NOTE

Cell wall-affecting antibiotics modulate natural transformation in SigH-expressing *Staphylococcus aureus*

Le Thuy Nguyen Thi [1], Veronica Medrano Romero [2] and Kazuya Morikawa [3,*]

[1] Ph.D. Program in Human Biology, School of Integrative and Global Majors, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, 305-8575, Japan; E-Mail: lenacns2005@gmail.com (L.T.N.T.)
[2] Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, 305-8575, Japan; E-Mail: veromedrom@gmail.com (V.M.R.)
[3] Faculty of Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, 305-8575, Japan; E-Mail: morikawa.kazuya.ga@u.tsukuba.ac.jp (K.M.)
***** Author to whom correspondence should be addressed;
E-Mail:morikawa.kazuya.ga@u.tsukuba.ac.jp (K.M.); Tel.: +81-29-853-3928; Fax: +81-29-853-3928.

Running title: antibiotics affecting transformation in *Staphylococcus aureus*

Keywords: *Staphylococcus aureus*/ competence/ transformation/ SigH/ antibiotics

Staphylococcus aureus is a major opportunistic human pathogen causing a broad spectrum of infections[1]. This bacterium has an extraordinary ability to rapidly acquire resistance to antibiotics, and methicillin-resistant strains (MRSA), the most common cause of nosocomial infections, are now spreading into the community[2, 3]. In *S. aureus* genome, many virulence and antibiotic resistance genes are found in mobile genetic elements[4 5], indicating that horizontal gene transfer (HGT) must play a critical role in the evolution. In general, Gram-positive bacteria have three HGT mechanisms: conjugation, transduction, and transformation[6]. Conjugation occurs in *S. aureus*, but it requires a series of *tra* genes or conjugative plasmids, which are not widespread among *S. aureus* strains[7, 8]. Transduction is thought to be more predominant, because most *S. aureus* isolates have prophages in their genome. The head size of phages limits the DNA length that can be transferred (c.a. 45kbp)[4], but a giant phage in the environment can transfer larger DNA fragments[9].

We recently demonstrated for the first time that *S. aureus* can develop natural genetic competence for DNA transformation in a manner dependent on SigH[10]. SigH is one of the alternative sigma factors. It associates with the core RNA polymerase, and renders the resultant holoenzyme the ability to recognize the promoter sequence and initiate the transcription of competence operons (*comG* and *comE* operons) that encode the machinery for DNA incorporation[11]. SigH expresses in a minor subpopulation by two distinct mechanisms[10]. One is short-junction duplication generating a new *sigH* fusion gene. SigH expression by short-junction duplication is spontaneous and its frequency is less than 10^{-5}. Alternatively, SigH is expressed by post-transcriptional regulation, and SigH-expressing cells increase up to ~ 10^{-2} in complete synthetic medium (CS2). Thus, SigH expression is limited to a small subpopulation, which was one of the reasons for the difficulty in experimental detection of transformation. The transformation frequency in SigH-expressing cells is experimentally detectable, but the efficiency varies depending on the culture conditions, suggesting that unknown factor(s) affect the transformation of SigH-expressing cells.

Drug resistance is initially manifested in the settings where antibiotics constitute a selective pressure[3]. However, the effects of antibiotics on *S. aureus* transformation have not been explored yet. Here, we describe the effects of antibiotics on the efficiency of transformation in SigH-expressing cells.

The SigH-expressing strain (N315ex w/oφ h,[10]) was used as the recipient. 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. SigH is expressed by a plasmid, pRIT-sigH[11]. Transformation assay was carried out as previously described with some modifications[10]. Briefly, log-phase cells were suspended in fresh tryptic soy broth (TSB) with or without the antibiotics to be tested. After 5h incubation, cells were washed and replaced with fresh medium. Ten µg of unmethylated pHY300 (Tet^R) purified from *E. coli* HST04 *(dam-/dcm-*) was added; methylation status does not affect transformation frequency (data not shown). Following 2.5 h incubation at 37˚C with shaking, transformants were selected in brain heart infusion (BHI)-agar medium supplemented with 5 µg ml^{-1} tetracycline. Some transformants were tested for the presence of the tet^R gene by colony PCR. In line with our previous study[10], no spontaneous Tet^R mutant was detected throughout this study. The transformation frequency was calculated as the ratio of total number of transformants to the total viable cells after the antibiotics treatment and incubation with DNA.

