The Structure of Bypass of Forespore C, an Intercompartmental Signaling Factor during Sporulation in *Bacillus*[*][S]

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Sporulation in *Bacillus subtilis* begins with an asymmetric cell division giving rise to smaller forespore and larger mother cell compartments. Different programs of gene expression are subsequently directed by compartment-specific RNA polymerase σ-factors. In the final stages, spore coat proteins are synthesized in the mother cell under the control of RNA polymerase containing σ^K, (Eσ^K). σ^K is synthesized as an inactive zymogen, pro-σ^K, which is activated by proteolytic cleavage. Processing of pro-σ^K is performed by SpoIVFB, a metalloprotease that resides in a complex with SpoIVFA and bypass of forespore (Bof)A in the outer forespore membrane. Ensuring coordination of events taking place in the two compartments, pro-σ^K processing in the mother cell is delayed until appropriate signals are received from the forespore. Cell-cell signaling is mediated by SpoIVB and BofC, which are expressed in the forespore and secreted to the intercompartmental space where they regulate pro-σ^K processing by mechanisms that are not yet fully understood. Here we present the three-dimensional structure of BofC determined by solution state NMR. BofC is a monomer made up of two domains. The N-terminal domain, containing a four-stranded β-sheet onto one face of which an α-helix is packed, closely resembles the third immunoglobulin-binding domain of protein G from *Streptococcus*. The C-terminal domain contains a three-stranded β-sheet and three α-helices in a novel domain topology. The sequence connecting the domains contains a conserved DISP motif to which mutations that affect BofC activity map. Possible roles for BofC in the σ^K checkpoint are discussed in the light of sequence and structure comparisons.

In response to starvation, *Bacillus subtilis* and its relatives have the remarkable capacity to abandon growth and embark on a developmental pathway that leads to the production of dormant spores that are resistant to a variety of physical stresses. Sporulation begins with an asymmetric septation, which gives rise to two cells of unequal size but with identical chromosomes. The smaller cell is called the forespore, as it is destined to mature into the resistant spore, whereas the larger compartment is referred to as the mother cell, because it subsequently engulfs the forespore and nurtures the latter during its development. In the final stages, the mother cell lyses, and the mature spore is released into the environment where it can remain dormant indefinitely, germinating when favorable conditions for growth are restored (1).

A hallmark of sporulation is the utilization of a series of spatially and temporally regulated RNA polymerase σ-factors to effect differential gene expression from the identical chromosomes present in the forespore and the mother cell. σ^F, σ^E, σ^G, and σ^K become activated sequentially as part of what has been termed a crisscross regulatory cascade (2). Activation of each component depends on the expression of genes under the regulation of the preceding σ-factor in the cascade and on signals being relayed between the two compartments. σ^F and σ^G become activated in the forespore, whereas σ^E and σ^K become active in the mother cell. The activation of these σ-factors is coordinated and linked to morphogenetic events to ensure that development proceeds appropriately. As a result, the sporulation process is punctuated by a series of checkpoints at which the activation of subsequent events is delayed until an appropriate set of cues has been received (3).

The last of the σ-factors to become activated is σ^K. RNA polymerase containing σ^K (Eσ^K) transcribes mother cell genes encoding spore coat proteins, as well as later-acting factors involved in the release of the spore from the mother cell and in spore germination. The σ^K checkpoint ensures that σ^K becomes active in the mother cell only at the appropriate stage of forespore development, which is ~3 h after the onset of sporulation (4, 5). Premature activation of σ^K, by as little as 30 min, leads to aberrant spore formation (6). Transcription of *sigK*, which encodes σ^K, takes place from a σ^E-dependent promoter and is confined to the mother cell (7). σ^K is translated as an inactive precursor with a 20-residue N-terminal pro-sequence that targets pro-σ^K to the outer forespore membrane and prevents it from binding to core RNA polymerase (5, 8). Removal of the pro-sequence to yield mature σ^K is catalyzed by SpoIVFB, an integral membrane metalloprotease found in the outer forespore membrane (9, 10). SpoIVFB-mediated processing of σ^K is under complex regulation involving mother cell and forespore-derived factors (5, 11, 12). It has been proposed that the mother cell proteins bypass of forespore (Bof)[5]A and SpoIVFA form a ternary complex with SpoIVFB in the outer forespore membrane (see Fig. 1*A*). In this com-

