SosA inhibits cell division in *Staphylococcus aureus* in response to DNA damage

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Abstract

Inhibition of cell division is critical for cell viability under DNA damaging conditions. In bacterial cells, DNA damage induces the SOS response, for which inhibition of cell division is an integral part. In the rod-shaped bacterium, *Escherichia coli,* the SOS controlled inhibitor SulA blocks division at an early step by binding to the prokaryotic tubulin homolog, FtsZ, but still allows lateral cell wall synthesis leading to cell filamentation. However, in coccoid bacteria, such as the human pathogen *Staphylococcus aureus*, the link between DNA damage and inhibition of cell division remains poorly understood. Here, we have identified and characterized the SOS-induced cell division inhibitor of *S. aureus* designated SosA. Upon DNA damage, wild-type cells increase in size, whereas those lacking *sosA* do not, with a concomitant decrease in viability. The 77 amino acid gene product of *sosA* is conserved among staphylococci and is predicted to be localized at the membrane. Functional studies showed that it does not interfere with early cell division events such as Z-ring formation but rather affects a later step, probably by interacting with known divisome components, in particular PBP1. Characterization of truncated SosA variants and mutant strains revealed that the extracellular C-terminus of SosA has dual functions in being required for cell division inhibition while simultaneously controlling SosA levels by acting as a likely target for degradation by the uncharacterized membrane protease CtpA. Our findings provide important insights into cell division regulation in coccoid bacteria that in turn may foster improved therapeutic interventions targeting this essential process.

Significance

Staphylococcus aureus is a clinically relevant bacterium and a model organism for cell division of spherical bacteria. We show that upon DNA damage *S. aureus* expresses SosA, an endogenous cell division inhibitor, causing cell swelling and cessation of the cell cycle at a stage prior to division plate completion. SosA appears to function by interference with the essential membrane-associated division protein PBP1. Cell division inhibition is strictly regulated and we identify the membrane protease CtpA as being involved in shut-down of SosA-mediated growth arrest. Membrane-bound carboxy-terminal processing proteases may constitute functional homologs in Gram-positive species of the Lon protease of Gram-negative bacteria, regulating cell division inhibition. Uncovering the molecular details of SosA function and regulation may be of therapeutic value.

Introduction

Bacteria multiply by coordinated and essential events of DNA replication and cell division, both representing important biological processes and valuable targets for antimicrobial therapy (1-4). In the event of DNA damage, activation of the SOS response ensures that cell division is delayed until the DNA is repaired. The SOS regulon is controlled by the conserved LexA repressor, which is de-repressed in response to RecA binding to single-stranded DNA (5-7). The SOS response has been mostly studied in *Escherichia coli* and among the LexA regulated genes is *sulA* encoding the cell division inhibitor that suppresses division by binding to FtsZ that at the division site acts as a scaffold for assembly of cell division components (4, 8-14). In rod-shaped bacteria, inhibition of

cell division leads to filamentation as a consequence of lateral peptidoglycan synthesis. Such a phenotype is also observed in *E. coli* mutants lacking the Lon protease for which SulA is a substrate (15, 16). For spherical cells such as *Staphylococcus aureus* little is known of how SOS induction is coupled to cell division. Neither specific posttranslational mechanisms for negative regulation of cell division inhibition have been identified in Gram-positive species at present.

S. aureus is a serious Gram-positive human pathogen that originally received its name from the grape like clusters of coccoid cells which result from the unique cell division process that occurs in three consecutive orthogonal planes (17-19). In this organism we have previously identified *lexA* and noted an open reading frame (designated *sosA*) that is divergently transcribed from *lexA* and is controlled by the LexA repressor and the SOS response (20-23). The location of *sosA* adjacent to *lexA* indicated that it might encode a cell division inhibitor as similar gene synteny has been observed for cell division inhibitors encoded by Gram-positive, rod shaped bacteria namely *Bacillus subtilis* (24), *Bacillus megaterium* (25), *Listeria monocytogenes* (26, 27), *Mycobacterium tuberculosis* (28) and *Corynebacterium glutamicum* (29) (Fig. 1*A*). The cell division inhibitors of these organisms display no similarity to SulA and within the group they generally show little homology with the exception of YneA from *L. monocytogenes* and ChiZ from *M. tuberculosis* (28) that resemble *B. subtilis* YneA. Commonly, however, they have a single membrane spanning segment, a predicted extracellular C-terminus and, with the exception of DivS from *C. glutamicum*, a LysM domain, a ubiquitous peptidoglycan binding motif (30, 31), suggesting that membrane and/or cell wall localization are common features characterizing Gram-positive cell division inhibitors.

The fundamental processes of staphylococcal cell division have been studied spatiotemporally using super-resolution microscopy techniques (18, 32) and recent efforts combining genetic approaches have unveiled the molecular mechanism in unprecedented detail (33, 34). Here, we demonstrate that *sosA* encodes the SOS inducible cell division inhibitor in *S. aureus* and document its impact on cell division following treatment with DNA damaging agents. Moreover, we identify a possible mechanism for control of endogenous cell division inhibition by proteolysis. Thus, we further our insight into these basic biological phenomena, which could improve our possibilities to develop new antimicrobials targeting the cell division machinery in *S. aureus*.

Results

The *sosA* gene is conserved in staphylococci. In *S. aureus*, the open reading frame located adjacent to *lexA* (divergently transcribed) was named *sosA* and hypothesized to encode an inhibitor of cell division (22) (Fig. 1*A*). The 77 amino acids long product has homology and 25-60% amino acid identity to proteins encoded by genes occupying the same chromosomal location in a range of staphylococci (Fig. 1*B* and *SI Appendix*, Fig. S1), while showing no clear homology to the known Gram-positive cell division inhibitors. Of note, the C-terminal part of SosA lacks a LysM domain, which is present in the cell division inhibitors YneA and ChiZ (35, 36). The N-terminal half of SosA includes a putative transmembrane (TM) domain and the C-terminal part is predicted to be located extracellularly (TOPCONS server (37)). Among the different staphylococcal species, a highly conserved sequence is located just C-terminally of the predicted TM domain and, although less striking, some conservation seems to exist also in the extreme C-terminus (Fig. 1*B*).

Genotoxic stress inhibits cell division in an SosA-dependent manner. To analyze whether SosA is an SOS-induced cell division inhibitor, we deleted *sosA* from 8325-4, the common laboratory *S. aureus* strain, and JE2, a derivative of the clinically relevant USA300 lineage. Growth and survival of these mutants was monitored upon challenge with a lethal concentration of the DNA damaging agent Mitomycin C (MMC). Within a 2 hours MMC killing experiment, wild type cells showed a 10-100 fold higher survival rate than the *sosA* mutant cells at each time point, whereas the optical densities of cultures of both cell types were comparable (Fig. 2*A*). Flow cytometry measurements of arbitrary cell size distributions revealed that treatment with MMC led to a 3 and 6 fold increase in forward light scatter (FSC-A) values in 8325-4 and JE2, respectively, while the cell size distribution of the *sosA* mutants remained essentially constant (Fig. 2*B*). The constant cell size of the *sosA* mutants during DNA damage could imply that these cells, in contrast to wild type cells, continue dividing. Indeed, the striking phenotype was confirmed by time-lapse microscopy, where wild type cells increase their size over time, occasionally ending up lysing, while the size of the mutant cells lacking *sosA* stays the same and cell division continues (Fig. 2*C* and Movie S1). The phenotypes of the 8325-4 *sosA* mutant were abrogated in a strain complemented with a chromosomal copy of *sosA* expressed from its native promoter (*SI Appendix*, Fig. S2). Thus, the SosA protein allows *S. aureus* to increase in cell size and maintain viability under DNA damaging conditions.

SosA inhibits cell division. If SosA indeed is a cell division inhibitor in *S. aureus*, it can be predicted that overexpression of the protein should be sufficient to inhibit growth under DNA non-damaging conditions. Hence, we expressed *sosA* episomally from an anhydrotetracycline (AHT)-inducible promoter and found that while the control vector does not affect cell viability in either the presence or absence of the inducer, the plating efficiency of cells with the plasmid for *sosA*

overexpression was markedly compromised under inducing conditions (Fig. 3*A*). When grown in liquid culture and compared to the control, there was a 2-3 fold increase in forward light scatter values, showing that the size of the *sosA*-expressing cells increased (Fig. 3*B*). Microscopy observations over time confirmed the slight increase in cell size and reduction in number of cells upon SosA overproduction (Fig. 3*C* and Movie S2). Interestingly, contrary to what was observed upon sustained DNA-damaging conditions (Fig. 2*B*), we found that ectopic induction of SosA caused only a transient increase in cell size (compare time points 45 and 90 min to 135 min in Fig. 3*B*), indicating the existence of a negative regulatory mechanism.

