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Phosphate starvation enhances the pathogenesis of *Bacillus anthracis*

Somya Aggarwal[1], Vikas Kumar Somani[1], Rakesh Bhatnagar*

Molecular Biology and Genetic Engineering Laboratory, School of Biotechnology, Jawaharlal Nehru University, New Delhi 110067, India

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ABSTRACT

Identifying the factors responsible for survival and virulence of *Bacillus anthracis* within the host is prerequisite for the development of therapeutics against anthrax. Host provides several stresses as well as many advantages to the invading pathogen. Inorganic phosphate (Pi) starvation within the host has been considered as one of the major contributing factors in the establishment of infection by pathogenic microorganisms. Here, we report for the first time that Pi fluctuation encountered by *B. anthracis* at different stages of its life cycle within the host, contributes significantly in its pathogenesis. In this study, Pi starvation was found to hasten the onset of infection cycle by promoting spore germination. After germination, it was found to impede cell growth. In addition, phosphate starved bacilli showed more antibiotic tolerance. Interestingly, phosphate starvation enhanced the pathogenicity of *B. anthracis* by augmenting its invasiveness in macrophages *in vitro*. *B. anthracis* grown under phosphate starvation were also found to be more efficient in establishing lethal infections in mouse model as well. Phosphate starvation increased *B. anthracis* virulence by promoting the secretion of primary virulence factors like protective antigen (PA), lethal factor (LF) and edema factor (EF). Thus, this study affirms that besides other host mediated factors, phosphate limitation may also contribute *B. anthracis* for successfully establishing itself within the host. This study is a step forward in delineating its pathophysiology that might help in understanding the pathogenesis of anthrax.

1. Introduction

Microbial pathogenesis is a consequence of host–microbe interactions. Both microbial as well as host factors are required for successful establishment of an infection (Casadevall and Pirofski, 2003; Casadevall et al., 2001). Thus, investigating factors which support the virulence of a pathogen would provide greater insight into the understanding of bacterial physiology for mining new targets for combating infections. Various stresses encountered by the pathogen within the host like iron depletion (Carlson et al., 2009; Cendrowski et al., 2004), oxidative stress (Cybulski et al., 2009; Kim et al., 2013), carbon dioxide stress (McKenzie et al., 2014) etc. have been well studied in a variety of pathogens. Besides stress, host may also contribute factors that can favor the survival and virulence of pathogens (Wang et al., 2002). Targeting such factors is of great significance for developing reliable treatment strategies to combat infections.

Bacillus anthracis, a potential biothreat, completes its infection cycle in various host niches where it encounters different environmental conditions like temperature fluctuation, osmolarity difference, nutrient limitation, highly oxidative conditions *etc.* Spore germination is an initial and crucial step in the infection by *B. anthracis*. It occurs inside the phagolysosomal compartment of macrophages having nutrient deprived environment. It also exerts oxidative and acidic stress on the invading pathogen (Fang, 2004; Garin et al., 2001). To adapt efficiently to this hostile environment, it switches on its gene regulation in a modular fashion (Storz and Hengge-Aronis, 2000). Transcriptional profiling of *B. anthracis* inside the alveolar macrophages revealed that genes for phosphate transport are upregulated during early stages of infection (Bergman et al., 2007). Genes encoding phosphate transporters have been found to be upregulated under phosphate starvation conditions in some bacteria (Rifat et al., 2009; Santos-Beneit et al., 2008). Hence, it can be inferred that during its intracellular phase of life cycle, *B. anthracis* faces low phosphate conditions inside

Abbreviations: Pi, inorganic phosphate; PDM, phosphate depleted medium; PBM, phosphate balanced medium; PRM, phosphate repleted medium; BHI, brain heart infusion media; PBS, phosphate buffer saline; MOI, multiplicity of infection; PA, protective antigen; LF, lethal factor; EF, edema factor; DMEM, Dulbecco's modified eagle's medium; MTT, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); ELISA, enzyme linked immunosorbent assay; HRP, horse radish peroxidase; CMS, culture media supernatant.

* Corresponding author. Tel.: +91 11 26704079; fax: +91 11 26742040.
E-mail addresses: somya.sbt@gmail.com (S. Aggarwal), vikassomani012@gmail.com (V.K. Somani), rakeshbhatnagar@jnu.ac.in (R. Bhatnagar).

[1] These authors contributed equally to this work.

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macrophages. After germination, vegetative bacilli lyse the host phagocytes and become exposed to blood where copious amount of toxins is secreted by them while they multiply resulting in bacteremia and toxemia, eventually leading to death. The phosphate concentration in blood ranges between 2.4 to 4.1 mg/dl ≈ 0.1 mM to 0.2 mM (Shenoy, 2009). Accumulation of the unusual pentanucleotide ppGpp is induced in *E. coli* under phosphate starvation (Spira et al., 1995). In *B. anthracis*, upregulation of this stringent alarmone ppGpp was observed at 0.2 mM phosphate concentration suggesting it a phosphate starved condition for *B. anthracis in vitro* (Van Schaik et al., 2007). Thus, it can be envisaged that *B. anthracis* encounters phosphate deprived conditions firstly inside the phagosomes and then, while circulating in the blood stream during the infection cycle. Therefore, it is imperative to investigate the effects of phosphate limitation on the life cycle of *B. anthracis*, which is expected to provide novel insights into underlying mechanisms of bacterial pathogenesis.

