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***Bacillus anthracis* Physiology and Genetics**

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Abstract

Bacillus anthracis is a member of the *Bacillus cereus* group species (also known as the “group 1 bacilli”), a collection of Gram-positive spore-forming soil bacteria that are non-fastidious facultative anaerobes with very similar growth characteristics and natural genetic exchange systems. Despite their close physiology and genetics, the *Bacillus cereus* group species exhibit certain species-specific phenotypes, some of which are related to pathogenicity. *B. anthracis* is the etiologic agent of anthrax. Vegetative cells of *B. anthracis* produce anthrax toxin proteins and a poly-D-glutamic acid capsule during infection of mammalian hosts and when cultured in conditions considered to mimic the host environment. The genes associated with toxin and capsule synthesis are located on the *B. anthracis* plasmids, pXO1 and pXO2, respectively. Although plasmid content is considered a defining feature of the species, pXO1- and pXO2-like plasmids have been identified in strains that more closely resemble other members of the *B. cereus* group. The developmental nature of *B. anthracis* and its pathogenic (mammalian host) and environmental (soil) lifestyles of make it an interesting model for study of niche-specific bacterial gene expression and physiology.

Keywords

anthracis; *Bacillus cereus* group; bacteriophage; plasmid; genetic exchange; virulence gene expression

1. Introduction

The etiologic agent of anthrax, *Bacillus anthracis*, is perhaps the most renowned pathogen of the *Bacillus* genus. Like all *Bacillus* species, *B. anthracis* is a Gram-positive spore-former that is commonly found in the soil. Unlike most other *Bacillus* species, respiratory, gastrointestinal, or cutaneous entry of *B. anthracis* spores into mammals can result in systemic infection and lethal disease. Spores are considered to be the predominant form of *B. anthracis* outside of a host, but upon infection, spores germinate to become vegetative cells that can replicate to high numbers in virtually all body tissues. Death of the host and contact of infected tissues with air results in a return to the spore form of the bacterium. As is true for all *Bacillus* species, *B. anthracis* spores are highly resistant to adverse environmental conditions, but it has been proposed that *B. anthracis* is more dependent than other *Bacillus* species on sporulation for species survival (Turnbull, 2002). Vegetative cells appear to survive poorly in simple environments including water and bulk soil (Lindeque and Turnbull, 1994; Saile and Koehler, 2006; Turnbull, 2002). Thus, the spore - vegetative cell – spore cycle is essential for the pathogenic lifestyle of this developmental bacterium.

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The evolution of *B. anthracis* and its relatedness to other *Bacillus* species is intriguing. *B. anthracis* is a member of the “group 1 bacilli” also known as the “*B. cereus* group”, which also includes *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanensis* (Ash and Collins, 1992; Ash et al., 1991; Helgason et al., 2000; Helgason et al., 2004; Lechner et al., 1998; Rasko et al., 2005; Tourasse et al., 2006; Turnbull, 1999). The *B. cereus* group species exhibit remarkably similar cell structure, physiology, and natural genetic exchange systems but they are distinct with regard to pathogenicity. In large part, species-specific pathogenicity is associated with plasmid content. *B. anthracis* is distinguished by its virulence plasmids pXO1 (182 kb), which contains the structural genes for the anthrax toxin proteins, *pagA* (PA), *lef* (LF) and *cya* (EF), (Koehler, 2002) and pXO2 (96 kb), which carries the biosynthetic operon for capsule, *capBCADE* (Candela et al., 2005; Makino et al., 1989; Okinaka et al., 1999a). Although pXO1 and pXO2 are considered to be specific to *B. anthracis*, there are some reports of rare *B. cereus* strains harboring plasmids with similarity to these plasmids (Avashia et al., 2007; Hoffmaster et al., 2004; Klee et al., 2006; Rasko et al., 2007). Some of these unusual strains have been isolated from humans and animals that succumbed to an anthrax-like disease. Phenotypes conferred by plasmid genes have been the focus of many investigations comparing *B. anthracis* to related species. Nevertheless, not all unique attributes of *B. anthracis* are plasmid-associated. Plasmid-cured strains of the species exhibit some species-specific phenotypes despite the striking degree of sequence similarity and gene synteny associated with the chromosomes (Rasko et al., 2005).

