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1 **The Secreted Tyrosine Phosphatase PtpA promotes**
2 ***Staphylococcus aureus* Survival in RAW 264.7 Macrophages**
3 **Through Decrease of the SUMOylation Host Response**

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16
17 **ABSTRACT**

18 *S. aureus* is a human pathogen that is extremely adaptable and is the cause of a variety of
19 nosocomial and community-acquired infectious diseases. During infection, *S. aureus* is able to
20 affect the host cell in many ways to enable its own multiplication, spread, and evasion of the
21 host immune defense. One of the mechanisms utilized by *S. aureus* to survive is to inhibit the
22 SUMOylation of host proteins in order to increase its intracellular survival and persistence. Here,
23 we show that the reduction in the levels of cellular SUMO-conjugated proteins in *S. aureus* strain

24 Newman-infected RAW 264.7 cells is associated with the *S. aureus* secreted protein tyrosine
25 phosphatase PtpA, which results in a reduction of Ubiquitin conjugating enzyme 9 (Ubc9) protein
26 level, the critical enzyme of the SUMOylation modification. In addition, we demonstrate that the
27 amino acid residue D120, which is essential for PtpA phosphatase activity, is required for this
28 reduction. This study shows for the first time that *S. aureus* strain Newman impedes via PtpA
29 the host SUMOylation response, which contributes to promote persistence of *S. aureus* within
30 the host.

31 32 **IMPORTANCE**

33 *S. aureus* uses numerous strategies to survive and persist in the intracellular environment of
34 professional phagocytes, including modulation of the SUMOylation process. This study aims to
35 understand how *S. aureus* alters host SUMOylation to enhance its intracellular survival in
36 professional phagocytes. Our results indicate that *S. aureus* strain Newman utilizes PtpA-driven
37 phosphorylation to decrease the amount of SUMOylated proteins in murine macrophages to
38 facilitate its survival in this immune cell type.

39 **Keywords:** *Staphylococcus aureus*; SUMOylation; secreted phosphatase; PtpA; macrophage
40 survival

41 **Running title:** PtpA promotes *Staphylococcus aureus* intramacrophagic survival

42 43 44 **INTRODUCTION**

45 Pathogenic bacteria often affect the host cell physiology during infection to enable their own
46 multiplication, spread, and evasion of the host immune defense (1). Post-translational
47 modifications (PTMs), which are essential for controlling the location, activity, and interaction of
48 proteins are often involved in these host-interaction processes upon infection (2). PTMs comprise
49 phosphorylation, acetylation, and methylation, as well as the incorporation of small polypeptides
50 like ubiquitin or ubiquitin-related proteins such as the Small Ubiquitin-like Modifiers (SUMOs). It
51

52 is known that a number of pathogens hijack PTMs for their own benefit; however, it is only
53 established that a small number of pathogenic microorganisms may interfere with the
54 SUMOylation pathway (2–6). SUMOylation is a type of reversible post-translational modification
55 that occurs in eukaryotic cells. In this regulation, a SUMO protein is covalently bound to its
56 target proteins (7), thereby influencing cellular functions such as DNA replication, the transcription
57 of genetic information, processing of RNA, and cell signaling (8, 9). Only very recently,
58 researchers started looking into the strategies utilized by pathogenic bacteria to alter
59 SUMOylation of host proteins, and our understanding of these processes is still limited (10). In a
60 recent study, we were able to show that the human pathogen *Staphylococcus aureus* inhibits the
61 SUMOylation of host proteins in order to increase its intracellular survival and persistence (11).
62 Additionally, a correlation between the decreased degree of SUMOylation and the reduction in
63 the amount of the SUMO-conjugating enzyme Ubc9 was observed. Moreover, artificial increased
64 SUMOylation in macrophages was shown to reduce the intracellular proliferation of bacteria,
65 whereas treatment with the SUMOylation inhibitor ML-792 led to an increase of the bacterial
66 survival within this immune cell type (11). Interestingly, human pathogens such as *Listeria*
67 *monocytogenes* (2) and *Yersinia pestis* (12), or the plant pathogen *Xanthomonas euvesicatoria*
68 (13) have been shown to release effectors that are able to elicit a general deSUMOylation. *S.*
69 *aureus* is an opportunistic human pathogen that is extremely versatile and the cause of a variety
70 of nosocomial and community-acquired diseases (14, 15). Pathogenicity of this bacterium is
71 largely attributed to its reservoir of virulence factors and regulatory elements (16, 17). The
72 bacterium is able to invade a variety of non-professional and professional phagocytic cells,
73 where it may persist for many days (18–20). PtpA is a low-molecular-weight protein tyrosine
74 phosphatase that is secreted by *S. aureus*. We have previously shown that PtpA is released
75 during growth and macrophage infection, and that deletion of *ptpA* reduces *S.*
76 *aureus* intramacrophage survival and infectivity (21). In this study, we show that a reduction in
77 the levels of cellular SUMO-conjugated proteins is associated with PtpA, which causes a

78 reduction of the Ubc9 level, the essential enzyme of the SUMOylation modification machinery. In
79 addition, we demonstrate here that the phosphatase activity is required for the PtpA-dependent
80 reduction in SUMOylation.

81 RESULTS

82 ***S. aureus* PtpA phosphatase activity is required for survival in murine macrophages.**

83 We recently showed that PtpA enhances the intracellular survival of *S. aureus* in murine
84 macrophages (21), but it is unknown yet, whether the phosphatase activity of PtpA is necessary
85 for this intramacrophage survival capacity of *S. aureus*. In order to test this, we created a PtpA
86 phosphatase deficient mutant in *S. aureus* strain Newman (Newman $\Delta ptpA::ptpA_{D120A}$), based
87 on earlier findings showing that residue D120 in the catalytic loop of PtpA is necessary for its
88 phosphatase activity (22). Next, we determined how cells of the *S. aureus* strains Newman (WT),
89 Newman $\Delta ptpA$, Newman $\Delta ptpA::ptpA$, and Newman $\Delta ptpA::ptpA_{D120A}$ survived inside of cells
90 of the murine macrophage cell line RAW 264.7. Intracellular CFU counts at T0 were similar
91 between all strains used to infect the RAW 264.7 cells, thus ruling out a phagocytosis defect of
92 the mutant (Fig. 1a). However, at 24 h post-Gentamycin treatment (pGt), intracellular bacteria
93 loads decreased significantly for Newman $\Delta ptpA::ptpA_{D120A}$, when compared to the WT (Fig.
94 1b). In line with our previous findings (21), survival rates of the $\Delta ptpA$ mutant also dropped to
95 around 50% of the survival rates seen in macrophages that had been infected with the WT and
96 the *cis*-complemented Newman $\Delta ptpA::ptpA$ strain, respectively (Fig. 1b). These findings
97 confirmed on the one hand the important role PtpA plays for the capacity of *S. aureus* to persist
98 within murine macrophages, and demonstrated on the other hand that the phosphatase activity
99 of PtpA is required for the intracellular survival of *S. aureus* strain Newman in murine
100 macrophages. To exclude that the decreased numbers of Newman $\Delta ptpA$ and Newman
101 $\Delta ptpA::ptpA_{D120A}$ cells seen in infected RAW 264.7 cells at 24 h pGt might be due to alterations
102 in bacterial cytotoxicity elicited by these strains during the intracellular passage, we next used the

