

Article

The Dual Role of RsiP in Regulating Virulence and Host Adaptation in *Bacillus anthracis*

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Abstract

Bacillus anthracis displays susceptibility to penicillin despite harboring a β -lactamase gene, a phenotype governed by the anti-sigma factor RsiP. While RsiP represses σ^P -dependent β -lactamase expression, its broader roles in physiology and virulence remain unclear. This study aimed to define the global regulatory functions of RsiP beyond antibiotic resistance. Deletion of *rsiP* significantly upregulated the *nprR* gene, which is an important quorum-sensing (QS) system regulator and enhanced protease secretion. The Δ *rsiP* mutant caused higher mortality in cellular and *Galleria mellonella* models and triggered elevated inflammatory cytokines (IL-6, IL-1 β , TNF- α , MIP-2) in macrophages models. Surprisingly, in DBA/2 mice models, Δ *rsiP* was attenuated, with increased host survival and reduced bacterial loads. Competitive indices (CI) confirmed fitness defects in mice (spleen CI = 0.39; liver CI = 0.42). These defects were not due to altered oxidative stress tolerance but were attributed to impaired macrophage internalization of Δ *rsiP* spores, reducing early colonization. Our findings indicate that RsiP not only modulates β -lactam resistance but also influences extracellular protease activity and host adaptation.

Keywords: *Bacillus anthracis*; anti-sigma factor RsiP; extracellular protease; host adaptation

1. Introduction

Bacillus anthracis is a spore-forming, Gram-positive, nonmotile bacterium that causes anthrax, an acute infectious zoonotic disease [1–3]. Due to the spores' ease of dispersal through air, soil, and food, *Bacillus anthracis* has historically been exploited as a biological weapon [4,5]. Its capacity to cause outbreaks frequently makes it a possible global public health concern [6].

The virulence of *B. anthracis* is primarily mediated by two plasmid-encoded factors [7]. The pXO1 plasmid harbors a pathogenicity island that contains the *pagA*, *lef*, and *cya* genes, which encode the three components of anthrax toxin: protective antigen (PA), lethal factor (LF), and edema factor (EF) [8]. The pXO2 plasmid carries the *capBCADE* operon responsible for the synthesis of the poly- γ -D-glutamic acid capsule, which confers resistance to phagocytosis [9–12].

Apart from the contribution of extrachromosomal elements to virulence, certain virulence factors are also encoded on the chromosome, including extracellular protease like



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Npr599, regulated by NprR [13]. The production of protease is regulated by the quorum-sensing (QS) system, and Npr599 is closely associated with this QS process [14]. Extracellular proteases are secreted virulence factors produced by bacteria that perform various functions, allowing them to target host cells or tissues directly or indirectly [15].

In contrast to the well-characterized virulence mechanisms, *B. anthracis* exhibits a perplexing phenotype regarding antibiotic resistance: it harbors a putative β -lactamase gene yet remains intrinsically sensitive to penicillin [16]. This apparent paradox suggests the involvement of a specific regulatory system—the sigma factor σ^P and the anti-sigma factor RsiP.

σ^P is a member of the extra cytoplasmic function (ECF) sigma factor subgroup within the σ^{70} family, which are responsible for recognizing and binding to the -10 and -35 promoter sequences, respectively [17]. Typically, most ECF sigma factors are sequestered by anti-sigma factors that bind to the sigma factor and inhibit its interaction with RNA polymerase [18]. To activate the sigma factor signaling cascade, these anti-sigma factors must be inactivated, enabling the sigma factor to associate with RNA polymerase and initiate transcription of target genes [19,20].

In *B. thuringiensis* and *B. cereus*, σ^P is inhibited by the anti- σ factor RsiP. In the exposure of ampicillin, the degradation of RsiP, a well-studied multistep process, leads to the release of σ^P and subsequent activation of β -lactamase gene transcription [21]. In *B. anthracis*, a single residue mutation in RsiP may block its degradation activity, explaining the susceptibility of anthrax strains to β -lactam antibiotics [22]. Therefore, the persistent presence of RsiP in *B. anthracis* may represent a deliberate forfeiture of penicillin resistance. However, the broader physiological implications of this stable, non-degraded RsiP, which functions as a constitutive inhibitor, particularly its impact on virulence expression, remain unknown. In this work, we constructed a *rsiP* deletion mutant (Δ *rsiP*) and evaluated its effects on gene expression, protein secretion, virulence phenotypes, and host immune responses. The study integrated in vitro experiments and in vivo models (e.g., mouse and insect systems) to comprehensively dissect the pleiotropic functions of RsiP.