SosA is a late stage cell division inhibitor. To pinpoint at which step of the cell division process the SosA-mediated cell division inhibition is taking place, we employed fluorescence microscopy to monitor cellular localization of FtsZ and EzrA, crucial cell division proteins in *S. aureus*, upon induction of SosA overproduction. Neither the *sosA* overexpression plasmid in non-inducing conditions (Fig. 4*A* and *B*) nor the control vector in non- and inducing conditions (*SI Appendix*, Fig. S3*A* and *B*) caused changes in cell morphology and protein localization. Although cell swelling was observed when *sosA* was overexpressed, in contrast to the cell division inhibitor SulA in *E. coli*, SosA does not affect localization of the cell division initiator FtsZ (Fig. 4*A*) or placement of the membrane-bound division protein EzrA (Fig. 4*B*), which is considered as an early recruited protein connecting the cytoplasmic division components with the peptidoglycan-synthesizing membrane-bound complex (38). To further explore the cellular consequences of SosA-mediated cell division inhibition, phenotypes of 5 min HADA labeled JE2/pSosA grown in the absence or presence of inducer (*SI Appendix*, Fig S3*C*) were examined. While in the absence of AHT 47% and 23% of cells, respectively, showed a ring (incomplete septum) or line (complete septum) of nascent peptidoglycan synthesis, there was accumulation (89%) of cells with incomplete septa and a

severe drop in cells with complete septa (1%) when *sosA* was overexpressed (Fig. 4*C*). Additionally, 1% of cells had an 'hourglass' shape after incubation with AHT. HADA marks regions of nascent peptidoglycan synthesis. Therefore, whole cell walls of cells overproducing SosA were labeled with a fluorescent NHS ester and examined by 3D structured illumination microscopy (3D-SIM). Microscopy visualization revealed that the majority of cells had only signs of septation - a 'piecrust' (18) (*SI Appendix*, Fig. S3*E*i), and showed that in an hourglass like shaped cell there was a gap in the septal peptidoglycan (*SI Appendix*, Fig. S3*E*ii). The fact that SosA does not affect localization of early cell division proteins, FtsZ and EzrA and the cell population becomes synchronized to a particular stage of the cell cycle (phase P2 (32)), suggests that SosA does not hinder initiation of septum formation but blocks progression of septum completion – a phenotype similar to *S. aureus* DivIB depleted cells (39). Although DivIB has been shown dispensable for FtsZ and EzrA localization to the septum and piecrust formation, its absence results in inhibition of septum progression and completion, and mislocalization of GpsB (39), a late cell division protein (39, 40). Again, under non-inducing conditions SosA did not alter cell morphology and GpsB-GFP localization (Fig. 4*D*) and the control vector did not have any effect in the presence and absence of AHT (*SI Appendix*, Fig. S3*D*). Interestingly, while causing visible cell enlargement in this reporter strain, SosA overproduction did not impair recruitment of GpsB to the mid-cell either (Fig. 4*D*), indicating that SosA acts on (a) later cell division component(s) than GpsB.

SosA interacts with PBP1. No direct interaction has been observed between the Gram-positive cell division inhibitors and FtsZ. Though poorly characterized mechanistically, they may suppress cell division at later stages such as by interacting with FtsI/FtsQ (for *M. tuberculosis* ChiZ)(35) or via delocalization of FtsL and/or DivIC (for *B. subtilis* YneA) (41). By employing a bacterial two-hybrid system approach based on cell division proteins from *S. aureus* (38) we sought to identify

possible *in vivo* interaction candidates for SosA. This analysis indicated that PBP1 and possibly also DivIC and PBP3 are likely targets for the activity of SosA (Fig. 5*A*). PBP1, which is a homolog of *E. coli* FtsI (42), is an essential component of the divisome that, in *S. aureus*, is considered to play a role in two distinct processes, septum formation and cell separation and it has been suggested that only the latter activity requires the transpeptidase (TP) activity of the enzyme (43). If indeed PBP1 is a target for SosA, it seemed possible that ectopic expression of *pbpA* (encoding PBP1) would mitigate the effect of concomitant *sosA* overexpression. Intriguingly, overproduction of PBP1 along with SosA was manifested as a synergistic lethal phenotype leading to considerable growth impairment even at low inducer concentrations, whereas the effects of SosA or PBP1 overproduction alone were negligible (Fig. 5*B*). Whilst this does not suggest a simple titration of SosA by PBP1, it does support the two-hybrid system evidence that SosA is mechanistically linked to PBP1. Interestingly, we find that SosA is also synergistically lethal when co-expressed with a mutant variant of PBP1 lacking TP activity (*SI Appendix*, Fig. S4). Hence, SosA obstructs septum formation progression and closure in *S. aureus.* This is likely mediated through PBP1 and the mechanism may be independent of the PBP1 TP activity.

The C-terminus of SosA is essential for function and possibly also required for autoregulation. The C-terminal part of SosA containing segments that appear highly conserved in staphylococci (Fig. 1*B*) was predicted in silico to be extracellularly exposed. First, we confirmed this prediction by constructing PhoA-LacZ reporter fusions (44) whereby fusion of the reporter chimera to the C-terminus of SosA confer a phosphatase-positive, β-galatosidase-negative phenotype in *E. coli*, indicative of translocation across the membrane (*SI Appendix*, Fig. S5). Next, aiming at a functional characterization of the SosA protein, we constructed a series of C-terminally truncated variants by successive elimination of ten amino acids (AAs) (Fig. 6*A*), resulting in four variants of SosA lacking

between 10 and 40 AAs C-terminally. Strikingly, we observed that truncation of the 10 C-terminal AAs (and to some extent also removal of 20 C-terminal AAs) generated a hyper-potent inhibitor severely compromising plating efficiency even at low inducer concentration (Fig. 6*B*). Time-lapse microscopy revealed that cells producing SosAd10 (SosA missing the last 10 AAs) had a dramatic phenotype. Prominent cell swelling and reduction of cell-cycle rate was observed (Fig. 6*C* and Movie S3). The phenotypes from the plating assay correlated with the cell size measurements by flow cytometry (*SI Appendix*, Fig. S6), which indicated that cells expressing the 10 AA truncated protein were stalled in the non-dividing state, resulting in abnormal cell size, whereas those expressing full length SosA were more transiently halted or delayed in cell division (*SI Appendix*, Fig. S6 and Movie S3). In contrast, deletion of 30 or 40 AAs from the C-terminal part eliminated the ability of SosA to inhibit cell division, as SosAd30 and SosAd40 gave comparable plating efficiency results to the control (Fig. 6*B*). This prompted us to investigate the relevance of the highly conserved residues located in the membrane-proximal part of the SosA C-terminal part by performing alanine substitutions within the protein. To this end, we used the hyper-active SosAd10 variant as a scaffold and found that while point mutations at AA positions 37 or 38 (E and Q) had no effect, point mutations at positions 40 or 41 (Y and E) caused partial inactivation of activity and mutation of residues 44 and 45 (D and H) completely abolished cell division inhibition activity of the protein (*SI Appendix*, Fig. S7). Importantly, the inactive variants SosAd40 and SosAd10(44A) are presumably localized to the membrane as are SosA and SosAd10 when evaluated by translocation of phosphatase activity (*SI Appendix*, Fig. S5). We conclude that conserved residues in the membrane-proximal segment of the extracellular C-terminal part of SosA are essential for cell division inhibition, while truncation at the extreme C-terminus of the protein augments activity.

SosA is regulated by the CtpA protease. We reasoned that one explanation for the severity of the 10 AA truncated SosA on cell division could be that the extreme C-terminus of SosA serves as an endogenous signal for proteolytic degradation, allowing the mutant protein but not wild type SosA to escape degradation and accumulate. We therefore searched the Nebraska Transposon Mutant Library (45) for *S. aureus* protease mutants, specifically focusing on proteases considered to be membrane-localized. As a potential candidate we identified a mutant in *ctpA* (Carboxyl-terminal protease A) and found that overproduction of the wild type SosA protein in a *ctpA* mutant background completely abolished cell viability, similarly to what was observed when overproducing the 10 AA truncated SosA protein in wild type *S. aureus* cells (Fig. 7*A*). In agreement with this result, we found that co-overproduction of SosA and CtpA in the *ctpA* mutant background alleviated the detrimental effect of SosA and that the degree of complementation depended on the relative expressional levels of the two proteins (*SI Appendix*, Fig. S8). Furthermore, substantiating the negative regulatory role played by CtpA with respect to SosA, we noted that the *ctpA* mutant was moderately more sensitive to MMC (measured as minimum inhibitory concentration) than wild type *S. aureus* but only when encoding also a functional *sosA* gene (Fig. 7*B*). Additionally, upon MMC-induced DNA damage, the SosA protein accumulated to higher levels in the *ctpA* mutant when compared to the wild type (Fig. 7*C*). Altogether, based on these results we propose that CtpA proteolytic activity relieves SosA-mediated cell division inhibition and allows growth to resume once DNA stress has ceased.

