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WalRK two component system of *Bacillus anthracis* responds to temperature and antibiotic stress

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ABSTRACT

WalRK Two Component System (TCS) of *Bacillus anthracis* forms a functional TCS. This report elaborates upon the WalRK genomic architecture, promoter structure, promoter activity and expression under various stress conditions in *B. anthracis*. 5′ RACE located the WalRK functional promoter within 317 bp region upstream of WalR. Reporter gene assays demonstrated maximal promoter activity during early growth phases indicating utility in exponential stages of growth. qRT-PCR showed upregulation of WalRK transcripts during temperature and antibiotic stress. However, WalR overexpression did not affect the tested antibiotic MIC values in *B. anthracis*. Collectively, these results confirm that WalRK responds to cell envelope stress in *B. anthracis*.

1. Introduction

Adaptability is a virtue microorganisms are endowed with, making it possible for them to survive in habitats which are too challenging for other living forms. A pre-requisite for survival in such environments is a precise ability to monitor environmental parameters. Bacteria have evolved elegant signaling mechanisms to sense their outside surroundings and channel the information to cell interior for an appropriate response. Two Component Systems (TCSs) are such a class of regulatory modules, functioning towards the coupling of signal sensing, signal transmission and response generation which effect adaptations to various environmental changes [1,2]. Most bacterial genomes code for multiple TCSs, each specific for a particular environmental signal [3]. Pathogenic organisms have additionally maneuvered these pathways for virulence-related gene regulation. TCSs can effect changes at the levels of gene expression, regulon expression, whole-cell phenotypic changes (motility, competence) and multi-cellular behavioral changes (biofilm, sporulation) [4]. Evolutionarily related bacteria inhabiting divergent environments also differ in TCSs regulation. To establish a successful infection, a pathogen must perceive the host environment and adapt appropriately, drawing attention to the role of TCSs in pathogenesis, which is recognized inadequately till now. TCSs are the predominant mode of environmental sensing in prokaryotes and ubiquitous in bacteria, with absolute absence from mammals, thereby also posing as attractive antimicrobial targets. In an effort to counter drug resistance, there is an increasing chorus to target pathways which are not bactericidal/bacteriostatic but antivirulent in nature. Such an inhibitor will entail a lessened ecological pressure and deter antimicrobial resistance development. Targeting of multiple TCSs in one single step provides one such strategy.

A TCS typically encodes a Histidine Kinase (HK), which senses the signal and transmits it to its cognate Response Regulator (RR), which mounts the required response. While HKs are mostly membrane proteins, with periplasmic and/or cytoplasmic signal sensing domains, RRs are cytoplasmic entities with a number of effector functions, dominated by DNA binding [1,2]. Signal sensing leads to activation and autophosphorylation of the HK followed by phosphoryl group transfer to its cognate RR. Phosphorylation brings about conformational changes in the RR, enhancing its DNA binding affinity so as to cause a concomitant change in gene expression. RR phosphorylation is further controlled by phosphatase activity of the HK, RR or specific aspartyl phosphatases [2].

Bacillus anthracis, the causative agent of anthrax, can lead to fatal infections in animals and humans [5]. It has a bimodal lifecycle-as dormant spores outside the host and metabolically active vegetative cells inside its host. The infective particles of anthrax are its spores. Their high resilience, infectivity combined with the steep mortality rate of the disease make anthrax a CDC Tier I biological threat. TCSs may play an important role in the

Abbreviations: TCS, two component system; HK, histidine kinase; RR, response regulator; RACE, rapid amplification of cDNA ends.

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adaptation responses of *B. anthracis* as evident by a higher number of TCS components encoded by its genome in comparison to the non-pathogenic *Bacillus subtilis* [6]. WalRK is one of the functional TCS of *B. anthracis* which may regulate multiple genes with functions ranging from structure, cell division, metabolism, transport and sporulation [6]. Its homolog in *S. aureus* has been implicated in multidrug resistance [7,8].