2. Materials and Methods

2.1. Bacterial Strains, Cell Lines, and Growth Conditions

To avoid biosecurity risks, we used the following attenuated plasmid-cured strains: A16D2 (pXO1+, pXO2−) which similar to the anthrax vaccine strain A16R used in China. The knockout strains constructed in this study were derived from a natural mutant isolated from an environmental surface, which carries a frame shift mutation resulting in the truncation of the RsiP protein to its first 30 amino acids [23]. Based on this mutation, the *rsiP* gene was knocked out (Δ *rsiP*) in the A16D2 strain using the previously reported CRISPR-Cas9 technique, thereby constructing a mutant retaining only the coding sequence for the first 30 amino acids of this gene [24]. The complementary strain (Comp1) of *rsiP* was constructed by electroporation of the plasmid pBE2-*rsiP* into the Δ *rsiP* mutant. Electroporation was conducted using a Gene Pulser Xcell system (Bio-Rad, Hercules, CA, USA) with the following parameters: 0.7 kV, 500 Ω , 25 μ F, and a 1 mm cuvette. For routine cultivation, strains were grown in LB medium at 37 °C with shaking (220 rpm). J774A.1 cell was acquired from ATCC (Manassas, VA, USA) and cultivated in DMEM + 10% heat-inactivated FBS with antibiotic-antimycotic at 37 °C and 5% CO₂.

2.2. RT-qPCR Analysis

Bacteria were cultured overnight at 37 °C under the following two conditions: in LB medium, and in LB medium containing 0.9% sodium bicarbonate within 5% CO₂. Total RNA was isolated from bacterial cells using Trizol reagent (Invitrogen, Carlsbad,

CA, USA). Following chloroform addition, cell lysis, and centrifugation, the RNA was collected at the aqueous interface and precipitated with isopropanol. Potential DNA contamination was removed by DNase I (Beyotime Biotech, Shanghai, China) treatment in the presence of $MgCl_2$. The purified RNA was then reverse-transcribed into cDNA using mRNA Universal SYBR qPCR Master Mix (Vazyme Biotech, Nanjing, China). Gene expression was normalized to *gatB_Yqey* and quantified using the $2^{-\Delta\Delta CT}$ method [25]. Primer sequences are listed in Table 1.

Table 1. Primers used throughout manuscript.

Target Gene	Primer F	Primer R	Gene Product
<i>sigP</i>	TCTGTCTGGCAGAACAAAGAGTT	CATCACTACCACCCTCCCCTA	Sigma factor
<i>bla1</i>	CGGCAGGGGTATTACTGCAA	GTCCCCCATCTGTCTAAGCG	Beta-lactamase
<i>nprR</i>	CGCTGGTTGTTCAAAGGACG	CGTGTCGCGAAAGAGTTTGG	Regulator of neutral protease
<i>npr599</i>	AAACAAGAAGCGAAACCGGC	TGCTCCGCGCGTATTATCTT	Neutral protease
<i>gatB_Yqey</i>	AGCTGGTCGTGAAGACCTTG	CGGCATAACAGCAGTCATCA	GatB_Yqey domain-containing protein (reference gene)

2.3. Extracellular Protease Activity Assay

We analyzed the extracellular protease activity by spreading 5 μ L of the LB culture (obtained from fresh colonies grown at 37 °C and 220 revolutions per minute) containing the bacterial strain A16D1, Δ *rsiP* on milk culture plates, respectively [26]. Following incubation at 37 °C, protease activity was phenotypically assessed by monitoring the formation of a clear hydrolytic halo around the colonies over time.

2.4. Western Blot Analysis

To prepare protein samples for Western blot analysis, the bacterial supernatant and cell pellets were processed separately. Proteins in the supernatant were concentrated by trichloroacetic acid precipitation. The cell pellets were lysed on ice for 30 min using a buffer containing 8 M urea, 1% (*w/v*) DTT, and a protease inhibitor cocktail (4693132001, Roche, South San Francisco, CA, USA), followed by centrifugation at $12,000 \times g$ for 15 min at 4 °C to collect the soluble fraction. Proteins (30 μ g per lane) were resolved by 12% SDS-PAGE and transferred onto a 0.45 μ m PVDF membrane (10600029, GE Healthcare, Dusseldorf, Germany). After blocking with skim milk for 1 h, the membrane was incubated with the following primary antibodies: rabbit anti-NprR, rabbit anti-Npr599. Following TBST washes, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence detection kit.

2.5. Spore Generation and Purification

Starter strains culture was inoculated into 3 mL of LB with shaking at 37 °C overnight. The next day, 2 mL aliquots were coated on LB agar at 30 °C for 15 days [27]. The spore was harvested in 10 mL of ice-cold sterile 10% glycerol. Spore preparations from multiple plates were merged, re-suspended at 70 °C for 30 min to kill any remaining vegetative cells, purified by diatrizoate sodium and meglumine diatrizoate, and washed 3 times in ice-cold sterile 10% glycerol.

2.6. Cell Counting Kit-8 Assay

J774A.1 cells were seeded in a 96-well plate and cultured for 24 h. After replacing the culture medium, the cells were subjected to various concentrations of spore suspensions prepared in DMEM for 10 h. Subsequently, 10 μ L of CCK-8 reagent (CK04-500T; DOJINDO, Kumamoto, Japan) was added to each well, followed by a 1 h incubation. Cell viability was then assessed by measuring the optical density with a microplate reader, thereby comparing the cytotoxicity of different spores.

2.7. *Galleria mellonella* Infection

The virulence of bacterial strains was assessed using a *Galleria mellonella* infection model [28,29]. Purified spores were quantified via plate counting and adjusted to a concentration of 5×10^8 spores/mL in PBS. A total of 60 larvae were randomly allocated into three groups ($n = 20$ per group). Each larva was injected in the third distal left proleg with 10 μ L of the respective spore suspension. Following injection, larvae were acclimated at room temperature for 30 min and then incubated at 37 °C. Survival was monitored at 6, 12, and 24 h post-infection, with mortality defined by the presence of melanization and absence of movement upon tactile stimulation.

