Peptidoglycan Crosslinking Relaxation Plays an Important Role in Staphylococcus aureus WalKR-Dependent Cell Viability
Aurelia Delauné, Olivier Poupel, Adeline Mallet, Yves-Marie Coïc, Tarek Msadek, Sarah Dubrac

To cite this version:
Aurelia Delauné, Olivier Poupel, Adeline Mallet, Yves-Marie Coïc, Tarek Msadek, et al.. Peptidoglycan Crosslinking Relaxation Plays an Important Role in Staphylococcus aureus WalKR-Dependent Cell Viability. PLoS ONE, Public Library of Science, 2011, 6 (2), pp.e17054. 10.1371/journal.pone.0017054 . pasteur-02870016

HAL Id: pasteur-02870016
https://hal-pasteur.archives-ouvertes.fr/pasteur-02870016
Submitted on 16 Jun 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution 4.0 International License
Introduction

Staphylococcus aureus is the most common Gram-positive bacterium causing both nosocomial and community-acquired infections, which extend from superficial skin lesions to life-threatening deep-tissue invasive diseases such as endocarditis, osteomyelitis and pneumonia [1]. Asymptomatic carriage of this pathogen, usually within the nares, is widespread and often linked to deep-tissue invasive diseases such as endocarditis, osteomyelitis [2,3]. Asymptomatic carriage of this pathogen, usually within the nares, is widespread and often linked to deep-tissue invasive diseases such as endocarditis, osteomyelitis and pneumonia [1]. Asymptomatic carriage of this pathogen, usually within the nares, is widespread and often linked to deep-tissue invasive diseases such as endocarditis, osteomyelitis and pneumonia [1]. Asymptomatic carriage of this pathogen, usually within the nares, is widespread and often linked to deep-tissue invasive diseases such as endocarditis, osteomyelitis and pneumonia [1]. Asymptomatic carriage of this pathogen, usually within the nares, is widespread and often linked to deep-tissue invasive diseases such as endocarditis, osteomyelitis and pneumonia [1].

Despite its status as a major human pathogen, many of the mechanisms involved in S. aureus virulence and fitness remain poorly understood. Two-component systems (TCSs) are key regulatory pathways allowing bacteria to adapt their genetic expression to environmental changes. Composed of a sensor histidine kinase, generally bound to the cell membrane, and its cognate response regulator, they act to control expression of specific sets of genes in answer to defined signals. Most S. aureus strains have 16 TCSs, with an additional one present in MRSA [4,5]. Although many of these systems remain to be characterized, several have been shown to play a role in virulence (AgrC/AgrA, SaeS/SaeR) [6], antibiotic resistance (VraS/VraR, GraS/GraR) [7–9], or adaptation to environmental changes such as oxygen pressure (SrrA/SrrB) [10]. Among the 16 TCSs common to all S. aureus strains, only one is essential for bacterial survival, the WalK/WalR system (aka YycG/YycF), highly conserved among low G+C % Gram-positive bacteria [11–13].

In previous work, we highlighted the major role the WalKR system plays in S. aureus peptidoglycan metabolism by controlling expression of most of the cell wall hydrolase genes [14]. Nevertheless, none of the identified WalKR-regulated genes appear to be essential, and the reason this signal transduction pathway is required for cell viability remains to be established. Cell wall metabolism is vital for bacterial fitness, playing an important role in cell shape and division, resistance to external stresses such as osmotic pressure, as well as host-pathogen interactions. While the role of cell wall plasticity in bacterial fitness is well documented, none of the S. aureus cell wall hydrolases have been characterized as essential for bacterial growth, presumably due to the high level of genomic redundancy in this pathogen, which produces several autolytic enzymes with similar activities (see MiST Database: http://genomics.ornl.gov/mist) [15,16]. The WalKR system has also been extensively studied in B. subtilis and S. pneumoniae, and...
shown to be involved in regulation of cell wall metabolism in these bacteria [13,17–19]. Furthermore, in S. pneumoniae, the essentiality of the WalKR system has been linked to the regulation of a single gene, pceB, encoding a putative cell wall amidase [17,18].

In this study, we tested whether overexpression of WalKR-regulated autolysin genes in a WalKR-independent manner could compensate for the essential nature of the system. Indeed, since the WalKR system controls the synthesis of most of the identified cell wall hydrolases, we reasoned that the essentiality of this system could be linked to global regulation of one type of autolytic activity rather than that of a single gene. This allowed us to show that only increased production of LytM or SsaA can restore cell viability in the absence of WalKR. Although the lytM and ssaA genes are not essential when WalKR is present, our results indicate that peptidoglycan crosslinking relaxation through crossbridge hydrolysis must play a crucial role in the essential nature of the WalKR system.

Results

WalKR depletion leads to cell wall thickening and a defect in cell division

We have previously shown that the WalKR system is a major regulator of cell wall degradation, and is also necessary for peptidoglycan biosynthesis and turn-over [14]. Since none of the identified WalKR regulon genes appear to be essential, this suggested that the WalKR requirement for cell viability could be specifically linked to its role in controlling cell wall metabolism. Cell wall plasticity is involved in daughter cell separation and mutant strains deleted for two of the major Staphylococcus aureus cell wall hydrolase genes (atlA and sle1) harbor an abnormal rough cell surface and a pronounced defect in cell separation [20,21].

In order to identify morphological changes associated with WalKR-depletion, we used electron microscopy to examine S. aureus cells where the wallRK operon is placed under the control of an IPTG-dependent inducible promoter (strain ST1000, Psplac-walRK) [12], grown in the presence or absence of IPTG. Although no gross morphological differences were observed by scanning electron microscopy (data not shown), ultrastructural analysis by transmission electron microscopy (TEM) showed significant changes. Indeed, when WalKR is produced, the ST1000 strain displays the typical diplococcal S. aureus morphology, with a single central division septum (Fig. 1A). However, as shown in Fig. 1B, WalKR-depleted cells exhibit abnormal and misplaced division septa, with the formation of several new septa before separation of the daughter cells (indicated by arrows, Fig. 1B). WalKR-depleted cells also displayed a rougher cell surface, with amorphous “fuzzy” extracellular material, and increased cell wall thickness, almost twice that of cells producing WalKR (58.56 ± 12.05 nm vs. 32.64 ± 4.54 nm, respectively, Student t test P-value <0.05) (Fig. 1A and 1B). Interestingly, all of these phenotypes are strikingly similar to those described for S. aureus atlA sle1 mutants [21] and VISA (vancomycin intermediate S. aureus) strains with reduced susceptibility to vancomycin [22].