[5] The abbreviations used are: Bof, bypass of forespore; HSQC, heteronuclear single quantum correlation; IgG, immunoglobulin G; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy.
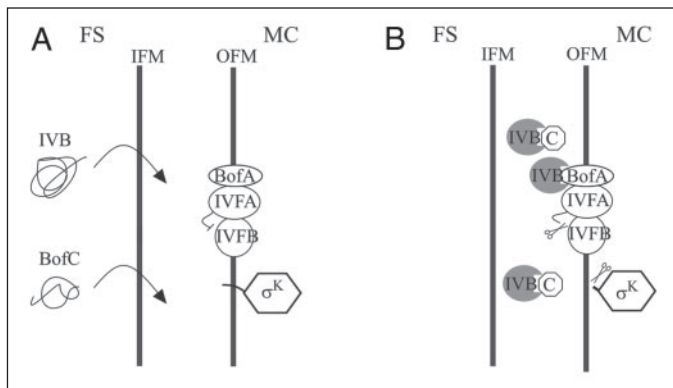
FIGURE 1. **Schematic illustration of the σ^K checkpoint.** *A*, BofA, SpoIVFA (*IVFA*), and SpoIVFB (*IVFB*) form a complex in the outer forespore membrane (*OFM*). Pro-σ^K is in the mother cell (*MC*) and tethered to the OFM. Its processing is delayed until a signal is received from the forespore (*FS*) in the form of SpoIVB (*IVB*) and BofC (*C*), which are secreted across the inner forespore membrane (*IFM*). *B*, SpoIVB binds to BofA and mediates cleavage of SpoIVFA. This relieves inhibition of SpoIVFB, which then cleaves pro-σ^K to generate the active transcription factor. BofC delay of SpoIVB signaling is represented here through SpoIVB-BofC complex formation, although it should be noted that the existence of this complex has not been established.

plex, SpoIVFB is stabilized and pro-σ^K cleavage is delayed (13, 14) until an appropriate signal is received from the forespore (9).

The forespore signal is provided by SpoIVB (15), a protein expressed at low levels under the control of σ^F and at augmented levels under σ^G control (16). SpoIVB contains a signal peptide, a PDZ domain, and a serine peptidase domain (17, 18). It has been proposed that SpoIVB is secreted into the intercompartmental space where it interacts with the BofA-SpoIVFA-SpoIVFB complex. This, in turn, leads to SpoIVB-mediated cleavage of SpoIVFA (19, 20) and relief of inhibition of pro-σ^K processing by SpoIVFB (21) (Fig. 1).

bofC was discovered as a gene whose deletion in a *spoIIIG* (which encodes σ^G) null mutant background restores signaling of pro-σ^K processing, giving rise to a Bof phenotype (4). This suggests that BofC is able to inhibit pro-σ^K processing by SpoIVB but only when the latter is present at the low concentrations produced by Eσ^F. *bofC* deletion in a wild-type background does not affect pro-σ^K processing (4).

BofC has no motifs to suggest a putative function nor does its sequence resemble any other proteins besides BofC orthologues. To gain further insights into forespore control of pro-σ^K processing in *B. subtilis*, we have embarked on structural studies of SpoIVB and BofC. Here we present the solution structure of BofC determined by NMR. A full list of chemical shifts and NMR assignments have been deposited in the BioMagResBank (accession number 6731).

MATERIALS AND METHODS

Purification of BofC—The plasmid pET28bBofC was provided by Dr. T. C. Dong, Royal Holloway, University of London. pET28bBofC contains the coding sequence for the full-length 170-residue BofC pre-protein. For overproduction of BofC, overnight cultures of *Escherichia coli* BL21(DE3) pET28bBofC were used to inoculate fresh LB medium containing 30 μg/ml kanamycin. The cultures were grown at 37 °C to an OD$_{600}$ of 0.6–0.7. Expression of recombinant protein was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 1 mM, and the cultures were grown at 30 °C for a further 3.5 h.

A 0.1 volume of Tris-HCl at 100 mM (pH 8.5) was added to the shaking flasks, and 10 min later, the cells were harvested by centrifugation. The cell pellet was resuspended in one-fifth of the original culture volume of a 40% sucrose, 30 mM Tris-HCl (pH 7.5), and 2 mM EDTA solution. After gentle swirling for 20 min at room temperature, the cells

were again harvested by centrifugation and resuspended in one-eighth the original volume of ice-cold water. Cellular material was removed in a further centrifugation step, and the supernatant representing the periplasmic fraction was reduced in volume by ultra filtration (Vivascience), exchanged into buffer A (20 mM Tris-HCl, pH 8, 10% glycerol, 5 mM EDTA, 10 mM NaCl) and loaded onto a Q-Sepharose column. The column was developed with a 10–400 mM NaCl gradient in buffer A. BofC eluted as a sharp peak at ~100 mM NaCl. The protein was subsequently purified to homogeneity by gel filtration on a Superdex 75 column in buffer A. The yield of BofC was 2–3 mg/liter of cell culture.

Labeling of Protein by ^{13}C and ^{15}N—For BofC structure determination by solution NMR, an overnight culture was grown at 37 °C in a minimal medium of the following composition: 27 mM Na$^+$/K$^+$ phosphate (pH 6), 2 mM NaCl, 2 mM MgSO$_4$, 0.1 mM CaCl$_2$, 1 mg/ml ^{15}NH$_4$Cl, 0.4% D-glucose (for ^{13}C^{15}N labeling, 0.2% D-[^{13}C]glucose was used), 30 μg/ml kanamycin, and 1 μg/ml of each of the vitamins riboflavin, niacinamide, pyridoxine monohydrochloride, and thiamine. Expression was induced at an OD$_{600}$ of 0.6–0.7 by the addition of isopropyl 1-thio-β-D-galactopyranoside to 1 mM, and the cells were cultured at 30 °C overnight. BofC was purified as described under "Purification of BofC."

