# Anthrax: Biology of *Bacillus anthracis*

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*Perhaps no other microorganism has received as much attention for its use as a potential agent for bioterrorism as Bacillus anthracis. In spite of the fact that the organism has been known for a very long time, limited progress has been made in developing a vaccine or understanding its biochemical and genetic properties. The genus Bacillus includes aerobic bacilli forming heat-resistant spores. B. anthracis are the only non-motile and the most pathogenic bacilli in this genus. Pulmonary anthrax can be caused by inhalation of just 10,000 spores of anthrax and is fatal unless treated immediately with antibiotics. Anthrax is actually a disease of herbivorous animals with humans getting infected by spores due to accidental entry into the body by contact with infected animals or contaminated animal products, insect bites, inhalation or ingestion. This lethality is principally due to the polysaccharide capsule that helps the bacterium to evade immune attack and the tripartite toxin that can kill the host depending on the mode of entry of the bacillus into the host and the host's immune status.*

## Microbiology

*Bacillus anthracis* are a large ($1-1.5\,\mu$m by $4-10\,\mu$m), aerobic, Gram-positive, non-motile, encapsulated, chain-forming, rod-shaped bacillus that form oval spores (Figure 1). The rectangular shape of the individual cell in the chain form gives rise to a boxcar-like appearance. The virulent strains of the bacillus give rise to rough, non-hemolytic to mild hemolytic colonies in blood agar, while smooth mucoid colonies are seen with avirulent strains. Box 1 provides some historical details about *B. anthracis*.

## Epidemiology

*B. anthracis* is geographically unconstrained, distributed well in almost all continents. All animals as well as man are susceptible to the bacillus to varying degrees. Herbivorous animals, both domestic and wild, like sheep, goat, horse, bison, etc. are the most susceptible. Animal anthrax has been a perennial problem in countries like Iran, Turkey, Sudan, Pakistan and parts of India. Animals get infected when they graze in an area contaminated with the spores of *B. anthracis*. Anthrax in herbivorous animals tends to be serious with terminal stages characterized by bleeding from the nostrils, mouth and bowel, contaminating the environment with the vegetative form of the bacillus, which sporulates and remains dormant till suitable climatic conditions and a host are provided. The carcasses of these dead animals act as an additional environmental hazard.

While humans are more resistant to infection than herbivores, the mechanisms underlying this phenomenon are unclear. The actual incidence of human cases annually is plagued by under-reporting and failure to receive proper medical attention; but is believed to be around 20,000–100,000. Human cases are usually agricultural (herding, farming, etc.) or industrial (fur, leather industry, etc.). Anthrax is a well-documented enzootic disease[1] (endemic equivalent in animals) occurring in animal populations throughout India and in the animal-rearing community. In 1990, an epizootic (epidemic equivalent in animals) of anthrax in sheep, leading to many fatal human anthrax cases was reported from Vellore in south India[1]. Box 2 describes the Sverdlovsk anthrax outbreak of 1979.



**Figure 1.** Gram-stained *Bacillus anthracis*. Characteristic boxcar appearance is shown.

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**Box 1.** History.

- *B. anthracis* was the first bacterium to be observed under a microscope[1] (Pollender, 1850).
- First communicable disease shown to be transmitted by inoculation of infected blood[1] (Davaine, 1850).
- First bacillus to be isolated in pure culture and shown to possess spores[1] (Koch, 1876). Koch's famous postulates are based on his study of anthrax bacillus.
- First bacterium to be used for preparation of an attenuated vaccine[1] (Pasteur, 1881).

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**Box 2.** Man-made disaster: Sverdlovsk outbreak of 1979.

In April 1979 there was an outbreak of anthrax in the then USSR, resulting in a loss of 66 lives, all of which were reported to be of gastrointestinal type of anthrax, arising from a contaminated meat source. Recent analysis of epidemiological data, the autopsy reports on the dead by Russian physicians and pathological reports reveal that most of the cases were of inhalation type of anthrax originating from a leak of an aerosol of anthrax from a military facility in the region of Sverdlovsk[27].

