Annu. Rev. Microbiol. 2001. 55:647–71

ANTHRAX

Michèle Mock and Agnès Fouet
Toxines et Pathogénie Bactérienne, (CNRS URA 2172), Institut Pasteur, Paris Cedex 15, France; e-mail: mmock@pasteur.fr; afouet@pasteur.fr

Key Words *Bacillus cereus* group, virulence, toxin, peptide capsule, spore

■ **Abstract** *Bacillus anthracis* was shown to be the etiological agent of anthrax by R. Koch and L. Pasteur at the end of the nineteenth century. The concepts on which medical microbiology are based arose from their work on this bacterium. The link between plasmids and major virulence factors of *B. anthracis* was not discovered until the 1980s. The three toxin components are organized in two A-B type toxins, and the bacilli are covered by an antiphagocytic polyglutamic capsule. Structure-function analysis of the toxins indicated that the common B-domain binds to a ubiquitous cell receptor and forms a heptamer after proteolytic activation. One enzyme moiety is an adenylate cyclase and the other is a Zn^{2+} metalloprotease, which is able to cleave MAPKKs. The capsule covers an S-layer sequentially composed of two distinct proteins. Knowledge of the toxins facilitates the design of safer veterinary vaccines. Spore-structure analysis could contribute to the improvement of human nonliving vaccines. The phylogeny of *B. anthracis* within the *Bacillus cereus* group is also reviewed.

CONTENTS

INTRODUCTION

Bacillus anthracis, the etiological agent of anthrax, is a gram-positive, nonmotile, aerobic, facultative anaerobic, spore-forming, rod-shaped bacterium. Dormant spores are highly resistant to adverse environmental conditions including heat, ultraviolet and ionizing radiation, pressure, and chemical agents. They are able to survive for long periods in contaminated soils and thus account for the ecological cycle of the organism. In a suitable environment, spores reestablish vegetative growth. However the bacilli are poor survivors (67), and it is unclear whether existence of a complete cycle, from germination to resporulation, occurs outside the host. Indeed the particular properties of *B. anthracis*, compared with those of other *Bacillus cereus*–group bacilli sharing the same ecological niche, are consistent with a life cycle that almost exclusively takes place in the mammalian host. Spores ingested by herbivores germinate within the host to produce the vegetative forms; these multiply and express their virulence factors, killing the host (Figure 1). The vegetative form is square ended and capsulated. Sporulating cells carry elliptic, centrally located spores. Bacilli shed by the dying or dead animal will sporulate on contact with air. Sporulation requires the presence of free oxygen, and the efficiency of the process is influenced by the environmental conditions. The proportion of cells that reach the ultimate stage, a dormant spore, is variable. The various steps of this cycle and current knowledge of the molecular mechanisms they involve are reviewed.

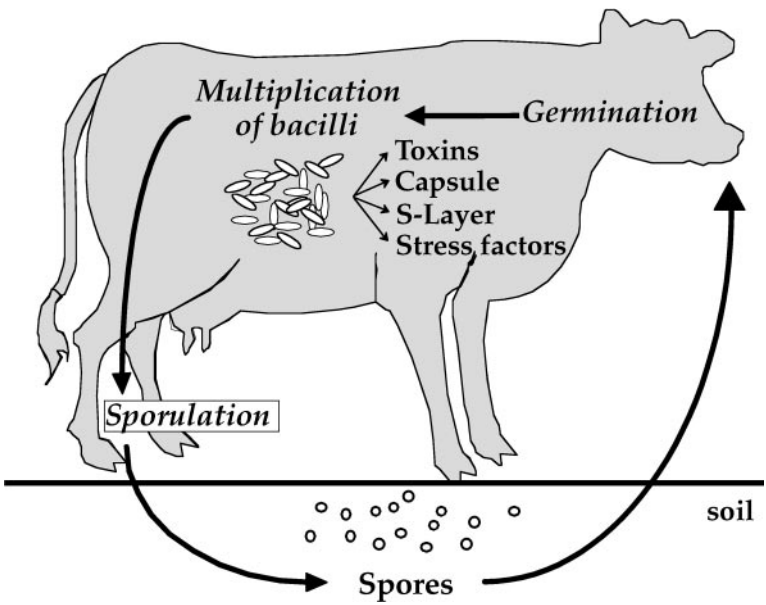

Figure 1 *Bacillus anthracis* cycle.

THE ORGANISM

The Disease and Early Steps of Infection

Anthrax is primarily a disease of herbivores, but all mammals, including humans, are susceptible. The disease is initiated by the entry of spores into the host body. This can occur via a minor abrasion, via an insect bite, or by eating contaminated meat or inhaling airborne spores. There are three types of human infection: cutaneous, gastrointestinal, and inhalational (pulmonary). Each form can progress to fatal systemic anthrax. The cutaneous form, the most common, is first manifested as a small pimple that develops, within a few days, into a painless black eschar characteristic of anthrax. The lesions are always accompanied by substantial edema. This form is easily diagnosed and can be treated with a variety of antibiotics. In gastrointestinal and inhalational forms, the illness is insidious at first, with mild symptoms of gastroenteritis, slight fever, and flu-like symptoms. Early diagnosis is difficult, and the disease abruptly develops into a systemic form that becomes treatment resistant and rapidly fatal, with shock-like symptoms, sepsis, and respiratory failure (115).

In animal models, the inhalational route and intradermal inoculation are extensively used. Whatever the route of infection, spores are taken up by macrophages and transported to the regional lymph nodes draining the site of inoculation. (66, 100). As the phagocytic capacity of the lymph node is overwhelmed, the infection extends to successive nodes, and the bacilli then enter the bloodstream. Early observations suggested that after inhalational exposure, spores could germinate within alveolar macrophages (100, 103). Efficient germination of *B. anthracis* spores of the Sterne strain was observed by confocal microscopy in the phagolysosome of alveolar macrophages. This phenomenon is associated with early expression of the toxin genes in the same cellular compartment (37). It is not known whether capsulation can start concomitantly within the macrophages. It is not known how the germinated spores circumvent the antimicrobial environment of the phagolysosome or how the vegetative forms subsequently escape from macrophage. Although *B. anthracis* is an extracellular pathogen, it nevertheless appears to require an intracellular step to initiate infection. Spore germination might be triggered within the macrophage by host-specific signals. A germination operon *gerX* has been found on a *B. anthracis* plasmid, pXO1 (Figure 2, and see virulence plasmids section); its deletion from the Sterne strain affects the germination in macrophages and in vivo (38). The deletion of *gerX* also reduced virulence in mice. The three predicted proteins, GerXA, GerXB, and GerXC, encoded by *gerX* are similar to *Bacillus subtilis*, *B. cereus*, and *Bacillus megaterium* germination proteins (11, 13, 52). These proteins may form a receptor specifically detecting germinants within the host. It is intersting to note that the *gerX* locus is the only germination operon known that is located on a virulence plasmid. There are also at least five other loci encoding germination-like proteins on the chromosome of *B. anthracis* (www.tigr.org; T. Read, personal communication).

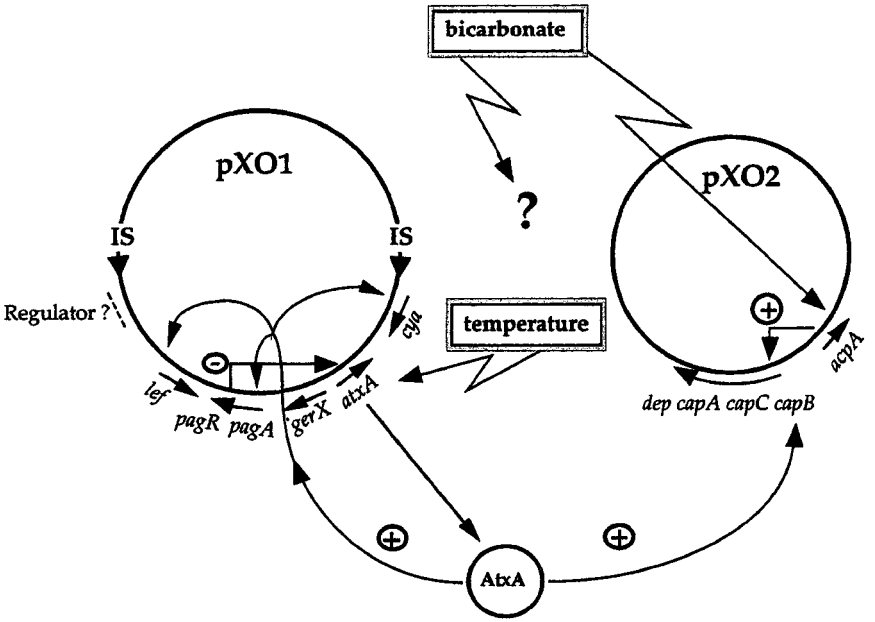

Figure 2 Schematic representation of *B. anthracis* plasmids and the virulence and germination genes they encode. The insertion sequences and the regulatory elements are shown. Question marks indicate putative or uncharacterized regulation.

The Virulence Plasmids

Fully virulent strains of *B. anthracis* carry two large plasmids, pXO1 and pXO2, that encode the primary virulence factors, toxin production and capsule formation, respectively (Figure 2). The complete DNA sequences of pXO1 and pXO2 are known. The GC content (33%) of these plasmids is similar to that of the *B. anthracis* chromosome.