103 lactate dehydrogenase (LDH) assay to determine the damage rates of the infected macrophage
104 cells. The LDH test is a classic assay for identifying cytotoxicity by evaluating the level of damage
105 to the cellular plasma membrane via the amount of LDH enzyme that is released into the culture
106 media (23). Notably, infection with all four *S. aureus* strains resulted in LDH release rates in the
107 culture supernatants over time that were comparable to the LDH release rates seen with
108 uninfected RAW 264.7 macrophages (Fig. 1c). This finding indicates that *S. aureus* strain
109 Newman and its *ptpA* derivatives are able to survive intracellularly in macrophages for extended
110 periods of time without inducing a clear cytotoxicity. This observation also ruled out that the
111 reduced bacterial cell numbers seen in Newman $\Delta ptpA$ and Newman $\Delta ptpA::ptpA_{D120A}$
112 infected RAW 264.7 cells at 24 h pGt were due to an elevated cytotoxicity of the internalized
113 bacterial cells. An elevated cytotoxicity would have led to an enhanced killing of the infected
114 macrophages and a subsequent release of bacterial cells into the extracellular milieu in which
115 large proportions of the released cell population would have been killed by the lysostaphin present
116 in the cell culture medium. To test whether complementation of the Newman $\Delta ptpA$ mutant with
117 *ptpA_{D120A}* might have an effect on the growth behavior, we also studied its growth in Tryptic
118 Soy Broth at 37°C and 225 rpm over time (Fig. 1d). The *in vitro* growth curves obtained with all
119 four Newman derivatives yielded rather comparable growth kinetics, except for a non-significant
120 reduced growth of the $\Delta ptpA::ptpA_{D120A}$ mutant during the exponential growth phase (*i.e.* 2-4
121 h).

122 ***S. aureus* PtpA phosphatase is involved in the decrease of host SUMOylation upon**
123 **infection.** Given the findings that a *ptpA* deletion or D120 mutation reduced the intramacrophage
124 survival of *S. aureus*, and based on our recent observations showing that *S. aureus* inhibits the
125 SUMOylation of host proteins in order to increase its intracellular survival and persistence in
126 macrophages (11), we wondered whether PtpA might be involved in the interference with the host
127 SUMOylation response of RAW 264.7 cells to *S. aureus* infection. In order to test this hypothesis,
128 we analyzed the amounts of SUMO1- and SUMO2/3-conjugated proteins in uninfected RAW

129 264.7 cells and infected RAW 264.7 cells at 24 h pGt. First, in comparison to non-infected cells,
130 macrophages that were infected with *S. aureus* Newman showed a significant and specific
131 decrease in the amount of SUMO1 (Fig. 2a) and SUMO2/3 (Fig. 2b) modified proteins, in
132 accordance with our previous observations (11). On the other hand, the global pattern of SUMO-
133 conjugated proteins were rather comparable to that of non-infected RAW 264.7 cells (ni) when
134 RAW 264.7 were infected with Newman $\Delta ptpA$ and $\Delta ptpA::ptpA_{D120A}$ cells, respectively (Fig.
135 2). In contrast, decreased SUMOylation profiles were again observed in RAW 264.7 cells infected
136 with the *cis*-complemented Newman $\Delta ptpA::ptpA$ derivative (Fig. 2). These data strongly suggest
137 that PtpA phosphatase activity plays a major role in the decrease in SUMOylation observed in
138 RAW 264.7 cells infected with *S. aureus*. As demonstrated in our previous study, *S. aureus*
139 Newman survival is decreased at 5 h post infection (11). However, despite of the high amount of
140 intracellular bacteria after 5 h of infection (around $5,75 \times 10^5$ CFU/mL, or 12 % survival
141 percentage), no reduction in the amount of SUMOylated proteins in WT-infected macrophages in
142 comparison with uninfected macrophages was observed. On the other hand, after 24 h of
143 infection, the intracellular survival of strain Newman was further reduced by 1 \log_{10} (around 2×10^4
144 CFU/mL, or 3% survival percentage) and a decrease in the amount of SUMOylated proteins in
145 these infected macrophages was observed (11). In addition, we can exclude the possibility that
146 differences in SUMOylation profile between macrophages infected with *S. aureus* strains
147 Newman or Newman $\Delta ptpA$ are due to the reduced number of intracellular bacteria. This
148 demonstration was performed by reducing the MOI of the wild-type strain (MOI 10) to half of the
149 one used for the mutant (MOI 20) as the survival default is about 50% between strains (Fig. 2c,
150 2d). These observations allow us to exclude the hypothesis that the absence of SUMOylation
151 reduction by *S. aureus* $\Delta ptpA$ is linked to a low quantity of intracellular bacteria after 24h of
152 infection.

153 ***S. aureus* PtpA reduces Ubc9 level in a transcriptional-independent manner.** After
154 demonstrating that PtpA is a critical factor for *S. aureus* intramacrophage survival and that it is

155 responsible for the reduction of SUMOylation in this immune cell type, we wondered how PtpA
156 might accomplish this regulation. One potential effector molecule might be the ubiquitin
157 conjugating enzyme 9 (Ubc9), which is the only E2 conjugating enzyme of the SUMOylation
158 machinery required for the SUMOylation to occur (9). Therefore, we started with measuring the
159 amount of the Ubc9 enzyme present in macrophages infected with our *S. aureus* Newman strain
160 set. We observed that the Ubc9 protein level in macrophages infected with *S. aureus* Newman or
161 the *ptpA*-complemented strain decreased by approximately 50% at 24 h pGt when compared to
162 uninfected cells (Fig. 3a). In contrast, no clear reductions in Ubc9 signals were observed in
163 macrophages infected with the Δ *ptpA* derivative and the PtpA phosphatase-inactive *ptpA_D120A*
164 derivative, respectively (Fig. 3a). Next, we utilized the proteasome inhibitor MG132 in order to
165 evaluate whether the proteasome had a role in Ubc9 reduction. These experiments revealed that
166 inhibition of proteasome activity by MG132 had no impact on the amount of Ubc9 (Fig. 3b), which
167 continued to drop in macrophages infected with *S. aureus* strains expressing a WT PtpA. In
168 addition, qRT-PCR was used to examine the levels of *ubc9* expression. Here, we found that *S.*
169 *aureus* infection indeed affected the expression of the Ubc9 enzyme at the mRNA level (Fig. 3c),
170 however, in a rather PtpA-independent manner. (Fig. 3c). Taken together, these findings suggest
171 that PtpA does have an effect on the quantity of Ubc9, though, without affecting the proteasome
172 and the transcriptional pathway, respectively.

