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The *Bacillus subtilis* Signaling Protein SpoIVB Defines a New Family of Serine Peptidases

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The protein SpoIVB plays a key role in signaling in the σE checkpoint of *Bacillus subtilis*. This regulatory mechanism coordinates late gene expression during development in this organism and we have recently shown SpoIVB to be a serine peptidase. SpoIVB signals by transiting a membrane, undergoing self-cleavage, and then by an unknown mechanism activating a zinc metalloprotease, SpoIVFB, which cleaves pro-σE to its active form, σE, in the outer mother cell chamber of the developing cell. In this work we have characterized the serine peptidase domain of SpoIVB. Alignment of SpoIVB with homologues from other spore formers has allowed site-specific mutagenesis of all potential active site residues within the peptidase domain. We have defined the putative catalytic domain of the SpoIVB serine peptidase as a 160-amino-acid residue segment at the carboxyl terminus of the protein. His236 and Ser378 are the most important residues for proteolysis, with Asp363 being the most probable third member of the catalytic triad. In addition, we have shown that mutations at residues Asn290 and His394 lead to delayed signaling in the σE checkpoint. The active site residues suggest that SpoIVB and its homologues from other spore formers are members of a new family of serine peptidases of the trypsin superfamily.
strain PY79 as a template. The primers were P1 (5'-H11032/H11002 testing for an AmyE
amE
locus of SC1836 cells as described in Materials and Methods.

Nonconservative mutants
NH1377
spolVBDL213
pNH1375 into SC1836
NH1320
spolVBDL240
pNH1308 into SC1836
NH1345
spolVBDL242
pNH1342 into SC1836
NH1346
spolVBDL243
pNH1343 into SC1836
NH1347
spolVBDL290
pNH1344 into SC1836
NH1380
spolVBKA321
pNH1376 into SC1836
NH1326
spolVBDL363
pNH1314 into SC1836
NH1328
spolVBKS378
pNH1316 into SC1836
NH1455
spolVBKA87
pNH1445 into SC1836
NH1330
spolVBDH394
pNH1318 into SC1836

Conservative mutants
NH1451
spolVBFH236
pNH1441 into SC1836
NH1452
spolVBDN242
pNH1442 into SC1836
NH1453
spolVBDN363
pNH1443 into SC1836
NH1454
spolVBSA378
pNH1444 into SC1836

a Mutations were created in pBluescript clones carrying the spoIVB gene, sequenced, and subcloned into pDG364 to give the plasmid clones indicated. These subclones were then linearized and DNA was introduced into the amyE locus of SC1836 cells as described in Materials and Methods.

Site-directed mutagenesis of putative peptidase catalytic residues. Figure 1 shows a sequence alignment of the C-terminal domains (residues 188 to 425 in B. subtilis SpoIVB) of SpoIVB homologues from four Bacillus species (B. subtilis, B. anthuracis, B. stearothermophilus, and B. halodurans). This region lies downstream of the PDZ domain (residues 102 to 186 in B. subtilis SpoIVB). The availability of sequences from such divergent sources aids the definition of strictly conserved residues. A single serine (Ser378) is conserved among these proteins. Proteolysis in vivo and in vitro is inhibited by the serine peptidase inhibitor diisopropylfluorophosphate and a mutation at this serine (SA378) inhibits autoproteolysis of SpoIVB (36). In addition, residues (GMSG) flanking this serine are conserved and are reminiscent of the SA clan of serine peptidases, which comprise the trypsin superfamily (1, 23) which has recently been reclassified as the clan PA(S) (24). As has been discussed previously, this is the most likely candidate for the active site serine (36). The catalytic domain of most (but not all) serine peptidases consists of a triad of three residues (histidine, aspartic acid, and serine), with their order and spacing defining the clan and family of the

Western blotting analysis. Samples (1 ml) were taken from sporulating cultures and cells were harvested by centrifugation and frozen in liquid N2. To break cells, pellets were suspended in 50 µl of TS buffer (25 mM Tris-HCl [pH 7.4], 0.1 M NaCl) containing lysosome (0.2 µg/ml) and incubated for 10 min on ice. Fifty microliters of 2X sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading dye was then added and the samples were sonicated for 10 s before gel loading (approximately 20 µl of sample/well). Immunoblotting of sporulating extracts with polyclonal antiserum to SpoIVB was performed as described previously (12, 36).