The pXO1 isolated from the Sterne strain (AF065404) (85) is 181,654 nucleotides long with 143 open reading frames (ORFs), covering about 61% of the DNA. pXO1 carries the structural toxin genes, *pagA*, *lef*, and *cya*; regulatory elements; a resolvase and a transposase; and *gerX*, a three-gene germination operon (38). All these genes map within a 44.8-kbp region flanked by inverted IS1627 elements: This region has been termed a pathogenicity island (Figure 2). It appears to be able to transpose because its inversion has been reported once in a laboratory strain (114). The plasmid also carries DNA topoisomerase (29) and 15 ORFs possibly involved in horizontal transfer.

pXO2 carries *capB*, *capC*, *capA*, and *dep*, all known to encode capsule synthesis and degradation (114), and *acpA*, which is a regulatory gene (Figure 2). pXO2, from a Pasteur strain, has been sequenced (AF188935) (R.T. Okinaka, personal communication). It is 96,231 bp long and contains 85 ORFs. Little is known about its putative functions.

Long before the discovery of plasmids, the attempts to attenuate the virulence of anthrax strains for vaccine purposes led to plasmid curing. The various attenuated strains obtained by Louis Pasteur had lost either one or both plasmids. Strains can be cured of plasmids by various laboratory conditions, such as growth at 43°C or in the presence of antibiotics. Curing of pXO1 is rare, and its spontaneous loss in environmental samples has not been reported. Various phenotypes, in addition to toxin production (77), have been linked to the presence of pXO1 (114). Cured cells sporulate earlier and at higher frequencies, and they grow more poorly on certain minimal media and are more sensitive to some bacteriophages. $pXO1^-$ strains are also less capsulated (see regulation section). Unlike pXO1, pXO2 is easily and spontaneously lost. $pXO1^+$ and $pXO2^-$ strains have been found in environmental samples and among virulent strains (117). The only phenotype associated thus far with pXO2 is capsulation, which is easy to detect by observing colony morphology. However, among $pXO2^+$ strains, uncapsulated mutants can be found at a rather high frequency (36). Thus pXO2 may be more susceptible to rearrangements than pXO1. It is interesting that pXO2 can be associated with the differences in virulence between wild-type strains (130, 133).

SURFACE STRUCTURES

The Exosporium

The spore surface provides the first interaction with the host. An exosporium structure has been observed outside the spore coat of some *Bacillus* and *Clostridium* species; *B. cereus* and *B. anthracis* have a loosely fitting exosporium (42, 128). In *B. cereus*, the major component of the exosporium is protein, but lipid (18%) and carbohydrate (20%) are also present (71). Two exosporium-specific major glycoproteins have been described (9, 32). The exosporium usually contains three hexagonal lattice layers (128, 129), and its surface is decorated with filamentous appendages (41). As with gram-negative pili, these filaments may be of importance for spore attachment to surfaces or ligands. The function of the exosporium is unknown, but it does not appear to be important either for dormancy or germination (33). It may act as a sieving barrier, preventing exoenzymic attack on the spore coat and cortex layers (83).

The Capsule

In addition to the cytoplasmic membrane and peptidoglycan layers found in all bacteria, *B. anthracis* bacilli have two other surface structures, namely a capsule and an S-layer. The *B. anthracis* parietal architecture is therefore complex, and indeed very few bacteria possess both these structures.

The first description of *B. anthracis* capsule goes back to M'Fadyean's work in 1903. Preisz described the relationship between in vivo formation of the capsule and *B. anthracis* virulence (91). *B. anthracis* capsule is currently considered to be one of its two major virulence factors (114 and references therein). The capsule

contributes to pathogenicity by enabling the bacteria to evade the host-immune defenses and provoke septicemia. Indeed, *B. anthracis* capsule inhibits phagocytosis (70, 91, 138), and it is a monotonous linear polymer that is only very weakly immunogenic (35). The characteristics of *B. anthracis* capsule therefore do not favor an immune response.

The capsule is a polymer of γ-D-glutamic acid (138). The molecular weight of the polyglutamic chains is between 20 and 55 kDa in vitro and estimated to be 215 kDa in vivo (35, 96). Although peptide capsule is not common in bacteria, some species, including *B. subtilis*, *B. megaterium*, and *Bacillus licheniformis*, synthesize glutamic acid polymers. However, this polymer can either constitute a capsule or be secreted into the medium (for a review, see 60). In *B. anthracis*, the glutamyl polypeptide only adheres to the cell by an as yet unknown mechanism. The biochemical structure of *B. anthracis* and *B. licheniformis* capsules are identical, and the same precursor is used for their synthesis, namely L-glutamic acid. This suggests that their biosynthesis pathways are also identical. In *B. licheniformis*, poly-γ-D-glutamic acid is synthesized by a membranous enzymatic complex in three steps. In brief, the polyglutamyl chains are extended by sequential addition of new D-glutamyl residues to the terminal amino group of a glutamyl residue of the acceptor.

B. anthracis capsule synthesis is encoded by the plasmid pXO2 (Figure 2) (36). Three pXO2 genes are sufficient for poly-γ-D-glutamic acid synthesis in *Escherichia coli* (70). These genes, *capB*, *capC*, and *capA*, encode enzymes of 44.8, 16.5, and 46.4 kDa, respectively. Their sequences suggest that they are membrane-associated enzymes. Three genes encoding a poly-γ-glutamate synthetic (PGS) system in a poly-γ-glutamic acid–secreting strain *B. subtilis* have been cloned (1). These genes, *pgsBCA*, are similar to the *B. anthracis capBCA* genes. It is interesting that the PGS system does not seem to include a component for glutamate isomerization; it requires the presence of a glutamate racemase to synthesize poly-γ-D-glutamic acid. By analogy, the situation might be similar for the *B. anthracis* CapBCA system. No specific enzymatic function has yet been assigned to any of these *B. anthracis* or *B. subtilis* proteins. A fourth gene in the *B. anthracis* cap locus, *dep*, is associated with depolymerization of the capsular polymer (120). *dep* appears to catalyze the hydrolysis of the poly-γ-D-glutamic acid polymer, thus controlling the size of the capsule.

It would be of great value to correlate the genetic information accumulated concerning *B. anthracis* capsule synthetic enzymes with the biochemical reactions needed for the synthesis of the cell-attached poly-γ-D-glutamic acid. The involvement of the putative racemase and the synthesis stereospecificity found in *B. anthracis* need to be analyzed. Furthermore, the precise localization of the capsule attachment site and the specific components, if any exist, and the specific mechanisms that ensure capsule adhesion to the bacteria will have to be elucidated.

S-Layer

The capsule is the outermost element of the cell wall. When *B. anthracis* does not produce its capsule, the exterior of the cell wall appears layered owing to

the S-layer (74). However, the presence of an S-layer is not required for a normal capsulation of *B. anthracis* bacilli, but it may modify its fine structure (74). The S-layer, overlaying the peptidoglycan, is composed of fragments displaying a highly patterned ultrastructure (33, 49). S-layers are proteinaceous paracrystalline sheaths that completely cover the cell surface. Most result from noncovalent, entropy-driven assembly of a single (glyco)protein protomer, constituting 5%–10% of total cell protein, on the surface of the bacteria. Its synthesis presumably requires an energy input from the bacteria. Various functions have been proposed for S-layers, including shape maintenance and molecular sieving, and they may be a virulence factor (for review, see 101). The *B. anthracis* S-layer has no influence on the 50% lethal dose in the animal models used. Nevertheless, the capsule and the S-layer seem to have a cumulative effect, increasing resistance to complement pathway-mediated defenses (95). Further analysis is required before the role of the S-layer becomes clear.

There are two abundant 94-kDa proteins, namely Sap (surface array protein) and EA1 (extractable antigen 1) (24), on the cell surface of *B. anthracis* (23, 75). The genes, *sap* and *eag*, have been identified, and they have been deleted together and independently (23, 75). In the capsulated strain, Sap and EA1 can each be the sole S-layer constituent, and there is no other S-layer component. The deduced amino acid sequences of Sap (814 residues) and EA1 (862 residues) have common features. Both proteins harbor a standard gram-positive signal peptide followed by two domains: The first, constituted of three SLH (S-layer homology) motifs, is discussed below; the second, the protease resistant core, may be the crystallization domain (73). The presence of a signal peptide and SLH motifs in these proteins is consistent with these proteins being localized on the bacterial surface. The existence of two S-layer genes may result in the simultaneous synthesis of both proteins or in the activation of the second gene if one is silenced (101 and references therein). In fact, Sap and EA1 are sequentially present at the cell surface; they are only found colocalized at the cell surface at an intermediary stage, when EA1 synthesis starts. Thus both are present while EA1 progressively replaces Sap. During the stationary phase, the cell surface does not expand, and the replacement of Sap by EA1 requires the release of Sap into the supernatant. EA1 drives this displacement. A developmentally regulated S-layer switch, from a "Sap–S-layer" to an "EA1–S-layer" has thus been suggested (T. Mignot, S. Mesnage, E. Couture-Tosi, M. Mock & A. Fouet, manuscript in preparation; E. Couture-Tosi, T. Mignot, S. Mesnage, H. Delacroix, M. Chami, A. Fouet & G. Mosser, manuscript in preparation). In vivo, EA1 and Sap are major surface antigens, implying that they are both synthesized in vivo (74, 75).

