The stringent response of *Bacillus anthracis* contributes to sporulation but not to virulence

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The Gram-positive, spore-forming pathogen *Bacillus anthracis* is the aetiological agent of anthrax. Its main virulence factors are two toxins and an anti-phagocytic capsule. When *B. anthracis* is grown in laboratory culture, the highest expression of the anthrax toxin genes occurs during entry into stationary phase, suggesting that nutrient limitation is an environmental cue which induces toxin production. A common bacterial response to starvation is the so-called stringent response, in which the hyperphosphorylated guanosine nucleotide (p)ppGpp is the effector molecule. In *Escherichia coli*, *Bacillus subtilis* and other bacteria, accumulation of this molecule leads to down-regulation of stable RNA synthesis and upregulation of the expression of genes involved in survival under nutrient-poor conditions. This study focuses on the stringent response of *B. anthracis*. We show that in *B. anthracis* the *relA* gene is responsible for the synthesis of (p)ppGpp and the stringent down-regulation of stable RNA synthesis upon starvation for the essential amino acids isoleucine, leucine and valine. The deletion of *relA* did not affect the expression of the virulence gene *pagA* or virulence in a mouse model of infection. In contrast, spore counts upon growth and sporulation in a defined medium were approximately 10 000-fold lower for the *relA* deletion mutant than for the parental strain. The contribution of the stringent response to efficient sporulation of *B. anthracis* is notable, as this suggests that the stringent response may contribute to the persistence of *B. anthracis* in the natural environment.

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INTRODUCTION

Bacillus anthracis is a Gram-positive sporulating bacterium, and causes the disease anthrax. The infectious cycle of *B. anthracis* starts when spores are taken up by a mammalian host. Upon germination of the spores inside the host, vegetative cells synthesize different virulence factors, by far the most important of which are two toxins and a capsule (Mock & Fouet, 2001). Binary combinations of protective antigen (PA) with either lethal factor (LF) or oedema factor (EF) constitute the toxins. PA, LF and EF are encoded by the genes *pagA*, *lef* and *cya*, respectively. These three genes are carried by pXO1, a 181 kb plasmid, and are organized as independent transcriptional units. The *B. anthracis* capsule is composed of poly- γ -D-glutamate. A five-gene operon, *capBCADE*, encodes the enzymes responsible for capsule synthesis and anchoring (Candela & Fouet, 2005; Candela *et al.*, 2005), and this operon is harboured by pXO2, a 96 kb plasmid. The main function of the capsule is the inhibition of phagocytosis during infection (Mock &

Fouet, 2001). Upon infection, the host dies from a combination of toxæmia and septicaemia. As *B. anthracis* mainly infects herbivores, vegetative cells generally find themselves in the soil upon the death of the host and the decomposition of its carcass (Dragon *et al.*, 2005). *B. anthracis* has a limited ability to grow in soil (Van Ness, 1971; Jensen *et al.*, 2003; Saile & Koehler, 2006) and vegetative cells of *B. anthracis* therefore form endospores, thereby completing the infectious cycle (Mock & Fouet, 2001).

Two important conditions favouring the expression of *B. anthracis* virulence factors are the presence of a CO₂–bicarbonate equilibrium and a temperature of 37 °C (Sirard *et al.*, 1994; Fouet & Mock, 1996). These conditions are specifically encountered in the mammalian host. *In vitro* studies in liquid media have revealed that the three toxin genes are coordinately regulated under these conditions. Their rate of transcription increases throughout exponential growth, reaching a maximum upon entry into stationary phase. The regulatory mechanisms that control toxin gene expression are not fully understood (Fouet & Mock, 2006). An important regulator is AtxA, which is encoded by a gene situated on pXO1. It activates toxin gene expression, capsule formation and other chromosomally or

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Abbreviations: ILV, isoleucine, leucine and valine; PA, protective antigen.

plasmid-encoded genes (Uchida *et al.*, 1993; Guignot *et al.*, 1997; Mignot *et al.* 2003; Bourgogne *et al.* 2003). The alternative sigma factor σ^H and the transition-state regulator AbrB have been shown to control *atxA* transcription and consequently toxin gene expression (Saile & Koehler, 2002; Strauch *et al.*, 2005; Hadjifrangiskou *et al.*, 2007). The mode of action of AtxA is still unclear, but recently it has been suggested that its activity can be increased or decreased by phosphorylation of two different histidine residues (Tsvetanova *et al.*, 2007).