Uncoupling cell wall hydrolase gene expression from WalKR-dependent regulation

Given the dramatic impact of WalKR-depletion on septum placement and cell separation, we reasoned that WalKR-
independent expression of one or more cell wall hydrolase genes might restore cell viability in the absence of WalKR. We therefore tested the effect of uncoupling expression of autolysin-encoding genes from WalKR-dependent regulation.

Peptidoglycan hydrolases include lytic transglycosylases cleaving the glycan strand, amidases that hydrolyze the amide bonds linking the stem peptides to the glycan strand, and glycol-glycid endopeptidases cleaving the S. aureus-specific pentaglycine interpeptide crossbridges [16]. As shown in Fig. 2, we chose to overexpress WalKR-regulated genes shown or presumed to be involved in cell wall degradation. We have recently shown that sle1 [21], encoding a CHAP-domain amidase (Cysteine, Histidine-dependent Amidohydrolases/Peptidases) [23,24], is a novel member of the WalKR regulon (A. Delaune et al., in preparation). The major autolysin gene, atlA, which encodes a bifunctional enzyme with amidase and glucosaminidase domains [25] was not included in this approach since we were unable to obtain an expression plasmid carrying this gene, presumably because its expression is deleterious for E. coli. Genes were cloned into plasmid pCN51, placing them under control of the Pcad cadmium chloride-inducible promoter [26] (see Materials and Methods). The resulting overexpression plasmids were then introduced into strain ST1000 (Pspac-walRK), whose growth is IPTG-dependent [12]. Growth of strain ST1000 remained IPTG-dependent in the presence of the control vector pCN51 and cadmium chloride (Fig. 3A and Fig. 4A, open symbols), indicating that neither the pCN51 vector nor the presence of cadmium chloride interfere with expression from the Pspac promoter.

Figure 2. Diagram of S. aureus peptidoglycan and cleavage sites for WalKR-regulon encoded cell wall hydrolases. Peptidoglycan cleavage sites for WalKR-regulon encoded cell wall hydrolases are indicated: A) glycan strand glycosidic bond (glucosaminidase: AtlA). B) glycan strand glycosidic bond (lytic transglycosylases: SceD and IsaA). C) N-Acetylmuramyl-L-alanyl amide bond (AtlA amidase domain, and the Sle1, SsaA, SAOUHSC_00671, _00773, _02576 and _02883 CHAP domain hydrolases). D) pentaglycine bridge glycol-glycid bond (LytM endopeptidase).

doi:10.1371/journal.pone.0017054.g002

Figure 3. Overexpression of lytM restores viability to cells starved for WalKR. Cells were grown in TSB +/− 1 mM IPTG and gene overexpression was performed using the multicopy pCN51 plasmid carrying a CdCl2 inducible promoter whose expression was induced using 0.25 μM CdCl2. A) Growth curves are depicted for derivatives of S. aureus strain ST1000 carrying a Pspac-walRK chromosomal fusion and the pCN51 control vector (ST1012, open symbols) in TSB-CdCl2 in the presence (#) or absence (elope) of 1 mM IPTG. Growth of the ST1000 derivatives overproducing the LytM glycyl-glycid endopeptidase (ST1002, ▲) or the inactive LytM H291A enzyme (ST1128, ■) were carried out in TSB-CdCl2 without IPTG. In the presence of IPTG, strains overproducing cell wall hydrolases grew similarly to the control ST1012 strain (data not shown). B) Relative levels of lytM transcripts were measured by qRT-PCR during growth of strains ST1012 (pCN51 control) or ST1002 (overexpressing lytM), in TSB-CdCl2. Expression levels were normalized using the 16S rRNA as an internal standard and are indicated as n-fold change, expressed as the means and standard deviations of quadruplicate experiments. C) Western blot showing LytM and LytM H291A overproduction. Cells were grown in TSB-CdCl2 with or without 1 mM IPTG, and the LytM and LytM H291A proteins were detected by Western blotting using purified polyclonal rabbit antibodies. The arrow indicates the position of the mature enzyme, with a predicted molecular mass of 32 kDa. Lanes: 1, ST1012; 2, ST1012 +IPTG; 3, ST1002 (lytM); 4, ST1002 +IPTG; 5, ST1128 (lytM* H291A); 6, ST1128 +IPTG; 7, Purified mature his-tagged LytM.

doi:10.1371/journal.pone.0017054.g003
The presence of 0.25 overexpression was performed using the multicopy pCN51 plasmid in or inactive SsaA C171S (ST1124, \textit{ssaA} starved for WalKR.

Regulation of lytic transglycosylase genes is not responsible for WalKR esssentiality

Lytic transglycosylases cleave the cell wall glycan strand. Lytic activity of IsaA and SceD has been demonstrated and we have shown that the corresponding genes are controlled by the WalKR system [14,27]. We cloned the \textit{isaA} and \textit{sceD} genes in the pCN51 vector, placing them under the control of the \textit{Pead} promoter. The resulting plasmids, pAD13 and pAD01, were introduced into strain ST1000 and IPTG-dependent growth was examined in the presence of CdCl2. WalKR-independent overexpression of \textit{isaA} or \textit{sceD} was verified by qRT-PCR, and did not allow growth of the \textit{Pspac-walRK} strain in the absence of IPTG (data not shown).

Production of the LytM glycyl-glycyl endopeptidase restores growth to WalKR-depleted cells

The \textit{S. aureus} NCTC 8325 core genome encodes three M23 peptidase domain proteins with putative glycyl-glycyl endopeptidase activity. Among them is the previously characterized LytM enzyme [28,29], whereas the other two, SAOUHSC_00174 and _02464, have not been studied. Transcription of \textit{lytM} is under the control of the WalKR system, and we have shown that WalKR binds directly to its target operator sequence upstream from the \textit{lytM} promoter [12,14]. The SAOUHSC_00174 and _02464 genes are not preceded by a WalR binding site, and we have shown that their expression is not controlled by the WalKR system (data not shown).

