Sortase D Forms the Covalent Bond That Links BcpB to the Tip of *Bacillus cereus* Pili*

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_Bacillus cereus_ and other Gram-positive bacteria elaborate pili via a sortase D-catalyzed transpeptidation mechanism from major and minor pilin precursor substrates. After cleavage of the LPXTG sorting signal of the major pilin, BcpA, sortase D forms an amide bond between the C-terminal threonine and the amino group of lysine within the YPKN motif of another BcpA subunit. Pilus assembly terminates upon sortase A cleavage of the BcpA sorting signal, resulting in a covalent bond between BcpA and the cell wall cross-bridge. Here, we show that the IPXTG sorting signal of BcpB, the minor pilin, is cleaved by sortase D but not by sortase A. The C-terminal threonine of BcpA is amide-linked to the YPKN motif of BcpA, thereby positioning BcpB at the tip of pili. Thus, unique attributes of the sorting signals of minor pilins provide Gram-positive bacteria with a universal mechanism ordering assembly of pili.

Sortases catalyze transpeptidation reactions to assemble proteins in the envelope of Gram-positive bacteria (1). Secreted proteins require a C-terminal sorting signal for sortase recognition such that sortase cleaves the substrate at a short peptide motif and forms a thioester–linked intermediate to its active site cysteine (2–4). Nucleophilic attack by an amino group within the bacterial envelope resolves the thioester intermediate, generating an amide bond tethering surface proteins at their C terminus onto Gram-positive bacteria (5). Four classes of sortases can be distinguished on the basis of sequence homology and substrate recognition (6, 7). Sortase A cleaves secreted protein at LPXTG sorting signals and recognizes the amino group of lipid II peptidoglycan precursors as a nucleophile (8, 9). Sortase B cleaves protein substrates at NPQTN sorting signals (10). This enzyme immobilizes proteins within fully assembled cell walls, utilizing the cell wall cross-bridge as a nucleophile (11). Sortase C cuts LPNTA sorting signals and anchors proteins to the peptidoglycan cross-bridges in sporulating bacteria (12, 13). Finally, sortase D catalyzes transpeptidation reactions in the assembly of pili (14, 15). Sortase D recognizes the amino group of lysine residues within the YPKN motif of pilin subunits as nucleophiles (16). The resultant sortase D-catalyzed amide bond links adjacent pilin subunits to grow the pilus fiber (16, 17).

Pili of Gram-positive bacteria comprised either two or three different pilin subunits synthesized as cytoplasmic precursors with N-terminal signal peptides and C-terminal sorting signals (P1 precursors) (14, 18). After translocation across the plasma membrane, P2 precursor species arise from removal of the signal peptide from P1 precursors by a signal peptidase (16). _Bacillus cereus_ pili are composed of two subunits; that is, the major pilin, BcpA, and the minor pilin, BcpB (15). In contrast to BcpA, which is deposited throughout the pilus, BcpB is found at fiber tip (15). Sortase D cleaves the BcpA LPXTG motif sorting signal between the threonine and glycine residues to form an amide bond to the ε-amino group of the lysine within the YPKN motif of adjacent BcpA subunits (16). However, sortase A also cleaves BcpA precursors, which are subsequently linked to the side chain amino group of meso-diaminopimelic acid within lipid II (19). The latter reaction serves to terminate fiber elongation, immobilizing BcpA pili in the cell wall envelope (19).

The conservation of sortase D, the YPKN motif, and C-terminal sorting signal in major pilin subunits suggest a universal pilus assembly mechanism among Gram-positive bacteria (14, 20). However, the molecular mechanism whereby bacilli deposit BcpB, the minor pilin, at the tip of BcpA pili is not known. Although the BcpB precursor harbors an N-terminal signal peptide and a C-terminal IPXTG sorting signal, it lacks the YPKN pilin motif of the major subunit (15). Furthermore, the substrate properties of the BcpB IPXTG sorting signal for the four classes of sortases expressed by bacilli has yet to be established.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The following plasmids contain _B. cereus_ pilus genes cloned under control of the *spaC* promoter of _pLM5_ (12) and were described previously: pIB39 _bcpA-srtD_, pIB12 _bcpA-srtD-bcpB_, pIB28 _bcpA-K162A-srtD-bcpB_, pIB32 _bcpA-srtD<sup>C200T</sup>-bcpB_ and pIB48 _bcpA<sub>LAVAA</sub>-srtD-bcpB_ (15). pIB182 encodes for _bcpA-srtD-bcpB<sub>AVIPNTGG719</sub>_, which contains a seven-residue deletion in BcpB, encompassing the IPXTG sorting signal motif and one residue flanking each end (VIPNTGG<sup>715</sup>). pIB12 was used as a template in site-directed mutagenesis with _Pfu_ polymerase, as described previously (15), with primer pairs P128 and P129 Table 1. pIB139 encodes for _bcpA-srtD-bcpB_ with a MH<sub>6</sub> tag inserted six nucleotides upstream from the codons encoding for the IPXTG motif in _bcpB_. pJB139 was created by quick change with primer pair P237/P238 and pJB12 template DNA. Amplified product was digested with NheI and ligated, and non-methylated plasmid DNA was digested with DpnI before transformation into _Escherichia coli_ DH5-α. pJB139 was amplified with primer pairs