Protein Analysis—Electrospray ionization mass spectrometry was performed using an API QSTAR liquid chromatography/tandem mass spectrometry system. Protein masses were predicted using the ExPASy ProtParam tool (us.expasy.org/tools/protparam.html).

Dynamic light scattering was performed on a ProteinSolutions DynaPro machine. Samples were centrifuged before injection into the cells, and the data were analyzed using the Dynamics software package, version 5.

Sedimentation equilibrium experiments were conducted at 20 °C on a Beckman Optima XL/A analytical Ultracentrifuge, using a Beckman cell with a 12-mm path length in an AN-50Ti rotor. Absorbance scans (at 280 nm) were taken at ~3 hourly intervals until sedimentation equilibrium was achieved. The data were analyzed using the Beckman Origin software.

NMR Spectroscopy—All spectra were recorded at 298 K on Bruker DRX700 and DRX900 spectrometers operating at 700 and 900 MHz proton frequencies, respectively. ^{15}N- and ^{13}C^{15}N-labeled samples were prepared in buffers containing 20 mM Na$_2$PO$_4$ (pH 6), 1 mM EDTA, and complete protease inhibitors (Roche Applied Science). For backbone and H$^\beta$/C$^\beta$ assignments, sequential (i-1) three-dimensional HNCO, CBCA(CO)NH, and HBHA(CO)NH in combination with bifurcate (i,i-1) three-dimensional HN(CA)CO, HNCA, HNCACB, and HN(CA)HA spectra were recorded. All spectra were processed in XWinNMR (Bruker, Biospin, Rheinstetten, Germany), whereas assignment was performed using PASTA (22) and AutoAssign (23) with peak lists generated within SPARKY (www.cgl.ucsf.edu/home/sparky). Sidechain assignment from three-dimensional C(CCO)NH total correlated spectroscopy, H(C)CH total correlated spectroscopy (employing 15 ms of flip-flop spectroscopy-16 (25) C,C-mixing each), and (H)CCH-correlated spectroscopy was assisted by in-house software (to be published). A two-dimensional H,H total correlated spectrum, employing 50 ms of XY16 mixing (26) with H-^{15}N suppression in the direct dimension was additionally recorded for the assignment of aromatic moieties.

Distance data were derived from four three-dimensional (HSQC)-NOESY-HSQC spectra, all recorded with a 100-ms NOE evolution time: H,NH-NOESY, H,CH-NOESY, (H)C,CH-NOESY and (H)C,NH-NOESY. For a verification of possible protein aggregation, a pseudo-two-dimensional diffusion-ordered spectroscopy experiment was measured employing double-pulsed field gradient echoes with variable

gradient strength and a diffusion delay of 150 ms. Results were referenced against the known value for the water diffusion rate at 298 K (2.3×10^{-9} m²/s) sampled with the same pulse sequence but with the final WATERGATE suppression module omitted.

NOE Analysis and Structure Calculations—Automatic NOE assignment and structure calculations were performed using the CANDID module of the program CYANA (27, 28). The quality of the structures was improved in an iterative procedure where CANDID runs were followed by manual analysis. Comparing the NOESY spectra to the preliminary structure subsequently allowed the assignment of missing resonances and improved the quality of the peak lists. Hydrogen bond restraints were defined when they were consistent with the secondary shift data, expected NOE contacts, and the calculated structure. Manual NOE peak assignments were generally not fixed in the CANDID runs but were used to create accurate spectrum-specific chemical shift lists to check the consistency of subsequent CANDID runs and to verify the manual assignments. The final CANDID run was performed using CYANA version 2.0 with Ramachandran and side-chain rotamer dihedral angle restraints in all but the last cycle. In the final cycle, fixed stereospecific assignments of prochiral groups were used when available.

The final set of NOE-based restraints determined by CANDID, in combination with restraints for 38 H-bonds and dihedral restraints for 90 residues from TALOS, were used in a water refinement run using crystallography NMR software (29) according to the standard RECOORD protocol (30). Structures were validated using WHATIF (31) and PROCHECK (32, 33).

RESULTS AND DISCUSSION

Production and Characterization of Recombinant BofC—BofC has an N-terminal signal sequence typical of proteins secreted from Bacillus by the Sec-type secretion system (34, 35). Fusion of this signal peptide sequence to a sequence encoding *E. coli* alkaline phosphatase produced alkaline phosphatase activity in the periplasm (36), implying that BofC can be translocated across the cytoplasmic membrane of *E. coli*. Osmotic shock was used here to demonstrate that BofC is directed to the periplasm of *E. coli*, consistent with the hypothesis that BofC is secreted into the intermembrane space between the mother cell and forespore in *B. subtilis.*

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) of purified BofC revealed a band toward the high end of the region demarcated by the 14–21.5-kDa standard markers. If BofC is cleaved by signal peptidase I upon secretion, the resulting polypeptide would have a predicted molecular mass of ~16.2 kDa (36). Electrospray ionization mass spectrometry suggested that the purified product expressed from pET28bBofC has a mass of 16,173 ± 2 Da, consistent with truncation of the N-terminal residues, to give a mature protein beginning at residue Ala[31] (data not shown). BofC-(31–170) has a calculated mass of 16,173 Da. Successive cycles of Edman degradation yielded the sequence Ala-Glu-Val-Glu-His corresponding to residues 31–35 of BofC and unambiguously identifying the product as BofC-(31–170). These data show that secretion of BofC from *E. coli* is accompanied by signal peptidase I cleavage. Henceforth, the BofC numbering will refer to the mature protein (residues 1–140) whose structure has been determined.

