Cell Biology: Research & Therapy

RESEARCH ARTICLE A SCITECHNOL JOURNAL

Effect of Bas2108-2109 Two Component System on Multicellular Behavior of *Bacillus anthracis*

Vatika Gupta[1,2], Kanika Jain[1], Pooja Gulati[1,2] and Rakesh Bhatnagar[1*]

Abstract

Bacillus anthracis, a threating bioterror agent causing anthrax is endowed with 41 Two-Component Systems (TCS). TCS in *B. anthracis* shares significant similarity with TCS present in *Bacillus subtilis*. We have recently reported BAS2108-2109 TCS as an ortholog of DegUS TCS and it was shown to be important in the regulation of proteases in *B. anthracis*. DegUS TCS have been implicated for their role in the regulation of multicellular behavior in other bacteria. In this study, the role of BAS2108-2109 TCS in regulating multicellular behavior in *B. anthracis* was investigated. *B. anthracis* overexpressing BAS2109 was found to exhibit retarded swarming motility, biofilm and pellicle formation indicating its role in complex colony formation. Overexpression of BAS2109 also augmented sporulation in *B. anthracis*, although it reduced spore germination. Invasion and adherence of the *B. anthracis* over-expressing BAS2109 was higher as compared to the wildtype, further suggesting the role of this system in the pathogenesis of *B. anthracis*. Together, our results demonstrate the role of BAS2108-2109 in controlling various multicellular behavioral responses in *B. anthracis*.

Keywords

Two-component system; *Bacillus anthracis*; DegUS; Multicellular behavior

Introduction

Multicellular behavior is a common strategy employed by bacteria to gain an adaptive advantage over unicellular form during diverse environmental conditions [1]. Bacteria differentiate and coordinate activity within a colony to manifest complex multicellular processes viz. quorum sensing, biofilm/pellicle formation, sporulation and swarming motility [2]. Bacterial species can enter into distinct, and often incompatible, multicellular states, via coordination of complex regulatory pathways [3]. They have evolved an intricate signal transduction mechanism, known as Two-Component System (TCS), for orchestrating the response to challenging environmental conditions [4]. Typically, TCS comprises a histidine kinase that is autophosphorylated in response to a stimulus, followed by subsequent phosphotransfer to the response regulator which controls the cellular adaptive response by modulating gene expression [4]. TCS is involved in fine-tuning of diverse genes such that it affects a wide array of cellular functions such as physiological processes, pathogenesis, antimicrobial resistance and multicellular behavior [4,5].

Anthrax is a fatal disease of antiquity that continues to impose a threat as a bioterror agent. The causative agent, *B. anthracis*, has a bimodal lifecycle that alternates between dormant environmental sporulation stage and rapidly growing vegetative bacilli in the mammalian hosts. For survival under such constantly changing conditions, *B. anthracis* might utilize TCS for adaptive responses as evidenced by the high number of TCS encoded by its genome [6]. Recently, BAS2108-2109 was identified as a functional TCS of *B. anthracis* involved in the regulation of its proteases [7]. Its homolog DegUS in *Bacillus subtilis* has been implicated in the regulation of several multicellular processes which include sporulation, swarming, protease production, competence, biofilm formation, and poly-γ-glutamic acid production. Similar to the regulatory roles of DegU, another homologous orphan response regulator, in the development of multicellular processes in *Listeria monocytogenes* has been well documented [2]. However, the role of BAS2108-2109 in regulating the multicellular behavior of *B. anthracis* is not yet explored. Deciphering how *B. anthracis* incorporate environmental and regulatory signals to orchestrate the complex processes which regulate its multicellular behavior will strengthen our understanding of the anthrax biology and hence, development of effective countermeasures. In the present study, we have investigated the influence of BAS2108-2109 TCS on the multicellular behavior of *B. anthracis*, in an endeavor to understand the regulatory role of this TCS in *B. anthracis*.