As shown in Fig. 3A, a \textit{Pspac-walRK} strain overexpressing \textit{lytM} (strain ST1002 carrying plasmid pSD3-13) grew as well in the absence of IPTG, i.e., without expression of \textit{walRK}, as the control strain (ST1000/pCN51) in the presence of IPTG, indicating that \textit{lytM} overexpression is able to fully restore growth and viability to cells starved for the WalKR system. We verified by quantitative real-time PCR (qRT-PCR) analysis that \textit{walKR} expression remained inducible by IPTG, and unexpressed in the absence of IPTG in the ST1012 (ST1000/pCN51) and ST1002 (ST1000/pSD3-13) strains grown in the presence of CdCl2 (data not shown).

Overexpression of \textit{lytM} and corresponding LytM overproduction were verified by qRT-PCR and Western blot analysis. As shown in Fig. 3B, pSD5-13 increases \textit{lytM} transcription approximately 50-fold, and this increased transcription is directly correlated to significant LytM protein accumulation (Fig. 3C, lane 1 compared to lane 3). We note that \textit{in vivo} levels of LytM protein are undetectable in the ST1000/pCN51 strain with or without WalKR production (Fig. 3C, lanes 1 and 2), although basal levels of the protein could be seen after overexposing the Western blot (data not shown). Although the genes encoding the other two M23 peptidase proteins (SAOUHSC_00174 and _02464) are not regulated by the WalKR system, we also tested their ability to compensate WalKR depletion, and showed that neither of the two proteins allowed growth of WalKR-depleted cells when overproduced (data not shown).

In order to determine whether the glycyl-glycyl endopeptidase enzymatic activity of LytM was specifically required for its capacity to restore viability to WalKR-depleted cells, we used site-directed mutagenesis to inactivate its catalytic site. The M23 peptidase domain of LytM extends from residues 208 to 309, and the peptidase domain proteins with putative glycyl-glycyl endopeptidase activity. Among them is the previously characterized LytM enzyme [28,29], whereas the other two, SAOUHSC_00174 and _02464, have not been studied. Transcription of \textit{lytM} is under the control of the WalKR system, and we have shown that WalKR binds directly to its target operator sequence upstream from the \textit{lytM} promoter [12,14]. The SAOUHSC_00174 and _02464 genes are not preceded by a WalR binding site, and we have shown that their expression is not controlled by the WalKR system (data not shown).

As shown in Fig. 3A, a \textit{Pspac-walRK} strain overexpressing \textit{lytM} (strain ST1002 carrying plasmid pSD3-13) grew as well in the absence of IPTG, i.e., without expression of \textit{walRK}, as the control strain (ST1000/pCN51) in the presence of IPTG, indicating that \textit{lytM} overexpression is able to fully restore growth and viability to cells starved for the WalKR system. We verified by quantitative real-time PCR (qRT-PCR) analysis that \textit{walKR} expression remained inducible by IPTG, and unexpressed in the absence of IPTG in the ST1012 (ST1000/pCN51) and ST1002 (ST1000/pSD3-13) strains grown in the presence of CdCl2 (data not shown).

Overexpression of \textit{lytM} and corresponding LytM overproduction were verified by qRT-PCR and Western blot analysis. As shown in Fig. 3B, pSD5-13 increases \textit{lytM} transcription approximately 50-fold, and this increased transcription is directly correlated to significant LytM protein accumulation (Fig. 3C, lane 1 compared to lane 3). We note that \textit{in vivo} levels of LytM protein are undetectable in the ST1000/pCN51 strain with or without WalKR production (Fig. 3C, lanes 1 and 2), although basal levels of the protein could be seen after overexposing the Western blot (data not shown). Although the genes encoding the other two M23 peptidase proteins (SAOUHSC_00174 and _02464) are not regulated by the WalKR system, we also tested their ability to compensate WalKR depletion, and showed that neither of the two proteins allowed growth of WalKR-depleted cells when overproduced (data not shown).

In order to determine whether the glycyl-glycyl endopeptidase enzymatic activity of LytM was specifically required for its capacity to restore viability to WalKR-depleted cells, we used site-directed mutagenesis to inactivate its catalytic site. The M23 peptidase domain of LytM extends from residues 208 to 309, and it has previously been shown that histidine residue 291 is crucial for LytM activity [30]. We placed the \textit{lytM} mutant allele (H291A) under control of \textit{Pead} in pCN51, generating plasmid pSD3-24. As shown in Fig. 3A, expression of \textit{lytM} cannot restore growth to cells lacking WalKR. In order to compare LytM and LytM H291A production \textit{in vivo} and to rule out the possibility that the inactive LytM H291A protein might be specifically targeted by proteases, we performed Western blot experiments with polyclonal LytM antibodies. As mentioned above, native levels of LytM are undetectable in the ST1000/pCN51 strain, however as shown in Fig. 3C introduction of pSD3-13 or pSD3-24 into strain ST1000 clearly led to significant overproduction of LytM (lanes 3 & 4) or LytM H291A (lanes 5 & 6), respectively.

LytM belongs to the lysostaphin-type peptidase family, whose prototype is the \textit{S. simulans}-produced lysostaphin, which also acts as a glycyl-glycyl endopeptidase, cleaving the pentaglycine cross-
bridge [31]. Since active recombinant lysostaphin is readily available [32], we tested the effect of adding sub-lethal concentrations (0.01 and 0.025 μM/ml; higher amounts lead to cell lysis) to the extracellular medium on growth of the ST1000 IPTG-dependent Pspac-walRK strain. There was no effect on the growth profile of the WalKR-depleted culture, indicating that extracellular glycyl-glycyl activity of lysostaphin cannot compensate for the absence of WalKR (data not shown).

We also tested the effect of adding purified LytM extracellularly, by purifying a His-tagged recombinant form of mature LytM, which was also unable to restore growth in the absence of WalKR, suggesting localization of the enzyme may be important (data not shown). In a reciprocal experiment, we cloned a DNA fragment encoding a recombinant active and secreted form of lysostaphin (lux7 allele, partially deleted in the propeptide encoding region) [33] under the control of the CdCl2-inducible promoter of pCN51, generating the lysostaphin production plasmid pAD08. The induction conditions used (0.25 μM CdCl2) allowed normal growth of strain ST1000/pAD08 in the presence of IPTG. However, unlike LytM, intracellular production of lysostaphin at sub-lethal levels under conditions of WalKR deletion did not restore cell growth, suggesting that LytM and lysostaphin may differ with respect to their role in cell wall metabolism (data not shown).