Discussion

Based on the results reported here, we propose the following model for regulation of cell division in the prolate spheroid bacterium *S. aureus* under SOS-inducing conditions (Fig. 8). Under normal

conditions, expression of *sosA* is repressed by the strong LexA repressor, but LexA is inactivated through autocleavage under SOS conditions, resulting in expression of *sosA*. It appears that the N-terminal autocleavage product of LexA needs to be further proteolytically processed for *sosA* expression to be fully induced, thus adding another layer of expressional control (22). Upon LexA derepression, SosA accumulates to levels inhibiting cell division. SosA does not cause FtsZ delocalization, nor does it prevent assembly of downstream components, EzrA and GpsB, representatives of early and late cell division components. The inhibitor appears to affect progression and completion of the septum formation via interaction with PBP1. As a consequence of SosA activity, cells appear synchronized at the stage of septum formation initiation, while an ongoing off-septal peptidoglycan synthesis leads to their cell enlargement (*SI Appendix*, Fig. S3*C*). Once conditions are favorable for continued growth, *sosA* expression is repressed and the membrane protease CtpA is involved in removal or inactivation of SosA activity, allowing growth resumption (Fig. 8).

The model illuminates several unanswered questions. It will be particularly important to determine exactly how SosA affects the function of PBP1. In this context it should be noted that our data fit well with the finding that genetic depletion of PBP1 causes cell enlargement and deficiency in septum completion (42). At present we cannot readily explain the peculiar finding that SosA and PBP1 seem to act in a synergistically lethal manner (Fig. 5*B*) but it seems possible that the two proteins together constitute a signal to halt septum formation beyond a certain point. Alternatively, the synergistic effects could depend on stoichiometry and physical accessibility in the sense that PBP1 would engage in a non-productive complex with SosA and as such more efficiently prevent access for residual and functional PBP1 to the site. We keep in mind that SosA may also interact with PBP3 and DivIC (Fig. 5*A*). Interaction between *S. aureus* divisome

components constitutes a complex web and in the bacterial two-hybrid study, PBP1 interacts with DivIC, which in turn engages in interaction with PBP3 (38). Thus, the inhibitory activity of SosA could be multifactorial.

Analysis of the AA sequence of SosA indicated that the N-terminal part contains a TM domain, while the C-terminal is located extracellularly. The anticipation that the TM domain promotes localization to the cellular membrane was supported by PhoA-LacZ fusions to SosA and to various point and deletion mutants of the protein (*SI Appendix*, Fig S5). The abrogation of activity by the 40 AAs truncation as well as by a single alanine substitution (D44A) suggests that this segment is essential for correct localization to the septum or, perhaps more interestingly, that the actual motif for cell division inhibition is localized at the exterior of the membrane. If indeed PBP1 interference lies within this segment of SosA, it could provide a novel paradigm for interference with penicillin-binding proteins beyond the transpeptidase-inhibitory activity exerted by β-lactam antibiotics.

Studies of bacterial cell division have benefitted from the development of genetic tools and highly sophisticated microscopic imaging technologies. However, most often bacteria grow as an unsynchronized population with individual cells being in different phases of cell division making it a challenge to put in order the temporal events during cell cycle progression, DNA replication, and division. Essentially, SosA is involved in halting the *S. aureus* cell cycle at a specific morphological stage, e.g., following the piecrust formation, and we anticipate that it may constitute a useful tool for the generation of synchronized staphylococcal cells for experimental purposes.

Endogenous cell division inhibition must be stringently regulated, as demonstrated by the pioneering work showing that SulA of *E. coli* is degraded by the Lon protease (16). Similarly, YneA

from *B. subtilis* was reported to be regulated by proteolysis (36) while a more temporary activity of the YneA homologue in *B. megaterium* has been suggested to be due to mRNA instability (25). We demonstrated that a minor truncation of the C-terminus of SosA yields a hyper-potent cell division inhibitor (Fig. 6), which led us to speculate that the extreme C-terminus serves as a signal for proteolysis. We identified a CtpA protease-deficient strain in which SosA appears to accumulate and has a more detrimental effect than in the wild type background. Bacterial carboxy-terminal proteases are poorly characterized, in particular with respect to their substrate preferences. The *S. aureus* CtpA protein is located to the cell membrane/cell wall fraction and is involved in stress tolerance and virulence in a mouse model of infection (46). We propose that CtpA specifically regulates SOS-induced cell division inhibition in staphylococci, likely via recognition of the C-terminus of SosA. Biochemical evidence for such a direct activity will be needed, and at present we cannot exclude an indirect effect. Interestingly, a point mutation in *B. subtilis* YneA at its extreme C-terminus generated a stabilized yet functional variant of the cell division inhibitor (36), suggesting that YneA and SosA may be degraded by similar mechanisms. Of note, carboxy-terminal proteases are ubiquitous in bacterial genera and homologs of CtpA are found in other Gram-positive species such as *B. subtilis* and *L. monocytogenes.* Hence, the CtpA-like proteases could represent functional homologs of the *E. coli* Lon protease, allowing negative regulation of endogenous cell division inhibition more broadly amongst Firmicutes or even Gram-positives.

Apart from being of basic biological interest, the bacterial cell division process may constitute an unexhausted source of novel therapeutic targets (1,2). We believe that SosA could be a natural scaffold that can point to new therapeutically accessible targets for interference with an essential process in *S. aureus* and other staphylococci.

Materials and Methods

Bacterial strains and plasmids used in this work. The bacterial strains and plasmids used in this study are listed in Table S1 and Table S2, respectively. *E. coli* strains were grown in Luria-Bertani medium (Oxoid) or on LB agar (Oxoid). *S. aureus* strains were grown in Tryptic Soy Broth (TSB) or on Tryptic Soy Agar (TSA) (both Oxoid). Ampicillin (100 µg/ml), chloramphenicol (10 µg/ml), or erythromycin (5 µg/ml) were added when appropriate. Anhydrotetracycline (AHT) (Sigma-Aldrich) and Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Thermo Scientific) were used as inducers for protein expression.

Construction of strains and plasmids. All oligonucleotides used are listed in Table S3. *S. aureus* 8325-4Δ*sosA* is a clean deletion of *sosA* (SAOUHSC_01334) in strain 8325-4 obtained by allelic replacement using the temperature-sensitive shuttle vector pIMAY (47). 1 kb regions up- and downstream of *sosA* were PCR amplified (Phusion Hot Start II DNA Polymerase, Thermo Scientific) using primer pairs Up-sosA_fw-KpnI/Up-sosA_rev and Dw-sosA_fw/Dw-sosA_rev-SacI, respectively, and subsequently joined in a spliced overhang PCR using Up-sosA_fw-KpnI/Dw-sosA_rev-SacI. The resulting deletion fragment was cloned into pIMAY via KpnI/SacI, generating pIMAY-Δ*sosA*, purified from *E. coli* DC10B and transformed into *S. aureus* subsequently maintained at 28 °C. Chromosomal integration of the plasmid was performed at 37 °C under selective conditions (chloramphenicol) followed by passage at 28 °C without antibiotic selection and final plating on TSA containing 500 ng/ml AHT for plasmid counterselection. Colonies were replica-plated to select for sensitivity towards chloramphenicol and successful allelic exchange were screened for by PCR amplification using primer pairs Ctrl_dsosA_F/Ctrl_dsosA_R positioned

outside *sosA* and Fwd_MCS/Rev_MCS targeting the vector, respectively. The allelic replacement procedure was identical for *S. aureus* JE2 to create JE2Δ*sosA*.

The chromosomal transposon insertion mutation in *ctpA* conferring erythromycin resistance was obtained from the Nebraska Transposon Mutant Library (NTML, NE847) (45) and was moved by transduction (phage Φ11) to *S. aureus* JE2, JE2Δ*sosA*, S. *aureus* 8325-4 and 8325-4Δ*sosA*, resulting in JE2-*ctpA*, JE2Δ*sosA*-*ctpA*, 8325-4-*ctpA* and 8325-4Δ*sosA*-*ctpA*, respectively. JE2-*ctpA*(−erm), an erythromycin-sensitive derivative, was generated by elimination of the transposon-encoded *ermB* in JE2-*ctpA* by allelic exchange using the pTnT vector using temperature-mediated chromosomal integration and *secY*-mediated counterselection as described before (48).