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## Mediation of pathogenicity

*B. anthracis* is an extracellular pathogen that multiplies very rapidly in the blood stream, attaining high density enough to make the host succumb to it. Anthrax is lethal because of (1) its poly(D)glutamate capsule[2], which resists phagocytosis and evades immune attack and (2) due to its toxin. The anti-phagocytic capsular material is made of a polymer of D-glutamic acid residues linked together by peptide bonds involving the gamma carboxyl group. In culture, the capsule does not form under ordinary conditions but only if incubated under 10–25% $CO_2$ or in the presence of bicarbonate[1]. Anthrax toxin is made up of three factors: protective antigen (PA), edema factor (EF) and lethal factor (LF). The combination of PA and EF is called edema toxin and that of PA and LF is called lethal toxin. EF or LF or EF and LF combinations are shown to be inactive.

The LF is a 776 amino acid protein that contains a putative zinc-binding site $[HEFGH/F]^{3,4}$ at residues 686 through 690, which is characteristic of metalloproteases. It is a highly specific protease that cleaves members of mitogen-activated protein kinase–kinase[3] (MAPKK) near the amino terminus, causing cessation of many signalling pathways involved in the MAPKK pathway. This characteristic feature has been exploited to show the inhibitory effect on tumorigenesis involving

ras-MAPKK pathway[5] in athymic mice (mice lacking T-cell function due to absence of thymus since embryonic stage). Thus there is a potential for it being used as an anti-cancer drug in the near future.

Recently, the crystal structure[6] of the LF has been resolved at 2.2 Å. The molecule has dimensions of 100 Å length and 70 Å width, made up of four domains, with domain I perched on top of the other three domains (Figure 2 *a* and *b*). The point of contact between domain I and rest of the molecule is through domain II, by means of charged polar and water-mediated interactions. Domain III shows sequence similarity to the VIP2 toxin of *Bacillus cereus*. Domain III is required for LF activity, because any mutation affecting the buried residues in this domain abrogates function. Domain IV carries the catalytic centre. Here a water molecule and three protein side chains coordinate a zinc ion tetrahedrally, in an arrangement typical of the thermolysin family. Histidine 686 and 690 in the HEXXH motif along with glutamate735 from helix 4$\alpha$6 form the coordinating residues in the structure.

The EF[7] is a calmodulin-dependent adenylate cyclase. This gets activated in the animal or human cell, which provides calmodulin and initiates conversion of ATP into cyclicAMP (cAMP). This elevation in the intracellular cAMP level results in fluid outpour into the extracellular spaces resulting in edema. The X-ray crystal structure of EF, which is bound to calmodulin as well as the unbound form, has been elucidated (Figure 3). On calmodulin-binding, one of the EF's helical domains undergoes a conformational change forcing the helix to undergo a 15° translation and 30° rotation away from the catalytic site and results in the active EF. His 351 in the molecule acts as the catalytic base. In addition, the activation site has a single metal ion well-positioned for catalysis. The carboxy-terminal of EF is sufficient to cause catalysis. The rate of conversion of ATP into cAMP by EF is 1000-fold higher than that caused by mammalian adenylate cyclases.