There are at least 18 ORFs with SLH sequences in *B. anthracis*. At least 15 on the chromosome, 2 on pXO1, and 1 on pXO2. SLH motifs have been found in many surface proteins from gram-positive bacteria, and it was suggested that they are responsible for cell-wall binding (69). This was confirmed by studies with *B. anthracis* S-layer proteins (10, 73, 76). Polypeptides composed of the three SLH motifs of EA1 or Sap are able to bind *B. anthracis*–purified cell wall, in vitro, with similar K_d (10, 73). This binding is to a secondary polymer, possibly the

neutral polysaccharide described by Erkwunife et al. (18), covalently linked to the peptidoglycan (72, 73). Chimeric genes encoding fusion proteins combining the SLH domains of EA1 or Sap and the mature form of normally secreted proteins (the *B. subtilis* levansucrase or a fragment of the *Clostridium tetanii* toxin) have been constructed and inserted into *B. anthracis* chromosome. The SLH domains are sufficient to anchor the chimeric proteins in their active form to the surface of the bacilli (73, 76). It is presumed that they do the same for the S-layer proteins.

The analysis of the cell wall partner of the SLH domain led to the identification of a bicistronic operon (*csaAB*) involved in SLH anchoring (72). *csaB* mutant is unable to anchor S-layer proteins and also many other SLH-containing proteins. The parental- and mutant-strain peptidoglycans are similar. The peptidoglycan-associated polysaccharides of the parental and mutant strains have the same molecular mass and the same composition. However, the parental polysaccharide possesses pyruvate residues that are absent from the mutant. Contrary to the pyruvylated form, the nonpyruvylated polysaccharide is unable to bind SLH domains. Therefore, CsaB is responsible for the pyruvylation of the polysaccharide and consequently for anchoring the numerous SLH-containing proteins of *B. anthracis*. The *csaB* mutant bacilli have a twisted filamentous morphology due to a greatly reduced autolytic activity (72). SLH-mediated cell wall anchoring is widespread among bacteria. Indeed, the *csaAB* operon is present in all bacterial species that synthesize SLH-containing proteins, whether gram positive or gram negative. Also, pyruvate is present in the cell wall of all tested bacteria containing SLH and *csaB* sequences.

TOXINS

The presence of toxins in filter-sterilized serum of guinea pigs was first demonstrated by Smith and coworkers (109). These toxins play a key role in the pathogenesis of anthrax and are composed of three proteins acting in binary combinations (111), now termed protective antigen (PA), for its ability to elicit a protective immune response against anthrax (34); lethal factor (LF); and edema factor (EF). Intravenous injection of PA + LF (LeTx, lethal toxin) provokes death of the animal (3), whereas intradermal injection of PA + EF (EdTx, edema toxin) produces edema in the skin (111). Separately, none of these proteins is toxic. They represent a unique variation of the A-B toxins (64). PA is the common cell-binding domain (B) able to interact with two different enzyme domains (A), EF and LF, that elicit cell damage.

In the past decade, an enormous amount of work has addressed the structural and functional analysis of these three secreted protein toxins and the role of EdTx and LeTx in pathogenesis. The structural genes, *pagA*, *lef*, and *cya*, encoding PA, LF, and EF, respectively, are all located on pXO1. The three genes have been cloned (81, 99, 124) and sequenced (5, 22, 132).

Functional Organization and Mode of Action of the Toxins

PROTECTIVE ANTIGEN The mature protein (PA83) is 735 amino acids (M_r 82,684). The crystal structure of monomeric PA83 has been determined at a resolution of 2.1 Å (89). The molecule is folded into four functional domains. Each domain is required for a particular step in the intoxication process. Domain 1 (residues 1–249) contains the proteolytic activation site. Cleavage of the PA monomer, in vitro by trypsin (64) or in vivo by cellular proteases, releases the subdomain 1a, which is the N-terminal fragment of 20 kDa (PA20). The subdomain 1b is part of the remaining 63-kDa fragment (PA63). PA63 can oligomerize into ring-shaped heptamers (79); this heptamer has also been crystallized, and its structure has been solved (89). Domain 2 (residues 250–487) is a β-barrel core containing a large flexible loop (residues 302–325) that has been implicated in pore formation (89). At low pH, the heptamer undergoes conformational changes and converts from prepore to pore. This involves a rearrangement of the seven loops of the heptamer that constitutes a 14-stranded β-barrel spanning the membrane. There is a chymotrypsin cleavage site (Phe^{313}-Phe^{314}) in this loop and it is required for toxicity (105). The heptamer forms cation-selective channels in artificial and cell membranes (26, 80). Domain 3 (residues 488–594) is the smallest of the four domains. It has a hydrophobic patch that is thought to be involved in protein-protein interactions (89). Domain 4 (residues 595–735) is required for binding to the receptor. It has limited contact with the other domains. Short deletions of the C-terminal end or of part of the solvant-exposed loop (704–722), or amino acids substitutions, reduce or eliminate the interaction of PA with the receptor. Monoclonal antibodies directed to this region can also block receptor binding (64).

The receptor of PA is partially proteinaceous and present on many cell types (21). However, it is still unidentified. CHO cells are EF sensitive, most commonly used to study the effects of EdTx. In contrast to EF, LF is active only on certain lines of macrophages (30). Sensitive macrophages lyse within 1–2 h of incubation with LeTx. Cytolysis has therefore been used as a convenient assay for LF activity, as it unambiguously reflects the activity of LeTx in animal models (64). A general model for toxin entry into host cells is presented in Figure 3.

The first step is the highly specific binding of PA to the cell surface receptor. On cell binding, PA is cleaved by furin or a furin-like protease (64). According to the cellular model, the cleavage occurs at the cell surface, but there is evidence that PA is also proteolyzed in the serum of infected animals (7, 25). The two possibilities are not mutually exclusive, and both native and cleaved PA are able to bind to the cell receptor, the subsequent steps being unaffected. The proteolytic activation of PA leads to the release of PA20 from the remaining PA63 receptor-bound fragment, exposing the binding site for EF and LF. This is a critical step in the intoxication: A deletion-mutant PA lacking the protease cleavage site is completely nontoxic to macrophages when administered with LF (64). The heptamer then

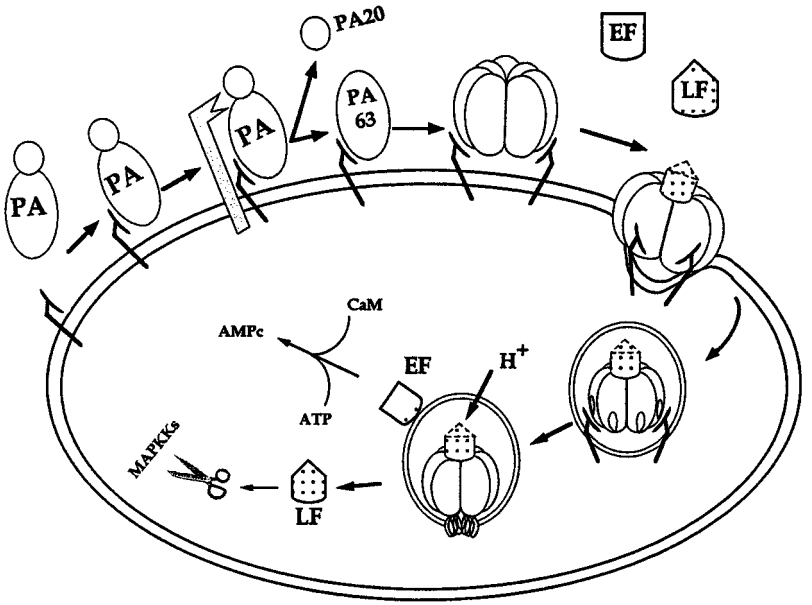

Figure 3 Cellular model of action of anthrax toxins.

binds competitively LF and EF. The receptor does not play a role in this interaction, the affinity of binding being similar whether PA is bound to the receptor or not (19). Monoclonal antibody studies suggest that two different sites on PA may be involved in the binding of LF and EF (68).

There is evidence that each PA63 monomer may bind one EF or LF, allowing the binding of seven molecules of LF and/or EF on a heptamer. This step is then followed by the internalization of the hetero-oligomeric complex. Oligomerization triggers receptor-mediated endocytosis of PA63 (4). EdTx and LeTx both require passage through an acidic vesicle for activity, and inhibitors of endosomal acidification or of endocytosis prevent toxicity (64). At acidic pH, the prepore converts to a pore and this transition is followed by or concommittant with insertion of the pore into the membrane, allowing the translocation of LF/EF. The two components may actively contribute to their translocation via the pore (58) with an, at least partial, unfolding (126, 134). In the final step, EF and LF reach their cytosolic targets.

EF and LF share similar sequence homology in their N-terminal 250 amino-acid regions. A 300-residue, truncated form of EF competes for PA binding (61), and mutagenesis in the amino-terminal domain of LF eliminates toxicity and PA binding (93). Fusion proteins containing the 254 N-terminal residues of LF promote a PA-mediated internalization of heterologous peptides (64, 78). The other parts of EF and LF are involved in the catalytic activities.

EDEMA FACTOR The mature protein is 767 residues. EF was the first of the two enzyme domains of anthrax toxins to be characterized. It is an adenylate cyclase converting intracellular ATP into cAMP, an activity that is dependent on the eucaryotic protein calmodulin (65). EdTx therefore provokes a substantial increase in intracellular cAMP levels. EdTx and the *Bordetella pertussis* calmodulin-dependent adenylate cyclase share three highly conserved sequences, including a 24–amino acid stretch (303–339) that carries the ATP-binding sequence, also present in the eukaryotic adenylate cyclase (22). This sequence is involved in catalysis (63, 136). The calmodulin binding site has not been properly defined; however, the peptide 499–532 and the C-terminal 150 residues are required (62, 82). EF can interact with lipids independently of pH (127), and unlike LF, EF remains associated to the vesicle membrane after translocation to the cytosol (39). It is very likely therefore that EF ressembles the membrane-associated eukaryotic adenylate cyclase.