Materials and Methods

Bacterial strains and growth conditions

B. anthracis Sterne (pXO1+ and pXO2-) was routinely grown in Brain Heart Infusion (BHI) medium (Difco, USA) at 37°C, 150 rpm. *B. anthracis* strain (BA-BAS2109) expressing BAS2109 under IPTG inducible Pspac promoter in pHCMC05 vector and *B. anthracis* strain (BA-pHCMC05) without insert pHCMC05 vector were used in the study [7]. Chloramphenicol was used, when required, at 5 µg/ml final concentration. When appropriate, Isopropyl-B-D-Thiogalactopyranoside(IPTG) was added to the medium at the final concentration of 1 mM.

Microscopy

Overnight grown culture of *B. anthracis* was centrifuged at 13,000 rpm for 10 min. The pellet was washed thrice with 1X PBS followed by fixing of cells with 4% paraformaldehyde at RT for 30 min. Afterward, the cells were washed thrice with 1X PBS. This was followed by thorough washing with 1X PBS thrice. Cells were visualized by Olympus Fluoview FV1000 laser scanning confocal microscope for any morphological changes. For Scanning Electron Microscopy (SEM), cells were fixed in 1%glutaraldehyde for 3 hours at RT. Following, cells were washed thrice with 1X PBS and samples were prepared for SEM analysis as described previously [8]. Cells were visualized using Zeiss EV040SEM at Advanced Instrumentation Research Facility (AIRF), JNU.

***Corresponding author:** Dr. Rakesh Bhatnagar, School of Biotechnology, Jawaharlal Nehru University, New Delhi, 110067, India, Tel: +91 11 26742040; E-mail: rakeshbhatnagar@jnu.ac.in

Received: February 26, 2020 **Accepted:** March 16, 2020 **Published:** April 06, 2020

Growth analysis

1% inoculum of BA-pHCMC05 and BA-BAS2109 was added in BHI broth respectively, and culture aliquots were taken after every half an hour till 10 hours. Absorbance at 600 nm (A_{600nm}) was recorded and the growth curve was plotted by using average A_{600nm} from three independent experiments with standard deviation.

Sporulation

10% of the overnight grown culture of *B. anthracis* was inoculated in the sporulation broth (HiMedia, India) at 37°C, 120 rpm for 5 days. Afterward, the culture was heated to 65°C for 60 min to kill vegetative bacilli. The spores were collected by centrifugation at 13,000 rpm for 10 min followed by thorough washing with 1X PBS thrice. Finally, spores were suspended in autoclaved miliQ and stored at -20°C till further use. The spore concentration was calculated by the serial dilution method. The CFU/ml from three independent experiments were plotted with the standard deviation.

Spore germination

The germination ability of the spores formed was assessed by inoculating 50×10^3 spores into the BHI broth and incubation at 37°C. The aliquots were taken after 15 min and 30 min of inoculation and subjected to 65°C for 60 min to kill any vegetative cells. The spore germination efficiency was calculated by the serial dilution method. CFU/ml was calculated for each condition and mean from three independent experiments was plotted.

Swarming assay

Swarm assay was performed on BHI plates containing 0.8% agar. *B. anthracis* cultures were grown till A_{600nm}~0.6 and used as inoculum. 10 µl of the culture was spotted in the center of the plates and were dried for 30 min in the laminar hood. The inoculated plates were incubated for 5 days at RT and the progression of the swarm was monitored in cms. Three independent experiments were performed in duplicates.