The PA is a protein having four domains. It binds to the plasma membrane of target cells by its carboxy-terminal domain (Figure 4). This is followed by cleavage of its N-terminal domain by a furin-like protease[8] present in the cell membrane of the host cell, into a smaller amino-terminal fragment PA20, which dissociates into the extracellular space. The human furin is a calcium-dependent serine endoprotease that recognizes the sequence Arg-X-X-Arg[9] and has been shown to cleave the PA *in vitro*. The larger fragment PA63, which is a 63 kD protein, remains on the cell surface. It undergoes heptamerization[10–13,] to display a high affinity binding site $(kD \sim 1\, nM)^{11}$ for a domain that is present in both EF and LF, and also serves as a specific receptor that mediates endocytic entry of EF or LF into target cells. Acidification of endosome results in formation of 14-stranded $\beta$-barrel structure[14] by the heptamer,
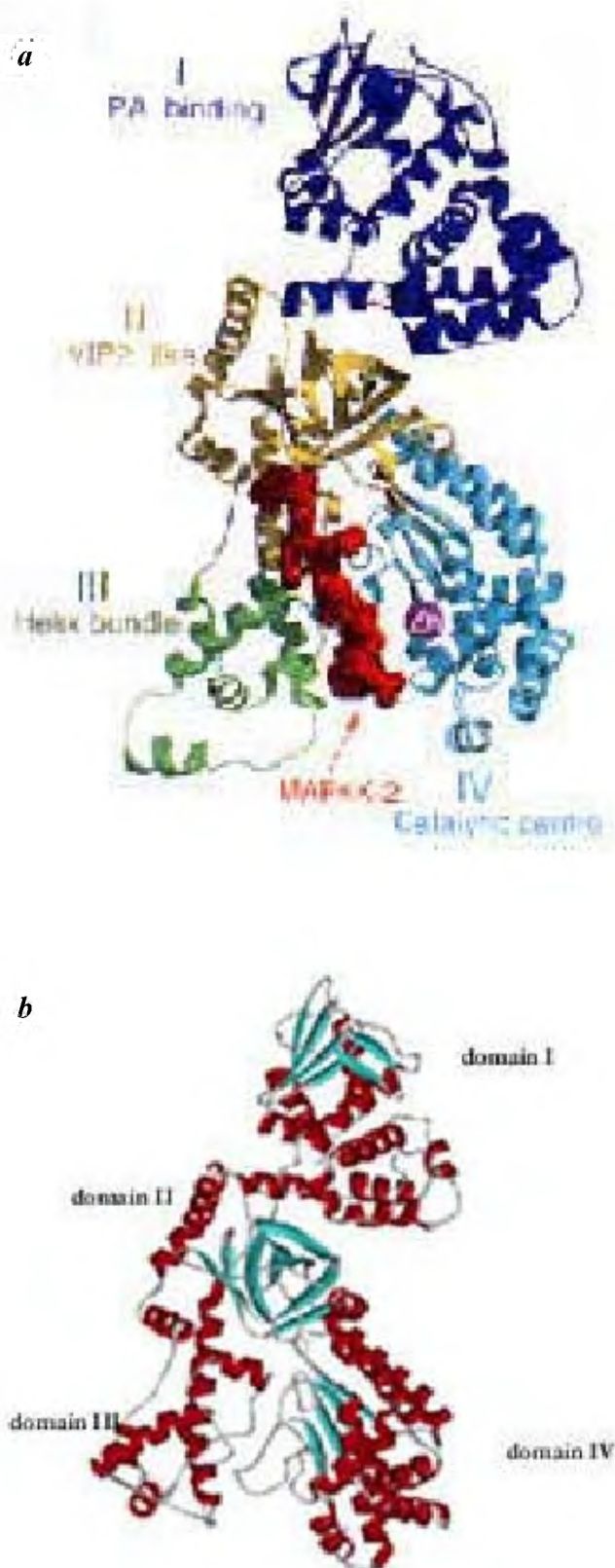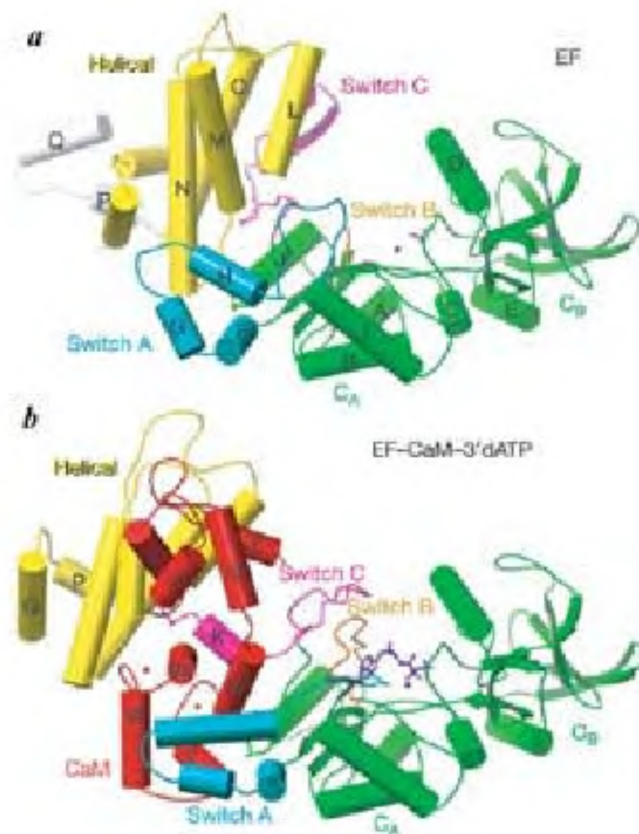
**Figure 3.** Secondary structures of EF: *a*, EF alone and *b*, EF-calmodulin (CaM)-3'deoxy complex. Switches A, B and C are segments which undergo large conformational changes on binding CaM, leaving other segments least affected. Switch A binds to CaM as well as the nucleotide, while switch C swings 33° on CaM-binding. Switch B is a disordered region which attains an orderly shape on CaM-binding. The deoxy ATP analogue is shown in purple (with permission from Drum *et al.*[7]).