The effects of antibiotics on transformation were tested by at least 3 independent experiments (Figure 1). Bacitracin reproducibly increased the transformation frequency at low concentrations, but showed suppressive effect at higher concentrations. D-cycloserin showed no significant effect. Transformants were rarely detected in 1 µg ml^{-1} cefazolin treatment (frequency 0.8 x 10^{-11}, n = 1; none detected, n =2). Oxacillin abolished transformation: no transformants were detected when cells were treated at ½ MIC of oxacillin. Mitomycin C suppressed transformation in a concentration-dependent manner. Ciprofloxacin, norfloxacin and streptomycin had no significant effect.

Vancomycin and fosfomycin increased the transformation frequencies (Figure 2a, 2b), and the effects were statistically significant ($p = 0.016$, $n = 9$ for vancomycin; $p = 0.012$, $n = 10$ for fosfomycin) (Figure 2c). SigH-expressing cells lacking the competence genes (N315ex w/oφ ΔcomEh, N315ex w/oφ ΔcomGh) generated no transformant in the presence of these antibiotics (n = 2), confirming that this is due to the transformation by natural genetic competence, rather than other horizontal gene transfer mechanisms[10]. The transformation frequency in the presence of fosfomycin was highly variable depending on the experiment (Figure 2c). This variation might be due to the killing effect of fosfomycin (Figure 2b).

Bacitracin, vancomycin, and fosfomycin, are cell wall-affecting antibiotics with distinct modes of action. Bacitracin binds to certain lipid carrier to block the supply of the cell wall components[12]. Vancomycin (glycopeptide antibiotics) binds to the peptidoglycan precursors, UDP-N-acetylmuramyl-pentapeptides, and inhibit transglycosylation reactions[13]. Fosfomycin inhibits UDP-N-acetylglucosamin enolpyruvoyl transferase (MurA) that is required for the first step in bacterial cell wall biosynthesis[14]. This is the first report on the positive effect of cell wall-affecting antibiotics on natural transformation among bacteria: e.g. in *Streptococcus pneumoniae*, mitomycin C and norfloxacin (quinolone) have positive effect on transformation, but the cell wall-affecting antibiotics such as vancomycin and ampicillin (β-lactam) have no effect[15]. We think that the staphylococcal response to cell wall-affecting antibiotics to induce natural transformation has an important significance with respect to *S. aureus* evolution. External physical damages to the cell wall (by silica beads or lysostaphin, an enzyme that cleaves *S. aureus* cell walls[16]) did not facilitate the transformation (data not shown). This suggests that effects of cell wall-affecting antibiotics on transformation involve certain complex cellular activity.

Low concentrations of bacitracin are often combined with other antibiotics in triple-antibiotic ointments used in the treatment of soft tissue infections[17]. The observed hormetic effect of bacitracin on transformation suggests that this antibiotic might accelerate the horizontal gene transfer in clinical settings. Vancomycin remains one of the effective resources for MRSA treatment, though we already have reports on vancomycin resistant *S. aureus* (VRSA)[18]. Fosfomycin previously selected resistant staphylococci (fosfomycin resistant *S. aureus* increased in Japanese hospitals in 1980's), but it is still among the choices for treatment, often by combination with other antibiotics[19]. We emphasize that the present study potentially raises a caution regarding medical prescription in the treatment of *S. aureus* considering the induction of horizontal gene transfer. The results presented in this study are limited to a single SigH-expressing strain: the effect of antibiotics on transformation efficiency may vary depending on growth conditions and strains, and next studies must address these points.