2.8. Cytokine/Chemokine Assay

Serum cytokine/chemokine levels were quantified using the ProcartaPlex Pro Mouse Cytokine/Chemokine Panel 1A 36-Plex (Invitrogen, EPX360-26092-901, Carlsbad, CA, USA) with 25 μ L of serum from each mouse, following the manufacturer's protocol. Measurements were performed on a Luminex 200 system, and data were analyzed with ProcartaPlex Analyst 1.0 software.

2.9. Growth Curve Analysis

Bacteria were grown overnight in Brain Heart Infusion (BHI) medium at 37 °C with shaking. The overnight culture was diluted to an OD600 of 1, which standardizes the initial cell density. Growth curves were generated using a Biotek Synergy H1 48-well plate reader (Jieling, Tianjing, China). OD600 measurements were taken every 5 min to monitor bacterial growth over time.

2.10. Virulence Studies

For the infection model, first, 7-week-old female DBA/2 mice were intraperitoneally injected with spores (approximately 1×10^7 viable bacteria, 100 μ L). Then, these mice were monitored multiple times every day. The time of death for each mouse was observed and recorded. The survival data were analyzed using the Kaplan–Meier curve, and statistical comparisons were made using GraphPad Prism software (version 10.0). All animal procedures were performed in accordance with the protocol approved by the Animal Welfare and Ethics Committee of the Academy of Military Medical Sciences (IACUC-SWGCYJS-2024-036).

2.11. Bacterial Load Measurement

Briefly, bacterial spore suspensions were adjusted to a concentration of 1×10^7 CFU/mL. DBA/2 mice were then administered an intraperitoneal injection of 100 μ L of this suspension. At 24 h post-injection, three mice were euthanized and subjected to dissection. The spleen and liver from each mouse were aseptically collected and homogenized in 1 mL of PBS. The resulting tissue homogenates were serially diluted, and 500 μ L aliquots of appropriate dilutions were spread onto LB agar plates. After incubation at 37 °C for 12 h, bacterial colonies were enumerated on plates containing between 30 and 300 colonies. The entire procedure was performed under sterile conditions.

2.12. Competitive Index of Dissemination

The competitive index (CI) was calculated by the following equation: (CFU/mL of $\Delta rsiP$) $\div$ (CFU/mL of A16D2). $\Delta rsiP$ /A16D2 spores were inoculated in 3 mL of BHI broth and incubated at 37 °C with shaking for 24 h. Following the incubation, the cultures were subjected to serial dilution and plated onto both plain BHI agar and BHI agar containing 20 µg/mL Ampicillin (BHI + Amp20). For the in vivo CI, 7-week-old female DBA/2 mice were injected subcutaneously with 10^6 spores (a mixture of A16D2 and $\Delta rsiP$) in 100 µL of $1 \times$ PBS. Twenty-four hours later, mice were humanely killed, and the liver and spleen of each mouse were pooled in 5 mL of PBS. The organs were homogenized with a handheld homogenizer, diluted in PBS, with each dilution plated on LB and LB + Amp20. The competitive index was calculated using the same method as described for in vitro experiments [28].

2.13. Evaluation of Spore Survival Rates Following Exposure to Hydrogen Peroxide

Spore suspensions were exposed to a liquid medium containing different concentrations of H_2O_2 , which ranged from 2.5 to 50 mmol/L [30]. For comparison, a control group was maintained in the same medium in the absence of H_2O_2 and handled in parallel. All samples were co-incubated at 37 °C for a duration of 30 min. Following the incubation period, the oxidative reaction was quenched by adding an equal volume of a 1.5% sodium thiosulfate solution [31]. Subsequently, all samples were subjected to a 10-fold serial dilution series using a sterile diluent. From each dilution tube, an appropriate volume was plated onto solid agar medium. After a defined cultivation period under suitable conditions, viable colonies were enumerated. The bacterial survival rate was calculated as the percentage of the mean colony-forming unit (CFU) count for each treatment group relative to the mean CFU count obtained from the untreated control group, using the formula: (Mean CFU of treated group/Mean CFU of control group) $\times$ 100%.

2.14. ROS Detection

J774A.1 macrophages were seeded in a 96-well plate and infected with bacterial spores at a MOI of 10. After 1 h of incubation, extracellular spores were removed by washing with PBS, followed by the addition of DMEM medium containing gentamicin to eliminate any remaining non-phagocytosed spores. Following PBS wash, the cells were incubated with 10 µM DCFH-DA in a 5% CO_2 incubator at 37 °C for 20 min [32]. During incubation, the plate was gently mixed at 5 min intervals to ensure even exposure of the cells to the probe. Finally, the cells were washed three times with PBS, and fluorescence was measured using a microplate reader.

