Plasmid-Encoded Autolysin in *Bacillus anthracis*: Modular Structure and Catalytic Properties

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A *Bacillus anthracis* virulence plasmid-encoded peptidoglycan hydrolase (AmiA) with sequence similarity to N-acetyl- and N-acetylmuramoyl-L-alanine amidases hydrolyzes peptidoglycan independently of cell wall binding. Residues H341, E355, H415, and E486 are absolutely required for catalysis. Many AmiA paralogs are fused to different sorting signals, suggesting that these modular proteins result from domain shuffling.

Peptidoglycan (or murein) is a polymer of amino sugars cross-linked by short polypeptides. This scaffolding molecule forms a bag-shaped exoskeleton enclosing the plasma membrane and ensures cellular integrity under hypoosmotic conditions and maintenance of cell shape (19). The peptidoglycan network constantly undergoes modifications, hydrolysis, insertion of new material, and release of old material throughout vegetative growth (for reviews, see references 1 and 8). In particular, correct cell wall assembly requires peptidoglycan hydrolase activity. Depending on their substrate specificity, peptidoglycan hydrolases are classified as N-acetylmuramidases, N-acetylg glucosaminidases, N-acetylmuramoyl-L-alanine amidases, or endopeptidases (6). Their functional redundancy makes their study difficult.

Only some peptidoglycan hydrolases can cleave the high-molecular-weight polymer and cause bacteriolysis. Such enzymes are potentially lethal and are called autolysins. It has been suggested that autolysins contribute to various fundamental steps of the bacterial life cycle: cell separation, cell wall turnover, peptidoglycan maturation, and cell differentiation (mother cell lysis and spore outgrowth) in spore-forming bacteria (17). There is growing evidence that in pathogens, some autolysins contribute to virulence: they play a role in adhesion and in the amplification of the inflammatory response by releasing muramyl peptides (7, 14).

In this paper, we report the existence of an autolysin in *Bacillus anthracis* (AmiA), encoded by the virulence plasmid that encodes capsule synthetic activity, pXO2. The key amino acids involved in the enzymatic activity of AmiA were identified by site-specific mutagenesis. In addition, we show that peptidoglycan hydrolysis by AmiA does not require cell wall binding, in contrast to the well-characterized pneumococcal amidase LytA.

Identification of the AmiA protein as a plasmid-encoded bimodular autolysin. The sequence of the *B. anthracis* virulence plasmid, pXO2, contains a gene (pXO2-42; accession number NP_053197) potentially encoding a peptidoglycan hydrolase that we renamed *amiA*. The lacZ reporter gene was fused to the *amiA* coding sequence at the *amiA* locus. β-Galactosidase assays of the corresponding strain showed that *amiA* is expressed constitutively throughout development (data not shown). In addition, the expression pattern was not modified by the addition of bicarbonate, a compound known to mimic host conditions in vitro (18).

Sequencing of *amiA* revealed that arginine 302 and serine 311 from the annotated sequence in the database are in fact a serine and a glycine, respectively. The deduced amino acid sequence of AmiA consists of 503 amino acids, including a putative signal sequence of 28 residues (16). Sequence comparison with protein data banks suggested that AmiA results from the fusion of two functional domains (Fig. 1A). The N-terminal domain is composed of three S-layer homology (SLH) repeats of about 50 residues involved in the cell wall targeting of several surface proteins in gram-positive bacteria (4, 10, 11, 13). The rest of the mature protein is organized in two subdomains: a 100-amino-acid region with no homology to any protein from data banks, and a region highly similar to numerous N-acetylmuramoyl-L-alanine amidases (EC 3.5.1.28; referred to herein as amidases). These enzymes cleave the amide bond between the lactyl group of the muramic acid residue and the α-amino group of the L-alanine residue of the stem peptide (6). Some peptidoglycan hydrolases amplify the inflammatory response by releasing muramyl peptides. In contrast, AmiA, which is predicted to hydrolyze the bond between the glycan and the peptide moiety, would not amplify this response and might even decrease the quantity of inflammatory substrate. This could enable *B. anthracis* to multiply actively before the host develops an immunological response.

