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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 ( $\Delta$ 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 ( $\Delta$ 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<sup>6</sup>/mL of human

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

47 **Kinetics of early biofilm formation.** Biofilm Ring Test® (BioFilm Control, Saint Beauzire, France) was used  
48 according to the manufacturer's instructions to evaluate early biofilm formation. *S. aureus* strains were grown  
49 at 37 °C for 6 h in BHI or in CWM. Standardized to an OD600 of  $1.00 \pm 0.05$ , the bacterial suspension was diluted  
50 1:250 to  $4.10^6$  CFU/mL. 1% magnetic beads were added to the bacterial suspension in BHI or CWM. 200 µL were  
51 added to a 96-well microplate in triplicate and incubated for 3 h at 37°C. The microplate was placed on a  
52 magnetic block for 1 min and scanned with a custom plate reader. The BFCE3 software calculated adhesion  
53 strength from images before and after magnetic attraction. A low BFI value (<2) indicates complete  
54 immobilization of beads due to sessile cells, while a high value (>7) indicates high mobility of beads under  
55 magnet action, designating no biofilm formation.

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

61  
62 **Cells infecting and viability assay.** Intracellular bacterial growth and cytotoxicity during macrophage (RAW  
63 264.7), keratinocyte (HaCaT) and osteoblast (MC3T3-E1) infections were quantified as previously described [9].  
64 Briefly, *S. aureus* strains were grown in TSB or CWM broth to mid-exponential phase growth prior to infection.  
65 In 24-well plates, RAW 264.7 cells (MOI 20:1), HaCaT cells (MOI 100:1), and MC3T3-E1 (MOI 100:1) were  
66 infected with *S. aureus* NSA1385 and  $\Delta$ rosa strains, then incubated for 1 h for RAW 264.7 cells, or 1h30 for HaCaT  
67 and MC3T3-E1 cells, at 37°C and 5% CO<sub>2</sub>. Cells were washed and the extracellular bacteria killed by gentamicin  
68 treatment (100 µg/mL) for 60 min and cells were washed twice with PBS (T0) and incubated in fresh medium  
69 with 5 µg/mL lysostaphin for 5, 24, and 48 h. Intracellular bacteria were counted by lysing infected cells with  
70 0.1 Triton X-100 in PBS. Lactate dehydrogenase (LDH) activity in cell culture supernatant was measured to  
71 quantify cytotoxicity using the CyQuant LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, Rockford, IL,  
72 USA) according to the manufacturer's instructions.

73  
74 **Statistical Analysis.** GraphPad software package Prism 9.5.5 was used to estimate the statistical significance of  
75 differences across groups, which is indicated in the relevant figure legends.