The current study reports the WalRK genomic architecture, promoter structure, promoter activity and expression under various stress conditions in *B. anthracis*, in an attempt to understand its regulation, especially in the backdrop of role in cell envelope stress and drug resistance.

2. Materials and methods

2.1. Strains, plasmids and biochemicals

Escherichia coli strain DH5α and avirulent strain of *Bacillus anthracis* Sterne 34F2 (pXO1^{+} pXO2^{-}) were used in this study. *E. coli* strain GM2929 (*dam*$^{-}$ *dcm*$^{-}$) was used for propagating and isolating DNA for *B. anthracis* electroporation. *E. coli* strains were grown in Luria Bertani (LB) medium and *B. anthracis* Sterne strain in Brain Heart Infusion (BHI) medium, supplemented with antibiotics ampicillin (100 μg/ml) and chloramphenicol (5 μg/ml) wherever required. pGEMT-Easy from Promega (Madison, WI, USA) was used for functional promoter assays in *E. coli*. An *E. coli-Bacillus* shuttle vector pHCMC05 comprising of an inducible Pspac promoter was donated by *Bacillus* Genetic Stock Center and used for homologous gene expression. All routine biochemicals and chemical reagents were from Sigma (St. Louis, MO, USA), Amresco (Solon, OH, USA) or USB (Affymetrix, Ohio, USA). Liquid chemicals were from Merck (Darmstadt, Germany).

2.2. RT-PCR

One-step RT-PCR (Qiagen) was performed using DNase (Ambion) treated total RNA, isolated using TRI reagent (Sigma–Aldrich), from exponentially growing *B. anthracis* (O.D.$_{600nm}$~0.6) cells as template and indicated primers (Table S1), as per the manufacturer's instructions. 1 μg of total RNA was used as the template with 50 pmoles of primers in a 50 μl reaction mix. A no template negative control and a genomic DNA (gDNA) contamination check control was included in the experiment. The reaction products were analyzed by 1% agarose electrophoresis and ethidium bromide staining.

2.3. 5′ RACE

To determine the *walRK* promoter, 5′ RACE (Rapid Amplification of cDNA Ends) system (Invitrogen) was used. Total RNA was isolated from exponentially growing (O.D.$_{600nm}$~0.6) *B. anthracis* cells using the TRI reagent and treated with DNase. A PCR check for gDNA contamination was performed using gene-of-interest specific primers. Using this RNA as the template, and two primers GSP1 and GSP2 spanning an internal site in the coding region of *walR*, 5′ RACE was performed using the manufacturer's protocol. 2 μg of total RNA was used as the template with 5 pmoles of GSP 1 for the first strand cDNA synthesis. The reaction products were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. The sequence of the product was confirmed by automated DNA sequencing.

2.4. WalRK functional promoter determination

The upstream regions of *walRK* were PCR amplified with the indicated primers using gDNA as the template. The amplicons were digested and ligated into a *Kpn*I and *BamH*I digested vector pLacZ, a promoterless pGEMT vector with a *lacZ* insert (a kind gift from Dr. Nirupama Banerjee, ICGEB, India), to obtain *walRK* promoter-*lacZ* transcriptional fusions. The various constructs obtained were RKProm+, RKPromTrunc1, RKPromTrunc2 and RKPromTrunc3 (Fig. 1). The RKProm+ - *lacZ* construct was subcloned into pHCMC05 also. The sequence of the constructs was confirmed by automated DNA sequencing. To check the functional nature of the constructs, they were transformed into DH5α cells and plated onto LB plates containing ampicillin and X-gal (40 μg/ml).