The maximal expression of toxin genes occurs at the end of exponential phase, upon entry into stationary phase, suggesting a link between nutrient limitation and toxin gene expression in *B. anthracis*. However, sporulation is also triggered by nutrient limitation, and these two stationary-phase responses appear to be mutually exclusive (White *et al.*, 2006). An important bacterial response to starvation is called the stringent response (for a recent review, see Braeken *et al.*, 2005). It is characterized by a rapid down-regulation of stable RNA synthesis upon amino acid starvation. The accumulation of the effector molecules ppGpp and pppGpp is an important hallmark of the stringent response. These nucleotides are synthesized by enzymic phosphorylation of GDP and GTP to ppGpp and pppGpp, respectively, using ATP as a phosphate donor. In *Escherichia coli*, in which the stringent response was first studied (Cashel *et al.*, 1996), the proteins SpoT and RelA catalyse this reaction. In Gram-positive bacteria, the protein RelA, which combines the functions of *E. coli* SpoT and RelA, appears to be the only protein involved in the production of (p)ppGpp (Mittenhuber, 2001). In the non-pathogenic Gram-positive model organism *Bacillus subtilis*, *relA* is necessary for efficient sporulation (Eymann *et al.*, 2001). In the non-sporulating Gram-positive pathogens *Mycobacterium tuberculosis*, *Listeria monocytogenes* and *Streptococcus mutans*, the deletion of the *relA* gene affects virulence properties (Taylor *et al.*, 2002; Dahl *et al.*, 2003; Lemos *et al.*, 2004).

In this study we generated a *relA* deletion mutant in *B. anthracis*, and using this mutant we were able to show that *relA* is essential for (p)ppGpp accumulation and down-regulation of stable RNA synthesis upon amino acid starvation. We could not detect a role for *relA* in the expression of *pagA* or in virulence in a mouse model of infection. However, sporulation in a defined medium was affected in the *relA* mutant, leading to approximately 10 000-fold lower spore counts for the *relA* mutant than for the parental strain. Our data indicate that the stringent response of *B. anthracis* may be important for the persistence and spread of *B. anthracis* in the environment by contributing to the process of sporulation.

METHODS

Strains and culture conditions. The strains used were *B. anthracis* 7702 (pXO1⁺, pXO2⁻), RPG1 (pXO1⁺, pXO2⁺, Tox⁻) and the *pagA-lacZ* and *atxA-lacZ* promoter reporter strains RBAF140 and

7702XFI, respectively (Collection de l'Institut Pasteur; Gimenez *et al.*, 2004; Sirard *et al.*, 1994; Guidi-Rontani *et al.*, 1999). For pre-cultures, strains were grown overnight in 20 ml brain heart infusion (BHI) broth with 0.5 % (v/v) glycerol in 100 ml Erlenmeyer flasks at 37 °C with rotary shaking at 150 r.p.m. Bacterial growth was determined by measuring OD₆₀₀ with an Ultrospec 3300pro spectrophotometer (Amersham Pharmacia Biotech) using cuvettes with a 1 cm path length. When cultures reached OD₆₀₀ >0.5, samples were diluted fourfold with the growth medium as diluent prior to the measurement.