Expression of SpoIVB templates in Escherichia coli. A pET22b clone (pΔIVB) containing a truncated SpoIVB template starting at codon Cys1 of the PE vector has been described (36). Identical clones were made carrying mutant spoIVB templates spoIVBN236 (pΔIVBN236), spoIVBDL242 (pΔIVBDL242), spoIVBDL363 (pΔIVBDL363), and spoIVBSK378 (pΔIVBSK378).

RESULTS

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peptidase (23). Clans indicate a common evolutionary origin, usually assigned following determination of three-dimensional structure, while families are primarily defined by sequence homology. The alignment reveals four conserved Asp residues (positions 213, 240, 242, and 363) that could form the acidic part of the catalytic triad. Only two His residues (positions 326 and 394) are conserved which could represent the base that enhances the nucleophilicity of the active site serine. Conserved lysine residues (positions 321 and 387) were also targeted for mutagenesis as they could potentially perform the role of a base. Examples include β-lactamase and Tsp, which usually assigned following determination of three-dimensional structure, while families are primarily defined by sequence homology. The alignment reveals four conserved Asp residues (positions 213, 240, 242, and 363) that could form the acidic part of the catalytic triad. Only two His residues (positions 326 and 394) are conserved which could represent the base that enhances the nucleophilicity of the active site serine. Conserved lysine residues (positions 321 and 387) were also targeted for mutagenesis as they could potentially perform the role of a base. Examples include β-lactamase and Tsp, which have a catalytic Ser-Lys dyad (1). Similarly, the conserved asparagine at position 290 was mutated, since Asn residues are known to be involved in oxyanion stabilization in some proteases (e.g., subtilisin) or are important in orienting catalytic activity. While families are primarily defined by sequence homology. The alignment reveals four conserved Asp residues (positions 213, 240, 242, and 363) that could form the acidic part of the catalytic triad. Only two His residues (positions 326 and 394) are conserved which could represent the base that enhances the nucleophilicity of the active site serine. Conserved lysine residues (positions 321 and 387) were also targeted for mutagenesis as they could potentially perform the role of a base. Examples include β-lactamase and Tsp, which have a catalytic Ser-Lys dyad (1). Similarly, the conserved asparagine at position 290 was mutated, since Asn residues are known to be involved in oxyanion stabilization in some proteases (e.g., subtilisin) or are important in orienting catalytic activity.

In vivo effects of SpoIVB mutations. All mutations were created in a pBluescript spoIVB clone, which carried the entire spoIVB gene. The mutated spoIVB clone was sequenced to verify the presence of a single base change and then subcloned into the plasmid pDG364. When linearized, this plasmid encoded SpoIVB activity (Table 2).

As shown in Table 2 there were three distinct classes of mutant phenotype. The first group (group I) consisted of spoIVBH236, spoIVBHF236, spoIVBDL242, spoIVBDL363, spoIVBSA378, and spoIVBSK378 mutants which caused a severe effect on spore formation, with the failure to produce heat- or lysozyme-resistant spores. Spore formation was arrested at stage III-IV with the production of limited numbers of phase-grey spores. This phenotype was essentially asporogenous and indistinguishable from the null spoIVB::spc mutant. The pDG364 plasmids carrying these mutant alleles were introduced into spo− cells (strain PY79) and in each case we found that the resulting phenotype was Spo−, showing that the mutation was recessive to the wild-type spoIVB gene when placed at its normal chromosomal position (data not shown).

The second phenotypic class (group II) was made up of strains carrying the spoIVB::spc mutants which caused a severe effect on spore formation, with the failure to produce heat- or lysozyme-resistant spores. Spores produced (approximately 30% fewer spores than in the wild-type strain). For these mutants we examined the capacity of spores to germinate and found that they were germination defective. Further analysis revealed that for spoIVBN290 this germination defect was temperature sensitive, since the mutant was unable to germinate properly at 37°C. In other work...
we have shown that bofA and bofB mutations (in BoFA and SpoIVFA, respectively) allow constitutive activation of SpoIVB and therefore pro-σK processing (8). This, in turn, leads to premature σK-controlled gene expression, a reduction in spore-forming efficiency, and for those spores that are formed (approximately 10% of the total CFU), a defective germination response. This phenotype is most probably caused by a defect in spore coat assembly, which is controlled by the σK regulon. We reasoned that the reduced sporulation efficiency and defective germination response of the NI290 and NH1320 strains that were phenotypically SpoK−/K+ (9), leads to premature germination (approximately 10% of the total CFU), a defective spore coat formation (30%), and secondly, expression of the σK-regulated gene expression, a reduction in spore-forming efficiency, and for those spores that are formed (approximately 10% of the total CFU), a defective germination response. This phenotype is most probably caused by a defect in spore coat assembly, which is controlled by the σK regulon. We reasoned that the reduced sporulation efficiency and defective germination response of the NI290 and HD394 mutants could be caused by a defect in signaling.