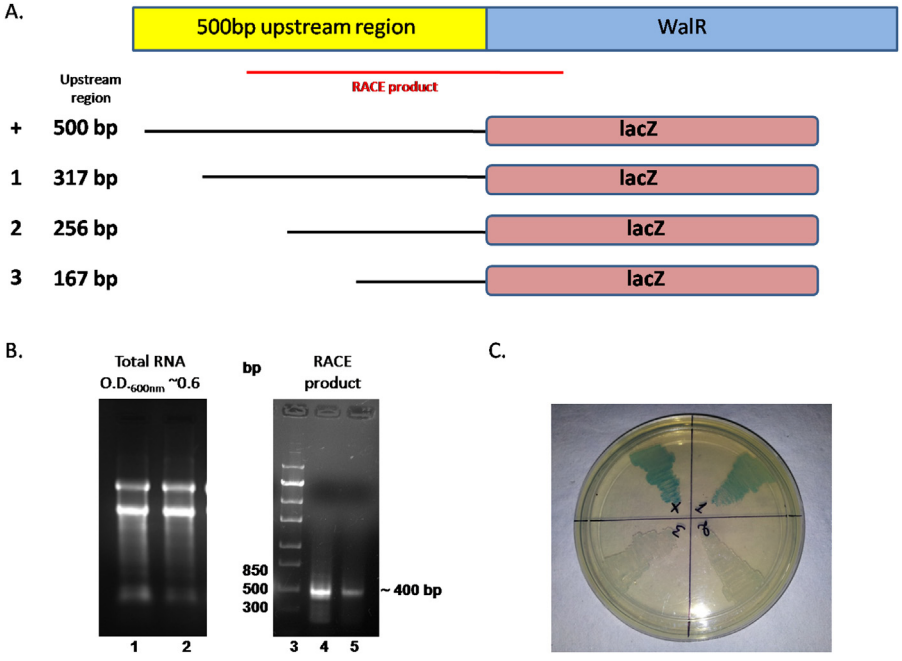

Fig. 1. 5′ RACE, *walRK* upstream deletion mutants and their activity analysis. (A). Schematic representation of *lacZ* transcriptional fusions with different *walRK* upstream regions. (B). Total RNA from *B. anthracis* (lanes 1 and 2) and *walR* 5′ RACE products (lanes 4 and 5), Lane 3-marker. (C). β-galactosidase activity of *walRK* upstream deletion mutants.

2.5. WalRK promoter activity with different phases of growth

RKProm+ - $lacZ_{pHCMC05}$ bearing *E. coli* strain was grown in LB medium containing ampicillin. Overnight cultures were diluted to 1% in fresh LB medium and 2 ml aliquots were withdrawn every hour for β-galactosidase (*lacZ*) activity, measured according to the method of Miller (Miller, 1992). The expression of β-galactosidase was reported in Miller units which is calculated as 1000 X (Absorbance at 420 nm)/ (Absorbance at 600 nm) X Time (min) X Volume (ml).

2.6. Minimum inhibitory concentration (MIC) determination for B. anthracis

To determine the antibiotic concentrations to be used for stress studies in *B. anthracis*, the MIC was determined for-vancomycin, fosfomycin and bacitracin-using the broth microdilution method [9] with slight modifications. BHI was used as the growth medium and absorbance was monitored spectrophotometrically. The 96 well microplates were read using a microplate reader (Tecan, Switzerland).

2.7. WalRK expression in E. coli and B. anthracis under stress

Overnight cultures were diluted to 1% in fresh BHI medium and grown at 37 °C till exponential phase ($O.D._{600nm}$~0.4). Once the optical density was reached, the culture was split into two parts-one was control which had no treatment and the other was experimental. The stress conditions were temperature (42 °C), osmotic (4 M NaCl) and ethanol (4% ethanol) and antibiotics-vancomycin, fosfomycin and bacitracin. For *E. coli*, the sub-inhibitory antibiotic concentrations were taken from literature while for *B. anthracis*, they were used as determined by the MIC experiments. Each stress treatment was for an h at 37 °C. For *E. coli*, WalRK expression was assessed by RKProm+ - $lacZ_{pHCMC05}$ activity of the promoter fusion as described above, while for *B. anthracis* qRT-PCR was done.