2.15. Cellular Spore Association Assay

J774A.1 cells were plated at a density of 1.0×10^5 cells per well in 24-well Corning cell bind plates and left to attach overnight. The next day, the cell monolayers were washed three times with $1 \times$ PBS. Spores were diluted to a multiplicity of infection (MOI) of 10:1 in 200 µL of DMEM containing 10% heat-inactivated FBS. This spore solution was then added to the monolayers and incubated at 37 °C in 5% CO_2 for 1 h. After incubation, the supernatants were removed, and the monolayers were washed three times with $1 \times$ PBS. Next, media with 20 µg/mL gentamicin was added and left for 1 h to kill any remaining extracellular bacteria. The monolayers were washed three more times with $1 \times$ PBS to remove the antibiotics and non-internalized spores. Cold sterile water was added to lyse the cells for 15 min. The resulting suspensions were diluted and plated on BHI agar to count the internalized bacteria. The initial spore inoculum was also plated to determine the number of spores used for infection and to calculate the internalization efficiency

for each strain. This experiment was conducted in triplicate across two independent experiments. The data from the five data points were averaged, and the standard deviation was calculated.

3. Results

3.1. The Deletion of *rsiP* Enhances Extracellular Protease Production and Activity

To further investigate the effects of *rsiP* on the expression of virulence factors, *rsiP* mutants were constructed based on previous studies, which reported that a *B. anthracis* mutant acquired β -lactam resistance due to the absence of the σ^P binding domain [33].

Real-time quantitative PCR (RT-qPCR) analysis revealed that the sigma factor *SigP*, β -lactamase gene *bla1* and metalloproteinase *nprR*, and *npr599*, were significantly upregulated in $\Delta rsiP$ (Figure 1A). This pattern was consistent under both air and CO₂ conditions, indicating that RsiP serves a negative regulatory function across various environments (Figure 1B). To further assess the impact of gene expression changes on protein levels, Western blot analysis and milk plate hydrolysis experiments were conducted. The results demonstrated that the expression levels of NprR and Npr599 proteins were significantly elevated in $\Delta rsiP$ strains compared to the wild type (A16D2) suggesting that the deletion of *rsiP* enhanced the secretion and activity of these factors (Figure 1C–E).

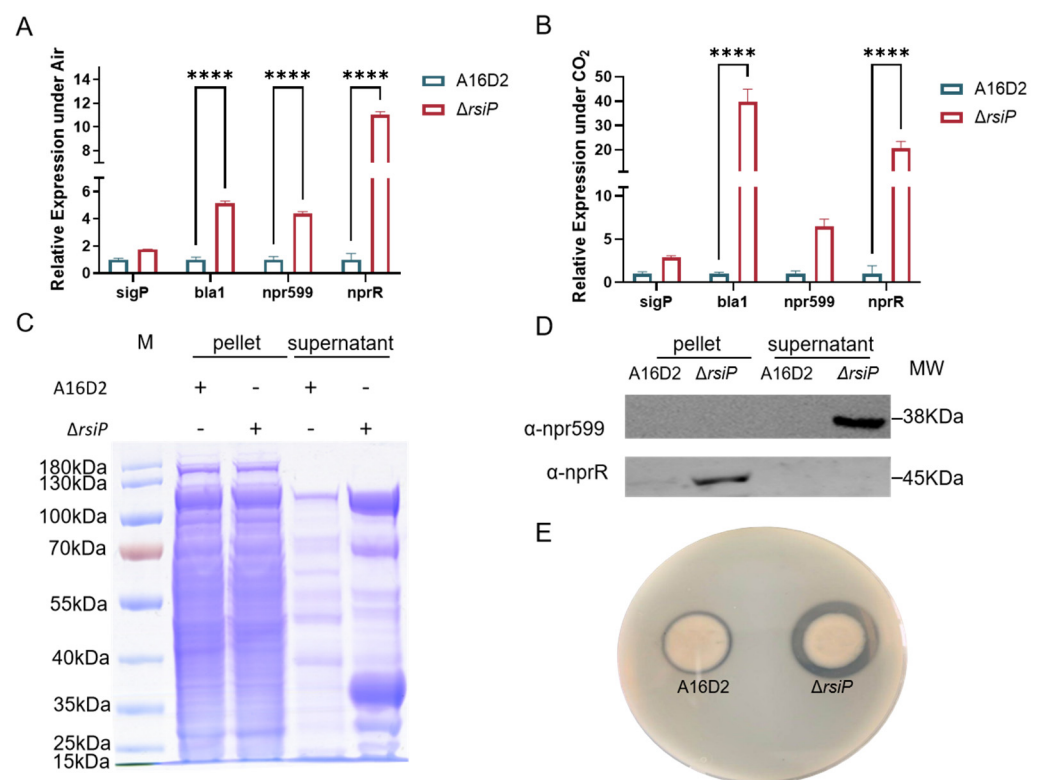


Figure 1. The deletion of *rsiP* upregulates extracellular protease genes, boosting their secretion and activity. (A) The expression levels of *SigP*, *bla1*, *nprR*, and *npr599* under air conditions. (B) The expression levels of these genes in a CO₂ environment. (C) SDS-PAGE Gel for separation of cellular and supernatant proteins from A16D2 and $\Delta rsiP$ Strains (D) Western blot analysis to assess the protein expression of NprR and Npr599. (E) Features extracellular proteolytic enzyme activity assays for the $\Delta rsiP$ strain, where colonies exhibiting significant enzymatic activity are marked by halos. Statistical significance was determined using Two-way ANOVA (**** $p < 0.0001$). All experiments were conducted with three biological replicates and repeated at least three times.