LETHAL FACTOR The mature protein contains 776 residues. There is a series of four imperfect repeats in the central part of the molecule, and deletions in this region render the protein unstable and inactive (93). The catalytic domain resides in the C-terminal part where a HExxH zinc-metalloprotease consensus sequence has been identified (residues 686–690) (57). Mutations of the critical residues abolish lethal toxin activity and binding of Zn^{2+} to LF, indicating that, like the clostridial botulinum and tetanus toxins, LF is a zinc protease. Recently, independently and by two different techniques, it has been reported that LF cleave the amino N terminus of mitogen-activated protein kinases (MAPKKs), Mek1 and Mek2 (17, 123). The MAPK pathway relays environmental signals to the transcriptional machinery in the nucleus and thus modulates gene expression via a burst of protein phosphorylation. Seven different MAPKKs have been identified (135), and LF cleaves all family members except Mek5 (88, 123). Although it is likely that the activity of LF on MAPKKs is important in anthrax pathogenesis, the cascade of events following the cleavage remains unclear. Macrophage cell lines resistant to the lytic effect of LeTx and peritoneal macrophages isolated from mouse strains insensitive to LeTx are nevertheless sensitive to the protease activity of LF on MAPKKs. This discrepency may indicate that there are other cytosolic targets of LF involved in cytotoxicity. There is evidence implicating the proteasome (113). Furthermore, a single gene, designated *Ltx1* on chromosome 11, determines the susceptibility of mouse macrophages to LeTx (98). Susceptibility was dominant to resistance, which suggests that resistance is caused by the absence of a molecule acting downstream from the activity of LF.

Toxins and Pathogenesis

The residual virulence of the Sterne strain is due to toxin production, and experimental infection at high doses provokes the two main toxin-related symptoms characteristic of anthrax: edema and shock-like death. Toxin mutant strains derived from the Sterne strain have been used to validate in vivo the various

proposed steps of the cellular model of action. Gene deletions or point mutations destroying the enzyme activities of EF and LF are sufficient to abolish edema and lethality, respectively (7, 90). Moreover EF- and LF-deficient strains were less effective at causing death and edema, respectively, than the parental EF + LF producing strain. These properties are evidence of in vivo synergy between EdTx and LeTx, and they indicate that it involves the toxin catalytic activities directly. Thus, toxins probably act together within the host. Mutants producing PA molecules deleted for the furin-cleavage site and for the chymotryspin-cleavage site in domain 2 are nonpathogenic. Thus, in vivo, PA can only be processed at these sites. Deletions of the PA-receptor domain, or of the loop involved in binding, abolish or reduce virulence. Furthermore, the properties of the PA mutant strains provide evidence that PA may be cleaved in serum by furin-like proteases and bind LF without necessarily interacting with its cell receptor (7).

Although macrophages are likely to be key mediators in toxin-induced shock, the molecular mechanisms and the sequence of events leading to death in cases of anthrax remain unclear and controversial. The signaling pathways involving Mek1, Mek2, and Mek3 play a crucial role in the activation of macrophages and are directly involved in the production of cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1, and IL-6. However, the links between the cleavage of MAPKKs by LF, macrophage lysis, and pathogenesis are far from being understood. Release of proinflammmatory cytokines by macrophages on treatment with LeTx could account for shock, and mice depleted of macrophages have been reported to be insensitive to LeTx challenge (43, 44). However there are conflicting data concerning the modulation of TNF-α and IL-1β by LeTx. Sublytic doses of LeTx induced production of these cytokines (44), but the absence of effect has also been reported. Moreover, LeTx inhibited the production of NO and TNF-α induced by lipopolysaccharides (20, 88). These discrepancies await clarification. EdTx is also able to influence cytokine production by increasing the amount of cAMP (50). It strongly reduces lipopolysaccharide-induced TNF-α production by cultured monocytes. EdTx inhibits the phagocytic and oxidative burst abilities (84) and stimulates chemotaxis in human neutrophils (125). This toxin can therefore increase host susceptibility to infection. Indeed, during the early steps of infection, a reduction in the release of proinflammatory mediators, owing to a synergystic effect of both toxins, may greatly facilitate bacterial proliferation. Later, during septicemia, large amounts of LeTx may trigger toxic shock and death.

REGULATORY PATHWAYS IN *B. ANTHRACIS*

Major Virulence Factor Regulation of Synthesis

Capsule and toxins are produced during multiplication of the vegetative form in the host (Figure 1). In vitro, their synthesis is induced by the addition of bicarbonate and

is temperature dependent, indicative of regulation processes. These environmental factors are signatures of the mammal host (107).

The expression of the three toxin genes is coordinately regulated by bicarbonate and temperature at the transcriptional level (2, 107). Similarly, bicarbonate but not temperature enhances *capB* transcription (27). The temperature dependence of the state of capsulation of the bacilli is probably due to the effect of temperature on enzymatic activities.

The existence of transcriptional activators was inferred, and consequently, there were attempts to discover them (2, 8). AtxA, a pXO1-encoded transcriptional activator for the expression of *pagA* was independently isolated by two different approaches (Figure 2; 59, 119). The presence of pXO1 in bacteria enhances capsule production (36), and AtxA acting on transcription is responsible for this increase (40, 121). Thus, genes belonging to different genetic elements, namely pXO1 and pXO2, have their transcription controlled by AtxA. The AtxA regulon, in which AtxA is the regulatory protein, can thus be defined (40). AtxA enhances *pagA* transcription in a heterologous host, *B. subtilis*, which suggests that it is not the bicarbonate intermediate (119). Also, AtxA synthesis is not affected by bicarbonate. Yet, in the absence of AtxA, when all other genetic elements are in their native loci, no bicarbonate induction of *pagA* transcription is observed (59). Furthermore, AtxA is required for the expression of the three toxin genes in vitro and in vivo (15, 119). A $pXO1^{+}$ $pXO2^{-}$ strain devoid of AtxA elicits a much weaker immune response than the parental strain to the toxin proteins. Moreover, it is avirulent in the mouse model of anthrax (15). Other pXO1-encoded genes are regulated by bicarbonate and by AtxA (48). The regulatory and physiological links, if any, between the bicarbonate and the AtxA controls should be analyzed.

The existence of a transcriptional repressor of the three toxin genes has been suggested by the description of a mutant in which these genes are strongly expressed in a bicarbonate-independent manner (119). It is unfortunate that this mutant has not been further characterized. There have been searches for other toxin gene regulators. *pagR*, the second gene of the *pag* operon, represses expression of the *pag* operon (46). PagR also influences the expression of other pXO1-encoded genes (47). The expression of *pagA* is highest during transition into the stationary phase, and AbrB, an ortholog of the *B. subtilis* transition-state regulator, is implicated in this regulation (T.M. Koehler, personal communication). A pXO2-encoded transcriptional activator of *capB*, AcpA, has been described to regulate capsule synthesis (122). Its own synthesis is, at the transcriptional level, bicarbonate dependent. The presence of AcpA is not required for the AtxA-dependent enhancement of *capB* expression (121). The cascade of regulators between external bicarbonate and the induction of transcription of bicarbonate-dependent genes needs to be unraveled.

S-Layer Regulation

The two S-layer proteins, Sap and EA1, are both synthesized in vitro and in vivo despite the energy consumption (74, 75). The presence of both proteins in all

media tested initially suggested a constitutive, nonregulated synthesis. However, Sap is found both on the cell surface and in the supernatant, whereas EA1 is exclusively cell anchored. Sap and EA1 are (see above) sequentially at the surface and are synthesized sequentially: Sap during exponential growth and EA1 during stationary phase. *sap* and *eag* are contiguous and in the same orientation on the chromosome. They are separated by a noncoding fragment of ~700 bp and are two independent transcriptional units (T. Mignot, S. Mesnage, E. Couture-Tosi, M. Mock & A. Fouet, manuscript in preparation). Sap is a bifunctional protein, being an S-layer protein and a DNA-binding protein. Indeed, Sap is a transcriptional repressor of *eag*. Thus *eag* is not expressed until *sap* expression is repressed. Therefore, Sap is responsible for the developmentally controlled synthesis of EA1.

σ^B Factor and Other Regulated Components

Environmental stimuli most probably change during infection and stressful conditions certainly occur. Late in the development of the disease, for instance, there is septicemia and the bacilli are in stationary phase. As the bacilli require external oxygen for sporulation, they have to survive as nongrowing vegetative cells. This may constitute a stressful situation for the bacilli. Such considerations led to the hypothesis that *B. anthracis* has a stress regulon. In gram-positive bacteria, the transcriptional factor σ^B and the genes it controls define such a regulon. The operon encoding the general stress-transcription factor σ^B and two proteins of its regulatory network, RsbV and RsbW, has been cloned from *B. anthracis* (28). The *B. anthracis sigB* operon contains three genes, whereas there are eight in *B. subtilis*. Nevertheless, the gene cluster is similar to the *B. subtilis sigB* operon, in both the primary sequence of the gene products and in the order of the three genes. The *B. anthracis* operon is preceded by a σ^B-like promoter sequence, the expression of which depends on an intact σ^B transcription factor. The *B. anthracis* and *B. subtilis* σ^B operons do not respond to the same stresses. For instance, during stationary phase, *B. anthracis* σ^B operon is transcribed later than that of *B. subtilis*. The differences presumably reflect physiological and ecological differences between these two bacilli. A *B. anthracis*-σ^B mutant is less virulent than the parental strain, which suggests that σ^B may confer an advantage in physiological conditions and indicates that σ^B is a minor virulence factor (28).

