INTRODUCTION

Staphylococcus aureus is a Gram-positive, spherical bacterium that divides in three consecutive, orthogonal planes (Monteiro et al., 2015; Pinho, Kjos, & Veening, 2013; Tzagoloff & Novick, 1977). As is the case for all bacteria, replicated chromosomes must be efficiently segregated to ensure accurate inheritance of genetic material (Badrinarayanan, Le, & Laub, 2015; Bloom & Joglekar, 2010; Graumann, 2014; Toro & Shapiro, 2010; Wang, Llopis, & Rudner, 2013). How this is achieved in dividing S. aureus cells is still poorly understood.

The prototypical DNA segregation system, parABS, was first identified and studied on prophage and Escherichia coli plasmids (Baxter & Funnell, 2014; Gerdes, Howard, & Szardenings, 2010; Hayes & Barilla, 2006; Oliva, 2016; Salje, 2010; Schumacher, 2012), however, homologues are also present on most bacterial chromosomes (Livny, Yamaichi, & Waldor, 2007). The parABS system consists of a Walker-type ATPase (ParA/Soj), a DNA-binding protein (ParB/Spo0J), and repetitive centromere-like sequences (parS) (Baxter & Funnell, 2014; Gerdes et al., 2010; Salje, 2010). Generally, ParB proteins bind specifically to parS sites that are located in the origin-proximal region to form a nucleoprotein complex (Breier & Grossman, 2007; Lin & Grossman, 1998; Minnen, Attaiech, Thon, Gruber, & Veening, 2011; Murray, Ferreira, & Errington, 2006). ParA proteins bind nonspecifically to nucleoid DNA and interact with parS-bound ParB proteins (Lutkenhaus, 2012; Vecchiarelli, Hwang, & Mizuuchi, 2013; Vecchiarelli, Neuman, & Mizuuchi, 2014). Recently, it was found that ParB binds cytidine triphosphate (CTP) and exhibits parS-dependent CTP hydrolysis to...
modulate parS binding and recruitment of ParA (Osorio-Valeriano et al., 2019; Soh et al., 2019). Interaction with ParB stimulates the ATPase activity of ParA and releases ParA from the ParB/parS complex, ultimately creating a gradient of ParA that drives DNA segregation toward the cell poles (Hatano & Niki, 2010; Sanchez, Rech, Gasc, & Bouet, 2013; Vecchiarelli et al., 2014).

An S. aureus Spo0J homologue exhibits 47% identity with Bacillus subtilis Spo0J. However, similar to Streptococcus pneumoniae, S. aureus does not encode a ParA/Soj homologue, suggesting that the mechanism of chromosome segregation in S. aureus may deviate from canonical mechanisms described thus far.

Studies in S. pneumoniae and B. subtilis show that the structural maintenance of chromosomes (SMC) protein is recruited to origin-proximal sites through interaction with ParB/Spo0J (Gruber & Errington, 2009; Minnen et al., 2011; Sullivan, Marquis, & Rudner, 2009). SMC (MukB in E. coli) is a condensin that, in complex with ScpA and ScpB, contributes to chromosome compaction and organization (Britton, Lin, & Grossman, 1998; Mascarenhas, Soppa, Strunnikov, & Graumann, 2002; Moriya et al., 1998; Wang, Tang, Riley, & Rudner, 2014). In the absence of both B. subtilis Spo0J and SMC, chromosome segregation defects are amplified compared to the absence of either gene alone, suggesting a role for Spo0J and SMC interaction in chromosome segregation (Britton et al., 1998; Lee & Grossman, 2006; Wang et al., 2014). A previous study has shown that SMC also contributes to staphylococcal chromosome segregation, since a significant proportion of cells lacking SMC were anucleate (Yu, Herbert, Graumann, & Götz, 2010). However, little is known about the functional significance of S. aureus Spo0J and its interactions, if any, with SMC.

Based on the function of ParB/Spo0J proteins, we sought to verify the role of Spo0J in staphylococcal chromosome segregation and its potential role in SMC function. We used super-resolution structured illumination microscopy (SIM) to confirm that Spo0J is indeed involved in S. aureus chromosome segregation, but found that it is not essential for growth and viability. We also show that Spo0J is required for correct localization of SMC and that both proteins act in concert to ensure efficient chromosome segregation, however, a role in cell division was not immediately evident. Our data indicate the involvement of additional interactions that influence S. aureus chromosome segregation and cell division, suggesting redundancy or overlapping roles of proteins involved in these essential processes.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and growth

Bacterial strains used in this study are listed in Table 1. Molecular cloning and plasmid propagation were performed using E. coli DC10B cells grown in Luria-Bertani (LB) broth (Sigma-Aldrich). PCR-based experiments were performed using E. coli RN4220 strains (Löfblom, S. aureus, and E. coli) grown in Brain Heart Infusion (BHI) agar (Becton Dickinson Difco™). Liquid cultures were routinely grown at 37°C with aeration, unless otherwise specified.

Plasmids were introduced into chemically competent E. coli DC10B or BTH101 cells using heat shock (Sambrook & Russell, 2001). Plasmids isolated from E. coli DC10B strains were introduced into S. aureus RN4220 cells via electroporation (Löfblom, Kronqvist, Uhlen, Ståhl, & Wernérus, 2007; Monk, Shah, Xu, Tan, & Foster, 2012). Where applicable, antibiotics were used at the following concentrations: 100 µg/ml ampicillin, 50 µg/ml kanamycin, 10 µg/ml erythromycin, and 15 µg/ml neomycin. Details on the construction of strains used in this study are provided in Appendix 1.

2.2 | Recombinant DNA techniques

Plasmids used in this study are listed in Table 1. Oligonucleotides were synthesized by Integrated DNA Technologies and are listed in Table A1. DNA modification enzymes were purchased from New England Biolabs.

Plasmids were constructed using standard molecular cloning techniques (Sambrook & Russell, 2001) with T4 DNA ligase or Gibson assembly (Gibson et al., 2009) using Q5 high-fidelity DNA polymerase, T5 exonuclease, and Taq DNA ligase. Plasmid DNA was isolated from E. coli DC10B cells using the QiaPrep Spin Miniprep kit (Qiagen). Staphylococcus aureus genomic DNA was isolated using the Purelink Genomic DNA Mini kit (Invitrogen), with the addition of 50 µg/ml lysostaphin (Sigma-Aldrich) for cell lysis at 37°C for 30 min. DNA purification was performed using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Details on the construction of plasmids used in this study are provided in Appendix 1. Plasmid sequences were verified by DNA sequencing (Fasmac).

2.3 | Bacterial growth assays

Overnight cultures of strains to be assayed were diluted 1:50 in fresh media (SSM9PR minimal media or TSB rich media) and grown at 37°C until OD_{600nm} ~ 0.05. Cultures were aliquoted in triplicate into separate wells of a 96-well microplate. Optical density was measured every 30 min at 595 nm using a MultiSk” Go microplate reader (Thermo Fisher Scientific) set at 37°C with moderate shaking. Experiments were performed in triplicate for at least three independent experiments.