3.2. Deletion of *rsiP* Enhances Virulence and Induces a More Robust Inflammatory Reaction in Cell

To evaluate virulence phenotypic changes, we infected J774A.1 cells with spores of the wild-type and $\Delta rsiP$ strains. Cytotoxicity assays demonstrated that $\Delta rsiP$ spores exhibited enhanced virulence in a concentration-dependent manner (Figure 2A). Macrophages serve as the first line of defense against inhaled pathogens [34]. To investigate the impact of the $\Delta rsiP$ strain on innate immune responses, we infected J774A.1 macrophage cells using spores at a multiplicity of infection (MOI) of 10 and assessed the expression of inflammatory cytokines. Compared to the wild-type, infection with the $\Delta rsiP$ spore led to significantly elevated induction of IL-6, IL-1 β , TNF- α and IL-18 at 12 h post-infection, with TNF- α showing a marked increase as early as 6 h (Figure 2B–E). In addition, the expression of the chemokine MIP-2 was increased by 3-fold (Figure 2F). These results indicate that the loss of *rsiP* triggers a more robust inflammatory response.

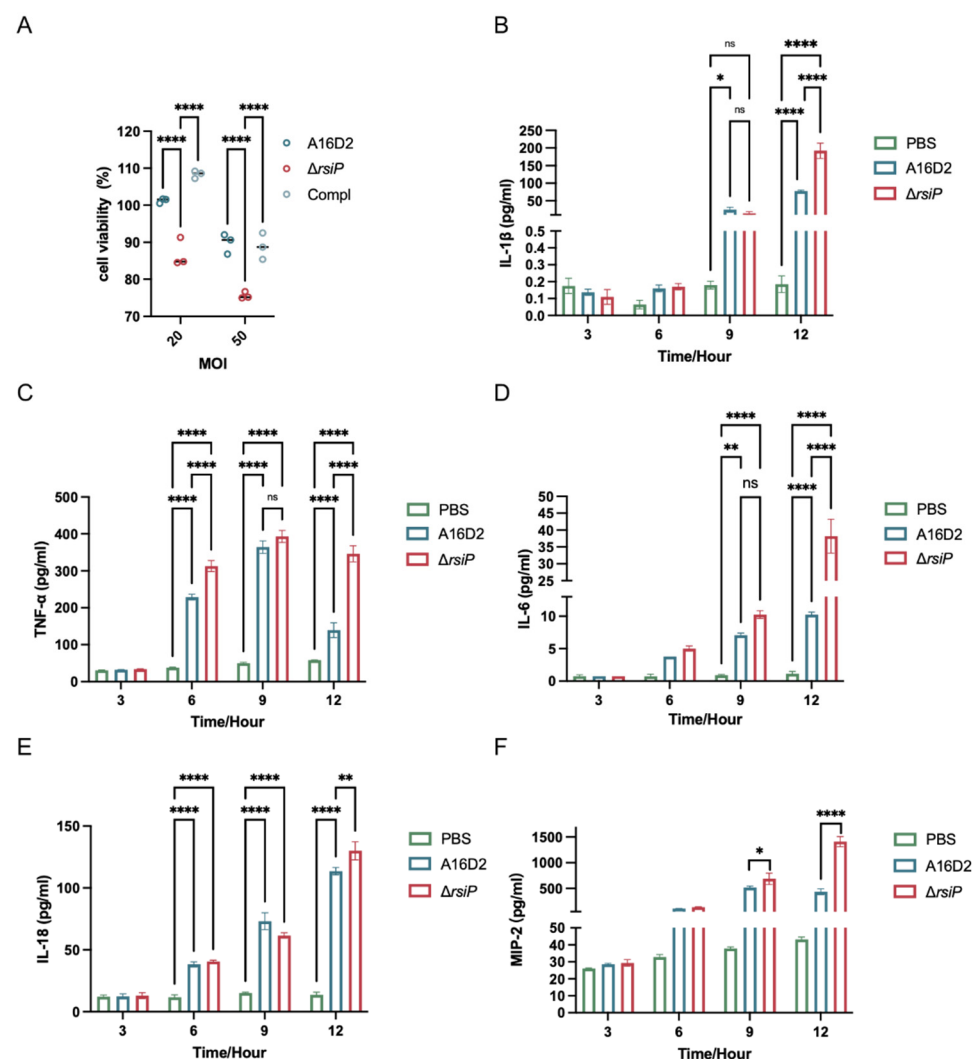


Figure 2. Deletion of *rsiP* enhances virulence and induces a more robust inflammatory reaction in cell. (A) Cell counting Kit-8 assay for A16D2, *rsiP* deletion and Compl in different MOI (n = 3). The inflammatory cytokine/chemokine responses in macrophages stimulated with *rsiP* mutant spores were examined. J774A.1 macrophage cells were co-cultured with specific mutant strains for durations of 3, 6, 9, and 12 h. Following these incubation periods, the culture supernatants were harvested for cytokine quantification using Luminex xMAP technology (n = 4); (B) TNF- α levels; (C) IL-1 β levels; (D) IL-6 levels; (E) IL-18 levels; (F) MIP-2 levels. Statistical significance was determined using Two-way ANOVA (* $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$).

