

Sodium Polyanethol Sulfonate Modulates Natural Transformation of SigH-Expressing Staphylococcus aureus

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journal or publication title	Current microbiology
volume	75
number	4
page range	499-504
year	2018-04
権利	(C) Springer Science+Business Media, LLC, part of Springer Nature 2017 This is a post-peer-review, pre-copyedit version of an article published in Current Microbiology. The final authenticated version is available online at: http://dx.doi.org/10.1007/s00284-017-1409-5
URL	http://hdl.handle.net/2241/00151563

doi: 10.1007/s00284-017-1409-5

1 Original paper

2 **Sodium Polyanethol Sulfonate modulates natural transformation of**
3 **SigH-expressing *Staphylococcus aureus***

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5

6 **Abstract**

7 Expression of genes required for natural genetic competence in *Staphylococcus aureus* is
8 controlled by an alternative transcription sigma factor, SigH. However, even in the SigH-
9 expressing cells, the DNA transformation efficiency varies depending on culture conditions.
10 We report here that cells grown in the competence-inducing medium (CS2 medium) exhibit
11 enlarged morphology with disintegrated cell walls. Notably, an autolysis inhibitor, Sodium
12 Polyanethol Sulfonate (SPS), facilitated transformation in CS2 medium in a dose dependent
13 manner, suggesting the involvement of the cell wall metabolism in transformation. However,
14 the transformation efficiency of cells grown in TSB was not improved by physical or
15 enzymatic damage on the cell walls.

16

17

18 *Keywords:* Sodium polyanethol sulfonate, *Staphylococcus aureus*; natural transformation;
19 cell wall.

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21

22 **1. Introduction**

23 *Staphylococcus aureus* is a Gram-positive bacterium that naturally inhabits our nasal
24 cavity. This bacterium is also an important human pathogen that can cause a broad
25 spectrum of infections. Treatment of *S. aureus* infections has been difficult because of
26 antibiotic resistance. Importantly, *S. aureus* has a prominent ability to acquire resistance
27 through horizontal gene transfer (HGT) [5].

28 Subpopulations of *Staphylococcus aureus* can develop the competence for natural
29 DNA transformation under the control of the alternative sigma factor, SigH [12, 13]. In
30 addition to SigH expression (and SigH dependent expression of the *comE* and *comG* operon
31 genes encoding the DNA incorporation machinery), environmental factors are thought to be
32 required for natural transformation, because the transformation frequency of SigH
33 expressing cells is variable depending on the culture conditions. Cell wall-affecting
34 antibiotics were also found to affect the transformation in SigH-overexpressing cells [17].

35 In this study, we found that the autolytic enzyme inhibitor, Sodium Polyanethol
36 Sulfonate (SPS), can affect the transformation efficiency of SigH-expressing *S. aureus*,
37 supporting the idea that cell wall metabolism is an important factor in the modulation of
38 transformation.

39

40 **2. Materials and methods**

41 *2.1. Bacterial strains*

42 The *S. aureus* strains used in this study are listed in Table S1 (Supplementary
43 material). The strain N315ex w/o ϕ h was used in most experiments. In this strain, the
44 prophage was eliminated to exclude the possibility of “pseudo-competence” DNA transfer

45 with the help of phage components, which is distinct from real competence [13]. SigH is
46 expressed by a plasmid, pRIT-sigH [12].

