JOURNAL OF PROTEOMICS 105 (2014) 242–265

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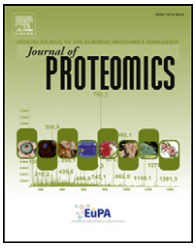

Delineating the effect of host environmental signals on a fully virulent strain of *Bacillus anthracis* using an integrated transcriptomics and proteomics approach☆

Gurudutta Panda[a], *Trayambak Basak*[b,c], *Pooja Tanwer*[a], *Shantanu Sengupta*[b,c], *Vítor A.P. Martins dos Santos*[d,e], *Rakesh Bhatnagar*[a,*]

[a]*Molecular Biology and Genetic Engineering Laboratory, School of Biotechnology, Jawaharlal Nehru University, New Delhi, 110067, India*
[b]*Genomics and Molecular Medicine Unit, CSIR-Institute of Genomics and Integrative Biology, New Delhi, 110020, India*
[c]*Academy of Scientific & Innovative Research, Delhi, India*
[d]*Systems and Synthetic Biology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands*
[e]*LifeGlimmer GmbH, Markelstrasse 38, Berlin 12163, Germany*

ARTICLE INFO

Available online 7 January 2014

Keywords:
Bacillus anthracis
iTRAQ
SCX-LC-MS/MS
Microarrays
Host associated lifestyle
Environmental perturbation
mRNA-protein correlation

ABSTRACT

Pathogenic bacteria sense the host environment and regulate expression of virulence-related genes. Environmental signals like temperature, bicarbonate/CO_2 and glucose induce toxin production in *Bacillus anthracis*, but the mechanisms by which these signals contribute to virulence and overall physiological adaptation remains elusive. An integrated, systems level investigation using transcriptomics and iTRAQ-based proteomics was done to assess the effect of temperature, bicarbonate/CO_2 and glucose on *B. anthracis*. Significant changes observed in amino acid, carbohydrate, energy and nucleotide metabolism indicates events of metabolic readjustments by environmental factors. Directed induction of genes involved in polyamine biosynthesis and iron metabolism revealed the redirection of cellular metabolite pool towards iron uptake. Protein levels of glycolytic enzymes, ptsH and Ldh along with transcripts involved in immune evasion (*mprF*, *bNOS*, Phospholipases and *asnA*), cell surface remodeling (*rfbABCD*, *antABCD*, and *cls*) and utilization of lactate (*lutABC*) and inositol showed constant repression under environmental perturbations. Discrepancies observed in mRNA/protein level of genes involved in glycolysis, protein synthesis, stress response and nucleotide metabolism hinted at the existence of additional regulatory layers and illustrated the utility of an integrated approach. The above findings might assist in the identification of novel adaptive strategies of *B. anthracis* during host associated survival and pathogenesis.

Biological Significance
In this study, the changes observed at both transcript and protein level were quantified and integrated to understand the effect of host environmental factors (host temperature, bicarbonate and glucose) in shaping the physiology and adaptive strategies of a fully virulent strain of *B. anthracis* for efficient survival and virulence in its host. Perturbations

☆ This article is part of a Special Issue entitled: Proteomics of non-model organisms.

* *Corresponding author*. Tel.: +91 11 26704079; fax: +91 26742040, +91 26704711(Lab).
E-mail address: rakeshbhatnagar@jnu.ac.in (R. Bhatnagar).

1874-3919/$ – see front matter
http://dx.doi.org/10.1016/j.jprot.2013.12.018

affecting toxin production were found to concordantly affect vital metabolic pathways and several known as well as novel virulence factors. These changes act as a valuable asset for generating testable hypotheses that can be further verified by detailed molecular and mutant studies to identify novel adaptive strategies of *B. anthracis* during infection.
Adaptation of an integrated transcriptomics and proteomics approach also led to the identification of discrepancies between mRNA/protein levels among genes across major functional categories. Few of these discrepancies have been previously reported in literature for model organisms. However their existence in *B. anthracis* and that too as a result of growth perturbations have not been reported till date. These findings demonstrate a substantial role of regulatory processes post mRNA synthesis via post transcriptional, translational or protein degradation mechanisms.
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1. Introduction

The ability of bacterial pathogens to promptly recognize and adapt to environmental factors encountered in the mammalian host is essential for its survival and multiplication. Recognition is accomplished through sophisticated sensing and signal transduction mechanisms like quorum sensing, two component systems and sugar transport mechanisms like phosphotransferase systems (PTS). These mechanisms activate a cascade of regulatory pathways that lead to the expression of virulence factors, metabolic readjustment, cell surface remodeling and other cellular adaptive responses necessary for survival in the host [1–4]. The environmental cues can be highly diverse based on the niche occupied by the pathogen in the host. It includes shift in temperature, presence of bicarbonate, availability of nutrients, varying levels of oxygen, oxidative stress and presence of several other physical and host response factors [5,6].