2.4 | Bacterial viability assays

Overnight cultures were diluted 1:50 in fresh media and grown to mid-exponential phase. Cultures were normalized to OD_{600nm} 1.0
and serially diluted in phosphate-buffered saline (PBS), after which 10 µl was spotted onto SSM9PR minimal media agar or BHI agar plates. Plates were incubated overnight at 37°C. Data were obtained from at least three independent experiments.

2.5 | Bacterial two-hybrid assays

*Escherichia coli* adenylate cyclase, *cya*, mutant cells (BTH101) were cotransformed with pairs of pKNT25 and pUT18 plasmid derivatives. Five single colonies of each strain were patched onto selective LB agar plates containing 100 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Sigma-Aldrich) and 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich). Plates were incubated at 30°C for 3 days. Blue patches indicate β-galactosidase activity from interaction between T25 and T18 fusions. Data shown are representative of three independent assays.

2.6 | Fluorescence microscopy

Overnight cultures of *S. aureus* cells were diluted 1:50 in fresh TSB with appropriate antibiotics and incubated at 37°C with aeration until mid-exponential phase (OD600nm ~ 0.4–0.6). For fluorescence localization of Spo0J-mRFPmars and SMC-GFP, cultures were grown at 30°C until OD600nm ~ 0.1–0.2. Expression of Spo0J-mRFPmars and SMC-GFP was induced for 2 hr with 2.5 ng/ml anhydrotetracycline and 0.1 mM IPTG, respectively. Where required, DNA was

### TABLE 1 Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant genotype or description | Reference |
|-------------------|----------------------------------|-----------|
| **Escherichia coli** |                                  |           |
| DC10B             | *E. coli* K-12 (DH10B) derivative. Δdcm damΔhsdRMS endA1 recA1 | Monk et al. (2012) |
| BTH101            | *F*, *cya*-99, araD139, galE15, galK16, rpsL1 (Strr), hsdR2, mcrA1, mcrB1 | Karimova et al. (1998) |
| **Staphylococcus aureus** |                              |           |
| RN4220            | Restrictionless derivative of NCTC 8325–4 | Kreiswirth et al. (1983) |
| HC061             | RN4220 Δspo0J                    | This study |
| HC080             | RN4220 Δsmc                      | This study |
| HC094             | RN4220 Δspo0J Δsmc               | This study |
| **Plasmids**      |                                  |           |
| pHCO52            | pSK1 ori, AmpR, NeoR, P\_xyl/tetO-spo0J-mRFPmars | This study |
| pHCO61            | pMAD containing *S. aureus* RN4220 spo0J upstream and downstream regions, AmpR, EryR | This study |
| pHCO67            | pSK41 ori, AmpR, EryR, P\_spac-smc-gfp | This study |
| pHCO80            | pMAD containing *S. aureus* RN4220 smc upstream and downstream regions, AmpR, EryR | This study |
| pHC102            | pSK41 ori, AmpR, EryR, P\_spac-gfp | This study |
| pHC111            | pKNT25 derivative encoding Spo0J-T25, KanR, P\_lac-spo0J-T25 | This study |
| pHC112            | pKNT25 derivative encoding SMC-T25, KanR, P\_lac-smc-T25 | This study |
| pHC114            | pUT18 derivative encoding Spo0J-T18, AmpR, P\_lac-spo0J-T18 | This study |
| pHC115            | pUT18 derivative encoding SMC-T18, AmpR, P\_lac-smc-T18 | This study |
| pKNT25            | Encodes T25 fragment (residues 1–224) of adenylate cyclase, CyaA, fused in frame downstream of MCS, KanR | Karimova et al. (1998) |
| pKT25-zip         | pKT25 derivative encoding the leucine zipper of GCN4, KanR, P\_lac-T25-zip | Euromedex |
| pUT18             | Encodes T18 fragment (residues 225–399) of adenylate cyclase, CyaA, fused in frame downstream of MCS, AmpR | Karimova et al. (1998) |
| pUT18C-zip        | pUT18C derivative encoding the leucine zipper of GCN4, AmpR, P\_lac-T18-zip | Euromedex |
| pMAD              | Allelic replacement vector; pBR322 and pE194ts origins of replication in *E. coli* and staphylococci, AmpR, EryR | Arnaud, Chastanet, and Débarbouillé (2004) |
| pSK9065           | *E. coli*–*S. aureus* shuttle vector carrying low-copy number *S. aureus* pSK1 origin of replication and anhydrotetracycline-inducible P\_xyl/tetO promoter, AmpR, NeoR | Brzoska and Firth (2013) |
| pSK9067           | *E. coli*–*S. aureus* shuttle vector carrying low-copy number *S. aureus* pSK41 origin of replication and IPTG-inducible P\_spac promoter and optimized lacOid operator, AmpR, EryR | Brzoska and Firth (2013) |

Note: AmpR, confers ampicillin resistance; EryR, confers erythromycin resistance; KanR, confers kanamycin resistance; NeoR, confers neomycin resistance; ori, origin of replication.
stained with 2.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI), cell membranes were stained with 5 μg/ml FM4-64 (Invitrogen), and cell walls were stained with an equal mixture of vancomycin and a vancomycin BODIPY FL conjugate (0.4 μg/ml, Invitrogen) for 2 min at room temperature. After washing with PBS, a small aliquot of cells was applied to an agarose pad containing 2% (w/v) agarose in TSB on a microscope slide, and then covered with a glass coverslip for imaging.

Super-resolution SIM imaging was performed using a ZeissELYRA PS.1 microscope equipped with a 100 × 1.46 NA alpha plan apochromat oil immersion objective and a pco.edge sCMOS camera. Fluorescence images were acquired sequentially using 200–300 ms exposure times per image, for a total of 15 images per SIM reconstruction. All imaging was performed at room temperature (~23°C). Raw data were reconstructed using the SIM algorithms in ZEN 2011 SP7 software (black edition, Carl Zeiss). Brightfield images were captured using widefield imaging mode. Images had a final pixel size of 25 nm × 25 nm.

Images were processed and analyzed using Fiji (Schindelin et al., 2012). Cell diameters were determined by measuring the distance between FM4-64 fluorescence peaks from line profiles drawn along the short axis of cells. Data were obtained from at least three independent experiments.

2.7 Western blot and immunodetection

Staphylococcus aureus cultures were grown using the growth and induction conditions described for fluorescence microscopy. Following induction for 2 hr, cells were harvested (1,800 g, 4°C, 10 min) and then resuspended 1:200 in lysis buffer (0.1 M NaCl, 0.1 M Tris-HCl, 0.01 M MgCl₂, pH 7.5) containing 1 × protease inhibitor cocktail (Roche) and 0.1 mg/ml DNaseI (Roche). Samples were electrophoresed on 4%–15% Tris-glycine polyacrylamide gradient gels and then transferred to 0.45 µm nitrocellulose membranes in Towbin buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3).

Blots were blocked with 5% (w/v) EasyBlocker (GeneTex) in TBST (25 mM Tris, 0.15 M NaCl, 0.05% Tween-20). Spo0J-mRFPmars was detected with a 1:2,000 dilution of anti-RFP or anti-mCherry antibodies (Abcam), and SMC-GFP and GFP were detected with a 1:3,000 dilution of anti-GFP antibodies (Abcam). Primary antibodies were detected with a 1:3,000 dilution of goat anti-rabbit HRP-conjugated antibodies (Bio-Rad). Antibodies were diluted in blocking buffer and incubated with blots for 1 hr at room temperature. Blots were washed three times with TSBT before and after blocking and antibody incubations. Secondary antibodies were detected using Clarity Western ECL substrate (Bio-Rad). Blots were imaged using a ChemiDoc gel imager (Bio-Rad).