All plasmid constructs mentioned below were cloned in E. coli IM08B (49) from where they were transformed into *S. aureus* strains. For complementation of the *sosA* knockout, the *sosA* gene with its native promoter was PCR amplified from strain 8325-4 using primers Up-sosA-promo_SalI and Dw-sosA_EcoRI and cloned into designated restriction sites in plasmid pJC1112 (50). The resulting plasmid (pJC1112-*sosA*) was transformed into the integration-proficient strain RN9011, creating RN9011-*sosA*-compl. when selected with erythromycin. The chromosomally integrated plasmid was transduced into 8325-4Δ*sosA*, resulting in 8325-4Δ*sosA*-compl. For *sosA* expression the *sosA* gene including its predicted ribosomal binding site was cloned into the BglII and EcoRI sites of pRAB12-*lacZ* (51) using primers Up-sosA_BglII/Dw-sosA_EcoRI, at the same time eliminating the *lacZ* reporter gene. The resulting plasmid was called pSosA. Generation of plasmids encoding C-terminally truncated variants of SosA was obtained by PCR amplification of DNA fragments using Up-sosA_BglII and downstream primers Dw-sosA(d10)_EcoRI to Dw-sosA(d40)_EcoRI, all equipped with premature stop codons. The PCR products were ligated into pRAB12-*lacZ* using BglII and

EcoRI cut sites to create pSosAd10, pSosAd20, pSosAd30 and pSosAd40. Single and double amino acid substitutions (alanine) in the SosAd10 protein were obtained by cloning commercially synthesized DNA fragments (Twist Bioscience) into pRAB12-*lacZ* using the same restriction sites. Plasmids for *pbpA* or *ctpA* expression, pPBP1 and pCtpA, were constructed by cloning the *pbpA* and *ctpA* genes behind the P_{spac} promoter in pSK9067 (52) using primer pairs pbpA_F-SalI/pbpA_R-EcoRI and ctpA_F-SalI/ctpA_R-EcoRI, respectively. A plasmid for *pbpA** expression was constructed by use of the same primer pair but using pMAD-PBP1* (53) as a template. For membrane topology analysis using pKTop (44), *sosA*, *sosAd10*, *sosAd10(44A)* and *sosAd40* were PCR amplified from respective expression constructs using primer SosA_F-BamHI and primers SosA_R-KpnI, SosAd10_R-KpnI or SosAd40_R-KpnI, respectively, and cloned in frame in front of the *phoA*-*lacZ* chimeric gene using BamHI/KpnI restriction sites.

In order to construct JE2 strains producing a fluorescent fusion of FtsZ, EzrA or GpsB in the presence of the *sosA* expression plasmid, JE2 pSosA was transduced with a lysate from SH4665 (SH1000 pCQ11-FtsZ-eYFP), JGL227 (SH1000 *ezrA*-*gfp*+) or JGL228 (SH1000 *gpsB*-*gfp*+), resulting in SJF4694 (JE2 pRAB12-*lacZ* pCQ11-FtsZ-eYFP), SJF4696 (JE2 pRAB12-*lacZ ezrA*-*gfp*+) and SJF4699 (JE2 pRAB12-*lacZ gpsB -gfp*+), respectively. Control strains SJF4693 (JE2 pRAB12-*lacZ* pCQ11-FtsZ-eYFP), SJF4696 (JE2 pRAB12-*lacZ ezrA*-*gfp*+) and SJF4699 (JE2 pRAB12-*lacZ gpsB*-*gfp*+) were constructed by a phage transduction of JE2 pRAB12-*lacZ* with lysates from SH4665 (SH1000 pCQ11-FtsZ-eYFP), JGL227 (SH1000 *ezrA*-*gfp*+) or JGL228 (SH1000 *gpsB*-*gfp*+), respectively. To induce FtsZ-eYFP production in SJF4693 and SJF4694, cells were grown in the presence of 50 µM IPTG.

In order to screen for interaction of SosA with cell division/cell wall synthesis proteins of *S. aureus*, *sosA* was amplified from *S. aureus* SH1000 genomic DNA using primers ALB133 and ALB134, and ligated into pUT18C using BamHI/EcoRI cut sites, resulting in pT18-SosA. All fusion constructs to T25 and control vectors were generated previously (38, 54-56, Table S2).

Determination of OD_{600} and CFU after MMC treatment. Strains were grown overnight on TSA plates and used for inoculating TSB and allowed to grow to OD_{600} = 0.05 when Mitomycin C (MMC from *Streptomyces caespitosus*, Sigma-Aldrich) was added. Cell density was monitored by OD_{600} measurements at intervals onwards and samples were withdrawn to determine culture colony forming units by serial dilution in 0.9% w/v NaCl and plating on TSA.

Flow cytometry. Cell size distributions of cultures were arbitrarily quantified by flow cytometry using the forward scatter signal (FSC-A) acquired on a BD Accuri™ C6 flow cytometer (BD Biosciences). Cell samples were diluted in 0.9% w/v NaCl to an approximate density of 10^6 cells/ml and sonicated briefly to allow acquisition of scatter signal from single cells predominantly. Sonication was performed with a Bandelin sonopuls HD2070/UW2070 apparatus (Bandelin electronics, Germany) fitted with the MS 73 probe. Ten pulses of 500 msec were given at 50% power. All flow cytometry experiments were independently repeated at least twice with similar results.

Plating assays. Spot dilution was used to evaluate plating efficiency of strains carrying various plasmid constructs. Strains were grown exponentially until an approximate OD_{600} of 0.5 under plasmid selective conditions. Strains were then ten-fold serially diluted in 0.9% w/v NaCl and positioned as 10 µl spots on TSA containing selective antibiotics with/without inducer at indicated

concentrations and incubated at 37 °C overnight. All spot plating assays were independently repeated with similar results.

Labelling *S. aureus* with HADA

Cells grown to mid-exponential phase (OD_{600} ~ 0.5) were incubated with 500 µM HADA at 37°C for 5 min. Cells were then washed by centrifugation and resuspension in PBS.

Labelling *S. aureus* with NHS ester

Labelling with NHS ester was performed as described before (33). Briefly, cells grown to mid-exponential phase (OD_{600} ~0.5) were collected by centrifugation and growth medium was discarded. Cells were resuspended in PBS containing 8 µg ml^{-1} Alexa Fluor 647 NHS ester (Invitrogen) and incubated at room temperature for 5 min. Cells were washed by centrifugation and resuspension in PBS.

Fixing

Cells were fixed by incubation in 4% (w/v) paraformaldehyde at room temperature for 30 min.

Widefield Epifluorescence Microscopy

Fixed cells were dried onto a poly-L-Lysine coated slide and mounted in PBS. Imaging was performed using either a Nikon Ti Inverted microscope fitted with a Lumencor Spectra X light engine or a v4 DeltaVision OMX 3D-SIM system (Applied Precision, GE Healthcare, Issaquah, USA).

3D Structured Illumination Microscopy

A high precision cover slip (High-precision, No.1.5H, 22x22mm, 170±5 µm, Marienfeld) was cleaned by sonicating in 1 M KOH for 15 min at room temperature. The coverslip was washed with water and incubated in 0.01% (w/v) poly-L-lysine solution (Sigma) for 30 min at room

temperature. The coverslip was rinsed with water and dried with nitrogen. Fixed cells were dried onto the poly-L-Lysine coated cover slip and mounted on a slide with SlowFade Diamond (Invitrogen). 3D SIM visualisation was performed using a v4 DeltaVision OMX 3D-SIM system (Applied Precision, GE Healthcare, Issaquah, USA) equipped with a Plan Apo 60x, 1.42 NA oil objective, using 1.514 immersion oil, a 642 nm laser and a standard excitation/emission filter set (683/40). For each z-section, a sample was imaged in 5 phase shifts and 3 angles. The z-sections were 0.125 nm in depth. Raw data were reconstructed with the Softworx software (GE Healthcare) using OTFs optimized for the specific wavelength and oil used.

Time-lapse microscopy. Strains were grown overnight in TSB medium at 37°C, then diluted 100 times in fresh TSB and grown until OD = 0.1. Cells were washed once with fresh TSB and spotted onto TSB-acrylamide (10%) pads previously incubated for 2h in TSB medium supplemented, when appropriate, with 0.04 µg/mL mitomycin C (MMC) or 200 ng/mL anhydrotetracycline (AHT). Pads were placed into a Gene frame (Thermo Fisher Scientific) and sealed with a cover glass. Phase contrast images were acquired on a DV Elite microscope (GE healthcare) equipped with a sCMOS (PCO) camera and a 100x oil-immersion objective. Images were acquired with 200 ms exposure time every 4 minutes for at least 6 h at 37 °C using Softworx (Applied Precision) software. Images were analyzed using Fiji (http://fiji.sc).

Western blot analysis. A custom peptide (EZBiolab) comprising AAs 35-77 of SosA was conjugated, through NH_2 (N-terminus) and NH_2 of lysines (K) to a KLH carrier protein using glutaraldehyde and used for antibody production in rabbits (Covalab). Bacterial cells were pelleted and frozen immediately at -80 °C before being lysed in TE-buffer by bead-beating (Fastprep-24™, MP Biomedicals). The protein concentration of the lysates was measured using the Qubit™ Protein

Assay Kit (ThermoFisher Scientific) and were normalized to equal amounts of protein, separated on NuPAGE® 4-12% Bis-Tris gels using MES buffer and the XCell sure-lock mini-cell system (ThermoFisher Scientific), transferred to a polyvinylidene difluoride membrane(PVDF) and probed with primary antibody diluted 1:500. Bound antibody was detected with the WesternBreeze® Chemiluminescent Kit, anti-rabbit according to the instructions from the manufacturer (ThermoFisher Scientific).