Bacillus anthracis, a Gram-positive spore-forming bacterium, is the etiological agent of anthrax. Anthrax is a lethal disease affecting both humans and animals, and can also be used as a major biological weapon [7]. The life cycle of *B. anthracis* is biphasic in nature: an infectious stage for multiplication in the host and a sporulation stage for persistence in the soil [8]. The fully virulent form of *B. anthracis* is described by the presence of two extra-chromosomal plasmids — pXO1 and pXO2 [7]. The genes encoding the tripartite anthrax toxin components — protective antigen (PA), lethal factor (LF) and edema factor (EF), are present on the pXO1 plasmid [9], whereas the genes encoding the biosynthetic operon of the poly-gamma-D-glutamate capsule is located on the pXO2 plasmid [10,11]. The toxin component PA is responsible for binding to the mammalian cell surface and delivering the other two components, either LF (a zinc metalloprotease) or EF (an adenylate cyclase) into the host cell [12]. The capsule is required to evade the host immune response [7,11]. Lethality is caused by the combined action of anthrax toxins (PA, LF and EF) leading to toxemia, and immune evasion causing uncontrolled bacterial growth leading to bacteremia and septicemia [13,14]. As systemic circulation/bloodstream is the major site for manifestation of systemic anthrax [15–17], the primary signals of host environment for *B. anthracis* includes the host body temperature (37 °C), presence of bicarbonate/CO_2 and glucose. These three well-known host associated factors enable *B. anthracis* to sense the imminent host environment and induce toxin production [18,19].

The switch in temperature from an environmental reservoir (22 °C–28 °C) to a warm-blooded host (37 °C) acts as an important signal for the pathogen to sense upon infecting a mammalian host [20]. Many pathogenic bacteria like *Shigella flexneri* [21], *Salmonella enterica* [22], *Escherichia coli* [21], *Borrelia burgdorferi* [23], *Bordetella pertussis* [24] and *Listeria monocytogenes* [25] up-regulate expression of virulence genes in response to the temperature shift experienced within the host by using molecular elements called 'thermosensors' [26]. In the case of *B. anthracis*, shift in growth temperature from 28 °C to 37 °C coordinately activates the expression of toxin genes by regulating the synthesis of anthrax toxin activator (atxA) gene, which acts as the master transcriptional regulator of both toxin and capsule genes in *B. anthracis* [19,27].

The bicarbonate/CO_2 based buffering system present in the mammalian host extracellular fluids, at concentrations ranging from 15–40 mM, also acts as a signal for the pathogen to sense [28,29]. The stimulation of bacterial virulence factors by bicarbonate/CO_2 has already been observed in several pathogenic bacteria, with examples including the toxic shock syndrome toxin 1 of *Staphylococcus aureus* [30], the cholera toxin of *Vibrio cholerae* O1 biotype E1 Tor [31] and the Mga virulence regulator of group A *streptococci* [32]. Unlike temperature, addition of bicarbonate/CO_2 (5% CO_2 and 0.8% bicarbonate) not only induces the expression of toxin genes but also stimulates capsule biosynthesis in *B. anthracis* [19,33].

The link between nutrient sensing and regulation of virulence genes has been recognized in several bacterial pathogens [34,35]. In the case of *B. anthracis*, the presence of glucose in the blood at concentrations ranging from 0.1 to 0.15% might represent an important signal during transition to the host systemic circulation to cause bacteremia and septicemia. A recent study also established glucose as an important host environment derived signaling molecule, which regulates toxin gene expression via the transcriptional regulator CcpA in *B. anthracis* [18].

The growth of *B. anthracis* at 37 °C in a 0.8% bicarbonate containing synthetic defined medium (R medium) and 5% CO_2 is reported to induce toxin production under laboratory settings [36]. In the present study, sequential environmental perturbations were carried out in the above described toxin inducing condition and the effect of temperature, bicarbonate