2.8 Immunoprecipitation

Immunoprecipitation experiments were performed on S. aureus cell lysates prepared as described for Western blotting. Cell lysates (~1.5 mg total protein) from S. aureus cells expressing Spo0J-mRFPmars (pHC052) and either GFP (pHC102) or SMC-GFP (pHC067) were incubated with pre-equilibrated GFP-Trap magnetic agaroose beads (Chromotek) for 1 hr at 4°C with gentle inversion. Beads were magnetically separated and washed twice with wash buffer (10 mM Tris-HCl, 150 mM NaCl, 0.5 mM EDTA, pH 7.5) at 4°C, before elution of bound complexes with elution buffer (50 mM Tris-HCl, 10 mM EDTA, 1% SDS, pH 8) at 65°C for 15 min. Eluates were magnetically separated from the beads and analyzed by Western blotting and immunodetection.

2.9 Statistical analyses

Analyses of statistical significance were performed using unpaired Student’s t tests. p values <.05 were considered statistically significant and are indicated with asterisks.

3 RESULTS

3.1 spo0J and smc are not essential for Staphylococcus aureus growth and viability

There is much variation in the functional significance of ParB and SMC in different bacteria. For example, parB is essential in Caulobacter crescentus, while its homologue spo0J is not essential in B. subtilis nor S. pneumoniae (Iretón, Gunther, & Grossman, 1994; Minnen et al., 2011; Mohl, Easter, & Gober, 2001). Additionally, smc is not essential in S. pneumoniae and C. crescentus, but deletion causes lethality under conditions promoting fast growth in B. subtilis (Gruber et al., 2014; Jensen & Shapiro, 1999; Minnen et al., 2011). In order to determine whether spo0J and smc are essential in S. aureus, we generated separate spo0J and smc deletions in the S. aureus RN4220 parental strain. Strains were constructed using an integrative pMAD derivative followed by double-crossover homologous recombination to generate markerless deletions at the spo0J or smc loci of the S. aureus RN4220 genome (Appendix 1). Furthermore, because spo0J and smc were shown to interact in B. subtilis and S. pneumoniae, we also constructed a Δspo0J Δsmc double mutant to test whether this interaction is essential in S. aureus.

The Δspo0J (HC061), Δsmc (HC080), and Δspo0J Δsmc (HC094) mutants showed similar growth rates to the RN4220 parental strain, with no difference in viability when grown at 37°C in TSB rich medium or SSM9PR minimal medium (Figure 1a–c). As described below, we showed that the lack of significant difference in growth and viability between the strains was not a result of suppressor mutations or polar effects from the gene deletions (Figure A1).
3.2 Spo0J and SMC are involved in Staphylococcus aureus chromosome segregation

The roles of Spo0J and SMC in chromosome segregation are well-studied in B. subtilis and S. pneumoniae. Perturbations to spo0J or smc result in defects in chromosome segregation, with a spo0J smc double mutant having a synthetic lethal phenotype in B. subtilis when grown in rich medium (Britton et al., 1998). Yu et al. (2010) showed that SMC is involved in S. aureus chromosome segregation, but we wondered whether spo0J also plays a role together with SMC. To this end, we performed SIM imaging to examine the DNA content of S. aureus RN4220, Δspo0J, Δsmc, and Δspo0J Δsmc derivatives stained with DAPI.

When grown at 37°C in TSB rich medium, 0.1% of the RN4220 parental strain contained cells that were anucleate, that is, without a nucleoid and, therefore, devoid of DAPI staining (Figure 2a,b). In comparison, 0.6% of Δspo0J and 1.1% of Δsmc cells were anucleate, which are both significantly greater than the frequency observed for the parental strain (p = .0241 and 0.0021, respectively) (Figure 2a,b), suggesting that spo0J and smc are required for normal chromosome segregation in S. aureus. Notably, the Δspo0J Δsmc double mutant showed a significantly higher proportion of anucleate cells compared to the parental strain and the Δspo0J and Δsmc single mutants (3.2% anucleate cells, p = .0017, 0.0032, and 0.0070, respectively) (Figure 2a,b), indicating an increased chromosome segregation defect in the absence of both spo0J and smc.

Defects in chromosome segregation are often associated with nucleoid abnormalities such as nucleoid condensation or bisection of unsegregated nucleoids by the division septum (Minnen et al., 2011; Veiga & G Pinho, 2017; Yu et al., 2010). However, in our study, we found no difference in the occurrence of nucleoid abnormalities between the parental and mutant strains and did not observe any instances of septum formation over unsegregated chromosomes.

3.3 Spo0J is required for SMC localization in Staphylococcus aureus

Our finding that Spo0J and SMC are both required together to ensure accurate chromosome segregation led us to speculate that S. aureus Spo0J and SMC might behave similarly to their counterparts in other bacteria. A significant role of ParB/Spo0J in S. pneumoniae, B. subtilis, and C. crescentus is to recruit the condensin, SMC, to origin-proximal sites, where ParB/Spo0J binds (Gruber & Errington, 2009; Minnen et al., 2011; Sullivan et al., 2009; Tran, Laub, & Le, 2017). To determine whether this interaction might also occur in S. aureus, we constructed Spo0J-mRFPmars and SMC-GFP fusion proteins so that their localizations could be visualized by SIM imaging. Both proteins were expressed from compatible, coresident, low-copy number plasmids in S. aureus. Spo0J-mRFPmars expression was induced with 2.5 ng/ml anhydrotetracycline from the P_xyl/tetO promoter, while SMC-GFP expression was induced with 0.1 mM IPTG from the P_spoc promoter. Importantly, both fluorescent fusions were functional under the induction conditions tested, since Spo0J-mRFPmars and SMC-GFP could complement the chromosome segregation defects...
FIGURE 2 Chromosome segregation in *Staphylococcus aureus* cells lacking Spo0J and SMC. (a) Super-resolution SIM images of mid-exponential phase *S. aureus* parental (RN4220), Δspo0J, Δsmc, and Δspo0J Δsmc strains. Cell membranes (yellow) were stained with the lipid dye FM4-64. DNA (cyan) was stained with DAPI. Left: merge of cell membrane and DNA fluorescence. Right: merge of DNA fluorescence and brightfield images. Arrowheads indicate anucleate cells. Scale bars = 1 µm. (b) Frequency of anucleate cells in the presence and absence of spo0J and smc, calculated from SIM fluorescence micrographs. Data represent averages of three independent experiments. n_{total} = 1,233, 2,552, 2,075, and 2,846 cells for RN4220, Δspo0J, Δsmc and Δspo0J Δsmc strains, respectively. Error bars indicate standard error of the mean. *p < .05, considered statistically significant (unpaired Student’s t test)
of the Δspo0J and Δsmc mutants, respectively (Figure A1). This further verifies that the growth, viability, and chromosome segregation phenotypes of the Δspo0J and Δsmc strains were the result of gene deletions and not other mutations or polar effects.