3.3. Deletion of *rsiP* Enhances Virulence in *Galleria Mellonella* Larvae

Galleria mellonella larvae (*G. mellonella*) is also a commonly employed model for assessing anthrax virulence [29]. We infected *G. mellonella* with spores of the wild-type and $\Delta rsiP$ strains. Infection experiments in *G. mellonella* further confirmed the higher lethality of the $\Delta rsiP$ strain, which was consistent with the cell results (Figure 3A,B).

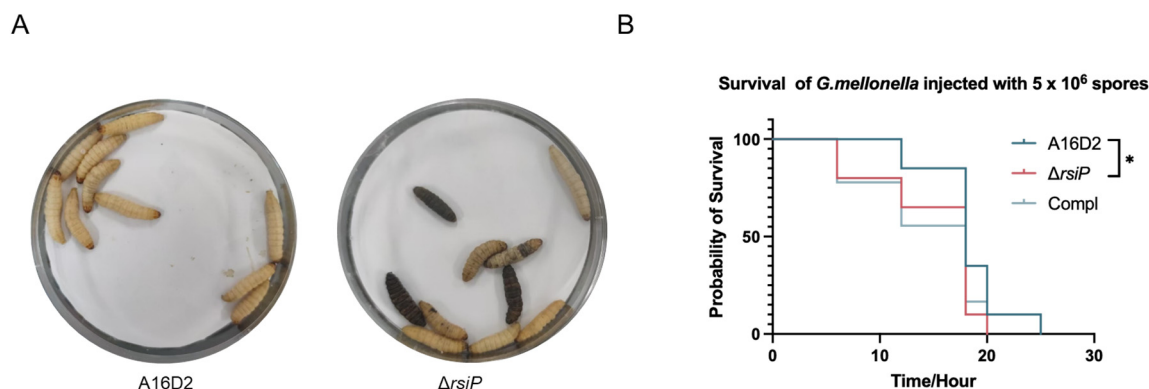


Figure 3. Deletion of *rsiP* enhances virulence in *G. mellonella*. (A) *G. mellonella* at 6 h post infection with spores. (B) *G. mellonella* survival curve. Statistical significance was determined using the Mantel–Cox log rank test. ($n = 20$, * $p < 0.05$).

3.4. Expression of *rsiP* Is Shown to Increase the Colonization Capacity and Competitive Fitness of *Bacillus anthracis* in Mice

To assess the role of *rsiP* during in vivo infection, we compared the pathogenicity of wild-type and $\Delta rsiP$ in a mouse model. Although no significant difference in vitro growth rate was observed between the two strains (Figure 4A), infection of DBA/2 mice with 10^7 CFU illustrated that the wild-type strain exhibited greater virulence, resulting in 100% mortality within 40 h (Figure 4B). Moreover, the bacterial burden in the spleens of mice infected with the wild-type strain was markedly higher than in the $\Delta rsiP$ group (Figure 4C). Competition index (CI) analysis was conducted to quantify the competitive fitness of the strains both in vitro and in vivo (Figure 4D). The in vitro mean CI was 0.95. In vivo assessment, mice were subcutaneously inoculated with a 1:1 mixture of the same spore preparations (10^6 spores per mouse). Spleens and livers were harvested 24 h post-infection for bacterial load analysis. The proportion of the $\Delta rsiP$ mutant was significantly lower than that of the wild-type strain in all organs examined, with mean CIs of 0.39 in the spleen and 0.42 in the liver. CI values consistently below 1 indicate that the wild-type strain possesses a superior capacity for survival, dissemination, or colonization in both cellular and host environments.

To further elucidate the cellular mechanisms underlying the aforementioned phenotypes, we hypothesized that the reduced competitive fitness of $\Delta rsiP$ might be attributed to the following factors: (1) an increased susceptibility to host-derived oxidative stress (e.g., reactive oxygen species or ROS generated during macrophage respiratory burst) [30] or an enhanced ability to provoke ROS production by macrophages, both of which ultimately compromise the survival of the mutant; (2) an altered efficiency in the recognition and internalization of spores by macrophages, which impairs the bacterium's ability to survive and replicate intracellularly during the early stages of infection.