47 2.2. Natural transformation assay

48 Transformation assay was carried out as previously described with some modifications [13].
49 Tryptic soy broth (TSB; Becton Dickinson Company), brain heart infusion broth (BHI; Becton
50 Dickinson Company), nutrient broth (Sigma) supplemented with 70 μM CaCl_2 (NBCaCl_2), and
51 the complete synthetic medium, CS2 (composition is available in [13]) were tested for the
52 efficiency of transformation in N315ex w/o ϕ h cells. Transformation protocol was same for
53 all the media tested. Glycerol stocks of *S. aureus* were inoculated in 5 ml of tryptic soy broth
54 with 12.5 $\mu\text{g}/\text{ml}$ chloramphenicol (TSBcm) and grown overnight at 37 °C with shaking at 180
55 rpm. Cells were collected by centrifugation and suspended into the appropriate medium to
56 be used (1:20 dilution in 10 ml medium), and grown for 8 hours. Sodium polyanethol
57 sulfonate (SPS) (Sigma) was added prior to the 8-hour growth. Medium was replaced with
58 fresh medium, and 10 μg of purified plasmid, pT181 from *S. aureus* COL, or pHY300 from *E.*
59 *coli* HST04 *dam*⁻/*dcm*⁻, was added. Following 2.5-hour incubation at 37 °C with shaking,
60 transformants were selected in BHI-agar medium supplemented with 5 $\mu\text{g}/\text{ml}$ tetracycline.

61 Transformants were tested for the presence of plasmid and for *tet*^R by colony PCR. In line
62 with our previous experiences, no spontaneous *tet*^R mutants were detected throughout the
63 study. Transformation frequency was calculated as the ratio of total number of
64 transformants to total colony forming unit (cfu) after the 2.5-hour incubation with DNA. Cfus
65 were counted on TSB-agar (TSA) plates.

66 2.3. Electron microscopy

67 Overnight cultures of bacteria (N315 h, N315 v, COL h, COL) in TSBcm or TSB were
68 inoculated into drug-free TSB or CS2 to a final optical density (OD₆₀₀) of 0.2. After 8-hour
69 culture, cells were harvested by centrifugation. After a complete wash in ice-cold PBS, cells
70 were fixed with 2 % glutaraldehyde in PBS followed by post fixation with 1 % OsO₄,
71 dehydrated, embedded and thin sectioned by conventional method described elsewhere [1].
72 The specimens were observed using a JEOL JEM-1400 electron microscope. The cell surface
73 roughness was evaluated from electron microscopy images by measuring the ratio of the
74 length of the cell surface to the corresponding linear distance (i.e., the ratio 1.0 means
75 completely smooth, and larger value means rough morphology).

76 2.4. Whole cell autolysis assay

77 Whole cell autolysis assay was performed as described by Mani *et al* [11]. Cells from the
78 overnight TSBcm culture of N315ex w/oϕ h were inoculated with 1:20 dilution in 10 ml TSB
79 or CS2 with or without 0.1 % SPS. For CS2, cells were washed once with fresh CS2 prior to
80 inoculation. After 8 hours (in CS2) or 5 hours (in TSB), cells were harvested by centrifugation.
81 Cells were washed twice with 10 ml of ice-cold water and resuspended in 10 ml of 0.05 M
82 Tris-HCl buffer (pH 7.2) containing 0.05 % (v/v) Triton X-100. Two hundred microliters of the
83 cell suspension was distributed into a 96-well plate, then incubated at 30 °C with shaking at
84 180 rpm. The changes in OD₆₀₀ were measured at 30 min intervals by using the EnSpire®
85 Multimode plate reader (PerkinElmer®).

86 2.5. Zymographic analysis

87 The supernatant from 8-hour culture of N315ex w/oϕ h in CS2 or 5-hour culture in TSB
88 was recovered by centrifugation at 6000 x g for 15 min at 4 °C, filtered through 0.22 µm
89 cellulose acetate membrane filter (Advantec®, Toyo Roshi Ltd.), and concentrated 10-fold

90 using Ultracel® 10K Centrifugal filter unit (Merck Millipore). Sodium dodecyl sulfate (SDS)
91 extracts were prepared as previously described [16]. Protein concentrations were
92 determined by the Bradford assay (Bio-Rad Laboratories). Twenty micrograms of total
93 proteins were analyzed by zymogram as described by Qoronfleh and Wilkinson using
94 *Micrococcus luteus* (Sigma) as a substrate [14].