Spo0J-mRFPmars formed fluorescent foci consistent with previously shown localization patterns and indicative of DNA-binding to origin-proximal sites (Pinho & Errington, 2004; Veiga, Jorge, & Pinho, 2011) (Figure 3a and Figure A2). SMC-GFP displayed similar localization patterns to Spo0J-mRFPmars, often with overlapping, or partially-overlapping, foci (Figure 3a). Our results suggest that S. aureus Spo0J and SMC likely localize to similar subcellular positions, consistent with potential complex formation.

If S. aureus Spo0J recruits SMC to origin-proximal sites as described in B. subtilis, S. pneumoniae, and C. crescentus (Gruber & Errington, 2009; Minnen et al., 2011; Sullivan et al., 2009; Tran et al., 2017), then we predicted that SMC-GFP might be mis-localized in the absence of Spo0J. Therefore, we endeavored to determine whether SMC-GFP localization is dependent on Spo0J. For this purpose, we expressed SMC-GFP in the S. aureus Δspo0J mutant strain and compared its localization to that in the parental RN4220 strain. Subsequent SIM imaging revealed that more than 90% of cells in the parental strain contained at least one fluorescent focus of SMC-GFP, with the majority (62%) containing two SMC-GFP foci per cell (Figure 3b,c). In contrast, SMC-GFP localization appeared more dispersed in the Δspo0J cells, with a significantly higher proportion of cells containing no SMC-GFP foci (50%, p < .0001), and an average of 0.96 foci per cell, compared to 2.1 foci in the parental strain (p < .0001, n_total = 1,307 and 1,345 cells for RN4220 and Δspo0J strains, respectively, Figure 3b,c). Western blotting using anti-GFP antibodies showed that the loss of SMC-GFP foci in the absence of spo0J was not due to the release of free GFP by proteolysis (Figure 3d). No proteolysis was also detected for Spo0J-mRFPmars (Figure 3d). Taken together, our results suggest that staphylococcal chromosome segregation involves the formation of complexes containing Spo0J and SMC and that Spo0J is required for correct localization of SMC in S. aureus.
3.4 | Spo0J and SMC form a complex in *Staphylococcus aureus* to mediate chromosome segregation

The increased proportion of anucleate cells in the Δspo0J Δsmc mutant and the dependence of SMC localization on Spo0J suggested potential interaction between Spo0J and SMC. Therefore, in order to verify whether Spo0J and SMC form a complex in *S. aureus*, we performed co-immunoprecipitation experiments on *S. aureus* RN4220 cells expressing Spo0J-mRFPmars and either GFP or SMC-GFP. Following nuclease digestion of genomic DNA, we used anti-GFP antibodies coupled to magnetic agarose beads to immunoprecipitate GFP or SMC-GFP and their interacting proteins. We were able to detect Spo0J-mRFPmars in the immunoprecipitated complexes isolated from *S. aureus* cells expressing both Spo0J-mRFPmars and SMC-GFP, but not from cells expressing Spo0J-mRFPmars and GFP (Figure 4a). Thus, Spo0J-mRFPmars was isolated specifically in the presence of SMC-GFP, indicating the formation of complexes containing Spo0J and SMC in *S. aureus*. Notably, the Spo0J-mRFPmars immunoprecipitated from cell lysates containing SMC-GFP was only weakly detected using anti-RFP antibodies, which may be indicative of weak or transient complex formation.

To test whether Spo0J and SMC might interact directly, we conducted bacterial two-hybrid assays using Spo0J and SMC fusions to the *Bordetella pertussis* adenylate cyclase (CyaA) fragments, T25 and T18 (Karimova, Pidoux, Ullmann, & Ladant, 1998). The assays revealed that in the *E. coli* cya mutant strain BTH101, β-galactosidase activity was detected from cells expressing the Spo0J-T25 and SMC-T18 fusions (blue patches), however, no activity was detected from cells expressing the reciprocal fusions, SMC-T25 and Spo0J-T18 (white patches) (Figure 4b). We do not exclude the possibility that an interaction between Spo0J and SMC, direct or indirect, may be dependent on Spo0J localization or DNA binding, or on the presence of other *S. aureus* proteins that were not present in the bacterial two-hybrid assays. The assays also showed self-interaction of Spo0J and SMC (Figure 4b), which is consistent with the fluorescent Spo0J-mRFPmars and SMC-GFP foci observed (Figure 3a).

3.5 | Absence of Spo0J and SMC does not interfere with normal *Staphylococcus aureus* cell division

Spatiotemporal coordination of chromosome segregation and cell division is paramount for successful cell growth. Impairment of *parB* in
cell membranes with the lipid dye FM4-64 and calculated the proportion of cells in each phase from SIM images. We observed no evidence of each phase of the cell cycle, we stained *S. aureus* (Monteiro et al., 2015). In order to provide an indication of the relative duration of each phase of the cell cycle, we categorized the cell cycle into three phases, characterized by cells that had no septum (Phase 1), an incomplete septum (Phase 2) or a complete septum (Phase 3) (Monteiro et al., 2015). In order to provide an indication of the relative duration of each phase of the cell cycle, we stained *S. aureus* cell membranes with the lipid dye FM4-64 and calculated the proportion of cells in each phase from SIM images. We observed no significant difference in the distribution of cells in the three cell cycle phases for the parental, Δspo0J, and Δsmc strains (Figure 5b). We did, however, observe a modest, but insignificant, increase in the proportion of cells in Phase 2 for the Δspo0J Δsmc double mutant (54 ± 3.6% compared to 42 ± 3.6% for the parental strain, mean ± SEM, *p* = .1032) (Figure 5b), potentially indicating slightly prolonged septum closure in these cells. Taken together, these results suggest that spo0J and smc do not significantly affect cell cycle progression and are not essential for normal cell division in *S. aureus*.

4 | DISCUSSION

In this study, we examined the functional significance of Spo0J and SMC in *S. aureus*, and found that both proteins are required for efficient chromosome segregation, which is consistent with their predicted roles. A single Δspo0J mutant resulted in 0.6% anucleated cells (Figure 2), which is similar to the frequency observed for *B. subtilis* and *S. pneumoniae* mutants (Ireton et al., 1994; Minnen et al., 2011). Our observations for the Δsmc mutant are also consistent with the previously shown contribution of SMC to *S. aureus* chromosome segregation (Yu et al., 2010).

Fluorescence localization of Spo0J-mRFPmars and SMC-GFP in *S. aureus* showed that the two proteins formed foci that often overlapped or partially overlapped, and were reminiscent of oriC localization (Pinho & Errington, 2004; Veiga et al., 2011) (Figure 3a). Importantly, SMC-GFP localization is dependent on Spo0J, since absence of spo0J resulted in a significant reduction in the number of staphylococcal cell division defects in *B. subtilis* correlate with elongated cells (Britton et al., 1998; Ireton et al., 1994; Lee & Grossman, 2006). In order to investigate the influence of Spo0J and SMC on *S. aureus* cell division, we sought to determine whether the chromosome segregation defects observed in the spo0J and smc mutants correspond to abnormalities in staphylococcal cell division.

