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The ROSA-like prophage colonizing *Staphylococcus aureus* promotes intracellular survival, biofilm formation and virulence in a chronic wound environment

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Abstract

The transition from colonization to invasion is critical in diabetic foot ulcer (DFU). *S. aureus* can colonize DFU, or invade the underlying tissues, causing serious infections. The ROSA-like prophage has previously been implicated in strain colonization characteristics of *S. aureus* isolates in uninfected ulcers. Here, we investigated this prophage in the *S. aureus* colonizing strain using an *in vitro* chronic wound medium (CWM) mimicking the chronic wound environment. CWM reduced bacterial growth and increased biofilm formation and virulence in a zebrafish model. Moreover, the ROSA-like prophage promoted intracellular survival of *S. aureus* colonizing strain in macrophages, keratinocytes and osteoblasts.

Keywords: Diabetic foot ulcer, colonizing *Staphylococcus aureus*, ROSA-like prophage, chronic wound medium

INTRODUCTION

Chronic wounds such as pressure ulcers, venous/arterial ulcers and diabetic foot ulcers (DFU) cause high morbidity and mortality [1]. *Staphylococcus aureus* is the most frequently isolated bacteria in polymicrobial DFU, leading to delayed wound healing [2]. This species colonizes approximately 30% of the human population and causes various clinical infections [3]. Therefore, it is crucial to differentiate between colonizers and pathogens causing infection in DFU [4–6]. One major difference between colonizing and infecting *S. aureus* strains isolated from DFUs is the presence of a stable prophage, named ROSA-like [7]. The integrated ROSA-like phage increased biofilm formation, reduced virulence, cytotoxicity and the number of viable intracellular bacteria in infected osteoblasts compared to ROSA-like-negative strains [7,8]. However, these studies were conducted under conditions not reproducing the chronic wound environment, which could affect *S. aureus* colonizing strain adaptation. In this study, we assessed whether the ROSA-like prophage was involved in the persistence and adaptive ability of *S. aureus* to the stressful conditions of DFU using an *in vitro* medium that closely mimics the chronic wound microbiological, cellular, and inflammatory environment [1].

METHODS

Bacterial strains, media and growth conditions. *S. aureus* DFU clinical isolate NSA1385 and its isogenic ROSA-like (Δ*rosa*) excision variant were previously published [5]. Briefly, NSA1385 was identified in an uninfected DFU, which did not progress to infection. The ROSA-like ejection variant (Δ*rosa*) was produced after iron-restricted growth. Sequencing demonstrated that the only difference between the two strains was the deletion of this phage [7]. *S. aureus* strains were grown in Tryptic Soy Broth (TSB) medium or Chronic Wound Medium (CWM) at 37°C and 225 rpm. We have previously described the *in vitro* CWM [1], comprising 79.5% Bolton broth, 0.5% hemolyzed human blood and 20% heat inactivated human serum, with 1.10^6/mL of human

keratinocyte debris (HaCaT cells), buffered to pH 8.0. Bacterial growth was observed using a microplate reader (Tecan, Lyon, France).

Kinetics of early biofilm formation. Biofilm Ring Test® (BioFilm Control, Saint Beauzire, France) was used according to the manufacturer's instructions to evaluate early biofilm formation. *S. aureus* strains were grown at 37 °C for 6 h in BHI or in CWM. Standardized to an OD600 of 1.00 ± 0.05, the bacterial suspension was diluted 1:250 to 4.10^6 CFU/mL. 1% magnetic beads were added to the bacterial suspension in BHI or CWM. 200 µL were added to a 96-well microplate in triplicate and incubated for 3 h at 37°C. The microplate was placed on a magnetic block for 1 min and scanned with a custom plate reader. The BFCE3 software calculated adhesion strength from images before and after magnetic attraction. A low BFI value (<2) indicates complete immobilization of beads due to sessile cells, while a high value (>7) indicates high mobility of beads under magnet action, designating no biofilm formation.