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P105/106 to alter the sorting signal of BcpA, creating pJB202 (bcpA_LAVAA-srtD-bcpB_MH6). P197/198 and P124/125 were used with template pJB202 to introduce two substitutions in bcpA (N163A and V170M), creating pJB213 (bcpA

| Primer | Restriction site | Nucleotide sequence (5′−3′) |
|--------|----------------|-----------------------------|
| 45     | None           | AAAAGTGGATATATTATTCAAGCAGAGGATTAGTATAAGGAAAGGTAAATTTATGTACTT |
| 46     | PstI           | AAAATCTAGTCACCACTTACGATACTT |
| 47     | NheI           | AAAAGTCGCTACATATCTTTTATTAAATAGGAAAGGTAAATTTATGTACTT |
| 88     | KpnI           | AAAATCTAGTCACAACCTTACGATACTT |
| 89     | KpnI           | AAAATCTAGTCACAACCTTACGATACTT |
| 105    | None           | AAAAGTGGATATATTATTCAAGCAGAGGATTAGTATAAGGAAAGGTAAATTTATGTACTT |
| 106    | None           | AAAATCTAGTCACCACTTACGATACTT |

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| 46     | PstI           | AAAATCTAGTCACCACTTACGATACTT |
| 47     | NheI           | AAAAGTCGCTACATATCTTTTATTAAATAGGAAAGGTAAATTTATGTACTT |
| 88     | KpnI           | AAAATCTAGTCACAACCTTACGATACTT |
| 89     | KpnI           | AAAATCTAGTCACAACCTTACGATACTT |
| 105    | None           | AAAAGTGGATATATTATTCAAGCAGAGGATTAGTATAAGGAAAGGTAAATTTATGTACTT |
| 106    | None           | AAAATCTAGTCACCACTTACGATACTT |

**BcpB Linkage to Pilus Tip**

The BcpB cell-wall sorting signal was amplified with primers P88/P89 with pSY40 or pSY41 to alter the sorting signal of BcpA, creating pJB202 (bcpA

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| Primer | Restriction site | Nucleotide sequence (5′−3′) |
|--------|----------------|-----------------------------|
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| 46     | PstI           | AAAATCTAGTCACCACTTACGATACTT |
| 47     | NheI           | AAAAGTCGCTACATATCTTTTATTAAATAGGAAAGGTAAATTTATGTACTT |
| 88     | KpnI           | AAAATCTAGTCACAACCTTACGATACTT |
| 89     | KpnI           | AAAATCTAGTCACAACCTTACGATACTT |
| 105    | None           | AAAAGTGGATATATTATTCAAGCAGAGGATTAGTATAAGGAAAGGTAAATTTATGTACTT |
| 106    | None           | AAAATCTAGTCACCACTTACGATACTT |

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|--------|----------------|-----------------------------|
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| 46     | PstI           | AAAATCTAGTCACCACTTACGATACTT |
| 47     | NheI           | AAAAGTCGCTACATATCTTTTATTAAATAGGAAAGGTAAATTTATGTACTT |
| 88     | KpnI           | AAAATCTAGTCACAACCTTACGATACTT |
| 89     | KpnI           | AAAATCTAGTCACAACCTTACGATACTT |
| 105    | None           | AAAAGTGGATATATTATTCAAGCAGAGGATTAGTATAAGGAAAGGTAAATTTATGTACTT |
| 106    | None           | AAAATCTAGTCACCACTTACGATACTT |