BofC migrated as a single band in native polyacrylamide gels (8.75%), suggesting a homogeneous preparation, an inference supported by dynamic light scattering experiments. The molecular mass of BofC was estimated to be 14 kDa from the retention volume of BofC relative to molecular weight standards during gel filtration. This indicates that BofC is a monomer, a result confirmed by sedimentation equilibrium experiments in the analytical Ultracentrifuge, which gave a molecular mass of 16,700 ± 100 Da. NMR diffusion-ordered two-dimensional spectroscopy measurements at 298 K also agreed well with the conclusion that BofC is a monomer (data not shown).

NMR Spectroscopy—Electrospray ionization mass spectrometry of ¹⁵N-labeled BofC revealed a molecular mass of 16,359 Da, suggesting 98% efficiency of labeling. Initial ¹⁵N-HSQC spectra were recorded at 700 MHz in buffers of varying salt concentrations and pH and at different temperatures (supplemental Fig. S1). For most conditions tested, the signal dispersion in this fingerprint spectrum was high and indicative of a well folded protein. Stability tests were carried out to optimize the buffer conditions. The doubly labeled sample had a predicted mass of 17,095 Da and a recorded mass of 17,081 Da, establishing again that labeling was almost complete.

Backbone resonances of BofC were assigned with the PASTA tool (22) using sequential C-α, C-β, C' and H-α chemical shift information obtained from the array of triple-resonance experiments outlined under "Materials and Methods" resulting in 97.6% complete assignment. Assignment of side chains was 94.2% complete; a small number of side-chain protons, generally the labile amino protons of arginine and lysine residues, remained unassigned due to weak intensity or ambiguity.

NOE Assignments and Structure Calculations—The first automatic NOE assignment and structure calculation using the CANDID module in CYANA was performed when the completeness of proton resonance assignments reached 79%. The N-terminal domain converged to the correct fold in the first run, whereas the C-terminal domain converged consistently to the correct fold only after the completeness reached 93%. The final completeness was 94.2% for non-exchanging protons.

The final set of NOE restraints, H-bond restraints, and dihedral restraints was used to generate water-refined structures following the RECOORD protocol. 100 structures were annealed from extended chains in vacuum, of which the 85 lowest energy structures were subjected to water refinement. Of these, the 25 lowest energy structures were selected for the final structure set. A summary of the results of the structure calculations is given in supplemental Table S1.

Description of the Secondary Structure and Fold—The secondary structure of BofC was determined from C-α, C-β, C', and H-α chemical shift indices (37–40) and backbone NOE patterns, indicating seven β-strands and four α-helices. The folding topology of the protein was obtained from NOE contacts between the β-strands, (Fig. 2A), revealing two separate domains. In addition to the four helices clearly predicted by the secondary structure data, a putative fifth helix (αB) was less clearly indicated. This helix was later confirmed during quantitative structure determination, and the fold could thus be viewed as comprising two domains: one mainly β (N-terminal domain) and the other $\alpha + \beta$ (C-terminal domain) linked by a long loop partially formed by helix αB (Fig. 2).