which assists translocation of the other two toxin components into the cytosol. There is evidence[10,15] to show that in low pH, the $2\beta_2$–$2\beta_3$ (residues 302–325) of PA63 come together to form the 14-stranded β-barrel structure (Figure 5).

The primary cell type affected in anthrax pathogenesis is the macrophage[16]. In this cell, the initial low levels of toxin cause inhibition of release, but not the synthesis of pro-inflammatory cytokines, tumour necrosis factor alpha (TNF-α) and nitrous oxide (NO). Once the concentration of the toxin builds up, it lyses the macrophage by an unknown mechanism, releasing the entire stock of stored cytokines in a short span of 2 h. These high levels of NO and TNF-α attained in the serum, manifest clinically as septic shock.

The receptor for anthrax toxin[17], ATR (anthrax toxin receptor), has been identified to be a 368 amino acid protein. ATR contains a single extracellular Von-Willebrand type-A (VWA) domain, which aids the binding of toxin to the cell surface. VWA domains are present in a variety of cell surface proteins like matri-



**Figure 2.** Stereo ribbon representation of LF; *a*, The MAPKK-2 substrate is shown as ball and stick model and the zinc ion is labelled (with permission from Pannifer *et al.*[6]); *b*, The four domains are clearly shown. The MAPKK2 substrate is not shown here (compare with Figure 2 *a*).

lins and integrins, and these domains play a crucial role in protein–protein interactions and constitute ligand-binding sites for integrins (designated as domain I). These domains carry the well-conserved MIDAS motif[11] (metal ion-dependent adhesion site), which is crucial for ligand binding through domain I.

The cytoplasmic tails of ATR and furin carry a similar acidic cluster motif which make both these proteins localize to the basolateral surface of the polarized cell[18]. This is important in that, activation of PA needs the help of furin, while its transport into the cell requires ATR, and both have to be present on the same side in a polarized cell for efficient translocation.

Box 3 describes properties of the anthrax bacillus which make it well-suited for biowarfare.

## Genomic stability of *B. anthracis*

*B. anthracis* is believed to be one of the few microorganisms whose genome is well conserved even from far-flung places[19]. Almost every sample collected from one place seems to be homogenous with the other. Ames, Vollum and Sterne are the only three strains