Disruption of staphylococcal cell division, for example, by impeding FtsZ, EzrA, Noc, or DnaK function, results in cell enlargement and aberrant cell division (Bottomley et al., 2017; Jorge, Hoicyk, Gomes, & Pinho, 2011; Lund et al., 2018; Monteiro et al., 2018; Pereira et al., 2016; Pinho & Errington, 2003; Veiga et al., 2011). Therefore, as an indicator of cell division, we measured the cell diameters of *S. aureus* cells grown at 37°C in TSB and stained with the lipid dye FM4-64. Crosses indicate mean; lower, middle, and upper lines of boxes indicate first quartile, median, and third quartile, respectively; ends of whiskers indicate 1.5IQR (interquartile range); circles indicate outliers. *n*total = 335, 346, 379, and 422 cells for RN4220, Δspo0J, Δsmc, and Δspo0J Δsmc strains, respectively. No significant difference was detected in cell diameter for Δspo0J, Δsmc, and Δspo0J Δsmc strains compared to RN4220 (*p* = .7803, 0.9252, and 0.1872, respectively, unpaired Student’s *t* test). Histograms showing the distribution of cell diameters are shown in Figure A3. (b) Cell cycle progression of *S. aureus* strains in the presence and absence of spo0J and smc. Mid-exponential phase *S. aureus* cells were stained with the lipid dye FM4-64, and the frequency of cells in Phase 1 (no septum), Phase 2 (incomplete septum) and Phase 3 (complete septum) of the cell cycle was determined using super-resolution SIM imaging. Graphical representations of the cell cycle phases are shown below the horizontal axis. Data represent averages of three independent experiments. *n*total = 581, 590, 643, and 646 cells for RN4220, Δspo0J, Δsmc, and Δspo0J Δsmc strains, respectively. Error bars indicate standard error of the mean.

C. crescentus blocks FtsZ ring assembly and subsequent cell division (Mohl et al., 2001), while chromosome segregation defects in *B. subtilis* overlap or partially overlap, and were reminiscent of oriC localization (Pinho & Errington, 2004; Veiga et al., 2011) (Figure 3a). Importantly, SMC-GFP localization is dependent on Spo0J, since absence of spo0J resulted in a significant reduction in the number of staphylococcal cell division (Mohl et al., 2001), while chromosome segregation defects in *B. subtilis* correlate with elongated cells (Britton et al., 1998; Ireton et al., 1994; Lee & Grossman, 2006). In order to investigate the influence of Spo0J and SMC on *S. aureus* cell division, we sought to determine whether the chromosome segregation defects observed in the spo0J and smc mutants correspond to abnormalities in staphylococcal cell division.

Disruption of staphylococcal cell division, for example, by impeding FtsZ, EzrA, Noc, or DnaK function, results in cell enlargement and aberrant cell division (Bottomley et al., 2017; Jorge, Hoicyk, Gomes, & Pinho, 2011; Lund et al., 2018; Monteiro et al., 2018; Pereira et al., 2016; Pinho & Errington, 2003; Veiga et al., 2011). Therefore, as an indicator of cell division, we measured the cell diameters of *S. aureus* cells to determine whether differences in cell size could be attributed to inefficient chromosome segregation. We found no significant difference in cell diameters between the parental and deletion strains (Figure 5a and Figure A3), which suggests that the absence of spo0J or smc, alone or together, does not affect *S. aureus* cell size.

We next investigated whether spo0J and smc play a role in cell cycle progression. We categorized the *S. aureus* cell cycle into three phases, characterized by cells that had no septum (Phase 1), an incomplete septum (Phase 2) or a complete septum (Phase 3) (Monteiro et al., 2015). In order to provide an indication of the relative duration of each phase of the cell cycle, we stained *S. aureus* cell membranes with the lipid dye FM4-64 and calculated the proportion of cells in each phase from SIM images. We observed no significant difference in the distribution of cells in the three cell cycle phases for the parental, Δspo0J, and Δsmc strains (Figure 5b). We did, however, observe a modest, but insignificant, increase in the proportion of cells in Phase 2 for the Δspo0J Δsmc double mutant (54 ± 4.3% compared to 42 ± 3.6% for the parental strain, mean ± SEM, *p* = .1032) (Figure 5b), potentially indicating slightly prolonged septum closure in these cells. Taken together, these results suggest that spo0J and smc do not significantly affect cell cycle progression and are not essential for normal cell division in *S. aureus*.
It should be noted that our observations were made on segregation of the bulk chromosome, and as such, we do not exclude the possibility that chromosomal loci might be disorganized or mis-segregated in the absence of either spo0J or smc. Taking this into account, we did not observe any bisection of the nucleoid by the division septum in any of the strains analyzed. These events were probably prevented by Noc, which prevents divisome assembly over the staphylococcal nucleoid (Veiga et al., 2011), and FtsK and SpoIIIE, which ensure that DNA is not guillotined by the division septum during staphylococcal cell division (Veiga & G Pinho, 2017; Yu et al., 2010). Indeed, an S. aureus smc spoIIIE double mutant showed aberrations in chromosome organization and content (Yu et al., 2010). Spo0J and SMC are therefore probably involved in the early stages of S. aureus chromosome segregation, immediately following DNA replication, presumably by acting at origin-proximal sites to segregate the origins.

Importantly, although we observed a significant proportion of anucleate cells in the absence of spo0J and smc, the defect in chromosome segregation was not sufficient to severely affect growth, viability, cell division, or cell cycle progression (Figures 1 and 5). However, it would not be surprising that S. aureus utilizes multiple complementary mechanisms, in addition to Spo0J and SMC, to ensure accurate chromosome segregation and coordination with cell division. These additional mechanisms warrant further investigation and may help to better understand how these essential processes are carried out in spherical staphylococci.

ACKNOWLEDGMENTS

We are grateful to Morten Kjos for kindly providing reagents for the bacterial two-hybrid system. Work in the Structural Cellular Biology Unit is supported by OIST core subsidy.

CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTION

Helena Chan: Conceptualization; Formal analysis; Investigation; Writing-original draft; Writing-review & editing. Bill Söderström: Supervision; Writing-review & editing. Ulf Skoglund: Supervision; Writing-review & editing.

ETHICS STATEMENT

None required.

DATA AVAILABILITY STATEMENT

Data from this study are available from the corresponding author on reasonable request.

ORCID

Helena Chan https://orcid.org/0000-0002-5194-0006

REFERENCES

Arnaud, M., Chastanet, A., & Débarbouillé, M. (2004). New vector for efficient allelic replacement in naturally nontransformable,
low-GC-content. Gram-positive bacteria. Applied and Environmental Microbiology, 70(11), 6887–6891.

Badrinarayanan, A., Le, T. B. K., & Laub, M. T. (2015). Bacterial chromosome organization and segregation. Annual Review of Cell and Developmental Biology, 31(1), 171–199.

Baxter, J. C., & Funnell, B. E. (2014). Plasmid partition mechanisms. Microbiology Spectrum, 2, 1–20. https://doi.org/10.1128/microbiolspec.PLAS-0023-2014

Ben-Yehuda, S., Fujita, M., Liu, X. S., Gorbatyuk, B., Skoko, D., Yan, J., ... Losick, R. (2005). Defining a centromere-like element in Bacillus subtilis by identifying the binding sites for the chromosome-anchoring protein RacA. Molecular Cell, 17(6), 773–782.

