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To cite this version:
Kazuya Morikawa, Aya Takemura, Yumiko Inose, Melody Tsai, Le Thuy Nguyen Thi, et al.. Expression of a Cryptic Secondary Sigma Factor Gene Unveils Natural Competence for DNA Transformation in Staphylococcus aureus. PLoS Pathogens, Public Library of Science, 2012, 8 (11), pp.e1003003. 10.1371/journal.ppat.1003003. pasteur-02869905

HAL Id: pasteur-02869905
https://hal-pasteur.archives-ouvertes.fr/pasteur-02869905
Submitted on 16 Jun 2020

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Expression of a Cryptic Secondary Sigma Factor Gene Unveils Natural Competence for DNA Transformation in Staphylococcus aureus

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Abstract

It has long been a question whether Staphylococcus aureus, a major human pathogen, is able to develop natural competence for transformation by DNA. We previously showed that a novel staphylococcal secondary sigma factor, SigH, was a likely key component for competence development, but the corresponding gene appeared to be cryptic as its expression could not be detected during growth under standard laboratory conditions. Here, we have uncovered two distinct mechanisms allowing activation of SigH production in a minor fraction of the bacterial cell population. The first is a chromosomal gene duplication rearrangement occurring spontaneously at a low frequency ($\leq 10^{-5}$), generating expression of a new chimeric sight gene. The second involves post-transcriptional regulation through an upstream inverted repeat sequence, effectively suppressing expression of the sight gene. Importantly, we have demonstrated for the first time that S. aureus cells producing active SigH become competent for transformation by plasmid or chromosomal DNA, which requires the expression of SigH-controlled competence genes. Additionally, using DNA from the N315 MRSA strain, we successfully transferred the full length SCCmeII element through natural transformation to a methicillin-sensitive strain, conferring methicillin resistance to the resulting S. aureus transformants. Taken together, we propose a unique model for staphylococcal competence regulation by SigH that could help explain the acquisition of antibiotic resistance genes through horizontal gene transfer in this important pathogen.

Introduction

Staphylococcus aureus, first discovered and described over 130 years ago [1,2], belongs to the low G+C % Gram-positive Bacilli class of Firmicutes that also includes Bacillus subtilis and Listeria monocytogenes. A commensal bacterium, often colonizing mammalian nasal cavities [3], S. aureus is also a major human pathogen causing a broad spectrum of infections ranging from food poisoning and superficial skin abscesses to more serious diseases such as pneumonia, meningitis, osteomyelitis, sepsis, toxic shock syndrome and sepsis [4]. It has acquired resistance to a wide variety of antibiotics [5,6], and methicillin-resistant strains (MRSA), the most common cause of nosocomial infections, are now spreading into the community [7].

S. aureus genome contains several mobile genetic elements such as transposons, bacteriophages, insertion sequences, pathogenicity islands and a staphylococcal cassette chromosome (SCC) [8,9], which carry many of the toxin and antibiotic resistance genes. This indicates that horizontal gene transfer (HGT), occurring in bacteria through multiple mechanisms [10], must play a critical role in the evolution of this major human pathogen. Conjugation in S. aureus requires a series of tra genes or conjugative plasmids, which are only found in certain isolates [11]. Most S. aureus strains are lysogenized with temperate phages, which can enter a lytic cycle that leads to generalized transduction when host DNA is mispackaged into some phages and transferred to a recipient cell upon the following infection. In addition, unusual phage-like infectious particles are involved in the efficient transfer of staphylococcal pathogenicity islands [12].

Natural genetic competence for transformation involves the binding and uptake of extracellular DNA [13]. Following a publication in 1972 reporting the existence of a transformation-like phenomenon in S. aureus [14], numerous reports investigating this process appeared over the ensuing decade. It was finally shown that this was in fact not natural genetic competence, but a type of HGT that requires contaminating phage tail fragments in the DNA preparation, which bind to the host cell and allow entry of DNA [15]. Thus, to date, bona fide DNA-mediated transformation of S. aureus by natural genetic competence has not yet been detected and remains something of a “holy grail”.

Despite this fact, orthologues of most of the competence genes encoding the DNA uptake machinery, such as the comG and comE genes, have been detected and remain something of a “holy grail”.
Author Summary

Staphylococcus aureus is a major human pathogen responsible for a broad spectrum of infections, emphasized by the emergence of multiple antibiotic-resistant strains with up to 60% of strains worldwide resistant to methicillin (Methicillin Resistant Staphylococcus aureus or MRSA). Indeed, MRSA-related infections are now one of the leading causes of death in the USA, highlighting the growing threat this bacterium poses to human health. Many bacteria have the ability to acquire novel genetic characteristics, including antibiotic resistance, through the uptake of extracellular DNA, a phenomenon known as natural genetic transformation or competence. We have shown that the SigH staphylococcal sigma factor is a likely key component for competence development, but that its gene is not expressed under standard laboratory conditions. Here, we have uncovered two distinct mechanisms allowing activation of SigH production in S. aureus: a chromosomal gene duplication rearrangement and post-transcriptional regulation through an upstream inverted repeat sequence. Importantly, we have demonstrated for the first time that S. aureus cells producing active SigH become competent for natural transformation by plasmid or chromosomal DNA, and we were able to confer methicillin resistance to a methicillin-sensitive strain by transformation with chromosomal DNA. SigH-dependent competence development in S. aureus could help explain the acquisition of antibiotic resistance genes and the rise of the so-called “superbug.”

operons [13,16], are present and conserved in staphylococcal genomes [17,18] (See Supplementary Material Figure S1). This has also been reported for several other supposedly non-competent bacterial species, such as Listeria monocytogenes or Lactococcus lactis [19–21], indicating that we do not yet fully understand the specific conditions required for competence development in these bacteria and that they may in fact be able to become competent.

We previously identified a novel alternative sigma factor in Staphylococcus aureus, SigH, and demonstrated that it associates with core RNA polymerase to specifically transcribe the comG and comE competence operon orthologues in S. aureus [18]. In addition to the primary vegetative sigma factor SigA [22], S. aureus has at least three alternative sigma factors: SigB [23–25], SigH [18], and SigS [26]. SigB is responsible for a variety of stress responses and is activated by a partner-switching regulatory mechanism [27], while the function of SigS remains elusive [26].

Staphylococcal SigH has a unique evolutionary characteristic in that it shares exceptionally low sequence similarity between different bacterial species [18,28]. Phylogenetic studies indicate that it belongs to a large group also including SigH of Bacillus subtilis [29], and ComX of Streptococcus pneumoniae [30]. These related sigma factors are widely distributed among Firmicutes, with diverse physiological roles: in B. subtilis, SigH (SpoOH) is required for transcription of several early sporulation genes [31,32] whereas in S. pneumoniae, ComX (SigX) directs the expression of late genetic competence genes in response to a peptide quorum-sensing regulatory pathway [30,33,34].

In S. aureus however, the sigH gene appears to be cryptic since its expression could not be detected under standard laboratory culture conditions, although its artificial overexpression in vivo induces expression of the comE and comG operons [18]. Because true bacterial cryptic genes are more akin to pseudogenes, likely to be lost through “use it or lose it” evolutionary constraints [35–37], the high conservation within S. aureus strains of SigH and the competence gene orthologues it controls suggested they must be expressed and play a role under certain specific growth conditions.

In this study, we wished to gain insight into staphylococcal competence development by identifying conditions where the SigH secondary sigma factor is active. We report the existence of two distinct mechanisms allowing activation of SigH production in a minor fraction of the cell population and have successfully demonstrated for the first time that S. aureus cells producing active SigH become competent for transformation by DNA, in a manner dependent upon SigH-controlled competence genes. We propose a model for staphylococcal competence regulation, and discuss its evolutionary differences with the known systems in B. subtilis and Streptococcus species as well as the crucial role competence likely plays in acquisition of antibiotic resistance genes.

Results

SigH active cells can be isolated and stably maintained

In order to isolate cells where SigH is produced and active, the pTet-rep positive selection double-reporter plasmid was constructed, using the tet tetracycline antibiotic resistance gene and the bgaB β-galactosidase gene from Bacillus stearothermophilus [38] (See Materials and Methods; Figure 1A). The SigH-dependent comG (SA1374) and comE (SA1418) promoters [18] were used to monitor SigH activity by creating transcriptional fusions with the tet and bgaB genes, respectively. Reporter strains carrying pTet-rep are sensitive to tetracycline and do not exhibit β-galactosidase activity, due to the absence of endogenous SigH activity in S. aureus under standard laboratory growth conditions. Cells in which SigH activity spontaneously occurs and is stably maintained for a number of generations are thus expected to give rise to tetracycline-resistant β-galactosidase positive colonies.

The pTet-rep plasmid was introduced into S. aureus strain RN4220, giving strain RNtet-rep, and stationary phase cultures were plated in the presence of 5 μg/ml tetracycline. The majority of the tetracycline resistant colonies were also β-galactosidase positive, and using this approach we obtained SigH active cells from all S. aureus strain backgrounds tested (N315, RN4220, 8325–2, MRSA408) with frequencies ranging from ca. 10−5−<10−9 depending on the experiment (See Supplementary Material Figure S2). We measured the frequency fluctuation using the classical Luria-Delbruck test [39] (Figure 1B). When the reporter strain was cultured in a single flask, aliquots gave rise to similar numbers of SigH active cells. In contrast, when grown as multiple independent cultures, the number of SigH active cells varied (Figure 1B and Supplementary Material Figure S2). These variations suggest that SigH activity results from a spontaneous event, rather than artificial induction by the presence of tetracycline.