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

## 79 RESULTS

### 80 ROSA-like prophage affects *S. aureus* growth, early biofilm formation and virulence in Chronic Wound 81 Media

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

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

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

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

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

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

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

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

142

## 143 Discussion

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

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

## 171 Notes

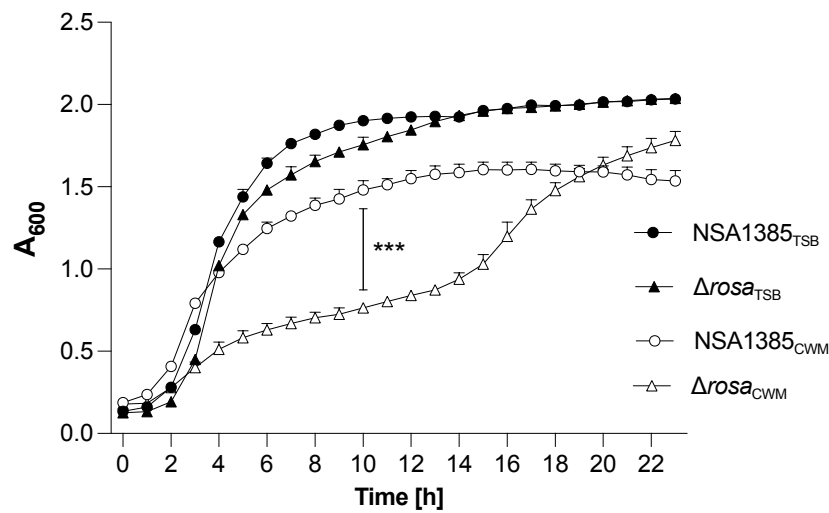
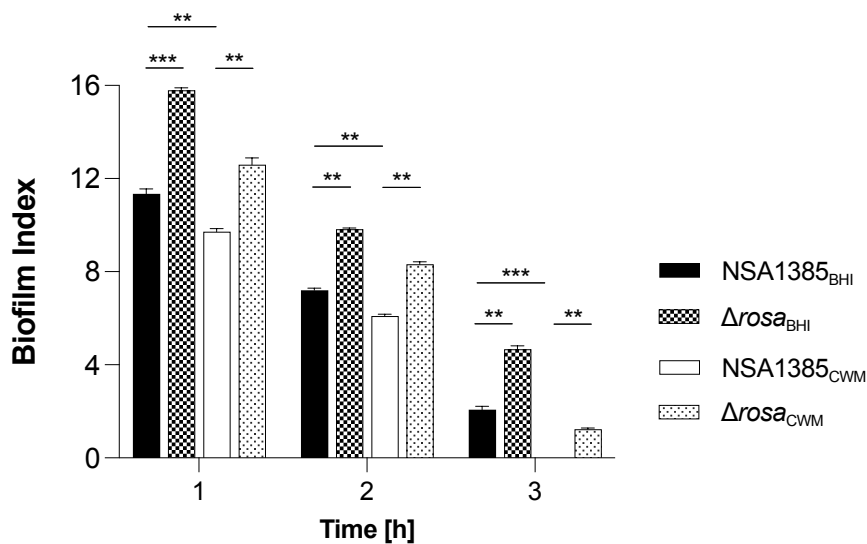
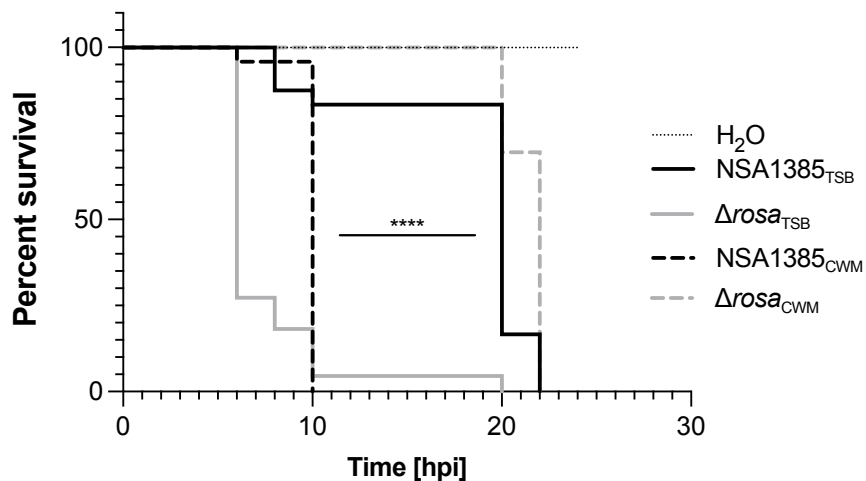
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176 *Potential conflicts of interest.* The authors declare no conflict of interest.