Ben-Yehuda, S., Rudner, D. Z., & Losick, R. (2003). RacA, a bacterial protein that anchors chromosomes to the cell poles. Science, 299(5606), 532–536.

Bloom, K., & Joglekar, A. (2010). Towards building a chromosome segregation machine. Nature, 463(7280), 446–456.

Bottomley, A. L., Liew, A. T. F., Kusuma, K. D., Peterson, E., Seidel, L., Foster, S. J., & Harry, E. J. (2017). Coordination of chromosome segregation and cell division in Staphylococcus aureus. Frontiers in Microbiology, 8, 1–13. https://doi.org/10.3389/fmicb.2017.01575

Bowman, G. R., Comolli, L. R., Zhu, J., Eckart, M., Koenig, M., Downing, K. H., ... Shapiro, L. (2008). A polymeric protein anchors the chromosomal origin/ParB complex at a bacterial cell pole. Cell, 134(6), 945–955.

Breier, A. M., & Grossman, A. D. (2007). Whole-genome analysis of the chromosome partitioning and sporeulation protein Spo0J (ParB) reveals spreading and origin-distal sites on the Bacillus subtilis chromosome. Molecular Microbiology, 64(3), 703–718. https://doi.org/10.1111/j.1365-2958.2007.05690.x

Britton, R. A., Lin, D.-C.-H., & Grossman, A. D. (1998). Characterization of a prokaryotic SMC protein involved in chromosome partitioning. Genes & Development, 12(9), 1254–1259.

Broszka, A. J., & Firth, N. (2013). Two-plasmid vector system for independently controlled expression of green and red fluorescent fusion proteins in Staphylococcus aureus. Applied and Environment Microbiology, 79(9), 3133–3136.

Dworkin, J., & Losick, R. (2002). Does RNA polymerase help drive chromosome segregation in bacteria? Proceedings of the National Academy of Sciences of the United States of America, 99(22), 14089–14094.

Ebersbach, G., Briegel, A., Jensen, G. J., & Jacobs-Wagner, C. (2008). A self-associating protein critical for chromosome attachment, division, and polar organization in caulobacter. Cell, 134(6), 956–968.

Gerdes, K., Howard, M., & Szardening, F. (2010). Pushing and pulling in prokaryotic DNA segregation. Cell, 141(6), 927–942.

Gibson, D. G., Young, L., Chuang, R.-Y., Venter, J. C., Hutchison Iii, C. A., ... Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. Nature Methods, 6(5), 343–345.

Graumann, P. L. (2014). Chromosome architecture and segregation in prokaryotic cells. Journal of Molecular Microbiology and Biotechnology, 24(5–6), 291–300.

Gruber, S., & Errington, J. (2009). Recruitment of condensin to replication origin regions by ParB/Spo0J promotes chromosome segregation in B. subtilis. Cell, 137(4), 685–696.

Gruber, S., Veening, J.-W., Bach, J., Blettner, M., Bramkamp, M., & Errington, J. (2014). Interlinked sister chromosomes arise in the absence of condensin during fast replication in B. subtilis. Current Biology, 24(3), 293–298.

Hatano, T., & Niki, H. (2010). Partitioning of P1 plasmids by gradual distribution of the ATPase ParA. Molecular Microbiology, 78(5), 1182–1198.

Hayes, F., & Barilla, D. (2006). The bacterial segregosome: A dynamic nucleoprotein machine for DNA trafficking and segregation. Nature Reviews. Microbiology, 4(2), 133–143.

Ireten, K., Gunther, N. W., & Grossman, A. D. (1994). spo0J is required for normal chromosome segregation as well as the initiation of sporulation in Bacillus subtilis. Journal of Bacteriology, 176(17), 5320–5329.

Jensen, R. B., & Shapiro, L. (1999). The Caulobacter crescentus smc gene is required for cell cycle progression and chromosome segregation. Proceedings of the National Academy of Sciences of the United States of America, 96(19), 10661–10666.

Jorge, A. M., Holczek, E., Gomes, J. P., & Pinho, M. G. (2011). EzaR contributes to the regulation of cell size in Staphylococcus aureus. PLoS ONE, 6, https://doi.org/10.1371/journal.pone.0027542

Jun, S., & Wright, A. (2010). Entropy as the driver of chromosome segregation. Nature Reviews Microbiology, 8(6), 600–607.

Karimova, G., Pidoux, J., Ullmann, A., & Ladant, D. (1998). A bacterial two-hybrid system based on a reconstituted signal transduction pathway. Proceedings of the National Academy of Sciences of the United States of America, 95(10), 5752–5756.

Kjos, M., & Veening, J.-W. (2014). Tracking of chromosome dynamics in live Streptococcus pneumoniae reveals that transcription promotes chromosome segregation. Molecular Microbiology, 91(6), 1088–1105.

Kreiswirth, B. N., Lofdahl, S., Betley, M. J., O’Reilly, M., Schlievert, P. M., Bergdoll, M. S., & Novick, R. P. (1983). The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature, 305(5936), 709–712.

Lee, P. S., & Grossman, A. D. (2006). The chromosome partitioning proteins Soj (ParA) and Spo0J (ParB) contribute to accurate chromosome partitioning, separation of replicated sister origins, and regulation of replication initiation in Bacillus subtilis. Molecular Microbiology, 60(4), 853–869. https://doi.org/10.1111/j.1365-2958.2006.05140.x

Lemon, K. P., & Grossman, A. D. (2001). The extrusion-capture model for chromosome partitioning in bacteria. Genes & Development, 15(16), 2031–2041.

Liew, A. T. F., Theis, T., Jensen, S. O., Garcia-Lara, J., Foster, S. J., Firth, N., ... Harry, E. J. (2011). A simple plasmid-based system that allows rapid generation of tightly controlled gene expression in Staphylococcus aureus. Microbiology, 157(3), 666–676.

Lin, D.-C.-H., & Grossman, A. D. (1998). Identification and characterization of a bacterial chromosome partitioning site. Cell, 92(5), 675–685.

Livny, J., Yamaichi, Y., & Waldor, M. K. (2007). Distribution of centromere-like parS sites in bacteria: Insights from comparative genomics. Journal of Bacteriology, 189(23), 8693–9703.

Löffblom, J., Kronqvist, N., Uhlén, M., Ståhl, S., & Wernérus, H. (2007). Optimization of electroprotonation-mediated transformation: Staphylococcus cannuis as model organism. Journal of Applied Microbiology, 102(3), 736–747.

Lund, V. A., Wacnik, K., Turner, R. D., Cotterrell, B. E., Walther, C. G., Fenn, S. J., ... Foster, S. J. (2018). Molecular coordination of Staphylococcus aureus cell division. eLife, 7, 1–31. https://doi.org/10.7554/eLife.32057

Lutkenhaus, J. (2012). The ParA/MinD family puts things in their place. Trends in Microbiology, 20(9), 411–418.

Mascarenhas, J., Soppa, J., Strunnikov, A. V., & Graumann, P. L. (2002). Cell cycle-dependent localization of two novel prokaryotic chromosome segregation and condensation proteins in Bacillus subtilis that interact with SMC protein. EMBO Journal, 21(12), 3108–3118. https://doi.org/10.1093/emboj/cdf314

Minnen, A., Attiaiech, L., Thon, M., Gruber, S., & Veening, J.-W. (2011). SMC is recruited to oriC by ParB and promotes chromosome segregation in Streptococcus pneumoniae. Molecular Microbiology, 81(3), 676–688.