95 2.6. Mechanical and enzymatic disruption of cell walls

96 Fastprep® (MP Biomedicals) was used to physically disrupt bacterial cell walls. A TSBcm
97 overnight culture of N315ex w/oϕ h was diluted 20-fold with fresh TSBcm (total 10 ml), and
98 cultured for 8 hours. Cells were harvested, suspended in 0.4 ml TSB, and transferred to 2 ml
99 tube containing 0.1 mm silica beads (MP Biomedicals). The tube was shaken in Fastprep® at
100 4 m/s for 10 sec, 20 sec and 30 sec, once or twice, at 4 °C. After beating, cells were collected
101 by centrifugation (10000 rpm, 10 min) and cell-bead mixture was resuspended in 1 ml TSB.
102 The cell suspension was transferred to a new tube, and 10 µg of purified plasmid pHY300
103 was added to test the transformation frequency.

104 For lysostaphin treatment, cells grown in 5 ml TSBcm were harvested at the mid-log
105 phase. Cells were then suspended in 10 ml TSB medium containing lysostaphin at different
106 concentrations and incubated for 5 hours at 37 °C with shaking. After treatment, cells were
107 washed and mixed with purified pHY300 for transformation.

108

109 3. Results

110 3.1. CS2 medium is important for the transformation of SigH-expressing cells

111 In the transformation protocol reported previously, SigH-expressing cells (N315ex

112 w/o ϕ h) were growth in CS2 medium [13]. The medium dependency was confirmed here
113 and summarized in Table 1. Transformation was undetectable (less than 10^{-11}) in TSB, BHI,
114 and NBCaCl₂ (Nutrient broth supplemented with 70 μ M CaCl₂) but it reached 10^{-9} order in
115 CS2 medium, when pT181 purified from *S. aureus* COL was used as the donor DNA. We also
116 tested a shuttle vector, pHY300, purified from *E. coli*, and observed the similar dependency
117 on the CS2 medium (Table 1).

118 *3.2. S. aureus cells in CS2 medium exhibit disintegrated cell walls*

119 N315 derivative strains including N315ex w/o ϕ h tend to generate cell debris in CS2
120 medium, but not in other media, irrespective of the presence of prophage and the SigH
121 expressing plasmid (data not shown). This was not the case in COL and COL h strains, of
122 which transformation was undetectable in the same CS2 protocol. We observed the strains
123 N315h (Fig. 1A, 1B), N315v (Fig. 1B), N315 (Fig. S1A, Supplementary material), and COL (Fig.
124 S1B, Supplementary material) by transmission electron microscopy. N315 overexpressing
125 SigH (N315 h) grown in CS2 medium (Fig. 1B), but not in TSB (Fig. 1A), exhibited irregular
126 morphology and enlarged cell size with partly disturbed cell wall. Such morphological
127 features were unique in N315 background (Fig. S1A), not observed in COL (Fig. S1B), and are
128 not attributed to the artificial expression of SigH, since cells carrying the vector control
129 (N315 v) showed similar changes when grown in CS2 medium (Fig. 1C).

130 *3.3. Inhibitor of murein hydrolases increases transformation in CS2 medium*

131 The autolytic rate of the cells grown in CS2 medium was higher than those grown in TSB
132 medium (Fig. 2A), and zymogram analyses indicated that autolytic enzymes in CS2 medium
133 are at a comparable level with TSB medium (Fig. 2B). We tested the effect of a murein

134 hydrolase inhibitor, sodium polyanethol sulfonate (SPS) [19]. The inhibitory effect of SPS on
135 the autolysis of the CS2-grown cells was confirmed as shown in Fig. 2A. Zymogram indicated
136 that the autolysin Atl (51 kDa and 62 kDa) and LytM (35 kDa) were strongly reduced by SPS
137 in the SDS extract, and it was undetectable in culture supernatant (Fig. 2B). Strikingly, SPS
138 increased transformation frequency in a dose-dependent manner in CS2 medium (Fig. 2C),
139 but not in TSB medium (Fig. 2D). The addition of 0.1 % SPS in CS2 medium resulted in 10-fold
140 increase in the transformation frequency ($p < 0.01$) (Fig. 2C).