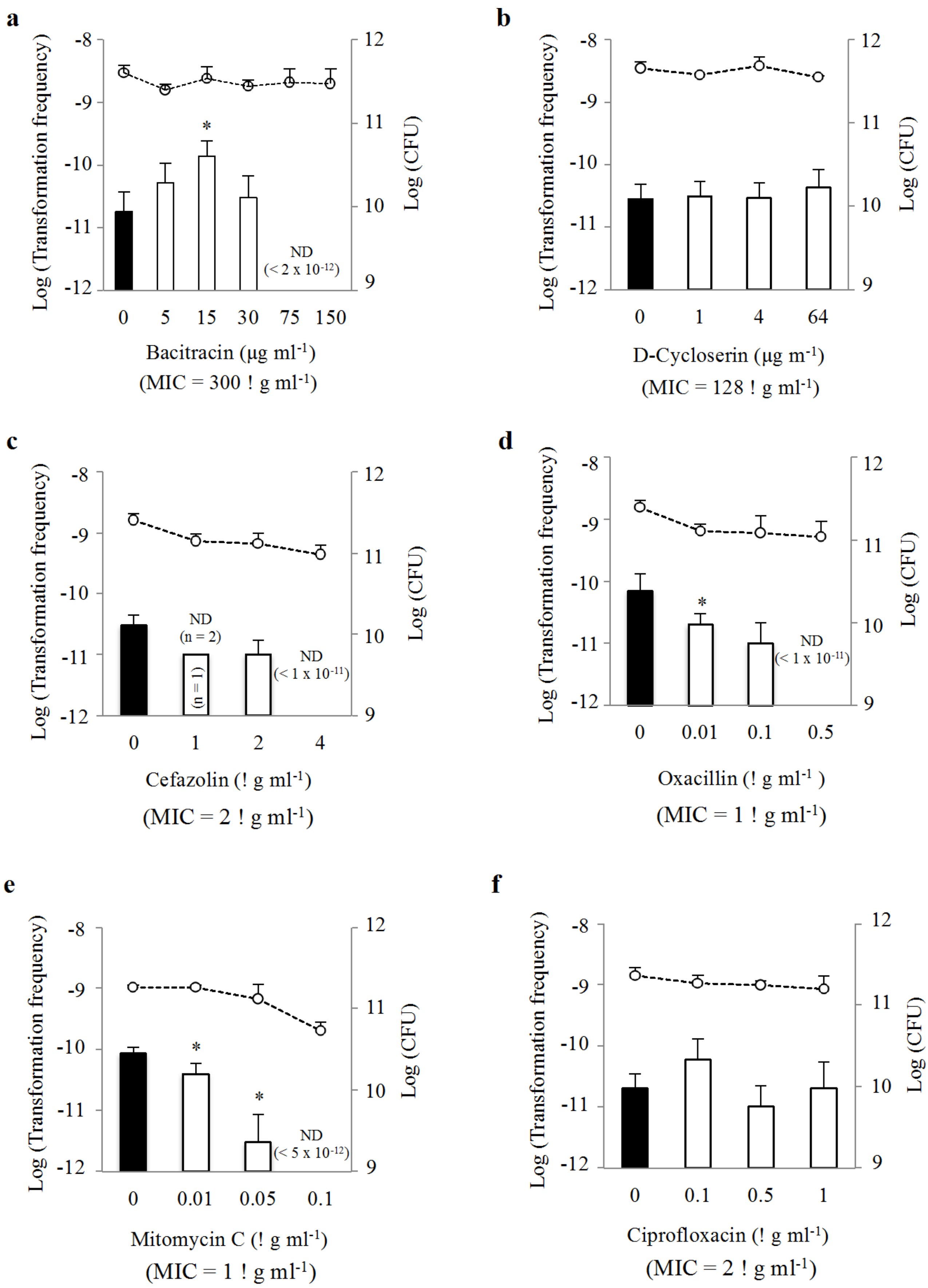
Acknowledgments

This work was supported by Takeda Science Foundation, Pfizer Academic Contributions, and JSPS KAKENHI Grant Number 25860313.