**ssaA** is the only WalKR-dependent CHAP domain amidase gene able to restore growth to a WalKR-depleted strain

CHAP domains are conserved in several peptidoglycan hydrolases, often associated with amidase activity [23,24,34,35]. Careful examination of the *S. aureus* NCTC 8325 genome [5] allowed us to predict 14 genes encoding CHAP domain proteins (SAOUHSC_00256, _00427 or slet, _00671, _00773, _01219, _01515, _02019, _02023, _02173, _02571 or ssaA, _02576, _02855, _02883, _02979). In agreement with the presence of our defined consensus WalR binding site DNA sequence, we have shown that six of these are controlled by the WalKR system: ssaA, SAOUHSC_00671, _00773, _02576, _02883 [14] and slet (A. Delaune et al., in preparation).

We tested the effect of overexpressing each of these six genes on growth of the ST1000 (Pspac-walRK) strain in the presence or absence of IPTG. Whereas WalKR-independent overexpression of slet, SAOUHSC_00671, _00773, _02576 or _02883, as verified by qRT-PCR, had no effect on IPTG-dependent growth (data not shown), we showed that expression of ssaA from the Pcad promoter (plasmid pAD12) fully restores growth of *S. aureus* in the absence of WalKR (Fig. 4A). While this work was in progress, a similar finding was independently observed by the group of Simon Foster (S. Foster, University of Sheffield, personal communication).

Growth of the ST1000 strain, starved for the WalKR system but overexpressing ssaA, was identical to that of the strain expressing walRK, and we measured ssaA expression from the Pcad promoter and showed that this overexpression does not interfere with regulated expression of walRK from the Pspac promoter which remained unexpressed in the absence of IPTG (Fig. 4B and data not shown respectively). As shown in Fig. 4B, ssaA transcription was increased approximately 30-fold in strain ST1123, carrying the pAD12 plasmid with the Pcad-ssaA fusion, as compared to the strain carrying the control pCN51 vector under the same culture conditions (TSB with 0.25 μM CdCl2), similar to the lytM overexpression levels shown in Fig. 3B.

CHAP domains are characterized by two highly conserved motifs containing cysteine, histidine and asparagine residues, all of which have been shown to be critical for enzymatic activity [35,36]. In order to verify whether the ability of SsaA to restore viability to WalKR-starved cells was linked to its enzymatic activity, we used site-directed mutagenesis to change cysteine residue 171 to serine, a mutation that has previously been shown to abolish the activity of PlyC, a bacteriophage lysin with a CHAP domain [37]. The mutated ssaA* (C171S) allele was placed under the control of the Pcad promoter and introduced into strain ST1000. As shown in Fig. 4A, overexpression of the ssaA* allele is not able to restore growth to a WalKR-depleted strain. We verified by qRT-PCR that the mutated ssaA* allele was overexpressed from the Pcad promoter at levels comparable to those of the native ssaA allele (data not shown). Taken together, these data indicate that SsaA is the only CHAP domain protein encoded by WalKR regulon genes able to restore growth to WalKR-depleted cells and that this is dependent on its enzymatic activity.

**SsaA and LytM are not sufficient to completely compensate for the absence of WalKR**

As shown above, WalKR-depleted cells exhibit abnormal division septa and a rougher cell surface. We therefore examined the morphology of cells overproducing LytM or SsaA in the absence of WalKR, comparing strains ST1012 (pCN51), ST1002 (pSD3-13) and ST1123 (pAD12) by TEM. As shown in Fig. 5A, overexpression of lytM or ssaA only partially restored cell morphology: although cell wall thickness was diminished, cells presented a separation defect and some abnormal and misplaced division septa remained present in WalKR-depleted LytM- or SsaA-overproducing strains even though growth was indistinguishable from that of cells producing WalKR. In order to test the viability of these abnormal cells, we used fluorescence microscopy and the Live/Dead BacLight™ bacterial viability assay on stationary phase cultures of the ST1000 strain carrying either the pCN51 control plasmid or the pSD3-13 and pAD12 plasmids allowing overexpression of lytM or ssaA, respectively. As expected, viability of strain ST1000 carrying the pCN51 vector remains strictly dependent on WalKR, and almost all of the cells died when starved for WalKR (red staining, Fig. 5B upper panel). As shown in Fig. 5B, the ST1000/pSD3-13 or pAD12 strains were still viable in the absence of WalKR (green stain) but formed aggregates characteristic of bacteria with a cell separation defect (upper panel). This defect is not linked to lytM or ssaA overexpression since when the WalKR system was produced, the cells were well separated (lower panel).

**Discussion**

We report here the first data establishing a direct link between WalKR-dependent regulation of cell wall metabolism and WalKR essentiality in *S. aureus*. The strategy of uncoupling expression of genes encoding cell wall hydrolases from WalKR-dependent activation allowed us to show that only two WalKR-regulon autolysin genes are able to compensate for the absence of this essential TCS, *lytM* and *ssaA*. Our results suggest that loss of cell viability following WalKR depletion can be compensated through peptidoglycan crosslinking relaxation.

LytM is produced with a signal peptide, suggesting that it is secreted, and carries a peptidase M23 domain similar to that of *S. simulans* lysostaphin [28]. LytM is a glycyl-glycyl endopeptidase, cleaving the pentaglycine interpeptide crossbridges of the *S. aureus* cell wall [29,30,38]. Cross-bridges stabilize the *S. aureus* cell wall and are essential since mutants impaired in their biosynthesis (*fmhB, femA, femB*) are non- or barely viable [39–41]. Furthermore, the pentaglycine interpeptide bridges are involved in exposure of cell surface proteins that are covalently anchored to them by

---

PLOS ONE | www.plosone.org 5 February 2011 | Volume 6 | Issue 2 | e17054
sortase-dependent cross-linking [42]. Several studies have established a link between the degree of crosslinking of peptidoglycan and methicillin resistance in a PBP2-dependent manner, as well as resistance to other unrelated classes of antibiotics such as glycopeptides [39,43]. Pentaglycine cross-bridges thus play key roles in bacterial fitness, antibiotic resistance and in virulence, through the control of surface protein display, so it is not surprising that their biosynthesis genes are essential.