141 To exclude the possibility that SPS somehow induced plasmid incorporation without
142 competence machinery, SigH-active cells lacking the *comE* or *comG* operon (N315ex w/o ϕ
143 Δ comE h, N315ex w/o ϕ Δ comG h) were tested. We detected no transformant of these
144 strains (data not shown). Thus, SPS can affect natural transformation in CS2 medium.

145 *3.4. Physical damage on cell wall does not facilitate transformation of SigH-expressing S.* 146 *aureus in TSB*

147 We addressed whether the increase in transformation of SigH-expressing cells would be
148 simply attributed to the physically disturbed cell wall. First, we tested the effect of physical
149 disruption by silica beads on the transformation of cells grown in TSB. Cfu was measured at
150 different time points of bead beating. Beating twice for 10, 20 and 30 seconds (10x2 sec,
151 20x2 sec and 30x2 sec) resulted in 30%, 45% and 60% reduction in cfu. None of the
152 treatments improved the transformation efficiency (Fig. S2A, Supplementary material). We
153 also tested the effect of lysostaphin, an enzyme that cleaves *S. aureus* cell wall [10]. We did
154 not observe any increase in the transformation frequency by lysostaphin treatment up to the
155 concentration that reduced the cfu by half (0.1 μ g/ml). Higher concentrations of lysostaphin

156 resulted in undetectable transformants due to cell death (Fig. S2B, Supplementary material).

157 Thus, external physical damage on the cell wall does not simply facilitate the transformation.

158

159 **4. Discussion**

160 Regulatory mechanisms and signals for the development of natural competence are
161 diverse among species [9]. In 2012, *S. aureus* natural transformation was firstly reported
162 where the cryptic alternative sigma factor, SigH, plays a key role [13]. The transcription
163 factor ComK was also found to enhance the expression of the SigH regulon [7]. However, the
164 regulation of competence development and the following transformation are still largely
165 unknown. The present study aimed to gain insight into the regulation of staphylococcal
166 competence, of which frequency is still low in laboratory settings. Although we had to use
167 the SigH expressing strain to evaluate the transformation frequency, we clarified the distinct
168 transformation efficiencies of SigH-expressing cells in different culture media, and firstly
169 described the effect of SPS on the transformation.

170 Transformation in SigH-expressing cells is facilitated in CS2 medium compared with other
171 complex rich media (BHI, TSB, LB). The growth rate decreases and the cell size increases in
172 CS2 medium (Fig. S3 and Fig. S4, Supplementary material). SigH in normal cell (N315ex) is
173 expressed in up to 10 % subpopulation in CS2 [13], but the increase of the cell size is
174 observed for almost all N315ex cells. In addition, the cell size increase was at comparable
175 level between N315ex, N315ex h and N315ex Δ sigH (Fig. S4, Supplementary material).
176 Therefore, such a morphological change is not under the control of SigH. The addition of
177 0.1 % SPS did not change the cell size in CS2 and TSB (Fig. S4, Supplementary material),
178 suggesting that impaired autolytic activity is not the sole reason for the cell size difference.

179 The slow growth during competence, and delayed cell division is also observed in *Bacillus*
180 *subtilis*, where ComGA and Maf interfere with rRNA synthesis and cell division, respectively
181 [8], [4]. It is thought that the arrest of growth could permit cells to express competence
182 machinery genes and internalize exogenous DNA. It could also permit the repairing of the
183 bacterial genome after the recombination during transformation [4, 8]. Whether the
184 enlarged cell size is a prerequisite for the staphylococcal transformation is elusive, but in
185 general, it is conceivable that optimal growth conditions where cells divide at high rate are
186 not suitable for competence development.