Bacterial two-hybrid assay. Interactions between SosA and proteins involved in cell division and cell wall synthesis of *S. aureus* were tested using *E.coli* BTH101 (Δ*cyaA*) cotransformed with pT18-SosA and a plasmid carrying a fusion of T25 with a cell division or cell wall synthesis protein on minimal medium as previously described (38).

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M.S.B., K.W., P.K., M.T.C., S.J.F., and H.I. conceived and designed the study. Experiments were performed by M.S.B., K.W., C.G., and A.L.B. M.S.B. K.W., C.G., G.L., D.F., J.-W.V., S.J.F., and H.I. contributed to analysis of data and drafting of the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

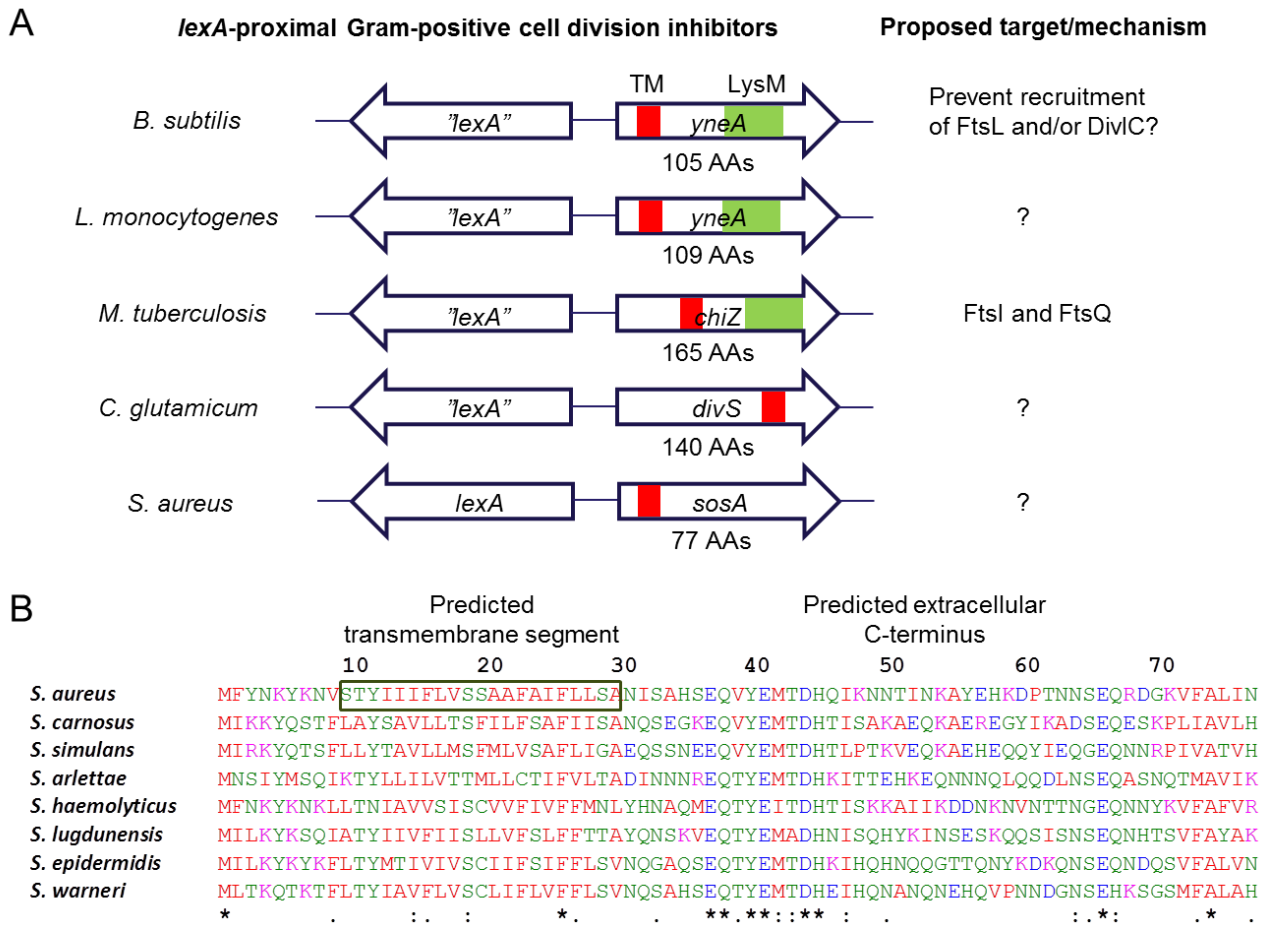

Fig 1 Gram-positive SOS-controlled cell division inhibitors. (*A*) Schematic representation of genes encoding characterized Gram-positive SOS-regulated cell division inhibitors (not drawn to scale) including the uncharacterized *sosA* from *S. aureus*. Despite considerable sequence divergence these genes are commonly chromosomally co-localized with *lexA* homologous genes. The cell division inhibitors carry a single transmembrane domain (TM) and several proteins have an additional LysM domain. (*B*) Alignment (CLUSTAL O(1.2.4)) of SosA sequences deduced from open reading frames next to *lexA* in *S. aureus* strain 8325-4 (YP_499864) and seven *Staphylococcus* species: *S. carnosus* (CAL27889), *S. simulans* (AMG96201), *S. arlettae* (EJY94737), *S. haemolyticus* (YP_253482), *S. lugdunensis* (YP_003471776), *S. epidermidis* (YP_188489), and *S. warneri* (EEQ79882). The proteins are 77 amino acids long and are characterized by a predicted

transmembrane segment at AAs 10-30 (for *S. aureus* SosA) and a predicted extracellular C-terminal (TOPCONS (37)) with considerable sequence conservation at the membrane-proximal portion (***** denotes fully conserved residues, : indicates conservation of residues with highly similar properties).

