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Phylogeny and functional conservation of σ^E in endospore-forming bacteria

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Conservation of the sporulation processes between *Bacillus* spp. and *Clostridium* spp. was investigated through evolutionary and complementation analyses of σ^E. Alignment of partial predicted σ^E amino acid sequences from three *Bacillus* spp., *Paenibacillus polymyxa* and five *Clostridium* spp. revealed that amino acid residues previously reported to be involved in promoter utilization (M124, E119 and N120) and strand opening (C117) are conserved among all these species. Phylogenetic analyses of various sigma factor sequences from endospore-forming bacteria revealed that homologues of σ^E, σ^K and σ^G clustered together regardless of genus, suggesting a common origin of sporulation sigma factors. The functional equivalence between *Clostridium acetobutylicum* σ^E and *Bacillus subtilis* σ^E was investigated by complementing a non-polar *B. subtilis* σ^E null mutant with the *spoIIG* operon from either *B. subtilis* (*spoIIG*^{Bs}) or *C. acetobutylicum* (*spoIIG*^{Ca}). Single-copy integration of *spoIIG*^{Bs} into the *amyE* locus of the σ^E null mutant completely restored the wild-type sporulation phenotype, while *spoIIG*^{Ca} only partially restored sporulation. Maximal expression of *spoIIG*^{Ca}*–lacZ* occurred approximately 12 h later than maximal expression of *spoIIG*^{Bs}*–lacZ*. Differences in temporal expression patterns for *spoIIG*^{Ca} and *spoIIG*^{Bs} in the *B. subtilis* background may at least partially explain the observed sporulation complementation phenotypes. This study suggests a common phylogenetic ancestor for σ^E in *Bacillus* spp. and *Clostridium* spp., although regulation of σ^E expression may differ in these two genera.

Keywords: sigma factors, sporulation, sigma E, *Bacillus* spp., *Clostridium* spp.

INTRODUCTION

Although *Bacillus* spp. and *Clostridium* spp. are evolutionarily distant, morphological examination suggests that these genera employ similar sporulation processes (Young & Cole, 1993). The sporulation process in *Bacillus* spp., particularly in *Bacillus subtilis*, has been extensively probed at the molecular level, resulting in identification of approximately 125 genes that contribute to endospore formation (Stragier & Losick, 1996). A similar understanding of the regulation of spore formation in the genus *Clostridium* is beginning to emerge.

Transcription of *B. subtilis* genes required for sporulation is principally regulated by a cascade of sigma factors including the sporulation-specific sigma factors σ^F, σ^E, σ^G and σ^K (Errington, 1993; Haldenwang, 1995; Stragier & Losick, 1996). Sigma factors are a class of proteins constituting essential dissociable subunits of prokaryotic RNA polymerase. They provide promoter recognition specificity, contribute to DNA strand separation, then dissociate from RNA polymerase core enzyme following transcription initiation (Aiyar *et al.*, 1994; Haldenwang, 1995; Helmann & Chamberlin, 1988).

In the predivisional *B. subtilis* cell, the two-cistron *spoIIG* operon comprised of *spoIIGA* and *spoIIGB* is transcribed by the σ^A holoenzyme (Baldus *et al.*, 1994; Buckner *et al.*, 1998). σ^E, encoded by *spoIIGB*, is synthesized as an inactive pro-protein (pro-σ^E) in the predivisional cell (Errington, 1993). Pro-σ^E becomes active by cleavage of approximately 27 amino acids from its amino terminus following asymmetric cell

The GenBank accession numbers for the sequences determined in this work are AF225461–AF225466.

division (Carlson *et al*., 1996; Peters *et al*., 1992). This SpoIIGA-mediated proteolytic processing event is triggered by the extracellular signal protein SpoIIR that is produced in the forespore under the control of σ^F (Hofmeister *et al*., 1995; Karow *et al*., 1995; Londono-Vallejo & Stragier, 1995). σ^E activity is confined to the mother cell, where it directs transcription of several genes including *spoIIID* (Errington, 1993). SpoIIID is a sequence-specific DNA-binding protein that regulates transcription of many genes of the σ^E and σ^K regulons (Halberg & Kroos, 1994; Halberg *et al*., 1995; Zhang *et al*., 1997).

Several lines of evidence suggest that various regulatory features of the sporulation pathway are conserved between *Clostridium* spp. and *Bacillus* spp. *Clostridium acetobutylicum* ORFs bearing significant identity to the *Bacillus* genes encoding the sporulation sigma factors σ^E, σ^G and σ^K have been identified (Sauer *et al*., 1994; Wong *et al*., 1995) and expression patterns of these genes have been shown to be similar to those of *B. subtilis* (Santangelo *et al*., 1998). As in *B. subtilis*, *C. acetobutylicum* has a two-cistron *spoIIG* operon comprised of *spoIIGA* and *spoIIGB*. In addition, genes bearing identity with *B. subtilis spoOA* and *spoIVB* have been identified in six *Clostridium* species (Brown *et al*., 1994).

An improved understanding of sporulation processes in *Clostridium* spp. will also provide insight into physiological aspects unique to this genus. For example, linkages appear to exist between sporulation and toxin production in *Clostridium* spp. To illustrate, Zhao & Melville (1998) found that the P_2 and P_3 promoters of the enterotoxin gene (*cpe*) of *C. perfringens* bear similarity to the consensus promoter sequence for *Bacillus* spp. σ^E-dependent genes.

This study was designed to further improve our comparative knowledge of the sporulation processes in *Bacillus* and *Clostridium*. We used *spoIIGB* sequences from various spore-formers to investigate evolutionary relationships among these bacteria. We also used σ^E to investigate the functional conservation of the sporulation pathway between *C. acetobutylicum* and *B. subtilis*.

METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Clostridium* spp. were grown anaerobically at 37 °C for 24–48 h in Reinforced Clostridial Medium (Difco), and *Bacillus* spp. were grown at 37 °C for 24–48 h in Nutrient Broth (Difco) or on Tryptose Blood Agar Base plates (TBAB; Difco). Recombinant *B. subtilis* strains were derived from PB2. *Escherichia coli* DH5α was used as a host for plasmid construction. The *E. coli–B. subtilis* shuttle vector pKSV7 (Smith & Youngman, 1992) was used to construct an internal deletion mutation in *B. subtilis spoIIGB*. The low-copy-number *B. subtilis* vector pPL703 (Duvall *et al*., 1983) was used for extrachromosomal complementation. Plasmid pDH32 was used to create chromosomal insertions bearing *spoIIG–lacZ* fusions at the *B. subtilis amyE* locus (Henner, 1990).