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| 88     | KpnI           | AAAATCTAGTCACAACCTTACGATACTT |
| 89     | KpnI           | AAAATCTAGTCACAACCTTACGATACTT |
| 105    | None           | AAAAGTGGATATATTATTCAAGCAGAGGATTAGTATAAGGAAAGGTAAATTTATGTACTT |
| 106    | None           | AAAATCTAGTCACCACTTACGATACTT |

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3 The abbreviations used are: GST, glutathione S-transferase; MALDI, matrix-assisted laser desorption ionization; HMW, high molecular weight; Ni-NTA, nickel-nitrilotriacetic acid; RP, reverse phase; HPLC, high performance liquid chromatography.
products were transferred to a polyvinylidene difluoride membrane, stained with Amido Black, and submitted for Edman degradation.

**Electron Microscopy**—Double labeling experiments with /H9251-BcpA antisera and 10-nm gold anti-rabbit IgG conjugates followed by /H9251-BcpB antisera and 15-nm gold anti-rabbit IgG conjugates were performed as described previously (15).

**RESULTS**

The Sorting Signal of the Minor Pilin Is Required for Its Assembly into Pili—When transformed with pJB12, a plasmid encoding the /B. cereus pilin operon (bcpA, srtD, bcpB), /B. anthracis Sterne forms pili comprising both the major pilin subunit, BcpA, and the minor pilin subunit, BcpB (Fig. 1A) (15). Lysates of bacilli were examined by SDS-PAGE and immunoblotting for the polymerization of pilin subunits. High molecular weight BcpAHMW and BcpBHMW species represent covalently linked pilin subunits within pilus fibers (Fig. 1B). As expected, /B. anthracis expressing bcpA and srtD but not bcpB (pJB39) formed exclusively BcpAHMW species, indicating that the major pilin is polymerized in the absence of BcpB (Fig. 1B) (15). Substitution of lysine 162 of BcpA (K of the YPKN pilin motif) with alanine (pJB28, bcpAK162A-srtD-bcpB) abrogates polymerization of both BcpAHMW and BcpB into high molecular weight species (BcpBHMW) and caused the accumulation of pilin precursors, BcpAP and BcpBP (Fig. 1B) (15). Deletion of the IPNTG peptide in the sorting signal of BcpB (pJB182, bcpA-srtD-bcpB/IPNTG) abolishes the formation of BcpBHMW without affecting BcpA polymerization (Fig. 1B). Thus, the BcpB C-terminal sorting signal is required for the incorporation of the minor pilin subunit into pili but is otherwise dispensable for the polymerization of BcpA.

The BcpB Sorting Signal Is Cleaved by Sortase D—To examine BcpB sorting signal cleavage, we generated a translational hybrid between the minor pilin 3′ coding end and the 5′ end of glutathione S-transferase (gst) (Fig. 2A). /B. anthracis Sterne (wild-type sortase A) and an isogenic srtA deletion variant

**FIGURE 1.** The IPNTG motif sorting signal of BcpB is required for the incorporation of BcpB into pili. A, schematic of plasmids expressing pilin genes under control of the isopropyl 1-thio-β-D-galactopyranoside-inducible Promoter. pJB12 expressed wild-type bcpA-srtD-bcpB, and pJB39 expressed bcpA-srtD-bcpB-IPNTG. The cell wall-sorting signal pentapeptide motif in BcpB was deleted in plasmid pJB182 (bcpA-srtD-bcpB/IPNTG). Cell wall sorting signals in BcpA and BcpB are colored red. The YPK pilin motif is transferred in BcpA is colored blue. B, /B. anthracis cell wall extracts were digested with mutanolysin. BcpA and BcpB pilus material was separated by SDS-PAGE and analyzed by immunoblotting with /H9251-BcpA and /H9251-BcpB antisera. BcpA and BcpB high molecular weight material (HMW) and precursor species (P) are indicated. The electrophoretic mobility of the marker is indicated.

