# Characterization of a delta *rod*A mutant in *Staphylococcus carnosus*

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# 1 ZUSAMMENFASSUNG

Bakterien können in einer Vielzahl von Formen erscheinen. Manche sind stäbchenförmig, manche geformt wie Spiralen andere bilden Filamente oder sogar komplizierte pilzartige Geflechte. Formgebend ist in Bakterien vor allem die Anordnung ihrer Zellwand dem sogenannten Murein oder Peptidoglykan, einer rigiden polymeren extrazellulären Struktur. Staphylokokken sind wie bereits in ihrem Name beschrieben, kleine kugelförmige Bakterien, die sich in Clustern anordnen können. Trotz seiner vergleichsweise einfachen Form besitzt das nicht pathogene Bakterium *Staphylococcus carnosus* drei Proteine, welche in stäbchenförmigen Bakterien mit dem Erhalt der Zylinderform des Mureins assoziiert werden.

Das Ziel der Arbeit war es diese sogenannten SEDS-Proteine (steht für shape elongation division and sporulation, also Form, Längenwachstum, Teilung und Sporenbildung) auf ihrer Funktion hin zu untersuchen. Von jedem der 3 in *S. carnosus* annotierten Gene (*fts*W1, *fts*W2, *rod*A) wurden Deletionsmutanten angefertigt und diese wurden biochemisch und morphologisch charakterisiert. Unter den getesteten Bedingungen, welche den Standardbedingungen im Labor entsprachen, zeigte lediglich die Deletion von *rod*A eine eindeutige phänotypische Veränderung gegenüber dem Wildtyp Stamm.

Die Wachstumsrate war verändert mit einer verkürzten lag-Phase und einem niedrigeren Endniveau. Diese Beobachtung und die frühzeitige, spontane Lyse des Mutanten-Stamms ließen darauf schließen, dass es auch Veränderungen in der Zellwand der Mutante gegeben hat. Lichtmikroskopische Untersuchungen der Zellen waren genauso wie die elektronenmikroskopischen unauffällig. Erst als mit fluoreszenzmikroskopischen Methoden der genaue Ort der Murein-Biosynthese sichtbar gemacht wurde, konnten Unterschiede in Form von einer diffusen statt septalen Verteilung des neu synthetisierten Mureins ausgemacht werden. Dies bestätigte sich nochmal durch Anwenden hochauflösender Fluoreszenzmikroskopie.

Des Weiteren wurde das Murein bzw. dessen Zusammensetzung biochemisch und chromatographisch untersucht. Murein besteht aus den zwei alternierenden Zucker N-Acetyl-Glukosamin und N-Acetyl-Muraminsäure, die ß-(1,4) glykosidisch verknüpft sind. Angehängt an die Muraminsäure ist eine Peptid-Kette mit 3 bis 5 Aminosäure, welche in *S. carnosus* wiederrum über eine aus 5 Glyzinen bestehende Interpeptid-

Brücke quervernetzt sein können. Man erhält durch Verdau mit dem Enzym Mutanolysin und Auftrennung über einer HPLC ein typisches Muropeptid-Muster, welches im Falle der Mutante eindeutig zu längeren Retentionszeiten verschoben war. Massenspektrometrische Analysen offenbarten den Einbau der Aminosäure Serin in die Muropeptide der Mutante. Diese ist nicht ungewöhnlich für Bakterien, zeigt aber, inwiefern die Deletion die Zellen beeinflusst. Dieser Fund wurde in einem speziell Zur Bestätigung dieses Fundes wurden die Aminosäuren der Stammpeptide des Mureins mit einer eigens dafür etablierten Methode untersucht, sowie eine zweite besser auflösende Massenspektrometrie durchgeführt. In beiden Fällen waren die Ergebnisse positiv für den Einbau von Serin in das Murein der Mutante. Eine weitere Auffälligkeit aus der Massenspektrometrie, nämlich das vermehrte Vorkommen von Tripeptiden, Mureinuntereinheiten mit lediglich drei Aminosäuren im Stammpeptid, und deren Abwesenheit in der Mutante führte zu dem Schluss, dass die Modifikation, die im Wild typ *S. carnosus* TM300 noch durchgeführt werden kann, in der *rod*A Deletion gestört ist. Das Enzym, das vermutlich für diese Modifikation verantwortlich ist, nennt man L, D-Carboxypeptidase und ist nur für zwei Staphylokokken Spezies annotiert, neben *S. carnosus* auch noch in *S. pseudointermidies*. Dies erklärt zum einem den Unterschied im Murein-Muster von *S. aureus* SA113 WT, der dieses Enzym nicht besitzt, zu *S. carnosus* TM300 zum anderen scheint in der *rod*A Deletion die Funktion des Enzyms beeinträchtigt zu sein, sei es durch den vermehrten Einbau von Serin, durch eine fehlende Aktivierung durch RodA oder durch fehlerhafte Lokalisation.