2. Distinguishing phenotypes

B. anthracis is best distinguished from related species by its ability to synthesize the anthrax toxin proteins and the poly-D-glutamic acid capsule. The bacterium produces these virulence factors in a number of complex and defined media. Optimal synthesis of the toxin proteins occurs during culture at 37°C in defined media containing glucose, as the carbon source, and bicarbonate (Leppla, 1988; Ristroph and Ivins, 1983). Capsule is produced at high levels in defined and complex media, but synthesis is dependent upon the presence of dissolved bicarbonate in the media (Green et al., 1985; Meynell and Meynell, 1964; Thorne et al., 1952). Toxin and capsule synthesis are highest as the culture transitions from exponential to stationary phase (Drysdale et al., 2004; Drysdale et al., 2005; Koehler et al., 1994; Leppla, 1988; Sirard et al., 1994). The “CO₂/bicarbonate effect” on toxin and capsule synthesis is long known (Gladstone, 1946; Puziss and Wright, 1954; Thorne, 1993). Steady state levels of toxin gene and *cap* operon transcripts increase up to 60-fold in response to this signal (Bartkus and Leppla, 1989; Drysdale et al., 2004; Green et al., 1985; Koehler et al., 1994; Sirard et al., 1994). Deletion of the genes encoding a bicarbonate transporter results in decreased uptake of bicarbonate and the absence of toxin gene induction, indicating that induction of the virulence genes requires transport of bicarbonate into cells (Wilson et al., 2008). Nevertheless, the molecular basis for the transcriptional response to the signal remains elusive. Temperature also affects toxin gene expression. Cells growing at 37°C exhibit 4- to 6-fold higher levels of toxin gene transcripts than cells growing at 28°C (Sirard et al., 1994), however, no temperature effect has been reported for capsule synthesis. Given the mammalian body temperature of 37°C and bicarbonate/CO₂ concentrations of 15 to 40 mM in the bloodstream (Lentner, 1981), these signals are likely significant during infection.

In addition to production of the anthrax toxin proteins and a poly-D-glutamic acid capsule, classic *B. anthracis* phenotypes include absence of β-hemolysis on sheep blood agar, absence of phospholipase C activity, lack of motility, susceptibility to penicillin, and sensitivity to the γ bacteriophage (Marston et al., 2006). Nevertheless, for some of these traits there are exceptions. Motile strains and strains that appear to produce flagella have been reported (Klee et al., 2006; Liang and Yu, 1999) although the genomes of sequenced strains, which are nonmotile and presumed nonflagellated, are devoid of some genes commonly associated with

flagella (Read et al., 2003). Rare penicillin-resistant isolates have also been reported (Cavallo et al., 2002; Klee et al., 2006; Mohammed et al., 2002; Odendaal et al., 1991). Sequenced *B. anthracis* strains, which are penicillin-susceptible, harbor two structural genes for β -lactamase proteins (Chen et al., 2003; Read et al., 2003). These *bla* genes are transcriptionally silent in prototypical strains, but constitutively expressed in a rare highly penicillin-resistant isolate (Chen et al., 2004; Ross et al., in press). Although susceptibility to γ phage is part of the standard protocol used by many diagnostic laboratories for identification of *B. anthracis*, non-susceptible *B. anthracis* strains and susceptible *B. cereus* strains have been described (Abshire et al., 2005; Davison et al., 2005; Klee et al., 2006).

3. Cell and colony morphology

In most growth conditions, *B. anthracis* cells have distinctive morphology (see figure 1). The vegetative cells are square-ended rods, approximately 1 by 5-8 μ m, with the propensity to form chains. The *B. anthracis* capsule is readily visualized microscopically using India Ink exclusion (Drysedale et al., 2004). *B. anthracis* generally grows as planktonic cells in liquid media, although formation of pellicles during static incubation and adherence to solid surfaces can occur (Charlton et al., 2007; Lee et al., 2007). *B. anthracis* forms long serpentine chains during the exponential phase of batch culture. In infected tissues, generally single cells or short chains of 2-3 cells are observed (Lincoln et al., 1965).