Mohl, D. A., Easter, J. Jr., & Gober, J. W. (2001). The chromosome partitioning protein, ParB, is required for cytokinesis in Caulobacter crescentus. Molecular Microbiology, 42(3), 741–755.

Monk, I. R., Shah, I. M., Xu, M., Tan, M.-W., & Foster, T. J. (2012). Transforming the untransformable: Application of direct
transformation to manipulate genetically Staphylococcus aureus and Staphylococcus epidermidis. Mbio, 3, 1–11. https://doi.org/10.1128/mBio.00277-11

Monteiro, J. M., Fernandes, P. B., Vaz, F., Pereira, A. R., Tavares, A. C., Ferreira, M. T., … Pinho, M. G. (2018). Peptidoglycan synthesis drives an FtsZ-treadmilling-independent step of cytokinesis. Nature, 554(7693), 528–532.

Moriyta, S., Tsujikawa, E., Hassan, A. K. M., Asai, K., Kodama, T., Murray, H., Ferreira, H., & Errington, J. (2006). The bacterial chromosome: Molecular and biological aspects. Molecular Microbiology, 29(1), 179–187.

Murray, H., Ferreira, H., & Errington, J. (2006). The bacterial chromosome segregation protein spo0J spreads along DNA from parS nucleation sites. Molecular Microbiology, 61(5), 1352–1361.

Oliva, M. A. (2016). Segregation complex formation during DNA trafficking in bacterial cell division. Frontiers in Molecular Biosciences, 3, 1–9. https://doi.org/10.3389/fmolb.2016.00051

Osorio-Valeriano, M., Altegoer, F., Steinchen, W., Urban, S., Liu, Y., Bange, G., & Thanbichler, M. (2019). ParB-type DNA segregation proteins are CTP-dependent molecular switches. Cell, 179(7), 1512–1524.

Pereira, A. R., Hsin, J., Kröl, E., Tavares, A. C., Flores, P., Hoiczyk, E., … Pinho, M. G. (2016). FtsZ-dependent elongation of a coccolid bacterium. Mbio, 7, 1–9. https://doi.org/10.1128/mBio.00908-16

Pinho, M. G., & Errington, J. (2003). Dispersed mode of Staphylococcus aureus cell wall synthesis in the absence of the division machinery. Molecular Microbiology, 50(3), 871–881.

Pinho, M. G., & Errington, J. (2004). A divIVA null mutant of Staphylococcus aureus undergoes normal cell division. FEMS Microbiology Letters, 240(2), 145–149.

Pinho, M. G., Kjos, M., & Veening, J. (2013). How to get (around) Mechanisms controlling growth and division of cocccid bacteria. Nature Reviews Microbiology, 11(9), 601–614.

Salje, J. (2010). Plasmid segregation: How to survive as an extra piece of DNA. Critical Reviews in Biochemistry and Molecular Biology, 45(4), 296–317.

Sambrook, J., & Russell, D. W. (2001). Molecular cloning: A laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanchez, A., Rech, J., Gasc, C., & Bouet, J. (2013). Insight into centromere-binding properties of ParB proteins: A secondary binding motif is essential for bacterial genome maintenance. Nucleic Acids Research, 41(5), 3094–3103.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., … Cardona, A. (2012). Fiji: An open-source platform for biological-image analysis. Nature Methods, 9(7), 676–682.

Schumacher, M. A. (2012). Bacterial plasmid partition machinery: A minimalistic approach to survival. Current Opinion in Structural Biology, 22(1), 72–79.

Soh, Y.-M., Davidson, I. F., Zamuner, S., Basquin, J., Bock, F. P., Taschner, M., … Gruber, S. (2019). Self-organization of parS centromeres by the ParB CTP hydrolase. Science, 366(6469), 1129–1133.

Sullivan, N. L., Marquis, K. A., & Rudner, D. Z. (2009). Recruitment of SMC by ParB-parS organizes the origin region and promotes efficient chromosome segregation. Cell, 137(4), 697–707.

Toro, E., & Shapiro, L. (2010). Bacterial chromosome organization and segregation. Cold Spring Harbor Perspectives in Biology, 2, a003049. https://doi.org/10.1101/cshperspect.a003049

Tran, N. T., Laub, M. T., & Le, T. B. K. (2017). SMC progressively aligns chromosomal arms in Caulobacter crescentus but is antagonized by convergent transcription. Cell Reports, 20(9), 2057–2071.

Tzagoloff, H., & Novick, R. (1977). Geometry of cell division in Staphylococcus aureus. Journal of Bacteriology, 129(1), 343–350.

Vecchiarelli, A. G., Hwang, L. C., & Mizuuchi, K. (2013). Cell-free study of F plasmid partition provides evidence for cargo transport by a diffusion-ratchet mechanism. Proceedings of the National Academy of Sciences of the United States of America, 110(15), E1390–E1397.

Vecchiarelli, A. G., Neuman, K. C., & Mizuuchi, K. (2014). A propagating ATPase gradient drives transport of surface-confined cellular cargo. Proceedings of the National Academy of Sciences of the United States of America, 111(13), 4880–4885.

Veiga, H., & G Pinho, M., (2017). Staphylococcus aureus requires at least one FtsK/SpoIIIE protein for correct chromosome segregation. Molecular Microbiology, 103(3), 504–517.

Veiga, H., Jorge, A. M., & Pinho, M. G. (2011). Absence of nucleoid occlusion effector Noc impairs formation of orthogonal FtsZ rings during Staphylococcus aureus cell division. Molecular Microbiology, 80(5), 1366–1380.

Wang, X., Llopis, P. M., & Rudner, D. Z. (2013). Organization and segregation of bacterial chromosomes. Nature Reviews Genetics, 14(3), 191–203.

Wang, X., Tang, O. W., Riley, E. P., & Rudner, D. Z. (2014). The SMC condensin complex is required for origin segregation in Bacillus subtilis. Current Biology, 24(3), 287–292.

Yamaichi, Y., Bruckner, R., Ringgaard, S., Möll, A., Cameron, D. E., Briegel, A., … Waldor, M. K. (2012). A multidomain hub anchors the chromosome segregation and chemotactic machinery to the bacterial pole. Genes & Development, 26(20), 2348–2360.

Yu, W., Herbert, S., Graumann, P. L., & Götz, F. (2010). Contribution of SMC (Structural Maintenance of Chromosomes) and SpoIIIE to chromosome segregation in staphylococci. Journal of Bacteriology, 192(15), 4067–4073.