# 2 SUMMARY

Bacteria can appear in various shapes. Some are rod-shaped some form spirals and other build filaments or even complex mycelium. The cell wall is responsible for the determination of the shape. The cell wall consists of peptidoglycan, which is an extracellular polymeric structure. Staphylococci are small spherical bacteria, which are known to form clusters. Despite maintaining this relatively simple form the non pathogenic *S. carnosus* possesses three so called SEDS proteins (shape, elongation, division and sporulation) usually associated with the rod from of other bacteria.

The aim of this study was the estimation of all the three annotated genes (*fts*W1, *ftsW*2, *rod*A) by creating deletions of these genes and by characterization of the deletion mutants. But under normal laboratory conditions only the Δ*rod*A deletion showed an obvious phenotype compared to the wild type. The growth rate was diminished but the lag-phase was also reduced. Regarding these results and a higher and earlier lysis rate in the Δ*rod*A deletion it was concluded that there were some alterations in the peptidoglycan of the mutant. Differences could not be observed in light or electron microscopy. However after labeling the sites of peptidoglycan biosynthesis with fluorescence markers, it occurred that the much defined localization of the peptidoglycan biosynthesis was disperse in the Δ*rod*A deletion. This could also be confirmed with super resolution fluorescence microscopy.

Experiments regarding the composition of the peptidoglycan were performed. Usually in staphylococci it consists of alternating residues of β-(1,4) linked *N*-acetylglucosamine and *N*-acetylmuramic acid. Attached to the *N*-acetylmuramic acid is a peptide chain of three to five amino acids. The glycan chains can be crosslinked by the interpeptide bridge containing of 5 glycine residues. Purification, digestion with the enzyme mutanolysine and separation via HPLC leads to a unique pattern of muropeptides. This pattern was shifted to higher retention times for the mutant and therefore the muropeptides were analyzed in detail by mass spectrometry. This revealed the incorporation of serine in the peptidoglycan of the mutant. Serine is not very unlikely to be found in the peptidoglycan, but unusual to result of a gene deletion. To confirm the serine incorporation the amino acids of the stem peptide were analyzed and a second MS analysis with a better resolution was performed. Both experiments gave positive results for serine in the mutant muropeptides. From the MS data there

was an interesting finding in the amount of tripeptides for the monomeric muropeptides of the *S. carnosus* TM300 WT to the penta- or tetrapeptides of the Δ*rod*A deletion. On the one hand this could explain the differences of the muropeptide pattern from *S. aureus* SA113 WT to *S. carnosus* TM300 WT, because there is an enzyme called L, D- carboxypepidase which specifically catalyzes the hydrolization of tetra- to tripeptides and can only be found in two of the annotated staphylococcal species, on the other hand the shifted muropeptide pattern of the mutant can be explained from the disfunction of this enzyme. The absence of RodA somehow reduces the L, D-carboxypepidase whether increased amount of serine turns the muropetides into a poor substrate for the enzyme or the RodA protein is needed for activation or correct localization of the L, D- carboxypepidase.

# 3 INTRODUCTION

## 3.1 *GENUS STAPHYLOCOCCI*

The genus Staphylococcus (Firmicutes; Bacilli, Bacillales, Staphylococcaceae) belongs to the Gram positive bacteria and are round shaped microorganisms, which often form groups of masses, hence their name: staphyle is ancient Greek for bunch of grapes and coccus is ancient Greek for round (Ogston, 1882). The facultative anaerobic, non-motile, and non sporeforming staphylococci have a low DNA G+C content (30-39 %) and a genome size of 2-3 Mbp in average (George, 1994; Kloos, 1998). Around 50 species and subspecies of this genus are described and classified into two groups: coagulase-negative (CNS) and coagulase-positive staphylococci (CPS) (Rosenstein and Götz, 2013). This classification is based on the enzyme coagulase which is secreted and leads to agglutination of blood plasma (Tobin *et al.*, 1994). Most important staphylococcal species were grouped based on DNA-DNA hybridization studies (Schleifer *et al.*, 1983; Münch, G. *et al.*, 2012) to the CNS and novobiocin-sensitive species groups *S. epidermidis* (*e. g. S. lugdunensis*, *S. capitis*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. warneri*) and *S. simulans* (*e. g. S. carnosus* and *S. simulans*), the CNS and novobiocin-resistant species groups *S. saprophyticus* (*e. g. S. cohnii*, *S. saprophyticus*, *S. xylosus*) and *S. sciuri*, as well as the CPS and novobiocinsusceptible species groups *S. intermedius* (*e. g. S. delphinii*, *S. intermedius, S. hyicus*) and their main representative *S. aureus* (Futatsugi *et al.*, 2013). This mapping is backed up by analysis of the major autolysines (Albrecht, 2012).