Three SigH active clones (designated A, B, and C) generated from strain RNtet-rep were chosen for further characterization of the stable SigH activation mechanism. Clones A and B exhibited similar β-galactosidase activities on X-gal plates, whereas clone C displayed higher levels. These SigH-positive phenotypes were not due to mutations in the reporter plasmids (Figure 1C). Indeed, the plasmids were cured from clones A, B, and C, to generate strains A2, B1, and C1, and a comE-bgaB transcriptional fusion reporter plasmid, pRIT-PcomE-bgaB, was then introduced into these cells by electroporation (Figure 1C). The A2, B1, and C1 transformants were β-galactosidase-positive (designated A2b, B1b and C1b, respectively), whereas the parental RN4220 strain transformed with pRIT-PcomE-bgaB was not (Figure 1C), indicating that the SigH activity detected in clones A, B and C was stably maintained throughout these procedures. However, rare revertant white colonies emerged after overnight cultures of strains A2b, B1b...
and C1b, sometimes due to a deletion in the reporter plasmid but often to actual loss of SigH activity (see below). The frequency of cells where SigH activity was lost after a single overnight culture was ca. $10^{-2}$ to $10^{-3}$. We observed no differences during growth in TSB medium between SigH active and SigH inactive cells.

Increased SigH activity is associated with a reversible gene duplication through a chromosomal rearrangement event

Southern blot analyses of the sigH locus were performed on genomic DNA of the A2, B1, and C1 strains displaying increased SigH activity (Figure 2A and 2B) as well as revertants from each clone that had lost SigH-dependent β-galactosidase activity after plasmid curing and introduction of the pRIT-PcomE-bgαB reporter plasmid (A2-r, B1-r, and C1-r are the revertants from A2b, B1b and C1b, respectively). When the upstream region of sigH was used as a probe (Figure 2A), there was no difference in pattern between the parental RN4220 and the SigH active strains. In contrast, when the sigH coding sequence was used as a probe (Figure 2B), an additional band (HindIII digest) or a slower migrating stronger signal (PvuII digest) was detected for all of the SigH active mutants. This result suggests that the sigH gene is

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**Figure 1. Positive selection for *Staphylococcus aureus* cells with active SigH.**

A) Positive selection tetracycline resistance (tet) reporter plasmid (pTet-rep). Reporter strains carrying pTet-rep were selected with 5 μg/ml tetracycline. cat: chloramphenicol resistance gene. bgαB: β-galactosidase gene. PcomG: promoter region of comG operon. PcomE: promoter region of comE operon. B) Fluctuation tests indicate that SigH activation occurs spontaneously. The y-axis represents the numbers of SigH active colonies detected from $10^9$ cfu. Cells were grown in drug-free TSB and then selected for tetracycline resistance. Open symbols: aliquots from a single flask. Closed symbols: independent cultures in separate test tubes. Diamonds: N315 pTet-rep, Triangles: RNtet-rep (RN4220 pTet-rep). C) SigH activity can be stably maintained through generations without selection pressure. pTet-rep was cured from SigH active cells, and pRIT-PcomE-bgαB was introduced. Cells were grown on TSA plates containing 100 μg/ml X-gal and 12.5 μg/ml chloramphenicol. Rare white cells (about 1%) that lost SigH activity were also observed (see text).

doi:10.1371/journal.ppat.1003003.g001
Natural Genetic Competence in S. aureus

A

| Hind III | Pvu II |
|---------|--------|
| RN4220  | RN4220 |
| A2      | A2-r   |
| A2      | A2-r   |
| B1      | B1-r   |
| B1      | B1-r   |
| C1      | C1-r   |
| C1      | C1-r   |

B

| Hind III | Pvu II |
|---------|--------|
| RN4220  | RN4220 |
| A2      | A2-r   |
| A2      | A2-r   |
| B1      | B1-r   |
| B1      | B1-r   |
| C1      | C1-r   |
| C1      | C1-r   |

C

WT(RN4220)

sigH

Pvu II

SA0491

rpmG

secE

nusG

SAS015

rplK

1 kbp

B1

AACTCAAG

Hind III

Pvu II

nusG + sigH fusion

C1

CAATGCA

Hind III

Pvu II

rplK + sigH fusion

D

MW marker

RN4220

RH

A2

A2-r

B1

B1-r

C1

C1-r

(kDa)

26

19

15

SigH
duplicated in the SigH active cells. Interestingly, the hybridization pattern for each of the revertants was identical to that of the RN4220 parental strain indicating that this is a fully reversible process. The duplication apparently occurs in close proximity to the sigH locus, because a stronger sigH signal was systematically observed in the PauII digests. This was confirmed by sequence analysis of the sigH region in strains B1 and C1.

SigH is produced as a fusion protein encoded by a new chimeric gene generated by tandem duplication through a spontaneous chromosomal rearrangement

The nucleotide sequence of the entire sigH locus was determined for the B1 and C1 clones (Figure 2C). In the B1 clone, a 1210 bp region was found to be tandemly duplicated. The duplicated region was flanked by the 8 bp direct repeat sequence AACTCAAG. This duplication did not affect the structure of the original sigH gene, but instead generated a new chimeric gene with the downstream nusG coding sequence, with an intact copy of nusG still present. The chimeric nusG-\(^2\)-sigH gene results from the precise in-frame fusion of codon 30 of the nusG coding sequence with codon 7 of sigH, such that the resulting chimeric NusG-\(^2\)-SigH protein has its first seven amino acids replaced with the first 50 from NusG. In the case of clone C1, the 7 bp direct repeat sequence CAATGCA flanked a 1888 bp tandemly duplicated region, generating a new chimeric rplK-\(^2\)-sigH gene, by fusing coding 42 of the downstream rplK gene with codon 16 of sigH, and retaining intact copies of sigH and rplK upstream and downstream of the duplication, respectively. This indicates that the duplication unit as well as the chimeric gene partner can vary, and that this process involves Short Junction (SJ) tandem duplication [40]. In both cases, the downstream gene’s promoter, ribosome binding site and translational initiation codon drove expression of the resulting chimeric sigH gene. Western blot analyses indicated that the chimeric SigH proteins migrate in SDS-PAGE with a higher apparent molecular mass than native SigH, which is entirely consistent with the expected sizes of the chimeric proteins as deduced from the nucleotide sequence (SigH: 23 kDa, NusG-\(^2\)-SigH: 28.3 kDa, RplK-\(^2\)-SigH: 25.5 kDa; Figure 2D). Other types of SJ duplications leading to chimeric SigH proteins were also identified and are shown in Figure S3 (See Supplementary Material). The sigH locus nucleotide sequence of the B1-\(^r\) and C1-\(^r\) revertants that had lost SigH-dependent \(\beta\)-galactosidase activity was also determined, showing that the reversion had restored the native single copy sigH sequence following loss of the duplication.

SigH is active in S. aureus under specific culture conditions

In an attempt to identify specific growth conditions that could lead to production of active SigH in S. aureus, a reporter plasmid expressing GFP under the control of the comG promoter was constructed as described in Materials and Methods (pRIT-com-gfp). As negative and positive controls, we used pRIT-gfp (no promoter) and pRIT-asp-gfp where the \(\sigma^H\)-dependent asp23 promoter drives GFP expression. These plasmids were introduced into S. aureus strain N315 and GFP expression was examined by Western blot analysis. As shown in Figure 3A, asp23 promoter activity was detected in standard laboratory media, such as BHI and RPMI 1640. In contrast, \(\sigma^H\)-dependent expression was undetectable throughout growth under these conditions (Figure 3A). We saw no effect on SigH activity when cultures were exposed to different compounds or stress conditions, including hypertonic shock (1 M KCl or 2 M NaCl, for 20 min), heat shock (46°C for 20 min), freeze-thaw cycle, treatment with detergents (CHAPS, NP40, Tween 20, Triton X-100), or antibiotics (bacitracin, cefazolin, gentamycin, oxacillin, penicillin G, tetracycline, vancomycin at the Minimal Inhibitory Concentrations for 20 min).

In contrast, as shown in Figure 3B, we could clearly detect SigH-dependent production of GFP when cells were grown aerobically in a complete synthetic medium (CS2 medium; see Materials and Methods and Supplementary Material Table S1). Similar results were obtained when cells were grown anaerobically in other synthetic media (CS1 medium, -GS medium; see Materials and Methods and Supplementary Material Figure S4). The negative control strain without the comG promoter reporter (N315 pRIT-gfp) showed no GFP production under any of these conditions.

Northern blot analysis allowed us to show that the endogenous S1365 gene, the last gene of the \(\sigma^H\)-dependent comG operon [18], is indeed actively transcribed under these SigH-activating growth conditions (Figure 3C). This transcription was lost in the NKSSh sigH mutant strain, and restored in the NKSSh complemented strain expressing sigH from a multicopy plasmid (Figure 3C).