PCR amplification and DNA sequencing. Crude bacterial cell lysates were prepared from vegetative cells by the method of Furrer *et al*. (1991). *spoIIGB* fragments (~ 364 bp) for phylogenetic analyses were amplified by hot-start 'touchdown' PCR (Roux, 1995) with the degenerate oligonucleotide primers SigE-F [5′-AAAGC(G/T)GT(A/T)AATAC(A/C)-TTT(A/G)ATCCA-3′] and SigE-R [5′-GA(C/T)TGAGA-(A/T)AT(A/C)CC(A/C)A(G/T)CATATC-3′]. This primer set was designed using the published sequences of *spoIIGB* from *B. subtilis* (Stragier *et al*., 1984), *B. thuringiensis* (Adams *et al*., 1991) and *C. acetobutylicum* (Wong *et al*., 1995). Amplification was performed in a Perkin Elmer 2400 thermal cycler using the following conditions: 1 cycle of 2 min at 94 °C; 21 cycles of 1 min at 94 °C, 1 min at the initial annealing temperature of 60 °C (which was decreased by 0·5 °C per cycle for 20 subsequent cycles) and 1 min at 72 °C; followed by 20 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C; and a hold at 72 °C for 5 min for all strains except *C. perfringens*, *B. polymyxa* and *C. bifermentans*. Annealing temperatures during the 21 'touchdown' cycles were decreased from 55 °C to 45 °C for *C. perfringens* and *B. polymyxa*, and from 45 °C to 35 °C for *C. bifermentans*. PCR was performed in a 50 µl volume containing 3·0 mM $MgCl_2$, 30 pmol of each primer and 2·5 U Amplitaq DNA polymerase (Perkin-Elmer).

PCR products were purified using the Wizard PCR preps DNA purification system (Promega) after excision of the band from a low-melting agarose gel (Fisher Scientific). The purified PCR products were cloned into the pCR2.1 vector and transformed into competent *E. coli* INVαF′ as specified in the TA Cloning Kit (Invitrogen). Plasmids were purified with the QIAprep spin plasmid purification kit (Qiagen) and at least two clones per bacterial strain were sequenced using the primers M13F and M13R. Sequencing was performed using an Applied Biosystems Division 373 A Stretch DNA sequencer (Perkin Elmer) at the Cornell Center of Advanced Technology.

Sequence alignments and phylogenetic trees. Alignments and phylogenetic analyses were performed using the programs of the DNAStar software package and the PHYLIP software package, version 3.57c (Felsenstein, 1989), respectively.

Bacterial transformation. Preparation and transformation of competent *B. subtilis* cells were performed following the two-step transformation procedure (Cutting & Horn, 1990). Transformants were selected on TBAB agar (Difco) plates containing 5 µg chloramphenicol ml^{-1} (for pDH32 and pKSV7) or 5 µg neomycin ml^{-1} (for pPL703).

Generation of the *B. subtilis* σ^E null mutant. A nonpolar internal deletion mutation in *spoIIGB* of *B. subtilis* PB2 was generated using splicing by overlap extension (SOEing) PCR (Horton *et al*., 1990) and the vector pKSV7 (Smith & Youngman, 1992). SOEing PCR primers were designed based on published sequences of *spoIIG* and *spoIIIG* of *B. subtilis* (GenBank accession nos X17344, X01180 and X57547). The primers SOE-A and SOE-B (Table 2) amplified a 423 bp fragment encoding portions of *spoIIGA* and *spoIIGB*. The primers SOE-C and SOE-D amplified a 420 bp fragment encoding portions of *spoIIGB* and *spoIIIG*. The two resulting PCR fragments served as template for a subsequent PCR amplification using SOE-A and SOE-D to yield an 843 bp fragment with an in-frame 603 bp deletion in *spoIIGB*. This PCR fragment was digested with *Xba*I and *Eco*RI and cloned into pKSV7. The resulting plasmid, pEFA60, was transformed

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source
Strains		
Bacillus megaterium 14581		ATCC
Paenibacillus polymyxa 12321		ATCC
B. thuringiensis 639		Cornell University
Clostridium sporogenes 795		DSM
C. bifermentans 630		DSM
C. butyricum M83		Cornell University
C. perfringens type A 8798 NC		Cornell University
C. acetobutylicum ATCC 824		ATCC
E. coli DH5α	*lacZ*Δ*M15 recA*1	Hanahan (1985)
Additional *B. subtilis* strains		
PB2	*trpC2*	Piggot (1973)
FSL-A2-030	*trpC2* Δ*sigE*	This study
FSL-A2-031	pEFA10 → FSL-A2-030	This study
FSL-A2-032	pEFA11 → FSL-A2-030	This study
FSL-A2-033	*amyE*::pEFA90 *trpC2*	This study
FSL-A2-034	*amyE*::pEFA90 *trpC2* Δ*sigE*	This study
FSL-A2-035	pEFA10 → FSL-A2-034	This study
FSL-A2-036	pEFA11 → FSL-A2-034	This study
FSL-A2-037	*amyE*::pEFA91 *trp2* Δ*sigE*	This study
FSL-A2-038	*amyE*::pEFA92 *trp2* Δ*sigE*	This study
Plasmids		
pEFA60	pKSV7 suicide vector harbouring a *spoIIGB* internal deletion mutation	This study
pEFA10	pPL703 harbouring *spoIIG*^{Ca}	This study
pEFA11	pPL703 harbouring *spoIIG*^{Bs}	This study
pEFA90	pDH32 harbouring *spoIIID* promoter from PB2	This study
pEFA91	pDH32 harbouring *spoIIG*^{Ca}	This study
pEFA92	pDH32 harbouring *spoIIG*^{Bs}	This study

into competent *B. subtilis* PB2. Subsequently, a chloramphenicol-resistant transformant was serially passaged in LB with 5 μg chloramphenicol ml^{-1} at 42 °C to direct chromosomal integration of the plasmid at the *B. subtilis spoIIG* locus by homologous recombination. Following the third passage, a loopful of culture was streaked onto TBAB with 5 μg chloramphenicol ml^{-1} and incubated at 42 °C. Colonies were passaged in LB (with no chloramphenicol) at 30 °C. Following every third passage, the culture was screened for plasmid excision by replica plating on TBAB with and without chloramphenicol. Allelic-exchange mutagenesis was confirmed by PCR amplification and by directly sequencing the PCR product.

Extrachromosomal complementation of the *B. subtilis* σ^E null mutant. We constructed derivatives of the low-copy-number *B. subtilis* plasmid pPL703 carrying the *spoIIG* operon from either *B. subtilis* (*spoIIG*^{Bs}) or *C. acetobutylicum* (*spoIIG*^{Ca}). *spoIIG* operons were amplified by PCR from chromosomal DNA preparations of *C. acetobutylicum* or *B. subtilis*, respectively, using primers CAF1 and CAR1 or BSF1 and BSR2 (Table 2). The purified PCR products were cloned into pPL703, creating pEFA10 and pEFA11, which were then transformed into competent cells of the *B. subtilis* σ^E null mutant FSL-A2-030 to generate strains FSL-A2-031 and FSL-A2-032, respectively. Plasmids were isolated using the Midi Kit (Qiagen), and recombinant DNA sequences were confirmed by sequencing.