Staphylococci are a wide spread species. The natural habitats of staphylococci are the skin, cutaneous glands and the mucosa of animals and humans. The micro flora of human skin is mainly composed of CNS, where *S. epidermidis* occurs as the most frequent and dominant colonizing species (Lleo *et al.*, 1990; Marples, 1998). CNS were classified over a long time as saprophytic and rarely pathogenic organisms. In the meantime *S. epidermidis*, *S. haemolyticus* and *S. saprophyticus* were classified as opportunistic pathogens and are one of the main causes of nosocomial, foreign object associated, and blood stream infections especially in immunosuppressed persons (Tobin *et al.*, 1994; von Eiff *et al.*, 1997; Zell *et al.*, 2008). In contrast to CNS, the CPS *S. aureus* strains are the major human pathogens that cause acute and inflammatory diseases. These are for example skin infections and invasive infections like

endocarditis, osteomylitis, pneumonia and sepsis. Furthermore, some *S. aureus* strains can lead to food poisonings, exfoliative dermatitis or toxic shock syndrome if producing the matching toxins. The main reasons for the pathogenicity of *S. aureus* are the various virulence factors such as extracellular enzymes (coagulase, DNase, fibrinolysin, hyaluronidase), exotoxines (α-, β-, γ-, δ-hemolysine, leukocidine, exfoliative toxin A and B, toxic-shock-syndrom-toxine-1), pyrogentic and superantigenes. Cell wall anchored surface proteins play an important role in tissue adhesion and immune evasion, and are also known as microbial surface components recognizing adhesive macromolecules (MSCRAMMs), *e. g.* protein A (SPA), fibrinogen-binding clumping factors ClfA/B; fibronectin-binding proteins FnbpA/B, or collagen adhesin Cna (Yokoe *et al.*, 2014). *S. aureus* also has the ability to proliferate extracellularly, as well as intracellularly.

In the past years, caused by an intensive use of antibiotic treatment in medical health care as well as livestock breeding, there was an increase in the number of antibiotic resistant staphylococci. This is a severe problem which is still growing and challenging for medical health care systems worldwide. Also an increase of antibiotic resistant CNS strains isolated from food was noticed (Heroven *et al.*, 2012). CNS, such as *S. carnosus* or *S. xylosus*, are used as food grade organisms, *e. g*. as meat starter cultures in food industry (Hammes and Hertel, 1998; Kengara *et al.*, 2013) .

## 3.2 *STAPHYLOCOCCUS CARNOSUS*

The species *Staphylococcus carnosus* was first isolated from raw meat and was named according to that. The coagulase negative staphylococcus (CNS) *S. carnosus* is an avirulent GRAS (generally regarded as safe) organism, which is widely used in food industry as meat starter culture or in biotechnology as cloning host. The natural habitat of *S. carnosus* is not known but it is speculated to be found on animal skin since it can be isolated from meat products or even fermented fish (Rosenstein and Götz, 2010).

The genome of *S. carnosus* TM300 has just recently been sequenced. It revealed a G+C content of 34.6% which is higher than the GC content of most other staphylococcal species like the pathogenic *S. aureus*, *S. epidermidis*, *S. saprophyticus* and *S. haemolyticus* but still in the low GC-cluster of the gram-positive bacteria. In addition the genome with its 2,56 Mbp and only 2474 open reading frames (ORF) is one of the smaller staphylococcal genomes. In contrast to the pathogenic staphylococci, the genome contains only one prophage (ΦTM300), but no mobile elements such as plasmids, insertion elements or transposons are present. 55 ORFs of *S. carnosus* TM300 are disrupted, which this species might afford because of the nutrient rich habitat. Two out of these disrupted genes are annotated as the global regulators *agrC* and *saeR* (Augustin et al., 1992). Agr (accessory gene regulator) and SaeR are responsible for the expression of exoproteins among other main virulence factors (Giraudo, 1999; Rogasch *et al.*, 2006; Prufer *et al.*, 2012; Schonberger *et al.*, 2012). Another disrupted gene is *ffh* which encodes a key protein for signal recognition (Munch *et al.*, 2012) and translocation of membrane proteins and secreted proteins to the cytoplasmic membrane that in *B. subtilis* is required for the "bacterial signal recognition particle" (SRP)-dependant pathway (Zanen *et al.*, 2006). The inactivation of thoses three genes might explain the small amount of extracellular proteins in *S. carnosus* TM300. On the other hand the genome encodes all necessary genes for dissimilatoric nitrate and nitrite reduction, two genes for catalases, which are suitable for hydrogen peroxide reduction (Schlag, S. *et al.*, 2008).