SigH is only active in a minor fraction of the cell population

Production of GFP in cells grown aerobically in CS2 medium was observed by confocal microscopy. Interestingly, only a small fraction of the cell population produced GFP when expression was driven from the SigH-dependent comG promoter (Figure 4A), with a frequency of 1.8±0.7% (at 7 h, n = 3). This is in clear contrast to the evenly distributed GFP expression from the SigB-dependent construct during growth in BHI (Figure 4B), whereas no GFP-positive cells were detected using the negative control strain (N315 pRIT-gfp).

SigH activation occurs through multiple pathways

We tested whether the cells producing GFP observed in Figure 4A resulted from transient inducible SigH activation during growth in CS2 medium by a mechanism distinct from stable activation through SJ tandem duplication. We therefore introduced chromosomal mutations inactivating the two potential translation initiation codons of sigH strain N315ex-sigH\(^H\); Figure 5A). These mutations should not affect SigH activation by SJ tandem duplication, which
does not require the native sigH translation initiation site. The two reporter plasmids, pMKcomGFP and pTet-rep were then introduced into the mutant strain. Western blot analysis showed that GFP production during growth in CS2 medium was abolished in this strain (Figure 5B), whereas as expected, stable SigH activation (emergence of tetR colonies) was observed in this mutant, but with only very few cells expressing GFP (Figure 5C). Thus, we conclude that two distinct mechanisms for activation of S. aureus SigH expression coexist: one requires the original translation initiation codon, and occurs stochastically during growth in chemically defined media, whereas the SJ-tandem duplication mechanism does not require the native sigH translation initiation sequences. The nucleotide sequence of the sigH locus from SigH active clones arising from the N315ex-sigH* mutant revealed SJ-tandem duplication events similar to those in the B1 and C1 clones (See Supplementary Material Figure S3).

Post-transcriptional control of sigH expression involves the 5'-UTR

Overexpression of sigH using a high-copy number plasmid (pRIT-sigHNH7) is not sufficient to induce SigH activity during growth in standard growth media such as BHI or LB (Figure 6A).

Figure 3. SigH is activated under specific growth conditions. A) Western blot analyses directed against GFP showing that SigH is not active in standard culture conditions. Strains N315aspGFP (asp23 promoter), N315comGFP (comG promoter) and N315-GFP (promoter-less) were grown aerobically in BHI or RPMI 1640 medium. B) Western blot against GFP showing that SigH activity is induced in CS2 medium. Incubation times (hours) are indicated above the panel. Positive control cells of N315aspGFP were grown in TSB for 10.5 h. C) Northern blot analysis confirming SigH-dependent expression of SA1365, one of the comG operon genes. Strains N315, NKSH (sigH inactivation mutant), and NKSHh (complemented strain) were grown in anaerobic and static conditions in -GS medium.
doi:10.1371/journal.ppat.1003003.g003
In this plasmid, \textit{sigH} is constitutively expressed from the \textit{spa} promoter (staphylococcal protein A gene), indicating that increased transcription of \textit{sigH} is not sufficient to produce active SigH. When this strain was grown in CS2 medium, accumulation of GFP was induced (Figure 6A), but again only in a minor fraction of the cell population (Figure 6C, top row), suggesting that some post-transcriptional regulatory mechanism limits SigH activation in the cell. Examination of the \textit{sigH} DNA sequence revealed a perfect 13 base inverted repeat (IR), lying just upstream from the translation start codon, within the 5' UTR region, likely sequestering the ribosome binding site within a stable stem-loop secondary structure (Figure 6B). We constructed a plasmid expressing \textit{sigH}, with one half of the IR deleted (Figure 6B, pRIT-sigHIRd). Interestingly, all of the cells were now able to express \(\sigma^H\)-dependent GFP (Figure 6C, middle row), both during growth in CS2 and TSB medium, indicating that this IR acts post-transcriptionally to negatively control SigH production. When we replaced the entire 5' UTR region with a synthetic DNA sequence containing a consensus ribosome binding site (GGGAGG) and replacing the TTG initiation codon with ATG (plasmid pRIT-sigH, Figure 6B), we again observed \(\sigma^H\)-dependent GFP expression in all of the cells (Figure 6C), confirming that \textit{sigH} translation is the limiting step, in agreement with the results shown above from the SJ-tandem duplication, where the native translation initiation sequences were replaced in the resulting chimeric genes. Northern blot analysis confirmed that each of the three \textit{sigH} expression plasmids allowed cells to accumulate similar amounts of \textit{sigH} mRNA during growth in TSB (Figure 6D), suggesting that the IR acts post-transcriptionally to negatively control SigH production.

![Figure 4](https://example.com/fig4.png)

**Figure 4.** \textbf{SigH is activated in a minor fraction of the cell population.} Confocal microscopy reveals that SigH-dependent GFP expression is limited to a minor fraction of the cell population. In N315comGFP, the GFP-positive frequency was 1.6±0.6% (mean ± SD, n = 10; Panel A), while 100% of N315aspGFP cells expressed GFP (Panel B). Left panels: Fluorescent GFP signals detected by confocal microscopy. Right panels: Phase contrast images. Middle: merged images. A) N315comGFP grown in CS2 medium. GFP expression is observed only in a minor fraction of the cell population. The maximum frequency of GFP-positive cells was about 1%. B) N315aspGFP grown in BHI medium. All cells express GFP.

doij:10.1371/journal.ppat.1003003.g004
confirming that post-transcriptional control of SigH production at the translational level is the limiting step.

**SigH production allows natural genetic transformation of *S. aureus* cells**

Efforts to detect natural genetic transformation in *S. aureus* have long been unsuccessful. Our findings indicate this is due at least in part to the fact that SigH production is restricted to a minor fraction of the cell population. Having established that growth in CS2 medium was conducive to production of active SigH, we used the strain overexpressing sigH from the pRIT-sigH plasmid and were able to establish for the first time a protocol through which reproducible natural transformation of *S. aureus* cells can be achieved (see Materials and Methods). Cells were grown in CS2 medium and incubated with pT181 plasmid DNA, and we obtained transformation efficiencies between $10^{-3}$ and $10^{-6}$ (Table 1), and could show that all the transformants were the recipient strain carrying the intact plasmid (Supplementary Material Figure S5).

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**Figure 5. Two distinct mechanisms are responsible for SigH activation.** The translation initiation codon of *sigH* is required for SigH activation in CS2 medium, but not for SJ-dependent SigH activation. A) Nucleotide sequence of the *sigH* translation initiation site region. The expected translation initiation codon, and the downstream in-frame TTG codon are underlined. Sequences for the wild type strain (N315ex; top), and the constructed mutant (N315ex-sigH*; bottom) are shown and the mutated residues are shown in lower case. B) Western blot analysis of SigH-dependent GFP production in WT (N315ex) and N315ex-sigH* carrying pMK3-com-gfp. Cells were grown in CS2 medium for the indicated times. No GFP signal was detected in the translation initiation codon mutant strain (N315ex-sigH*). C) Rare GFP expressing cells are still detectable in N315ex-sigH* carrying pMK3-com-gfp.

doi:10.1371/journal.ppat.1003003.g005
In order to establish that natural genetic competence requires not only SigH, but the σH-dependent competence gene orthologues as well, we introduced the SigH expression plasmid into strains lacking either the comE or comG operon (N315Δcom Eh and N315Δcom Gh, respectively). We carried out three independent experiments testing competence of these strains using plasmid pT181 and could not detect any transformants (less than 10^-10; Table 1), indicating that, as expected, both the comE and comG operons are required for natural genetic competence in S. aureus, in addition to SigH.

In order to determine whether natural transformation also occurs using chromosomal DNA, we tested transfer of the MRSA SCCmec element from strain N315, which carries the aadD gene conferring kanamycin resistance, located 3.44 kb away from the meca oxacillin resistance gene (See Supplementary Material Figure S6). The recipient strain, N315ex-h (Km8, Ox8), is a N315 derivative that has lost the SCCmec region and carries the pRIT-sigH plasmid. Cells were grown in CS2 medium, incubated with purified N315 genomic DNA, and transformants were selected for kanamycin resistance (100 μg/ml). PCR analysis revealed that approximately 90% of the kanamycin resistant colonies contained the aadD gene (43/47), suggesting the remainder were spontaneous resistant mutants (Supplementary Material Figure S6A). The aadD transformation efficiency was 3.1x10^-8, and the meca gene was detected in 37 out of the 43 aadD positive clones, demonstrating that co-transformation using chromosomal DNA can be achieved for two distant markers.

Long PCR analysis allowed us to show that these transformants in fact carry the entire 52 kbp SCCmecII element (Supplementary Material Figure S6B). The experiment was repeated twice and gave similar results.

We also tested whether HGT can occur between different cells. We found that the pT181 plasmid can be transferred from COL to N315h when these strains were co-cultured in CS2 medium (Table 1). The efficiencies varied depending on the experiments, likely due to the long co-cultivation time (8 hours). The median transformation efficiency from COL to N315h was 7.2x10^-8 (min = 6.6x10^-9, Q1 = 4.1x10^-8, Q3 = 1.8x10^-7, max = 9.0x10^-7; n = 12). We could also detect rare HGT to wild type N315 (one transformant in 10 independent experiments).