187 SPS affects cell wall turnover due to the inhibition of the cross wall separation [19].
188 Although the direct mechanism by which SPS inhibits the release of autolytic enzymes is not
189 well understood yet, it was suggested that SPS binds to wall teichoic acids and changes the
190 molecular conformation of the cell wall matrix resulting in the inhibition of not only the
191 septum but also the peripheral wall autolytic enzymes. This binding might cause a
192 considerable shift in the net charge of the cell wall since SPS is negatively charged [19].
193 Therefore, in the present study, we will not conclude that the effect of SPS on the
194 transformation is through the inhibition of autolytic enzymes: it might be due to such a
195 drastic change of the physical characteristics of the cell walls or other unknown reasons.
196 Nonetheless, taken together with our previous report that some cell-wall affecting
197 antibiotics modulate transformation of SigH-expressing cells [17], it is likely that cell wall
198 metabolism plays an important role in natural genetic competence in *S. aureus*. The
199 importance of cell wall metabolism in transformation is also recognized in other Gram-
200 positive species including *B. subtilis*, *S. pneumoniae* and other streptococci [2, 15].

201 The physical disruption of the cells did not increase the transformation efficiency in TSB

202 medium. This suggests that the increase in transformation frequencies of SigH-expressing
203 cells cannot simply be attributed to the damages in the cell wall alone. In this context, it is
204 valuable to note that the ComC disulfide bond is necessary for the maturation of ComG
205 pseudopilin [18]. A study in *S. pneumoniae* reported that competence accessories (EndA
206 nuclease and the DNA receptor ComEA) are recruited near the septum and the DNA uptake
207 could occur at this position [3]. Thus, the present data is consistent with the idea that the
208 DNA uptake would be finely controlled through such multiple processes.

209 In conclusion, the present study added new information that SPS can facilitate natural
210 transformation in SigH-expressing *S. aureus*. Together with our previous report that cell-wall
211 affecting antibiotics can affect transformation [17], this study supports the idea that cell wall
212 metabolism plays an important role in the DNA incorporation process by the competence
213 machinery expressed by SigH.

214

215 **Acknowledgements**

216 We thank Ms. Junko Sakamoto for electron microscopy. This work was supported by
217 Takeda Science Foundation, Pfizer Academic Contributions, and The Waksman Foundation of
218 Japan.

219

220 **Conflict of interest**

221 The authors declare no conflict of interest.

222

223

224 **References**

225

226 [1] Anderson RC, Haverkamp RG, Yu PL. Investigation of morphological changes to
227 *Staphylococcus aureus* induced by ovine-derived antimicrobial peptides using TEM and AFM.
228 FEMS Microbiol Lett 2004;240:105-10.

229 [2] Bayles KW. The biological role of death and lysis in biofilm development. Nature reviews.
230 Microbiology 2007;5:721-6.

231 [3] Berge MJ, Kamgoue A, Martin B, Polard P, Campo N, Claverys JP. Midcell recruitment of
232 the DNA uptake and virulence nuclease, EndA, for pneumococcal transformation. PLoS
233 Pathog. 2013;9:e1003596.

234 [4] Briley K, Jr., Prepiak P, Dias MJ, Hahn J, Dubnau D. Maf acts downstream of ComGA to
235 arrest cell division in competent cells of *B. subtilis*. Molecular microbiology 2011;81:23-39.

236 [5] Cafini F, Romero VM, Morikawa K. Chapter 4: Mechanisms of horizontal gene transfer, in
237 The rise of virulence and antibiotic resistance in *Staphylococcus aureus*. . InTech, 2017.

238 [6] Dubrac S, Boneca IG, Poupel O, Msadek T. New insights into the Walk/WalR (YycG/YycF)
239 essential signal transduction pathway reveal a major role in controlling cell wall metabolism
240 and biofilm formation in *Staphylococcus aureus*. Journal of Bacteriology 2007;189:8257-69.

241 [7] Fagerlund A, Granum PE, Havarstein LS. *Staphylococcus aureus* competence genes:
242 mapping of the SigH, ComK1 and ComK2 regulons by transcriptome sequencing. Molecular
243 microbiology 2014;94:557-79.

244 [8] Hahn J, Tanner AW, Carabetta VJ, Cristea IM, Dubnau D. ComGA-RelA interaction and
245 persistence in the *Bacillus subtilis* K-state. Molecular microbiology 2015;97:454-71.