173
174 **Modulating the level of SUMOylation confirms the role of PtpA to promote**
175 **intracellular survival of *S. aureus* Newman.** In our next series of experiments, we made use of
176 RAW 264.7 cells overexpressing SUMO1 or SUMO3 moieties to artificially increase the level of
177 SUMOylated proteins in order to substantiate the role of PtpA in the host SUMOylation response
178 to *S. aureus* infection. When these SUMO1 or SUMO3 overexpressing macrophages were
179 challenged with *S. aureus* Newman and the quantity of viable intracellular bacteria was counted
180 after 24 hours post infection, a clear decrease in the intracellular persistence rate of this strain
181 was observed in SUMO1 or SUMO3 overexpressing RAW 264.7 cells, when compared to control

182 macrophages expressing GFP (Fig. 4a). Notably, a rather similar effect was noticed with the *cis*-
183 complemented $\Delta ptpA::ptpA$ derivative, whereas SUMO1 or SUMO3 overexpressing RAW 264.7
184 cells challenged with the $\Delta ptpA$ strain presented considerably lower survival rates. We observed
185 4.6- and 4-fold reductions between SUMO1 or SUMO3 overexpressing macrophages in
186 comparison to GFP overexpressing macrophages infected with the $\Delta ptpA$ mutant while 3.2-fold
187 and 1.7-fold reductions between SUMO1 or SUMO3 overexpressing macrophages and GFP
188 overexpressing macrophages infected with the WT. A similar trend was also noticed for SUMO1
189 or SUMO3 overexpressing RAW 264.7 cells infected with the *ptpA_D120A* mutant. These results
190 confirm, as previously observed, that an increase in SUMOylation in host cells has a negative
191 impact on the ability of *S. aureus* to survive inside of macrophages (11), and, importantly,
192 demonstrate that PtpA expression is necessary to minimize the SUMOylation host response in
193 order to improve *S. aureus* long-term survival. In addition, the function of PtpA in the regulation
194 of *S. aureus* survival inside of macrophages that had been pretreated with an inhibitor of the
195 SUMOylation machinery targeting the SUMO-activating enzyme E1, which is a heterodimer of the
196 SAE1/SAE2 subunits enzyme, was addressed (24). Macrophages that were treated with the
197 inhibitor ML-792 exhibited a substantial increase in the amount of intracellular bacterial
198 cells regardless of the strains used to infect the treated macrophages (Fig. 4b). According to these
199 findings, treatment with the ML-792 inhibitor is able to restore *S. aureus* survival regardless of
200 PtpA..

201

202 **DISCUSSION**

203 SUMOylation is an important post-translational modification system deployed by eukaryotes
204 to modulate diverse cellular mechanisms (9). Only lately, researchers started to look whether
205 pathogenic bacteria might make use of this system for their own benefit, and our understanding
206 of these interactions is still limited (25, 26). Some pathogens interfere with the host SUMOylation
207 machinery (26). The enteropathogenic species *Salmonella* Typhimurium interferes with the

208 SUMOylation host response by inducing the overexpression of two host microRNAs that post-
209 transcriptionally reduce Ubc9 expression (4). *Shigella flexneri* is responsible for the modification
210 of SUMO-conjugated proteins involved in the regulation of mucosal inflammation and epithelial
211 infiltration, respectively (3, 27). Adherent-invasive *Escherichia coli* (AIEC) are thought to restrict
212 autophagy by altering the host's SUMOylation, thus enabling intracellular proliferation (5). By
213 reducing the induction of host inflammatory pathways, *Klebsiella pneumoniae*
214 diminishes SUMOylation to enhance its infectivity (6). Bacteria such as *Xanthomonas*
215 *euvesicatoria* (13) and *Yersinia pestis* (12) have been shown to release effectors that are able to
216 imitate host deSUMOylases, which in turn induces deSUMOylation of host proteins. The pore-
217 forming toxin listeriolysin (LLO) produced by *Listeria monocytogenes* was demonstrated to modify
218 the host SUMOylation response by degrading Ubc9 (2). More recently, we demonstrated that *S.*
219 *aureus* reduces the SUMOylation response in macrophages, thereby promoting its intracellular
220 persistence within this immune cell type (11). However, the bacterial effector(s) that modulate the
221 host SUMOylation response following *S. aureus* ingestion remained unknown yet. In this study,
222 we show that (i) the secreted protein tyrosine phosphatase PtpA is associated with the reduction
223 of the SUMOylation response in murine macrophages to promote its intracellular persistence, (ii)
224 the PtpA phosphatase function is required for the modulation of the SUMOylation response, (iii)
225 Ubc9 levels are markedly decreased in a PtpA-dependent manner, (iv) the intracellular survival
226 of *S. aureus* Δ *ptpA* cells is significantly decreased in macrophages overexpressing SUMO1 or
227 SUMO3, suggesting the involvement of PtpA in this SUMO-dependent regulation, and (v) when
228 macrophages were treated with the SUMOylation inhibitor ML-792, Δ *ptpA* mutants were able to
229 survive inside of macrophages to a similar extent as WT cells. Our findings suggest that PtpA is
230 required for a global deSUMOylation in host cells, at least at a later stage of infection (*i.e.* 24 h
231 pGt), by lowering the level of Ubc9 to promote its intracellular survival. Furthermore, in a previous
232 study, we demonstrated that *S. aureus* PtpA is significantly secreted in macrophages 18 h after
233 infection that could correlate with a late decrease of SUMOylation involving PtpA at 24h post-

234 infection (21). However, as treatment with the proteasome inhibitor MG132 had no effect on the
235 amount of Ubc9 in RAW 264.7 cells infected with *S. aureus* strain Newman, we assume that the
236 Ubc9 degradation seen in murine macrophages infected with *S. aureus* Newman cells expressing
237 a functional PtpA is independent of the proteasome. Our qRT-PCR studies suggest furthermore
238 that PtpA does not markedly affect Ubc9 expression, at least on the transcriptional level. As
239 other virulence effectors released by bacterial pathogens have been demonstrated to
240 downregulate Ubc9 (5), it was already suggested that interfering with host SUMOylation via this
241 critical enzyme of the SUMO machinery is a mechanism utilized by many different kinds of
242 pathogenic bacteria (26), and the same seems to hold truth for *S. aureus*. However, the PtpA-
243 dependent target(s) that is/are responsible for the reduction of Ubc9 seen in *S. aureus*-infected
244 macrophages still remain to be identified, and the specific mechanism of action to be defined.
245 Another limitation of this study is that we tested the impact of *S. aureus* PtpA on host cell
246 SUMOylation only with strain Newman yet, a cytotoxic and mouse pathogenic laboratory strain
247 that features a couple of uncommon characteristics such as the rare *saeS^P* allele leading to a
248 constitutive expression of the *sae* system, and truncations of the fibronectin-binding proteins
249 important for host cell invasion (doi: 10.1099/mic.0.2007/012245-0; doi: 10.1128/IAI.72.12.7155-
250 7163.2004; DOI: <https://doi.org/10.1128/jb.00476-17>). Given that the intracellular survival
251 phenotype of *S. aureus* in professional and non-professional phagocytic cells strongly depends
252 on the host cell type and the infecting bacterial strain (doi: 10.1016/j.cmi.2016.06.020;
253 doi:10.1128/IAI.00704-15; DOI: 10.3390/toxins11030135), it would be interesting to investigate,
254 if different *S. aureus* strains would generate a similar SUMO-response, as well as to test the
255 survival of *S. aureus* in human-derived macrophages such as human peripheral blood-derived
256 monocytic macrophages or THP-1 cells.