References

1. Lowy FD. *Staphylococcus aureus* infections. *The New England journal of medicine.* 339(8):520-532 (1998)
2. McCallum N, Berger-Bächi B, Senn MM. Regulation of antibiotic resistance in *Staphylococcus aureus*. *International journal of medical microbiology : IJMM.* 300(2-3):118-129 (2010)
3. Chambers HF, Deleo FR. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nat Rev Microbiol.* 7(9):629-641 (2009)
4. Lindsay JA. Genomic variation and evolution of *Staphylococcus aureus*. *Int J Med Microbiol.* 300(2-3):98-103 (2010)

5. Malachowa N, DeLeo FR. Mobile genetic elements of *Staphylococcus aureus*. *Cell Mol. Life. Sci.* 67(18):3057-3071 (2010)
6. Chen I, Dubnau D. DNA uptake during bacterial transformation. *Nature reviews. Microbiology.* 2(3):241-249 (2004)
7. Smith TL, Jarvis WR. Antimicrobial resistance in *Staphylococcus aureus*. *Microbes. Infect.* 1(10):795-805 (1999)
8. Clewell DB, An FY, White BA, Gawron-Burke C. *Streptococcus faecalis* sex pheromone (cAM373) also produced by *Staphylococcus aureus* and identification of a conjugative transposon (Tn918). *Journal of bacteriology.* 162(3):1212-1220 (1985)
9. Uchiyama J, et al. Intragenus generalized transduction in *Staphylococcus* spp. by a novel giant phage. *The ISME journal.* 8(9):1949-1952 (2014)
10. Morikawa K, et al. Expression of a cryptic secondary sigma factor gene unveils natural competence for DNA transformation in *Staphylococcus aureus*. *PLoS Pathog.* 8(11):e1003003 (2012)
11. Morikawa K, et al. A new staphylococcal sigma factor in the conserved gene cassette: functional significance and implication for the evolutionary processes. *Genes Cells.* 8(8):699-712 (2003)
12. Drablos F, Nicholson DG, Ronning M. EXAFS study of zinc coordination in bacitracin A. *Biochim Biophys Acta.* 1431(2):433-442 (1999)
13. Reynolds PE. Structure, biochemistry and mechanism of action of glycopeptide antibiotics. *Eur J Clin Microbiol Infect Dis.* 8(11):943-950 (1989)
14. Skarzynski T, Mistry A, Wonacott A, Hutchinson SE, Kelly VA, Duncan K. Structure of UDP-N-acetylglucosamine enolpyruvyl transferase, an enzyme essential for the synthesis of bacterial peptidoglycan, complexed with substrate UDP-N-acetylglucosamine and the drug fosfomycin. *Structure.* 4(12):1465-1474 (1996)
15. Prudhomme M, Attaiech L, Sanchez G, Martin B, Claverys J-P. Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*. *Science (New York, N.Y.).* 313(5783):89-92 (2006)
16. Kumar JK. Lysostaphin: an antistaphylococcal agent. *Applied microbiology and biotechnology.* 80(4):555-561 (2008)
17. Suzuki M, et al. Antimicrobial ointments and methicillin-resistant *Staphylococcus aureus* USA300. *Emerg Infect Dis.* 17(10):1917-1920 (2011)
18. Kali A. Antibiotics and bioactive natural products in treatment of methicillin resistant *Staphylococcus aureus*: A brief review. *Pharmacognosy reviews.* 9(17):29-34 (2015)
19. Michalopoulos AS, Livaditis IG, Gougoutas V. The revival of fosfomycin. *International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases.* 15(11):e732-739 (2011)