SsaA carries a carboxy-terminal CHAP domain, and its ortholog in S. epidermidis is highly antigenic and was recovered from whole cells and culture supernatant, suggesting it is also secreted [44]. SsaA has been linked to the hypersensitivity to macrolide-lincosamide-streptogramin B antibiotics of a thermosensitive walRK mutant in S. aureus, though the underlying molecular mechanism is unknown [45]. CHAP domains are conserved in a subset of peptidoglycan hydrolases [23,24,34,35] and we have identified 14 genes encoding CHAP-domain proteins in the S. aureus NCTC 8325 genome [5], and shown that six of these are regulated by the WalKR system [14] and (A. Delaune et al., in preparation). Of these, only ssaA is able to compensate for the absence of WalKR when it is overexpressed (Fig. 4). Among the bacterial CHAP proteins of S. aureus, only Sle1, which acts as a N-acetylmuramyl-L-alanyl amidase, has been characterized [21].

Degradation of peptidoglycan is critical, since cell wall plasticity is required for cell shape and cellular division. AtlA, one of the major S. aureus autolysins, is a bifunctional enzyme with amidase and glucosaminidase domains, and localizes in rings associated with division septa, consistent with its requirement for daughter cell separation by splitting the crosswall [46-48]. The Sle1 amidase also appears to play a crucial role in splitting the septum during cell division, since a skl mutant displays a higher number of septa per cell but without subsequent cell separation [21]. Interestingly, strains with mutations inactivating either atlA or skl are viable and their growth rates are normal, with only an atlA skl mutant presenting impaired growth [21].

We have shown here that WalKR depletion leads to a rougher cell surface, abnormal division septa and a defect in cell separation, but that cell viability is restored when LytM or SsaA are overproduced. Little is known about the role of these two autolytic enzymes in bacterial fitness. Unlike AtlA, LytM is not localized around the division septa, but distributed over the cell surface, suggesting that its role could be more general, involved in cell wall plasticity rather than cell division [29]. SsaA localization has never been studied. A ΔlytM mutant had not been described in the literature, however we constructed a mutant strain in which the entire lytM coding sequence was deleted and have shown that this mutant strain has no growth defect in rich medium, and behaves as the parental strain with respect to lysostaphin sensitivity and resistance to high osmolarity (data not shown). Essentiality of ssaA was initially controversial since although a mutant strain had been generated some time ago [45] a recent report on a genome-wide screening for essential genes in S. aureus using transposon-mediated differential hybridization suggested that it might be essential [49]. We constructed a ∆ssaA mutant as well as a ΔlytM ∆ssaA mutant (Strains ST1158 & ST1164; Table 1), and both grew as well as the parental strain, and did not display increased sensitivity to Triton X-100-induced lysis (data not shown).

Although LytM is the only glycyglycyl endopeptidase characterized so far in S. aureus [28,29], genome scanning allowed us to predict two more chromosomal genes encoding potential glycyl-glycyl endopeptidases as they share the LytM Pfam M23 peptidase domain (SAOUHSC_00174, and _02464). This likely functional redundancy could explain why the ΔlytM mutant has no obvious phenotype. The SAOUHSC_00174 and _02464 genes do not have a WalR binding site in their upstream region, their expression does not appear to be controlled by WalKR and their overexpression did not restore growth to cells depleted for WalKR (data not shown). The two genes appear to be expressed at a very low basal level and their roles in cell wall metabolism, if any, remains to be established.

Another well-known glycyglycyl endopeptidase cleaving the pentaglycine interpeptide crossbridge is lysostaphin, produced by S. simulans [50]. LytM and lysostaphin are described as having similar enzymatic activities, but we have shown that lysostaphin, added extracellularly or produced within the cells, was not able to restore viability to WalKR-depleted bacteria. This could suggest that location/targeting of LytM may be crucial or that LytM enzymatic activity could have a mild effect, allowing the cell wall to gain enough plasticity to restore growth, whereas the effect of lysostaphin has been shown to be drastic as it leads to cell lysis by forming holes in the cell wall [51]. Since distribution over the cell surface has been observed for LytM, it seems unlikely that localization is crucial for its activity [29]. The main enzymatic activities of LytM and lysostaphin are the same, i.e. glycyglycyl endopeptidase, but while they share a
catalytic domain, they also contain other domains as well, in particular the amino-terminal half of LytM (residues 26 to 212) whose specific role remains to be established. We and others have shown that LytM is not efficient in lysing S. aureus cells (data not shown) while it can cleave pentaglycine bridges and modify cell wall thickness without disrupting it [28,30,38].

As we have shown here, overexpression of either lytM or ssaA restored cell viability, but only partially compensated cell morphology in the absence of WalKR, with a cell separation defect and abnormal, incomplete or misplaced division septa still present (Fig. 5). This partial restoration is very likely due to the fact that WalKR controls expression of both aldA [14] and sde1 (A. Delaune et al., in preparation), since their expression is lowered in cells starved for WalKR and these remaining phenotypes are highly similar to those of a sde1 aldA mutant [21]. The initial finding that both LytM and SsaA can compensate for the absence of WalKR when they are overproduced was puzzling at first, since their enzymatic activities were thought to be distinct. Indeed, SsaA, with a CHAP domain, was annotated as an amidase, cleaving the N-acetylmuramoyl-L-alanyl amide bond, whereas LytM is a glycyglycyl endopeptidase. However, CHAP domains are in fact associated with two different types of peptidoglycan cleavage activities: N-acetylmuramoyl-L-alanyl amide as well as D-alanyl-glycyl endopeptidase activity [23,24,34,35]. This latter activity, effectively cleaving between the pentaglycine crossbridge and the stem peptide, is formally equivalent to that of LytM, since the end result of hydrolysing either the D-alanyl-glycyl or glycyl-glycyl bonds will lead to pentaglycine croslinking relaxation and release of a polyglycine extremity. Whereas bacterial CHAP domains are usually located at the carboxy-terminus, many S. aureus bacteriophage-encoded endolysins also have CHAP domains but at the amino-terminus [35]. Two of these endolysins have been well characterized: O11 LytA, and phage K LysK, sharing a tripartite organization: an amino-terminal CHAP domain, a central amidase2 domain, and a SH3b cell wall-binding domain. Both LytA and LysK have two distinct demonstrated enzymatic activities: a N-acetylmuramyl-L-alanyl amidase activity conferred by the amidase2 central domain and a D-alanyl-glycyl endopeptidase activity involving the CHAP domain [52,53]. Our results, showing that SsaA behaves differently from the other WalKR-regulon encoded CHAP domain proteins, strongly suggest that the SsaA CHAP domain is also endowed with D-alanyl-glycyl endopeptidase activity, which could explain why it can act as well as LytM in restoring cell viability in the absence of WalKR.