Biofilm formation

Biofilm was developed under static conditions as described earlier [8]. Briefly, mid-exponential phase BA-pHCMC05 and BA-BAS2109 cells were washed and used as inoculum and incubated for 5 days at 37°C. Media was replaced every 24 hours till 144 hours such that the attached cells are left undisturbed and planktonic cells are removed. Crystal violet staining was used for the quantification of the biofilm [8]. The media containing the planktonic cells was removed carefully from each well and were washed with 1X PBS. The biofilm cells were fixed with 70% ethanol at RT for 15 min. This was followed by washing with 1X PBS. Afterward, 100 µl crystal violet (0.1%) was added to each well and kept at RT for 15 min. The plate was thoroughly washed with water followed by drying. The crystal violet was solubilized by the addition of 100 µl of 30% acetic acid at RT for 15 min. The resulting solution was transferred to a fresh 96 well plate and the absorbance was recorded at 550 nm. The well containing 30% acetic acid was used as the blank.

Pellicle formation

Pellicle was achieved in glass tubes under static conditions. 1:100 dilution of overnight grown *B. anthracis* was used as inoculum and the tubes were kept at RT for 5 days under static conditions. A thick white opaque layer of cells of *B. anthracis* representing the pellicle layer was visualized at the liquid-air interface. Similarly, a pellicle for *B. anthracis* overexpressing BAS2109 was observed.

In vitro adherence and invasion studies

RAW 264.7 (mouse macrophages) cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM, Sigma) containing 10% Fetal Bovine Serum (FBS, Gibco) and antibiotics *viz.* penicillin, streptomycin and amphotericin B (HiMedia). For association and invasion studies, 2×10^6 cells were seeded per well in a 6 well plate in DMEM without antibiotics and incubated overnight in 5% CO_2 at 37°C. Infection with *B. anthracis* and BA-BAS2109 cells was done as described previously [9]. Overnight grown cultures of *B. anthracis* and BA-BAS2109 were washed with antibiotics free DMEM. The RAW 264.7 cells were infected with vegetative bacilli at a multiplicity of infection (MOI) of 1 for 1 hour. Afterward, media was removed to clear unbound bacteria and cells were washed with 1X PBS. The number of associated bacteria was evaluated by cell lysis with chilled PBS followed by dilution plating on BHI agar. Internalized bacteria were determined by the removal of unbound bacteria followed by washing of the macrophages. The cells were further incubated for 1 hour with serum and antibiotic-free DMEM containing 20 µg/ml gentamycin. After 1hour, cells were washed and lysed with chilled 1XPBS and the number of internalized bacteria was determined by a serial dilution plating method.

Statistical methods

For all experiments, statistics were performed using GraphPad Prism 5 software. The standard deviation was calculated from datasets representing three independent experiments. Statistical significance, p-value, was calculated by unpaired student's t-test.

Results

Bas2109 affects cell size but does not affect growth

The effect of overexpression of BAS2109 on *B. anthracis* physiology was initially analyzed on the morphology and growth of the bacilli. The effect of BAS2109 overexpression on the morphology of *B. anthracis* was examined by visualization of cells using confocal microscopy. Interestingly, BAS2109 overexpressing bacilli were found to have a reduced size as compared to BA-pHCMC05 and also, inhibited chain formation as observed in wild type *B. anthracis* (Figure 1A). The difference in size was also observed in SEM analysis (Figure 1B). The growth pattern of BA-pHCMC05 and BA-BAS2109 was also monitored and it was observed that both the cultures had similar growth rate thereby suggesting that the overexpression of BAS2109 does not affect the growth of *B. anthracis in vitro* under the studied conditions (Figure 1C).

High levels of BAS2109 promote sporulation, however, it reduces spore germination

To assess whether BAS2109 has a role in the sporulation process, sporulation efficiency of BA-BAS2109 and BA-pHCMC05 were compared. Spore titre in terms of colony-forming units per ml (CFU/ml) was plotted concerning the cultures. Overexpression of BAS2109 in *B. anthracis* resulted in about 46% increase in the sporulation titer as compared to BA-pHCMC05 (Figure 2A). The effect of BAS2109 on spore germination was also calculated. The spores formed by BA-BAS2109 germinated slowly for both 15 min and 30 min in comparison to BA-pHCMC05 (Figure 2B).