2.8. Transcriptome studies under stress conditions: qRT-PCR

For each stress treatment, an aliquot was taken and plated on BHI plates to check for contamination, if any and also for CFU calculation and comparison with the control, as an indication of stress induction. Total RNA was isolated for each stress treatment and checked for gDNA contamination with gene-specific primers, followed by cDNA preparation from 1 μg of DNase treated RNA. qRT-PCR was performed with control and stress condition samples as described before [6]. A no-stress control was used as the calibrator with *dna gyrase* as the endogenous control. Quantification of the relative changes in gene expression was performed using the $2^{-\Delta\Delta Ct}$ method. The data was plotted as mean of relative quantitation, from three independent runs with SEM.

2.9. Electroporation of B. anthracis competent cells with DNA

The electrocompetent *B. anthracis* cells were made using the protocol of Koehler et al. [10]. The DNA to be used for electroporation was isolated from dam- dcm- strain of *E. coli* GM2929 to increase the transformation efficiency. Around 10 μg of DNA was added to a 200 μl aliquot of cells in a pre-chilled 0.2 cm electrode gap cuvette (Bio-Rad) and electroporation was done at 2.0 kV, 200 Ω, 25 μF (mean time-constant > 8 ms) using a Bio-Rad Gene Pulser with a pulse controller and capacitance extender. An aliquot of cells was taken before electroporation to check for viable cell count. After giving the pulse, the cells were incubated on ice for 5 min and immediately diluted with 1 ml of BHI medium with 10% glycerol, 0.4% glucose, and 10 mM $MgCl_2$. The mixture was incubated at 37 °C for 1.5 h to allow expression of antibiotic resistance genes prior to plating on selective medium. The plates were incubated at 37 °C for 20 h.

2.10. WalR overexpression in B. anthracis

WalR ORF was amplified from the *B. anthracis* gDNA using forward and reverse primers with flanking *BamH*I and *Xba*I sites and ligated into double digested pHCMC05 vector to obtain PspacWalR construct. The sequence of the ORF was confirmed by automated DNA sequencing. This PspacWalR construct was electroporated in *B. anthracis* and the transformants were screened by colony PCR using *cat* cassette amplification. Positive transformants were grown in the presence of 1 mM IPTG supplemented BHI medium. Semi quantitative RT-PCR was done to check for *walR* overexpression, using *dna gyrase* as the loading control, followed by intensity analysis using Image J software.

3. Results and discussion

3.1. B. anthracis WalRK forms an operon with six other genes

WalRK forms a functional TCS in *B. anthracis* [6]. The HK and RR genes of any typical TCS occur in an operon. The genomic organization of WalK (BAS5318) and WalR (BAS5319) depicted the two genes to be part of an operon with six other genes, as per the DOOR database (Figure S1A) [11]. Apart from *walR* and *walK*, the rest of the genes from the operon are annotated as: BAS5317-hypothetical protein, BAS5316-hypothetical protein, BAS5315-metallo-beta-lactamase family protein, BAS5314-serine protease, BAS5313-hypothetical protein and BAS5312-rRNA large subunit methyltransferase, as per the KEGG database. WalR precedes WalK in the

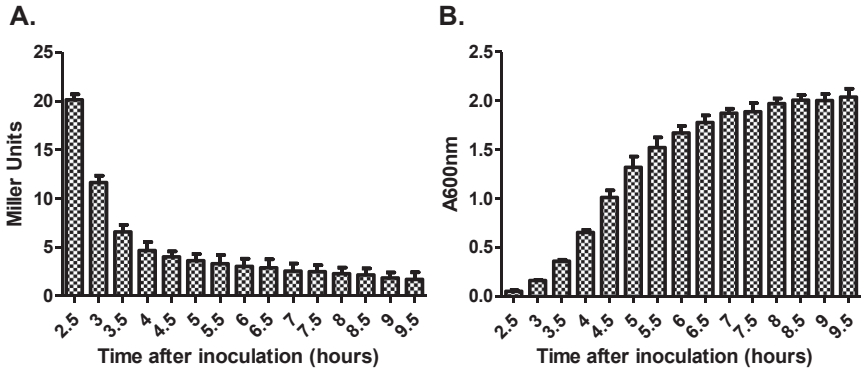

Fig. 2. WalRK promoter activity along the growth curve. (A). β-galactosidase activity (B). Growth curve, of RKProm+ - *lacZ* transcriptional fusion in *E. coli*. Mean with SEM values, from three independent experiments are shown.