Generation of *relA* deletion mutants in *B. anthracis*. The *relA* mutant was first made in strain 7702 and was constructed by allelic replacement of a 1.6 kb internal fragment of *relA* with a non-polar kanamycin-resistance cassette. To obtain this mutant a 2.8 kb fragment containing the *relA* gene with 0.5 kb up- and 0.1 kb downstream flanking regions was PCR-amplified using the primers Rel5' (5'-CGCATTAGAAGTAGGATTTGCGCCAGTTCG-3') and Rel3' (5'-CCGAACGGAATTTGTCCTACGATCTCACCG-3'). This fragment was cloned into pCR2.1 (Invitrogen), resulting in pRel10. This vector was then digested with *SpeI*, followed by blunting of the overhang with Vent DNA polymerase, and *PstI* digestion. The resulting product was cloned into pUC18, which was digested by *SmaI* and *PstI*, resulting in pRel15. Subsequently, a 1.5 kb kanamycin-resistance cassette, which was obtained by *Clal* digestion from pAT21 (Trieu-Cuot & Courvalin, 1983) and subsequent blunting of overhangs, was inserted in pRel15, which was digested by *BamHI* and *EcoRV* and blunted. This replaces a 1.6 kb fragment of the *relA* gene with the resistance cassette. PCR analysis was performed on kanamycin-resistant clones to select for the vector with the kanamycin-resistance cassette in the same orientation as the *relA* gene. This vector was named pRel27, and was subsequently digested by *EcoRI* and *SphI*. The fragment containing the *relA* gene and the kanamycin-resistance vector was cloned into the conjugative plasmid pATAS28, resulting in pRel37. This vector was used to generate deletion mutants of *relA* by filter mating (Trieu-Cuot *et al.*, 1987; Pezard *et al.*, 1991) of *B. anthracis* 7702 with *E. coli* HB101 carrying the plasmids pRK24 and pRel37. Deletion of *relA* in *B. anthracis* was confirmed by PCR analysis using primers on the kanamycin-resistance cassette and outside the cloned fragment containing the *relA* gene (data not shown). The *relA* deletion was transferred to other genetic backgrounds by phage transduction with CP51, as described elsewhere (Green *et al.*, 1985). The *relA* deletion mutant in the 7702 background was termed 7RelK.

Development of a defined medium for *B. anthracis*. A defined low-phosphate medium (DLPM) for culturing *B. anthracis* was based on a medium developed elsewhere for *Bacillus cereus* (Buono *et al.*, 1966). The DLPM medium for *B. anthracis* contained MOPS (40 mM, pH 7.4) as buffering agent, 0.2 mM of each amino acid, 0.1 mM K₂HPO₄, 0.8 mM MgSO₄, 0.04 mM MnCl₂, 0.2 mM NaCl, 10 mM KCl, 0.2 mM CaCl₂, 0.05 mM ZnSO₄, 0.04 mM FeCl₃, 20 mM glucose and 1 mg thiamine hydrochloride l⁻¹. As an inoculum, 1 ml of an overnight culture in BHI + 0.5 % glycerol was washed twice with 1 ml DLPM and resuspended in an equal volume of DLPM. Subsequently, 20 ml DLPM was inoculated with 20 µl of the washed overnight culture and cultured at 37 °C as described above.

Measurement of RNA synthesis. RNA synthesis by *B. anthracis* was determined by measuring the incorporation of [³H]uridine, essentially as described by Lewis *et al.* (2000). *B. anthracis* was grown in DLPM to OD₆₀₀=0.15. Subsequently 2 ml aliquots of the culture were filtered using hydrophilic Durapore membrane filters (0.45 µm pore-size, Millipore). The filters were then resuspended in 2 ml pre-warmed DLPM or DLPM with the amino acids isoleucine, leucine and valine (ILV) omitted (DPLM-ILV). In addition, the medium

contained [^3H]uridine (10 μCi ; 370 kBq) and cold uridine (0.1 mM). Aliquots of 250 μl were removed and added to 1 ml ice-cold 10% TCA. Samples were left on ice for at least 30 min, after which total RNA was collected by filtration on Whatman GF/C glassfibre disks. Subsequently, filters were washed twice with 1 ml ice-cold 10% TCA and once with 3 ml 100% ethanol. [^3H]Uridine incorporation was determined by scintillation counting.