The only other bacterium in which essentiality of the WalKR system can be bypassed by expression of a regulon gene is Streptococcus pneumoniae. In Streptococci, WalKR essentiality is slightly different than in S. aureus or B. subtilis since only the gene encoding the response regulator is essential [13,54,55]. Transcriptome analyses have shown that WalKR regulates expression of several genes involved in cell wall degradation [17]. Among them is pscB, which encodes a CHAP domain protein, thought to have amidase activity, and involved in cell seption during division [17]. Interestingly, the group of Malcolm Winkler (Indiana University) was able to show that constitutive expression of the essential pscB gene is sufficient to bypass the requirement of WalR for cell viability in S. pneumoniae [17]. In agreement with our results, WalKR essentiality in S. pneumoniae as in S. aureus appears linked to regulation of a gene involved in cell wall hydrolisis, and in both cases a gene encoding a CHAP domain protein (PcsB or SsaA, respectively) is involved.

The essential nature of the WalKR system has made it a highly attractive target for novel antimicrobial compounds [56,57] and recent results indicate that this TCS is specifically activated in S. aureus during nasal colonization [38,39], suggesting it may play an important role in host-pathogen adaptation and virulence and emphasizing the importance of understanding its requirement for cell viability.

We have shown here that the genes encoding LytM and SsaA are not essential in S. aureus, but that peptidoglycan croslinking relaxation through pentaglycine cross-bridge cleavage is important

### Table 1. Strains and plasmids used in this study.

| Strain or plasmid | Description | Source or reference* |
|-------------------|-------------|----------------------|
| RN4220 | Restriction-deficient transformation recipient strain | [68] |
| ST1000 | RN4220 Pspac-walRK | [12] |
| ST1002 | Pspac-walRK pSD3-13 | pSD3-13ST1000 |
| ST1012 | Pspac-walRK pCN51 | pCN51ST1000 |
| ST1020 | Pspac-walRK pAD01 | pAD01ST1000 |
| ST1031 | ΔlytM | pAD07RN4220 |
| ST1057 | Pspac-walRK pAD04 | pAD01ST1000 |
| ST1069 | Pspac-walRK pAD06 | pAD01ST1000 |
| ST1081 | Pspac-walRK pAD08 | pAD01ST1000 |
| ST1083 | Pspac-walRK pAD09 | pAD01ST1000 |
| ST1123 | Pspac-walRK pAD12 | pAD01ST1000 |
| ST1124 | Pspac-walRK pSD3-25 | pSD3-25ST1000 |
| ST1128 | Pspac-walRK pSD3-24 | pSD3-24ST1000 |
| ST1133 | Pspac-walRK pAD13 | pAD01ST1000 |
| ST1134 | Pspac-walRK pAD14 | pAD01ST1000 |
| ST1135 | Pspac-walRK pAD15 | pAD01ST1000 |
| ST1158 | ΔssaA | pAD18RN4220 |
| ST1164 | ΔlytM ΔssaA | pAD18ST1031 |

* Arrows indicate plasmid introduction by electroporation.
doi:10.1371/journal.pone.0017054.t001
in restoring cell viability in the absence of WalKR. Cell wall degradation is an essential process, with cross wall splitting required during cell division and peptidoglycan expansion requiring the release of free extremities to add new cell wall monomers through the activity of penicillin binding proteins. As shown in this paper, overproduction of either LytM or SsaA restores the viability of WalKR-depleted cells but not normal cell separation. We can speculate that the enzymatic activities of SsaA and LytM allow normal cell wall enlargement by increasing free polyglycine extremities, allowing cell wall biosynthesis through transpeptidase reactions. Future work will aim at understanding why this is so important in a context in which most of the cell wall hydrolases are down-regulated.

Materials and Methods

Bacterial strains and growth media

Escherichia coli K12 strain DH5α™ [F− (φ80dlacZAM15) Δ (lacZYA-argF) U169 recA1 endA1 hsdR17 (rK−, mK+) phoA supE44 λ− thi-1 gyrA96 relA1] (Invitrogen) was used for cloning experiments, and E. coli strain BL21 λ, DE3 [60] (Novagen) for protein overproduction and purification. E. coli strains were grown in LB medium and ampicillin (100 μg/ml) was added when required. Staphylococcus aureus strains and plasmids used in this study are listed in Table 1. S. aureus strain ST1000 (RN4220 P_bac-wardRK) [12] and derivatives described in Table 1 were grown in Trypticase Soy Broth (TSB; Difco) supplemented with chloramphenicol (10 μg ml−1), erythromycin (1 μg ml−1) and IPTG (1 mM) when required. Cadmium chloride (CdCl2) was added at a final concentration of 0.25 μM for expression from the Pead promoter. Bacterial growth experiments were carried out in microtiter plates (100 μl culture volume) and incubated in a Synergy 2 thermoregulated spectrophotometer plate reader (BioTek) using the Gen5™ Microplate Software (BioTek). E. coli and S. aureus strains were transformed by electroporation using standard protocols [61] and transformants were selected on LB or Trypticase Soy Agar (TSA; Difco) plates, respectively, with the appropriate antibiotics.

DNA manipulations

Oligonucleotides used in this study were synthesized by Sigma-Proligo and their sequences are listed in Table S1. S. aureus chromosomal DNA was isolated using the MasterPure™ Gram-positive DNA purification Kit (Epicentre Biotechnologies). Plasmid DNA was isolated using a QIAprep Spin Miniprep kit (Qiagen) and PCR fragments were purified using the Qiaquick PCR purification kit (Qiagen). T4 DNA ligase and restriction enzymes (New England Biolabs), PCR reagents and Pdox thromostable DNA polymerase (Roche) were used according to the manufacturer’s recommendations. Nucleotide sequencing of plasmid constructs was carried out by Beckman Coulter Genomics.