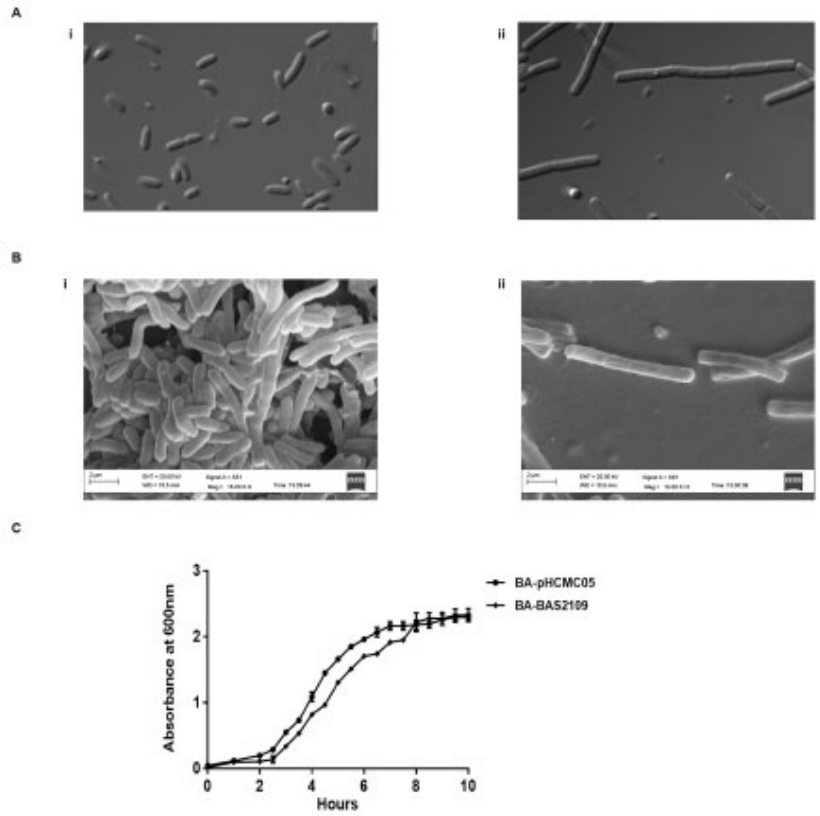

Figure 1: Effect of overexpression of BAS2109 on *B. anthracis* morphology and 338 growth. **(A):** Cell size of BA-BAS2109 (i) and BA-pHCMC05 (ii) as observed by confocal 339 microscopy; **(B):** Cell size of BA-BAS2109 (i) and BA-pHCMC05 (ii) as observed by 340 SEM. Both confocal microscopy and SEM analysis revealed a reduction in the size of 341 cells overexpressing BAS2109 as compared to the wild-type strain; **(C):** Graph showing 342 growth kinetics of BA-BAS2109 and BA-pHCMC05. No significant difference was 343 observed on the growth pattern of BA-BAS2109 and BA-pHCMC05.