Determination of intracellular (p)ppGpp levels. *B. anthracis* cultures were grown in DLPM to $\text{OD}_{600}=0.03$. Next, 10 μCi ^{32}P -labelled H_3PO_4 was added to 1 ml of culture, followed by further incubation at 37 °C until the cultures reached OD_{600} 0.15. Cultures were then filtered and resuspended in DLPM-ILV containing 10 μCi ^{32}P -labelled H_3PO_4 . Immediately prior to filtration ($t=0$) and 5, 10, 20, 30 and 40 min after resuspension of the filters in the growth medium, 20 μl aliquots were removed and added to 20 μl 12 M formic acid. After three freeze-thaw cycles, 5 μl aliquots were analysed on PEI/cellulose TLC plates (Macherey-Nagel) with 1.5 M KH_2PO_4 as developing solution. Labelled nucleotides were visualized by autoradiography on film and Phosphorimager screens. To identify labelled nucleotides, ^{32}P -labelled formic acid extracts of *E. coli* MG1655 were prepared as described below, and these were run adjacent to the *B. anthracis* extracts on the TLC plates. *E. coli* was grown in DLPM to $\text{OD}_{600}=0.15$, after which cells were resuspended in DLPM from which all nitrogen sources were removed (DLPM-N) and incubated at 37 °C for 10 min. Aliquots (20 μl) were removed before and after resuspension in DLPM-N, and labelled nucleotides were extracted as outlined above.

β -Galactosidase assays. Expression of *pagA* and *atxA* during growth at 37 °C in R medium containing 0.6% (w/v) sodium bicarbonate was determined as previously described (Sirard *et al.*, 1994) by using *pagA-lacZ* and *atxA-lacZ* promoter reporter constructs integrated on pXO1 in both parental and *relA*-deleted backgrounds.

Animal experiments. Six female OF/1 (6–8 weeks old) mice (Charles River Laboratories) were injected subcutaneously with 5×10^4 , 5×10^5 or 5×10^6 spores per mouse of either *B. anthracis* 7702 or 7RelK. Survival of the mice during infection was scored daily for 15 days. Kaplan–Meier survival curves were drawn and analysed using log rank analysis with GraphPad Prism 4 software (GraphPad Software). LD_{50} values were estimated by the Reed and Muench method (Reed & Muench, 1938).

Determination of sporulation of *B. anthracis*. Strains were grown in DLPM at 37 °C with shaking at 150 r.p.m. At regular intervals,

1 ml samples were taken and viable counts were determined using unheated (for total viable counts) and heated (65 °C, 20 min; for spore counts) samples. Serial dilutions of the samples in physiological saline (0.9% NaCl) were plated on BHI plates. Plates were incubated for 18 h at 37 °C.

RESULTS

relA-dependent (p)ppGpp accumulation and stringent response

The gene encoding (p)ppGpp synthetase was identified in *B. anthracis* by searching the *B. anthracis* genome (Read *et al.*, 2003) for genes encoding homologues of RelA from *B. subtilis* and *L. monocytogenes* (Wendrich & Marahiel, 1997; Taylor *et al.*, 2002). The protein encoded by gene BA4637 is 77 and 70% identical, respectively, to these two genes, and BA4637 was therefore termed *relA*. A deletion mutant for the *relA* gene, termed *B. anthracis* 7RelK, was constructed by allelic replacement of a 1.6 kb internal fragment of BA4637 with a kanamycin-resistance cassette.

For our studies we developed a defined medium for *B. anthracis* (DLPM; see Methods). Amino acid auxotrophies of *B. anthracis* 7702 were determined by removing single amino acids from DLPM. By this ‘drop-out’ approach, the amino acids glycine, isoleucine, leucine, methionine, phenylalanine, serine and valine were found to be essential for growth of *B. anthracis* 7702. The deletion of *relA* did not affect amino acid auxotrophies (data not shown).