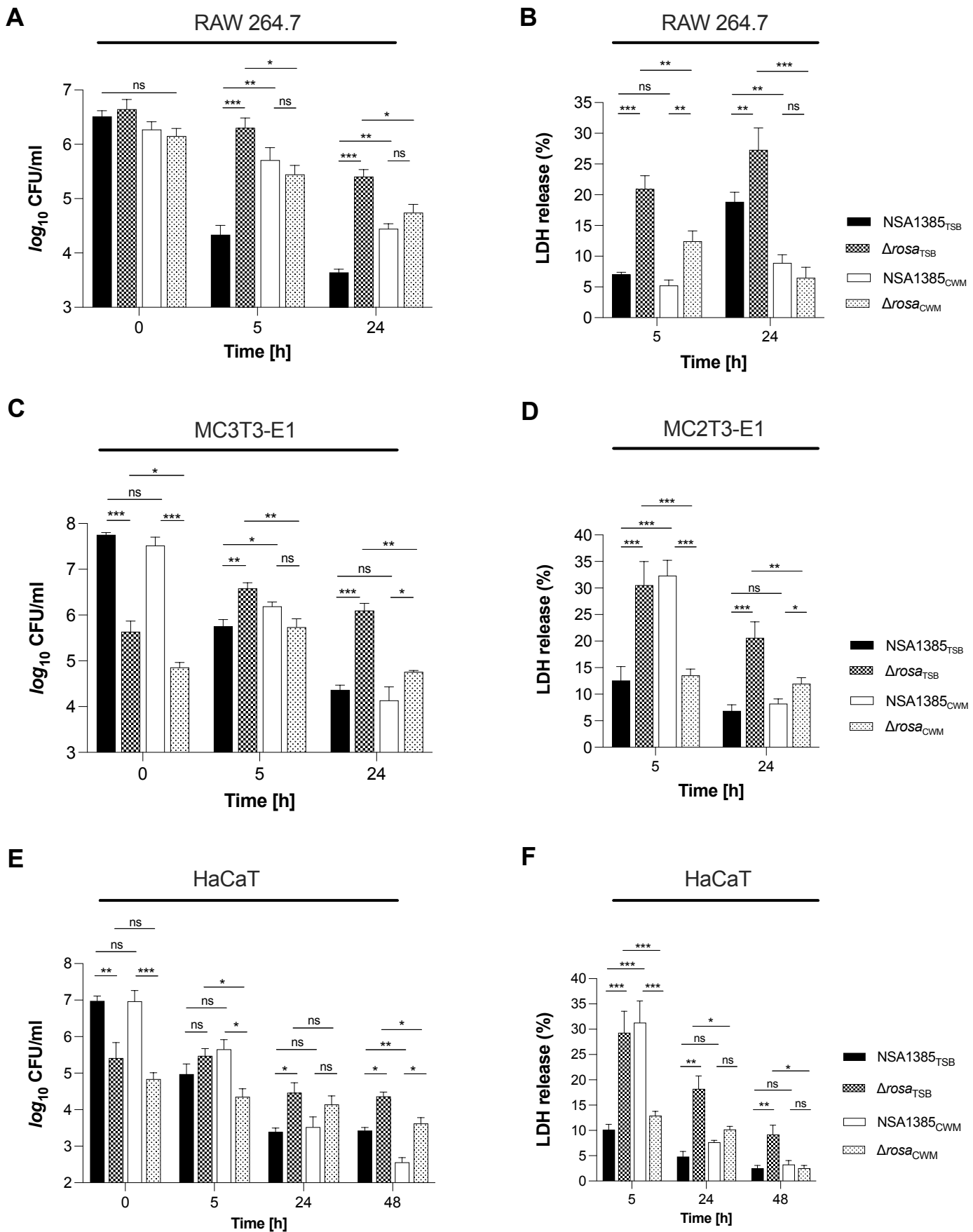
## 178 References

- 179 1. Pouget C, Dunyach-Remy C, Bernardi T, et al. A Relevant Wound-Like in vitro Media to Study Bacterial  
 180 Cooperation and Biofilm in Chronic Wounds. *Front Microbiol* [Internet]. 2022 [cited 2023 Jan 10]; 13. Available  
 181 from: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.705479>
- 182 2. Durand BARN, Pouget C, Magnan C, Molle V, Lavigne J-P, Dunyach-Remy C. Bacterial Interactions in  
 183 the Context of Chronic Wound Biofilm: A Review. *Microorganisms*. Multidisciplinary Digital Publishing  
 184 Institute; 2022; 10(8):1500.
- 185 3. Lavigne J-P, Hosny M, Dunyach-Remy C, et al. Long-Term Intrahost Evolution of *Staphylococcus aureus*  
 186 Among Diabetic Patients With Foot Infections. *Front Microbiol* [Internet]. 2021 [cited 2023 Jan 14]; 12. Available  
 187 from: <https://www.frontiersin.org/articles/10.3389/fmicb.2021.741406>
- 188 4. Sotto A, Richard J-L, Jourdan N, et al. Miniaturized oligonucleotide arrays: a new tool for  
 189 discriminating colonization from infection due to *Staphylococcus aureus* in diabetic foot ulcers. *Diabetes Care*.  
 190 2007; 30(8):2051–2056.
- 191 5. Sotto A, Lina G, Richard J-L, et al. Virulence Potential of *Staphylococcus aureus* Strains Isolated From  
 192