Peptidoglycan hydrolysis by AmiA is independent of cell wall binding. The full-length mature AmiA protein (pQAMI41) and its C-terminal domain (pQAMI61) were produced in *Escherichia coli* as histidine-tagged polypeptides (Fig. 1B). To construct pQAMI41, oligonucleotides His_AMI up and His_AMI down (Table 1) were used to amplify the sequence encoding the full-length AmiA protein from *B. anthracis* strain 4229 DNA. The corresponding PCR product was digested with *Bam*HI and *Sph*I and ligated to pQE30 which had been similarly digested. pQAMI61 was constructed by the same strategy with the aim of expressing the catalytic domain from AmiA alone, using oligonucleotides Ami-N and Ami-C (Table 1). The corresponding crude extracts (Fig. 2A) were
then used in zymogram assays with purified *B. anthracis* cell wall as the substrate (Fig. 2B). The enzymatic activities of AmiA and AmiA-related recombinant proteins were assayed by renaturing polyacrylamide gel electrophoresis (PAGE) (zymogram assay), as described by Foster (5). Full-length AmiA was able to hydrolyze *B. anthracis* high-molecular-weight peptidoglycan polymer, showing that it is an autolysin (Fig. 2B, lane 2). As expected, the truncated version of AmiA, devoid of the SLH domain, was unable to bind to purified cell walls (data not shown). Zymogram assays, however, revealed that it was active against *B. anthracis* cell walls, suggesting that cell wall binding is not required for enzyme activity (Fig. 2B, lane 3). Both the full-length protein and C-terminal domain of AmiA were enzymatically active when cell walls from a *csaB* mutant of *B. anthracis* were used as the substrate (Fig. 2C). In the *csaB* mutant, the peptidoglycan-associated polysaccharide is not substituted by pyruvate, abolishing the binding of SLH-containing proteins to the cell wall (12). This confirmed that the activity of AmiA does not require cell wall binding and that the negative charge of the peptidoglycan-associated polysaccharide due to pyruvate is not required for AmiA activity in vitro.

The only amidase extensively described to date is LytA from *Streptococcus pneumoniae* (9). The N-terminal domain of LytA harbors the enzymatic activity, and its C-terminal domain is involved in the interaction with choline-substituted lipoteichoic acids (2). Interestingly, and unlike *B. anthracis* AmiA, cell wall binding of LytA is required for enzymatic activity. The activity of LytA is indeed dramatically reduced when its capacity to interact with cell walls is abolished (10,000-fold reduction of activity when the choline from lipoteichoic acids is replaced with ethanolamine and 100,000-fold reduction when the N-terminal catalytic domain is produced alone [15]). It has been postulated that choline is an allosteric ligand of LytA, responsible for the conversion of the low-activity form of the enzyme (E form) to the fully active form (C form) (2). Our results show that these characteristics of *S. pneumoniae* LytA are not true for *B. anthracis* AmiA, since the full-length AmiA protein and the C-terminal catalytic module in isolation have similar activities against wild-type cell wall. To our knowledge, the ability of AmiA to cleave peptidoglycan in vitro independently of its binding to the cell wall is an original property for a peptidoglycan hydrolase from a gram-positive organism. This unique feature may explain why the catalytic module of AmiA is widespread among bacteria (see below).

**Identification of residues crucial for AmiA activity.** Multiple-sequencealignments were applied to the following protein sequences: *B. anthracis* 4229, accession number NP_053197; *S. pneumoniae* LytA, accession number NP_324923.