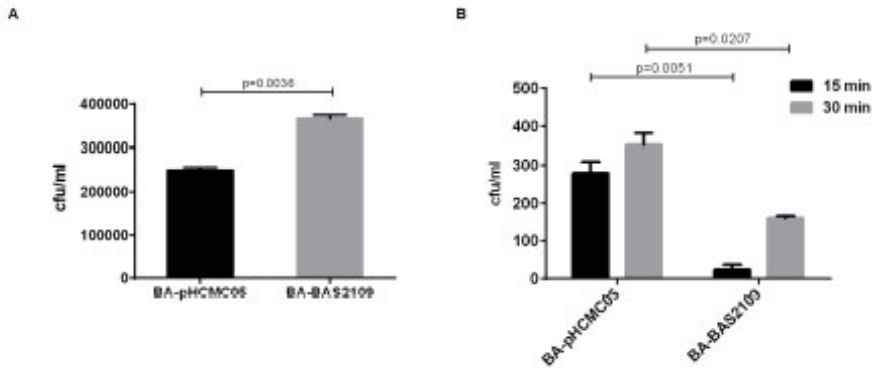

Figure 2: Influence of Bas2109 on sporulation. **(A):** Graph depicting sporulation 345 efficiency of BA-BAS2109 and BA-pHCMC05 which shows an increase in the spore 346 forming ability of *B. anthracis* overexpressing BAS2109; **(B):** Graph representing 347 germination efficiency of spores formed by `BA-BAS2109 and BA-pHCMC05. A 348 reduction in the germination of spores formed by BA-BAS2109 was observed as 349 compared to the wild-type. Statistical significance (p-value) is shown in the graphs.

BAS2109 influences swarming motility of *B. anthracis*

To understand the role of BAS2109 in coordinating complex colony architecture, swarm assay was performed with *B. anthracis* overexpressing BAS2109 and BA-pHCMC05. The cultures were spotted at the center of BHI plates with 0.8% agar and after 5 days, the progression of the swarm was monitored in mms. BA-BAS2109 showed delayed swarming motility as compared to BA-pHCMC05. BA-BAS2109 formed a swarm with a diameter of 2.85 ± 0.176 cms while BA-pHCMC05 formed 3.45 ± 0.098 cms swarm (Figure 3). The difference in swarming motility was found to be statistically significant with a p-value of 0.0032.

BAS2109 reduces biofilm and pellicle formation

The effect of BAS2109 on biofilm formation was quantified by the CV staining of the attached biofilm cells. A substantial decrease (~55 %) in biofilm formation of *B. anthracis* overexpressing BAS2109 as compared to BA-pHCMC05 was observed following CV stain analysis suggesting that high levels of BAS2109 inhibit biofilm formation (Figure 4A). Further, the role of BAS2109 in pellicle formation was also assessed since pellicle is a kind of biofilm that usually forms at the liquid-air interface under static conditions. Overexpression of BAS2109 in *B. anthracis* significantly reduced pellicle formation as compared to BA-pHCMC05 (Figure 4B).

High levels of BAS2109 promote adherence and invasion in *B. anthracis*

Adherence and invasion are important for the pathogenesis of any bacteria. Thus, the effect of overexpression of BAS2109 on

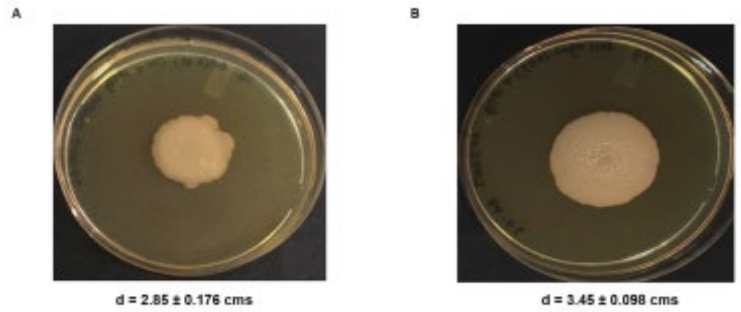

Figure 3: Effect of Bas2109 on swarming motility in *B. anthracis*. Reduced swarming 351 motility was observed for BA-BAS2109 **(A):** in comparison to BA-pHMC05 **(B):** Average 352 diameter for swarming from three independent experiments are mentioned below the 353 respective cultures.