B. anthracis generally produces off-white colonies that have irregular edges and a rough “ground glass” appearance when incubated in conditions that are not conducive for capsule formation. The colonies adhere firmly to the agar surface and transfer using a loop or toothpick can result in strings of cells that can be drawn up and remain perpendicular to the agar surface without support. Specific differences in the degree of tenacity have been associated with genotypic groups (Smith et al., 2000). Colonies on solid media that facilitate capsule synthesis appear extremely mucoid, reflecting the large capsule which can be more than 3 μ m in thickness (Drysedale et al., 2004).

Upon nutrient starvation, vegetative cells can develop into the dormant spore form of the bacterium. Prolonged culture of *B. anthracis* in a variety of media can facilitate spore formation (Leighton and Doi, 1971; Schaeffer et al., 1965; Tarr, 1933; Thorne, 1962; Turnbull et al., 2007). Phase microscopy of sporulating cells reveals forespores appearing as oval refractile bodies of 1- to 1.5- μ m in diameter, that are central or subterminal within the mother cell and do not cause significant swelling of the mother cell (Battisti et al., 1985). A distinguishing characteristic of the spores of *B. anthracis* and related species is the exosporium, an outermost layer that is not present on spores of many other *Bacillus* species. The exosporium has been visualized using electron crystallography and atomic-force microscopy (Ball et al., 2008; Chada et al., 2003).

4. Growth requirements

B. anthracis is a facultative anaerobe that can multiply readily in a variety of common laboratory media using multiple sugars and amino acids as carbon sources (Charlton et al., 2007; Puziss and Wright, 1959). Most studies of carbohydrate metabolism have been performed to determine relationships between sugar utilization and toxin synthesis. The precise nutritional requirements of *B. anthracis* are not clear and appear to vary between strains. Methionine and thiamine are required for growth, but growth in glucose-salts medium containing methionine and thiamine requires supplementation with multiple amino acids, which most likely serve as sources of nitrogen (Thorne, 1993). Hydrolyzed casein medium containing glucose, adenine, uracil, thiamine, tryptophan, cysteine, glycine, and various salts is used frequently for investigations of *B. anthracis* physiology and gene expression (Thorne and Belton, 1957). Defined medium comprised of glucose, thiamine, uracil, adenine, guanine,

calcium and multiple amino acids are also used (Brewer et al., 1946; Ristroph and Ivins, 1983). The most minimal defined medium, XO, contains glucose, ferric chloride, thiamine, glutamic acid, glycine, methionine, proline, serine, threonine, ammonium sulfate, magnesium sulfate, manganese sulfate, potassium phosphate, and sodium citrate (Hoffmaster and Koehler, 1997). The optimal growth temperature for *B. anthracis* is 37°C and the bacterium is unable to grow at temperatures above 43°C. Cell doubling times during growth in complex media at 37°C range from 30- to 60-min (Dai and Koehler, 1997; Passalacqua et al., 2007; Yang and Miller, 2008).

Iron acquisition by *B. anthracis* has been the subject of recent investigations. Like most mammalian pathogens, *B. anthracis* has multiple means of obtaining iron that is tightly bound to carrier and storage proteins in host tissues. In response to low iron availability, *B. anthracis* secretes the siderophores bacillibactin and petrobactin (Cendrowski et al., 2004; Koppisch et al., 2005; Koppisch et al., 2008). These ferric iron chelators bind iron with very high affinity and transport it into the bacterium via substrate-binding proteins (SBPs) and other ABC transporter components. *B. anthracis* can also scavenge iron from heme and heme-containing proteins, including hemoglobin, via iron-regulated surface determinant (Isd) proteins (Gat et al., 2008; Maresso et al., 2006; Maresso et al., 2008; Skaar et al., 2006). The three *B. anthracis* proteins that bind heme, IsdX1 (also known as IsdJ), IsdX2 (also known as IsdK), and IsdC (Maresso et al., 2006; Maresso et al., 2008) contain NEAT (near iron transporter) domains that are associated with binding to iron-containing ligands (Andrade et al., 2002). IsdC is bound covalently to the cell envelope by the transpeptidase SrtB while IsdX1 and IsdX2 are found in cell-free culture supernates (Skaar et al., 2006).