Knowing which proteins are induced in the host would greatly advance the understanding of anthrax physiopathology. For instance, extracellular proteins certainly play a role in the host-pathogen interaction. Extracytoplasmic factors (ECF), which are in fact sigma factors, are involved in the transcription of many extracellular proteins (51 and references therein). A preliminary analysis of the nearly completed *B. anthracis* genome sequence reveals the presence of ECFs. Their regulons merit a thorough analysis. With the progress of the genome sequencing and the transcriptome and proteomic studies that will follow, many other proteins synthesized in vivo should soon be identified.

ANTHRAX PROPHYLAXIES

The Vaccines

Anthrax was the first disease for which the principle of a bacterial vaccination was found to be effective. In the famous field trial of Pouilly Le Fort in 1881, the attenuated *B. anthracis* strain, obtained by Pasteur, was the first live vaccine to be used successfully. The Sterne strain, isolated in 1937 (112), is cured of pXO2 and consequently is toxinogenic and noncapsulated. The live vaccine is still used for veterinary purposes (115). This strain yields satisfactory protection results, and its worldwide use has greatly contributed to control the incidence of anthrax in livestock and wild animals. Nevertheless, there are some safety concerns hindering its use in sensitive animal species due to residual virulence. The development of recombinant genetics in *B. anthracis* has led to the appearance of modern alternatives. Sterne-strain derivatives producing genetically detoxified EF and LF offer safer, nontoxic, live vaccine candidates (7, 90). The use of such strains would also prevent environmental contamination with strains harboring wild-type pXO1. These studies have also highlighted the potential of *B. anthracis* for delivering foreign antigens in vivo. Various strategies have been used to induce specific protective immunity: Production of antigens under the control of the *pag* promoter (106, 108), anchoring at the bacterial surface by fusion to the SLH motifs of EA1 (76), and presentation to the immune system via PA through fusion to LF254 (6) have all proven to be efficient. Moreover, the response can be directed toward humoral or cellular immunity if the recombinant strain also produces listeriolysin O (106). *B. anthracis* is therefore a potential vehicle for multivalent veterinary live vaccines.

Because of its residual virulence, the live vaccine is not considered suitable for human use in Western countries. In the 1950s, after toxin discovery, nonliving vaccines were developed in the United Kingdom and in the United States. They are both PA-based cell-free vaccines from culture supernatants of the Sterne strain (31, 116). Multiple immunizations are required and there are cases of reactogenicity. An old field study indicated that the vaccine provides protection to an exposed susceptible human population. Recombinant PA can now be produced from different heterologous organisms, including *B. subtilis* (116), and the safety and consistency of the PA preparations has been improved. Highly purified PA preparations and recombinant PA have been tested in various animal models, including mice, guinea pigs, rabbits, and monkeys (31, 116, 137), in combination with various adjuvants (31, 53). However, PA vaccines provide less protection than live spore vaccines against lethal challenge with several strains of *B. anthracis*. Moreover, there was no direct correlation between anti-PA titers and protection (118, 131). Therefore, other antigens in addition to PA and/or cellular immunity may be required for full protection. Recent studies point to spore antigens provided by live spore vaccines (12).

B. anthracis Detection, Phylogeny, and Ecology

B. anthracis is found in soil and in the same ecological niche as are strains of *B. cereus*. All these strains belong to the *B. cereus* group, which also comprises *B. thuringiensis*. The distinction between these species is rather difficult. The simplest markers for distinguishing these strains are in fact attributable to their plasmids. For instance, capsulation of *B. anthracis* gives rise to mucoid colonies on given growth media. However, these distinction traits are not satisfactory for two major reasons. First, they depend on the growth media used. Second, and even more important, pXO2 is a plasmid that can be lost naturally (117). The cured isolates are not as virulent but should still be identified because they are *B. anthracis*, and their detection indicates the probable presence of fully virulent strains in the same sample. Other techniques have been developed, such as biochemical character analysis (lectins, fatty acids, glycosidase activities) (for references see 114) and phage typing (97 and references therein). Lack of motility and susceptibility to penicillin have been widely used, but the existence of *B. anthracis* strains that cannot be identified by these tests have been reported, rendering these approaches unreliable. During the past decade, the threat of *B. anthracis* as a biological weapon has revitalized the search for rapid and reliable methods of detection and identification. A randomly amplified polymorphic DNA marker has been described, but it is uninformative about the plasmid content and consequently the strain virulence (14). Ribotyping and more or less random polymerase chain reaction techniques have also been compared, and not one of the methods has been developed into a perfect tool (104). A *B. anthracis*–specific chromosomal DNA marker has been isolated, and a multiplex polymerase chain reaction analysis has been established based on this and plasmid markers (86, 94). The combination is perfectly specific for virulent *B. anthracis* and also indicates its virulence state. However, the chromosomal marker was also found in other strains from the *B. cereus* group, implying that nonvirulent *B. anthracis* strains cannot be unambiguously discriminated from other *B. cereus* strains with this tool (87).

DNA-DNA hybridization experiments suggested that *B. anthracis* and *B. cereus* were more closely related than the taxonomic classification implied (54). Multilocus enzyme electrophoresis and multiple-locus sequence typing showed that some *B. cereus* strains are more closely related to *B. anthracis* than to other *B. cereus* strains (45). Consequently, *B. anthracis* should be considered a lineage of *B. cereus* and not an independent species.

According to molecular analyses, *B. anthracis* is extremely monomorphic and thus constitutes only a single lineage of *B. cereus*. This has been confirmed by many groups while developing identification techniques. The sequence diversity at two loci has been studied in detail. One locus is chromosomal and overlaps a chromosomal "hyper" variable ORF of *B. anthracis*, and the other is a plasmid-encoded toxin gene. Both show little diversity (0.125% and 0.21% diversity per nucleotide, respectively) (92, 102). The monomorphism was further demonstrated

by the amplified fragment length polymorphism (AFLP) method (55). Genome-wide analysis of 357 AFLP characters, covering 6.3% of the genome, revealed only rare AFLP marker variation. Thus, AFLP is an extremely powerful tool for strain identification. Indeed, *B. anthracis* may be the most genetically uniform bacterial species known. Nevertheless, two distinct genetic subgroups (now termed A and B) were identified by analysis of 79 *B. anthracis* isolates. However, because the molecular basis of the variation was unknown, phylogenic conclusions can only be drawn with caution. Another molecular typing system was therefore developed based on variable-number tandem repeat (VNTR) (56). Multiple-locus VNTR analysis (MLVA) using eight VNTR regions of the *B. anthracis* genome, including one for each plasmid, was tested using 426 strains isolated worldwide. The VNTR markers frequently identified multiple, from 2 to 9, alleles. Eighty-nine distinct genotypes clustering into six major genetic groups were defined. They fall into two major phylogenetic groups that are identical to the A and B lineages defined by AFLP analysis. Group A is widely distributed geographically, whereas group B seems to be limited to a few regions (56). The association between endemicity of *B. anthracis* and soil characteristics such as calcium content and pH has been established (16). MLVA in Southern Africa correlated to soil analysis, indicates that group A strains are associated with broader ranges of soils than are group B strains (110). The differences in geographic diversity may be a consequence of ecological factors affecting strain persistence differentially. This may involve effects of the soil environment on sporulation efficiency and inhibition of germination. The existence of isolates from one group or the other may also be linked to germination and multiplication rates in particular mammals. MLVA appears to be a good tool for exploring the epidemiology and ecology of anthrax. The biological basis of the test and the products encoded by the VNTR harboring genes should therefore be studied thoroughly.

CONCLUDING REMARKS

In the very near future, the *B. anthracis* genome sequence will be completed. This will open up the fields of transcriptome and proteomics. Moreover, progress in these areas will benefit from the data expected shortly from parallel work in *B. cereus*. The fallout will include a better understanding of the persistence and virulence factors common to the *B. cereus* group and of those specific to *B. anthracis*. An exciting field of research concerning the *B. anthracis* host relationship is the molecular characterization of the complex toxin-related pathogenesis. Moreover, there will be an explosion in information about the exact contribution of surface structures to survival, germination, and bacillus multiplication. The combination of progress in our understanding of these two issues, toxin and surface, should greatly improve our ability to design safe and efficient acellular vaccines. *B. anthracis*, with which medical microbiology began, could very well continue to be the flagship of progress in both medical and environmental microbiology.

ACKNOWLEDGMENTS

We would like to thank M. Ferrand and M. Lévy for secretarial assistance and help with the figures, respectively.