![Germination phenotype](Image)

**FIG. 2.** gerE-lacZ expression in spoIB mutants. β-Galactosidase synthesis was measured at the indicated times following the initiation of sporulation in cells carrying the SPλgerE-lacZ reporter phage. In each case congenic strains were used and the relevant alleles (at the amrE locus) are given here (see Table 1 for complete genotypes). (A) •, NH578, spoIB⁺; ○, NH577, spoIBΔ::spc; △, NH1326, spoIVBDL363; ▲, NH1328, spoIVBSK378; □, NH1320, spoIVBH236; ■, NH1346, spoIVBDL242. (B) •, NH578, spoIB⁺; ○, NH577, spoIBΔ::spc; △, NH1345, spoIVBDL240; ▲, NH1347, spoIVBN1290; □, NH1330, spoIVBHD394; △, NH1377, spoIVBDL213; ○, NH1380, spoIVBKA321. (C) •, NH578, spoIB⁺; ○, NH577, spoIBΔ::spc; ■, NH1451, spoIVBF236; □, NH1452, spoIVBDN242; △, NH1453, spoIVBDN363; Δ, NH1454, spoIVBSA378; ●, NH1455, spoIVBKA387.

### TABLE 2. spoIVB alleles

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant allele</th>
<th>Mutation</th>
<th>Heat resistance</th>
<th>Lysozyme resistance</th>
<th>Germination phenotype</th>
<th>Signaling</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH577</td>
<td>spoIVBΔ::spc</td>
<td>insertion-deletion</td>
<td>$4.9 \times 10^{-5}$</td>
<td>$1.56 \times 10^{-2}$</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>NH578</td>
<td>spoIB⁺</td>
<td>Wild type</td>
<td>88</td>
<td>54.4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NH1377</td>
<td>spoIVBDL213</td>
<td>Asp213 to Leu</td>
<td>98</td>
<td>94.5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NH1320</td>
<td>spoIVBH236</td>
<td>His236 to Asn</td>
<td>$1.18 \times 10^{-4}$</td>
<td>$1.12 \times 10^{-2}$</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>NH1451</td>
<td>spoIVBF236</td>
<td>His236 to Phe</td>
<td>$2.3 \times 10^{-4}$</td>
<td>$6.9 \times 10^{-3}$</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>NH1345</td>
<td>spoIVBDL240</td>
<td>Asp240 to Leu</td>
<td>95.7</td>
<td>55.7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NH1346</td>
<td>spoIVBDL242</td>
<td>Asp242 to Leu</td>
<td>$1.12 \times 10^{-3}$</td>
<td>$2.19 \times 10^{-2}$</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>NH1452</td>
<td>spoIVBDN242</td>
<td>Asp242 to Asn</td>
<td>87.6</td>
<td>45</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NH1347</td>
<td>spoIVBN1290</td>
<td>Asn290 to Ile</td>
<td>28.9</td>
<td>29</td>
<td>+ (30°C), – (37°C)</td>
<td>Delayed</td>
</tr>
<tr>
<td>NH1380</td>
<td>spoIVBKA321</td>
<td>Lys321 to Ala</td>
<td>86</td>
<td>88</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NH1326</td>
<td>spoIVBDL363</td>
<td>Asp363 to Leu</td>
<td>$1.54 \times 10^{-4}$</td>
<td>$3.7 \times 10^{-2}$</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>NH1453</td>
<td>spoIVBDN363</td>
<td>Asp363 to Asn</td>
<td>77.6</td>
<td>42</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NH1328</td>
<td>spoIVBSK378</td>
<td>Ser378 to Lys</td>
<td>$1.76 \times 10^{-4}$</td>
<td>$1.77 \times 10^{-2}$</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>NH1454</td>
<td>spoIVBSA378</td>
<td>Ser378 to Ala</td>
<td>$3.25 \times 10^{-4}$</td>
<td>$3.26 \times 10^{-3}$</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>NH1455</td>
<td>spoIVBKA387</td>
<td>Lys387 to Ala</td>
<td>68.1</td>
<td>57</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NH1330</td>
<td>spoIVBHD394</td>
<td>His394 to Asp</td>
<td>26.7</td>
<td>34.7</td>
<td>– (30°C), – (37°C)</td>
<td>Delayed</td>
</tr>
</tbody>
</table>

*a* See Table 1.