5. Bacteriophage

Bacteriophage of *B. anthracis* comprise a genetically diverse group, including members of the *Siphoviridae*, *Podoviridae*, *Myoviridae*, and *Tectiviridae* families (Ackermann et al., 1978; Inal and Karunakaran, 1996; Minakhin et al., 2005; Nagy et al., 1976; Schuch and Fischetti, 2006; Sozhamannan et al., 2008; Walter and Baker, 2003). The γ phage is the most well-studied *B. anthracis* phage due to its diagnostic use and more recently, the proposed use of the lysin enzyme PlyG for decontamination (Fischetti, 2006; Kikkawa et al., 2008; Schuch et al., 2002). γ is morphologically similar to the *Siphoviridae* family, possessing a long contractile tail and an icosohedral head. The double-stranded DNA phage has a 37-kb genome, encoding 53 ORFs (Schuch and Fischetti, 2006). γ phage is actually a lytic variant of the temperate phage, W, a phage first identified in an atypical "*B. cereus*" isolate (Brown and Cherry, 1955; Fouts et al., 2006; McCloy, 1958). The γ phage is lytic on capsulated and noncapsulated *B. anthracis* cells, unlike phage W which is relatively inefficient for lysis of capsulated cells. Although γ is generally considered to be *B. anthracis*-specific, some nonsusceptible *B. anthracis* strains and susceptible non-*B. anthracis* isolates have been reported (Abshire et al., 2005; Davison et al., 2005; Klee et al., 2006; Schuch et al., 2002). Susceptible strains produce a cell-wall anchored protein, GamR (Gamma Receptor), which is associated with phage binding (Davison et al., 2005). Interestingly, there is some evidence of lysogenic conversion by γ or related phage. The γ phage DNA sequence contains a gene encoding the fosfomycin-resistance protein Gp41 and it has been suggested that resistance to this soil antibiotic may play a role in the survival of vegetative *B. anthracis* cells outside of the host (Schuch and Fischetti, 2006).

Bacteriophage with generalized transducing capability have served as useful tools for creation of recombinant *B. anthracis* strains. The transducing phage CP-51 and CP-54 were originally isolated from the soil as phage that could infect *B. cereus* (Thorne, 1968; Thorne, 1978). Transducing phage TP-21 was originally identified in *B. thuringiensis subsp. kurstaki* as a plasmid and shown subsequently to exist as a plasmid prophage (Thorne, 1993). The host

ranges of CP-51, CP-54, and TP-21 extend to multiple strains of *B. anthracis*, *B. cereus*, and *B. thuringiensis* (Ruhfel et al., 1984; Thorne, 1968; Thorne, 1978; Thorne, 1993; Yelton and Thorne, 1970). CP-51 and CP-54 have a similar, but not identical structure and are serologically related (Thorne, 1978; Thorne and Holt, 1974; Yelton and Thorne, 1970). Despite the value of these transducing phage for genetic studies, they remain relatively uncharacterized.

Finally, genome sequence analyses have revealed the presence of apparent intact and defective prophage (Inal and Karunakaran, 1996; Read et al., 2003; Schuch and Fischetti, 2006; Sozhamannan et al., 2006). Potential phenotypes conferred by the prophage have not been discerned, but the prophage sequences can facilitate distinction of *B. anthracis* from other *B. cereus* group species.

6. Plasmids

Since the discovery of pXO1 and pXO2 in the 1980s (Green et al., 1985; Mikesell et al., 1983; Uchida et al., 1985), the plasmids have served as the hallmark genetic feature of *B. anthracis*. Yet given the safety and regulatory concerns related to use of fully virulent *B. anthracis* strains in the laboratory, many investigators employ attenuated strains harboring only one of the two virulence plasmids. “Sterne” type strains, so named because of a strain first described by M. Sterne in 1939 (Sterne, 1939), carry pXO1 but are missing pXO2. These strains include Sterne, 34F2, STI, Weybridge, and others. “Pasteur” type strains (Mikesell et al., 1983), such as 6602 and 4229, harbor pXO2 but are pXO1-negative. pXO1 and pXO2 are stably maintained by *B. anthracis* growing *in vivo* during infection. During culture in the absence of any apparent selective pressure, spontaneous loss of pXO2 occurs more frequently than loss of pXO1 (Green et al., 1985; Thorne, 1985). The stability of pXO1 may be related to a plasmid-encoded type 1 topoisomerase (Fouet et al., 1994). *B. anthracis* can be cured of either plasmid during culture at 43°C or in the presence of sub-lethal concentrations of novobiocin (Chen et al., 2004; Green et al., 1985; Mikesell et al., 1983; Thorne, 1985).