**Box 3.** Anthrax and biowarfare.

Perhaps *B. anthracis* is the only microorganism that appears to have been used in recent act of bioterrorism. Every time a war takes place, the anthrax bacillus surfaces in the media as the prime biowarfare agent, only to disappear after the war. Properties[2] that make the anthrax bacillus well-suited for biowarfare are many. Its spores are highly resistant to physical and chemical agents; it lives as long as 60 years to perhaps a few centuries[19] in soil, has a long shelf life and can be easily dispersed in air to cause the most serious form of anthrax (pulmonary anthrax), which is highly fatal (60–80%). Dispersion does not require excessive sophistication or skill. Being an uncommon disease in humans (actually a disease of herbivorous animals), especially in urban areas, physicians may fail to consider anthrax in clinical situations.
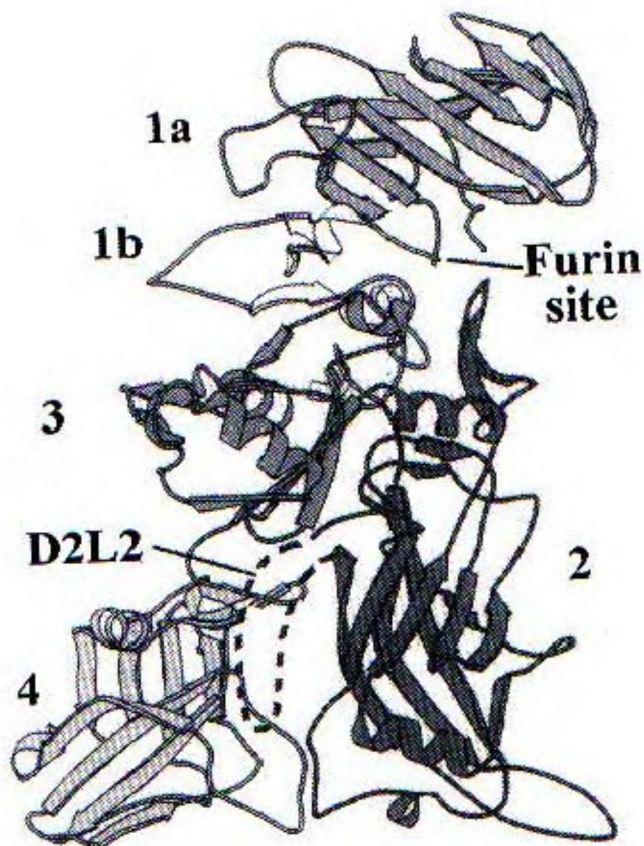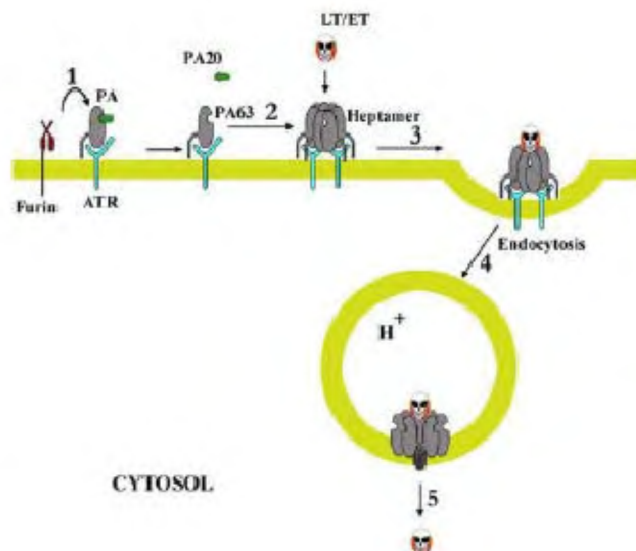
**Figure 5.** Model of anthrax toxin entry into the cell; 1, PA binds to its receptor ATR and is acted upon by the furin present in host cell membrane; 2, The smaller fragment PA20 is lost and the larger fragment PA63 undergoes heptamerization and binds to either LF or EF; 3, The heptamerized PA63 along with the bound EF /LF is internalized; 4, Acidification of endosome causes a conformational change; 5, Translocation of LF/ EF into the cytosol.

**Figure 4.** Ribbon diagram of native PA. The site of cleavage by furin is shown. (with permission from the Miller *et al.*[12]).

known so far, with no possible clue to differentiate one from the other, except for their virulence properties (Sterne is an avirulent strain used in vaccine preparation). This uniformity in its genome is believed to have arisen as a result of its unique life-cycle. Terminally ill animals dying of anthrax, ooze with infected blood from the nose, mouth and bowels, when the vegetative forms of the bacillus sporulate and remain dormant – for dec-

ades to centuries – till a new cycle starts. So the possibility of genetic variations has come down drastically, due to this so-called 'time capsule effect'. Another reason could be that unlike the human genome, which is re-shuffled during sexual reproduction, the anthrax genome is stable as they replicate asexually.

Box 4 provides a brief summary of the anthrax genome project.

The anthrax toxin is encoded by a temperature-sensitive plasmid[20] and loss of this plasmid makes the strain avirulent. The plasmid pX01 (110 MDa) harbours the genes for the toxin [$lef$ (LF), $pagA$ (PA), and $cya$ (EF)] and pX02 (60 MDa) carries genes for capsule synthesis ($capB$, $capC$, $capA$) and $dep$ for the capsule degradation. By culturing the bacilli, at 42–43°C, the toxin-producing plasmid (pX01) can be inactivated (this is the technique originally used by Pasteur for production of the anthrax vaccine).