*b* See Fig. 1.

*c* Heat or lysozyme resistance of cultures 24 h after the initiation of sporulation in DS medium (16). Values are expressed as percentages of CFU per milliliter in the untreated culture.

*d* Five-day-old colonies grown on sporulation agar were tested for their capacity to germinate normally on tetrazolium agar plates as described previously (16). All strains that were phenotypically SpoK− were not tested for a germination phenotype (ND).

*e* Signaling was defined according to the following two criteria: firstly, formation of pigmented colonies (Pig+) associated with the production of the αK-expressed CotA protein (30), and secondly, expression of the αK-controlled reporter gene, gerE-lacZ, in cells containing an SPλgerE-lacZ lysogen (see Fig. 2) as described previously (9).
agreement with previous work, while for spo cells, gerE-lacZ expression started at about 3.5 to 4 h after the initiation of spore formation. Our results define three clear classes of mutant gene expression, with (i) a block in σ^K-directed gene expression, (ii) no effect on σ^K-directed gene expression, and (iii) delayed and reduced levels of expression. These mutant phenotypes correspond exactly to the groups classified above according to their ability to sporulate.

The group I mutant strains with a null sporulation phenotype have essentially abolished or severely reduced gerE-lacZ expression (Fig. 2A and C). The second group comprised the mutants with no detectable sporulation defect. These strains expressed gerE-lacZ at the same time and at the same levels as in spo^+ cells (Fig. 2B and C). The third group caused a clear delay (1 h) and reduction in the level of gerE-lacZ expression (Fig. 2B). As mentioned above, defective signaling can lead to defects in spore coat assembly and spore germination. Here, gene expression was delayed, in contrast to mutations in bofA and bofB that allow constitutive processing of pro-σ^K, leading to premature σ^K-directed gene expression. Although other alleles within spoIVB have been shown to lead to delayed signaling (14, 17), the clear delay seen in the spoIVBHN394 and spoIVBNI290 mutations reinforces the notion that the role of this checkpoint is as a timing mechanism. That signaling does occur accounts for the levels of phase-bright spores that are produced.

**Proteolysis of spoIVB.** To determine the effect of our collection of mutations on proteolysis we examined spoIVB during spore formation using a polyclonal antiserum (Fig. 3). Previous work has shown that SpoIVB is synthesized as a 50-kDa polypeptide at about the second hour of sporulation (36). Starting at hour 3, SpoIVB is cleaved into a number of products (approximately 46, 45, and 44 kDa), one or more of which is the active form that signals SpoIVFB-mediated processing of pro-σ^K. Almost simultaneously, these forms are subject to secondary proteolysis, which presumably inactivates the active forms by cleavage to 42- and 40-kDa species which are then cleaved further. During sporulation, then, in wild-type cells, we see the 50-kDa species briefly but at hour 3 autopro- teolysis has begun producing the active species as well as the inactive forms.

The effects on SpoIVB proteolysis are shown in Fig. 3. It must be stressed that these Western blots are particularly difficult to interpret due to the appearance of intermediate species in the blots and our accuracy in sampling. Regardless, we have repeated these blots at least twice and are confident of the following classifications by residue.
(i) **His236 and Ser378.** Compared to *spo*<sup>+</sup> cells, in HN236 (Fig. 3A), HF236 (Fig. 3B), SK378 (Fig. 3A), and SA378 (Fig. 3B) mutants, the 50-kDa SpoIVB protein was cleaved less quickly and appeared to be maintained longer in its full-length form. Compared to *spo*<sup>+</sup> cells the 50-kDa species appeared to undergo proteolysis approximately 30 min later as judged by the appearance of the 40- to 42-kDa species. This corresponds to hour 3 using our sampling regimen shown in Fig. 3. We must emphasize that differences in actual processing time compared to previous studies (36) are empirical and must be taken in the context of processing relative to that in *spo*<sup>+</sup> cells. With some mutants such as KA387 (Fig. 3B) and DL213 (Fig. 3A) there appeared to be slightly more of the 50-kDa species than with *spo*<sup>+</sup> cells at the same time points. However, processing initiated at the same time as in *spo*<sup>+</sup> cells (2.5 h). To confirm that proteolysis was defective in these mutant proteins, we expressed the HN236 and SK378 SpoIVB mutants from pET28b expression vectors in *E. coli* BL21(DE3) and examined expressed proteins by SDS-PAGE. As shown in Fig. 4, expression led to the accumulation of a stable, full-length 50-kDa SpoIVB species. In contrast, expression of a wild-type SpoIVB template produced little or no detectable SpoIVB protein as it is subject to rapid self-cleavage in *E. coli* (36).