The toxin and capsule genes are the most thoroughly investigated plasmid genes, but functions of some other plasmid genes and structural features of the plasmids have been reported. DNA sequence annotation of pXO1 indicates 203 open reading frames (ORFs) (Okinaka et al., 1999b). An 8.7-kb element resembling a class II cointegrative transposon lies within a 44.8-kb pathogenicity island on the plasmid (Van der Auwera and Mahillon, 2005). The element, TnXO1, carries genes for a transposase and a site-specific recombinase in addition to a germination operon, *gerX*, that is associated with germination in phagocytes (Guidi-Rontani et al., 1999). The pathogenicity island also includes *pagA*, *lef*, and *cya*, the structural genes for the toxin proteins; *atxA*, a major virulence gene regulator (Koehler et al., 1994; Uchida et al., 1993); *bslA*, encoding an adhesion protein located in the S-layer of vegetative cells (Kern and Schneewind, 2008); and other ORFs. Comparison of the pXO1 DNA sequences of multiple *B. anthracis* strains reveals one strain in which the pathogenicity island is inverted (Thorne, 1993), but overall, the pXO1 DNA sequence appears to be stable.

Investigations aimed at identifying the pXO1 origin of replication (*ori*) and genes required for plasmid replication have resulted in dissimilar models (Pomerantsev et al., 2009; Robertson et al., 1990; Tinsley and Khan, 2006). A 5-kb region of pXO1 cloned in an *E. coli* vector was reported to facilitate replication of the plasmid in *B. anthracis* (Tinsley and Khan, 2006). The recombinant plasmid contains a 158-bp region, considered to be an *ori*, and four ORFs, *pXO1-43* to *pXO1-46*. RepX, encoded by *pXO1-45*, belongs to the tubulin family of cytoskeletal proteins that are associated with cell division and DNA segregation. Purified RepX can polymerize in a GTP-dependent manner and has been shown to form polymers *in vivo* in *B. anthracis*, properties consistent with a role in plasmid segregation and partitioning (Akhtar et al., 2009). More recently, another group described an 8.8-kb mini-pXO1 plasmid that

replicates in *B. anthracis* and *B. cereus* (Pomerantsev et al., 2009). The mini-replicon does not contain the previously implicated pXO1 sequences. Rather, it carries two ORFs, *pXO1-14* and *pXO1-16*, which appear to be essential for replication. ORF14 is predicted to encode a protein with a helix-turn-helix motif. ORF16 is the putative replication initiator Rep protein. The region upstream of *pXO1-16* bears the attributes of the *ori* from theta-replicating plasmids, which include an exclusively A+T-containing segment, five 9-bp direct repeats, an inverted repeat, and a σ^A -dependent promoter.

Sequence annotation of the smaller *B. anthracis* virulence plasmid, pXO2, reveals 110 ORFs, including the *capBCADE* operon that encodes proteins required for capsule synthesis, assembly, and transport (Candela et al., 2005; Green et al., 1985; Makino et al., 1989; Okinaka et al., 1999a), the *cap* regulatory genes *acpA* and *acpB* (Drysdale et al., 2004; Vietri et al., 1995), and an L-alanine amidase gene *amiA* (Mesnage and Fouet, 2002; Rollins et al., 2005). A 2.4-kb region of pXO2 cloned into an *E. coli* vector is sufficient for replication in *B. anthracis* (Tinsley et al., 2004). The cloned DNA carries a putative 60-bp *ori* located immediately downstream of a gene encoding the replication initiation protein, RepS. Recombinant RepS protein binds to a DNA fragment corresponding to the *ori*. Like pXO1, pXO2 appears to replicate via a theta-type mechanism with replication proceeding unidirectionally from the origin of replication (Tinsley et al., 2004).

Plasmids with high DNA sequence similarity and synteny to pXO1 and pXO2 have been identified in strains of other *B. cereus* group species (Hoffmaster et al., 2004; Hu et al., 2006; Hu et al., 2009; Rasko et al., 2007; Van der Auwera and Mahillon, 2008). In most reported cases, these pXO1-like and pXO2-like plasmids have not been shown to carry the pXO1 pathogenicity island or the *cap* region of pXO2. Notable exceptions include one clinical *B. cereus* isolate associated with a human illness resembling anthrax, that carries a pXO1-like plasmid with 99.6% similarity to pXO1 (Hoffmaster et al., 2004). Also, clinical isolates from wild great Apes that died of an anthrax-like disease do not bear classic phenotypes associated with *B. anthracis*, but harbor pXO1- and pXO2-like plasmids and produce protective antigen and capsule. Multilocus sequence typing of these strains indicates that they are closely-related to *B. anthracis* and two uncommonly virulent *B. cereus* and *B. thuringiensis* isolates (Klee et al., 2006).