Zebrafish killing assay. The zebrafish model was employed for bath immersion infection to assess *S. aureus* virulence. NSA1385 and Δ*rosa* strains were grown in TSB or CWM overnight at 37°C. Cultures were diluted, centrifuged, and reconstituted in fish water at 2.10^7 bacteria/mL. Embryos dechorionated at 48 hpf were bath immersed and infection was performed as previously described [9]. Bath immersion was performed on injured embryos 2 days after fertilization while the mouth was closed and the viability monitored for 24 h.

Cells infecting and viability assay. Intracellular bacterial growth and cytotoxicity during macrophage (RAW 264.7), keratinocyte (HaCaT) and osteoblast (MC3T3-E1) infections were quantified as previously described [9]. Briefly, *S. aureus* strains were grown in TSB or CWM broth to mid-exponential phase growth prior to infection. In 24-well plates, RAW 264.7 cells (MOI 20:1), HaCaT cells (MOI 100:1), and MC3T3-E1 (MOI 100:1) were infected with *S. aureus* NSA1385 and Δ*rosa* strains, then incubated for 1 h for RAW 264.7 cells, or 1h30 for HaCaT and MC3T3-E1 cells, at 37°C and 5% CO_2. Cells were washed and the extracellular bacteria killed by gentamicin treatment (100 µg/mL) for 60 min and cells were washed twice with PBS (T0) and incubated in fresh medium with 5 µg/mL lysostaphin for 5, 24, and 48 h. Intracellular bacteria were counted by lysing infected cells with 0.1 Triton X-100 in PBS. Lactate deshydrogenase (LDH) activity in cell culture supernatant was measured to quantify cytotoxicity using the CyQuant LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, RockFord, IL, USA) according to the manufacturer's instructions.

Statistical Analysis. GraphPad software package Prism 9.5.5 was used to estimate the statistical significance of differences across groups, which is indicated in the relevant figure legends.

Ethical statement. All animal experiments were conducted in conformity with European Union guidelines for the care and use of laboratory animals at the University of Montpellier as previously described [9].

RESULTS

ROSA-like prophage affects *S. aureus* growth, early biofilm formation and virulence in Chronic Wound Media

The bacterial growth of *S. aureus* NSA1385 and Δ*rosa* strains was compared in CWM versus TSB standard medium. *S. aureus* NSA1385 and Δ*rosa* growth curves showed no significant differences in TSB (Figure 1A). However, both strains grew slower in CMW in comparison to TSB, with a significant delay for Δ*rosa* in CWM compared to NSA1385 ($p < 0.001$) (Figure 1A). Therefore, the ROSA-like prophage is directly involved in the growth rate delay in a chronic wound environment.

Next, we assessed the impact of the ROSA-like prophage on early biofilm formation using the Biofilm Ring test. *S. aureus* NSA1385 formed a biofilm faster than the Δ*rosa* variant in both media and even faster in CWM than in the classical BHI medium (BFI = 9± 0.2 vs. 11± 0.1 and 12± 0.2 vs. 16± 0.3, for NSA1385 and Δ*rosa*, respectively) ($p < 0.01$). The difference remained significant at 2 h and 3 h ($p < 0.001$), confirming the role of the ROSA-like prophage in promoting biofilm formation and especially in a chronic wound environment (Figure 1B).

The impact of the ROSA-like prophage on bacterial virulence after growth in CWM was studied in the zebrafish model [9]. Strains grown in TSB showed over 90% mortality with Δ*rosa* as early as 10 hpi, while 70% of embryos survived following NSA1385 infections at 21 hpi. Interestingly, opposite results were observed after growth in CWM, as larvae infected with NSA1385 were all dead at 10 hpi, while embryos infected with Δ*rosa* survived until 21 hpi. The control group showed no mortality (Fig 1C). Therefore, the ROSA-like prophage increases virulence in a chronic wound environment.

ROSA-like phage is involved in the intracellular persistence of *S. aureus* in chronic wound environment.