Figure 2

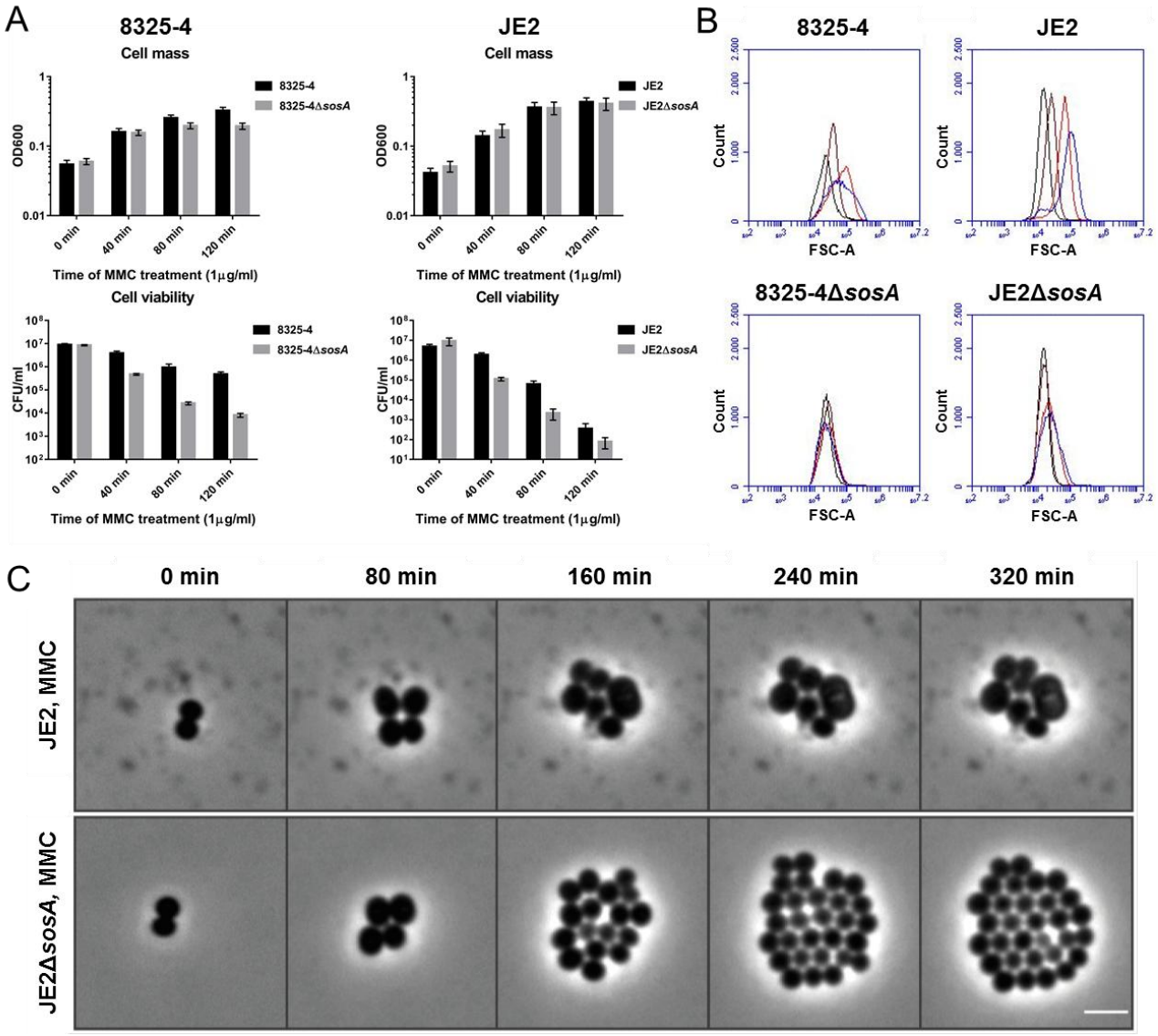

Fig 2 The *sosA* gene supports survival of *S. aureus* subjected to lethal DNA damage and is involved in bacterial swelling. (*A*) Culture optical density at 600 nm and cell viability of *S. aureus* strains 8325-4 and JE2 in comparison with their respective *ΔsosA* mutants upon challenge with a lethal dose of mitomycin C (MMC, 1 µg/ml) for 2 hours. Error bars represent the standard deviation from three biological replicates. (*B*) Cell size of 8325-4 and JE2 wild type and *ΔsosA* mutants during the course of the mitomycin C killing experiment estimated arbitrarily by flow cytometry (FSC-A). Cells were grown exponentially prior to MMC addition at an OD_{600} of 0.05. Samples were taken after 0 (black), 40 (brown), 80 (red) and 120 (blue) minutes of incubation with MMC. (*C*) Effect of MMC treatment (0.04 µg/mL) on cell shape and cell number of JE2 wild type and JE2Δ*sosA* as visualized by time-lapse phase contrast microscopy. Scale bar 2 µm.

Figure 3

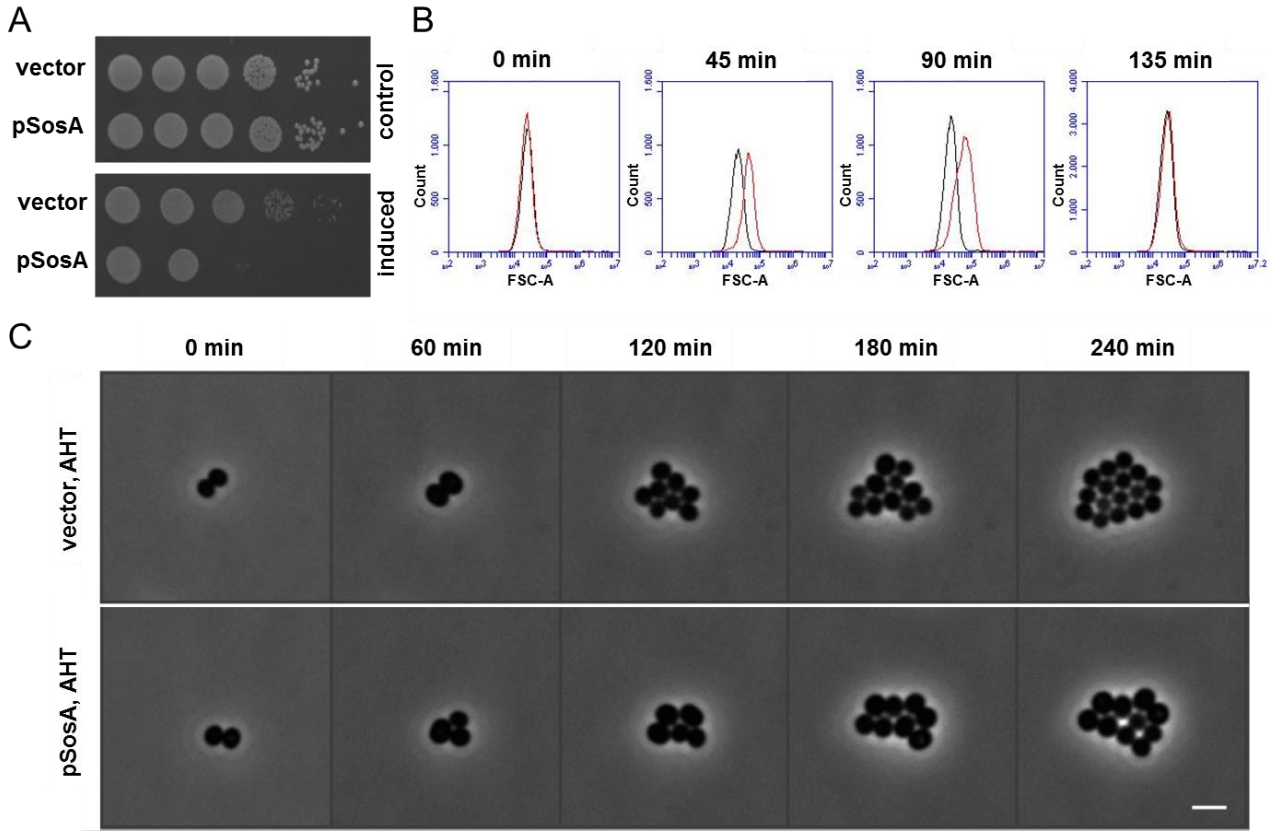

Fig 3 Expression of *sosA* alone interferes with *S. aureus* growth. (*A*) The effect of controlled expression of *sosA* on the ability to form colonies was assessed in *S. aureus* RN4220. Strains carrying either the vector control (vector) or *sosA* under an anhydrotetracycline (AHT)-inducible promoter (pSosA) were grown exponentially to an OD_{600} of 0.5, serially 10-fold diluted and plated on TSA plates in the presence (induced) or absence (control) of TSA+300 ng/ml AHT. The plates were incubated overnight at 37 °C and imaged. (*B*) Evaluation of cell size distribution by flow cytometry (FSC-A) of *S. aureus* RN4220 containing the control vector (black) or pSosA (red). Cells were grown exponentially prior to induction with 100 ng/ml AHT. At indicated time points cells were collected and analyzed by flow cytometry. (*C*) Visualization of cell size increase and reduction

of cell number of JE2/pRAB12-*lacZ* (control) and JE2/pRAB12-*sosA* in presence of 200 ng/mL AHT by time-lapse phase contrast microscopy at 37°C. Scale bar 2 µm.