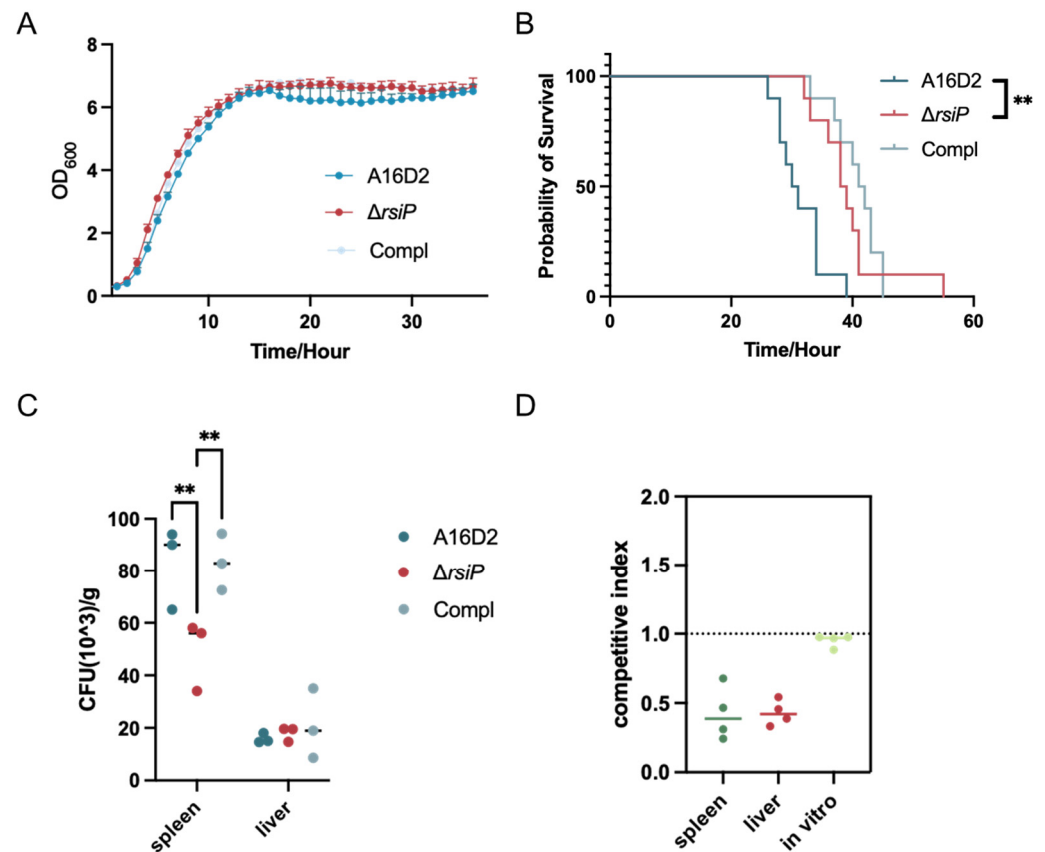


Figure 4. Virulence of A16D2 and $\Delta rs iP$ strains. (A) Time-growth curve. (B) Survival curve of mice infected by intraperitoneal injection of the various spores. Each experimental group included 10 mice. Statistical significance was determined using the Mantel–Cox log rank test. (C) Organs were collected, homogenized, and plated for the determination of CFU. Statistical significance was determined using two-way ANOVA (** $p \leq 0.01$). (D) Competition Index analysis.

To verify these hypotheses, this study first assessed the direct resistance of spores to oxidative stress. A16D2 and $\Delta rs iP$ spores were exposed to different concentrations of hydrogen peroxide (H_2O_2). The results showed no significant difference in survival rates between the two strains, indicating that RsiP deletion does not directly affect the intrinsic tolerance of spores to exogenous H_2O_2 (Figure 5A). In the macrophage infection model, although the level of ROS produced by host cells stimulated by $\Delta rs iP$ spores was not statistically different from that induced by A16D2 (Figure 5B), the efficiency of spore internalization was significantly reduced: the number of A16D2 spores phagocytosed by macrophages was approximately 1.5-fold higher than that of $\Delta rs iP$ (Figure 5C). These findings suggest that the reason for the attenuated competitive fitness of $\Delta rs iP$ in the host environment is not due to altered oxidative stress tolerance, but rather closely related to the decreased efficiency of host cell recognition and internalization. After internalization by macrophages, A16D2 spores can germinate more rapidly and enter the proliferation stage, thereby establishing a higher bacterial load in the early stages of infection. This is consistent with the result that $\Delta rs iP$ exhibits lower bacterial loads in host organs, indicating that the reduced internalization efficiency directly impairs the early establishment and subsequent dissemination of the bacterium in the host.

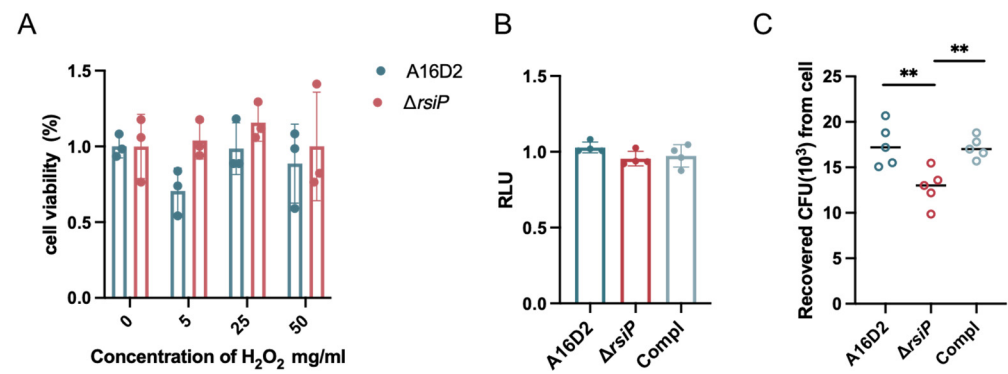


Figure 5. Assessing spore resilience to oxidative Stress, macrophage ROS Production, and cellular internalization. (A) Survival rates of spores after exposure to H₂O₂ (n = 3); (B) Production of ROS in macrophages stimulated by spores (n = 4); (C) The verification of intracellular internalization was conducted using One-way ANOVA test to evaluate the significance of differences in intracellular internalization ability, and was marked as follows (n = 5): ** indicates $p \leq 0.01$.