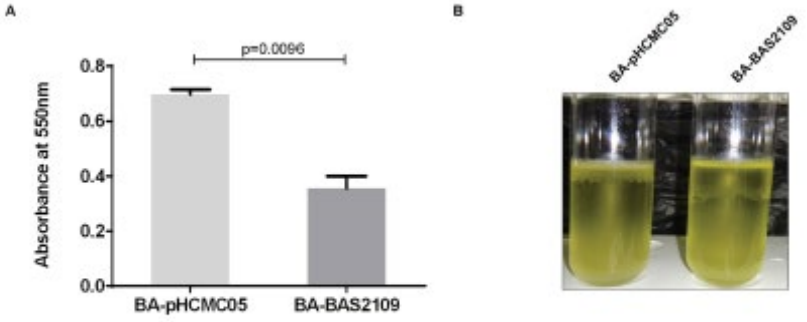

Figure 4: Biofilm and pellicle formation in *B. anthracis*-Bas2109. **(A):** Graph depicting 355 reduction in biofilm formation of BA-BAS2109 as compared to BA-pHCMC05 as 356 16 quantified by CV staining; **(B):** Pellicle formation as observed for BA-BAS2109 and BA-357 pHCMC05. A reduced pellicle for BA-BAS2109 was observed in comparison to BA-358 pHCMC05.

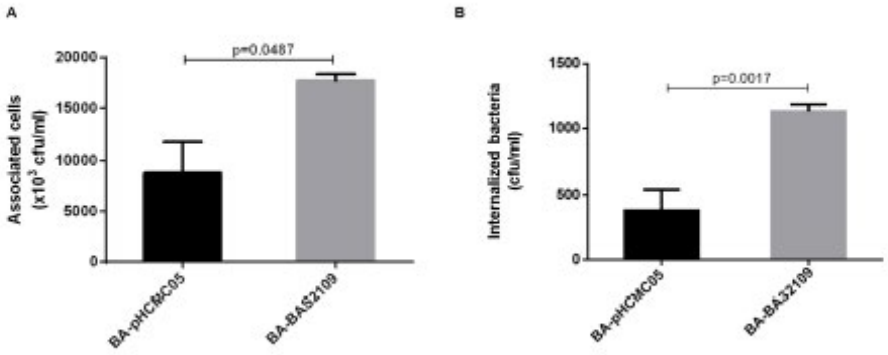

Figure 5: Effect of Bas2109 on the invasion and adherence efficiency of *B. anthracis* 360 *in vitro*. **(A):** Graph depicting association of BA-BAS2109 and BA-pHCMC05 with RAW 361 264.7 macrophages. BAS2109 overexpressing cells were found to have increased 362 adherence in comparison with wild-type; **(B):** Graph showing invasion efficiency in terms 363 of CFU/ml for BA-BAS2109 and BA-pHCMC05 wherein an increase in invasion ability 364 of cells overexpressing BAS2109 was observed. Statistical significance (p-value) is 365 indicated above the graphs.

the adherence and invasion efficiency of *B. anthracis* was analyzed *in vitro*. *B. anthracis* overexpressing BAS2109 showed ~1.5 times more association in comparison to BA-pHCMC05 (Figure 5A). Furthermore, the internalization of BA-BAS2109 was found to be significantly higher (~2 times) as compared to BA-pHCMC05 (Figure 5B).

Discussion

Bacteria regulate multicellular behavior such as swarming motility and pellicle/biofilm formation by incorporating environmental signals via an intricate regulatory network. TCS in bacteria are often involved in regulating such phenomena under a fluctuating environment [4]. Recently, BAS2108-2109 gene pair was identified as a functional TCS of *B. anthracis* involved in the regulation of its proteases during nutrient deprivation, presence of CO_2, and during pellicle formation [7]. DegUS, a homolog of BAS2108-2109 TCS, in *B. subtilis* and DegU of *L. monocytogenes* have been shown to regulate multicellular processes apart from their role in protease regulation [2], thus the role of BAS2108-2109 TCS in regulating different multicellular behaviors of *B. anthracis* was assessed.