Single-copy chromosomal complementation of the *B. subtilis* σ^E null mutant. Derivatives of the vector pDH32 were constructed for integration of *spoIIG*^{Bs} or *spoIIG*^{Ca} into the *amyE* chromosomal locus of the *B. subtilis* strain FSL-A2-030 (Δ*sigE*) (Henner, 1990). DNA fragments containing *spoIIG* were amplified with primers CAF1 and CAR6 for *C. acetobutylicum* and with primers BSF1 and BSR9 for *B. subtilis* PB2 (Table 2). The purified fragments were cloned into *Eco*RI–*Bam*HI sites of pDH32, generating *lacZ* transcriptional fusions in the resulting plasmids, pEFA91 and pEFA92. These plasmids were amplified in *E. coli* DH5α, linearized with *Pst*I and transformed into competent *B. subtilis* FSL-A2-030 cells with selection for chloramphenicol resistance, generating strains FSL-A2-037 (*C. acetobutylicum spoIIG*::*amyE*) and FSL-A2-038 (*B. subtilis spoIIG*::*amyE*). Disruption of the *amyE* locus was determined by flooding TBAB plates containing 1% soluble starch with Lugol's iodine: Amy$^+$ colonies were surrounded by a zone of clearing in the medium; Amy$^-$ colonies were not. Expression of the resulting *spoIIG–lacZ* fusions was monitored by measuring β-galactosidase activity (Miller, 1972).

Sporulation assay. *B. subtilis* cells were inoculated into 10 ml

Table 2. Oligonucleotide primer sequences

Primer	Sequence (5′ → 3′)	Location (and GenBank accession no.)
SOE-A*	GTCCTAGAG GAT TTT CGG AAC AGC CGA	891–908 bp (X17344)
SOE-B†	CTCAAGCCGCGAAATATA CAT CAG CAG CTT ATA CCA	1296–1313 bp (X17344)
SOE-C	TAT ATT TCG CGG CTT GAG	833–850 bp (X01180)
SOE-D‡	GGAATTCCA GCC GAC TTG AAA TAA G	343–360 bp (X57547)
CAF1‡	GGAATTCGA TCC AGA TGC AAA CAT AAT	1–20 bp (Z23079)
CAR1§	CCAATGCATTGGTTCTGCAGTTCC TTT GAA ATA ATA GCA TTA A	2046–2065 bp (Z23079)
BSF1‡	GGAATTCGA TCG TCC GAG ATG ATT ATG	1–20 bp (X17344)
BSR2¶	TTCCGCGGCCGCTATGGCCGACGTCGACCC TAC AGG AGC TGC TGT ACA	112–131 bp (X57547)
BSF8‡	GGAATTCCT GTT GAC GTA GGA CAT GTC	10311–10331 bp (Z82987)
BSR8‖	CGCGGATCC TGT AAT CGT GCA CAC CAC TC	10670–10690 bp (Z82987)
CAR6‖	CGCGGATCC CCT TTG AAA TAA TAG CAT TAA	2046–2065 bp (Z23079)
BSR9‖	CGCGGATCC CCT ACA GGA GCT GCT GTA CA	112–131 bp (Z57547)

* The *Xba*I restriction site (and additional sequence included to optimize cleavage; this also applies in footnotes marked ‡, §, ¶ and ‖) incorporated into this primer to facilitate cloning is underlined.

† The overhang complementary to SOE-C is underlined.

‡ The *Eco*RI restriction site incorporated into this primer to facilitate cloning is underlined.

§ The *Pst*I restriction site incorporated into this primer to facilitate cloning is underlined.

¶ The *Sal*I restriction site incorporated to facilitate cloning is underlined.

‖ The *Bam*HI restriction site incorporated to facilitate cloning is underlined.

Schaeffer's 2 × SG liquid medium (Leighton & Doi, 1971) supplemented with 25 mg tryptophan ml^{-1}, grown to mid-exponential phase with vigorous shaking (300 r.p.m.) at 37 °C, diluted 1:25 into a final volume of 50 ml of fresh 2 × SG and returned to the incubator shaker. Samples were removed after 24 h for parallel assessments of viable counts and of chloroform-resistant cells. Chloroform (25 µl) was added to 2·0 ml samples, then the treated cultures were held at room temperature without shaking for an additional 24 h. Serial dilutions of samples taken both before and after chloroform treatment were plated on 2 × SG. Viable cell counts before and after chloroform treatment were used to calculate the sporulation efficiency for each strain: sporulation efficiency = (no. of viable cells after chloroform treatment) × 100/(no. of viable cells before treatment) (Boor, 1994). Presence of spores was also verified using phase-contrast microscopy.

Construction of transcriptional *lacZ* fusions. Transcriptional *lacZ* fusions were constructed to investigate the functional activity of *C. acetobutylicum* σ^E or *B. subtilis* σ^E in the extrachromosomally complemented *B. subtilis* σ^E null mutant. For this purpose, a 359 bp DNA fragment containing the σ^E-dependent promoter region of *B. subtilis spoIIID* was amplified by PCR with primers BSF8 and BSR8 (Table 2). The purified PCR product was cloned into pDH32, yielding pEFA90 (Table 1). After confirming the correct insert by sequencing, the plasmid was linearized and transformed into competent *B. subtilis* PB2 and FSL-A2-030 (Δ*sigE*) cells with selection for chloramphenicol resistance, generating strains FSL-A2-033 and FSL-A2-034. These strains were also screened for AmyE phenotype as described above. Strain FSL-A2-034 was then transformed with either pEFA10 ($spoIIG^{Ca}$) or pEFA11 ($spoIIG^{Bs}$) to generate strains FSL-A2-035 and FSL-A2-036. All resulting strains were assayed for β-galactosidase activity (Miller, 1972).

RESULTS

PCR amplification and sequence analysis of σ^E

We designed degenerate PCR primers based on sequences that were highly conserved among available *spoIIGB* sequences (*B. subtilis*, *B. thuringiensis* and *C. acetobutylicum*) but that were unlikely to hybridize with nucleotide sequences of other σ^{70} family members. The resulting primers, SigE-F and SigE-R, amplified an ~ 364 bp nucleotide fragment corresponding to the following σ^E regions: partial 2.3 and 4.2 regions and complete 2.4, 3.1 and 4.1 regions as described by Lonetto *et al.* (1992). The predicted amino acid sequences for these regions share 83·3 %, 90·0 %, 70·6 %, 63·9 % and 68·8 % identity, respectively, among the three proteins (data not shown). SigE-F and SigE-R were used to amplify and sequence the internal *spoIIGB* fragment from *B. megaterium*, *Paenibacillus polymyxa*, *C. bifermentans*, *C. butyricum*, *C. perfringens* and *C. sporogenes* (GenBank accession nos AF225461–AF225466). In addition to these partial σ^E sequences, σ^E sequences from *B. subtilis*, *B. thuringiensis* and *C. acetobutylicum* were obtained from GenBank (accession nos X01180, X56697 and U07420, respectively) for inclusion in our sequence and phylogenetic analyses. Fig. 1 shows the multiple alignment of the partial sequences of the σ^E proteins. σ^E partial sequence identities among *Bacillus* spp., *Clostridium* spp. and *P. polymyxa* ranged between 67·0 and 100 %. Identical σ^E partial sequences were found in *B. thuringiensis* and *B. megaterium*.