257 Our findings show that a catalytically active form of the PtpA tyrosine phosphatase is
258 required to induce the host SUMOylation reduction, thus we hypothesize that a Tyr-
259 phosphorylation-dependent control mechanism may be involved. As the *ptpA* deletion mutant

260 displayed no clear effect on *ubc9* transcription, post-translational regulation seems to be the most
261 likely mode of regulation. One possible mechanism might be that PtpA affects the
262 phosphorylation status of Ubc9, which in turn influences the stability of the protein (28). However,
263 due to the fact that PtpA is a tyrosine phosphatase and that Ubc9 is known to be phosphorylated
264 on threonine residues (29, 30), it is unlikely that PtpA directly dephosphorylate Ubc9, but might
265 influence the phosphorylation status of Ubc9 by a cascade of kinases that are phosphorylated
266 on tyrosine residues. One putative candidate for such a scenario is the Ser/Thr protein kinase Akt
267 that is activated through tyrosine phosphorylation (31), and directly phosphorylates Ubc9 at
268 Thr35, favoring the SUMO-charged form of Ubc9 (29). Another candidate would be cyclin-
269 dependent kinase 1 (Cdk1, also known as cell division cycle 2 [Cdc2]), which in cooperation with
270 cyclin B fosters Ubc9 phosphorylation at Ser71 to enhance Ubc9 stability and SUMOylation
271 activity [DOI: 10.1371/journal.pone.0034250; DOI: 10.1002/hep.25701; DOI:
272 10.1016/j.ajpath.2016.05.007]. As Cdk1 activity itself is regulated by protein tyrosine and
273 threonine phosphorylation (reviewed in [DOI: 10.1083/jcb.200812045]), one may speculate that
274 PtpA might affect Ubc9 stability via Cdk1 dephosphorylation at Tyr15.

275 Another potential candidate by which PtpA might affect SUMOylation is SUMO-specific
276 protease 1 (SEN1), which acts as an endopeptidase to generate mature SUMO for protein
277 conjugation and as an isopeptidase to remove conjugated SUMO from targets [DOI:
278 10.1074/jbc.M702444200]. SEN1 was recently identified as a substrate of protein tyrosine
279 kinase Lck, which phosphorylates SEN1 at Tyr270, thereby rendering its endo- and isopeptidase
280 activities [DOI: 10.3389/fcell.2021.789348]. Lck was originally described as lymphocyte-specific
281 kinase but was also found in RAW 264.7 cells [DOI: 10.1093/toxsci/kfj096].

282 Predictably, low levels of Ubc9 are accompanied by decreased amounts of SUMOylated
283 proteins. In this context, one would expect to observe an accumulation of unconjugated SUMO
284 moieties. However, this was not the case in our Western blot analyses, as the fast migrating band
285 (~20 kDa) reactive to the anti-SUMO antibodies was always weak to undetectable in our

286 immunoblots of *S. aureus* Newman-infected cells, and on a comparable level to the corresponding
287 signals seen on immunoblots performed with cell lysates of uninfected RAW 264.7 cells, or of
288 those infected with the PtpA-defective strains (data not shown). These observations suggest that
289 *S. aureus* PtpA might also interfere with host cell SUMOylation by affecting the availability of
290 SUMO, potentially via interference with the Lin-28/let-7 pathway (32).

291 In conclusion, the current work shows for the first time that the secreted phosphatase PtpA
292 is capable of reducing the Ubc9 conjugation enzyme level to impede host SUMOylation response
293 in *S. aureus* Newman infected murine macrophages, thus promoting the intracellular survival of
294 the ingested bacterial cells in this immune cell type. SUMOylation crosstalk during bacterial
295 infection represents a promising area of research that will not only enhance our knowledge on
296 how SUMOylation occurs in cells, but may also reveal potential targets for therapeutic treatment
297 against *S. aureus* infections and persistence at long-term infections.

298 **MATERIALS AND METHODS**

299 **Bacterial strains and growth conditions.** Strains and plasmids used in this study are listed
300 in Table 1. Sequencing was used to confirm all mutant strains and plasmids used for this study.
301 Strains of *Escherichia coli* were cultivated at 37°C in LB medium with the addition of 100 mg/ml
302 ampicillin when needed. *S. aureus* isolates were either cultured in Tryptic Soy Broth (TSB; Becton
303 Dickinson) at 37°C and 225 rpm with a culture to flask volume of 1:10, or plated on Tryptic Soy
304 Agar (TSA; Becton Dickinson) supplemented with 10 mg/ml erythromycin when required.
305 Bacterial growth in 96-well plates was observed using a microplate reader (Tecan, Lyon, France).

306
307 **Construction of the *S. aureus* ptpA cis-complementation strain Newman**
308 **ptpA::ptpA_D120A.** For cis-complementation of the *ptpA* mutation in strain Newman Δ *ptpA* with
309 a *ptpA* derivative harboring the D120A exchange, the vector pEC1_*ptpA* (22) was used as
310 template to generate the suicide plasmid pEC1_*ptpA_D120A* by using the QuikChange Site-

311 Directed Mutagenesis Kit (Agilent Technologies) with the primer #1559 (5'-
312 GGAAGAGAGTGATGTACCAGCTCCATACTACACGAATAATT-3'). Plasmid pEC1_ *ptpA_D120A*
313 was then electroporated into the strain RN4220 Δ *ptp*, a marker-free Δ *ptpA* variant of *S. aureus*
314 strain RN4220, which was previously constructed (22). The RN4220 derivative that integrated
315 pEC1_ *ptpA_D120A* was subsequently used as a donor for phage transducing the cis-integrated
316 pEC1_ *ptpA_D120A* genome region into Newman Δ *ptpA* (22), thereby replacing the *aphAIII*-
317 tagged *ptpA* deletion with the *ptpA_D120A* derivative. Replacement of the *ptpA* deletion with
318 *ptpA_D120A* in Newman Δ *ptpA::ptpA_D120A* was confirmed by sequencing.

319

320 **Macrophages culture and infection.** The murine macrophage cell line RAW 264.7 (mouse
321 leukemic monocyte macrophage, ATCC TIB-71), was cultured in Dulbecco's modified Eagle's
322 medium (DMEM) (ThermoFisher Scientific), which was augmented with 10% foetal bovine serum
323 and kept at 37°C in a humidified atmosphere containing 5% carbon dioxide. Lentiviral
324 transduced Raw264.7 cell lines that expressed 6His-tagged SUMO1 and SUMO3 proteins
325 were previously generated (11). RAW 264.7 cells (5×10^5 cells/mL, in 24 well plates) were
326 challenged with *S. aureus* at a MOI of 20:1 (bacteria:cells), and the cell mixtures were then
327 incubated for 1 h at 37°C and 5 % CO₂. Residual extracellular bacteria were eliminated by
328 incubating the RAW 264.7 cells with gentamicin (100 µg/mL) for 30 minutes after the cells had
329 been washed once with PBS. Following gentamicin treatment, macrophages were washed twice
330 with PBS (T0), and subsequently incubated for 24 h in DMEM in presence of 5 µg/mL lysostaphin
331 (AMBI Products LLC, USA). Afterwards, macrophages were lysed by 0.1% Triton X100 treatment
332 (T24), and serial dilutions of the lysates plated on TSA plates, which were incubated for 24 h at
333 37°C. The number of bacterial colonies at T24/number of bacterial colonies at T0 x 100 percent
334 was used to calculate the survival rate of bacteria.