Plasmid and mutant construction

Plasmid pCN51 carrying the Pead-cadC promoter module (cadmium chloride-inducible promoter and the CadC repressor gene), was used for gene expression in S. aureus [26]. Plasmid pET28/16, a derivative of plasmid pET28a (Novagen), was used for protein overexpression and purification [62]. The pMAD allelic replacement vector [63] was used to generate the ΔlytM and ΔssaA mutant strains.

To uncouple gene expression from WalKR-dependent activation, DNA fragments corresponding to the coding sequences of cell wall hydrolase genes with their associated ribosome-hinding sites were generated by PCR using S. aureus RN4220 chromosomal DNA and the oligonucleotide pairs listed in Table S1, introducing BamHI and EcoRI sites at the 5′ and 3′ ends of the DNA fragments, respectively. The lytM* (encoding the LytM H291A protein) and ssaA* (encoding the SsaA C171S protein) mutant alleles were generated by site-directed mutagenesis using strand-overlap extension PCRs (SOE-PCR) [64]. Briefly, two DNA fragments overlapping at their ends were synthesized by PCR using oligonucleotide pairs OSA215/OSA259 and OSA260/OSA217 for lytM* and OAD042/OAD0265 and OAD042/OAD043 for ssaA* and fused by SOE-PCR using the external oligonucleotides, (OSA215/OSA217 for lytM*, OAD042/OAD043 for ssaA*) introducing BamHI and EcoRI sites at the 5′ and 3′ ends of the DNA fragments. The DNA fragments were cloned between the corresponding restriction sites of plasmid pCN51 in order to place gene transcription under the control of the Pead promoter. The resulting plasmids are listed in Table 1. DNA ligations were transformed into E. coli DH5α™ and the nucleotide sequences of the DNA inserts were determined before introducing the plasmids into the S. aureus ST1000 strain.

For intracellular production of lysostaphin in S. aureus, a 897-bp DNA fragment encoding an active recombinant form of lysostaphin (ls7) was generated by PCR using plasmid pCXSls7 [33], and oligonucleotides OP274 and OSA252 (see Table S1), and cloned between the BamHI and EcoRI sites of the pCN51 vector, resulting in plasmid pAD08.

Mature LytM (without its signal peptide, residues 26 to 316) was overproduced in E. coli using plasmid pAD05, constructed by cloning a 887-bp PCR-generated NovL/XloI DNA fragment corresponding to lytM (oligonucleotide pair OAD027/OAD018) between the corresponding restriction sites of plasmid pET28/16, replacing the stop codon with an XloI restriction site. This allows the creation of a translational fusion adding six histidine residues to the carboxy-terminus of the corresponding proteins, placing expression of the genes under the control of a T7 bacteriophage promoter.

For generation of a Δytm mutant strain, two DNA fragments, of 615 and 804 bp, were generated by PCR using oligonucleotides OAD003/OAD004 and OAD013/OAD014, respectively (see Table S1), corresponding to the DNA regions located immediately upstream and downstream from the lytM gene. These DNA fragments were cloned in tandem in two consecutive steps, between the BamHI and BglII restriction sites of the pMAD vector, resulting in plasmid pAD07. The plasmid was introduced by electroporation into S. aureus strain RN4220, and transformants were selected at 30°C on TSA plates containing erythromycin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (100 μg/ml). Integration and excision of pAD07 were then performed as previously described [63], yielding strain ST1031 (ΔlytM mutant).

The same approach was used to obtain the ΔssaA mutant. Oligonucleotides OAD064/OAD065 and OAD066/OAD067 were used to generate by PCR two DNA fragments of 559 bp and 607 bp respectively (Table S1). These fragments, corresponding to the upstream and downstream regions of the ssaA gene, were cloned between the BamHI and NdeI restriction sites of the pMAD vector to construct pAD18. The plasmid was then introduced into S. aureus strain RN4220 or ST1031 (ΔlytM). Integration and excision of pAD18 were performed as previously described [63], yielding strains ST1158 (RN4220 ΔssaA) and ST1164 (ΔlytM ΔssaA). The markerless gene deletions were confirmed by PCR amplification.

Total RNA extraction

Strains were grown in TSB- CdCl2 (0.25 μM) +/− 1 mM IPTG at 37°C, with aeration until OD600 = 1. Cells were pelleted by centrifugation (2 min, 20,800 x g) and immediately frozen at
Viability testing

Bacteria were stained using the LIVE/DEAD BacLight™ viability assay L-7012 (Molecular Probes, Invitrogen, Carlsbad, CA). This stain distinguishes live cells from dead bacteria, based on membrane integrity and two nucleic acid stains. The green fluorochrome (SYTO 9) can penetrate intact membranes while the larger red fluorochrome (propidium iodide) only penetrates compromised membranes of dead bacteria. Bacterial cultures were grown in TSB CdCl₂ (0.25 μM) +/- IPTG (1 mM). At appropriate optical densities, cultures were washed 3 times in 0.9% NaCl and concentrated in 0.9% NaCl between 2 to 10 times depending on the initial optical densities. Staining and fluorescent microscopic observations were then carried out as specified by the manufacturer.

Electron microscopy

For ultrastructural analyses, bacteria were grown in TSB +/- 1 mM IPTG and 0.25 μM CdCl₂ when indicated until OD₆⁰₀ = 1. Bacteria were fixed overnight at 4°C with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. Fixed samples were treated with 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, for 1 h, dehydrated in ethanol and embedded in epoxy resin. Ultrathin sections were stained with 2% uranyl acetate and lead citrate and examined using a Jeol JEM1010 transmission electron microscope (Jeol, Tokyo, Japan) at 80 kV and an Eloise MegaView III camera (Eloise SARL, Roissy, France).

Supporting Information

Table S1 Oligonucleotides used in this study. (DOC)

Acknowledgments

We would like to thank Fritz Götz for the kind gift of plasmid pCXlss7 and many helpful suggestions, as well as Marie-Christine Prévost (Institut Pasteur, Ultrastructural Microscopy Platform), François Baleux (Institut Pasteur, Chemistry of Biomolecules), Ivo Gomperts-Boneca and Simon J. Foster for helpful discussion.