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                          2.3 (76.9%)      2.4 (64.7%)           3.1 (50.0%)
                          ___________ ___________________ ____________________
B. subtilis               EKKIKLATYASRCIENEILMYLRRNNKIRSEVSFDEPLNIDVDGNELLLSDVLG  158
B. thuringiensis          ....................H......N.........................  158
B. megaterium             ....................H......N.........................  158
P. polymyxa               ........................S.T.T.....................M.  158
C. bifermentans           N.N.....................K...KKT.........V.L.........I..  158
C. butyricum              ........................S..KA.I..Y................I..  158
C. perfringens            ........................S.VKA.I..Y................I..  158
C. sporogenes             D........................S..KA.I..Y................I..  158
C. acetobutyricum         T........G..............S.VKA.I..Y..........K.....I..  158
                                     1 23   4

                                                    4.1 (50.0%)      4.2 (66.6%)
                          ___________________ ____________________ _____________
B. subtilis               TDDDIITKDIEANVDKKLLKKALEQLNEREKQIMELRFGLVG-EEEKTQKDVA  210
B. thuringiensis          .........L..T..RH..M...H...D............A.-G.........  210
B. megaterium             .........L..T..RH..M...H...D............A.-G.........  210
P. polymyxa               .EN.T.YRN..EQ..R...H...DK.TD..RL........QD-G.........  210
C. bifermentans           .EN.E.Y.L..EEI..D..VM..DR.SD............ASKGN.R...E..  211
C. butyricum              .EN.TVYNL..DE...Q..VM..KS..D...E.VR.....N.-TR.....X..  210
C. perfringens            .....VYNL..DE...E..FT.MKN.SN...E.V......C.-YK.....E..  210
C. sporogenes             ..N.EVYNL..DE...Q..LL.MKK......E.VR.....N.-KK.....E..  210
C. acetobutylicum         ..N.CVYNL..GE...Q..LF..KK......R.V...Y..T.-VG.....E..  210
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Fig. 1. Alignment of σ^E partial amino acid sequences (amino acids 105–210 according to the numbering for *B. subtilis* pro-σ^E) from *B. subtilis* (Stragier *et al.*, 1984) (GenBank accession no. X01180), *B. thuringiensis* (Adams *et al.*, 1991) (GenBank accession no. X56697), *B. megaterium*, *P. polymyxa*, *C. bifermentans*, *C. butyricum*, *C. perfringens*, *C. sporogenes* and *C. acetobutylicum* (Wong *et al.*, 1995) (GenBank accession no. U07420). The *B. subtilis* sequence is shown on top. For the other species, amino acids are given if they differ from the *B. subtilis* sequence. The subregions of σ^E are indicated above the alignment (Lonetto *et al.*, 1992). Amino acid conservation, calculated as percentage of conserved residues in all species for the number of residues in a given region, is indicated for each region. The number 1 below the alignment in subregion 2.3 indicates a conserved cysteine which is thought to be involved in DNA strand opening (Jones & Moran, 1992), and the numbers 2, 3 and 4 below the alignment in subregion 2.4 indicate conserved glutamic acid, asparagine and methionine, respectively, which are involved in promoter recognition (Diederich *et al.*, 1992; Jones & Moran, 1992; Tatti *et al.*, 1991; Tatti & Moran, 1995).

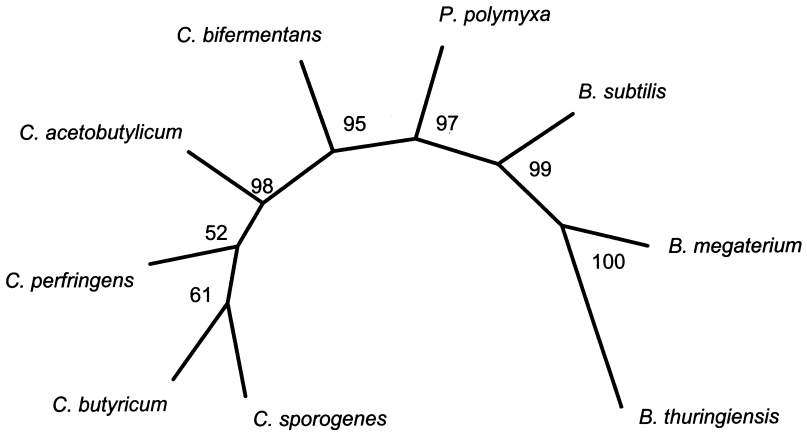

Fig. 2. Unrooted tree (100 replicates) for σ^E partial amino acid sequences constructed by the parsimony method using the programs SEQBOOT, Protpars, Consensus and Drawtree in the software package PHYLIP (Felsenstein, 1989). The numbers at the nodes of the tree represent the bootstrap values for each node. σ^E partial sequences used for this analysis are shown in Fig. 1.

Phylogenetic analysis

σ^E amino acid sequences (Fig. 1) were used for phylogenetic analyses by the parsimony method using SEQBOOT, Protpars, CONSENSE and Drawtree in the software package PHYLIP (Felsenstein, 1989). The resulting phylogenetic tree (Fig. 2) groups *Bacillus* spp. and *Clostridium* spp. separately from *P. polymyxa* and *C. bifermentans*. A phylogenetic tree (Fig. 3) generated by parsimony analysis of 16S rRNA GenBank sequences for the species represented in Figs 1 and 2 revealed clustering patterns very similar to those seen with σ^E.