335

336 **Immunoblotting.** Infected macrophages were lysed in 100 μ L of 2.5X Laemmli buffer,
337 boiled for 10 min at 95°C, sonicated for 10 sec at 50 % amplitude of a 20 kGz sonifier (DIGITAL,
338 Model 450-D, BRANSON) and centrifuged for 1 min at 12000 x g. Proteins were separated on
339 SDS-PAGEs, transferred to PVDF membranes, and analyzed by Western-blotting using an anti-
340 SUMO1 (#21C7, Developmental Studies Hybridoma Bank) or anti-SUMO2/3 antibody (#8A2,
341 Developmental Studies Hybridoma Bank) as primary antibody, and a HRP-coupled donkey-anti-
342 mouse antibody as secondary antibody (Jackson ImmunoResearch, Interchim, France). The
343 immunoblots were detected with the Enhanced Chemiluminescence Detection kit (ChemiDoc™,
344 BioRad) and quantified using Image Lab software (BioRad).

345

346 **Quantitative RT-PCR (qRT-PCR).** Total RNAs were extracted using the RNeasy® plus Mini
347 kit (Qiagen, GmbH, Germany) following the manufacturer's instructions. To measure the levels of
348 mRNA expression, one μ g of total RNA was reverse-transcribed using the SuperScript III®
349 Reverse Transcriptase kit from Invitrogen. Using SYBR Green qPCR Master Mix (Roche) and
350 specific primers (Table 2), quantitative RT-PCR (qRT-PCR) was carried out using a LightCycler
351 480 (Roche, France). As internal controls for mRNA quantification, the mouse β -actin gene was
352 utilized. Using the Ct technique, the fold-induction was determined as follows: $\Delta\Delta Ct = (Ct \text{ target}$
353 $\text{gene} - Ct \text{ internal control}) \text{ treatment} - (Ct \text{ target gene} - Ct \text{ internal control}) \text{ non-treatment}$, and
354 the final data were derived from $2^{-\Delta\Delta Ct}$.

355

356 **Statistical Analyses.** The statistical significance of changes between groups was
357 determined using the GraphPad software package Prism 9.4.0. P values < 0.05 were considered
358 statistically significant.

359

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366 and L.G.-Z; writing—original draft preparation, V.M., M.B., P.S. and L.G.-Z. All authors have read
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455

456 FIGURES LEGENDS

457 **Figure 1: Impact of PtpA on survival and cytotoxicity of *S. aureus* in macrophages. (a, b)**

458 Long-term survival of *S. aureus* in infected macrophages. Cells of *S. aureus* strains Newman
459 (WT; black bar), Newman $\Delta ptpA$ (white bar), Newman $\Delta ptpA::ptpA$ (grey bar) and Newman
460 $\Delta ptpA::ptpA_{D120A}$ (hatched bar) were used to infect RAW 264.7 macrophages at MOIs of 20
461 and co-incubated for 1 h at 37°C before extracellularly remaining bacterial cells were killed by
462 gentamicin/lysostaphin treatment. Bacteria were counted on plates after macrophage lysis with
463 Triton X100 (0.1%) at 24 hours pGt and represented as CFU enumeration (a) or survival rates

464 expressed in relation to the number of intracellular bacterial cells counted just after Gentamicin
465 administration and normalized to the survival rates seen with WT-infected macrophages at 24 h
466 pGt (b). Data show means and standard deviations (n=4). **, $p < 0.01$; ns, not significant (Mann-
467 Whitney *U* test). (c) LDH release was evaluated using the CyQUANT test kit after macrophage
468 cells were infected with bacteria at a MOI of 20 for 24 hours. The cells were seeded in a 96-well
469 plate for 25 min and the LDH release determined. The data on four biological replicates are shown
470 relative to the one hundred percent positive control corresponding to total cell lysis with Triton
471 X100 (1%). Non-infected RAW 264.7 cells served as negative control (ni). ns, not significant
472 (Mann-Whitney *U* test) (d) *In vitro* growth kinetics. Growth of *S. aureus* strains Newman (black
473 symbols), Newman $\Delta ptpA$ (white symbols), Newman $\Delta ptpA::ptpA$ (grey symbols) and Newman
474 $\Delta ptpA::ptpA_D120A$ (hatched symbols) were performed in TSB at 37°C and 110 rpm in 96-well
475 plates using a microplate reader (Tecan, Lyon, France). Data represent the mean A_{600} readings
476 at the time points indicated (n=3). ns, not significant (One-way ANOVA test).

477 **Figure 2:** PtpA decreases SUMO-conjugated proteins in *S. aureus*-infected macrophages.
478 Immunoblot analyses of the levels of SUMO1 (a), SUMO2/3 (b), and GAPDH in the lysates of *S.*
479 *aureus*-infected macrophages at 24 h pGt. Immunoblot analyses of the levels of SUMO1 (c),
480 SUMO2/3 (d) and GAPDH in the lysates of *S. aureus*-infected macrophages at 24 h pGt using
481 different MOI (MOI 10 for WT strain and MOI 20 for $\Delta ptpA$, $\Delta ptpA::ptpA$ and $\Delta ptpA::ptpA_D120A$).
482 Using Image Lab software (ChemiDoc), SUMO1 and SUMO2/3 smears were quantified from four
483 different experiments and normalized to the GAPDH signals (lower panels). The fold change
484 charts display the proportion of SUMOylated proteins recovered from infected cells in comparison
485 to the quantity of SUMOylated proteins in non-infected (ni) control macrophages. The data
486 represented are the mean \pm SD of four biological experiments. ** $p < 0.01$; * $p < 0.05$; ns, not
487 significant (Kruskal-Wallis test followed by Dunn's post hoc test; only differences between non-
488 infected and infected cells are shown).

489

490 **Figure 3:** *S. aureus* PtpA reduces Ubc9 level but not *ubc9* transcription. Immunoblot analysis of
491 Ubc9 levels in lysates of macrophages not treated **(a)**, or treated with MG132 for 3 hours prior to
492 infection **(b)**, and infected with *S. aureus* strains for 24 h post-gentamicin treatment. ni,
493 noninfected control cells. Ubc9 bands were quantified from four independent experiments and
494 normalized to β actin or GAPDH levels. The graph represents fold changes compared to non-
495 infected cells (bottom panel). * $p < 0.05$; ns, not significant (Kruskal-Wallis test followed by Dunn's
496 post hoc test). **(c)** The influence of PtpA on the transcription of *ubc9*. qRT-PCR was used to
497 perform quantitative assessments of the *ubc9* transcript in *S. aureus* cells that had been cultured
498 for 24 hours after being treated with gentamicin. Quantification of transcription rates was done in
499 relation to the transcription of β act (in copies per copy of β actin), which was used as the standard,
500 and normalized to the transcript rates seen with the uninfected controls. The data are provided
501 as mean + SD of four separate biological experiments. * $p < 0.05$; ns, not significant (Kruskal-
502 Wallis test followed by Dunn's post hoc test).