The N-terminal domain is composed of a four-stranded β-sheet covered by an α-helix. The β-sheet has a β2-β1-β4-β3 topology as illustrated in Fig. 2A. Strands β1 and β2 and strands β3 and β4 are connected by β-turns, whereas strands β2 and β3 are joined by an α-helix that runs across one face of the β-sheet. The N-terminal domain is very well defined by a large number of NOE contacts. A sequence of 11 residues links the N- and C-terminal domains. The C terminus of this linker region includes helix αB. This helix is very short, consisting of five residues, and is separated from the core of the C-terminal domain by further unstructured residues, effectively continuing the linker. The C-terminal domain comprises three α-helices and three short anti-parallel β-strands. The β-sheet has the strand order β5-β6-β7. The three helices pack in a somewhat loose arrangement around this sheet. The
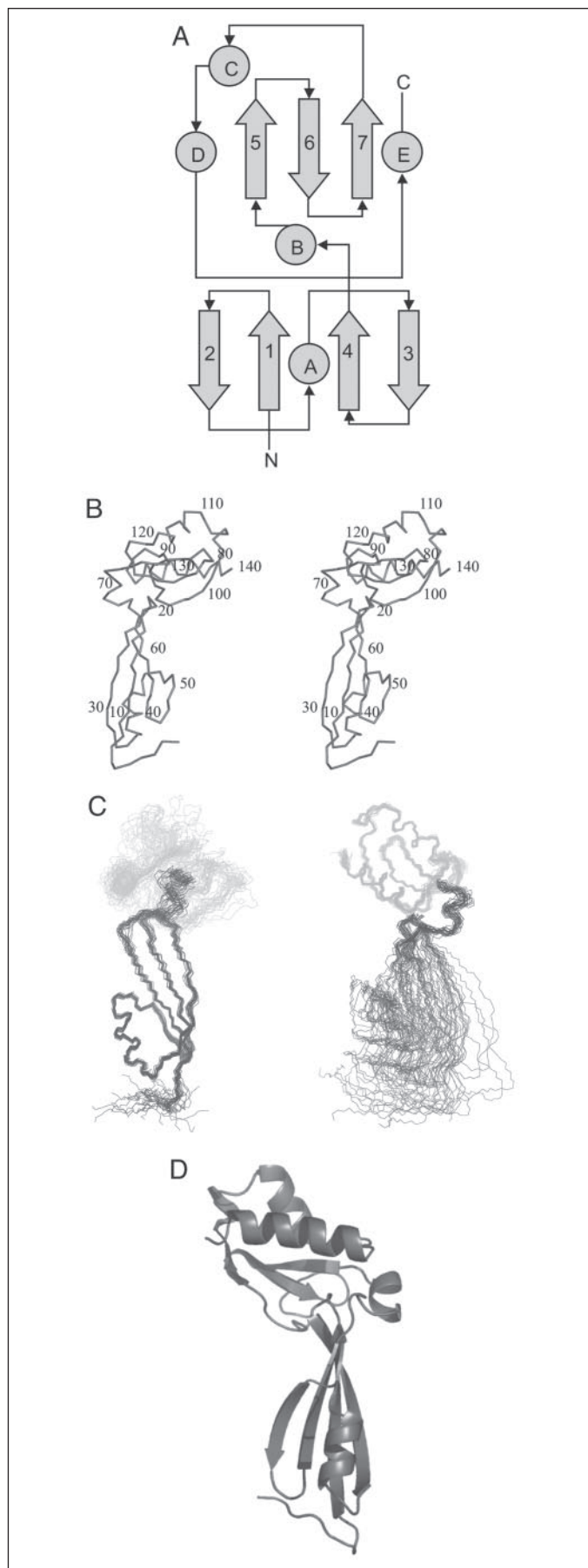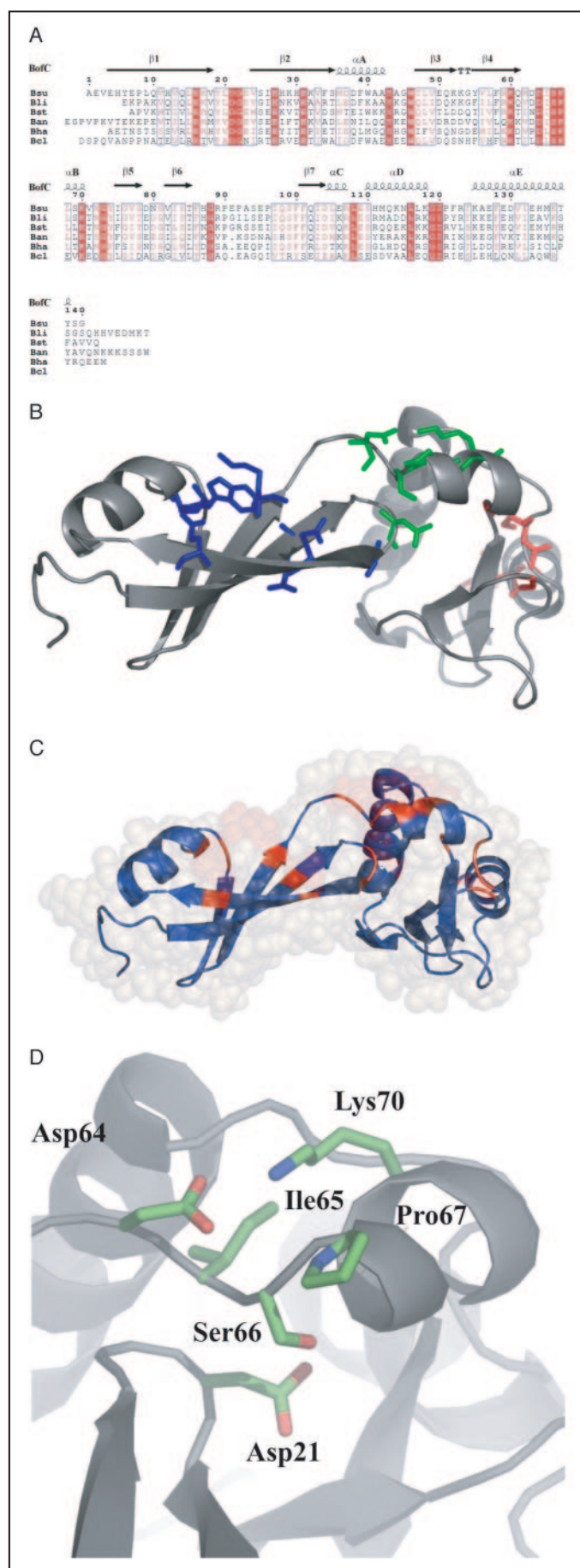
longest of the helices, αE, runs almost parallel to the sheet, and helix αD runs roughly perpendicular to the sheet with its C terminus located near to strand β5 of the sheet.