S. aureus survival within phagocytic and non-phagocytic cells was analyzed in RAW 264.7 murine macrophages, MC3T3-E1 murine osteoblasts, and HaCat human keratinocytes grown in TSB or CWM.

RAW 264.7 macrophages were used to phagocytose *S. aureus* and viable bacteria were recovered at 5 h and 24 h post-Gentamicin treatment (pGt). At T0, *S. aureus* NSA1385 and Δ*rosa* had similar numbers of phagocytosed bacteria, indicating that neither the ROSA-like prophage nor the CWM affected macrophage phagocytosis (Figure 2A). However, the Δ*rosa* strain had a higher viable intracellular bacterial count than NSA1385 in both media at T0, 5 and 24 h pGt suggesting that the ROSA-like prophage was involved in decreasing *S. aureus* intramacrophagic survival. Interestingly, intracellular *S. aureus* NSA1385 significantly enhanced its intramacrophagic survival when cultured in CWM versus TSB ($p< 0.01$), indicating that the ROSA-like prophage was critical for *S. aureus* colonizing strain adaptation to macrophage survival in chronic wound environment (Figure 2A).

We used the LDH assay to determine whether the higher survival of *S. aureus* NSA1385 in CWM was related to a lower cytotoxicity of the infected macrophage cells. Infection with *S. aureus* NSA1385 cultured in TSB or CWM enhanced LDH release over time in infected Raw 264.7 macrophages (Figure 2B). However, NSA1385 generated less LDH when grown in CWM versus TSB ($p< 0.01$). This suggested that *S. aureus* NSA1385 grown in CWM had a distinct intracellular fate versus TSB growth, as bacteria survived for extended periods within macrophages, associated with lower levels of cytotoxicity. Therefore, the inability of macrophages to eliminate intracellular *S. aureus* NSA1385 in a chronic wound environment may be a serious defect of the host innate immunity, leading to intracellular reservoirs of colonizing *S. aureus*. Moreover, the ROSA-like prophage affected macrophage cytotoxicity by decreasing LDH over time in Δ*rosa*-infected cells grown in CWM versus TSB ($p< 0.001$) (Figure 2B).

In non-phagocytotic cells, *S. aureus* is known to proliferate and persist intracellularly [10]. To test whether the chronic wound environment influenced survival with ROSA-like prophage in infected osteoblasts and keratinocytes, we allowed osteoblast cells to phagocytose *S. aureus* and viable bacteria were recovered at T0, 5 and 24 h pGt. At T0, *S. aureus* NSA1385 had significantly more phagocytosed bacteria compared to Δ*rosa* in both media ($p< 0.001$), indicating that the ROSA-like prophage increased osteoblast phagocytosis independently of the CWM (Figure 2C). In comparison to the NSA1385 strain, the Δ*rosa* strain had a higher percentage of infecting inoculum that persisted within osteoblasts at T5h pGt ($p< 0.01$), with the difference becoming more pronounced at T24 ($p< 0.001$) (Figure 2C). Interestingly, this behavior was associated with impairment of NSA1385 to multiply and persist within osteoblasts over time, despite higher phagocytosis. In contrast, the Δ*rosa* variant was unaffected, and internalized Δ*rosa* CFUs grew normally [8]. However, growth of *S. aureus* NSA1385 in CWM increased its survival at T5h versus in TSB ($p< 0.05$), but by T24h no difference was observed in intracellular counts (Figure 3C). However, intracellular bacteria of the Δ*rosa* strain grown in CWM were significantly lower over time in comparison to TSB ($p< 0.01$), suggesting that the CWM reduced Δ*rosa* intracellular survival. Overall, these results indicated that the ROSA-like prophage decreased the intracellular survival of the NSA1385 colonizing strain independently of the chronic wound environment, but the Δ*rosa* variant was sensitive to this environment. Moreover, an increased cytotoxic response over time against osteoblasts was observed with NSA1385 strain grown in TSB and CWM, in accordance with their increased intracellular survival (Figure 2D). The non-phagocytic human keratinocytes cell line HaCaT showed similar results (Figure 2E,F). Overall, our data indicated that in non-phagocytic cells, the CWM had no impact on phagocytosis defect but promoted intracellular survival of the NSA1385 strain and increased its related cytotoxicity.