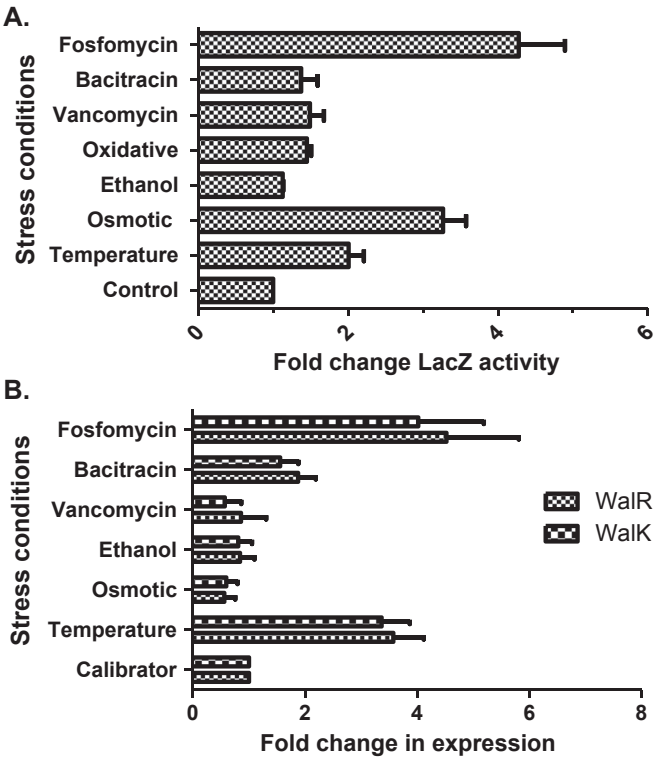

Fig. 3. WalRK expression under various stress conditions. (A). β-galactosidase activity of WalRK promoter in *E. coli*. (B). Fold change in *walR* and *walK* transcripts upon stress induction. Mean with SEM values, from three independent experiments are shown.

genomic organization and the two ORFs have only a three nucleotide spacing between them (Figure S1C). The WalR ORF has a strong ribosome binding site sequence (AGGAGG), nine nucleotides upstream its initiation codon, which adheres to the consensus sequence perfectly whereas WalK ORF has an imperfect ribosome binding site (AGCAGG), with one mis-match from the consensus sequence. To experimentally validate the operon organization of *walRK*, RT-PCR was done and the resultant amplicon size of ~1.3 kb indicated towards the presence of a single transcript for both of them, (Lane 2, Figure S1B) establishing that *walR* and *walK* are transcribed as a contiguous unit during exponential growth and are part of an operon, conforming to a typical TCS organization.

The WalRK operon consists of six other genes. Conserved domain analysis of the hypothetical proteins of the operon categorizes BAS5317 to YycH superfamily and BAS5316 to YycI superfamily, both of which have been shown to dispense regulatory role towards WalK activity in *B. subtilis* [12]. BAS5313 codes for a conserved hypothetical protein, exclusively present in Firmicutes. Sequence analysis could detect transcription terminators downstream of BAS5314, BAS5313 and BAS5312 (DOOR database). The essentiality of WalK for bacterial survival has been linked to the presence of YycH and YycI in its operon, such that WalK has been seen to be essential in bacteria which also have YycH and YycI, for instance, *B. subtilis* and *S. aureus* [13]. Extending the observation, WalK may be essential for *B. anthracis* survival as well, however, experimental validation of the same is in progress.