In both *E. coli* and *B. subtilis* the removal of essential amino acids has been found to lead to the accumulation of (p)ppGpp (Cashel *et al.*, 1996; Ochi *et al.*, 1981). During exponential growth in DLPM and upon starvation for ILV, the accumulation of (p)ppGpp in *B. anthracis* was measured by TLC analysis (Fig. 1). In *B. anthracis* 7702, spots corresponding to (p)ppGpp could not be identified during exponential growth in DLPM. Five minutes after ILV starvation, (p)ppGpp accumulated. The levels of (p)ppGpp appeared to decrease somewhat over time. In

Fig. 1. *relA*-dependent accumulation of (p)ppGpp in *B. anthracis* upon starvation for ILV. *B. anthracis* 7702 and 7RelK were grown in DLPM to mid-exponential phase. Cultures were then filtered and the filters containing the cells were resuspended in DLPM-ILV. At the indicated times, aliquots were removed from the cultures, and ^{32}P -labelled nucleotides were extracted and analysed by TLC. To identify labelled nucleotides of *B. anthracis*, ^{32}P -labelled extracts of nitrogen-starved (DLPM-N) *E. coli* MG1655 were run adjacent to the *B. anthracis* extracts on the TLC plates. Results shown are representative of two independent experiments.

7RelK, spots corresponding to (p)ppGpp were not present during exponential growth and did not appear upon starvation for ILV.

The defining characteristic of the stringent response is the down-regulation of RNA synthesis upon starvation for amino acids (Cashel *et al.*, 1996). In *B. anthracis* the removal of ILV leads directly to a rapid down-regulation of RNA synthesis as measured by [³H]uridine incorporation in RNA (Fig. 2). In 7RelK, however, RNA synthesis was unchanged upon starvation for ILV. We conclude that *B. anthracis* exhibits a stringent response on starvation for essential amino acids and that this response is mediated through the *relA* gene, which is responsible for the accumulation of (p)ppGpp.

Deletion of *relA* does not affect virulence

To assess the effect of the *relA* gene on the transcription of *pagA*, which encodes the toxin component PA, we deleted the *relA* gene in strain RBAF140 (Sirard *et al.*, 1994). This strain is isogenic to 7702, but carries a transcriptional fusion of the promoter of *pagA* with a *lacZ* reporter gene on pXO1. The β -galactosidase activities of RBAF140 and its *relA* mutant were determined during growth in R medium supplemented with 0.6% sodium bicarbonate. During growth in R medium, *pagA* transcription reached maximum levels in *B. anthracis* RBAF140 in early stationary phase (Fig. 3). The deletion of *relA* did not influence the transcription of *pagA* during growth in R medium. The transcription of the *atxA* gene was also not significantly affected by the deletion of *relA* (data not shown). The virulence of the *relA* mutant was compared to that of the

Fig. 2. The *relA*-dependent stringent response of *B. anthracis*. The synthesis of stable RNA was measured by [³H]uridine incorporation in TCA-precipitable material in *B. anthracis* 7702 (circles) and 7RelK (squares) during exponential growth in DLPM (closed symbols) and DLPM-ILV (open symbols). Results shown are representative of three independent experiments.

Fig. 3. Transcription of *pagA* in the wild-type strain and its isogenic *relA* deletion mutant. *B. anthracis* RBAF140 (circles) and its *relA* mutant (squares) were grown in R medium containing 0.6% sodium bicarbonate under a 5% CO₂ atmosphere. Growth was followed by OD₆₀₀ measurements (open symbols) and transcription of *pagA* was determined by measuring β -galactosidase activity (closed symbols). Results shown are representative of three independent experiments.

parental 7702 strain in a mouse model of anthrax. Estimated LD₅₀ values were essentially the same for the 7702 and 7RelK strains, at 2.5×10^4 and 2.4×10^4 spores per mouse, respectively. Both these results demonstrate that *relA* and the stringent response do not play a significant role in the virulence of *B. anthracis*. As capsulation was not affected by the deletion of *relA* in the capsulated RPG1 strain (data not shown), it was deemed unethical to perform animal experiments with RPG1 and its *relA* mutant, as capsulation is the sole determining factor of virulence in non-toxinogenic strains (Welkos *et al.*, 1993).