503

504 **Figure 4: Impact of SUMOylation over-expression or inhibition on intracellular survival of**
505 ***S. aureus ptpA* strain derivatives.** **(a)** Intracellular survival of *S. aureus* strains in macrophages
506 overexpressing SUMO1 or SUMO2/3 *versus* control macrophages (GFP vector). Intracellular
507 bacteria were counted after cell lysis and relative survival is presented as the ratio of intracellular
508 bacteria at 24 h post-gentamicin compared to cells transfected with an empty GFP-vector,
509 considered as 100%. **(b)** Macrophages pretreated with ML-792 at 0.5 μ M or DMSO were infected
510 with *S. aureus* strains. Numbers of intracellular bacteria recovered from macrophages at 24 h pGt
511 were counted and are presented as the ratio of intracellular bacteria compared to cells pretreated
512 with DMSO and infected with strain Newman (WT), which were considered as 100%. ** $p < 0.01$;
513 * $p < 0.05$; ns, not significant (Mann-Whitney *U* test).

514

515

517 **Table 1.** Strains and plasmids used in this study

518

Strain	Description ¹	Reference or source
<i>S. aureus</i>		
Newman	Laboratory strain, wildtype	
Newman $\Delta ptpA$	Newman $\Delta ptpA::lox66-aphAIII-lox71$; Kan ^R	(22)
Newman $\Delta ptpA::ptpA$	Newman $\Delta ptpA$ derivative cis-complemented with pEC1_ <i>ptpA</i> -Flag; Erm ^R	(22)