### TABLE 1. Bacterial strains, plasmids, and oligonucleotides used

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant properties*</th>
<th>Reference or source</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>E. coli</td>
<td>Host strain for general cloning, Tet*&lt;sup&gt;t&lt;/sup&gt;</td>
<td>Stratagene</td>
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<tr>
<td>M15(pREP4)</td>
<td>Expression strain for pQE30 derivatives, Kan*&lt;sup&gt;t&lt;/sup&gt;</td>
<td>Qiagen</td>
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<td><strong>B. anthracis</strong></td>
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<tr>
<td>SM11</td>
<td>S-layer-deficient strain (pXO1&lt;sup&gt;−&lt;/sup&gt;, pXO2&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>11</td>
</tr>
<tr>
<td>SM95 (<strong>acsB</strong>)</td>
<td>Anchoring-deficient strain (pXO1&lt;sup&gt;−&lt;/sup&gt;, pXO2&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>12</td>
</tr>
<tr>
<td>ATCC 4229</td>
<td>Capsulated strain (pXO1&lt;sup&gt;+&lt;/sup&gt;, pXO2&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Laboratory stock</td>
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<td><strong>Plasmids</strong></td>
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<td>pQE30</td>
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<td>This work</td>
</tr>
<tr>
<td>pQAMI61</td>
<td>pQE30 derivative for overexpression of AmiA catalytic domain</td>
<td>This work</td>
</tr>
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<td><strong>Oligonucleotides</strong></td>
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<td>AMI-N</td>
<td>TTTGGATCCATGTACATGAACAGACATTTCATTACATAC</td>
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<tr>
<td>AMI-C</td>
<td>TTTGCATGCTCTATTCATTTAGTAATAAGCAGACATGTTATGACCTTT</td>
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<tr>
<td>HIS AMI up</td>
<td>CTTCAGGATCCAAAAGACATTTACTGATGTACAA</td>
<td></td>
</tr>
<tr>
<td>HIS AMI down</td>
<td>GTITAGCAATGCTTAAATAGGTTGCCGGCCCFFTAAGGCAG</td>
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* The restriction sites in the primer sequences are underlined
Bacillus subtilis 168, Q02114; Anabaena sp. strain PCC7120, AAF33754.1; Bacillus halodurans C-125, BAB07384.1; Synechocystis sp. strain 6803, S75217; Bacillus polymyxa subsp. colistinus, BAA90649.1; bacteriophage SSP1, T42311; Bacillus licheniformis, P37134; Deinococcus radiodurans R1, D75402; Helicobacter pylori 26695, D64616; Treponema pallidum, B71348; Aquifex aeolicus, G70445; E. coli K12, P36548; Campylobacter jejuni NCTC 11168, CAB73523.1; Mycobacterium tuberculosis Rv3717, F70795; Buchnera sp. APS, BAB13265.1; Pseudomonas aeruginosa PA01, AAG08923.1; Staphylococcus aureus SR17238, BAA23140.1; Haemophilus influenzae Rd KW20, P44493; Xylella fastidiosa 9a5c, AAF83569.1; Streptomyces coelicolor A3, CABB3433.1; Mycobacterium leprae, CAA19159.1; Vibrio cholerae N16961, AAF93517.1; and Clostridium perfringens, S49554. They revealed highly conserved amino acids in AmiA and its paralogs (data not shown). We studied these conserved residues by systematic site-directed mutagenesis in AmiA and activity assays using *B. anthracis* peptidoglycan (Fig. 2D). Site-directed mutagenesis was carried out by PCR-based mutagenesis of pQAMI41, using two complementary mutation-harboring oligonucleotides for each mutant and the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, Calif.). PCR products and the inserts of all resulting plasmids were sequenced to check that only a single codon was modified. Sodium dodecyl sulfate (SDS)-PAGE analysis of the 10 mutants obtained showed that all the corresponding proteins were synthesized in similar quantities, suggesting that none of them was unstable (data not shown). Zymogram analysis led to the classification of the mutants into four major groups (Fig. 2D): the first group (S413A and P481A) had activity similar to that of the wild-type enzyme; the second group (R382A and L484G) had activity that was significant but weaker than that of AmiA; the third group (D338A and R399A) had only weak activity; and the fourth group (H341A, E355A, H415A, and E486A) had no detectable activity. Presumably, these four mutations identify residues essential for catalysis.

Little is known about the catalytic mechanism of amidases. The four residues of AmiA essential for catalysis (H341, E355, H415, and E486) are dispersed throughout the AmiA sequence and extremely well conserved in all the paralogs analyzed. To our knowledge, only one study describes the catalytic residues of another amidase, the T7 lysozyme (3). However, no sequence similarity was found between AmiA and T7 lysozyme. The elucidation of the three dimensional structure of AmiA would thus reveal whether the catalytic residues are found in a spatial configuration similar to that described for other peptidoglycan hydrolases.

Versatility of the AmiA catalytic domain. The distribution of AmiA-like amidases among prokaryotes was investigated. We found AmiA paralogs in more than 30 bacterial species, including both gram-positive and gram-negative species, as well as a few among phages.

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one is devoid of any cell wall-targeting domain but contains a hydrophobic sequence towards its N terminus, suggesting that it is targeted to the cytoplasmic membrane. It is interesting that two of the cell wall-targeted enzymes, including AmiA, also contain 100 amino acids between the SLH and the catalytic domains (Fig. 1A). These 100 residues were highly conserved between the two AmiA paralogs but had no similarity with other sequences from data banks. They may function as a spacer, allowing greater flexibility of the catalytic domain or interactions with other components involved in cell wall metabolism.

The occurrence of AmiA-related proteins in chromosomally and plasmid- and phage-encoded forms is an illustration of genome plasticity. In addition, the fusion of the AmiA catalytic domain to various sorting signals showed that there has been extensive domain shuffling during evolution, extending the repertoire of AmiA function. The sorting of the AmiA catalytic module to different subcellular compartments is indeed likely to allow a single enzymatic activity to have different physiological roles.

We thank Richard Okinaka for making the amiA sequence available before its release in the data bank and Michèle Mock, in whose laboratory this work was carried out. We are also indebted to Jean-Luc Mainardi, Laurent Gutmann, and Tahar Ait-Ali for fruitful discussions and constant support. We also thank the reviewers for interesting suggestions concerning the manuscript.

ADDITIONAL IN PROOF

Residues equivalent to those described as crucial in this study were also found in Bacillus subtilis CwlC N-acetylmuramoyl-