As shown above, natural competence requires both SigH and the SigH-dependent operons comE and comG, which suggests this is an active physiological process and not a physical artefact due to phage tail fragments. Since strains COL and N315 each have resident prophages (φL54a and φN315, respectively), we wished to definitively rule out any possible contribution of phage-mediated “pseudo-transformation”. We therefore eliminated the φN315 prophage from N315ex to generate N315ex w/oφ as described in Materials and Methods. As shown in Table 1 (last two columns), strain N315ex w/oφ carrying pRIT-sigH was transferrable with pHY300 plasmid DNA isolated directly from an E. coli dam^- dcm^- strain (see Materials and Methods), while the vector control strain, N315ex w/oφ pRIT3H, gave no detectable transformants. This experimental system does not contain any phage particles or phage

| Table 1. Transformation frequencies of S. aureus strains. |
|----------------------------------------------------------|
| **Recipient strain**                                     |
| N315h | N315Δcom Eh | N315Δcom Gh | N315 | N315ex w/oφ | N315ex w/oφ |
|        | pRIT-sigH | pRIT3H |
| **Donor** |
| Plasmid DNA (10 μg pT181) |
| ND | ND | ND | ND | ND |
| (n = 1) | (n = 3) | (n = 3) | (n = 3) | (n = 1) |
| 4.0x10^-8 ± 3x10^-8 |
| Plasmid DNA (10 μg pHY300) |
| ND | ND | ND | ND | ND |
| (n = 5) | (n = 9) | (n = 4) | (n = 9) | (n = 3) |
| 1.5x10^-8 ± 6.3x10^-7 |
| Frequencies = number of transformants/cfu of recipient strain; mean ± SD. |
| ND: none detected. |
| doi:10.1371/journal.ppat.1003003.g006 |

Figure 6. SigH activity is restricted to a minor fraction of the cell population through post-transcriptional regulation. A) Overexpression of sigH mRNA is not sufficient for SigH activation. N315ex carrying pMKcomGFP and pRIT-sigHNH7 was grown in CS2, LB, or BHI medium aerobically at 37°C for indicated time periods, and analyzed by Western blot. Only growth in CS2 medium led to GFP production. B) Sequences of the 5′-UTR region in each sigH-expressing plasmid. Arrows show the 13 bp inverted repeat, and the translation initiation codon is underlined. pRIT-sigHNH7 carries the native nucleotide sequence. The inverted repeat sequence was partially deleted in pRIT-sigHIRd, whereas it was entirely removed and the SD sequence replaced with a consensus ribosome binding site in pRIT-sigH. C) Deletion of one half of the inverted repeat allows all cells to produce active SigH. N315ex derivatives carrying the plasmids indicated on the left were grown in CS2 or TSB at 37°C with shaking. Note that all cells of N315ex carrying pRIT-sigHIRd show GFP signals, even in TSB. D) Northern blot analysis confirmed the accumulation of sigH mRNA in cells carrying all of the different sigH expression plasmids. N315ex cells carrying the designated plasmid together with pMKcomGFP, were grown in TSB medium. Exponentially growing cells (OD600nm = 0.5) were used for Northern blot analysis as described in Materials and Methods. The lower panel shows the EtBr stained agarose gel as a loading control.

doi:10.1371/journal.ppat.1003003.t001

ND: none detected.
genes, indicating that SigH-dependent natural competence is phage-independent.

We also demonstrated that unlike natural genetic competence, phage-dependent pseudo-transformation does not require the competence machinery. Indeed, in strain RN4220, which has no resident prophages, we did not obtain any transformants using chromosomal DNA (See Supplementary Material Figure S7). In contrast, when we used RN4220 lysogenized with phage φ11 and the CaCl2 washing procedure, we obtained transformants at a very low frequency (between $10^{-9}$–$10^{-5}$) through phage-mediated “pseudo-transformation” (See Supplementary Material Figure S7). In this same strain, when we deleted either the comG or comE genes (strains RKCG and RKCE, respectively; See Materials and Methods and Table 2), there was no significant difference in the number of transformants obtained (See Supplementary Material Figure S7). This indicates that the comG and comE operons are not required for phage-mediated “pseudo-transformation”, in stark contrast to their essential role in SigH-dependent natural genetic competence as shown in Table 1.

Taken together, our results have allowed us to demonstrate for the first time that natural genetic competence develops in a SigH-dependent manner in *Staphylococcus aureus*, allowing transformation by extracellular plasmid or chromosomal DNA as well as HGT between different strains.

**Discussion**

Since F. Griffith's pioneering discovery of DNA-mediated transformation in *Streptococcus pneumoniae* [41], natural genetic competence in low GC % Gram-positive bacteria has been extensively studied in *Bacillus subtilis* and *S. pneumoniae* and shown to involve the assembly of a complex DNA-binding and uptake machinery, made up of a competence pseudolipid and a DNA translocase [13,16]. During the 1970’s, although several reports described “pseudo-transformation” of *S. aureus*, this was revealed to be due in fact to contaminating phage tail fragments mediating DNA entry and HGT [15]. Despite many subsequent attempts, natural genetic competence was never successfully demonstrated in *S. aureus* even though sequence analysis readily reveals that its genome carries a practically full repertoire of the required competence gene orthologues, suggesting that specific conditions must exist allowing natural transformation by DNA in *S. aureus*. These genes include those encoding the pseudolipid proteins (major pseudolipin ComGC, minor pseudolipins ComGD and ComGE, prepilin peptidase ComC, the ComGB membrane protein and ComGA NTPase) as well as the DNA translocase apparatus (ComEA membrane DNA receptor, ComEC cytoplasmic channel and ComFA ATP-binding protein), displaying amino acid sequence identities ranging from 20 to 70% with the corresponding proteins of *S. pneumoniae* or *B. subtilis* (See Supplementary Material Figure S1).

Strong similarities and interesting differences exist between the competence pathways of *B. subtilis* and *S. pneumoniae*. Indeed, although in both cases the initial triggering event involves a peptide quorum-sensing two-component signal transduction pathway controlling expression of competence genes encoding the DNA uptake machinery, the steps in between are quite different [16,42,43]. In *Streptococcus* species, competence genes are regulated by ComX (aka SigX) [30], a secondary sigma factor related to staphylococcal SigH [18] and encoded by duplicated genes (comX1 and comX2) whose expression is directly controlled by the ComDE two-component system [30]. Interestingly, in *B. subtilis*, late competence genes are transcribed by the vegetative form of RNA polymerase holoenzyme, $E_{75}$, and instead positively controlled by a specific transcription activator, ComK [44]. Other similarities between the two bacteria include the fact that many additional factors play a part in the production of active ComK or ComX, both of which involve two-component signal transduction networks [42,43,45] and the post-transcriptional control of the levels of these two regulatory proteins by the Clp ATP-dependent protease [43,45–51].

In *S. aureus*, the situation appears to be more closely related to that of *S. pneumoniae*. Indeed, although a protein bearing some similarities to ComK is present (SA0892), we have previously identified the SigH secondary sigma factor, analogous to ComX, and shown that it acts specifically to direct transcription of the comE and comE operons that encode orthologues of the DNA uptake machinery [18].

Whereas in *S. pneumoniae* all of the cells become competent for a short period in time, in *B. subtilis* only a maximum of 10% of the cell population achieves competence [43], a fact that has been attributed to the positive autoregulatory feedback loop controlling comK expression, generating a heterogeneous bistable response in the cell population [52–54]. However, it is important to recall that natural undomesticated strains of *Bacillus subtilis* are in fact considered to be non-competent [55], and that transformation of *B. subtilis* at levels of 10% could be demonstrated only for a few strains isolated following extensive UV and X-ray mutagenesis [56,57], with the highly transformable 168 strain then chosen for most studies [58]. *B. subtilis* strains derived from 168 rapidly became “domesticated” once exposed to the accelerated lifestyle imposed in the laboratory, accumulating multiple mutations affecting competence development and biofilm formation [59–61].

Thus, the situation for *Staphylococcus aureus* appears highly reminiscent of that of undomesticated *B. subtilis*, with a cryptic DNA uptake apparatus presumably allowing only a very low level of natural transformation in its natural habitat, with the possibility that specific conditions may be required for competence development. Limiting the number of competent recipient cells in a population would be important to sustain genome integrity, minimizing risks and maximizing evolutionary gain by allowing only a fraction of the cells to access genetic variability. Among the many barriers to uptake of foreign DNA, restriction-modification systems are known to play an important role [62–64], and tight control of competence gene expression is also required to limit potentially detrimental HGT with other species [43]. In this respect it is tempting to speculate, given the observed population heterogeneity with respect to SigH activity, that natural competence in *S. aureus* has evolved as a bet hedging strategy [65], with most of the cells protected against the dangers of HGT, while a fraction are able to increase genetic variability through natural genetic competence.

As ComK and SigX (ComX) are the end products of the regulatory cascades controlling competence development in *B. subtilis* and *S. pneumoniae*, several attempts have been made to overproduce these proteins in non-competent bacteria in order to obtain genetic transformation. In *Streptococcus pyogenes*, which is not known to become competent, SigX has been shown to control expression of femB and cinA [50], as well as competence gene orthologues [66]. Likewise, overproduction of SigX in *Lactococcus lactis* also led to increased expression of competence gene orthologues [20]. The recent discovery among *Streptococcus* species of a second quorum-sensing pathway allowing activation of sigX expression [67–69] has led to the suggestion that members of the pyogenic streptococci group may in fact be able to develop competence under specific conditions [68,70].