7. Natural genetic exchange

Inter- and intra-species transduction and conjugation methods were crucial for early investigations of *B. anthracis* physiology and genetics. Before the advent of molecular biology methods and DNA sequencing, similarities in the *B. cereus* group species chromosomes were demonstrated by co-transduction of linked markers. Mating experiments in which conjugative plasmids transferred between species, and in some cases mobilized pXO1, pXO2, and other non-conjugative plasmids, revealed plasmid-specific phenotypes.

Plasmids pXO1 and pXO2 can be transferred in intra- and inter-species matings by conjugative plasmids originally found in *B. thuringiensis*. *B. cereus* group species harboring plasmids pXO12 and pXO14 can mobilize small selectable plasmids, such as the tetracycline-resistance plasmid pBC16, in inter-species matings with frequencies up to 10^{-2} transconjugants per donor (Battisti et al., 1985; Reddy et al., 1987). Co-transfer of the conjugative plasmid with the selected plasmid approaches frequencies of up to 80%. *B. anthracis* transconjugants carrying pXO12 or pXO14 can serve as donors of pXO1 and pXO2 (Green et al., 1985; Green et al., 1989; Reddy et al., 1987). Transfer of the virulence plasmids appears to occur via conduction. Co-integrates of pXO12 and pXO1 involving the insertion element IS4430 form in the donor strain and are resolved, sometimes improperly, in ex-conjugants (Green et al., 1989). pXO12 can also transfer pBC16 between *B. anthracis* strains co-cultured in a plant/soil system,

suggesting that conjugative transfer of the plasmids may occur in nature (Saile and Koehler, 2006).

Although pXO1 and pXO2 are not self-transmissible, it is notable that they carry homologues of genes encoding type IV secretion system (T4SS) machinery, a system that mediates delivery of protein and DNA substrates from donors to recipients during conjugative transfer. However, the T4SS gene homologues on pXO1 display low levels of homology to known T4SS genes and are physically scattered throughout the plasmid (Grynberg et al., 2007). The “transfer region” of pXO2, is very similar to that of pXO2-like conjugative plasmids pAW63 and pBT9727 originally found in *B. thuringiensis* strains (Van der Auwera et al., 2005). Yet some of the transfer genes on pXO2 carry nonsense mutations or frameshifts (Hu et al., 2009).

The most widely employed transducing bacteriophage of the *B. cereus* group is CP-51. The phage was instrumental in demonstrating that pXO2 is required for capsule synthesis (Green et al., 1985). Propagation of CP-51 on pXO1- pXO2+ Pasteur-type strains results in phage lysates that can transduce pXO2 to pXO2- *B. anthracis* and *B. cereus* at a frequency of about 10^{-8} transductants per plaque-forming unit. Cap+ isolates can be selected readily from transduction mixtures using phage CP-54. CP-54, which can infect *B. anthracis* and *B. cereus*, lyses non-capsulated cells, but does not adsorb to capsulated cells (Green et al., 1985). CP-51-mediated transduction of smaller plasmids and chromosomal genes occurs at higher frequencies of up to 10^{-5} transductants per PFU respectively (Ruhfel et al., 1984). The 60-MDal size estimate for CP51 DNA reported by Yelton and Thorne (Yelton and Thorne, 1971) is in agreement with the lower frequency of pXO2 transduction and likely indicates that the size of the plasmid (96 kb) approaches the limit for DNA packaging by the phage.