Tertiary Structure and Domain Interactions—NMR data give insight into both structure and dynamics. Often the presence of NOE contacts implies rigidity in the structure, whereas their absence indicates flexibility. From the experimental NMR data, the N-terminal domain of BofC is the better defined substructure, with an almost complete assignment of chemical shifts and a large number of NOE contacts defining the structure, with an average of 7.05 NOE contacts/residue. For the C-terminal domain, the resonance assignment is somewhat less complete, contributing to a less dense network of NOE connectivities, with an average of 4.35 NOE contacts/residue. Nonetheless, its structure is well defined, and the final structure ensemble displays very little dispersion (backbone root mean square deviation of 1.5 Å) (supplemental Table S1). Only the extensive loops connecting the elements of the secondary structure (three β-strands and three α-helices) show more substantial scattering, indicative of flexibility. Two adjacent loops near the C terminus (residues 86–100 and 119–124), located between β-strands 6 and 7 and between helices αD and αE, closely approach the N-terminal domain (Fig. 2). The loop sequence between β6 and β7 is poorly conserved, suggesting that it is not functionally important. By contrast, the sequence at the beginning of the loop between helices αD and αE is conserved; this is remarkable, as the sequences of the helices themselves are poorly conserved except for some residues at the C terminus of helix αD. These regions might form a functionally important conserved interaction surface. We cannot exclude the importance of this region for structural integrity, but no NOE contacts were found between this surface-exposed region and the N-terminal domain.

The linker region between the domains is poorly defined by the NMR data. Only a few long range NOE contacts could be unambiguously identified between the domains, i.e. between residues 19–24 (loop between β1 and β2) in the N-terminal domain and residues 97, 98, 125, 128, and 129 of the C-terminal domain. These contacts are, however, located quite close to the linker, whereas contacts farther away and thus with a larger structural "lever" are absent. Least squares superposition of the N-terminal domain structures (10–60) revealed a backbone root mean square deviation for the C-terminal domain (74–138) of 4.59 Å. Thus, the relative orientation of the domains appears quite flexible, within the steric restrictions imposed by the linker region. It could therefore be considered that both domains function in a way that does not require a specific relative orientation. To test this hypothesis, we expressed the individual N-terminal domain (1–62) and compared its ^{15}N HSQC spectrum to that of full-length BofC under identical conditions. The substantial overall congruence in both ^{15}N HSQC spectra proved the important point that the isolated N-terminal domain adopts the same fold as in the full-length protein. Apart from the expected shifts at the C terminus of the N-terminal domain (Lys[60] and Gln[61]) that run into the linker region, the only significant spectral changes were in the turn between strands β1 and β2, with the largest shifts occurring for Leu[20] and Asp[21]. This exactly correlates with the region identified as proximal to the interdomain interface from the NOE data (see above), whereas the absence of any other shift changes corroborates the conclusion that no further parts of the N-terminal domain (apart from the linker) contact the C-terminal domain. Analogous experiments with the

FIGURE 2. **The three-dimensional structure of BofC.** *A*, topology diagram with strands as *arrows* and helices as *circles*. *B*, stereo C-α representation with every tenth residue numbered. *C*, ensemble of the 25 lowest energy NMR solution structures superposed on the N-terminal domain (*left*) and the C-terminal domain (*right*). *D*, *Ribbon* diagram of the lowest energy structure of BofC. *B* and *D* were drawn in Pymol (www.pymol.org).

FIGURE 3. *A*, sequence alignment of BofC homologues. The alignment was generated by MultAlign (prodes.toulouse.inra.fr/multalin/multalin.html). Invariant residues have a *red* background, and conserved residues are *boxed*. The secondary structure elements of BofC are displayed *above* the alignment. The numbering refers to the mature form of BofC from *B. subtilis*. *Bsu*, *B. subtilis*; *Bli*, *B. licheniformis*; *Bst*, *Bacillus stearothermophilus*; *Ban*, *B. anthracis*; *Bha*, *B. halodurans*; *Bcl*, *B. clausii*. *B*, representative lowest energy NMR structure solution for BofC with the side chains of conserved residues represented as *sticks*; *blue* in cluster 1: Glu[16], Gly[22], Glu[27], Glu[31], Tyr[43], Trp[46], and Lys[60] in the N-terminal domain; *green* in cluster 2: Asp[21], Asp[64], Ser[66], Pro[67], and Lys[70] in the linker region; and *red* in cluster 3: Gly[73], Gly[88], Leu[108], Gly[119], and Ile[120] in the C-terminal domain are shown. The structure is orientated with the N-terminal domain to the *left side*. *C*, surface representation, in the same orientation as *B*. Conserved residues are colored *red*. *D*, the linker region between the domains with residues 64, 65, 66 and 67; the DISP motif, along with Asp[21] and Lys[70], in *stick* representation. This figure was drawn using PyMol (www. pymol.org).

C-terminal domain were frustrated by the finding that this domain is insoluble when expressed in isolation. We cannot entirely rule out the possibility that minor local conformational changes occur in the C-terminal domain when present in isolation.