In a similar approach, overproduction of the ComK transcription activator from *B. subtilis* was used to induce competence in
**Table 2. Staphylococcus aureus strains and plasmids used in this study.**

| Strain or plasmid | Description | Source |
|-------------------|-------------|--------|
| **Strains** | | |
| COL | MRSA, carrying tetracycline resistance plasmid pT181 | [86] |
| COLw/oQ | COL strain cured of the QL54a prophage | This study |
| N315 | pre-MRSA, KmR, ErmR | [87] |
| N315h | N315 carrying pRIT-sigH | [18] |
| N315comGFP | N315 carrying pRIT-com-gfp | This study |
| N315aspGFP | N315 carrying pRIT-asp-gfp | This study |
| N315-GFP | N315 carrying pRIT-gfp | This study |
| N315ex | SCCmec cured derivative of N315, KmR | [88] |
| N315ex-dr | N315ex pTet-rep pMK3-com-gfp | This study |
| N315ex-sigH* | sigH translation initiation site mutant of N315ex | This study |
| N315ex-h | N315ex carrying pRIT-sigH | This study |
| N315ex w/oQ | N315ex cured of the QL54a prophage | This study |
| N315ex w/oQh | N315ex w/oQ carrying pRIT-sigH | This study |
| N315ex w/oQv | N315ex w/oQ carrying pRIT-sigH | This study |
| NKSH | sigH disruptant of N315 | This study |
| NKSHh | NKSH carrying pRIT-sigH | This study |
| N315ΔcomE | N315 ΔcomE mutant | This study |
| N315ΔcomEh | N315 ΔcomE pRIT-sigH | This study |
| N315ΔcomG | N315 ΔcomG mutant | This study |
| N315ΔcomGh | N315 ΔcomG pRIT-sigH | This study |
| 8325-4 | nonlysogenic derivative of strain 8325N | [89] |
| RN4220 | derivative of 8325-4, restriction minus, modification plus | [90] |
| RH | RN4220 carrying pRIT-sigH | This study |
| RNNet-rep | RN4220 carrying pNet-rep | This study |
| RKCG | RN4220 ΔcomG mutant, CmR | This study |
| RKCE | RN4220 ΔcomE mutant, CmR | This study |
| A, B, C | SigH active mutants derived from RNNet-rep | This study |
| A2, B1, C1 | plasmid cured strains from A, B, and C | This study |
| A2b, B1b, C1b | A2, B1, C1 carrying pRITPcomEbga | This study |
| A2-r, B1-r, C1-r | SigH inactive revertant from A2b, B1b and C1b | This study |
| MRSA408 | MRSA, clinical isolate in Japan | [80] |
| **Plasmids** | | |
| pHY300PLX | shuttle vector, ori-pAMr-1, AmpR (E. coli), TelR (S. aureus) | Takara, Japan |
| pT7gfp | source of gfp gene, AmpR (E. coli) | [81] |
| pMK3 | shuttle vector, ori-pE194, AmpR (E. coli), KmR (S. aureus) | [82] |
| pMK3-com-gfp | PcomG-gfp transcriptional fusion in pMK3 | This study |
| pRIT5H | shuttle vector, ori-pC194, AmpR (E. coli), CmR (S. aureus); used to make pRIT derivatives | [80] |
| pRIT-sigH | sigH overexpressing plasmid, Pisa, SD sequence modified into SigA type sequence | [18] |
| pRIT-sigHNH7 | sigH overexpressing plasmid, Pisa | This study |
| pRIT-sigHNHd | sigH overexpressing plasmid, half of IR deleted, Pisa | This study |
| pRIT-com-gfp | PcomG-gfp transcriptional fusion | This study |
| pRIT-asp-gfp | Pasp23-gfp transcriptional fusion | This study |
| pRIT-gfp | promoter-less gfp | This study |
| pRIT-PcomE-bgaB | PcomE-bgaB transcriptional fusion | This study |
| pNet-rep | PcomG-tet, PcomE-bgaB; see Figure 1A | This study |
| pMAD | ori-pE194r; AmpR (E. coli), ErmR (S. aureus), Pcpd8-bgaB | [79] |
| pMAD-tet | pMAD derivative, AmpR (E. coli), ErmR, TETR (S. aureus) | This study |
| pMADtetcomGII | vector for deletion of comG locus, AmpR (E. coli), ErmR, TETR (S. aureus) | This study |
otherwise non-competent bacteria. This approach was successful both in undomesticated strains of B. subtilis [55] as well as in Bacillus cereus, previously considered to be non-competent [71,72]. Interestingly, B. cereus carries two copies of the comK gene, reminiscent of the comX situation in S. pneumoniae, although ComK1 and ComK2 appear to play different roles [73]. A recent report reveals that reconstitution of an intact comK gene in Listeria monocytogenes, leads to expression of competence gene orthologues which play a role in phagosomal escape and virulence, although genetic competence was not tested [74].

In this study, we have successfully shown for the first time that S. aureus can develop natural genetic competence for transformation by plasmid or chromosomal DNA, albeit in a minor fraction of the cell population, and that this requires both SigH and the competence genes it controls. In addition to stochastic activation of SigH production, which occurs under specific nutritional conditions during growth in chemically defined CS2 medium, we also showed that expression of sigH is controlled post-transcriptionally and that this process involves an inverted repeat sequence that likely acts to sequester the sigH ribosome binding site (Figure 7). We cannot exclude that this post-transcriptional control may involve a small regulatory non-coding RNA molecule. Using a positive selection screen, we could show that short junction (SJ) tandem duplication [40] occurred through a chromosomal rearrangement, duplicating the sigH gene as a translational fusion with either of the downstream rpsG or rplK genes and effectively relieving expression of the chimeric gene from the post-transcriptional control of the sigH 5’ UTR IR element (Figure 7). This process does not appear to be RecA-dependent, since we saw no significant difference in SJ duplication frequencies when cells were treated with Mitomycin C, an efficient inducer of the SOS response (data not shown).

SJ-duplication alters DNA information but is generally unstable and easily cured at higher frequencies than the duplication event, as we show here. To the best of our knowledge, this type of SJ-duplication in the sigH locus is the first example in bacteria that generates an in-frame chimeric fusion for gene activation. It remains to be determined whether this type of SJ duplication can occur at any place in the genome in addition to the sigH locus. We suggest that the sigH gene may be considered as a type of “contingency” locus [75], with a higher mutation rate allowing adaptation to specific deleterious conditions by favoring HGT through DNA uptake. This could explain the mechanism generating what we have termed the “similarity gulf” [20]: although the sigH gene itself is poorly conserved between different bacterial species, it is nevertheless embedded within a conserved surrounding genetic structure across several bacteria [28].

In S. aureus, sigH has also been reported to direct transcription of phage integrase genes to stabilize the lysogenic state [76]. In the present study, we have shown that SigH is required for competence development in a minor fraction of the cell population. SigH may also protect the subpopulation from phage-induced lytic death, allowing survivors to utilize dead cell materials, including DNA, with a higher probability of acquiring new genes through HGT.

Our demonstration that natural genetic transformation of S. aureus cells occurs in a SigH-dependent manner helps provide an explanation for the notorious acquisition of antibiotic resistance genes by this major pathogen. Indeed, even at very low natural competence levels, the selective pressures would ensure the survival and rapid spread of strains acquiring antibiotic resistance genes, as currently observed for MRSA strains [7].

Importantly, we showed that a large chromosomal region conferring methicillin resistance (SCCmec typeII) could be transferred by transformation. Although it has been suggested that SCC elements less than 45 kb in size may be transferred by phage transduction, no transfer mechanism is known for larger elements such as SCCmec typeII. However, it should be noted that we used N315ex cell as the recipient, which had lost the SCCmec element, resulting in a MSSA phenotype. The transfer of SCCmec into naive MSSA strains needs to be tested, but will require optimization of the transformation protocol. Further efforts will be aimed at improving transformation frequencies in S. aureus reference strains and understanding the molecular mechanisms involved in the transcriptional and post-transcriptional constraints that have evolved to limit SigH activity to a minor fraction of the cell population. The analysis of the IR sequence in the sigH mRNA will also likely play a key role in unraveling the molecular switch involved in controlling competence development.

### Materials and Methods

#### Bacterial strains and culture conditions

S. aureus strains and plasmids used in this study are listed in Table 2. S. aureus was grown in Brain Heart Infusion (BHI) medium, Trypticase Soy Broth (TSB), Nutrient Broth No. 2 (OXOID) supplemented with 3.6 mM CaCl₂ (NBCaCl₂), RPMI 1640, or complete synthetic media (CS1, -GS, and CS2). CS1 medium was based on a previous report [77]. The -GS medium was the same as CS1, omitting glycine and serine. CS2 synthetic medium was the same as HHWm medium [78] with the following modifications: 30 mg/l guanine, 15 mg/l adenine hemi-sulfate, 8.9 mg/l CaCl₂, 0.08 mg/l CuSO₄, 0.17 mg/l ZnSO₄, 0.12 mg/l CoCl₂·6H₂O, 0.12 mg/l Na₂MoO₄·2H₂O (See Supplementary Material Table S1 for full composition).