(ii) **Asp213 (Fig. 3A), Asp240 (Fig. 3A), Lys321 (Fig. 3A), and Lys387 (Fig. 3B).** Processing in these mutants appeared to be normal in terms of the onset of proteolysis and clearance of the 50-kDa species. Our interpretation is also based on the ability of these mutants to signal processing of pro-α<sup>K</sup> normally.

(iii) **Asp242.** We are unable to detect any SpoIVB DL242 mutant protein in *B. subtilis* (Fig. 3A). We have repeated this experiment a number of times and conclude that this mutant protein is unstable and presumably is rapidly cleared by secondary proteolysis. We confirmed this, in part, by expressing the SpoIVB DL242 protein in *E. coli* (Fig. 4), which showed that the mutant protein behaves differently from the wild-type protein, accumulating as a 35- to 40-kDa species. The aberrant molecular mass of this SpoIVB DL242 species implies that self-cleavage is defective and/or the protein is unstable. The latter deduction could arise if the structure of the mutant polypeptide is abnormal. This corresponds with our results showing that signaling is defective in *spoIVBDL242* cells. In contrast, a conservative amino acid substitution at this position (Asp242 to Asn) allows normal signaling in the α<sup>K</sup> checkpoint, and proteolysis of the SpoIVB DN242 protein appears normal.

(iv) **Asp290 (Fig. 3A) and His394 (Fig. 3A).** Processing of these SpoIVB mutant proteins appeared essentially normal, although there was perhaps a slight accumulation of the 50-kDa species in *spoIVBD394* cells. Although signaling is delayed in *spoIVBNI290* and *spoIVBHD394* mutants, we assume that the subtle change or delay in SpoIVB processing is not detectable by the 30-min sampling regimen used here.

(v) **Asp363.** Proteolysis appeared to be delayed by 30 min in *spoIVBDL363* mutant cells (Fig. 3A). Also, there appeared to be considerably less SpoIVB protein detectable by Western blotting. Expression of SpoIVB DL363 in *E. coli* showed a clear accumulation of a full-length 50-kDa precursor, showing that processing was defective. In contrast, processing in the mutant with a conservative replacement (SpoIVB DN363) was normal (Fig. 3B), as was signaling of pro-α<sup>K</sup> processing.

**DISCUSSION**

Our analysis of potential catalytic residues that comprise the serine peptidase domain has provided strong evidence for His236 and Ser378 as components of the active site, since substitution at these positions prevents signaling of pro-α<sup>K</sup> processing as well as inhibiting the proteolytic activity of SpoIVB. As mentioned below it must be stressed that proteases can retain residual activity even when active site residues are altered (20). The order and spacing of His236 and Ser378 agree with serine peptidases of the trypsin superfamily (1, 23) and make the essential catalytic unit approximately 160 amino acid residues contained at the C terminus of SpoIVB. The GMSG motif surrounding Ser378 closely resembles trypsin, but it is a distinct variant as this family typically has a residue with negative charge (Asp or Glu) preceding the active site serine (1, 23). In contrast, there is little similarity in sequence around the active site SpoIVB His236 residue with...
other trypsin family members, suggesting that SpoIVB must be a member of a new family of the PA(S) clan.