a
Log (Transformation frequency)
Log (CFU)
*
ND
(< 2 x 10-12)
0 5 15 30 75 150
Bacitracin (µg ml-1)
(MIC = 300 ! g ml-1)
b
Log (Transformation frequency)
Log (CFU)
0 1 4 64
D-Cycloserin (µg m-1)
(MIC = 128 ! g ml-1)
c
Log (Transformation frequency)
Log (CFU)
ND
(n = 2)
(n = 1)
ND
(< 1 x 10-11)
0 1 2 4
Cefazolin (! g ml-1)
(MIC = 2 ! g ml-1)
d
Log (Transformation frequency)
Log (CFU)
*
ND
(< 1 x 10-11)
0 0.01 0.1 0.5
Oxacillin (! g ml-1)
(MIC = 1 ! g ml-1)
e
Log (Transformation frequency)
Log (CFU)
*
*
ND
(< 5 x 10-12)
0 0.01 0.05 0.1
Mitomycin C (! g ml-1)
(MIC = 1 ! g ml-1)
f
Log (Transformation frequency)
Log (CFU)
0 0.1 0.5 1
Ciprofloxacin (! g ml-1)
(MIC = 2 ! g ml-1)

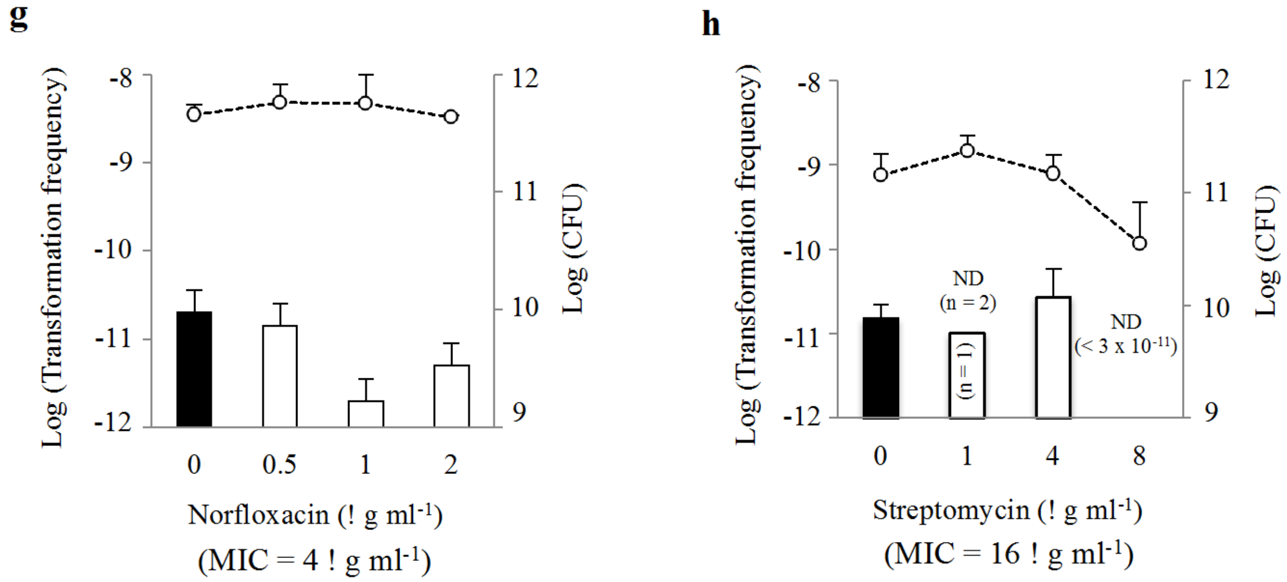


Figure 1. Effects of antibiotics on transformation. N315ex w/oφ h was exposed to different concentrations of antibiotics followed by the transformation with 10 µg of pHY300 plasmid. Bars: Log_{10}(Transformation frequencies); dotted lines: Log_{10}(CFU). Average values of at least 3 independent experiments are shown with SD. ND: none detected. *: $p<0.05$ by Student's T-test for log values of frequencies. MIC values of antibiotics were determined by microdilution method using TSB.