When using synthetic media, cells were collected from overnight cultures by brief centrifugation, and washed with the appropriate medium to be inoculated. For static anaerobic growth, a 2-ml Eppendorf tube was filled with the medium, and capped to prevent air exchange. For anaerobic growth with shaking, a
100-ml flask containing 50 ml medium was placed in a polyethylene bag together with an AnaeroPack (Mitsubishi Gas Chemical).

Construction of a positive selection dual reporter plasmid

Oligonucleotides used in this study are listed in Table 3. The comE promoter region was amplified with oligonucleotides comEpMlu and comEpKpn2, digested with MluI and KpnI, and cloned between the corresponding restriction sites of the pMAD shuttle vector [79], replacing the clpB promoter with the comE promoter. The resultant PcomE-bgaB transcriptional fusion cassette was excised by BamHI and SstI digestion, and cloned into the SphI site of pRIT5H [80] by blunt ligation to generate the pRIT-PcomE-bgaB plasmid. The resultant PcomE-bgaB transcriptional fusion cassette was excised by BamHI and SstI digestion, and cloned into the SphI site of pRIT5H [80] by blunt ligation to generate the pRIT-PcomE-bgaB plasmid. The tet gene was amplified from pHY300PLK (Takara) with oligonucleotides tetF(Eco) and tetR(Pst), digested by EcoRI and PstI, and ligated between the EcoRI - PstI sites of pRIT-PcomE-bgaB (See Supplementary Material Figure S8 for plasmid maps of pMAD-PcomE and pRIT-PcomE-bgaB). The resulting plasmid was then treated with NdeI and EcoRI, and ligated with the comG promoter fragment (PcomG), amplified with PcomG(SalI) and PcomG(EcoRI), and digested with NdeI - EcoRI. The ligation mixture was used directly to transform strain RN4220 by electroporation to generate strain RNtet-rep. The structure of the resultant tet-reporter plasmid (pTet-rep) is illustrated in Figure 1A and confers resistance to 12.5 μg/ml chloramphenicol.

Isolation of SigH active cells and revertants

RNtet-rep and other reporter strains were grown in BHI containing 12.5 μg/ml chloramphenicol at 37°C overnight. 100 μl aliquots were inoculated into 100 ml drug-free TSB and grown at 37°C with shaking until the stationary phase. About 2×10^9 cells and tetracycline (5 μg/ml) were mixed into TSB-agar (TSA) at 50°C. The mixture was immediately poured into sterile plates and solidified at room temperature. The plates were incubated at 37°C for 2 days. Colonies were replicated on TSA plates containing 5 μg/ml tetracycline and 100 μg/ml X-gal. Percentages of blue colonies were determined to calculate the SigH activation frequency.

Three SigH active clones generated from RNtet-rep were selected for further analysis. To eliminate the reporter plasmid, they were submitted to a few cycles of growth at 42°C and 37°C in drug free TSB medium, and plated onto TSA containing 100 μg/ml X-gal. White colonies with the expected phenotypes (tet S,c mS) were selected as plasmid cured strains, and designated A2, B1, and C1. pRIT-PcomE-bgaB was introduced into by electroporation into these strains to confirm stable SigH activation, giving strains

Figure 7. Competence development in Staphylococcus aureus involves two distinct mechanisms. A rare SJ-duplication mechanism generates a new chimeric sigH gene, and SigH is produced as a fusion protein. The duplication is cured at a high frequency, concomitant with the loss of gene duplication. Under the specific culture conditions used, SigH was expressed stochastically at a frequency of ca. 10^{-2}, through a post-transcriptional regulatory mechanism. The inverted repeat sequence upstream of the translation initiation site prevents sigH expression, likely forming a secondary structure trapping the ribosome binding site, and/or serving as a post-transcriptional regulatory target, restricting SigH activation to a minor fraction of the cell population. SigH active cells express genes for DNA-binding and uptake machinery and become competent for DNA transformation.

doi:10.1371/journal.ppat.1003003.g007
| Name         | Nucleotide sequence               |
|--------------|-----------------------------------|
| 1765R        | TTTCGTGGCCACCAACCCACCCACCCTTTTT |
| 1765F        | CCTTGGTTCTGATTCGGAAAGGGTTTGGAA  |
| 1792ssbR     | TTTGCTGCACATCTTGGACATGTCCAGAAG  |
| 1792ssbF     | ATTATTCTGATTCGGAAAGGGTTTGGAA   |
| 3.0-R        | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| 492uF        | CCTTGGTTCTGATTCGGAAAGGGTTTGGAA |
| 492uR        | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| 492uRf       | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| 492uRfF      | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| 492uRfR      | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| 492uRfRf     | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| 492uRfRfF    | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| agr-up-f     | TATATATATATATATATATATATATATAT |
| agr-up-r     | TATATATATATATATATATATATATATAT |
| agr-D-F(ecOR) | GCCGAATTCATGTCCAGAAGGGTTTGGAA |
| agr-down-r(bgl2) | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| ccrA-F       | ACATATATATATATATATATATATATATAT |
| ccrA-R       | CTATATATATATATATATATATATATATAT |
| comEpMu      | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| comEpKp2     | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| comG1(SalI)  | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| comG2(BglII) | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| down-att     | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| ErmA1        | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| ErmA2        | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| gfp-F(BamHI) | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| gfp-R(PstI)  | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| H1p          | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| H2p          | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| H5p          | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| IFcomG1       | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| IFcomG2       | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| lena007       | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| lena008       | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| mecAF         | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| mecAR         | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| NKcomG-U1(Bam) | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| NKcomG-U2(Bam) | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| NKcomG-DF(Hind) | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| NKcomG-D(Hind) | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| NKcomE-UF(Bam) | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| NKcomE-UR2(Bam) | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| NKcomE-DF(Hind) | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| NKcomE-DR(Hind) | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| PcomGe(EcoR)  | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| PcomGe(Sallp) | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| SA0492Rp      | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| SA1374up-f(Ndel) | TTTTGAATTCCTTTGACATGTCCAGAAG  |
| SA1374up-R(BamHI) | TTTTGAATTCCTTTGACATGTCCAGAAG  |
Table 3. Cont.

| Name               | Nucleotide sequence |
|--------------------|--------------------|
| SA1986up-f(Ndel)   | TTTCCATATGTTATTTAAACATCGTGACAGCCAG |
| SA1986up-r(Bam)    | CCGGGATCCAGGATAAAAGTTTTAAGTCGT |
| sigH(D(Sal))      | CCTAAGTCGACTTCTCAAAACATTTT |
| sigH(f(Eco))      | CGAATTCCTTGAAATTTTGAATGTAAC |
| sigH(F(+))        | GTCTAGAAGATAGAAGGGTTAGT |
| sigH(f(Eco))      | AAGAATTCTTAAATTTTGAATAGG |
| NKS H-F(BamHII)   | CCGGGATCCATATTGCAACCTCAAGCA |
| NKS H-R(HindIII)  | CCCAAGCTTCTATTTGTCACACATTAATAA |
| tetF(Eco)         | CTGAATTCATATTGCAAGGGTTGAA |
| TetF(Sal)         | CATATTGTCGACTAATGATGAAATACAG |
| TetR(EcoR)        | GGAATTCCTGTATATAAAAAAGGATCAAT |
| TetR(Xho)         | GAACTCGAGTTATAAAAAAGGATCA |
| the(AttR)         | AACTGCGATTATAAAAAAGGATCA |
| up-att            | CGAATTCGGAACTTGATGATGAAAA |
| Xsa u 235         | GGAATTCAGGGCTCGCACA |

doi:10.1371/journal.ppat.1003003.t003

A2b, B1b, and C1b. In order to isolate revertants of SigH activity, strains A2b, B1b, and C1b were grown in drug free BHI medium, and plated on TSA containing 100 μg/ml X-gal and 12.5 μg/ml chloramphenicol. White colonies were tested by Southern blot analysis for the loss of sigH duplication.

Fluctuation test

Strains RNtet-rep and N315 carrying pTet-rep were grown overnight at 37°C in TSB containing 12.5 μg/ml chloramphenicol. A 1.4 μl aliquot was used to inoculate 140 ml of drug-free TSB. 2 ml aliquots were distributed into 15~20 test tubes and grown overnight at 37°C, while 100 ml was grown in a single 500-ml flask. One ml of the culture was harvested from each test-tube or flask, and mixed with TSA containing 5 μg/ml tetracycline at 50°C. Numbers of SigH active cells (tetR, β-galactosidase positive) were then determined as described above.

Southern hybridization analysis

*S. aureus* genomic DNA was purified using standard procedures. One μg of DNA was digested with HindIII or PstII and separated by electrophoresis on a 1% agarose gel. The separated DNA fragments were transferred to a Hybond-N membrane (Amer sham Biosciences) by Southern blot. Analysis was carried out using the AlPhos Direct labeling kit and the CDP star detection reagent system according to the manufacturer's instructions (Amer sham Biosciences). DNA fragments used to prepare the probes were amplified by PCR with oligonucleotides H1p and H2p for the upstream region, and H5p and SA0492Rp for the sigH coding sequence.