Author Contributions

Conceived and designed the experiments: AD SD TM. Performed the experiments: AD OP AM Y-MC SD. Analyzed the data: AD OP AM SD TM. Contributed reagents/materials/analysis tools: AD OP AM Y-MC SD. Wrote the paper: AD SD TM.
32. Schneewind O, Fowler A, Faulk RF (1995) Structure of the cell wall anchor of surface proteins in Staphylococcus aureus. Science 268: 103–106.
33. Maidhof H, Reinicke B, Blument P, Berger-Bachi B, Labischinski H (1991) fadD, which encodes a factor essential for expression of methicillin resistance, affects glycine content of peptidoglycan in methicillin-resistant and methicillin-susceptible Staphylococcus aureus strains. J Bacteriol 173: 3507–3513.
34. Lang S, Livesey MA, Lambert PA, Littler WA, Elliott TS (2000) Identification of a novel antigen from Staphylococcus epidermidis. FEMS Immunol Med Microbiol 29: 211–220.
35. Martin PK, Bao Y, Boyer E, Winterberg KM, McDowell L, et al. (2002) Novel locus required for expression of high-level macroline-lincosamide-streptogramin B resistance in Staphylococcus aureus. J Bacteriol 184: 5610–5613.
36. Baba T, Schneewind O (1999) Targeting of multiple virulence determinants to the cell division site of Gram-positive bacteria: repeat domains direct autolysin to the equatorial surface ring of Staphylococcus aureus. Embo J 17: 4639–4646.
37. Sugai M, Komatsuzawa H, Akiyama T, Hong YM, Oshida T, et al. (1995) Identification of endo-beta-N-acetylglucosaminidase and N-acetylmuramyl-L-alanine amidase as cluster-dispersing enzymes in Staphylococcus aureus. J Bacteriol 177: 1491–1496.
38. Yamada S, Sugai M, Komatsuzawa H, Nakashima S, Oshida T, et al. (1996) An autolysin ring associated with cell separation of Staphylococcus aureus. J Bacteriol 178: 1565–1571.
39. Chaudhuri RR, Allen AG, Owen PJ, Shalom G, Stone K, et al. (2009) Comprehensive identification of essential Staphylococcus aureus genes using Transposon-Mediated Differential Hybridisation (TMDH). BMC Genomics 10: 291.
40. Schindler CA, Schuennert VT (1964) Lysostaphin: A new bacteriolytic agent for the lysis of staphylococci. Proc Natl Acad Sci U S A 51: 114–119.
41. Francius G, Domenech O, Mingeot-Leclercq MP, Dufrene YF (2008) Direct observation of Staphylococcus aureus cell wall digestion by lysostaphin. J Bacteriol 190: 7904–7909.
42. Becker SC, Dong S, Baker JR, Foster-Frey J, Pritchard DG, et al. (2009) LysK CHAP endopeptidase domain is required for lysis of live staphylococcal cells. FEMS Microbiol Lett 294: 52–60.
43. Navarre WW, Ton-Thañ H, Faull FK, Schneewind O (1999) Multiple enzymatic activities of the muramidase hydrolase from staphylococcal phage phi11. Identification of a D-alanyl-glycine endopeptidase activity. J Biol Chem 274: 13547–13556.
44. Throup JP, Koerber KK, Bryant AP, Ingram KA, Chalker AF, et al. (2000) A genomic analysis of two-component signal transduction in Staphylococcus pneumoniae. Mol Microbiol 35: 566–576.
45. Wagner C, Saisset Ad A, Schonfeld HJ, Kamber M, Lange R, et al. (2002) Genetic analysis and functional characterization of the Staphylococcus pneumoniae vic locus. J Bacteriol 184: 969–976.
46. J Bacteriol 177: 1491–1496.
47. Becker SC, Dong S, Baker JR, Foster-Frey J, Pritchard DG, et al. (2009) LysK CHAP endopeptidase domain is required for lysis of live staphylococcal cells. FEMS Microbiol Lett 294: 52–60.
48. Navarre WW, Ton-Thañ H, Faull FK, Schneewind O (1999) Multiple enzymatic activities of the muramidase hydrolase from staphylococcal phage phi11. Identification of a D-alanyl-glycine endopeptidase activity. J Biol Chem 274: 13547–13556.
49. Throup JP, Koerber KK, Bryant AP, Ingram KA, Chalker AF, et al. (2000) A genomic analysis of two-component signal transduction in Staphylococcus pneumoniae. Mol Microbiol 35: 566–576.
50. Wagner C, Saisset Ad A, Schonfeld HJ, Kamber M, Lange R, et al. (2002) Genetic analysis and functional characterization of the Staphylococcus pneumoniae vic locus. J Bacteriol 184: 969–976.
51. Francius G, Domenech O, Mingeot-Leclercq MP, Dufrene YF (2008) Direct observation of Staphylococcus aureus cell wall digestion by lysostaphin. J Bacteriol 190: 7904–7909.
52. Becker SC, Dong S, Baker JR, Foster-Frey J, Pritchard DG, et al. (2009) LysK CHAP endopeptidase domain is required for lysis of live staphylococcal cells. FEMS Microbiol Lett 294: 52–60.
53. Navarre WW, Ton-Thañ H, Faull FK, Schneewind O (1999) Multiple enzymatic activities of the muramidase hydrolase from staphylococcal phage phi11. Identification of a D-alanyl-glycine endopeptidase activity. J Biol Chem 274: 13547–13556.
54. Throup JP, Koerber KK, Bryant AP, Ingram KA, Chalker AF, et al. (2000) A genomic analysis of two-component signal transduction in Staphylococcus pneumoniae. Mol Microbiol 35: 566–576.
55. Wagner C, Saisset Ad A, Schonfeld HJ, Kamber M, Lange R, et al. (2002) Genetic analysis and functional characterization of the Staphylococcus pneumoniae vic locus. J Bacteriol 184: 969–976.
56. J Bacteriol 177: 1491–1496.
57. Becker SC, Dong S, Baker JR, Foster-Frey J, Pritchard DG, et al. (2009) LysK CHAP endopeptidase domain is required for lysis of live staphylococcal cells. FEMS Microbiol Lett 294: 52–60.
58. Navarre WW, Ton-Thañ H, Faull FK, Schneewind O (1999) Multiple enzymatic activities of the muramidase hydrolase from staphylococcal phage phi11. Identification of a D-alanyl-glycine endopeptidase activity. J Biol Chem 274: 13547–13556.
59. Throup JP, Koerber KK, Bryant AP, Ingram KA, Chalker AF, et al. (2000) A genomic analysis of two-component signal transduction in Staphylococcus pneumoniae. Mol Microbiol 35: 566–576.