3.2. WalRK functional promoter is located within 317 bp from WalR start codon

5′ RACE was done to determine the functional WalRK promoter using total RNA from exponentially growing *B. anthracis* cells. The RACE product obtained covered 259 bp upstream of the translation start codon. Keeping the position of the RACE product in mind, several promoter-lacZ transcriptional fusions were created to locate the functional promoter. A series of stepwise deletion mutants of varying lengths of WalRK promoter region were cloned upstream of a promoterless lacZ containing vector (Fig. 1). The functional nature of each reporter construct was examined by plating them on an LB agar plate containing ampicillin and X-gal. Two of the constructs, RKProm+ spanning 500 bp and RKPromTrunc1 covering 317 bp upstream of WalR start codon, produced blue colonies indicating they could express the β-galactosidase enzyme while the other two constructs, RKPromTrunc2 and RKPromTrunc3 covering 256 and 167 bp upstream of WalR, respectively, could not produce the reporter enzyme giving rise to white colonies. This confirmed that the WalRK functional promoter is located in the 317 bp region upstream of the WalR start codon.

A manual inspection of the region upstream of the RACE product yielded a putative −10 box (TATAGA) at −278 and −35 box (TTGAAG) at −297 positions conforming to the consensus sequences of sigma-A type promoter, however, the spacing between them was a non-canonical 13 bp. The transcription start site could be mapped to an "A" at −271. Sigma-A type promoters are characteristic of housekeeping genes, suggesting that WalRK activity is vital for cell survival in *B. anthracis*.

3.3. WalRK promoter activity is maximal in early stages of growth

The RKProm+ - *lacZ* construct was cloned into an *E. coli-Bacillus* shuttle vector, pHCMC05, to assess its activity in both heterologous and homologous host. To study the expression of WalRK promoter along the course of growth curve in heterologous host *E. coli*, RKProm+ - *lacZ* transcriptional fusion was transformed in *E. coli* DH5α cells which were observed for β-galactosidase activity. The promoter activity was found to be maximal in the early phases of growth reaching 20 Miller units within 2.5 h of inoculation. However, as the growth progressed, the activity started to decrease reaching close to 5 Miller units in the mid exponential phases, further dropping down to 2 Miller units in the later phases of growth (Fig. 2). This signifies that the promoter exhibits maximal activity during the early phases of growth. The RKProm+ - *lacZ* construct could not be successfully electroporated into *B. anthracis*, probably owing to its larger size (since the smaller size empty vector was readily electroporated). As a result, the WalRK promoter activity could not be determined in the homologous host.

The pattern of WalRK promoter activity suggests functional significance during stages of exponential growth. The predicted regulon of WalRK in *B. anthracis* does comprise of genes spanning diverse functions [6], particularly metabolism and transport, whose expression is required in exponential phases of growth, justifying the higher activity early on in growth.

3.4. WalRK expression changes in response to temperature and antibiotic stress

WalR regulon in related organisms portrays it to regulate genes involved in cell wall metabolism. This suggests towards a possibility that WalK may sense a signal that depicts the cell wall status and emanates from conditions of cell wall stress/non-stress [14–16]. Cell envelope stress response is primarily orchestrated by TCSs and extracytoplasmic function sigma factors in bacteria. The predicted number for both in *B. anthracis* is significantly higher than *B. subtilis* [17]. In case of TCSs, the cell envelope perturbation is sensed by the HK and relayed on to the cell interior through RR phosphorylation. The nature of cues implying cell envelope perturbation can be myriad requiring multiple TCS, each specific for

Table 1
MIC of different antibiotics for *Bacillus anthracis* before and after WalR overexpression.

Antibiotic	MIC determined for *Bacillus anthracis* Sterne strain (μg/ml)	MIC determined for *B. anthracis* Sterne strain overexpressing WalR (μg/ml)
Fosfomycin	700–800	600–700
Bacitracin	800–900	Not determined
Vancomycin	2	1

a distinct signal, for its management. In order to identify the stress conditions that may affect the regulation of WalRK, expression of RKProm+ - *lacZ* construct in *E. coli* was monitored under a number of stress stimuli, namely, temperature, osmotic, ethanol, oxidative and antibiotic, which impose a distinctive kind of cell wall stress. The antibiotics were used at a sub-inhibitory concentration, in accordance with the reported MIC values for *E. coli*. More than 2-fold change in β-galactosidase activity was observed during conditions of temperature, osmotic and fosfomycin stress (Fig. 3A).