Newman $\Delta ptpA::ptpA_D120A$	Newman $\Delta ptpA$ derivative cis-complemented with pEC1_ <i>ptpA_D120A</i> -Flag; Erm ^R	This study
RN4220 $\Delta ptpA$	RN4220 $\Delta ptpA::lox72$	(22)
RN4220 $\Delta ptpA_lox_aph$	RN4220 $\Delta ptpA::lox66-aphAIII-lox71$	(22)
<i>E. coli</i>		
IM08B	<i>E. coli</i> DC10B derivative harboring <i>hsdS</i> of <i>S. aureus</i> strain NRS384, Δdcm	(33)
TOP10	<i>E. coli</i> derivative ultra-competent cells used for general cloning	Invitrogen
Plasmids		
pEC1	pUC19 derivative containing the 1.45-kb <i>Clal</i> <i>erm</i> (B) fragment of Tn551	(34)
pEC1_ <i>ptpA_D120A</i> -Flag	pEC1 with a 1.4-kb fragment covering the <i>ptpA</i> ORF including a C-terminal flag tag and the aspartate 120 mutated to alanine, and 0.7-kb of the upstream region.	This study

¹Erm^R, erythromycin-resistant; Kan^R, kanamycin-resistant

519

520

521 **Table 2.** qRT-PCR primer used in this stud

522

Gene target	Primer	Sequence (5'-3')
<i>βactin</i>	forward	AGCCATGTACGTAGCCATCC
	reverse	CTCTCAGCTGTGGTGGTGAA
<i>ubc9</i>	forward	CCTCAGCCGCCTTGCGCAGGA
	reverse	ACTGTGCCAGAAGGATACACG

FIGURE 1

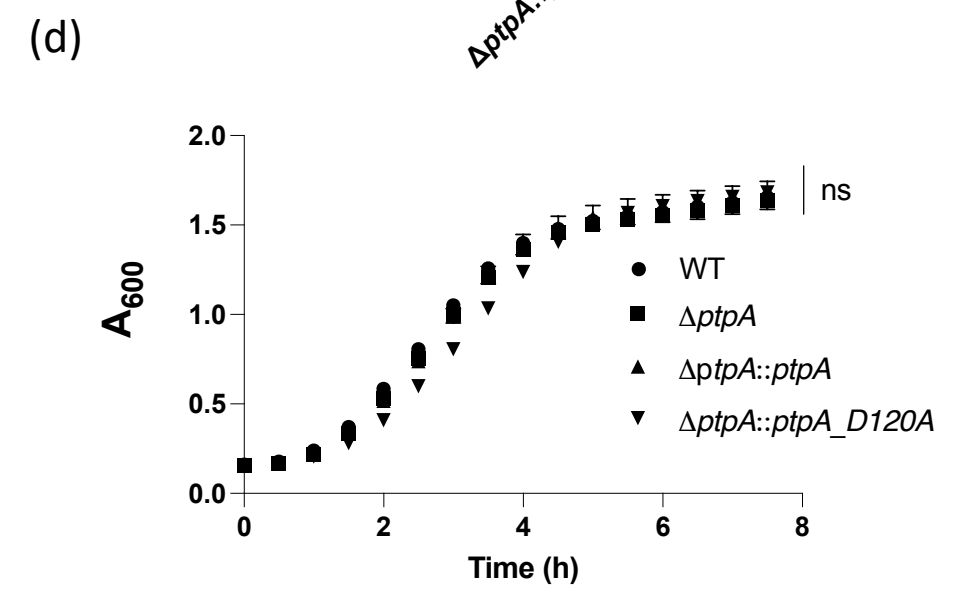
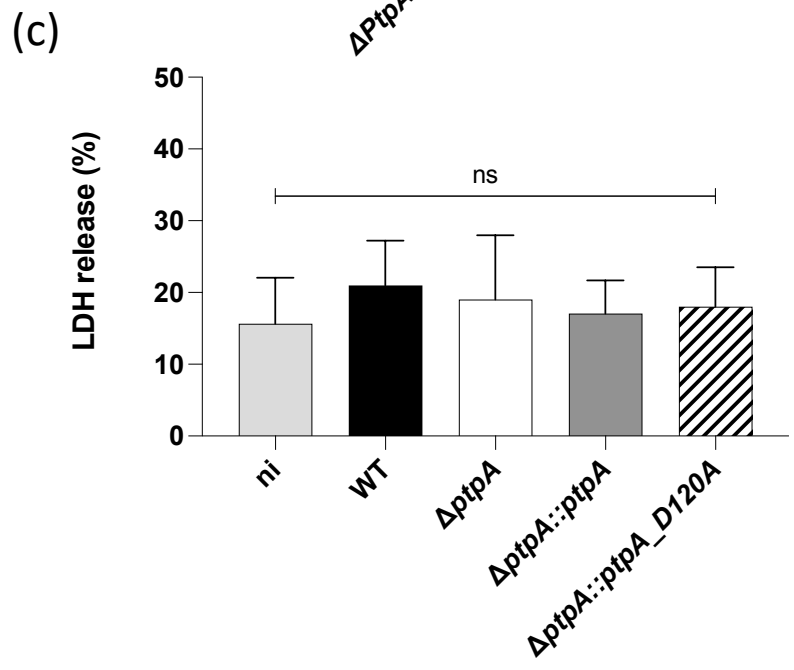
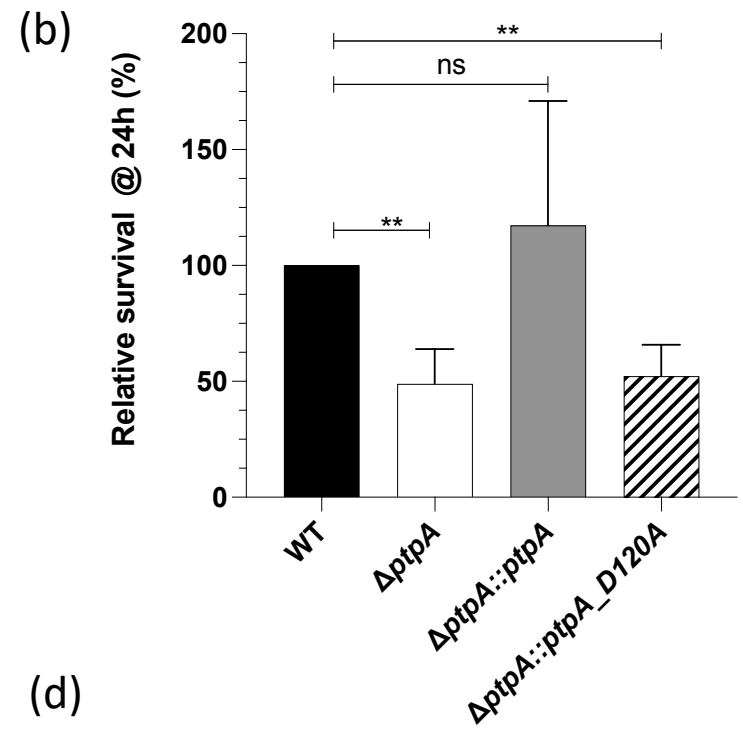
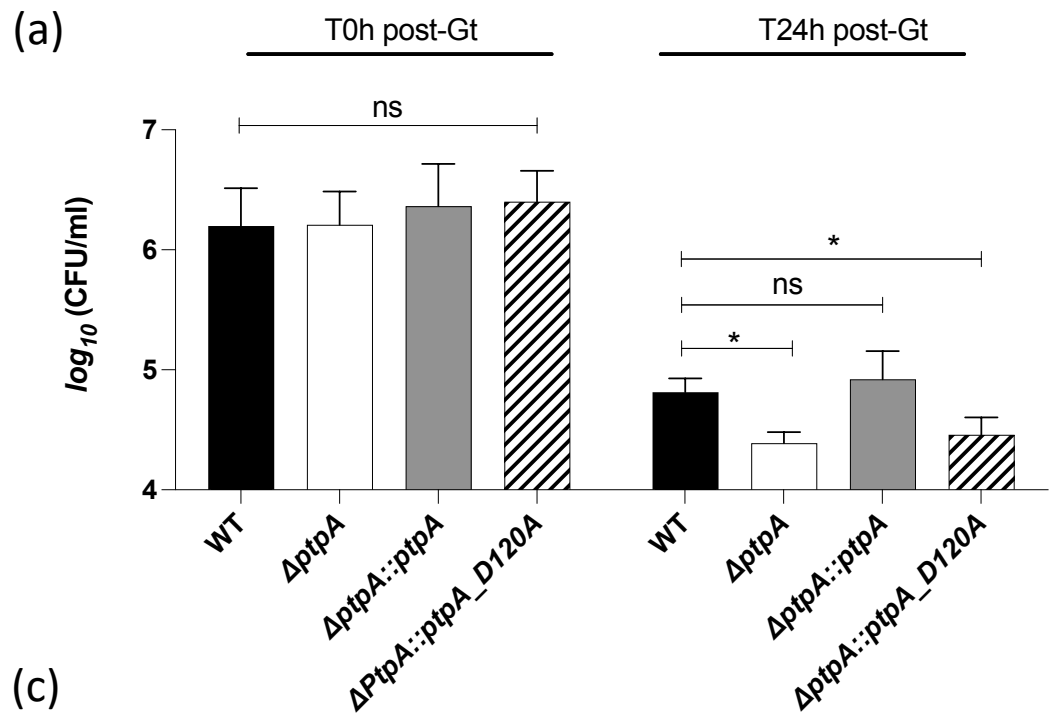


