824(pMSPOA) strain, as would have been expected from *spo0A* overexpression. Taken together, these data suggest that the regulation by Spo0A of two related but very different processes (sporulation versus solvent production) is a balancing act when viewed from the solvent production point of view. Very little or no expression of Spo0A would not be sufficient for induction of solvent formation genes, while high-level expression of Spo0A would result in accelerated sporulation and commitment into differentiated cellular forms that do not produce solvents. Thus, optimal production of solvents may require a low to medium level of Spo0A expression.

We have previously reported host-plasmid interactions (including those from plasmid pIMP1) resulting in positive effects on solvent production in low-pH (4.5) fermentations (41). Here, at pH 5.0, the effect on solvent production was not positive overall, although the sustained solvent production by both strains 824(pMSPOA) and 824(pIMP1) might have eventually resulted in solvent concentrations similar to or higher than that produced by the parental strain. The effect of plasmid pIMP1 was, however, positive in accelerating the sporulation process compared to that of the parental strain.

Although the *aad-ctfA-ctfB* transcript levels in strain 824(pMSPOA) were higher than those in strain 824(pIMP1), there were no significant changes in the maximum level of *adc* transcripts (Fig. 5C). Expression of the solvent formation genes *aad-ctfA-ctfB* and *adc* and sporulation-specific genes *sigE-sigG* occurred earlier in strain 824(pMSPOA) than in plasmid control strain 824(pIMP1). These data and the fact that lack of *spo0A* expression in SKO1 does not completely abolish either *sol* locus gene (*aad-ctfA-ctfB* and *adc*) expression (Fig. 4B) or solvent production (Fig. 3) suggest that Spo0A/Spo0A~P may be one of many regulators of solvent gene expression. Examples of this type of multiregulator control of gene expression are known in many organisms, notably in *B. subtilis*. The activating effect of Spo0A~P has to counteract several negative regulators, like SinR and Hpr, that prevent sporulation. In fact, most *abrB*-repressed *B. subtilis* genes are also controlled by one or more regulators that allow the cell to respond to multiple physiological conditions (36).

The delayed expression of *sigG* compared to that of *sigE* (Fig. 5D) is consistent with primer extension analysis experiments (32). The presence of low levels of a 2.4-kb transcript (Fig. 5D) early in the culture possibly containing all three genes (*spoIIGA-sigE-sigG*) is contrary to reported primer extension analysis data (32) but similar to what has been reported in *B. subtilis* (8). In *B. subtilis*, the SigG protein is apparently translated not from this long message but rather from the delayed short *sigG* message (8). Inability to detect any intact *sigE-sigG* message in the parental strain is probably because *spoIIAG-sigE* and *sigG* are expressed late and at low levels. In fact, previous attempts to detect these messages by Northern analysis in *C. acetobutylicum* DSM 1731 were not successful and necessitated the use of primer extension analysis (32). Lack of any detectable expression of a *sigE-sigG* message in SKO1 may, indeed, reflect the effect of *spo0A* inactivation. These data also further support the conclusion that Spo0A overexpression does, indeed, accelerate and enhance sporulation.

A second thiolase gene (*thlB*) (45) in *C. acetobutylicum* is located on the pSOL1 plasmid and shows very low expression as part of a three-gene operon yielding large-size (1.7- and 2.7-kb) transcripts. In contrast, the chromosomal monocistronic gene *thl* we used in this study produces a 1.4-kb transcript. Thus, there is no ambiguity regarding the interpretation of our *thl* data.

The high viscosity of 824(pMSPOA) cultures might be due to earlier and increased accumulation (as suggested by the morphological studies) of granulose, a storage polysaccharide produced during the stationary phase of parental-strain cul-

tures. Solvent accumulation dissolves this material and prevents an increase in culture viscosity.

The gene inactivation method used in this study to create SKO1 was conceptualized and implemented without difficulty. In fact, SKO1 was isolated during the first use of the technique. More than two dozen colonies where obtained that met the antibiotic resistance requirements for a potential gene inactivation product. Of these isolates, three were used in PCR experiments to determine the size of the *spo0A* region. All three of the selected isolates showed evidence that a genetic change (an insertion) occurred in the *spo0A* region based on the size of the PCR products. Only one of these three isolates (SKO1) was used in sequencing reactions to determine the precise nature of the genetic change. The targeted gene inactivation technique presented in this work would be beneficial to genetic studies with clostridia, and efforts to use it to inactivate other genes are warranted.

The findings presented here provide support for the hypothesis that Spo0A acts as a multifunctional regulatory protein that is crucial for transcription of solvent formation and sporulation genes. Our data suggest that its effect on solvent formation is a balancing act in regulating sporulation versus solvent gene expression. Northern analysis data show that the regulation of *spo0A* expression is similar to that in *B. subtilis*, despite the different numbers of 0A boxes found in the promoter regions of the corresponding *spo0A* genes. Our data also show that Spo0A apparently regulates other sporulation genes (here the *spoIIGA*, *sigE*, and *sigG* genes) in a manner similar to that in *B. subtilis*.

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