To check the same in homologous host, transcript levels of *walR* and *walK* were checked by qRT-PCR under similar conditions of stress. Early log phase cells were used for this analysis as the WalRK promoter was found to be maximally active during this growth period. Oxidative stress was not tested with current qRT-PCR setup since *dna gyrase* itself shows upregulation under oxidative stress [18]. The broth microdilution method was adopted to determine the MIC of various chosen antibiotics for *B. anthracis* Sterne and the results are presented in Table 1. These antibiotics were chosen for their ability to inhibit cell wall synthesis at different steps. The relative changes in gene expression were quantitated by comparative Ct method (Fig. 3B). The fold change and nature of change (whether induction or repression) was similar for both the genes, adding authenticity to the analysis. More than 2-fold change in the transcripts was observed after exposure to higher temperature and fosfomycin. Immunoblotting was done to confirm these findings, however, bands obtained for WalR were too faint to preclude comparative intensity analysis.

There was a good correlation between reporter gene assay and the qRT-PCR data, except for osmotic stress. Exposure to 4 M NaCl resulted in a lag phase before growth could resume in both *E. coli* and *B. anthracis* resulting in very low absorbance values which might be responsible for this discrepancy. WalRK induction was observed under conditions of high temperature but not osmotic or ethanol stress by qRT-PCR. A *B. subtilis* strain with a WalR H215P thermosensitive mutation leads to appearance of empty cells at non-permissive temperatures and adds credence to this observation [19]. Osmotic stress certainly perturbs the cell envelope, yet, an osmotic shock will not always mount a cell envelope stress response [17]. However, growth at higher temperatures can trigger an envelope stress. Notably, in *B. subtilis*, the transcription of heat-inducible class I genes, which include classical chaperones, is sigma-A dependent [20]. The antibiotics tested in this study perturb the cell wall biosynthesis, however, it was fosfomycin, which brought about a noticeable upregulation in WalRK expression. Fosfomycin inhibits the first committed step of bacterial cell wall biosynthesis by inhibiting the enzyme enolpyruvyl transferase, and hence, is a broad spectrum antibiotic [17].

3.5. *WalR overexpression in B. anthracis doesn't affect fosfomycin and vancomycin MIC*

WalR ORF was placed under the control of an IPTG inducible Pspac promoter in pHCMC05 to study the effect of its overexpression on fosfomycin and vancomycin MIC in *B. anthracis*. The construct was introduced into *B. anthracis* (Fig. 4A) and overexpression was checked by RT-PCR and immunoblotting. However, only a weak overexpression of ~1.7-fold was obtained in comparison to non-electroporated *B. anthracis* (Fig. 4B), which was not