## Clinical manifestations

There is no evidence of person-to-person transmission of anthrax. Quarantine of affected individuals is not recommended. The mode of infection determines the clinical manifestations. Skin contact causes cutaneous anthrax, ingestion causes gastrointestinal anthrax and inhalation causes pulmonary anthrax. Symptoms prior to the onset of fulminant disease may be mild or absent, such as low-grade fever, malaise or mild gastrointestinal abnormalities.

### Cutaneous anthrax

When spores are introduced into the skin through cuts or abrasions, it results in cutaneous anthrax. A pimple forms at the site of inoculation. Soon it becomes a pustule, which ulcerates and is followed by edema, necrosis and blackish eschar termed as a malignant pustule. Fatality is about 10–20%. This has been also called 'Hide

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**Box 4. Anthrax genome project.**

The first anthrax genome project on the Ames strain is nearing completion and is likely to be out soon. The recent spate of terrorist attacks, has paved the way for the first ever genome project triggered by crime – the second anthrax genome project[19], taken up at The Institute for Genome Research (TIGR). The second anthrax genome project will give a better understanding of the anthrax diversity and also in investigation of bioterrorism. Unlike the first project, which skipped the sequencing of plasmids pXO1 and pXO2 (which harbour the virulence genes), the second project encompasses them in its study.

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Porter's disease', as workers in the leather industry carrying hides on their shoulder or by other unprotected means are more frequently affected.

### Gastrointestinal anthrax

It is characterized by fever, nausea, vomiting, abdominal pain and bloody diarrhea. Fatality is about 50%.

### Pulmonary anthrax

Symptoms mimic viral respiratory disease and make early diagnosis difficult. It is characterized by fever, dyspnoea, stridor, hypoxia and hypotension, leading to death within 24 h. Fatality is 60–80%. This has been also called 'wool sorter's disease', as people in the wool-sorting industry are more at risk of getting this form of anthrax from spores present in the woolen fur.

## Diagnosis and management of anthrax

Laboratory diagnosis[1] is based on several techniques given below.

### Gram staining

Anthrax is an aerobic, Gram-positive, rod-shaped bacillus occurring in chains with a propensity to form spores under unfavourable conditions.

### Gelatin stab culture

It forms a characteristic 'inverted fir tree' appearance with slow liquefaction commencing from the top.

### Low power microscopy

It gives a medusa-head appearance[2], while in high power microscopy it gives boxcar appearance[20]. (Also called bamboo-stick appearance.)

### Mc' fadyean's reaction

When stained with polychrome methylene blue, the capsular material attains an amorphous purplish colour around the bacillus; this phenomenon is called Mc' Fadyean's reaction[20].

### Phage lysis

Anthrax bacillus is highly susceptible to gamma bacteriophage. This property is used to distinguish anthrax bacillus from other anthracoid bacilli.

## String of pearl reaction

In penicillin-containing medium, cells become large and spherical resembling a string of pearls[1]. This characteristic reaction differentiates *B. anthracis* from *B. cereus*, which is another aerobic spore-bearer.

Direct fluorescent antibody staining, DNA probing and PCR are recent methods to identify the bacillus rapidly. Variable number of tandem repeats[21] (VNTR) is a region where a short stretch of nucleotide sequence gets repeated few or more times. The number of times a sequence is repeated is conserved in a closely-related strain. Based on VNTR sequences, homology between two strains isolated from the same or different places can be determined. For instance, during an outbreak from a common source, the VNTR sequences are likely to be the same from different samples.

Box 5 mentions scientists involved in anthrax research.

Penicillin is the drug of choice[2], if no antibiotic resistance is suspected. On the other hand, ciprofloxacin[22] is said to be the safest drug in suspected anthrax cases, where infection by genetically engineered (penicillin-resistant) strains is large. Other drugs in use are erythromycin, tetracycline and chloramphenicol. In the later stages of the disease, antibiotic therapy can destroy the bacilli, but the toxin cannot be neutralized. Therefore, initiation of early treatment is very important. Thus if diagnosed early and treatment initiated soon, anthrax is not a real danger.

## Spore facts

Spores, though resistant to many substances, can be killed by 0.05% hypochlorite[20] solution (1-tablespoon bleach per gallon of water). Fumigation of rooms is carried out using chlorinedioxide gas for a period of 24 h and swabs are taken to confirm that the bacillus is completely destroyed. Steam sterilization and incineration

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**Box 5.** Scientific sleuths after anthrax.