Deletion of *relA* diminishes sporulation

To study the effect of *relA* on sporulation, *B. anthracis* 7702 and 7RelK were grown in DLPM, and at regular intervals, total viable counts and spore counts were determined (Fig. 4). The wild-type strain sporulated readily and efficiently in this medium, reaching spore titres of 3.6×10^6 ml⁻¹ after only 14 h. After 24 h and at later time points, both the spore counts and the total viable counts for 7702 were essentially the same at 2.4×10^7 ml⁻¹, indicating complete sporulation. Final spore counts in the 7RelK culture were only 1×10^3 ml⁻¹. The remaining vegetative cells of 7RelK started to die off after 48 h of growth in DLPM, presumably due to their inability to adapt to and survive in nutrient-poor conditions.

Fig. 4. Effect of the deletion of *relA* on sporulation of *B. anthracis*. Strains 7702 (circles) and 7RelK (squares) were grown in DLPM at 37 °C. Total viable counts (closed symbols) and spore counts (open symbols) were determined. The results are the mean of three independent experiments and SD is indicated by the error bars.

DISCUSSION

In this article we show that *B. anthracis* exhibits a rapid down-regulation of RNA synthesis upon starvation of the essential amino acids ILV. Upon removal of these amino acids, the hyperphosphorylated nucleotides ppGpp and pppGpp accumulate, and the gene *relA* was determined to be responsible for the biosynthesis of (p)ppGpp. Deletion of the *relA* gene did not affect the production of virulence factors or virulence in a mouse model, but had a large effect on sporulation.

Whereas it has long been known that in *B. anthracis* entry into the stationary growth phase coincides with maximal production of toxin components (Sirard *et al.*, 1994), the environmental cues that lead to this response are still unknown. On the basis of our findings, starvation for amino acids and the resulting stringent response can be ruled out as a trigger that leads to the production of the *B. anthracis* virulence factors. However, it cannot be ruled out that other regulatory mechanisms that react to amino acid levels have a role in controlling the production of virulence factors in *B. anthracis*.

It appears that in both *B. anthracis* and the Gram-positive model organism *B. subtilis*, an important function of the stringent response is conserved, i.e. priming of cells that undergo nutrient starvation to go into sporulation. In non-spore-forming pathogenic Gram-positive bacteria, the effects on virulence do not appear to be a consequence of a stringent response-dependent upregulated expression of specific virulence factors, but rather a more pleiotropic

effect on microbial metabolism, which can result in lower growth rates or the loss of certain phenotypes (such as biofilm formation) that can confer a competitive advantage during infection (Taylor *et al.*, 2002; Dahl *et al.*, 2003; Lemos *et al.*, 2004). As no major effect of the deletion of *relA* on both the production of virulence factors and the growth kinetics of *B. anthracis* was observed, our finding that the *relA* deletion mutant is as virulent as the parental *B. anthracis* strain is not unexpected.

Interestingly, the sporulation of *B. anthracis* was strongly affected in a *relA* deletion mutant. This shows that the stringent response of *B. anthracis* is important in signalling nutrient starvation and priming the cells to sporulate. The mechanistic pathway linking the stringent response to sporulation remains to be determined in *B. anthracis*, and may involve a decrease in GTP levels due to the inhibitory action of (p)ppGpp on the enzyme inosine monophosphate dehydrogenase, which has been shown to be a target for (p)ppGpp in several bacteria, including *B. subtilis* (Ochi *et al.*, 1981; Ochi, 1987; Kasai *et al.*, 2006).

The important role of the stringent response in the sporulation of *B. anthracis* has implications for the understanding of the mechanisms that are involved in the infectious cycle of *B. anthracis*, since the spore is the persistent form of *B. anthracis* in the natural environment and is the infectious form that is needed to cause the disease anthrax in the mammalian host. Consequently, the stringent response appears to contribute significantly to the ecological success of *B. anthracis* as a mammalian pathogen.

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