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* or *virA* with either *lef* or *virF*, 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-β-1,6-glucosamine. 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 toxaemia and septicemia. 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 CO$_2$–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 plasmid-encoded virulence factors.

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.
plasmid-encoded genes (Uchida et al., 1993; Guignot et al., 1997; Mignot et al. 2003; Bourgogne et al. 2003). The alternative sigma factor σH 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 (Tsytanov, 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+), RGP1 (pXO1+, pXO2+, Tox+) and the pagA–lacZ and atxA–lacZ promoter reporter strains RBAP140 and 7702XF1, 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 Rel’5’–CCGATTAGAAGTGGATTGGCCGCTTGCG-3’ and Rel3’–CCGAGGGAATTGTCCTACAGTCTacCGG-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 PsiI digestion. The resulting product was cloned into pUC18, which was digested by Smal and PsiI, resulting in pRel15. Subsequently, 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 SplI. The fragment containing the relA gene and the kanamycin-resistance vector was cloned into the conjugal 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 (DPLM) for culturing B. anthracis was based on a medium developed elsewhere for Bacillus cereus (Buono et al., 1966). The DPLM medium for B. anthracis contained MOPS (40 mM, pH 7.4) as a 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 1–1. As an inoculum, 1 ml of an overnight culture in BHI + 0.5% glycerol was washed twice with 1 ml DPLM and resuspended in an equal volume of DPLM. Subsequently, 20 ml DPLM 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 DPLM 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 prewarmed DPLM or DLEPM with the amino acids isoleucine, leucine and valine (ILV) omitted (DLEPM-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 glass fibre 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 OD<sub>600</sub>=0.03. Next, 10 μCi [32P]-labelled H<sub>3</sub>PO<sub>4</sub> was added to 1 ml of culture, followed by further incubation at 37 °C until the cultures reached OD<sub>600</sub> 0.15. Cultures were then filtered and resuspended in DLPM-ILV containing 10 μCi [32P]-labelled H<sub>3</sub>PO<sub>4</sub>. 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 M KH<sub>2</sub>PO<sub>4</sub> as developing solution. Labelled nucleotides were visualized by autoradiography on film and Phosphorimager screens. To identify (p)ppGpp spots corresponding to (p)ppGpp could not be identified 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.

**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 (Caskey 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](image-url)
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 \[^{3}H\]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 \(\beta\)-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 parental 7702 strain in a mouse model of anthrax. Estimated LD\(_{50}\) values were essentially the same for the 7702 and 7RelK strains, at \(2.5 \pm 10^4\) and \(2.4 \pm 10^4\) 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 \(3.6 \times 10^6\) 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 \(2.4 \times 10^7\) ml\(^{-1}\), indicating complete sporulation. Final spore counts in the 7RelK culture were only \(1 \times 10^7\) 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.
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 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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REFERENCES


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