How to cite this article: Chan H, Söderström B, Skoglund U. SpoOJ and SMC are required for normal chromosome segregation in Staphylococcus aureus. MicrobiologyOpen. 2020;9:e999. https://doi.org/10.1002/mbo3.999
APPENDIX 1
CONSTRUCTION OF PLASMIDS

pHC052 (p<sub>xyt</sub>spo0J-mRFPmars). A 0.85 kb fragment containing Staphylococcus aureus RN4220 spo0J was PCR-amplified from S. aureus genomic DNA using primers spo0J_Sall(F) and spo0J_SacI(R), and then restricted with Sall and SacI. The restricted fragment was purified and ligated with Sall and SacI-digested pSK9065 vector DNA using T4 DNA ligase.

pHC061 (Δspo0J). pHC061 was constructed using Gibson assembly (Gibson et al., 2009). Regions of ~2.5 kb upstream and downstream of S. aureus RN4220 spo0J were PCR-amplified from S. aureus genomic DNA using primer pairs spo0J_up(F)/spo0J_up(R) and spo0J_dwn(F)/spo0J_dwn(R), respectively. pMAD vector DNA was PCR-amplified using primers pMAD_dwn(F) and pMAD_up(R). Amplicons were digested with DpnI to remove methylated template DNA, and then the spo0J upstream region, downstream region, and pMAD vector DNA were ligated for 1 hr at 55°C in the presence of 100 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 200 µM of each of the four dNTPs, 10 mM DTT, 5% (w/v) PEG-8000, 1 mM NAD, 3.8 U/ml T5 exonuclease, 23.8 U/ml Q5 DNA polymerase, and 3.8 U/µl Q5 DNA ligase.

pHC067 (P<sub>spac</sub>-smc-gfp). A 3.6 kb fragment containing S. aureus RN4220 smc was PCR-amplified from S. aureus genomic DNA using primers smc_RBS_BamHI(F) and smc_SmaI(R), and then restricted with BamHI and SmaI. The restricted fragment was purified and phosphorylated with T4 polynucleotide kinase, then ligated with BamHI and SmaI-digested pSK9067 vector DNA using T4 DNA ligase.

pHC080 (Δsmc). pHC080 was constructed using Gibson assembly (Gibson et al., 2009). Regions of ~2.5 kb upstream and downstream of S. aureus RN4220 smc were PCR-amplified from S. aureus genomic DNA using primer pairs smc_up(F)/smc_up(R) and smc_dwn(F)/smc_dwn(R), respectively. pMAD vector DNA was PCR-amplified using primers pMAD_dwn(F) and pMAD_up(R). Amplicons were digested with DpnI to remove methylated template DNA, and then the smc upstream region, downstream region, and pMAD vector DNA were ligated for 1 hr at 55°C in the presence of 100 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 200 µM of each of the four dNTPs, 10 mM DTT, 5% (w/v) PEG-8000, 1 mM NAD, 3.8 U/ml T5 exonuclease, 23.8 U/ml Q5 DNA polymerase, and 3.8 U/µl Q5 DNA ligase.

pHC102 (P<sub>spac</sub>-gfp). A 0.7 kb DNA fragment containing gfp was amplified from pSK9067 using primers gfp_Sall(F) and gfp_EcoRI(R) and then restricted with SalI and EcoRI. The restricted fragment was purified and then ligated with SalI and EcoRI-digested pLOW vector DNA (Liew et al., 2011) using T4 DNA ligase.

Oligonucleotide Sequence a

| Oligonucleotide | Sequence<sup>a</sup> |
|----------------|----------------------|
| gfp_Sall(F)    | cgcgtcagagctgataatattatgtggtttcctttaaaaggaaggaac |
| gfp_EcoRI(R)   | cgccgaatctgatttataaagttgtccatacgg |
| pMAD_dwn(F)    | gattccgatcgcccgagcggaggg |
| pMAD_up(R)     | ccattgccagcatgatagctgcg |
| smc_BamHI(F)   | gcggcgatatctgcttccttccttcataacacatc |
| smc_KpnI(R)    | cgccggatatctgcttccttccttcataacacatc |
| smc_RBS_BamHI(F) | gcggcgatatctgcttccttccttcataacacatc |
| smc_dwn(F)     | cttttaaatgtgataaagagggattttagatgagttggaagctgacatag |
| smc_dwn(R)     | tccagcctgctgcggcagatgctttagctgtgatctctctcttccttccttcataacacatc |
| smc_Smal(R)    | gcgcggccggccgcatgatgcgcgagcagtcctgtctctctcttccttccttcataacacatc |
| smc_up(F)      | actagacagacatctatgcatgctgtcagctgctgttctgcagctctgactcag |
| smc_up(R)      | tgcttcatctgtgcagcattcattaatttctctccttccttccttcataacacatc |
| spo0J_BamHI(F) | cgccggatatctgcttccttccttcataacacatc |
| spo0J_Sall(R)  | cgccgtcagagctgataatattatgtggtttcctttaaaaggaaggaac |
| spo0J_dwn(F)   | aactgttataagatatattatttgctctagcgagagttgaagttgtaacttataaagatcttgcagctcattagctgatctttgctcatgatctgatctttgctcatgatctgatctttgctcatgatc |
| spo0J_dwn(R)   | tccagcctgctgcggcagatgctttagctgtgatctctctcttccttccttcataacacatc |
| spo0J_SacI(R)  | gcggcgatatctgcttccttccttcataacacatc |
| spo0J_SalI(F)  | cgccgtcagagctgataatattatgtggtttcctttaaaaggaaggaac |
| spo0J_up(F)    | actagacagacatctatgcatgctgtcagctgctgttctgcagctctgactcag |
| spo0J_up(R)    | atataatagttaactatttcatcactgtgaagttggattataaatctctcttataacag |

*Oligonucleotides are presented 5’-3’, left to right. Restriction sites are underlined. Ribosome binding sites are highlighted in bold. Linker sequences are italicized.*

TABLE A1 Oligonucleotides used in this study
A 0.85 kb DNA fragment containing S. aureus spo0J was amplified from S. aureus RN4220 genomic DNA using primers spo0J_BamHI(F) and spo0J_SacI(R) and then restricted with BamHI and SacI. The restricted fragment was purified and then ligated with BamHI and SacI-digested pKNT25 or pUT18 vector DNA using T4 DNA ligase.

**CONSTRUCTION OF STAPHYLOCOCCUS AUREUS DELETION STRAINS**

Electrocompetent S. aureus RN4220 cells were electroporated with the integration plasmid pHC061 or pHC080 to generate spo0J...
(HC061) and smc (HC080) deletions, respectively. Electroporated cells were incubated at 30°C on BHI agar containing 10 µg/ml of erythromycin and 100 µg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (BHI/Em/X-Gal) to select for transformants. Blue colonies were grown in TSB containing 10 µg/ml of erythromycin at 43°C for ~8 hr, and then streaked on BHI/Em/X-Gal agar. Plates were incubated at 43°C to promote vector integration into the chromosome via homologous recombination. Single blue colonies were selected and continuously subcultured approximately six times at 30°C in TSB without antibiotic selection to promote double cross-over and plasmid loss. The final subculture was serially diluted in PBS, spread on BHI/X-Gal agar plates, and incubated at 30°C. Single colonies were replica-patched onto BHI/X-Gal agar plates with and without erythromycin selection. White patches that were erythromycin-resistant, indicating plasmid excision from the chromosome, were selected and screened for gene deletion. Gene deletions were verified by PCR of isolated genomic DNA.

The S. aureus Δspo0J Δsmc double mutant (HC094) was generated in HC061 (Δspo0J) using the integrative plasmid, pHC080, to generate an additional smc deletion as described above.