246 [9] Johnston C, Martin B, Fichant G, Polard P, Claverys JP. Bacterial transformation:
247 distribution, shared mechanisms and divergent control. Nat. Rev. Microbiol. 2014;12:181-96.

248 [10] Kumar JK. Lysostaphin: an antistaphylococcal agent. Applied microbiology and
249 biotechnology 2008;80:555-61.

250 [11] Mani N, Tobin P, Jayaswal RK. Isolation and characterization of autolysis-defective
251 mutants of *Staphylococcus aureus* created by Tn917-lacZ mutagenesis. J. Bacteriol.
252 1993;175:1493-9.

253 [12] Morikawa K, Inose Y, Okamura H, Maruyama A, Hayashi H, Takeyasu K, et al. A new
254 staphylococcal sigma factor in the conserved gene cassette: functional significance and

255 implication for the evolutionary processes. *Genes to cells : devoted to molecular & cellular*
256 *mechanisms* 2003;8:699-712.

257 [13] Morikawa K, Takemura AJ, Inose Y, Tsai M, Nguyen Thi le T, Ohta T, et al. Expression of
258 a cryptic secondary sigma factor gene unveils natural competence for DNA transformation
259 in *Staphylococcus aureus*. *PLoS Pathog.* 2012;8:e1003003.

260 [14] Qoronfleh MW, Wilkinson BJ. Effects of growth of methicillin-resistant and -susceptible
261 *Staphylococcus aureus* in the presence of beta-lactams on peptidoglycan structure and
262 susceptibility to lytic enzymes. *Antimicrobial agents and chemotherapy* 1986;29:250-7.

263 [15] Ranhand JM. Autolytic activity and its association with the development of competence
264 in group H streptococci. *Journal of bacteriology* 1973;115:607-14.

265 [16] Sugai M, Akiyama T, Komatsuzawa H, Miyake Y, Suginaka H. Characterization of sodium
266 dodecyl sulfate-stable *Staphylococcus aureus* bacteriolytic enzymes by polyacrylamide gel
267 electrophoresis. *J. Bacteriol.* 1990;172:6494-8.

268 [17] Thi le TN, Romero VM, Morikawa K. Cell wall-affecting antibiotics modulate natural
269 transformation in SigH-expressing *Staphylococcus aureus*. *The Journal of antibiotics*
270 2016;69:464-6.

271 [18] van der Kooi-Pol MM, Reilman E, Sibbald MJ, Veenstra-Kyuchukova YK, Kouwen TR,
272 Buist G, et al. Requirement of signal peptidase ComC and thiol-disulfide oxidoreductase
273 DsbA for optimal cell surface display of pseudopilin ComGC in *Staphylococcus aureus*. *Appl*
274 *Environ Microbiol* 2012;78:7124-7.

275 [19] Wecke J, Lahav M, Ginsburg I, Kwa E, Giesbrecht P. Inhibition of wall autolysis of
276 staphylococci by sodium polyanethole sulfonate "liquoid". *Archives of Microbiology*
277 1986;144:110-5.

278 **Table 1** Transformation frequencies of N315ex w/oϕ h in different media

Donor DNA	Medium			
	TSB	BHI	NBCaCl ₂	CS2
pT181	ND (n = 2)	ND (n = 2)	ND (n = 2)	4.0 ± 3.0 × 10 ⁻⁹ (n = 11)
pHY300	5.0 ± 4.7 × 10 ⁻¹¹ (n = 32) ND (n = 2)	5.0 ± 6.0 × 10 ⁻¹¹ (n = 2)	3.0 ± 1.4 × 10 ⁻¹¹ (n = 2)	1.6 ± 1.4 × 10 ⁻⁹ (n = 8)