We have identified one Asp residue, Asp363, for which mutation to Leu leads to a lack of SpoIVB self-cleavage and signaling properties. Another Asp residue, Asp242, when mutated to Leu also blocked signaling yet failed to accumulate any protein at all, suggesting that the mutation rendered the protein unstable. We have noticed a DxxLL motif which is present in a number of serine peptidase families, especially those of the PA(S) clan (23, 24), and is found at position 363, and we take this as evidence that the SpoIVB serine peptidase has a catalytic triad with Asp363 as its third member. Intriguingly, a conservative change at this position does not disrupt proteolysis of the SpoIVB protein. The acidic residue is the least important component of the active site triad in serine peptidases, and Asp is replaced by Glu in carboxyl peptidase (37) and aspartyl dipeptidase (13). In the case of cytomegalovirus protease, histidine acts as the acid in a unique Ser-His-His triad. Mutation of cytomegalovirus protease His157 reduces activity only 5- to 10-fold (33). In contrast, catalysis by trypsin-like enzymes is severely impaired when the acidic residue is replaced by mutation, suggesting that the negative charge of the Asp side chain balances the positive charge that develops on the histidine during catalysis (6). In some systems, however, the Asp simply serves to maintain the correct position of the active site histidine. Indeed, in cysteine proteases, a conserved Asn residue (e.g., Asn175 in papain) serves this very purpose (35) and Asn156 appears in the hydrolase catalytic triad of E. coli outer membrane phospholipase A (15). We assume that the role of Asp363 is to stabilize the appropriate His263 rotamer or tautomer in SpoIVB catalysis, a function that could potentially be performed by Asn. The unusually close spacing of Asp363 to Ser378, its ability to be replaced by Asn, and the lack of a consensus motif around His236 all argue that SpoIVB represents a new family of serine peptidases.

One observation we have made here is that mutations that block signaling of pro-$\sigma^K$ processing underwent proteolysis in B. subtilis to yield intermediate forms. We have not noticed this before with a different spoIVBSA378 mutant which was placed at the spoIVB locus (36). We believe that one possible explanation is that even though an active site residue is mutated, the protease can still retain residual proteolytic activity (20). Despite mutation of all three members of the catalytic His-Asp-Ser triad of subtilisin, enzymatic activity is still approximately $10^3$ to $10^4$ times the noncatalyzed rate (5). Our in vivo work shows that changes at His236, Asp363, and Ser378 do appear to allow limited self-cleavage of SpoIVB to forms of approximately 46, 45, and 44 kDa. We had previously predicted that
these might represent active forms but we must now question this model. It is possible, though, that the 44- to 46-kDa species observed in these mutants are actually inactive forms brought about by incorrect cleavage or by secondary proteolysis. Alternatively, residual enzyme activity is sufficient to permit self-cleavage but at levels too low to permit signaling. Our work does show clearly, though, that all nonsignaling mutants show delayed cleavage of the full-length form of SpoIVB, and we wonder whether signaling can occur only during a precise window of time concurrent with release of SpoIVB from the inner forespore membrane by self-cleavage. This latter event would occur following the first cut, and for signaling this is presumably then the most important event, enabling release of SpoIVB. In the nonsignaling mutants, though, we reason that self-processing is substantially reduced, leading to a delayed appearance of intermediate species, at which time the SpoIVFB processing complex is no longer responsive (or competent) to cleave pro-αK.

The results of mutation at residues Asn290 and His394 are more difficult to interpret. Our work suggests that proteolysis of these mutants was less potent, leading to delayed signaling. Possible explanations include roles for these residues in ground state substrate binding or stabilization of the reaction transition state by forming an oxyanion hole reminiscent of Asn155 in subtilisin. Mutation of this sidechain leads to a 200- to 300-fold reduction in turnover number of this serine peptidase (4). A similar role for Asn290 might explain a delay in signaling.

We are presently attempting to determine the crystal structure of SpoIVB, which will enable us to test our prediction that Asp363 is suitably placed to comprise the third member of a catalytic triad and to assess the importance of residues Asn290 and His394 in SpoIVB function. We will also explore the role of the PDZ domain in relation to the peptidase activity. A survey of bacterial proteins with PDZ domains suggests that of 187 non-SpoIVB bacterial PDZ-containing proteins, remarkably only 9 are predicted not to contain transit peptides or transmembrane segments. More surprising is that only 28 are not associated with a peptidase activity, i.e., 85% of all known PDZ-containing bacterial proteins are also peptidases (Fig. 5), indicating that they have evolved a specialized role in association with peptidase activity due to their ability to traverse a membrane for (i) localization and (ii) substrate specificity, and thus are ideal for the signaling function of SpoIVB.

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