In conclusion, our experimental NMR data indicate that the subdomain interface is not very extensive and is largely restricted to the linker region, including loop $\beta1$-$\beta2$ in the N-terminal domain and the loop between $\beta6$ and $\beta7$ and the N terminus of αE in the C-terminal domain. Nonetheless, the observed interdomain NOE contacts near the linker limit the amplitude of possible domain reorientations and motional degrees of freedom.

The N-terminal Domain Has a Protein G-like Fold—A DALI search (41) of the protein structure data base shows that the N-terminal domain (residues 1–63) of BofC is topologically identical to the third immunoglobulin G-binding domain of protein G from Streptococcus, (Protein Data Bank (PDB) code 2igd; Z-score = 4.3, for superposition of 50 (of 61 total) equivalent C-α atoms with a root mean square deviation of 2.7 Å) (supplemental Fig. S2*A*). Protein G belongs to a large and diverse group of cell surface-associated proteins that bind to immunoglobulins (42). The IgG-binding domain is a highly conserved sequence of ~60 residues and this sequence similarity is reflected in a common structure. These IgG-binding domains are usually present in multiple copies within an individual protein G molecule, but they retain high affinity for IgG even when expressed individually (43).

Protein G is secreted by *Streptococcus* as part of a defense mechanism against phagocytosis by the host organism. By binding to the constant domains of IgG, protein G blocks the interaction of the immunoglobulin with complement proteins, preventing phagocytic cells bearing C3 receptors from taking up the complex for intracellular processing and degradation (44). This provides the bacteria with a mechanism for evading the opsonizing action of complement and increasing virulence by enhancing the capacity of the bacteria to survive in the host organism.

By analogy with protein G, we anticipate that the N-terminal domain of BofC may be a mediator of protein-protein interactions. However, the identity of the surfaces involved cannot straightforwardly be inferred. One reason for this is that structural studies have shown different surfaces on the IgG-binding domain of protein G forming interactions with IgG according to whether Fab or Fc fragments are being studied (supplemental Fig. S2). To test whether BofC binds to IgG, we carried out IgG-Sepharose affinity column chromatography. BofC was not retained on this column in contrast to protein G, which was tested in parallel. It is of further interest that the IgG-binding domains of protein G also bind to α_2-macroglobulin, a proteinase inhibitor in human plasma. This observation has led to the suggestion that protein G may be involved in proteolytic events at the cell surface (45), thus presenting another interesting parallel with the proposed role of BofC in pro-σ^K processing.

The C-terminal Domain Has a Unique Topology—The C-terminal domain of BofC (residues 61–140) seems to have a new and unique fold.

No significant structural homologues were found from a DALI search of the Protein Data Bank. The highest Z-scores obtained were ≤2. The closest structural match was murine-enabled/VASP homology 1 (EVH1) domain (46), which plays a role in the spatial control of actin assembly (PDB code 1evh; Z-score = 2.0, for superposition of 43 (of 111 total) equivalent C-α atoms and a root mean square deviation of 2.8 Å). The similarity occurs in the region of the three-stranded anti-parallel β-sheet (supplemental Fig. S2B). In both BofC and the EVH1 domain, an α-helix runs parallel to the face of this β-sheet. However, the interposing elements between β-sheet and α-helix are different in the two structures. The functionally conserved residues in the EVH1 domain, required for binding its polyproline peptide ligand, lie in a region that has no counterpart in the BofC domain.

Invariant Residues Map to Three Clusters on the Structure—A BLAST search with the sequence of BofC from *B. subtilis* as the search string identifies orthologues in all of the other *Bacillus* species whose genome sequences have been completed. BofC was not found in the genome of *Clostridium difficile* in which orthologues of the other known pro-σ^K processing components are also missing (24). An alignment of the amino acid sequence of BofC from *B. subtilis* with orthologues from *Bacillus anthracis* (representative of the *B. anthracis*, *Bacillus cereus*, *Bacillus thuringiensis* group), *Bacillus licheniformis*, *Bacillus halodurans*, and *Bacillus clausii* is shown in Fig. 3*A*. Conservation of the sequence in this type of alignment usually points to residues that play structurally or functionally important roles. Mapping of invariant residues onto the three-dimensional structure of BofC (Fig. 3, *B* and *C*), reveals three clear clusters: cluster 1 in the N-terminal domain, cluster 2 in the segment that connects the two domains, and cluster 3 in the C-terminal domain.

Cluster 1 consists of residues exposed on the surface of the β-sheet crossed by the α-helix. Of the seven invariant residues in this cluster, it is striking that three have acidic side chains, Glu^{16}, Glu^{27}, and Glu^{31}. In contrast, the invariant residues in cluster 3, within domain 2, tend to have apolar side chains and to cluster in the core of the domain, perhaps indicating a structural role.