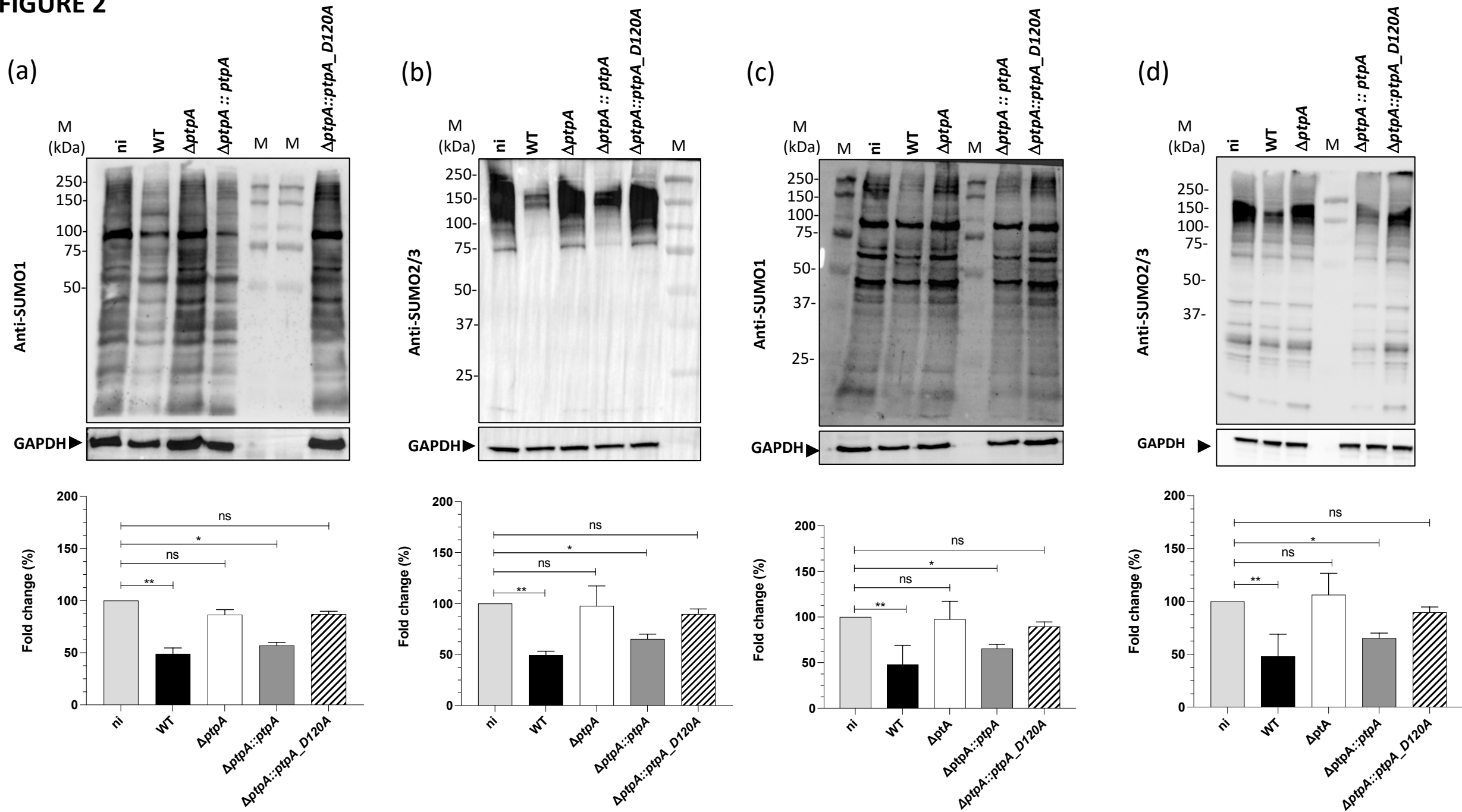
FIGURE 2

FIGURE 3

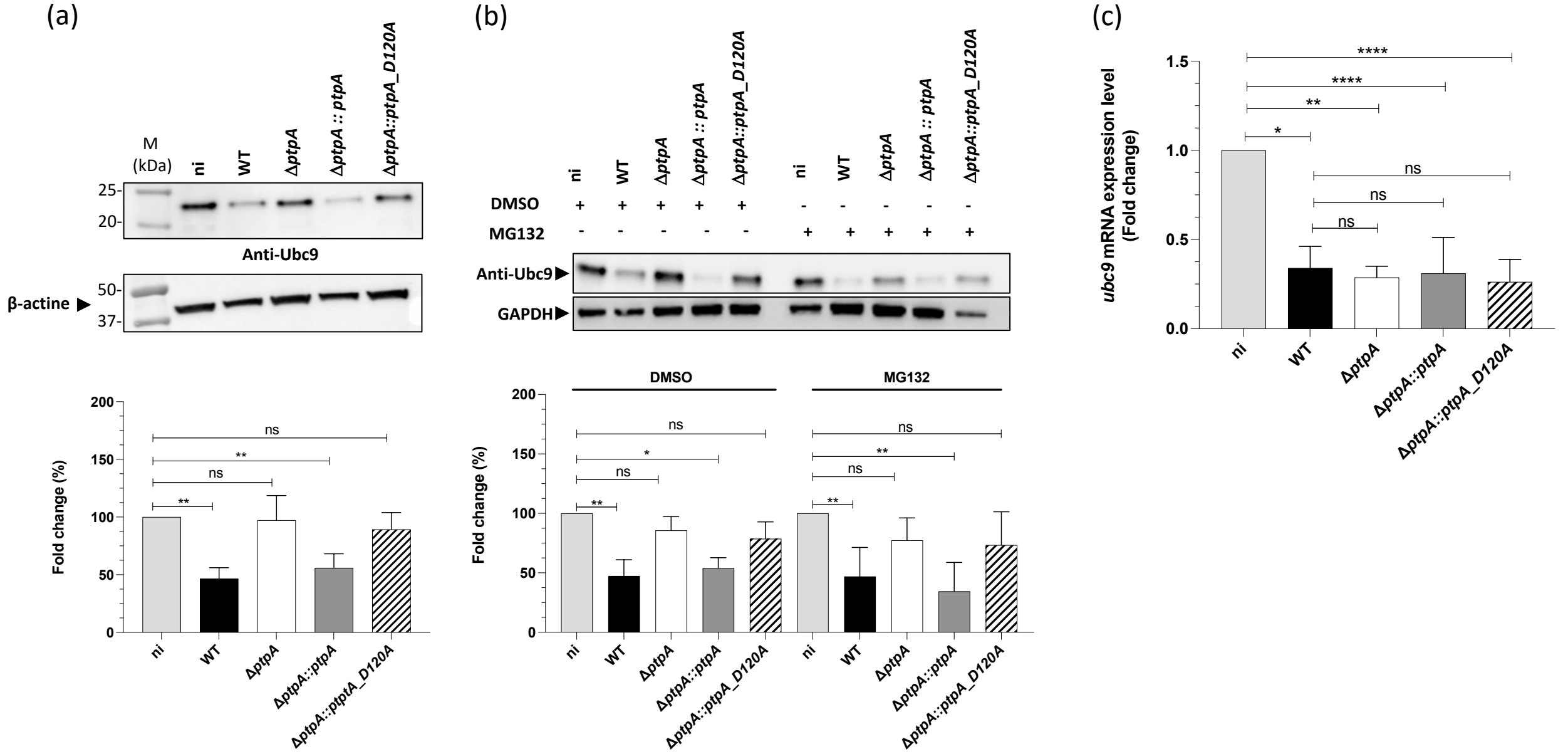
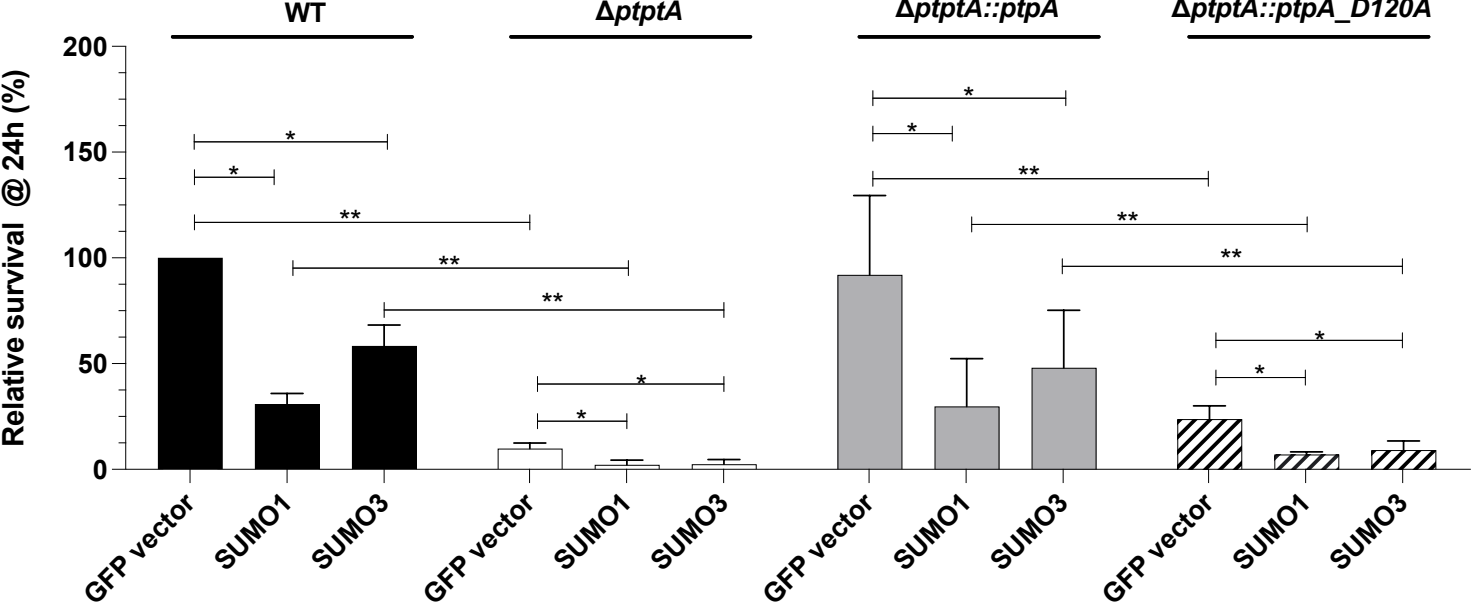


FIGURE 4

(a)



(b)