Discussion

In DFU, *S. aureus* experiences stress conditions including increased glucose concentration, lower temperature, lower tissue oxygenation, and exposure to antibiotics [11]. Environmental stresses can cause cellular damage in bacteria and impair macromolecule function, essential for survival and growth [12]. Prophages provide numerous advantages to endure these challenging conditions [13]. Here, we reported that the ROSA-like prophage of *S. aureus* colonizing strain, isolated from DFU, promoted bacterial growth, biofilm formation, persistence and virulence in chronic wound environment.

Most *S. aureus* isolates carry multiple prophages, affecting virulence, toxin production, immune evasion, and host preference. These genetic elements carry environmentally regulated adaptive genes [14]. For instance, prophages in *Vibrio parahaemolyticus* improve ultraviolet sensitivity, DNA methylase activity, quorum sensing, and resistance to environmental stress [12]. Integration of phage Min27 into *Escherichia coli* genome increases swimming motility, allowing bacteria to grow faster, move to high-nutrient areas and avoid harmful environments [15]. Moreover, removing all nine prophages from *E. coli* reduced resistance to oxidative, osmotic, and acid stress [13]. Here, ROSA-like prophage improved bacterial growth in CWM, as well as rapid biofilm formation, consistent with the NSA1385 colonizing phenotype. In addition, larvae infected with NSA1385 grown in CWM had increased mortality rate, confirming the impact of DFU environment on ROSA-like prophage's role in bacterial virulence. Furthermore, studies have shown that prophages help negative regulation of cellular inflammatory response, improving bacterial survival in the host through phagocytosis evasion [12]. Interestingly, our findings showed no difference for the colonizing strain's phagocytosis. We thus hypothesized that the mechanisms for *S. aureus* proliferation are either to multiply and hide in cells without killing them, or to improve other infecting strains' ability to evade the immune system and infiltrating deeper tissues. However, while these data point to virulence factors being influenced by environmental stress, a study on the colonizing strain pre-cultivated in a wound-like medium versus infecting strains isolated from DFU found no difference in any of the studied genes [11]. Only after a long exposure did the colonizing strain NSA1385 increase *fnbpA* gene expression and decrease *hla* gene expression [11]. Better understanding the role of the ROSA-like prophage in *S. aureus* behavior under stress will help develop new strategies to prevent and treat infections.

Notes

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Potential conflicts of interest. The authors declare no conflict of interest.