4. Discussion

This study investigated the role of RsiP in virulence regulation of *B. anthracis*. Our current results show that $\Delta rs iP$ significantly upregulated the expression of *sigP*, *bla1*, metalloprotease genes *nprR* and *npr599* (Figure 1A,B) under both ambient air and CO₂ conditions, which indicated that RsiP not only modulates β -lactam resistance but also influences extracellular protease activity. The extracellular protease activity of the *nprR* gene deletion mutant is significantly reduced, demonstrating that NprR affects the expression of Npr599 through a complex regulatory network [35,36]. Therefore, the deletion of RsiP affects the expression of *nprR* and *npr599* may via a σ^P -dependent pathway, thereby altering the extracellular protease activity profile of *B. anthracis*.

At the protein level, Western blot and milk plate hydrolysis assays confirmed that the secretion and activity of NprR and Npr599 were significantly enhanced in the $\Delta rs iP$ strain (Figure 1C–E), consistent with the gene expression results. The upregulation of virulence-associated proteases may directly contribute to bacterial pathogenicity, for instance, by degrading host matrices or suppressing immune responses [37,38].

Regarding virulence phenotypes, $\Delta rs iP$ spores enhanced virulence in both the murine macrophage J774A.1 cell line and the *G. mellonella* infection model and induced a more robust inflammatory response in cell. Previous reports indicated that when macrophages were infected with purified *B. anthracis* spores alone, TNF- α was released as early as 5 h following infection [39], which is consistent with our findings (Figure 2C).

Surprisingly, however, despite the stronger virulence of $\Delta rs iP$ in cell and in *G. mellonella*, the wild-type strain demonstrated higher lethality and organ bacterial loads in a murine systemic infection model (Figure 4B,C). Similar phenomena have been reported for other pathogens, where hyperactivation of immunity may conversely promote pathogen clearance [40,41]. Secondly, *G. mellonella* primarily relies on innate immune mechanisms, including humoral and cellular immunity (e.g., hemolymph and phagocytic cells) [42]. The $\Delta rs iP$ mutant may have lost its ability to effectively suppress adaptive immunity in mammalian hosts, but could still exhibit enhanced virulence in the innate immune environment of *G. mellonella*, where its specific virulence factors remain functional. Notably, the attenuation of virulence in the $\Delta rs iP$ mutant was moderate. This is consistent with the function of RsiP as a transcriptional regulator, whose primary role is the fine-tuning of virulence gene expression rather than serving as a structural component of the virulence machinery. Therefore, its deletion attenuates, but does not completely abrogate, the pathogenic process. The partial restoration of virulence in the complementary strain in *G. mellonella* and mouse

models (Figures 3B and 4B), alongside its clear rescue of competitive fitness (Figure 4D), suggests that the regulatory network involving RsiP may be embedded in a more complex system in vivo. The incomplete phenotypic reversal could be influenced by several factors. This highlights that the role of fine-tuning regulators like RsiP must be assessed within the adaptive landscape of the host environment. Future studies, such as transcriptomic profiling of strains recovered from infection, will be valuable to elucidate such potential compensatory mechanisms.

Moreover, CI analysis revealed that $\Delta rsiP$ was less competitive than the wild type both in vitro and in vivo (spleen and liver), with $CI < 1$, indicating impaired colonization and dissemination capacity. Based on the established finding that the inactivation of *cpdB* enhances cell adhesion by inhibiting the expression of the extracellular protease Npr599 [15], our results indicate that the deletion of *rsiP* conversely suppresses spore adhesion and upregulates Npr599 expression, which consequently leads to a reduced internalization rate and ultimately impairs competitive invasiveness.

It is noteworthy that the complemented strain in this study did not fully recapitulate all phenotypes of the WT strain. Specifically, in terms of virulence in both the *Galleria mellonella* (Figure 3B) and mice (Figure 4B), the complemented strain exhibited only partial restoration or even paradoxical effects. Although the complementing plasmid is designed to restore the function of the target gene, its expression level in the complemented strain may differ from that of the endogenous gene in the wild-type strain [43,44]. Therefore, the phenotypic discrepancies between the complemented and wild-type strains, rather than undermining the conclusions of this study, highlight the complexity of RsiP-mediated regulation in the in vivo environment. Future research should employ chromosomal in situ complementation or transcriptomic analysis in the complemented strain background to more precisely dissect the functional network of RsiP. Nonetheless, the current complementation experiments have adequately confirmed that the core phenotypes observed in the $\Delta rsiP$ were indeed directly attributable to the absence of RsiP.

In summary, RsiP acts as an intricate regulator of virulence in *B. anthracis*. It influences early interactions between the bacterium and the host immune system, playing a vital role in determining bacterial colonization capacity and ultimately pathogenicity. This study provides a new perspective for a deeper understanding of the pathogenic mechanisms of *B. anthracis*, and RsiP may represent a potential target for intervention.

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