Visit the Annual Reviews home page at www.AnnualReviews.org

LITERATURE CITED

1. Ashiuchi M, Soda K, Misono H. 1999. A poly-γ-glutamate synthetic system of *Bacillus subtilis* IFO 3336: gene cloning and biochemical analysis of poly-γ-glutamate produced by *Escherichia coli* clone cells. *Biochem. Biophys. Res. Commun.* 263:6–12
2. Bartkus JM, Leppla SH. 1989. Transcriptional regulation of the protective antigen gene of *Bacillus anthracis*. *Infect. Immun.* 57:2295–300
3. Beall FA, Taylor MJ, Thorne CB. 1962. Rapid lethal effects in rats of a third component found upon fractionating the toxin of *Bacillus anthracis*. *J. Bacteriol.* 83:1274–80
4. Beauregard K, Collier RJ, Swanson JA. 2000. Proteolytic activation of receptor-bound anthrax protective antigen on macrophages promotes its internalization. *Cell. Microbiol.* 2:251–58
5. Bragg TS, Robertson DL. 1989. Nucleotide sequence and analysis of the lethal factor gene (lef) from *Bacillus anthracis*. *Gene* 81:45–54
6. Brossier F, Weber-Levy M, Mock M, Sirard J-C. 2000. Protective antigen-mediated antibody response against a heterologous protein produced *in vivo* by *Bacillus anthracis*. *Infect. Immun.* 68: 5731–34
7. Brossier F, Weber-Levy M, Mock M, Sirard J-C. 2000. Role of toxin functional domains in anthrax pathogenesis. *Infect. Immun.* 68:1781–86
8. Cataldi A, Labruyère E, Mock M. 1990. Construction and characterization of a protective antigen-deficient *Bacillus anthracis* strain. *Mol. Microbiol.* 4:1111–17
9. Charlton S, Moir AJ, Baillie L, Moir A. 1999. Characterization of the exosporium of *Bacillus cereus*. *J. Appl. Microbiol.* 87:241–45
10. Chauvaux S, Matuschek M, Beguin P. 1999. Distinct affinity of binding sites for S-layer homologous domains in *Clostridium thermocellum* and *Bacillus anthracis* cell envelopes. *J. Bacteriol.* 181:2455–58
11. Clements MO, Moir A. 1998. Role of the *gerI* operon of *Bacillus cereus* 569 in the response of spores to germinants. *J. Bacteriol.* 180:6729–35
12. Cohen S, Mendelson I, Altboum Z, Kobiler D, Elhanany E, et al. 2000. Attenuated nontoxinogenic and nonencapsulated recombinant *Bacillus anthracis* spore vaccines protect against anthrax. *Infect. Immun.* 68:4549–58
13. Corfe BM, Sammons RL, Smith DA, Mauël C. 1994. The *gerB* region of the *Bacillus subtilis* 168 chromosome encodes a homologue of the *gerA* spore germination operon. *Microbiology* 140:471–78
14. Daffonchio D, Borin S, Frova G, Gallo R, Mori E, et al. 1999. A randomly amplified polymorphic DNA marker specific for the *Bacillus cereus* group is diagnostic for *Bacillus anthracis*. *Appl. Environ. Microbiol.* 65:1298–303
15. Dai Z, Sirard J-C, Mock M, Koehler TM. 1995. The *atxA* gene product activates transcription of the anthrax toxin genes and is essential for virulence. *Mol. Microbiol.* 16:1171–81
16. Dragon DC, Rennie RP. 1995. The ecology of anthrax spores: tough but not invincible. *Can. Vet. J.* 36:295–301

17. Duesbery NS, Webb CP, Leppla SH, Gordon VM, Klimpel KR, et al. 1998. Proteolytic inactivation of MAP-Kinase-Kinase by anthrax lethal factor. *Science* 280:734–37
18. Ekwunife FS, Singh J, Taylor KG, Doyle RJ. 1991. Isolation and purification of cell wall polysaccharide of *Bacillus anthracis* (Δ Sterne). *FEMS Microbiol. Lett.* 82:257–62
19. Elliott JL, Mogridge J, Collier RJ. 2000. A quantitative study of the interactions of *Bacillus anthracis* edema factor and lethal factor with activated protective antigen. *Biochemistry* 39:6706–13
20. Erwin JL, Little SF, Friedlander AM, DaSilva L, Bavari S, Chanh TC. 2000. *Macrophage-derived cell lines do not express pro-inflammatory cytokines after exposure to anthrax lethal toxin.* Presented at ASM, Gen. Meeting, 100th, Los Angeles
21. Escuyer V, Collier RJ. 1991. Anthrax protective antigen interacts with a specific receptor on the surface of CHO-K1 cells. *Infect. Immun.* 59:3381–86
22. Escuyer V, Duflot E, Sezer O, Danchin A, Mock M. 1988. Structural homology between virulence-associated bacterial adenylate cyclases. *Gene* 71:293–98
23. Etienne-Toumelin I, Sirard J-C, Duflot E, Mock M, Fouet A. 1995. Characterization of the *Bacillus anthracis* S-layer: cloning and sequencing of the structural gene. *J. Bacteriol.* 177:614–20
24. Ezzell JW Jr, Abshire TG. 1988. Immunological analysis of cell-associated antigens of *Bacillus anthracis*. *Infect. Immun.* 56:349–56
25. Ezzell JW Jr, Abshire TG. 1992. Serum protease cleavage of *Bacillus anthracis* protective antigen. *J. Gen. Microbiol.* 138:543–49
26. Finkelstein A. 1994. The channel formed in planar lipid bilayers by the protective antigen component of anthrax toxin. *Toxicology* 87:29–41
27. Fouet A, Mock M. 1996. Differential influence of the two *Bacillus anthracis* plasmids on regulation of virulence gene expression. *Infect. Immun.* 64:4928–32
28. Fouet A, Namy O, Lambert G. 2000. Characterization of the operon encoding the alternative s^B factor from *Bacillus anthracis* and its role in virulence. *J. Bacteriol.* 182:5036–45
29. Fouet A, Sirard JC, Mock M. 1994. *Bacillus anthracis* pXO1 virulence plasmid encodes a type 1 DNA topoisomerase. *Mol. Microbiol.* 11:471–79
30. Friedlander AM. 1986. Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. *J. Biol. Chem.* 261:7123–26
31. Friedlander AM, Pittman PR, Parker GW. 1999. Anthrax vaccine: evidence for safety and efficacy against inhalational anthrax. *JAMA* 282:2104–6
32. Garcia-Patrone M, Tandecarz JS. 1995. A glycoprotein multimer from *Bacillus thuringiensis* sporangia: dissociation into subunits and sugar composition. *Mol. Cell. Biochem.* 145:29–37
33. Gerhardt P. 1967. Cytology of *Bacillus anthracis*. *Fed. Proc.* 26:1504–17
34. Gladstone GP. 1946. Immunity of anthrax: protective antigen present in cell-free culture filtrates. *Br. J. Exp. Pathol.* 27:393–410
35. Goodman JW, Nitecki DE. 1967. Studies on the relation of a prior immune response to immunogenicity. *Immunology* 13:577–83
36. Green BD, Battisti L, Koehler TM, Thorne CB, Ivins BE. 1985. Demonstration of a capsule plasmid in *Bacillus anthracis*. *Infect. Immun.* 49:291–97
37. Guidi-Rontani C, Pereira Y, Ruffié S, Sirard J-C, Weber-Levy M, Mock M. 1999. Identification and characterization of a germination operon on the virulence plasmid pXO1 of *Bacillus anthracis*. *Mol. Microbiol.* 33:407–14
38. Guidi-Rontani C, Weber-Levy M, Labruyère E, Mock M. 1999. Germination of *Bacillus anthracis* spores within

alveolar macrophages. *Mol. Microbiol.* 31:9–17
39. Guidi-Rontani C, Weber-Levy M, Mock M, Cabiaux V. 2000. Translocation of *Bacillus anthracis* lethal and odema factors across endosome membranes. *Cell. Microbiol.* 2:259–64
40. Guignot J, Mock M, Fouet A. 1997. AtxA activates the transcription of genes harbored by both *Bacillus anthracis* virulence plasmids. *FEMS Microbiol. Lett.* 147:203–7
41. Hachisuka Y, Kojima K, Sato T. 1966. Fine filaments on the outside of the exosporium of *Bacillus anthracis* spores. *J. Bacteriol.* 91:2382–84
42. Hachisuka Y, Kozuka S, Tsujikawa M. 1984. Exosporia and appendages of spores of *Bacillus* species. *Microbiol. Immunol.* 28:619–24
43. Hanna P. 1998. Anthrax pathogenesis and host response. *Curr. Top. Microbiol. Immunol.* 225:13–35
44. Hanna PC, Acosta D, Collier RJ. 1993. On the role of macrophages in anthrax. *Proc. Natl. Acad. Sci. USA* 90:10198–201
45. Helgason E, Økstad OA, Caugant DA, Johansen HA, Fouet A, et al. 2000. *Bacillus anthracis, Bacillus cereus* and *Bacillus thuringiensis*—one species on the basis of genetic evidence. *Appl. Environ. Microbiol.* 66:2627–30
46. Hoffmaster AR, Koehler TM. 1999. Autogenous regulation of the *Bacillus anthracis* pag operon. *J. Bacteriol.* 181:4485–92
47. Hoffmaster AR, Koehler TM. 1999. Control of virulence gene expression in *Bacillus anthracis*. *J. Appl. Microbiol.* 87:279–81
48. Hoffmaster AR, Koehler TM. 1997. The anthrax toxin activator gene *atxA* is associated with CO_2-enhanced non-toxin gene expression in *Bacillus anthracis*. *Infect. Immun.* 65:3091–99
49. Holt SC, Leadbetter ER. 1969. Comparative ultrastructure of selected aerobic spore-forming bacteria: a freeze-etching study. *Bacteriol. Rev.* 33:346–78
50. Hoover DL, Friedlander AM, Rogers LC, Yoon I-K, Warren RL, Cross AS. 1994. Anthrax edema toxin differentially regulates lipopolysaccharide-induced monocyte production of tumor necrosis factor alpha and interleukin-6 by increasing intracellular cyclic AMP. *Infect. Immun.* 62:4432–39
51. Huang X, Gaballa A, Cao M, Helmann JD. 1999. Identification of target promoters for the *Bacillus subtilis* extracytoplasmic function sigma factor, sigma W. *Mol. Microbiol.* 31:361–71
52. Irie R, Fujita Y, Kobayashi M. 1996. Nucleotide sequence and gene organization of the *gerK* spore germination locus of *Bacillus subtilis* 168. *J. Gen. Appl. Microbiol.* 42:141–53
53. Ivins B, Fellows P, Pitt L, Estep J, Farchaus J, et al. 1995. Experimental anthrax vaccines: efficacy of adjuvants combined with protective antigen against an aerosol *Bacillus anthracis* spore challenge in guinea pigs. *Vaccine* 13:1779–84
54. Kaneko T, Nozaki R, Aizawa K. 1978. Deoxyribonucleic acid relatedness between *Bacillus anthracis* and *Bacillus cereus* and *Bacillus thuringiensis*. *Microbiol. Immunol.* 22:639–41
55. Keim P, Kalif A, Schupp J, Hill K, Travis SE, et al. 1997. Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. *J. Bacteriol.* 179:818–24
56. Keim P, Price LB, Klevytska AM, Smith KL, Schupp JM, et al. 2000. Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. *J. Bacteriol.* 182:2928–36
57. Klimpel KR, Arora N, Leppla SH. 1994. Anthrax toxin lethal factor contains a zinc metalloprotease consensus sequence which is required for lethal toxin activity. *Mol. Microbiol.* 13:1093–100