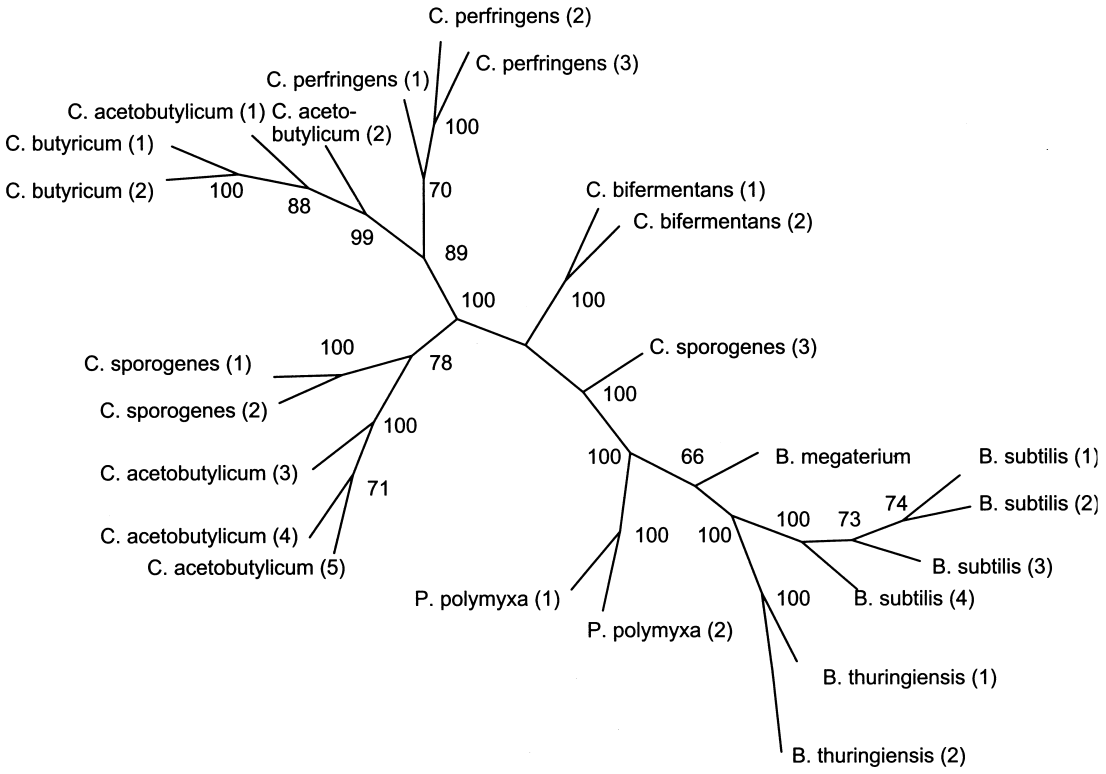

Fig. 3. Unrooted tree (100 replicates) for 16S rRNA sequences constructed by the parsimony method using SEQBOOT, Dnapars, Consensus and Drawtree in the software package PHYLIP (Felsenstein, 1989). The numbers at the nodes of the tree represent the bootstrap values for each node. 16S rRNA sequences used for this analysis are from the following species (GenBank accession numbers are shown in parentheses): *B. subtilis* (1, D64126; 2, D88802; 3, X60646; 4, D84213), *B. thuringiensis* (1, D16281; 2, X55062), *B. megaterium* (D16273), *P. polymyxa* (1, X60632; 2, D16276), *C. bifermentans* (1, X75906; 2, X73437), *C. perfringens* (1, M59103; 2, M69264; 3, Y12669), *C. acetobutylicum* (1, X81021; 2, U16147; 3, U16166; 4, X78070; 5, X78071), *C. butyricum* (1, X68177; 2, X68176), *C. sporogenes* (1, X68189; 2, M59115; 3, L09175).

Where available, multiple 16S rRNA sequences were included in this phylogenetic analysis.

To further probe the evolutionary relationships of sporulation sigma factors in *Clostridium* spp. and in *Bacillus* spp., we performed a phylogenetic analysis of all sigma factor sequences reported for the genera *Bacillus*, *Paenibacillus* and *Clostridium*, including the partial σ^E sequences described here. The resulting tree constructed by the parsimony method is shown in Fig. 4. Homologues of sporulation specific sigma factors (σ^E, σ^K and σ^G) from *Bacillus* and *Clostridium* spp. always formed coherent clusters distinct from other sigma factors. Similarly, σ^A from *B. subtilis* and from *C. acetobutylicum* formed a distinctive coherent cluster.

Construction of a *B. subtilis* σ^E null mutant

A non-polar *B. subtilis* σ^E null mutant (FSL-A2-030) containing an internal 603 bp deletion in *spoIIGB* (entire ORF $\sim$ 717 bp; Stragier & Losick, 1996) was constructed by allelic-exchange mutagenesis. The mutant phenotype was confirmed by the dual loss of capacities to form spores and to express the σ^E-dependent *spoIIID* promoter–*lacZ* fusion. Specifically, <10 chloroform-resistant-viable FSL-A2-030 cells ml^{-1} were present after exposure to chloroform ($<10^{-7}$% sporulation efficiency). Furthermore, no phase-bright spores were observed by phase-contrast microscopy following growth of the *B. subtilis* σ^E mutant on 2 × SG plates for 10 d.

Extrachromosomal complementation of the *B. subtilis* σ^E null mutant

To assess σ^E function, we determined and compared sporulation efficiencies for the *B. subtilis* strains FSL-A2-030 (Δ*sigE*), FSL-A2-031 (pEFA10 → FSL-A2-030), FSL-A2-032 (pEFA11 → FSL-A2-030) and the wild-type PB2 (Fig. 5). The sporulation efficiencies for the strains bearing pEFA10 (*spoIIG*^{Ca}) and pEFA11 (*spoIIG*^{Bs}) (FSL-A2-031 and FSL-A2-032) were $\sim$ 0·2% for each complemented strain. Restoration of spore production in these strains was verified by phase-contrast microscopy. Phase-bright spores were present in both strain FSL-A2-031 and strain FSL-A2-032 following growth on 2 × SG plates for 10 d.

σ^E activity in these recombinant strains was monitored by measuring β-galactosidase activity expressed from