Construction of GFP reporter strains

A 746 bp DNA fragment carrying the gfp (green fluorescent protein) gene with its Shine Dal gino sequence, GGGAGG, was amplified by PCR from the pT7gfp vector [81] using oligonucle otides gfp-F(BamHII) and gfp-R(PstI). Promoter regions were PCR-amplified from N315 using oligonucleotides SA1374up-F(NdeI) and SA1374up-R(BamHII) for comG (positions −253 to +65 from the translational start site), or SA1986up-f(Ndel) and SA1986up-r(Bam) for asp23 (−208 through +65). The promoter region and gfp DNA fragments were digested with the appropriate restriction enzymes and cloned together between the NdeI-PstI sites of the pRIT3H shuttle vector. The resultant plasmids, pRIT-com-gfp and pRIT-asp-gfp, carry transcriptional fusions between the comG or asp23 promoter regions and the gfp gene. These plasmids were introduced into *S. aureus* N315 by electroporation to generate strains N315comGFP and N315aspGFP. The negative control plasmid, pRIT-gfp, has no promoter sequence.

To construct pMK3-comGFP, the comG promoter and gfp region from pRIT-com-gfp was amplified by PCR with oligonucleotides PcomG(Sal)p and gfp-R(PstI), and ligated between the SalI-PstI sites of the pMK3 shuttle vector [82].

Western blot analysis

Total protein extracts from *S. aureus* cells were prepared as previously described [18], except that cells were suspended in a buffer containing 20 mM Tris-Cl (pH7.5), 5 mM EDTA, 1 mM PMSF, and 0.05 mg/ml lysostaphin. After cell wall degradation, SDS was added at a final concentration of 1%. Fifteen μg of total proteins were separated by SDS-PAGE and transferred onto PVDF membranes. Western blot detection of SigH was previously described [18]. For GFP detection, the membrane was incubated with 2 μg/ml of BD Living Colors Peptide Antibody (BD Biosciences) for 16–20 h at 4°C. After a wash with TBST, the membrane was incubated in the presence of 5 μg/ml of Goat Anti-Rabbit IgG Antibody conjugated with HRP (Promega) at 25°C for 30 min. Immuno-reactive bands were detected using the ECL plus Western Blotting Detection System (Amersham Biosciences). For Figures 5C and 6B the blot was treated with 10 μg/ml Anti-GFP IgY (Aves Labs) as the first antibody and 0.7 μg/ml of Anti-Chicken IgY HRP Conjugate (Promega) as the second antibody by using the SNAPid Protein Detection System (Millipore), and signals were detected using the Amersham ECL Western Blotting Detection System (GE Healthcare).
Confocal microscopy
Confocal imaging of cells was performed using a confocal laser-scanning microscope (TCS-SP2, Leica Microsystems). GFP was excited at 488 nm using the blue laser and fluorescence images were collected using the green channel. All fluorescence images were obtained with the same settings. Serial optical sections were obtained at about 0.6 μm intervals and three of them per image were reconstituted using the LCS Lite software (Leica Microsystems).

Construction of the NKSH sigH inactivation mutant
The sigH gene was inactivated by insertional mutagenesis in strain N315 using the method previously described [83]. The sigH insertional vector was constructed using the pKILts plasmid and a sigH DNA fragment (nucleotides -1 to +473) amplified by PCR with oligonucleotides NKSH-F(BamHI) and NKSH-R(HindIII). The correct homologous recombination in the resulting sigH inactivation mutant, strain NKSH, was confirmed by PCR and Southern blot analysis. The pRIT-sigH [18] plasmid was introduced into NKSH sigH mutant to generate the complemented strain NKSHh.

Construction of sigH expression plasmids
Construction of pRIT-sigH was previously described [18]. The pRIT-sigHNNH7 plasmid was constructed by inserting a PCR fragment amplified using oligonucleotides sigH-(Eco) and sigH-D(Sal) between the EcoRI-SalI sites of pRIT5H. For pRIT-sigHNRd construction, primers sigH2f(Eco) and sigH-D(Sal) were used.

Northern hybridization analysis
Cells were harvested by centrifugation at 5,000 × g at 4 °C. Total RNA was extracted as previously described [83]. For Figure 6D, total RNA was treated with TurboDNA-free (Ambion) to eliminate any trace of the vector DNA. Fifteen μg (for Figure 3C) or 5 μg (for Figure 6D) of total RNA was separated on a 1% agarose-formamide denaturing gel and transferred onto a Hybond N+ membrane (Amersham Biosciences). In Figure 3C, DNA fragments prepared by PCR from the N315 genomic DNA were labeled with [α-32P]-dCTP by random priming using the Ready-to-Go DNA labeling Beads (Amersham Biosciences), and used as probes. Hybridizations were carried out at 60 °C in a solution containing 5× SSPE, 5× Denhardt’s solution, 0.5% SDS, and 20 μg/ml of salmon sperm DNA, and the final washing condition was at 50 °C in 0.1× SSC and 0.1% SDS. In Figure 6D, probe preparation and detection was carried out using AlkPhos Direct Labelling and Detection System with CDP-Star (GE Health Care). The sigH PCR fragment amplified with sigH-F(−) and SA0492Rp was used as a probe.

Site-directed mutagenesis of sigH translation initiation codons
Potential translation initiation codons (TTG) of sigH were changed to Ochre stop codons (TAA) by PCR-based site-directed mutagenesis [illustrated in Figure 5B]. Two oligonucleotides, 492aRbgII and 492dBrgII, were designed to include these mutations and a BglII site. The tet gene was amplified with oligonucleotides Tet-F(Sal) and Tet-R(EcoRI) using pHY300PLK (Takara) as a template, and ligated between the EcoRI-SalI sites of pMAD to generate pMADtet (See Supplementary Material Figure S8 for plasmid map). Oligonucleotides 492ufSmal & 492uRbgII were used to amplify the sigH upstream region, and 492dFbgII & 492dRbgII for the downstream region. These fragments were sequentially ligated between the Smal-BglII sites of pMADtet to generate the pMADtet492 targeting plasmid. This was introduced into N315sex by electroporation. Mutants (tetracycline sensitive, β-galactosidase negative) were selected as previously described [79], and designated N315ex-sigH*. The introduced mutations were confirmed by direct sequencing analysis.

Construction of comG and comE mutants
Deletion/replacement mutants of the comG and comE regions were constructed by double-crossover homologous recombination. The targeting cassettes were constructed using plasmid pKILts-cat [84]. Oligonucleotide pairs used to amplify DNA fragments encompassing the comG (from SA1374 through SA1365) and comE regions (from SA1410 through SA1416) were: NKcomG-U1(Bam) and NKcomG-U2(Bam) for the comG upstream region; NKcomG-Df(Hind) and NKcomE-Df(Hind) for the comE downstream region; NKcomE-U(Bam) and NKcomE-UR2(Bam) for the comE upstream region; NKcomE-Df(Hind) and NKcomE-DR(Hind) for the comE downstream region (Table 3). The comG targeting cassette constructed in pKILts-cat was amplified by PCR with oligonucleotides comG1(Sal) and comG2(Bgl) and cloned between the SalI-BglII sites of pMAD [79]. The comG and comE deletion/replacement mutant strains (RKCG and RKCE) were then constructed in strain RN4220 by integration/excision as previously described [79,84]. The absence of each region was confirmed by PCR and Southern blot analysis.

Markerless comG and comE deletion mutants of N315 were also constructed using the pMAD vector. The chloramphenicol resistance gene was first eliminated from pMADcomG by inverse PCR using oligonucleotides IFComG1 and IFComG2, followed by self-circularization using the In-Fusion system (Clontech). The tetracycline resistance gene was amplified from pHY300PLK using oligonucleotides TetF(Sal) and TetR(Xho), and inserted into the SalI site to generate the comG locus targeting vector, pMADtetcomGII. To construct the comE targeting vector, the upstream and downstream regions were amplified by PCR using oligonucleotides 2EUf(Eco) and 2EUR(Bgl) (upstream), and 2EDf(Bgl) and 2EDR(Bgl) (downstream). These fragments were sequentially inserted into the EcoRI-BglII sites and the BglII site of pMAD-tet, respectively, to generate pMADcomEII. Plasmids were introduced into strain N315 by electroporation, after passing through strain RN4220. Mutants (tetracycline sensitive, β-galactosidase negative) were selected as described above. The N315ΔcomX and N315ΔcomG mutants lack the same region deleted in strains RKCG and RKCE, respectively, but have no antibiotic resistance marker at the deleted locus.

Natural transformation of S. aureus cells
The φL54a prophage was cured from the COL strain by ultraviolet light treatment as described [83]. The resulting strain, COLw/oφ, was verified for φL54a susceptibility and restored lipase activity as described. Plasmid pT181 DNA was then purified from strain COLw/oφ, using the QiAfilter Plasmid Midi kit (QIAGEN).

Competent S. aureus recipient cells were prepared by overnight growth in TSB containing chloramphenicol (12.5 μg/ml) with shaking at 37 °C. Cells were harvested from five hundred μl of overnight culture, washed with CS2 medium, resuspended in 10 ml of CS2 medium and grown at 37 °C with shaking. After 8 hours, cells were harvested by centrifugation, and resuspended in 10 ml of fresh CS2 medium. Ten μg of plasmid DNA (pT181 or pHY300 isolated from E. coli HST04 dam(-)/dcm-) was added to the suspension, and incubation was pursued at 37 °C with shaking for 2 hours. Cells were mixed into melted BHI-agar pre-cooled to
55°C together with 5 μg/ml tetracycline and 5 μg/ml erythromycin, and incubated at 37°C for 2 days. Colonies were tested for their characteristic to confirm that they were bona fide recipient cell transformants (e.g. kanamycin resistance and plasmid species). For transformation with chromosomal DNA, 10 μg of N315 genomic DNA was added to the cells. Transformants were selected with 100 μg/ml kanamycin (see Supporting Material Figure S3).