279

280 Mean frequency ± SD. ND: none detected (c.a. <10⁻¹¹)

281

282

283

284 **Figure Captions**

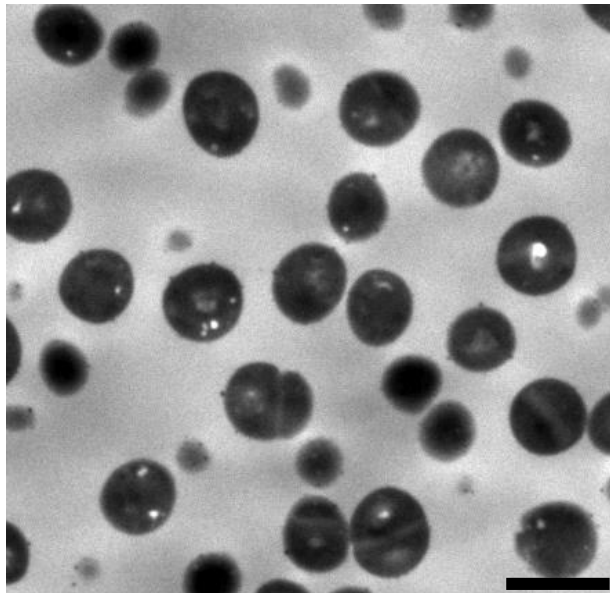
285 **Fig. 1** Transmission electron microscopy of cells grown in CS2 medium and TSB medium.
286 N315 overexpressing SigH (N315 h) grown in TSB (A) and CS2 (B); (C) N315 carrying the
287 control vector (N315 v) grown in CS2. Scale bar = 1 μ m. The cell surface roughness (mean
288 ratio \pm SD, see Material and method) in N315 h in TSB was 1.1 ± 0.03 , while the ratios were
289 1.25 ± 0.07 for N315 h in CS2 ($p < 0.01$), and 1.21 ± 0.04 for N315 v in CS2.

290

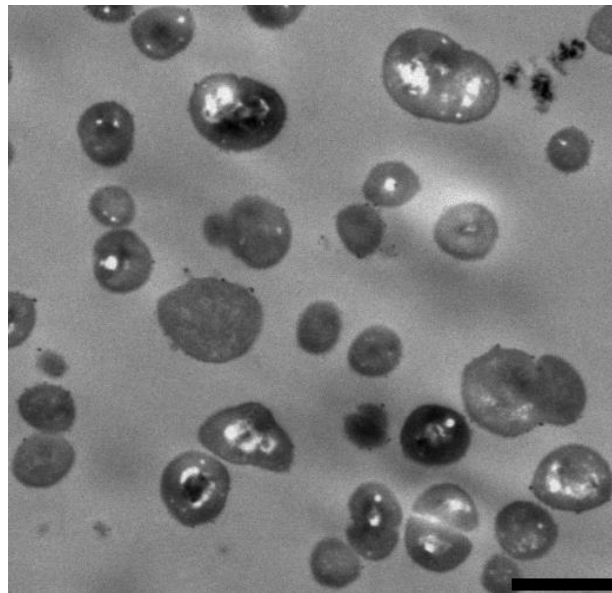
291 **Fig. 2** Effects of autolytic activity on *S. aureus* transformation. (A) Whole cell autolysis of cells
292 grown in CS2 and TSB with (+) or without (-) 0.1 % SPS. Data represent the averages of two
293 independent experiments. (B) Zymogram analysis of murein hydrolases in the culture
294 supernatant and SDS extract. Cells were grown in CS2 or TSB with or without 0.1% SPS. 138
295 kDa: uncleaved autolysin Atl; 115 kDa: intermediately processed Atl; 51 kDa: completely
296 processed endo- β -N-acetylglucosamidase; 62 kDa: completely processed N-acetylmuramoyl-
297 L-alanine amidase; 35 kDa: LytM [6]. (C) Effects of SPS on transformation in CS2 medium.
298 Mean and SD values are shown (** $p < 0.01$; * $p < 0.05$; $n = 4$). (D) SPS has no positive effect
299 on transformation in TSB medium ($n = 3$). ND: none detected. Bars: Log_{10} (Transformation
300 frequency); dotted lines: Log_{10} (cfu)

301

A



B



C