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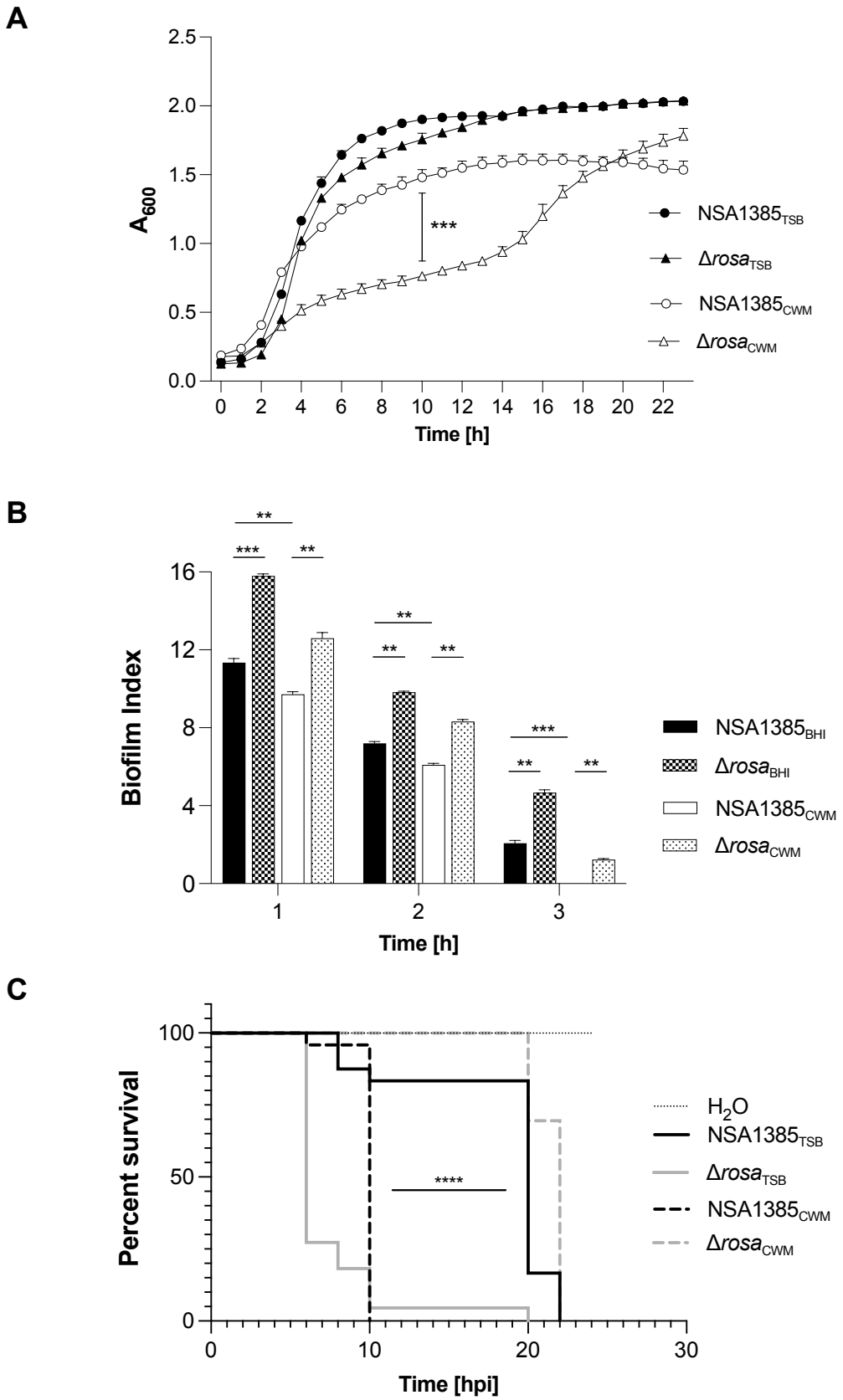

Figure 1. Growth, biofilm formation and virulence of *S. aureus* in chronic wound media (CWM) (**A**) Growth kinetics of NSA1385 (black circles) and Δ*rosa* (black triangles) strains in TSB; NSA1385 (white circles) and Δ*rosa* (white triangles) strains in CWM. At each time point (n= 3), the data show the mean A_{600} readings SD. Welsh's t test, *** p< 0.001. (**B**) Kinetics of biofilm formation determined by BioFilm Ring Test®. Comparisons were performed with t-test, *** p< 0.001, ** p< 0.01. (**C**) Kaplan–Meier representation of the survival of embryos injured in the tail fin at 48 h post-fertilization (hpf) in a bath infected with *S. aureus* strains at 2.10^7 CFU/mL grown in TSB or CWM or "fish water" (negative control). The proportion of surviving embryos (n= 24 for each, indicative of five separate experiments) is used to express the results.