Figure 4

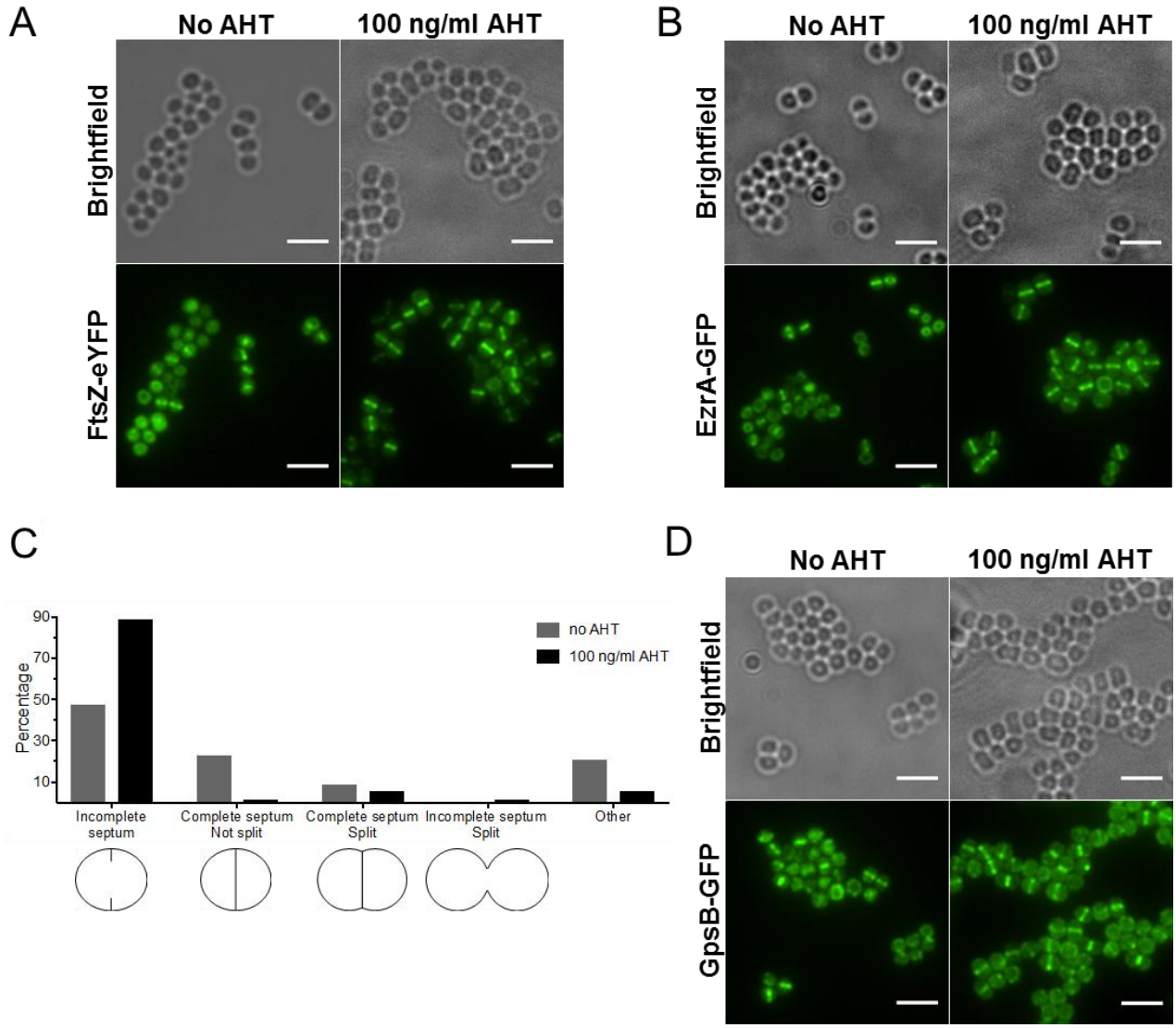

Fig 4 SosA does not impair localization of cell division proteins and septum formation initiation. (*A*) Localization of FtsZ-eYFP in SJF4694 (JE2 pSosA pCQ11-FtsZ-eYFP) grown in the absence and presence of 100 ng/ml AHT for 45 min. Fluorescence images are average intensity projections. Scale bars 3 µm. (*B*) EzrA-GFP localisation in SJF4697 (JE2 pSosA *ezrA-gfp+*) grown in the absence

or presence of 100 ng/ml AHT for 45 min. Fluorescence images are average intensity projections. Scale bars 3 µm. (*C*) Percentages of JE2 pSosA cells exhibiting incomplete, complete, complete split or incomplete split septa (n=573 for no AHT, n=607 for 100 ng/ml AHT; organisms either isolated or coadhered in pairs; other - staining in indistinct shape) after incubation with or without AHT for 45 min. (*D*) GpsB-GFP localisation in SJF700 (JE2 pSosA *gpsB-gfp*+) grown in the absence or presence of 100 ng/ml AHT for 45 min. Fluorescence images are average intensity projections. Scale bars 3 µm.

Figure 5

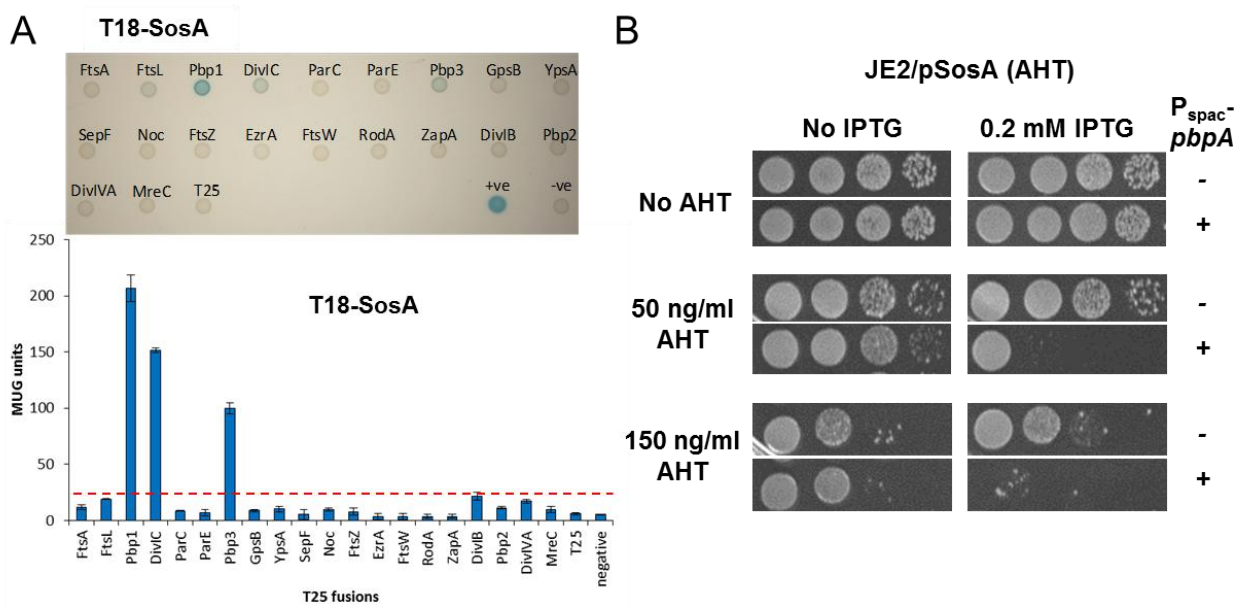

Fig 5 SosA interacts with the central divisome component PBP1. (*A*) Analysis of protein interaction between SosA and a panel of *S. aureus* cell division or cell wall synthesis proteins using the bacterial two-hybrid system. T18 and T25 are domains of an adenylate cyclase enzyme, 'T18-SosA' and 'T25 fusions' labels indicate the fusions between the C-terminus of the T18 adenylate cyclase fragment and the N-terminus of SosA and the T25 fusions to the cell division proteins. *Top*:

Interactions between *S. aureus* cell division proteins fused to T25 and *S. aureus* SosA fused to T18. 10 µl of a 1:100 dilution of overnight culture of co-transformed BTH101 were spotted onto minimal medium containing 150 µg/ml X-gal and incubated at 30 ^{o}C for 36 h. +ve, T25-zip + T18-zip; -ve, pKT25 + pUT18C. *Bottom*: β–Galactosidase activity of interactions between *S. aureus* T18~SosA and cell division proteins (T25 fusions). Activity is displayed as the mean of three independent measurements of β–galactosidase activity, in MUG (4-methylumbelliferyl-β-D-galactopyranoside) units, for each co-transformant. Error bars represent the standard deviation. Positive interactions are considered to be at least four times higher than the activity level for the negative control and this cut-off level is represented by the red line in the bar chart. (*B*) Assessment of the plating efficiency of *S. aureus* JE2 upon concomitant overexpression of *sosA* (pSosA, AHT-inducible) and *pbpA* (pPBP1) controlled by IPTG (+). (-) denotes the control vector for PBP1. Cells were grown exponentially to an OD_{600} of 0.5, serially 10-fold diluted and plated on TSA plates containing indicated inducer concentrations. The plates were incubated overnight at 37 ^{o}C and imaged.