**FIGURE 2.** Cleavage of BcpB-GST by sortase D and the contribution of sortases to pilus assembly. A, the diagram displays the precursor (P1) of BcpB-GST and the signal peptide (P2) and sortase-cleaved products (M). B and C, pilus formation from BcpA and BcpB substrates was examined by immunoblotting with α-BcpA and α-BcpB antisera. BcpA and BcpB high molecular weight material (HMW) and precursor species (P) are indicated. The electrophoretic mobility of the marker is indicated. D, cytoplasmic and membrane fractions were examined by immunoblotting with /H9251-SrtD, /H9251-SrtA, or /H9251-GST sera. Electrophoretic mobility of the molecular weight marker, pilin precursors (P1/P2), and mature cleavage products (M) on SDS-PAGE is indicated. E, bacilli were examined by immunogold labeling with α-BcpA serum and viewed by transmission electron microscopy. Scale bars, 500 nm.
BcpB Linkage to Pilus Tip

Figure 3. IsdX1-BcpBSS-GST is cleaved by sortase D. A, IsdX1-BcpAASS-GST is a translational hybrid between IsdX1 (green), the cell wall sorting signal of BcpA (red; IsdX1-BcpAASS-GST), or the cell wall sorting signal of BcpB (blue; IsdX1-BcpBSS-GST) and glutathione S-transferase. IsdX1-HybridSS-GST is a hybrid between IsdX1, the cell wall sorting signal of BcpA with alteration of the LPVTG motif to IPNTG, and glutathione S-transferase. The diagram displays the precursor (P1) of IsdX1ASS-GST and the signal peptidase (P2) and sortase-cleaved products (M). B, sortase cleavage products were detected in urea-SDS-released cytoplasmic and membrane fractions by immunoblotting with α-GST antiserum. Anti sera raised against B. cereus sortase D and B. anthracis sortase A allowed for their detection by immunoblotting. Labels indicate the sortase (srtA, srtD, or none) and substrate (isdX1ASS-GST with BcpA, BcpB, or hybrid cell wall-sorting signals) expressed in each strain. C, Bacillus cell wall extracts were digested with mutanolysin. IsdX1 anchoring was analyzed by immunoblotting with α-IsdX1 antibodies. D, affinity chromatography of IsdX1-BcpBSS-GST from bacilli on glutathione-Sepharose revealed P1/P2 and mature (M) species, the latter of which was analyzed by Edman degradation. The experimentally determined amino acid sequences is printed in blue.
anchoring of IsdX1 could not be detected in bacilli expressing either IsdX1-HybridSS-GST or IsdX1-BcpBSS-GST (Fig. 3C). Lysates of B. anthracis Sterne expressing IsdX1-BcpBSS-GST were subjected to affinity chromatography on glutathione-Sepharose. Eluate was analyzed by Coomassie-stained SDS-PAGE, which revealed the P1/P2 precursors (50 kDa) and the mature sortase D-derived C-terminal cleavage fragment of IsdX1-BcpBSS-GST (31 kDa, Fig. 3D). The 38-kDa polypeptide in the eluate likely represents B. anthracis GST; this species did not react with antibodies against IsdX1 or E. coli GST (Fig. 3B). Edman degradation of the 31-kDa C-terminal sortase D cleavage product of IsdX1-BcpBSS-GST generated the amino acid sequence GGSGTTIFY, which matches the predicted sequence between the threonine and the glycine of its IPNTG sorting signal (Table 2). Thus, sortase D cleaves the BcpB sorting signal between the threonine and the glycine of its IPNTG sorting signal.

**Sortase D Incorporates BcpB into Pili of B. anthracis**—We asked whether BcpB is incorporated into pili in the absence of sortase A, i.e. when these fibers cannot be immobilized in the cell wall envelope. B. anthracis (srtA::ermC) was transformed with pJB12 (bcpA, srtD, bcpB) or pJB39 (bcpA, srtD) (Fig. 4A). The srtA mutant bacilli released pili derived from either plasmid into the culture medium (Fig. 4C). Pili in the medium were precipitated with trichloroacetic acid and examined by immunoblot for pilus assembly in sortase A-mutant bacilli. pJB39 (39) expresses genes under control of the P chromosomal promoter were analyzed by immunoblot for pilus assembly in sortase A-mutant bacilli. pJB39 (39) expresses bcpA-srtD (Fig. 3A). As expected, immunoblotting of B. anthracis (pJB48) cell wall extracts did not reveal polymerized BcpA/HMW or BcpB/HMW (Fig. 5B) (15). Nevertheless, B. anthracis (pJB48) accumulated a 180-kDa species immunoreactive against both BcpA and BcpB sera (15). We reasoned these species must represent the sortase D transpeptidation product BcpB-BcpA_LAVAA (15). If so, purification and biochemical analysis of this transpeptidation product may reveal the chemical bond that tethers BcpB to BcpA pili.