8. Recombinant methods

There are no reported methods for generation of naturally competent cells of *B. anthracis*, but bifunctional plasmid vectors containing replication origins for Gram-negative and Gram-positive species can be introduced into *B. anthracis* using electroporation and an *E. coli* - *B. anthracis* conjugal mating system. Selectable markers commonly used in *B. anthracis* include genes encoding erythromycin, kanamycin-, and spectinomycin-resistance (Koehler, 2002). Highest electroporation frequencies are achieved when the plasmid DNA is isolated from DNA methyltransferase-deficient (*dam*) *E. coli* strains or from *B. subtilis* 168, a *Bacillus* strain that lacks adenine methylation activity (Marrero and Welkos, 1995). In the conjugal system, shuttle vectors containing the transfer origin of the IncP plasmid RK2 are mobilized by self-transmissible IncP plasmids that are co-resident in the donor strain (Cataldi et al., 1990; Pezard et al., 1991).

Numerous methods for generating mutations in the *B. anthracis* genome have been published. Integrative vectors, carrying *B. anthracis* DNA sequences but lacking a replication origin functional in *B. anthracis*, can be transferred to *B. anthracis* using the RK2 system with selection for plasmid-encoded antibiotic resistance (Cataldi et al., 1990; Pezard et al., 1991). Electroporation of unstable and temperature-sensitive plasmids into *B. anthracis* facilitates insertional mutagenesis and allelic replacement (Bongiorni et al., 2007; Chen et al., 2004; Fisher and Hanna, 2005; Janes and Stibitz, 2006; Saile and Koehler, 2002). The Cre-loxP system, first employed in *E. coli* to excise antibiotic resistance genes after their introduction into specific loci (Palmeros et al., 2000), has been developed for use in *B. anthracis* (Pomerantsev et al., 2006) and can be used to generate very large deletions in the virulence plasmids (Pomerantsev et al., 2009). Random mutagenesis employing mini-Tn10 and *mariner*-based transposon systems have proved useful in genetic screens for germination, toxin gene expression, and other phenotypes (Barua et al., 2009; Day et al., 2007; Tam et al., 2006; Wilson et al., 2007).

9. Toxin and capsule gene regulation

A pXO1-encoded protein, AtxA (for anthrax toxin activator) is a global regulator of transcription in *B. anthracis* (see figure 2). AtxA has a strong positive effect on the toxin genes, *pagA*, *cya*, and *lef*, and the capsule biosynthetic operon, *capBCADE*, and varying control of many other genes on the *B. anthracis* plasmids and chromosome (Bourgogne et al., 2003; Drysdale et al., 2004; Guignot et al., 1997; Hoffmaster and Koehler, 1997; Sirard et al., 2000; Uchida et al., 1997). In agreement with strong regulation of the toxin and capsule genes, an *atxA*-null mutant is highly attenuated for virulence in a murine model for anthrax (Dai et al., 1995). The molecular mechanism for AtxA protein function is unknown; there are no reports of direct interaction of AtxA with any of its target genes. AtxA exerts its affect on *capBCADE* expression via control of *acpA* (for anthrax capsule activator), a pXO2 gene located 5' of the *cap* operon (Drysdale et al., 2004; Guignot et al., 1997; Uchida et al., 1997; Vietri et al., 1995). Transcriptional read-through of *capBCADE* results in increased expression of the 3' proximal gene *acpB*, which affects *cap* operon expression in a positive feedback loop (Drysdale et al., 2005). Interestingly, AtxA, AcpA, and AcpB have sequence and functional similarity, yet while AtxA has far-reaching effects on *B. anthracis* gene expression, AcpA and AcpB effects are much more limited. PagR is another regulator that functions downstream of AtxA. The *pagR* gene is cotranscribed with the protective antigen gene in the AtxA-regulated *pagAR* operon (Hoffmaster and Koehler, 1999). PagR is a weak repressor of *pagAR* and thus serves in a negative feedback loop. In addition to autogenous control of *pagAR*, PagR regulates expression of the chromosome genes *sap* and *eag* which encode surface proteins of vegetative cells. PagR is a member of the ARSR family of transcriptional regulators and binds DNA specifically in the promoter regions of its target genes to repress transcription, in the cases of *pagAR* and *sap*, and activate transcription, in the case of *eag* (Mignot et al., 2003).

Mechanistic predictions for AtxA function have been made using *in silico* protein modeling programs. AtxA is an approximately 56-kD protein with a calculated isoelectric point of 9.5. Winged-helix and helix-turn-helix motifs are found at the amino-terminus of the protein, suggesting that AtxA has DNA-binding activity, however specific binding of AtxA to the promoter regions of target genes has not been shown. Although apparent transcriptional start sites and *cis*-acting sequences have been established for the highly AtxA-regulated toxin genes, sequence similarities are not apparent in the control regions. *In silico* modeling and *in vitro* gel mobility experiments indicate significant curvature associated with DNA in AtxA-regulated promoters, suggesting that the topology of promoter DNA may play a role in AtxA-mediated control of gene expression (Hadjifrangiskou and Koehler, 2008).

