Original paper

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

Abstract

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

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

1. Introduction

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

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

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

2. Materials and methods

2.1. Bacterial strains

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

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

2.2. Natural transformation assay

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

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

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 (OD_{600}) 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 % OsO_4,
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 ^{o}C with shaking at
84 180 rpm. The changes in OD_{600} 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 ^{o}C, filtered through 0.22 µm
89 cellulose acetate membrane filter (Advantec®, Toyo Roshi Ltd.), and concentrated 10-fold

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

2.6. Mechanical and enzymatic disruption of cell walls

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

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

3. Results

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

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

w/oϕ h) were growth in CS2 medium [13]. The medium dependency was confirmed here and summarized in Table 1. Transformation was undetectable (less than 10^{-11}) in TSB, BHI, and $NBCaCl_2$ (Nutrient broth supplemented with 70 μM $CaCl_2$) but it reached 10^{-9} order in CS2 medium, when pT181 purified from *S. aureus* COL was used as the donor DNA. We also tested a shuttle vector, pHY300, purified from *E. coli*, and observed the similar dependency on the CS2 medium (Table 1).

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

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

3.3. Inhibitor of murein hydrolases increases transformation in CS2 medium

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

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

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

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

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

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

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

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

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

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

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Conflict of interest

The authors declare no conflict of interest.

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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)	$4.0 \pm 3.0 \times 10^{-9}$ (n = 11)
pHY300	$5.0 \pm 4.7 \times 10^{-11}$ (n = 32) ND (n = 2)	$5.0 \pm 6.0 \times 10^{-11}$ (n = 2)	$3.0 \pm 1.4 \times 10^{-11}$ (n = 2)	$1.6 \pm 1.4 \times 10^{-9}$ (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 μm. The cell surface roughness (mean
288 ratio ± 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

Figure

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Fig. 1

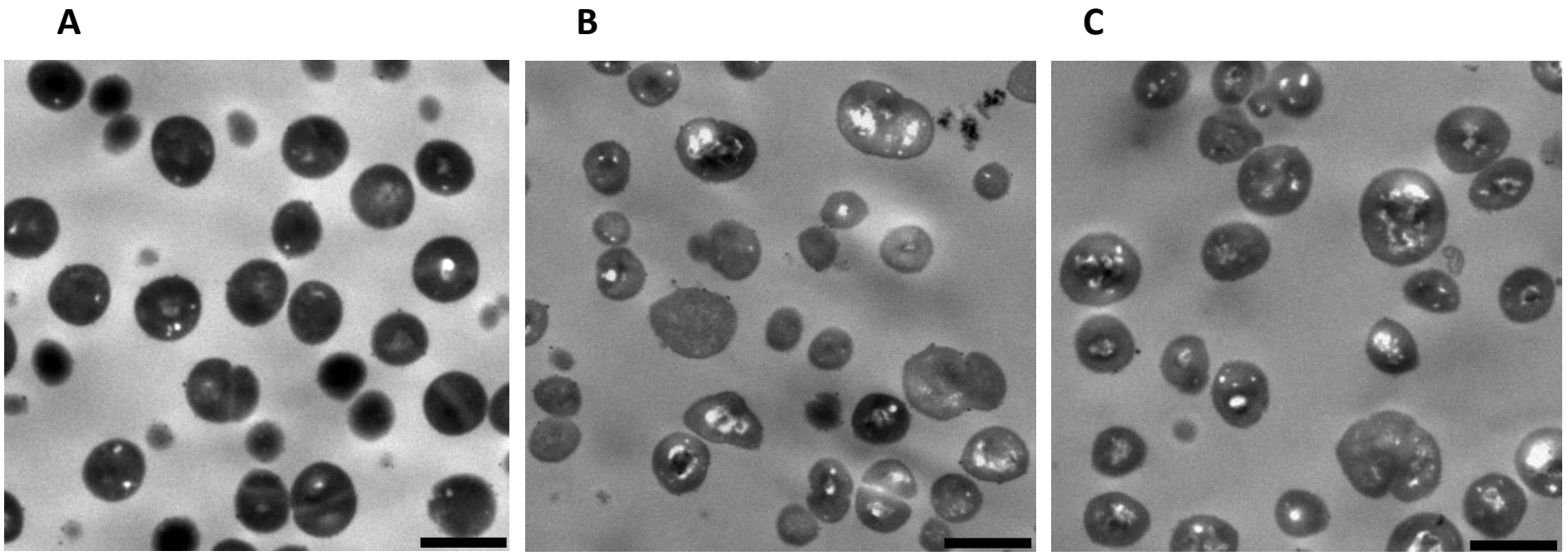

Fig. 2