![FIGURE 4. SrtA is not required for the incorporation of BcpB into pili fibers. A. plasmids expressing pilin genes under control of the P promoters were analyzed by immunoblot for pilus assembly in sortase A-mutant bacilli. PJB39 (39) expresses bcpA-srtD, whereas spJB12 (12) also expresses bcpB (see Fig. 1 for the schematic). Pili released into the medium were precipitated, separated by SDS-PAGE, and immunoblotted with α-BcpA (A) and α-BcpB (B). BcpA and BcpB high molecular weight material (HMW) and precursor species (arrowheads) are indicated. The electrophoretic mobility of the marker is indicated. C, pili released into the medium or attached to cells were labeled with α-BcpA serum and 15-nm gold antibody conjugate followed by α-BcpB serum and 15-nm gold conjugate. Arrowheads indicate 15-nm gold particles detected in pili from B. anthracis (srtA::ermC) (pJB12). Scale bars, 200 nm.](image-url)

**TABLE 2**

| Cycle | Amino acid |
|-------|------------|
| 1     | Gly (15.28) |
| 2     | Gly (14.83) |
| 3     | Ser (7.39)  |
| 4     | Gly (12.14) |
| 5     | Thr (17.77) |
| 6     | Thr (17.21) |
| 7     | Ile (14.10) |
| 8     | Phe (11.06) |
| 9     | Tyr (10.45) |

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BcpB Linkage to Pilus Tip

BcpB₅₆-H₆-BcpALAVAA in cell wall extracts from bacilli harboring pJB202 or pJB213 (Fig. 5).

Bacillus extracts containing BcpB₅₆-H₆-BcpALAVAA, LAVAA were subjected to affinity chromatography on Ni-NTA-Sepharose (Fig. 6A). The eluate was analyzed by Coomassie-stained SDS-PAGE, which demonstrated purification of the BcpB₅₆-BcpALAVAA transpeptidation product (Fig. 6A). Eluted transpeptidation product was cut at methionyl residues with cyanogen bromide (CNBr), and cleavage products were subjected to a second round of Ni-NTA affinity chromatography (Fig. 6A). Eluted peptides were further purified by RP-HPLC with UV detection at 215 nm (Fig. 6B). The ion signal at 2764.68 m/z was identified as the branched peptide HHHH-HWVIPNTYPKAEIKRGM⁺ containing the tryptophan oxidation product hydroxytryptophan (calculated monoisotopic m/z 2764.41) (Fig. 6C). An additional cluster of peaks at 2782.69 m/z is thought to represent the branched peptide containing homoserine instead of homoserine lactone, methionyl reaction products that are acquired during CNBr cleavage (Fig. 6C) (16).

The ion signal at 2764.68 m/z was fragmented via collisionally activated dissociation, and fragment ion spectra confirmed the predicted structure of the branched peptide displayed in Fig. 6D and Table 3. The b₅₂ and b₆₂ fragment ions revealed that the YPKN motif lysine residue 162 of BcpA participates in an amide bond with the C-terminal threonine (residue 717) of BcpB (Lys₁₆₂–Thr₇₁₇) (Table 3; Fig. 6D). The branched peptide was further analyzed by Edman degradation, which released...
two amino acids per cleavage during the first two reaction cycles (Fig. 6D; Table 4). In the third cycle, histidine was identified; however lysine (Lys162) was not released during Edman degradation, as this residue is engaged in an amide bond with BcpB T717 (Fig. 6C; Table 4). In cycle 7 Edman degradation released the lysine of Lys166, which is not amide-bonded to BcpA (Fig. 6D, Table 4). Tryptophan was not identified in cycle 7 because this residue is degraded during the Edman cycle (Table 4) (19). In summary, these results demonstrate that BcpB and BcpA are linked via an amide bond between the C-terminal threonine of BcpB and the ε-amino group of lysine within the YPKN motif of BcpA (Lys162–Thr717) (Fig. 6D).