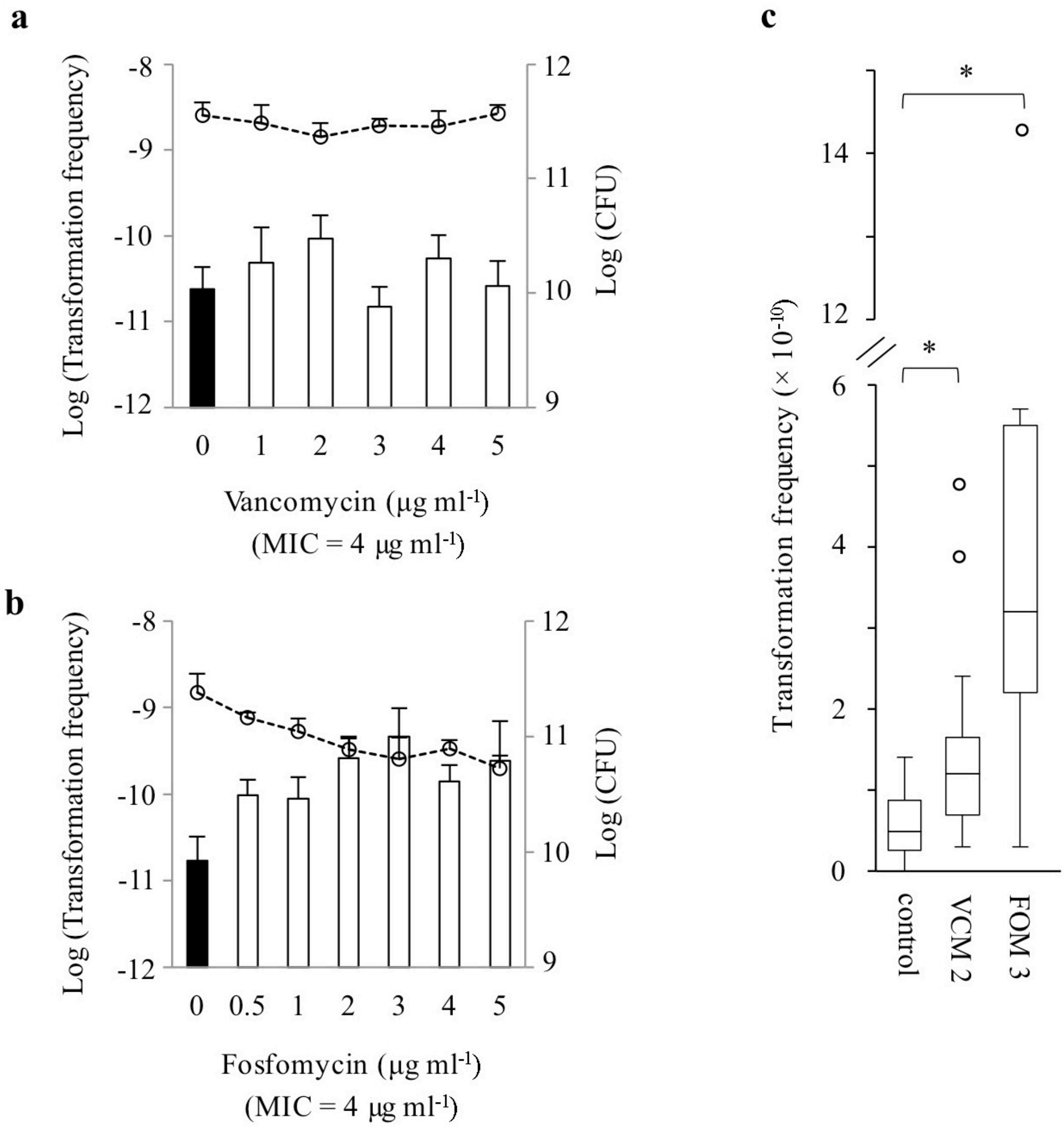


Figure 2. Effects of vancomycin (a) and fosfomycin (b) on transformation. (c) Repeated tests of the effects of 2 μg ml^{-1} vancomycin (VCM 2) and 3 μg ml^{-1} fosfomycin (FOM 3) on transformation frequencies are shown by box-plot. Boxes span the upper and lower quartile, lines inside the boxes indicate median, whiskers present the maximum and minimum values within the 1.5 interquartile range, empty circles represent data points that are outside of this range. control (no antibiotics) n = 10; VCM2 n = 9; FOM3 n = 10; * : $p < 0.05$.