In order to elucidate the role of phosphate starvation on establishment of anthrax, the effect of phosphate on various attributes of *B. anthracis* physiology and virulence within the host was studied. We found that phosphate starvation is one of the major contributing factors in establishment of anthrax by favoring spore germination, survival and virulence of the pathogen within the host. To best of our knowledge, this is the first detailed study on the contribution of Pi in establishment of *B. anthracis* infection.

2. Materials and methods

2.1. Bacterial strains and growth conditions

All experiments were done with the Sterne $34F_2$ ($pXO1^+$ $pXO2^-$) strain of *B. anthracis*. For primary culture, vegetative cells of *B. anthracis* Sterne were grown in Brain Heart Infusion (BHI) media at 37 °C with shaking at 150 rpm. For different phosphate conditions, phosphate depleted medium (PDM), phosphate balanced medium (PBM) and phosphate repleted medium (PRM) containing 0.2 mM, 3 mM and 5 mM of KH_2PO_4 respectively, was prepared as described previously (Van Schaik et al., 2007). Apart from phosphate, the media contained 40 mM MOPS, 0.2 mM of each amino acid. 0.8 mM $MgSO_4$, 0.04 mM $MnCl_2$, 0.2 mM NaCl, 10 mM KCl, 0.2 mM $CaCl_2$, 0.05 mM $ZnSO_4$, 0.04 mM $FeCl_3$, and 20 mM glucose. The pH was adjusted to 7.4 using KOH.

2.2. Growth analysis of B. anthracis under different phosphate conditions

For analyzing the growth of *B. anthracis* Sterne *in vitro* under different phosphate conditions, overnight grown culture in BHI was used for inoculation. Cells were washed in phosphate-free buffer as described earlier (Van Schaik et al., 2007), and inoculated in PDM, PBM and PRM. Aliquots were taken after every hour upto 10 h. Optical density was measured periodically at 600 nm with necessary dilution.

2.3. Antibiotic susceptibility studies

Minimum inhibitory concentration (MIC) of *B. anthracis* under different phosphate conditions was determined by using broth dilution method (Wiegand et al., 2008) in 96 well microtitre plate. Briefly, twofolds dilutions of antibiotics were made in PDM and PRM. Cells grown in BHI up to exponential phase were taken as primary culture. Before inoculating in different media, cells were first washed with phosphate free buffer to remove media components, if any. Both PDM and PRM with or without antibiotics were inoculated with 3×10^4 bacilli (per well) and allowed to grow under static conditions for 16–20 h at 37 °C. Among DNA gyrase inhibiting antibiotics, ciprofloxacin and ofloxacin were used, and among protein synthesis inhibitors, tetracycline and erythromycin were used, in the range of (0–1 μg/ml). MIC of each antibiotic was taken as the last concentration which prevented growth of the pathogen and was determined by measuring OD_{600} using Tecan Sunrise microplate reader.

2.4. Microscopic studies

B. anthracis cells were grown overnight in PDM, PBM and PRM at 37 °C and shaking at 150 rpm. Cells were washed thrice with 1× phosphate buffer saline (PBS). Cells were then fixed with 4% paraformaldehyde (in PBS) for 20 min at room temperature, washed thoroughly with PBS. Washed cells was then incubated with DAPI (4′,6-diamidino-2-phenylindole) at 5 μg/ml for 5 min at room temperature (RT) to stain nuclear DNA followed by washing twice with PBS. Finally, the cells were mounted on a glass slide and visualization was done by using Olympus FluoView FV1000 Laser Scanning Confocal Microscope. Cellular dimensions were measured by using ImageJ software. Final value of cell size was taken as average with standard deviation of nearly 10–15 cells from each condition.

2.5. Germination assay

B. anthracis spores were prepared by inoculating 10% of overnight grown culture of *B. anthracis* in sporulation broth (HiMedia). The culture was kept at 37 °C, shaking at 120 rpm for 5 days. The culture was subjected to heat at 65 °C for 30 min to kill vegetative cells. Spores were then centrifuged and pellet was washed with autoclaved MQ water. Concentration of spores was calculated by serial dilution method. To assess the effect of phosphate on germination, 5×10^4 spores were inoculated into PDM, PBM and PRM and allowed to grow at 37 °C. Aliquots were taken after every 15 min till 60 min and heat treatment was given at 65 °C for 30 min to kill germinated spores or vegetative bacilli. Germination efficiency was determined by calculating the CFU/ml obtained by plating the samples in different dilutions.

2.6. Infection studies in vitro

Mouse macrophage cell line, RAW 264.7, was maintained in DMEM (Sigma) with 10% heat inactivated fetal bovine serum (Gibco) containing appropriate concentrations of penicillin, streptomycin and amphotericin B (HiMedia). For experiment, cells were seeded in serum and antibiotic free DMEM, at a density of 2×10^6 cells per well in 6 well dishes and kept overnight in a humidified 5% CO_2 incubator. Infection with vegetative bacilli was done as described previously (Russell et al., 2007). Briefly, overnight grown bacilli from PDM, PBM and PRM were washed with serum and antibiotic free DMEM and allowed to infect macrophage cells at multiplicity of infection (MOI) 1 for 1 h. The media containing unbound bacilli was removed and washed twice with 1× PBS. To determine the number of associated bacteria (extracellular and intracellular), the cells were lysed with chilled PBS and the dilutions were plated on BHI agar. For assessing internalized bacteria, washed macrophages were incubated further with incomplete DMEM with gentamycin (20 μg/ml) for 1 h at 37 °C/5% CO_2, Cells were washed again followed by lysis with chilled 1× PBS. Appropriate dilutions of lysate were plated on BHI agar for CFU count.

Macrophages morphology was also monitored at every step using Olympus IX-71. Viewing was done using 60× objective.