- 193 Diabetic Foot Ulcers: A new paradigm. *Diabetes Care*. **2008**; 31(12):2318–2324.
- 194 6. Sotto A, Richard J-L, Messad N, et al. Distinguishing colonization from infection with *Staphylococcus*  
195 *aureus* in diabetic foot ulcers with miniaturized oligonucleotide arrays: a French multicenter study. *Diabetes*  
196 *Care*. **2012**; 35(3):617–623.
- 197 7. Messad N, Prajsnar TK, Lina G, et al. Existence of a Colonizing *Staphylococcus aureus* Strain Isolated in  
198 Diabetic Foot Ulcers. *Diabetes*. **2015**; 64(8):2991–2995.
- 199 8. Rasigade J-P, Dunyach-Rémy C, Sapin A, et al. A Prophage in Diabetic Foot Ulcer–Colonizing  
200 *Staphylococcus aureus* Impairs Invasiveness by Limiting Intracellular Growth. *J Infect Dis*. **2016**; 214(10):1605–  
201 1608.
- 202 9. Ahmad-Mansour N, Plumet L, Huc-Brandt S, et al. Investigating Pathogenicity and Virulence of  
203 *Staphylococcus pettenkoferi*: An Emerging Pathogen. *Int J Mol Sci*. Multidisciplinary Digital Publishing Institute;  
204 **2021**; 22(24):13614.
- 205 10. Josse J, Velard F, Gangloff SC. *Staphylococcus aureus* vs. osteoblast: relationship and consequences in  
206 osteomyelitis. *Front Cell Infect Microbiol* [Internet]. **2015** [cited 2021 Mar 17]; 5. Available from:  
207 <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4660271/>
- 208 11. Pouget C, Gustave C-A, Ngba-Esebe C, et al. Adaptation of *Staphylococcus aureus* in a Medium  
209 Mimicking a Diabetic Foot Environment. *Toxins*. Multidisciplinary Digital Publishing Institute; **2021**; 13(3):230.
- 210 12. Pazhani GP, Chowdhury G, Ramamurthy T. Adaptations of *Vibrio parahaemolyticus* to Stress During  
211 Environmental Survival, Host Colonization, and Infection. *Front Microbiol*. **2021**; 12:737299.
- 212 13. Wang X, Kim Y, Ma Q, et al. Cryptic prophages help bacteria cope with adverse environments. *Nat*  
213 *Commun*. Nature Publishing Group; **2010**; 1(1):147.
- 214 14. Wendling CC, Refardt D, Hall AR. Fitness benefits to bacteria of carrying prophages and prophage-  
215 encoded antibiotic-resistance genes peak in different environments. *Evol Int J Org Evol*. Wiley-Blackwell; **2021**;  
216 75(2):515.
- 217 15. Li D, Qian X, Liu X, et al. orf6 and orf10 in Prophage phiv142-3 Enhance the Iron-Acquisition Ability  
218 and Resistance of Avian Pathogenic *Escherichia coli* Strain DE142 to Serum. *Front Vet Sci* [Internet]. **2020** [cited  
219 2023 Feb 27]; 7. Available from: <https://www.frontiersin.org/articles/10.3389/fvets.2020.588708>
- 220

**A****B****C**

**Figure 1. Growth, biofilm formation and virulence of *S. aureus* in chronic wound media (CWM)** (A) Growth kinetics of NSA1385 (black circles) and  $\Delta$ rosa (black triangles) strains in TSB; NSA1385 (white circles) and  $\Delta$ 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<sup>®</sup>. 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<sub>TSB</sub> (black),  $\Delta$ rosa<sub>TSB</sub> (dotted black), NSA1385<sub>CWM</sub> (white) and  $\Delta$ rosa<sub>CWM</sub> (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.