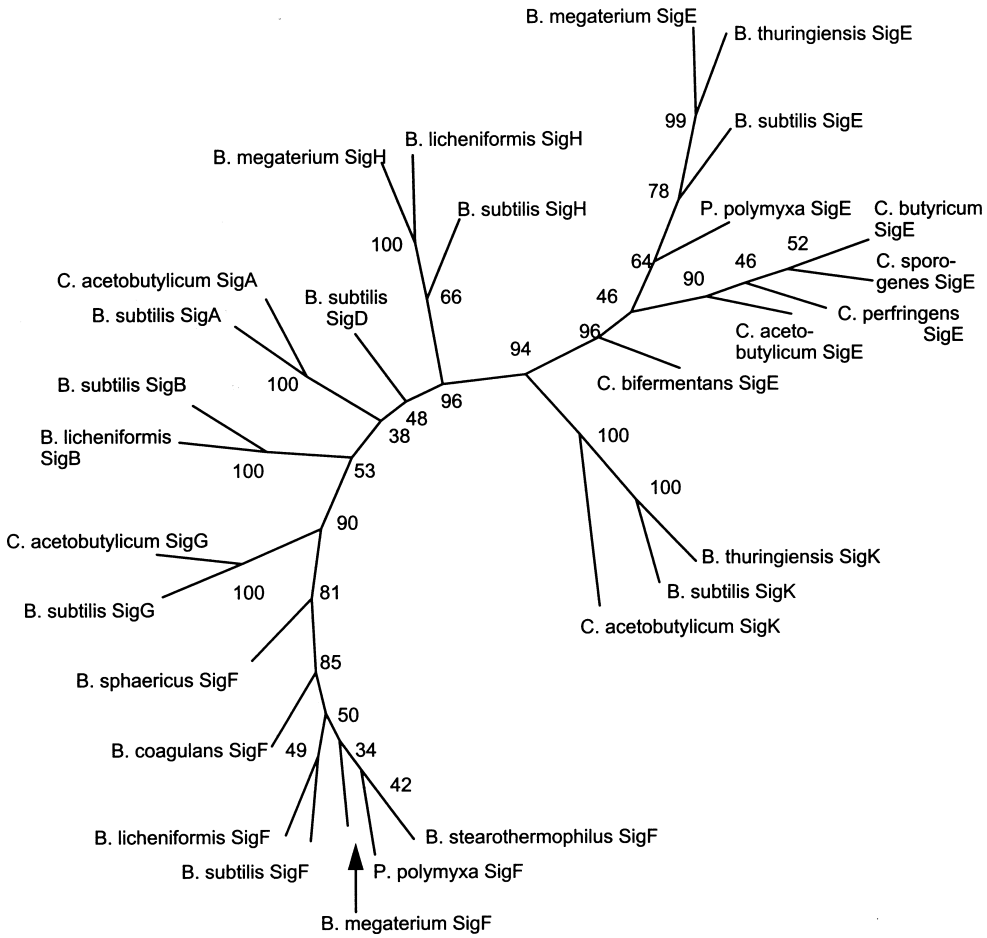

Fig. 4. Unrooted tree (100 replicates) for partial amino acid sequences for various sigma factors from spore-forming bacteria constructed by the parsimony method using the programs SEQBOOT, Protpars, Consensus and Drawtree in the software package PHYLIP (Felsenstein, 1989). The numbers at the nodes of the tree represent the bootstrap values for each node. σ^E sequences are as described in Fig. 1. The remaining sigma factor sequences obtained from GenBank were (GenBank accession numbers are given in parenthesis): *B. coagulans* SigF (Z54161), *B. licheniformis* SigH (M29694), *B. licheniformis* SigB (AF034567), *B. licheniformis* SigF (M25260), *B. megaterium* SigF (X63757), *B. megaterium* SigH (X59070), *B. sphaericus* SigF (L47359), *B. stearothermophilus* SigF (L47360), *B. subtilis* SigA (X03897), *B. subtilis* SigB (M34995), *B. subtilis* SigD (M20144), *B. subtilis* SigF (M15744), *B. subtilis* SigG (X57547), *B. subtilis* SigH (M29693), *B. subtilis* SigK (constructed from GenBank entries M23103 and M19299; Lonetto *et al.*, 1992), *B. thuringiensis* SigK (X56696), *C. acetobutylicum* SigA (Z23080), *C. acetobutylicum* SigG (Z23079), *C. acetobutylicum* SigK (L23317) and *P. polymyxa* SigF (L47358).

σ^E-dependent *spoIIID–lacZ* fusions. We constructed *spoIIID* promoter region–*lacZ* fusions in both the mutant and wild-type σ^E backgrounds (strains FSL-A2-033 and FSL-A2-034). In the wild-type strain containing a *spoIIID* promoter–*lacZ* fusion (FSL-A2-033), β-galactosidase activity was detected within 2 h of entry into stationary phase. β-Galactosidase activities measured in FSL-A2-030 (Δ*sigE*) and FSL-A2-034 (*amyE*::pEFA90 Δ*sigE*) were indistinguishable from the background β-galactosidase activity produced by PB2 for at least 6 h after entry into stationary phase. FSL-A2-035 and A2-036 (which each bore extrachromosomal copies of *spoIIGB* from *C. acetobutylicum* or *B. subtilis*, respectively) produced β-galactosidase activity levels only slightly higher than that of PB2 (data not shown).

Chromosomal complementation of the *B. subtilis* σ^E null mutant

spoIIG^{Bs} and *spoIIG*^{Ca} were integrated into the *B. subtilis amyE* locus utilizing pDH32-derived plasmids pEFA91 and pEFA92, respectively, to allow single-copy complementation of the *spoIIGB* null mutation and to generate *spoIIGB–lacZ* fusions. Expression of these fusions in strains FSL-A2-037 and FSL-A2-038 was monitored through β-galactosidase activity measurement, as shown in Fig. 6. Both strains showed detectable β-galactosidase activity in mid-exponential phase. β-Galactosidase activity in FSL-A2-038 (*amyE*:: *spoIIG*^{Bs}) peaked at 3 h after entry into stationary phase. FSL-A2-037 (*amyE*::*spoIIG*^{Ca}) produced 18–85% of the

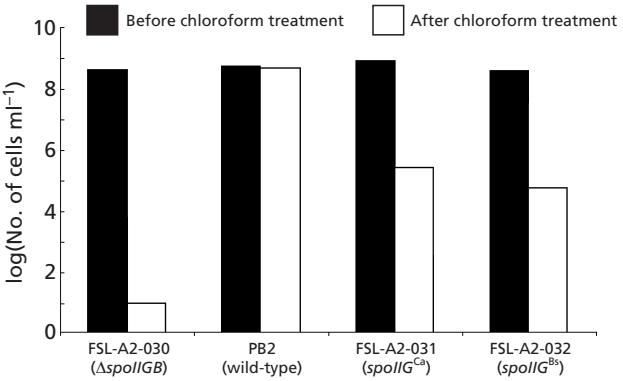

Fig. 5. Chloroform survival of 1-d-old *B. subtilis* cultures as a measure of sporulation capability. Strains were: FSL-A2-030 (Δ*spoIIG*); PB2 (*B. subtilis* wild-type); FSL-A2-031 (*B. subtilis* Δ*spoIIG* complemented with *spoIIG*^{Ca} on a low-copy-number plasmid); and FSL-A2-032 (*B. subtilis* Δ*spoIIG* complemented with *spoIIG*^{Bs} on a low-copy-number plasmid).

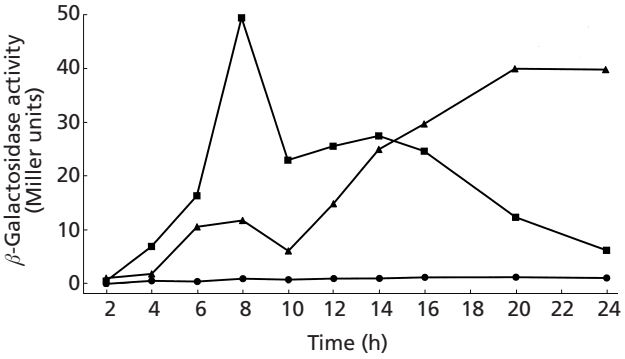

Fig. 6. β-Galactosidase activity for strains PB2 (●; wild-type), FSL-A2-037 (▲; *B. subtilis* Δ*spoIIGB* complemented with *spoIIG*^{Ca}–*lacZ* integrated in single copy at the *amyE* locus); and FSL-A2-038 (■; *B. subtilis* Δ*spoIIGB* complemented with *spoIIG*^{Bs}–*lacZ* integrated in single copy at the *amyE* locus). $t = 0$ designates the point at which mid-exponential-phase cultures were diluted into fresh medium to synchronize cultures. Onset of stationary phase for all cultures occurred at $t = 5$ h.