Strain-to-strain plasmid transformation
Cells were grown overnight in TSB with shaking at 37°C. One hundred μl of donor (COL) and 400 μl of recipient cells (N315 derivative) were mixed and washed with CS2 medium. Cells were resuspended in 10 ml of CS2 medium, and grown at 37°C for 8–10 hours with shaking. CFU values of N315 derivatives (larger colonies than COL derivatives) after co-cultivation were counted on drug-free BHI-agar plate. Co-cultivated cells were mixed into melted BHI-agar pre-cooled to 55°C together with 5 μg/ml tetracycline and 5 μg/ml erythromycin, and incubated at 37°C for 2 days. Colonies were tested for susceptibility to kanamycin to verify the transformants and kanamycin resistant clones were regarded as N315 derivatives.

Elimination of φN315 from strain N315ex
The φN315 prophage is integrated at an att site located within the hlb β-hemolysin gene, inactivating the gene and abolishing beta-hemolysis. We used the pMAD system to precisely excise φN315 from the N315 genome. A set of primers, up-att and down-att, was designed upstream and downstream of hlb (See Table 3). The target region encompassed by these primers is 46 kbp when hlb was designed upstream and downstream of β-hemolysin. We used the pMAD system to precisely excise φN315 (See Table 3). The latter fragment was amplified from N315 genomic DNA and cloned in a minor fraction of the cell population, and that the intact hlb gene can be amplified by PCR (data not shown). The latter fragment was amplified from N315 genomic DNA and cloned between the EcoRI and BglII sites of pMAD-tet, generating pMAD-tet-att. The plasmid was then introduced into strain N315ex and the phage-cured N315ex strain was selected by repeated integration/excision cycles of pMAD-tet-att as previously described [79]. In addition, beta-hemolysin activity was also monitored to verify the successful phenocconversion. The absence of the SA1765 and SA1792 (ssb) phage genes in the resultant N315ex w/oφ was confirmed by PCR (primers 1765F and 1765R for SA1765 and 1792ssbR and 1792ssbF for ssb) and Southern blot analysis using the 1765F-1765R PCR-generated DNA fragment as a probe.

Pseudo-Transformation assays
Phage particle-dependent pseudo-transformation assays were carried out based on the CaCl2 washing method previously described [77] with some modifications. In brief, S. aureus cells were grown in TSB medium at 37°C overnight with shaking (180 rpm, BR-23UM: TAIITEC). Cells were recovered by centrifugation and washed once with 0.1 M Tris-malate (pH 7.0). The cells were resuspended in 0.1 M Tris-malate (pH 7.0) supplemented with 0.1 M CaCl2. 24 μg of purified N315 genomic DNA was added to 1 ml of the cell suspension, and incubated at room temperature for 40 min. Cells were recovered by centrifugation and suspended in drug-free BHI medium. Following 1 h incubation at 37°C with shaking, cells were mixed with molten BHI-agar medium pre-cooled to 55°C and supplemented with 5 μg/ml erythromycin and poured into plates. After two days incubation at 37°C, colonies were counted and checked for the presence of the em gene by PCR with primers ErmA1 and ErmA2.

Supporting Information
Figure S1 Comparison of competence genes and their organization in Staphylococcus aureus, Bacillus subtilis and Streptococcus pneumoniae.
(TIF)
Figure S2 Summary of the frequencies of SigH active TetR BgaB-positive colonies in several independent experiments and different genetic backgrounds. Reporter strains carrying the positive selection tetracycline resistance (tet) reporter plasmid (pTet-rep) were grown in drug-free TSB and then selected with 5 μg/ml tetracycline. Shown is the summary of independent experiments from different cultures, while the data in Figure 1B is from a single overnight culture showing a lower fluctuation.
(TIF)
Figure S3 Nucleotide sequences of SJ duplication junctions in sigH chimeric gene fusions.
(TIF)
Figure S4 Western blot analysis of SigH-dependent GFP production under different growth conditions. A Western blot analysis of SigH-dependent GFP production in strain N315comGFP. Cells were grown at 37°C in -GS or CSM medium for 24 or 48 hours with or without shaking under aerobic (upper panel) or anaerobic (lower panel) conditions. Results with the highest induction efficiency are shown, with a tendency indicating that static growth rather than shaking could enhance SigH-dependent GFP production. The removal of glycine and serine from CS1 medium improved SigH activation (-GS medium). B Time course of GFP induction in N315comGFP cells during anaerobic static growth in -GS medium. Cells were grown in separate tubes and harvested at the indicated times, suggesting that GFP induction seems to be a specific cellular response rather than a spontaneous event.
(TIF)
Figure S5 Plasmid profile verification of S. aureus transformants. S. aureus cells were transformed with plasmid pT181, and plasmids were purified from twelve transformants using the GeneElute Plasmid Miniprep Kit (Sigma) and submitted to agarose gel electrophoresis. Strain N315 carries an endogenous 24.6 kb plasmid, pN315. Strain N315h carries the pRTIsigH SigH production plasmid in addition to pN315. M: λ HindIII DNA fragments.
(TIF)
Figure S6 PCR-based verification of SCCmecII element in S. aureus chromosomal DNA transformants. A) PCR verification of transformants for the horizontal transfer of chromosomal determinant. The SCCmecII element of N315 carries the adaD gene for kanamycin resistance (within pUB110), as well as the mecA gene conferring oxacillin (OX) resistance, located 3.44 kilobase pairs apart. Strain N315ex is an N315 derivative that has lost the complete SCCmecII region. N315ex-h cells (N315ex strain carrying the pRTIsigH plasmid; Km3, OX8) were transformed with purified N315 genomic DNA. Transformants were first selected for kanamycin resistance (100 μg/ml). The presence of the adaD gene was tested by PCR using primers lena007 and lena008 (top panel, typical profiles of selected colonies). Forty-three out of forty-seven kanamycin resistant colonies carried the adaD gene (c.a. 90%), with the remaining 4 likely spontaneous mutants. The adaD transformation efficiency was 3.1 × 10^{-6}. The presence of the mecA gene was verified by PCR using oligonucleotides mecAF and mecAR (bottom panel), and...
was detected in 37 out of the 43 andD-positive transformants. Positive and negative controls were carried out using chromosomal DNA from the donor and recipient strains (N315 and N315sex, respectively; last two lanes). B) Long PCR experiments confirming transfer by transformation of the entire SCCmeII element in seven transformants where andD and mecA were both present. Primer locations are shown on the SCCmeII map. All of the transformants gave signals with the expected size. Chromosomal DNA from strains N315 and N315sex-h was used for positive and negative controls, respectively. M: λ HindIII, ME2: 1 kb ladder (NEB). (TIF)

Figure S7 Phage-dependent “pseudo-transformation” in S. aureus does not require the comG and comE operons, unlike natural genetic competence. Experiments were carried out based on the method previously described [77] with some modifications. In brief, S. aureus cells were grown in TSB medium at 37°C overnight with shaking (180 rpm). Cells were recovered by centrifugation, washed once with 0.1 M Tris-malate (pH 7.0) and resuspended in 0.1 M Tris-malate (pH 7.0) supplemented with 0.1 M CaCl2. 24 µg of the N315 purified genomic DNA was added to 1 ml of cell suspension, and cells were incubated at room temperature for 40 min. Cells were recovered by centrifugation, suspended in drug-free BHI medium and incubated for 1 h at 37°C with shaking (100 rpm). Cells were mixed with BHI-agar medium pre-cooled to 55°C and supplemented with 5 µg/ml erythromycin and poured into plates. After two days incubation at 37°C, colonies were counted and checked for the presence of the emr gene by PCR with primers ErmA1 and ErmA2. (TIF)

Figure S8 Plasmid maps of plasmids pMAD-PcomE, pRT-PcomE-bgaB, and pMAD-tet. Plasmids pMAD-PcomE and pRT-PcomE-bgaB are intermediates used for constructing plasmid pTet-rep. Plasmid pRT-PcomE-bgaB was used as a reporter to follow SigH activity in Figure 1C. The PspA promoter sequence is located between the Ndel and EcoRI sites, and hence does not affect expression of the PcomE-bgaB transcriptional fusion. Plasmid pMAD-tet was modified from pMAD for use in S. aureus strains resistant to erythromycin. (TIF)

Table S1 Composition of CS2 medium. (PDF)

Acknowledgments
We thank Prof. Takashi Shina (Kyoto Prefectural University, Japan) for pT7gfp. We are grateful to Ms. Sayaka L. Takeshita, Mr. Shoshei Oheda, Atsushi Maruyama, Dr. Ryosuke L. Ohnwa, Dr. Shinji Saito (University of Tsukuba, Japan), Dr. Sarah Dubrac, Olivier Poupel, and Dr. Michel Débarbouille (Institut Pasteur, France) for experimental support and helpful discussions.

Author Contributions
Conceived and designed the experiments: KM TM. Performed the experiments: KM AJT MT LTN. Analyzed the data: KM YI TO TM. Wrote the paper: KM TM.

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