### Table 3

Summary of ions produced during MS/MS of the mutanolysin-released m/z 2764.68 parent ion

| m/z   | Observed | Calculated |
|-------|----------|------------|
| 70.14 | 70.07    | 0.07       | Immonium | Pro or Arg |
| 84.15 | 84.08    | 0.07       | Immonium | Lys        |
| 86.18 | 86.10    | 0.09       | Immonium | Ile        |
| 110.16| 110.07   | 0.09       | Immonium | His        |
| 129.20| 129.10   | 0.08       | Immonium | Lys        |
| 136.16| 136.08   | 0.08       | Tyrochrome |          |
| 230.29| 230.11   | 0.18       | i         |            |
| 233.19| 233.13   | 0.06       | a         |            |
| 247.24| 247.13   | 0.11       | a         |            |
| 275.23| 275.13   | 0.11       | b         |            |
| 302.28| 302.15   | 0.13       | i         |            |
| 312.24| 312.16   | 0.09       | i         |            |
| 313.29| 313.15   | 0.14       | i         |            |
| 340.25| 340.14   | 0.11       | i         |            |
| 384.26| 384.19   | 0.07       | a         |            |
| 386.25| 386.21   | 0.03       | y         |            |
| 412.32| 412.18   | 0.13       | b         |            |
| 439.34| 439.21   | 0.13       | i         |            |
| 477.32| 477.20   | 0.12       | i         |            |
| 514.26| 514.31   | -0.05      | y         |            |
| 549.38| 549.24   | 0.14       | b         |            |
| 576.39| 576.27   | 0.13       | i         |            |
| 627.47| 627.39   | 0.07       | y         |            |
| 658.46| 658.31   | 0.15       | a         |            |
| 686.43| 686.30   | 0.13       | b         |            |
| 713.45| 713.33   | 0.12       | i         |            |
| 756.44| 756.44   | 0.02       | y         |            |
| 795.45| 795.37   | 0.09       | a         |            |
| 823.55| 823.36   | 0.19       | b         |            |
| 827.58| 827.47   | 0.10       | y         |            |
| 963.55| 963.47   | 0.08       | i         |            |
| 997.63| 997.44   | 0.19       | a         |            |
| 1025.62| 1025.44 | 0.18      | b         |            |
| 1097.71| 1096.51 | 1.20      | i         |            |
| 1124.70| 1124.50 | 0.19      | i         |            |
| 1209.88| 1209.59 | 0.28      | i         |            |
| 1215.89| 1215.68 | 0.21      | i         |            |
| 1237.83| 1237.59 | 0.24      | i         |            |
| 1316.92| 1316.73 | 0.19      | i         |            |
| 1413.98| 1413.75 | 0.23      | i         |            |
| 1511.16| 1510.80 | 0.35      | i         |            |
| 1528.14| 1527.83 | 0.32      | i         |            |
| 1531.25| 1531.72 | -0.47     | i         |            |
| 1641.18| 1640.91 | 0.27      | i         |            |
| 1741.21| 1739.98 | 1.23      | i         |            |
| 1942.49| 1942.05 | 0.44      | i         |            |
| 2079.61| 2079.11 | 0.50      | i         |            |
| 2138.62| 2138.02 | 0.60      | i         |            |
| 2216.77| 2216.17 | 0.59      | i         |            |
| 2235.67| 2235.16 | 0.52      | i         |            |
| 2251.66| 2251.11 | 0.56      | i         |            |
| 2354.38| 2353.23 | 1.15      | i         |            |
| 2491.31| 2490.29 | 1.01      | i         |            |

- a The difference between observed and calculated monoisotopic m/z values.
- b i denotes internal ions.
- c Trp is modified as hydroxytryptophan. M* represents a homoserine lactone residue.

### Table 4

Edman degradation of BcpB-BcpA branched peptide

| Cycle | Amino acid | ε-Arg |
|-------|------------|-------|
| 1     | His (10.32), Tyr (13.93) |
| 2     | His (9.68), Pro (8.56)   |
| 3     | His (7.39)               |
| 4     | His (6.77), Ala (8.59)   |
| 5     | His (7.14), Glu (6.77)   |
| 6     | His (6.44), Ile (5.79)   |
| 7     | Lys (3.51)                |
| 8     | Val (2.41), Arg (24.00)  |
| 9     | Gly (2.29)                |
| 10    | Ala (2.53)                |
BcpB Linkage to Pilus Tip