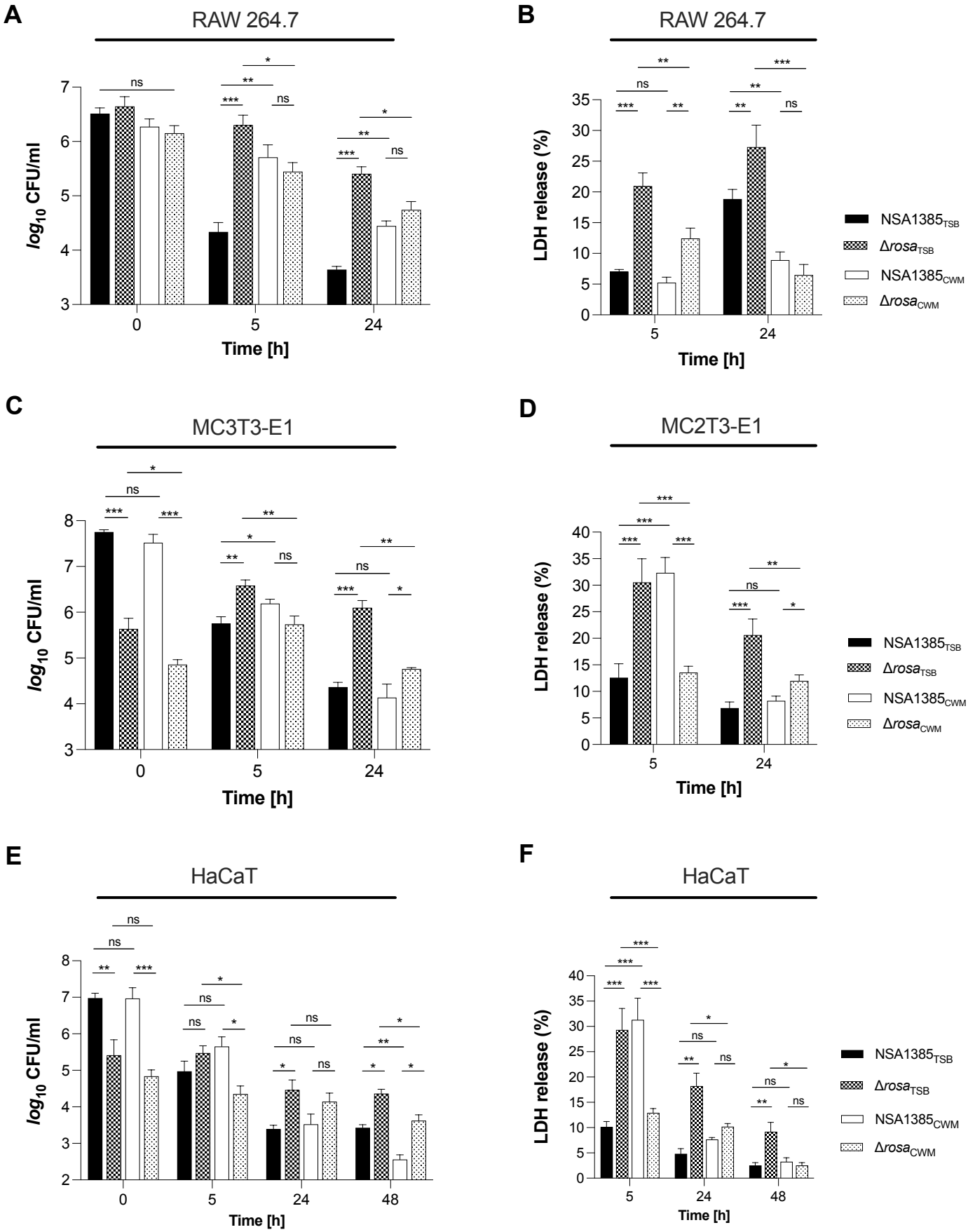

Figure 2. *S. aureus* survival in infected cells and influence of chronic wound environment. $NSA1385_{TSB}$ (black), $\Delta rosa_{TSB}$ (dotted black), $NSA1385_{CWM}$ (white) and $\Delta rosa_{CWM}$ (dotted white) bacteria were used to infect RAW 264.7 macrophages **(A)**, MC2T3-E1 osteoblasts **(C)** and HaCaT keratinocytes cells **(E)**. The average and standard deviation (SD) of five different experiments are represented. **(B, D, and F)** LDH release was measured using the CyQUANT assay kit. Data are expressed relative to the 100% positive control (n=3 biological repeats). A two-way ANOVA test was used to establish statistical significance, with * $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$; ns, not significant.