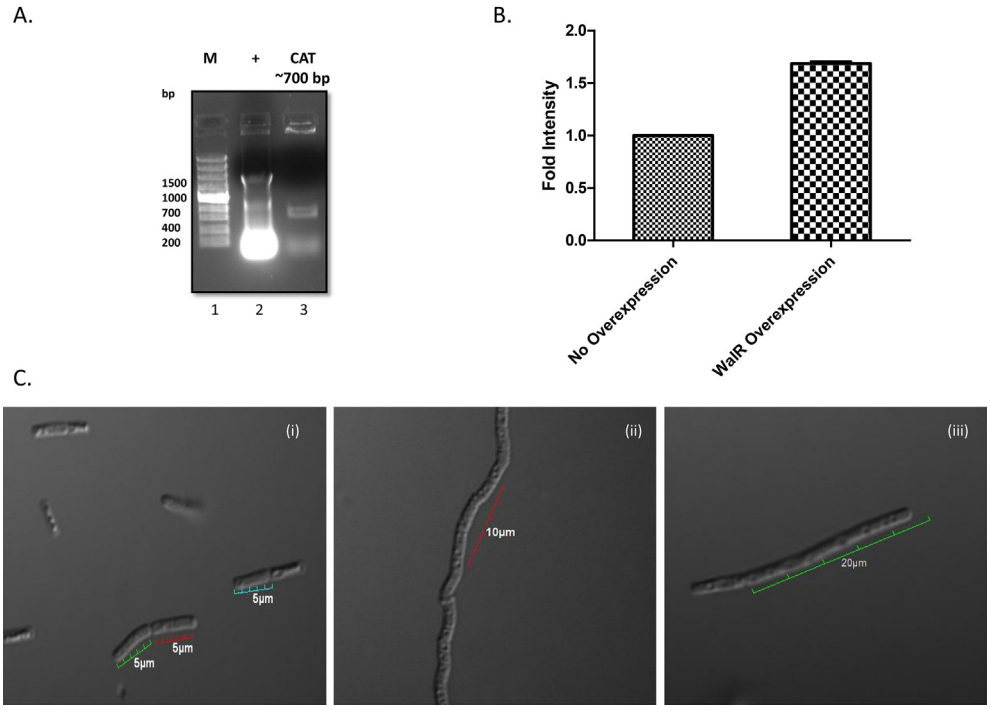

Fig. 4. WalR overexpression in *B. anthracis*. (A). Successful electroporation is shown as the *cat* cassette amplification at ~700 bp from electroporated *B. anthracis* (lane 3). "+"-PCR positive control and "M"-marker. (B). Fold change in WalR expression in electroporated *B. anthracis* by RT-PCR. (C). DIC microscopy shows cell elongation in a proportion of WalR overexpressing cells (ii) & (iii) compared to non-electroporated *B. anthracis* (i). Size measurement between neighboring septa is shown.

discernible in immunoblots as well. Differential Interference Contrast (DIC) microscopy revealed a difference in the morphology of the electroporated cells where overexpression resulted in cell elongation (Fig. 4C). To rule out if this was an artifact of electroporation, only vector electroporated cells were also observed by DIC microscopy confirming that the morphological change was a result of WalR overexpression. Such an elongation phenotype is typical of cells where peptidoglycan hydrolases are misregulated [21]. WalR has been proposed to positively regulate EA1 and FtsE in *B. anthracis*. EA1 has been shown to have murein hydrolase activity in *B. anthracis* while FtsE has been observed to regulate peptidoglycan hydrolases in *B. subtilis* [6], whose aberrant regulation can result in such a cytological change. Moreover, in *B. subtilis* FtsE depletion results in shorter than wildtype cells [22]. This confirmed that the weak WalR overexpression in *B. anthracis* was indeed functional; however, it conferred no variation in MIC for fosfomycin and vancomycin (Table 1).

It has been proposed that WalRK regulates cell wall homeostasis in Firmicutes [13,16]. Cell wall acting antibiotics may disturb this homeostasis, modulating WalRK activity, by inducing a cell wall stress response. Nevertheless, distinct cues probably exist since WalRK induction wasn't seen in all the antibiotic stresses. A number of reports link vancomycin resistance in *S. aureus* to WalRK [7,8]. However, we did not observe any WalRK upregulation during vancomycin stress in *B. anthracis* or alteration in vancomycin MIC after WalR overexpression, suggesting that either a different mechanism or more subtle changes in WalRK activity may be responsible for such a phenotype in *B. anthracis*. Alternatively, the achieved level of overexpression could be too low for such a phenotype. Next, the maximal WalRK induction could be seen during temperature and fosfomycin stress in both the reporter gene and qRT-PCR assays. Also, transcriptomic data in *B. anthracis* shows WalRK upregulation during oxidative stress [18]. Hence, we may conclude that WalRK expression responds to cell envelope stress, imposed by temperature, fosfomycin and possibly hydrogen peroxide, in *B. anthracis* through an upregulation.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.159.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.159.

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