DISCUSSION

Many Gram-positive bacteria elaborate pili and thereby adhere to and invade host tissues or form biofilms (23). Minor pilin subunits are critically important for bacterial adherence to host cell surfaces, which is also a prerequisite for invasion or biofilm formation (24–27). For example, the minor pilin subunit of Streptococcus agalactiae provides for bacterial adherence to the brain endothelium as well as pulmonary epithelial cells (28, 29). Corynebacterium diptheriae assemble pili from three subunits, the major pilin SpaA, and two minor pilins, SpaC and SpaB (14). SpaC is deposited at the tip of SpaA pili, whereas SpaB is found in regular intervals along the pilus shaft (14). Both SpaC and SpaB are required for corynebacterial adherence to pharyngeal cells, a process that does not depend on the SpaA subunit (30). Another minor pilin subunit, RrgA of Streptococcus pneumoniae, promotes bacterial adherence to respiratory epithelia (31). RrgA has been reported to bind to the extracellular matrix components fibronectin, collagen I, and laminin (32). Finally, Group A streptococcal pilin engage the scavenger receptor gp340 on pharyngeal cells to promote adherence and aggregation (33). Although minor pilins appear generally involved in pilus-mediated adherence, there are exceptions to this, as the major pilin subunit of S. pneumoniae pilus islet-2 (PI-2) mediates bacterial adherence to lung epithelial cells (34).

We have focused on B. cereus and its close relative B. anthracis to study the assembly of pili in Gram-positive bacteria. Bacilli form pili from two subunits, the major pilin BcpA and the minor pilin BcpB. We show here that the sorting signal of the minor pilin is recognized by sortase D, which subsequently cleaves its substrate between the threonine and the glycine residues of its IPNTG motif. The product of this reaction, a thioester-linked acyl enzyme, is resolved by the nucleophile attack of the ε-amino group of lysine within the YPKN pilin motif of BcpA. The aforementioned reaction is not absolutely dependent on the LPVTG sorting signal of BcpA (15). We took advantage of this observation and purified the sortase D-catalyzed transpeptidation product BcpB<sub>H6</sub>-BcpA<sub>LAVAA</sub> using a BcpA sorting signal variant that cannot be used as substrate for further polymerization. Mass spectrometry and Edman degradation of affinity-purified BcpB<sub>H6</sub>-BcpA<sub>LAVAA</sub> revealed the amide bond that tethers the minor pilin subunit to the tip of BcpA pili.

The observation that the IPNTG sorting signal of BcpB is recognized by sortase D, but not by sortase A, provides a compelling argument for a model that may be universally applicable for pilus assembly in Gram-positive bacteria. Biochemical analysis of sortase-catalyzed transpeptidation reactions revealed that resolution of acyl intermediates is the rate-limiting activity of sortase (35). Thus, if BcpB were the preferred substrate of sortase D, its intermediates could only be resolved by the nucleophilic attack of the amino group of BcpA, generating BcpB-BcpA transpeptidation products and positioning BcpA at the tip of pili. Sortase D can only accept one nucleophile, the ε-amino group of lysine within the YPKN pilin motif of BcpA. As a consequence, sortase D cleavage of BcpB-BcpA must be followed by further polymerization with major pilin subunits, resulting in the formation of BcpB-BcpA<sub>n+1</sub>. In contrast to BcpB, the BcpA LPVTG sorting signal can be cleaved by both sortase D and sortase A, the latter of which recognizes the side chain amino group of m-diaminopimelic acid within lipid II as a nucleophile to resolve its acyl intermediates (19). This reaction was recently demonstrated by studying the sortase A-catalyzed cell wall anchor structure of the major pilin subunit BcpA (19). Thus, competition between two transpeptidases, sortase D and sortase A, for the same pilin subunit can be viewed as a determinant for both pilus length and cell wall anchoring of fully assembled fibers. We propose that this model may be universally applicable to the assembly of pili in all Gram-positive bacteria. Pili in B. cereus, Actinomyces spp., and some group A streptococcal isolates are formed from two pilin subunits (36, 37). However, other Gram-positive bacteria assemble pilus from three subunits (38). C. diptheriae pili are formed via polymerization of the major subunit, SpaA. One of the minor subunits, SpaC, is deposited at the tip, whereas another minor pilin, SpaB, is incorporated at regular intervals along the shaft of polymerized SpaA (14). Recent work suggests that SpaB encompasses a side chain amino group that functions as a nucleophile, producing reiterative covalent links between SpaA and SpaB subunits (39). Nevertheless, it is still not clear whether pilin-specific sortases recognize the side chain amino groups of both major and minor pilins as nucleophiles to resolve their intermediates. Furthermore, the amide bonds that are formed during the assembly of pili comprising three subunits must be revealed.

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BcpB Linkage to Pilus Tip

MAY 8, 2009 • VOLUME 284 • NUMBER 19
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