β-galactosidase activity of FSL-A2-038 between 3 and 8 h after entry into stationary phase, with a peak of activity at 15 h after entry into stationary phase.

Sporulation efficiencies were determined for strains FSL-A2-037 (*amyE*::*spoIIG*^{Ca}), FSL-A2-038 (*amyE*::*spoIIG*^{Bs}), FSL-A2-030 (Δ*sigE*) and PB2 (wild-type) (Fig. 7). FSL-A2-038 and wild-type PB2 both produced similar numbers of cells from 1-d-old cultures (approx. 10^8 ml^{-1}) before and after chloroform treatment, achieving 82% and 84% sporulation efficiencies, respectively. Strain FSL-A2-037 produced 10^2 chloroform-resistant cells ml^{-1} from 1-d-old cultures (representing 0·0001% sporulation efficiency).

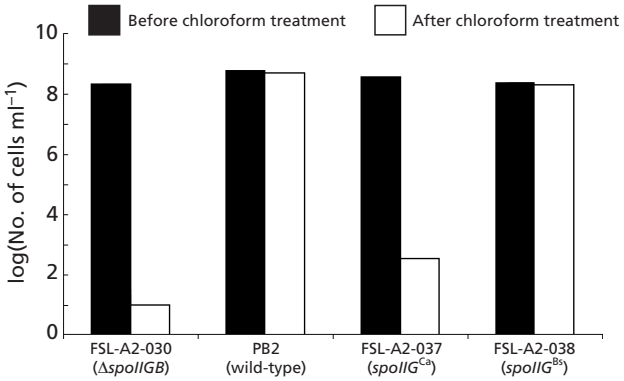

Fig. 7. Chloroform survival of 1-d-old *B. subtilis* cultures as a measure of sporulation capability. Strains were: FSL-A2-030 (Δ*spoIIG*); PB2 (*B. subtilis* wild-type); FSL-A2-037 (*B. subtilis* Δ*spoIIG* complemented with *spoIIG*^{Ca} integrated in single copy at the *amyE* locus); and FSL-A2-038 (*B. subtilis* Δ*spoIIG* complemented with *spoIIG*^{Bs} integrated in single copy at the *amyE* locus).

DISCUSSION

The capacity to form endospores unifies a group of otherwise diverse bacterial genera. A cascade of sigma factors, including σ^E (encoded by *spoIIGB*), directs gene expression during endospore formation. We probed the conservation of the sporulation pathways between the genera *Bacillus* and *Clostridium* through evolutionary and complementation analyses of alternative sigma factors, focusing on σ^E.

Sequence analyses

σ^E is a member of the σ^{70} family of proteins. Each of these σ proteins is comprised of four regions, which are believed to have distinctive functions and which are conserved to varying degrees among all family members (reviewed by Lonetto *et al.*, 1992). Alignment of the three complete pro-σ^E sequences from *B. subtilis*, *B. thuringiensis* and *C. acetobutylicum* obtained from GenBank showed amino acid identities ranging from 90% for region 4.2 to 63·0% for region 1.2 (data not shown). Notably, while the N-terminal amino acid sequence which is cleaved from the pro-σ^E protein to create the active σ^E protein (27 amino acids in each *Bacillus* species; 23 amino acids in *C. acetobutylicum*) (Carlson *et al.*, 1996; Peters *et al.*, 1992) is poorly conserved among these three organisms (21·4 %), the amino acid sequence flanking the cleavage site (YYIGG, amino acids 27–31 in *B. subtilis*) is exactly conserved among all three species.

The partial σ^E sequences obtained in this work as well as the σ^E sequences from *B. subtilis*, *B. thuringiensis* and *C. acetobutylicum* obtained from GenBank were combined for further sequence and phylogenetic analyses. The multiple alignment of the partial σ^E sequences (Fig. 1) includes the C-terminal portion of subregion 2.3 to the

beginning of subregion 4.2 of the σ^{70} family of proteins (Lonetto *et al*., 1992). Subregion 2.3 is proposed to be involved in catalysing DNA melting during open promoter complex formation (Helmann & Chamberlin, 1988). Jones & Moran (1992) demonstrated that a substitution of cysteine to arginine at position 117 in subregion 2.3 of *B. subtilis* σ^E conferred many of the characteristics expected of a sigma factor defective in DNA strand opening. This cysteine at position 117 is conserved among all of the σ^E sequences from the *Bacillus*, *Clostridium* and *Paenibacillus* species examined in this study.

Subregion 2.4, which is involved in RNA polymerase holoenzyme promoter utilization specificity, recognizes the −10 region of the promoter sequence (Lonetto *et al*., 1992). For *B. subtilis* σ^E, the methionine at position 124 was shown to interact with the nucleotide at position −13 in the −10 region of the *spoIID*, *spoIIID* and *cotEP1* promoters (Diederich *et al*., 1992; Jones & Moran, 1992; Tatti *et al*., 1991). Also, the glutamic acid residue at position 119 and the asparagine at position 120 appear to interact with nucleotides in the −10 region of σ^E-dependent promoters (Tatti & Moran, 1995). The corresponding methionine, glutamic acid and asparagine residues are conserved among all the species that we analysed. Our findings provide further evidence of evolutionary conservation of sporulation mechanisms among members of the diverse genera *Bacillus*, *Paenibacillus* and *Clostridium* (Sauer *et al*., 1994; Wong *et al*., 1995).

Phylogenetic analysis

The phylogenetic tree (Fig. 2) generated by parsimony analysis of the partial σ^E amino acid sequences grouped *Bacillus* spp. and *Clostridium* spp. into distinct clusters separate from *P. polymyxa* and *C. bifermentans*. Collins *et al*. (1994) had previously classified *C. bifermentans* into a cluster (cluster XI) distinct from *C. butyricum*, *C. acetobutylicum*, *C. perfringens* and *C. sporogenes* (cluster I) based on 16S rRNA analyses. These authors observed that *C. bifermentans* formed a distinct subgroup along with several other *Clostridium* spp. within cluster XI, suggesting that this subgroup may represent a different genus. These findings are consistent with our σ^E analysis, which confirmed a unique phylogenetic position for *C. bifermentans*. Our data also confirmed the status of *P. polymyxa* as a representative of a genus separate from *Bacillus*.