Just recently, few notable differences among different strains in different isolates have been detected based on VNTR repeats. The painstaking work of Paul Keim from Northern Arizona University has led to the understanding of the importance of VNTR repeats in tracking the possible source of spores. His team along with Dr Wilson's team has established the presence of 89 unique genomes by DNA finger-printing[21] of 426 isolates obtained from possible sources around the world. The count keeps adding up everyday. It is said that the resolution attained by fin-ger-printing will permit pinpointing the source beyond ambiguity in future.

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**Box 6.** Sporulation.

Sporulation occurs only in the presence of oxygen! Hence any animal that is believed to have died of anthrax is not taken for post-mortem[23], fearing that oxygen in the air entering inside the animal, on dissection, may promote spore formation. If no post-mortem were to be carried out, the vegetative form of the bacillus disintegrates along with the carcass, as it is not as resistant as a spore.

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are also used to destroy the bacillus. Box 6 briefly describes sporulation.

Vaccines composed of killed bacilli and/or capsular antigens produce no significant immunity. As already mentioned, the plasmid pXO1 codes for toxin and plasmid pXO2 codes for the capsule. A nonencapsulated toxigenic strain has been used effectively in livestock. The attenuated live vaccine strain developed by Sterne[23] in 1937, which is still the basis of most anthrax vaccines for livestock lacks pXO2 and is therefore $Cap^-$ $Tox^+$ (i.e. lacks capsule, but has toxin). The Sterne strain of *B. anthracis* produces sublethal amounts of the toxin that induces formation of protective antibody. The human anthrax vaccine is a preparation of PA, obtained from the culture filtrate of an avirulent noncapsulated strain of *B. anthracis*, which produces PA during active growth.

Protective immunity against anthrax requires antibodies against components of anthrax toxin, especially PA. Both the noncellular human vaccines and live-spore animal vaccines confer protection by eliciting antibodies to protective antigen. The poly-D-glutamic acid capsule of *B. anthracis* is poorly immunogenic, and antibodies to the polysaccharide and other components of the cell wall are not protective. The protection afforded by such vaccines apparently is related primarily to antibodies specific for the protective antigen component of the toxin.

## Newer modalities of treatment

Recently, a synthetic, polymeric, polyvalent inhibitor (PVI) that interacts specifically with heptameric PA63 and blocks its interaction with EF and LF has been reported by Collier *et al.*[24]. The efficacy of this PVI in inhibiting the action of anthrax toxin suggests that in future such anti-toxins can be used for treatment.

Sellman *et al.*[25] and Olsnes and Wesche[4] have identified a dominant negative mutant form of PA that co-assembles with the wild type of EF/LF and efficiently prevents their translocation into the cytosol from the endosome. This mutant strongly inhibited the action of the toxin in cell culture and in an animal intoxication model using rats, suggesting that it could be used in therapy.

Recently, Williamson *et al.*[26] have shown that only certain domains within the PA, especially in domain IV, carry the immuno-dominant protective epitopes in the entire molecule and are responsible for protective immunity.

One major concern regarding the use of antibiotics is the emergence of resistant strains of the pathogen. Vaccination is a possible alternative but cannot be applied to all diseases, either because of its short-term protection or the disease *per se* may be very rare, as in the case of anthrax. It is therefore very important to be able to treat bacterial infections as soon as they appear, with therapeutics other than antibiotics. Hence the work by Sellman *et al.*[25] is an important step forward in the progress of alternative pharmacotherapeutics.

The identification of the receptor for anthrax toxin ATR[17] paves the way for newer modalities of treating anthrax. Receptor blockade may soon be the favoured mode of neutralizing the toxin-induced lethal effects. It has also been proposed that the soluble VWA/I domain of ATR can inhibit toxin action. Coupled with the use of the cloned receptor as a tool for identifying inhibitors of PA–receptor interaction, the future scenario in treating anthrax appears quite promising.

The recent attacks using anthrax spores have exposed the vulnerability as well as the inadequacy[29–32] of the biodefence. Some basic questions regarding anthrax still remain unanswered. As far as the metabolism of poly(D)glutamate capsule is concerned, nothing much is known in spite of it being a virulence factor specific for anthrax bacillus. Maybe a drug targeted against one of the metabolic enzymes in this pathway could complement or could well become the first line of management of anthrax.

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