Two motifs for phosphotransferase system regulation domains (PRDs) are located centrally in the AtxA amino acid sequence and have been implicated in AtxA function (Tsvetanova et al., 2007). The phosphotransferase system (PTS) is responsible for uptake of specific sugars by many bacterial species (Gorke and Stulke, 2008) and PRDs are common to some bacterial proteins that respond to carbohydrate availability. Specific histidine residues in the apparent PRDs of AtxA can be phosphorylated *in vivo*; phosphorylation of H199 in PRD1 has been reported to positively affect AtxA activity, while phosphorylation of H379 has a negative impact on AtxA function (Tsvetanova et al., 2007). It is intriguing that carboxy-terminal region of AtxA bears some similarity to part of the EIIB protein of the PTS. EIIB phosphorylates sugars passing through the EIIC channel (Deutscher et al., 2006; Zeng and Burne, 2009). Thus, the presence of PRDs and an EIIB-like region in AtxA suggests a relationship between AtxA activity and sugar uptake in *B. anthracis*.

Certain signals and *trans*-acting factors have been reported to affect *atxA* gene expression. AtxA protein and *atxA* transcript levels are 5- to 6-fold greater in cultures grown at 37°C compared to 28°C (Dai and Koehler, 1997). Control of *atxA* expression has also been tied to

cytochrome c biogenesis. Mutants harboring insertions in genes encoding two heme-dependent small c-type cytochromes, CccA and CccB, and other components of the cytochrome c biogenesis pathway show decreased *atxA* expression (Wilson et al., 2009). The influence of c-type cytochromes on *atxA* expression indicates a potential connection between cellular redox state and virulence gene regulation in *B. anthracis*. The only *trans*-acting factor known to interact directly with the *atxA* promoter to affect transcription is AbrB. AbrB binds to DNA sequences overlapping the putative SigA recognition sequence for the major transcriptional start of the gene (Strauch et al., 2005), suggesting that the regulatory protein competes with RNA polymerase for promoter binding. During growth in batch culture, AbrB represses *atxA* transcription, exerting the greatest effect during exponential growth (Saile and Koehler, 2002). The well-characterized AbrB homologue from *B. subtilis*, is a “transition state regulator” that affects timing of expression for multiple genes in that species. Thus, it appears that AbrB is at least in part, responsible for growth phase control of *atxA* expression (Saile and Koehler, 2002).

10. Conclusion

Research efforts concerning *B. anthracis* have focused on attributes related to pathogenicity. Certainly, specific investigations of *B. anthracis* physiology and genetics as related to virulence will further progress toward development of new therapeutics and vaccines. However, independent of its “Select Agent” status, *B. anthracis* can be viewed as an outstanding model organism for studies of microbial ecology, evolution, cell development, and host-pathogen interactions. Further investigations, including functional and comparative genomic studies, will continue to increase knowledge of niche-specific lifestyles of this interesting bacterium.

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Figure 1.

B. anthracis cell morphology. (A) *B. anthracis* cells cultured on NBY medium containing 0.8% sodium bicarbonate and incubated at 37°C in an atmosphere containing 5% CO₂. Inserts show cells viewed in India Ink. Top, parent strain. Bottom, *capBCADE*-null mutant. (B) Fescue seedling in soil inoculated with *B. anthracis* spores and incubated at 30°C. Insert shows vegetative cells of *B. anthracis* in the rhizosphere of plant roots visualized using fluorescent microscopy as described (Saile and Koehler, 2006). (C) *Macaca fascicularis* (Cynomolgus macaque) animals. Insert shows blood smear taken from an infected animal with prominent bacteremia.

Figure 2. AtxA and virulence factor synthesis. Motifs apparent in the AtxA amino acid sequence are: WH, winged-helix; HTH, helix-turn-helix; PRD1 and PRD2, Phosphotransferase system Regulated Domains. PDGA, poly-D-glutamic acid. EIIB, Enzyme IIB similarity.