Perhaps the most striking of the three clusters is cluster 2. Segments of polypeptide that link domains in multidomain proteins are often associated with variability in sequence. This is because their role is usually limited to tethering functional domains to one another in a single molecule. In fact, hypervariable regions within otherwise well conserved orthologous sequences are often used to identify putative domain boundaries in proteins. The strong conservation of sequence and the obvious $^{64}DISP^{67}$ motif in the segment that constitutes the linker in the structure of BofC suggest a more active role. It may, for example, act as a linear epitope in binding to another protein. Alternatively, it may be a recognition sequence for a specific protease. Asp^{21} located in domain 1 and Lys^{70} in the C-terminal domain are seen in the structure to belong to cluster 2, around the linker region. Lys^{70} may interact with Asp^{64} (Fig. 3*D*). The interdomain contacts made by these conserved residues with the linker domain restrict the relative orientation of the two subdomains with respect to each other, possibly leading to the formation of a cleft between the two subdomains formed by the C-terminal end of the loop between β6 and β7 (IQSFF), and the loop between β1 and β2 (YLDGD) and the linker region (DISP). A genetic screen identified Ser^{66} as an important residue for BofC function (see next paragraph), arguing that the integrity of the linker region, and thereby the relative positioning of the two subdomains, is crucial for its function.

Mutagenesis Considerations—*bofC* was identified as a gene whose mutation in a *spoIIIG* (which encodes σ^G) null mutant background restores signaling of pro-σ^K processing. The first characterized *bofC* allele, *bofC1,* contains a missense mutation that results in the substitu-

tion of Ser^{66} of mature BofC by phenylalanine (4). Interestingly, this serine is part of the conserved DISP motif and situated in the interdomain linker. Subsequently, a *bofC* insertion mutant was constructed, *bofC::neo* by insertion of a neomycin resistance cassette at a unique restriction enzyme cleavage site in *bofC,* overlapping the Ser^{66} codon of the open reading frame (4). Thus a protein fragment would be expressed and presumably secreted across the inner forespore membrane to produce a polypeptide consisting of residues 1–66 of the mature BofC. The structure presented here shows that this truncated form encompasses all of the N-terminal domain and part of the interdomain linker. It is therefore likely that BofC-(1–66) is a stable folded entity.

The *bofC1* and *bofC::neo* alleles are distinguishable from a subsequently constructed *bofC* null mutant in which the whole gene is deleted, in that the former are partially active as negative regulators of intercompartmental signaling of pro-σ^K processing. Thus, in *spoIIIG* strains containing *gerE-lacZ* fusions, σ^K-directed expression of β-galactosidase occurs 3 h earlier and reaches higher levels in the *bofC* null background (*bofCΔ::neo*) than in the truncated or mutated *bofC* alleles (*bofC::neo* and *bofC1*). This result implies that both domains of BofC contribute to the inhibition of signaling of pro-σ^K processing. In the *spoIIIG* background, intact BofC blocks signaling completely, whereas BofC-(1–66) only delays signaling. One interpretation of the equivalent effects of the *bofC1* and *bofC::neo* alleles is that the S66F mutation nullifies the contribution of the C-terminal domain.

The Role of BofC in the σ^K Checkpoint—The role of BofC in the σ^K checkpoint remains unclear. The present study reveals that BofC is made up of two separate domains, one of which resembles an IgG-binding domain. It is tempting to use this structural similarity to infer a similarity in function and suggest that BofC participates in protein-protein interactions following its passage through a cell membrane. Its most probable interaction partners would be other components of the pro-σ^K processing system with SpoIVB being the primary candidate, because it, alone among this group, is transcribed by Eσ^G. The levels of SpoIVB and BofC in sporulating cells suggest that the fate of each molecule is dependent on their mutual interactions (36). Thus (i) there are increased levels of BofC in mutants unable to synthesize SpoIVB, (ii) there are decreased levels of active SpoIVB in *bofC* null mutants, and (iii) overproduction of BofC inhibits SpoIVB autoproteolysis and delays pro-σ^K processing (36).

SpoIVB undergoes complex post-translational processing involving secretion across the inner forespore membrane, autoproteolysis in *trans* to release the zymogen into the intercompartmental space, and autoproteolysis in *cis* to produce the mature pro-σ^K signaling species. It has been proposed that mature SpoIVB binds the C terminus of BofA as a prelude to cleaving SpoIVFA. This, in turn, releases SpoIVFB from its inhibition by SpoIVFA and BofA and allows it to cleave and activate pro-σ^K (21). BofC could inhibit signaling by forming a complex with SpoIVB in which interactions with other proteins are blocked or in which it acts as a competitive inhibitor of proteolysis. The implied importance of exposed residues in the BofC linker peptide would be consistent with this mode of action.

Biochemical experiments have so far failed to establish interactions of BofC with either the isolated PDZ domain of SpoIVB (21) or with intact SpoIVB, although performed with a mutant in which the active site serine residue is mutated to alanine (data not shown). However, the complex environment of the intercompartmental space and the flanking membranes involved in the σ^K checkpoint make the conditions under which these two proteins would interact extremely difficult to mimic *in vitro*. It is also possible that the inhibitory effect of BofC on SpoIVB-mediated signaling depends on the presence of other components of the pro-σ^K processing complex. Just as PDZ domain-mediated

binding of SpoIVB to BofA leads to the SpoIVB cleavage of SpoIVFA (20, 21), it is possible that SpoIVB-BofC interactions depend on the prior engagement of one or both components with SpoIVFA, SpoIVFB, or BofA. The observation that BofC is a two-domain protein with few constraints on the relative orientation of its domains is consistent with a possible function that involves interactions with a pair of protein partners.

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