58. Kochi SK, Schiavo G, Mock M, Montecucco C. 1994. Zinc content of the *Bacillus anthracis* lethal factor. *FEMS Microbiol. Lett.* 124:343–48
59. Koehler TM, Dai Z, Kaufman-Yarbray M. 1994. Regulation of the *Bacillus anthracis* protective antigen gene: CO_2 and a *trans*-acting element activate transcription from one of two promoters. *J. Bacteriol.* 176:586–95
60. König H, Niemetz R. 1997. Polyglutamate surface polypeptides in Bacteria and Archae. *FEMS Microbiol. Rev.* 20:36–39
61. Labruyère E, Kochi S, Mock M. 1994. Functional analysis of the NH_2-terminal region of the *Bacillus anthracis* edema factor. In *Bacterial Protein Toxins*, ed. J Freer, R Aitken, JE Alouf, G Boulnois, 73:271–72. FEMS Symp.
62. Labruyère E, Mock M, Ladant D, Michelson S, Gilles AM, et al. 1990. Characterization of ATP and calmodulin-binding properties of a truncated form of *Bacillus anthracis* adenylate cyclase. *Biochemistry* 29:4922–28
63. Labruyère E, Mock M, Surewicz WK, Mantsch HH, Rose T, et al. 1991. Structural and ligand-binding properties of a truncated form of *Bacillus anthracis* adenylate cyclase and of a catalytically inactive variant in which glutamine substitutes for lysine-346. *Biochemistry* 30:2619–24
64. Leppla S. 1995. Anthrax toxins. In *Bacterial Toxins and Virulence Factors in Disease*, ed. J Moss, B Iglewski, M Vaughan, AT Tu, 8:543–72. New York/Hong Kong: Basel
65. Leppla SH. 1982. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations in eukaryotic cells. *Proc. Natl. Acad. Sci. USA* 79:3162–66
66. Lincoln RE, Hodges DR, Klein F, Mahlandt BG, Jones WI Jr, et al. 1965. Role of the lymphatics in the pathogenesis of anthrax. *J. Infect. Dis.* 115:481–94
67. Lindeque PM, Turnbull PC. 1994. Ecology and epidemiology of anthrax in the Etosha National Park, Namibia. *Onderstepoort J. Vet. Res.* 61:71–83
68. Little SF, Novak JM, Lowe JR, Leppla SH, Singh Y, et al. 1996. Characterization of lethal factor binding and cell receptor binding domains of protective antigen of *Bacillus anthracis* using monoclonal antibodies. *Microbiology* 142:707–15
69. Lupas A, Engelhardt H, Peters J, Santarius U, Volker S, Baumeister W. 1994. Domain structure of the *Acetogenium kivui* surface layer revealed by electron crystallography and sequence analysis. *J. Bacteriol.* 176:1224–33
70. Makino SI, Uchida I, Terakado N, Sasakawa C, Yoshikawa M. 1989. Molecular characterization and protein analysis of the *cap* region, which is essential for encapsulation in *Bacillus anthracis*. *J. Bacteriol.* 171:722–30
71. Matz LL, Beaman TC, Gerhardt P. 1970. Chemical composition of exosporium from spores of *Bacillus cereus*. *J. Bacteriol.* 101:196–201
72. Mesnage S, Fontaine T, Mignot T, Delepierre M, Mock M, Fouet A. 2000. Bacterial SLH domain proteins are noncovalently anchored to the cell surface via a conserved mechanism involving wall polysaccharide pyruvylation. *EMBO J.* 19:4473–84
73. Mesnage S, Tosi-Couture E, Fouet A. 1999. Production and cell surface anchoring of functional fusions between the SLH motifs of the *Bacillus anthracis* S-layer proteins and the *Bacillus subtilis* levansucrase. *Mol. Microbiol.* 31:927–36
74. Mesnage S, Tosi-Couture E, Gounon P, Mock M, Fouet A. 1998. The capsule and S-layer: two independent and yet compatible macromolecular structures in *Bacillus anthracis*. *J. Bacteriol.* 180:52–58
75. Mesnage S, Tosi-Couture E, Mock M, Gounon P, Fouet A. 1997. Molecular characterization of the *Bacillus anthracis* main S-layer component: evidence that it

is the major cell-associated antigen. *Mol. Microbiol.* 23:1147–55
76. Mesnage S, Weber-Levy M, Haustant M, Mock M, Fouet A. 1999. Cell surface-exposed tetanus toxin fragment C produced by recombinant *Bacillus anthracis* protects against tetanus toxin. *Infect. Immun.* 67:4847–50
77. Mikesell P, Ivins BE, Ristroph JD, Dreier TM. 1983. Evidence for plasmid-mediated toxin production in *Bacillus anthracis*. *Infect. Immun.* 39:371–76
78. Milne J, Blanke SR, Hanna PC, Collier RJ. 1995. Protective antigen-binding domain of anthrax lethal factor mediates translocation of a heterologous protein fused to its amino- or carboxy-terminus. *Mol. Microbiol.* 15:661–66
79. Milne J, Furlong D, Hanna PC, Wall JS, Collier RJ. 1994. Anthrax protective antigen forms oligomers during intoxication of mammalian cells. *J. Biol. Chem.* 269:20607–12
80. Milne JC, Collier RJ. 1993. pH-dependent permeabilization of the plasma membrane of mammalian cells by anthrax protective antigen. *Mol. Microbiol.* 10:647–53
81. Mock M, Labruyère E, Glaser P, Danchin A, Ullmann A. 1988. Cloning and expression of the calmodulin-sensitive *Bacillus anthracis* adenylate cyclase in *Escherichia coli*. *Gene* 64:277–84
82. Munier H, Bouhss A, Krin E, Danchin A, Gilles A-M, et al. 1992. The role of histidine 63 in the catalytic mechanism of *Bordetella pertussis* adenylate cyclase. *J. Biol. Chem.* 267:9816–20
83. Nishihara T, Takubo Y, Kawamata E, Koshikawa T, Ogaki J, Kondo M. 1989. Role of outer coat in resistance of *Bacillus megaterium* spore. *J. Biochem.* (*Tokyo*) 106:270–73
84. O'Brien J, Friedlander A, Dreier T, Ezzell J, Leppla S. 1985. Effects of anthrax toxin components on human neutrophils. *Infect. Immun.* 47:306–10
85. Okinaka RT, Cloud K, Hampton O, Hoffmaster AR, Hill KK, et al. 1999. Sequence and organization of pXO1, the large *Bacillus anthracis* plasmid harboring the anthrax toxin genes. *J. Bacteriol.* 181:6509–15
86. Patra G, Sylvestre P, Ramisse V, Thérasse J, Guesdon JL. 1996. Isolation of a specific chromosomic DNA sequence of *Bacillus anthracis* and its possible use in diagnosis. *FEMS Immunol. Med. Microbiol.* 15:223–31
87. Patra G, Vaissaire J, Weber-Levy M, Le Doujet C, Mock M. 1998. Molecular characterization of *Bacillus* strains involved in outbreaks of anthrax in France in 1997. *J. Clin. Microbiol.* 36:3412–14
88. Pellizzari R, Guidi-Rontani C, Vitale G, Mock M, Montecucco C. 1999. Anthrax lethal factor cleaves MKK3 in macrophages and inhibits the LPS/IFNγ-induced release of NO and TNFα. *FEBS Lett.* 462:199–204
89. Petosa C, Collier RJ, Klimpel KR, Leppla SH, Liddington RC. 1997. Crystal structure of the anthrax toxin protective antigen. *Nature* 385:833–38
90. Pezard C, Berche P, Mock M. 1991. Contribution of individual toxin components to virulence of *Bacillus anthracis*. *Infect. Immun.* 59:3472–77
91. Preisz H. 1909. Experimentelle Studien Åber Virulenz, Empfänglichkeit und Immunität beim Milzbrand. *Zeitschr. Immunität. -Forsch.* 5:341–452
92. Price LB, Hugh-Jones M, Jackson PJ, Keim P. 1999. Genetic diversity in the protective antigen gene of *Bacillus anthracis*. *J. Bacteriol.* 181:2358–62
93. Quinn CP, Singh Y, Klimpel KR, Leppla SH. 1991. Functional mapping of anthrax toxin lethal factor by in-frame insertion mutagenesis. *J. Biol. Chem.* 266:20124–30
94. Ramisse V, Patra G, Garrigue H, Guesdon J-L, Mock M. 1996. Identification and characterization of *Bacillus anthracis* by multiplex PCR analysis of sequences