To verify the σ^E phylogenetic analysis, 16S rRNA GenBank sequences for the corresponding species were also analysed by the parsimony method. The resulting phylogenetic tree (Fig. 3) revealed clustering patterns very similar to those seen with σ^E. With the exception of *C. acetobutylicum* and *C. sporogenes*, multiple sequences from a given species generally clustered closely. The five *C. acetobutylicum* 16S rRNA sequences formed two different clusters, with three sequences clustering with *C. sporogenes* and two sequences clustering with *C. butyricum*. These findings are consistent with a previous phylogenetic analysis that reported distinctively different *spoOA* sequences from two *C. acetobutylicum* strains, with one sequence clustering with those of *C. butyricum* (Brown *et al*., 1994). Our findings provide further evidence in support of the reclassification of strains currently recognized as *C. acetobutylicum* into two phylogenetically distinct lineages and, perhaps, as two different species (Keis *et al*., 1995;Wilkinson *et al*., 1995). Two of the *C. sporogenes* 16S rRNA sequences clustered together, while a third *C. sporogenes* sequence (GenBank accession no. L09175) clustered most closely with the *Bacillus* cluster. These findings may suggest the existence of two phylogenetically distinct lineages of *C. sporogenes*.

The data presented here, as well as previous studies that identified homologous genes encoding sporulation-specific proteins in *Clostridium* spp. and in *Bacillus* spp. (e.g. *spoOA*; Brown *et al*., 1994) suggest a common evolutionary ancestor of the sporulation pathways in these two distinctive genera. As an alternative hypothesis, the sporulation pathways and the underlying genes could have evolved independently in these two genera. To further probe these two hypotheses, we performed a phylogenetic analysis of all sigma factor sequences reported for the genera *Bacillus*, *Paenibacillus* and *Clostridium*, including the partial σ^E sequences described here. The resulting tree constructed by the parsimony method is shown in Fig. 4. Homologues of sporulation-specific sigma factors (σ^E, σ^K and σ^G) from *Bacillus* and *Clostridium* spp. always formed coherent clusters distinct from other sigma factors. These findings suggest a common origin of sporulation sigma factors in the endospore-forming *Bacillus* and *Clostridria* rather than independent evolution of these proteins in these genera.

Functional analysis of σ^E conservation

To further probe the evolutionary conservation between the sporulation pathways, specifically σ^E, in *Clostridium* and *Bacillus* spp., we performed complementation analyses with the *spoIIG* alleles from *B. subtilis* and *C. acetobutylicum*.

Extrachromosomal complementation. Derivatives of the low-copy-number plasmid pPL703 were used for extrachromosomal complementation of the *B. subtilis* σ^E null mutant. Both complemented strains, FSL-A2-031 ($spoIIG^{Ca}$) and FSL-A2-032 ($spoIIG^{Bs}$), showed only partial recovery of the capacity to form spores in 1-d-old cultures (approx. 0·2 % sporulation efficiencies). σ^E activity was assessed by measuring expression from the σ^E-dependent *spoIIID* promoter–*lacZ* fusion. The wild-type strain FSL-A2-033 (PB2 bearing a *spoIIID–lacZ* fusion) yielded β-galactosidase activity within 2 h after the onset of stationary phase growth, consistent with previous findings (Tatti *et al*., 1991). Strains FSL-A2-035 and FSl-A2-036, which bore the *spoIIID–lacZ* fusion along with *spoIIG* from either *C. acetobutylicum* or *B. subtilis*, respectively, yielded β-galactosidase activities at

levels only slightly higher than that produced by the negative control strain, PB2. These results support previous reports of the ability of low levels of *spoIIG* activity (~17% of normal) to permit sufficient formation of endospores to allow spore detection under phase-contrast microscopy and production of viable colonies following chloroform treatment of 1-d-old cultures (Schyns *et al*., 1997). The absence of full recovery of the wild-type phenotype by the two complemented strains, and particularly by the *spoIIG*^{Bs}-complemented strain, is likely a consequence of our extrachromosomal complementation strategy. Adams *et al*. (1991) hypothesized that incomplete complementation of a *B. subtilis* σ^K defect with a pPL703 derivative bearing *B. thuringiensis* σ^{29} resulted from physiologically inappropriate gene copy numbers. In our study, it is possible that the presence of multiple *spoIIG* copies disrupted optimal levels of gene expression required for spore formation.

Chromosomal complementation. To probe the influence of *spoIIG* copy number on complementation of the σ^E null mutation, we performed additional complementation studies using pDH32 derivatives to integrate a single copy of either the *B. subtilis* or the *C. acetobutylicum spoIIG* operon into the chromosomal *B. subtilis amyE* locus of the σ^E null mutant, FSL-A2-030. Construction of pDH32 derivatives created *spoIIG–lacZ* fusions that allowed us to monitor *spoIIG* expression in the *B. subtilis* background. Measurement of β-galactosidase activity from these fusions showed that expression of *spoIIG*^{Ca}*–lacZ* was delayed in comparison with that of *spoIIG*^{Bs}*–lacZ*, peaking at 15 h rather than 3 h after entry into stationary phase (Fig. 6). Colony recovery following chloroform treatment revealed that *B. subtilis spoIIG* restored the wild-type phenotype, while *C. acetobutylicum spoIIG* yielded only a low level of complementation (0·0001% sporulation efficiency) in 1-d-old cultures.

Differences in *spoIIG*^{Bs} and *spoIIG*^{Ca} promoter regions may be at least partially responsible for the observed differences in transcription patterns directed by these constructs in the *B. subtilis* genetic background. In *B. subtilis*, the *spoIIG* operon has a σ^A-dependent promoter (Kenney *et al*., 1988) that is activated by SpoOA~P during the onset of sporulation (Baldus *et al*., 1994; Buckner *et al*., 1998). The promoter region of the *C. acetobutylicum spoIIG* operon was recently mapped by primer extension analysis (Santangelo, 1998). The deduced -35 and -10 σ^A recognition sequences in this operon differ from those in *B. subtilis* by 2 and 1 nucleotides, respectively, and the spacing between them consists of 21 nucleotides instead of the 23 present in the *B. subtilis spoIIG*. Further, two SpoOA-binding sites centred at -87 and -35 exist upstream of *B. subtilis spoIIG* (Baldus *et al*., 1994; Satola *et al*., 1991, 1992) but only one possible site was deduced in the *C. acetobutylicum* homologue (Wong *et al*., 1995).

Transcription and activation of σ^E is very complex (Stragier & Losick, 1996). Our findings suggest that regulation of *spoIIG* expression may differ between *Clostridium* spp. and *Bacillus* spp., as *spoIIG*^{Ca} is expressed later than *spoIIG*^{Bs} in the *B. subtilis* genetic background. It is also possible that *spoIIG*^{Ca} does not fully complement the B. subtilis σ^E null mutation as a consequence of inefficient association of *C. acetobutylicum* σ^E with the *B. subtilis* RNA polymerase or due to an inability of *C. acetobutylicum* σ^E*–B. subtilis* RNA polymerase holoenzyme to efficiently transcribe from σ^E-dependent promoters in *B. subtilis*. Genetic analyses of *C. acetobutylicum–B. subtilis* hybrid *spoIIG* sequences will provide insight into these hypotheses.

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