Figure 6

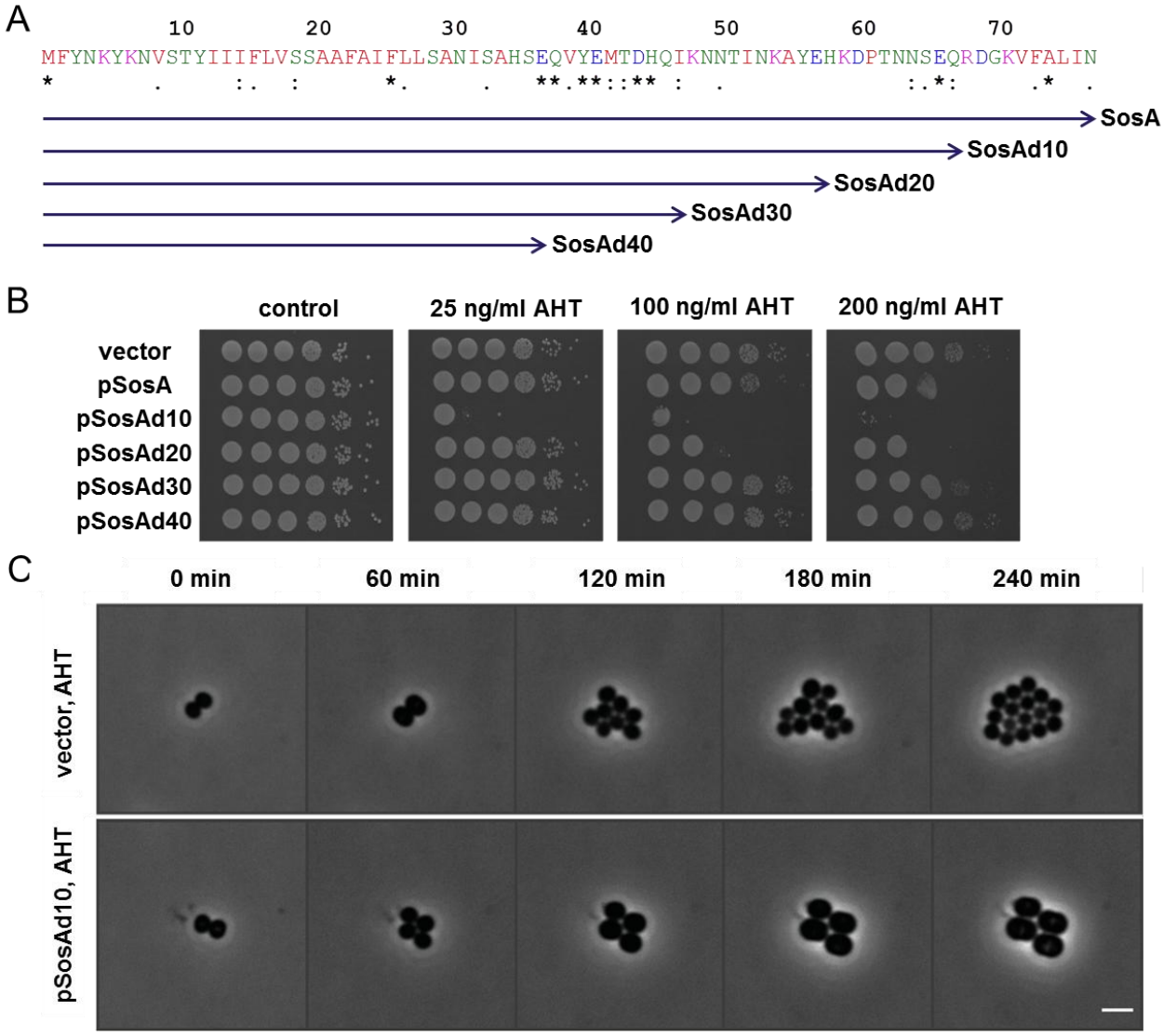

Fig 6 The effect of C-terminal truncations of SosA on inhibitory activity of the protein. (*A*) Schematic representation of the different truncated SosA constructs. Full length SosA is a 77 amino acid peptide. SosAd10 lacks the extreme C-terminal 10 amino acids, while SosAd40 is SosA truncated of almost its entire extracellular C-terminal part. Indicated conserved residues (*****) originate from the alignment in Figure 1. (*B*) Activity of the constructs was assessed in *S. aureus* RN4220 and compared to the vector control (vector). Cells were grown exponentially to an OD_{600}

of 0.5, serially 10-fold diluted and plated on TSA (control) or TSA plus inducer (AHT) at indicated increasing concentrations followed by incubation overnight at 37 °C. (C) Visualization of drastic cell size increase and reduction of cell number of JE2/pRAB12-*lacZ* (control) and JE2/pRAB12-*sosAd10* in presence of 200 ng/mL AHT by time-lapse phase contrast microscopy at 37°C. Scale bar 2 µm.

Figure 7

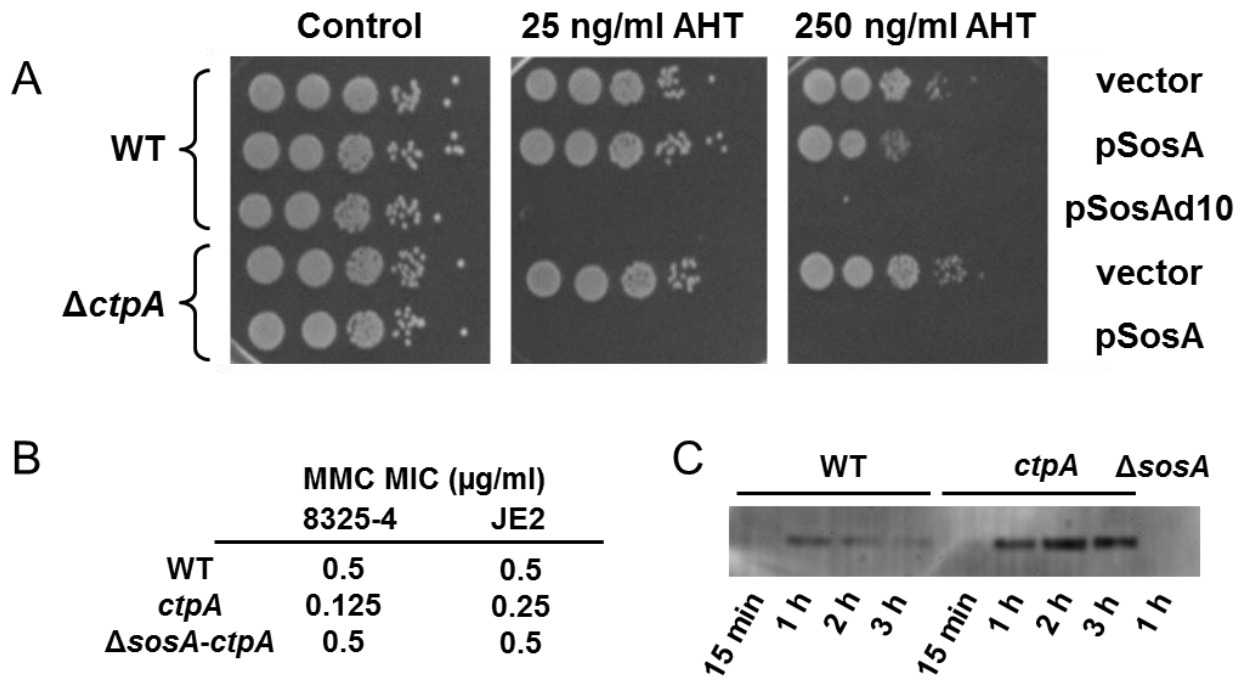

Fig 7 CtpA negatively regulates SosA. (*A*) Hypersusceptibility of an *S. aureus* JE2 *ctpA* mutant to SosA-mediated growth inhibition. Expression of *sosA* (pSosA) in wild type *S. aureus* JE2 (WT) and the corresponding JE2-*ctpA* mutant (Δ*ctpA*) were compared and referenced to the vector control (vector) and expression of the hyperactive SosAd10 variant (pSosAd10) in the wild type. Cells were grown exponentially to an OD_{600} of 0.5, serially 10-fold diluted and plated on TSA (Control) or TSA plus inducer (AHT) at indicated concentrations. The plates were incubated overnight at 37 °C and imaged. (*B*) Comparison of mitomycin C (MMC) minimum inhibitory concentration (MIC) for *S.*

aureus 8325-4 and JE2 wild type, *ctpA* and Δ*sosA-ctpA* strains. The MICs were obtained by broth dilution. (*C*) Western blot of accumulated SosA in *S. aureus* 8325-4 wild type (WT) and the *ctpA* mutant at indicated time points after addition of 0.5 µg/ml MMC to exponentially growing cells. The *S. aureus* 8325-4Δ*sosA* strain was used as a control.

Figure 8

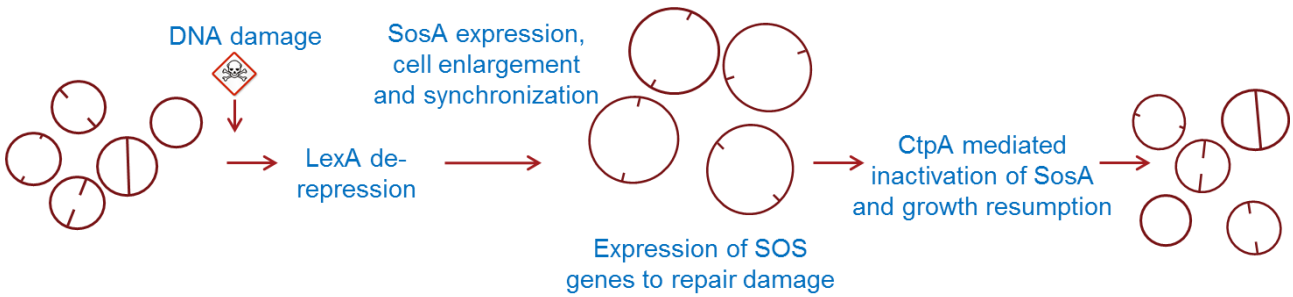

Fig 8 Proposed model for a regulated survival strategy of *S. aureus* upon DNA damage. As part of the SOS response SosA is produced and, by a membrane-localized activity, corrupts cell division activity via interaction with the essential divisome component PBP1. In effect, cells are still able to initiate septum formation but unable to complete it. This provides a spatio-temporal window for paralleled SOS response-regulated DNA repair activity. At the same time cell size increases due to off-septal activity of peptidoglycan synthesis enzymes. As the SOS response diminishes, the cellular concentration of SosA is lowered, directly or indirectly, by the proteolytic activity of CtpA. At this stage septa are allowed to complete and normal growth/division continues.

SI Appendix: 3 supplementary tables, 8 supplementary figures, 3 videos