on plasmids pXO1 and pXO2 and chromosomal DNA. *FEMS Microbiol. Lett.* 145:9–16
95. Ray KC, Mesnage S, Washburn R, Mock M, Fouet A, Blaser M. 1998. *Complement binding to Bacillus anthracis mutants lacking surface structures.* Presented at ASM Gen. Meet., 98th, Atlanta
96. Record BR, Wallis RG. 1956. Physicochemical examination of polyglutamic acid from *Bacillus anthracis* grown *in vivo*. *Biochem. J.* 63:443–47
97. Redmond C, Henderson I, Turnbull PCB, Bowen J. 1996. Phage from different strains of *Bacillus anthracis*. In *Anthrax: Proc. Int. Work.*, ed. PCB Turnbull, Salisbury Med. Bull., Spec. Suppl. 87:60–63
98. Roberts JE, Watters JW, Ballard JD, Dietrich WF. 1998. *Ltx1*, a mouse locus that influences the susceptibility of macrophages to cytolysis caused by intoxication with *Bacillus anthracis* lethal factor, maps to chromosome 11. *Mol. Microbiol.* 29:581–91
99. Robertson DL, Leppla SH. 1986. Molecular cloning and expression in *Escherichia coli* of the lethal factor gene of *Bacillus anthracis*. *Gene* 44:71–78
100. Ross JM. 1957. The pathogenesis of anthrax following the administration of spores by the respiratory route. *J. Pathol. Bact.* 73:485–94
101. Sára M, Sleytr UB. 2000. S-layer proteins. *J. Bacteriol.* 182:859–68
102. Schupp JM, Klevytska AM, Zinser G, Price LB, Keim P. 2000. *vrrB*, a hypervariable open reading frame in *Bacillus anthracis*. *J. Bacteriol.* 182:3989–97
103. Shafa F, Moberly BJ, Gerhardt P. 1966. Cytological features of anthrax spores phagocytized *in vitro* by rabbit alveolar macrophages. *J. Infect. Dis.* 116:401–13
104. Shangkuan YH, Yang JF, Lin HC, Shaio MF. 2000. Comparison of PCR-RFLP, ribotyping and ERIC-PCR for typing *Bacillus anthracis* and *Bacillus cereus* strains. *J. Appl. Microbiol.* 89:452–62
105. Singh Y, Klimpel KR, Arora N, Sharma M, Leppla SH. 1994. The chymotrypsin-sensitive site, FFD^{315}, in anthrax toxin protective antigen is required for translocation of lethal factor. *J. Biol. Chem.* 269:29039–46
106. Sirard J-C, Fayolle C, de Chastellier C, Mock M, Leclerc C, Berche P. 1997. Intracytoplasmic delivery of listeriolysin O by a vaccinal strain of *Bacillus anthracis* induces CD8-mediated protection against *Listeria monocytogenes*. *J. Immunol.* 159:4435–43
107. Sirard J-C, Mock M, Fouet A. 1994. The three *Bacillus anthracis* toxin genes are coordinately regulated by bicarbonate and temperature. *J. Bacteriol.* 176:5188–92
108. Sirard J-C, Weber M, Duflot E, Popoff MR, Mock M. 1997. A recombinant *Bacillus anthracis* strain producing *Clostridium perfringens* Ib component induces protection against iota toxins. *Infect. Immun.* 65:2029–33
109. Smith H, Keppie J, Stanley JL. 1955. The chemical basis of the virulence of *Bacillus anthracis*. V. The specific toxin produced by *Bacillus anthracis in vivo*. *Br. J. Exp. Pathol.* 36:460–72
110. Smith TJ, Blackman SA, Foster SJ. 2000. Autolysins of *Bacillus subtilis*: multiple enzymes with multiple functions. *Microbiology* 146:249–62
111. Stanley JL, Smith H. 1961. Purification of factor I and recognition of a third factor of the anthrax toxin. *J. Gen. Microbiol.* 26:49–66
112. Sterne M. 1937. Avirulent anthrax vaccine. *Onderstepoort J. Vet. Sci. Animal Ind.* 21:41–43
113. Tang G, Leppla SH. 1999. Proteasome activity is required for anthrax lethal toxin to kill macrophages. *Infect. Immun.* 67:3055–60
114. Thorne CB. 1993. *Bacillus anthracis*. In *Bacillus subtilis and Other Gram-positive Bacteria*, ed. AL Sonenshein, JA Hoch, R Losick, pp. 113–24. Washington, DC: ASM

115. Turnbull PCB. 1991. Anthrax vaccines: past, present and future. *Vaccine* 9:533–39
116. Turnbull PCB. 2000. Current status of immunization against anthrax: old vaccines may be here to stay for a while. *Curr. Opin. Infect. Dis.* 13:113–20
117. Turnbull PCB, Hutson RA, Ward MJ, Jones MN, Quinn CP, et al. 1992. *Bacillus anthracis* but not always anthrax. *J. Appl. Bact.* 72:21–28
118. Turnbull PCB, Leppla SH, Broster MG, Quinn CP, Melling J. 1988. Antibodies to anthrax toxin in humans and guinea pigs and their relevance to protective immunity. *Med. Microbiol. Immunol.* 177:293–303
119. Uchida I, Hornung JM, Thorne CB, Klimpel KR, Leppla SH. 1993. Cloning and characterization of a gene whose product is a *trans*-activator of anthrax toxin synthesis. *J. Bacteriol.* 175:5329–38
120. Uchida I, Makino S, Sasakawa C, Yoshikawa M, Sugimoto C, Terakado N. 1993. Identification of a novel gene, *dep*, associated with depolymerization of the capsular polymer in *Bacillus anthracis*. *Mol. Microbiol.* 9:487–96
121. Uchida I, Makino S-I, Sekizaki T, Terakado N. 1997. Cross-talk to the genes for capsule synthesis of *Bacillus anthracis* by *atxA*, the gene encoding *trans*-activator of anthrax toxin synthesis. *Mol. Microbiol.* 23:1203–13
122. Vietri NJ, Marrero R, Hoover TA, Welkos SL. 1995. Identification and characterization of a *trans*-activator involved in the regulation of encapsulation by *Bacillus anthracis*. *Gene* 152:1–9
123. Vitale G, Pellizzari R, Recchi C, Napolitani G, Mock M, Montecucco C. 1998. Anthrax lethal factor cleaves the N-terminus of MAPKKs and induces Tyrosine/Threonine phosphorylation of MAPKs in cultured macrophages. *Biochem. Biophys. Res. Commun.* 248:706–11
124. Vodkin MH, Leppla SH. 1983. Cloning of the protective antigen gene of *Bacillus anthracis*. *Cell* 34:693–97
125. Wade BH, Wright GG, Hewlett EL, Leppla SH, Mandell GL. 1985. Anthrax toxin components stimulate chemotaxis of human polymorphonuclear neutrophils. *Proc. Soc. Exp. Biol. Med.* 179:159–62
126. Wang J, Mushegian A, Lory S, Jin S. 1996. Large-scale isolation of candidate virulence genes of *Pseudomonas aeruginosa* by *in vivo* selection. *Proc. Natl. Acad. Sci. USA* 93:10434–39
127. Wang JM, Hartling JA, Flanagan JM. 1997. The structure of *clpp* at 2.3 angstrom resolution suggests a model for ATP-dependent proteolysis. *Cell* 91:447–56
128. Warth AD. 1978. Molecular structure of the bacterial spore. *Adv. Microbiol. Physiol.* 17:1–47
129. Wehrli E, Scherrer P, Kubler O. 1980. The crystalline layers in spores of *Bacillus cereus* and *Bacillus thuringiensis* studied by freeze-etching and high resolution electron microscopy. *Eur. J. Cell. Biol.* 20:283–89
130. Welkos SL. 1991. Plasmid-associated virulence factors of non-toxigenic (pXO1$^-$) *Bacillus anthracis*. *Microb. Pathog.* 10: 183–98
131. Welkos SL, Friedlander AM. 1988. Comparative safety and efficacy against *Bacillus anthracis* of protective antigen and live vaccines in mice. *Microb. Pathog.* 5:127–39
132. Welkos SL, Lowe JR, Eden-McCutchan F, Vodkin M, Leppla SH, Schmidt JJ. 1988. Sequence and analysis of the DNA encoding protective antigen of *Bacillus anthracis*. *Gene* 69:287–300
133. Welkos SL, Vietri NJ, Gibbs PH. 1993. Non-toxigenic derivatives of the Ames strain of *Bacillus anthracis* are fully virulent for mice: role of plasmid pXO2 and chromosome in strain-dependent virulence. *Microb. Pathog.* 14:381–88

134. Wesche J, Elliott JL, Falnes PO, Olsnes S, Collier RJ. 1998. Characterization of membrane translocation by anthrax protective antigen. *Biochemistry* 37:15737–46
135. Widmann C, Gibson S, Jarpe MB, Johnson GL. 1999. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol. Rev.* 79:143–80
136. Xia Z, Storm DR. 1990. A-type ATP binding consensus sequences are critical for the catalytic activity of the calmodulin-sensitive adenylyl cyclase from *Bacillus anthracis*. *J. Biol. Chem.* 265:6517–20
137. Zaucha GM, Pitt LM, Estep J, Ivins BE, Friedlander AM. 1998. The pathology of experimental anthrax in rabbits exposed by inhalation and subcutaneous inoculation. *Arch. Pathol. Lab. Med.* 122:982–92
138. Zwartouw HT, Smith H. 1956. Polyglutamic acid from *Bacillus anthracis* grown *in vivo*: structure and aggressin activity. *Biochem. J.* 63:437–54