The potential role of this TCS in the regulation of the multicellular behavior of *B. anthracis* was analyzed by overexpressing the regulatory protein of the TCS, BAS2109, in *B. anthracis*. It has been previously demonstrated that DegUS in *B. subtilis* is involved in sporulation. At high levels of DegU, cellular differentiation is shifted towards sporulation by modulating levels of the transcription factor, Spo0A [10]. Since spores are the infectious particles of the disease and thus, it is vital to study its regulatory network. Therefore, we analyzed the role of BAS2109 on the sporulation process and an increase in sporulation efficiency was observed in cells overexpressing BAS2109. However, the mechanism is not fully understood since Spo0A was not found to be a direct target of BAS2109, but it might interact with Spo0A via different pathways or BAS2109 might have evolved other regulatory pathways in *B. anthracis* to regulate sporulation [7]. Further studies might provide an insight into the precise mechanism employed by BAS2109 for the coordination of such processes in *B. anthracis*. The spores formed by BAS2109 overexpressing strain were found to germinate at a slower rate in comparison to the wild-type or vector control. This suggested that BAS2109 is also involved in the maintenance of the sporulation stage in addition to the early sporulation initiation.

The role of BAS2109 on different multicellular processes, including biofilm, pellicle and swarming, in *B. anthracis*. Overexpression of BAS2109 resulted in retarded biofilm and pellicle formation signifying the significant role of BAS2109 in these processes. A smaller swarm was observed for cells over-expressing BAS2109 which further indicated the role of BAS2108-2109 TCS in the coordination of multicellular behavior. Previous studies in *B. subtilis* have also confirmed the role of DegUS TCS in the regulation of swarming where high levels of phosphorylated DegU was shown to activate transcription of proteases such as aprE, nprB, and bpr which results in complete abrogation of swarming motility [11,12]. Since high levels of BAS2109 also increases protease gene expression therefore it may also result in the abrogation of complex colony formation in *B. anthracis*. During unfavorable conditions, cells from a sessile community, such as biofilm, tend to leave the community and under such circumstances, DegU regulatory network act as an escape mechanism that increases proteolytic activity resulting in an exodus of bacteria from the sessile community [12-14]. The increase in protease production causes dissociation of the cells from the extracellular matrix of the biofilm or other complex colony formation [15]. Thus, BAS2109 act in a similar manner as DegU where it regulates protease expression and thus, the complex colony architectures in *B. anthracis*. Along with controlling complex colony architecture, DegU has been implicated in the control of virulence in *L. monocytogenes*. To further explore the role of BAS2108-2109 in virulence of *B. anthracis,* invasion and adherence were also examined. Overexpression of BAS2109 resulted in an increased association of *B. anthracis* with the macrophages and a higher number of *B. anthracis* invaded the macrophages. These suggested that BAS2109 is involved in the pathogenesis of *B. anthracis*.

Overall, we establish BAS2108-2109 TCS as the regulator of multicellular behavior *viz.* sporulation, biofilm, pellicle formation and swarming motility in *B. anthracis*. This study also provides presumptive evidence for the role of BAS2108-2109 TCS in the pathogenesis of *B. anthracis* and hence can be targeted for the development of new antimicrobials for treatment of anthrax.

Conclusion

The results shown in this study validates the role of BAS2108-2019 TCS in the regulation of various multicellular behaviour in *B. anthracis*. The effect of BAS2109 on sporulation and spore germination further indicate its role in survival and persistence of the bacilli in the environment. Further, invasion studies hint towards significance of this system in the host-pathogen interaction and may be explored for developing treatment therapies for anthrax.

Acknowledgement

VG acknowledges ICMR-Senior Research Fellowship. RKB acknowledges JC Bose fellowship. We acknowledge Dr. Alisha Dhiman, SBT, JNU, for providing BA-pHCMC05.

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Author Affiliations

Top

[1]*Molecular Biology and Genetic Engineering Laboratory, School of Biotechnology, Jawaharlal Nehru University, New Delhi, India*

[2]*Medical Microbiology and Bioprocess Technology Laboratory, Department of Microbiology, Maharshi Dayanand University, Rohtak, Haryana, India*