To meet the requirements for a starter culture *S. carnosus* has to survive in a high salt environment and therefore possesses various osmoprotective systems and sodium-proton-antiporter. Additionally the genome analysis revealed a gene for the ornithin-decarboxylase, an enzyme synthesizing the biogenetic amines putrescine and cadaverine out of e.g. meat. These substances are natural metabolic products and synthetic precursors for alkaloides and polyamines as well as parts of ribosomes but toxic in larger amounts. Genes which are not present in *S. carnosus* are the *ica*-genes and so it cannot perform the PIA dependent biofilm formation. Despite of having three putative hemolysis and two exotoxine genes there is no hemolysis activity detectable in *S. carnosus* (Rosenstein *et al.*, 2009)

### *3.2.1* S. CARNOSUS *AS EXPRESSION SYSTEM*

In biotechnology it is common to produce peptides and proteins in microorganisms. Many recombinant proteins are produced in the Gram-negative organism *Escherichia coli*. But occasionally the overproduction in *E. coli* leads from misfolding to aggregation known as inclusion bodies (Müller *et al.*, 2012; Siemens *et al.*, 2012); Furthermore, part of the outer membrane of *E. coli* the lipopolysaccharide or endotoxine can cause severe problems, if the protein product is contaminated. Even small amounts of endotoxine can lead to inflammatory reactions in animal and human patients and the so called endotoxic shock can cause severe tissue damage or might even be lethal (Anspach, 2001; Ogikubo *et al.*, 2004; Boddy *et al.*, 2012).

Gram-positive bacteria are a good alternative to be used as overexpression systems as they lack the endotoxin. Another advantage of their usage is the structure of the cell envelope with only an inner cytoplasmic membrane and a thick murein layer, which allows the secretion of proteins directly to the supernatant. In contrast, Gram-negative bacteria possess two membranes that secreted proteins have to pass. *Bacillus subtilis* is often used as an expression system. However, fast degradation of secreted proteins by exoproteases is one major disadvantage and is making the system unsuitable for the overproduction and secretion of proteins. Nevertheless, protease free *B. subtilis* strains are available which are used in biotechnology. In previous studies, only low exoproteolytic activity was observed in *S. carnosus* TM300 (Götz, F. *et al.*, 1985; Liebl and Götz, 1986; Meens *et al.*, 1997). This might be due to the missing or defect regulators for exoproteins, such as AgrC, SaeR and Ffh (Rosenstein *et al.*, 2009). In contrast to the pathogenic staphylococci, no virulence factors are present in *S. carnosus*, neither secreted nor anchored to the cell wall peptidoglycan. Therefore, *S. carnosus* can serve as a system for the secretion and overproduction of proteins.

In addition *S. carnosus* only possesses a small amount of IgG binding proteins unlike *S. aureus* with huge amounts of Protein A. This finding means less trouble with all immunological based experiments such as immune-precipitation, western blots and many more frequently used in research. Another advantage of working with *S. carnosus* is the consistent development of expression systems for the induction of gene expression, the secretion of proteins and the secretion and anchoring of proteins to its cell wall (Wieland *et al.*, 1995; Peschel *et al.*, 1996; Strauss and Gotz, 1996; Hansson *et al.*, 2002; Wernerus *et al.*, 2003; Krismer *et al.*, 2012).To accelerate

complicated cloning, shuttle plasmids are available that can be used in both, *E. coli* and staphylococci (Brückner *et al.*, 1993; Wernerus *et al.*, 2003; Corrigan and Foster, 2009; Arnold *et al.*, 2012; Jarius *et al.*, 2012; Munch *et al.*, 2012).

## 3.3 *THE CELL WALL OF STAPHYLOCOCCI*

The murein or peptidoglycan (PG) sacculus is the shape and structure giving element of the bacterial cell, which in the case of the well-studied *S. aureus* is composed of a thick murein layer (~40 layers, 20-80 nm), covalently anchored and attached proteins as well as wall teichoic acids (WTA) (Strecker *et al.*, 2012). The chemical composition of peptidoglycan is well known. It is composed of glycan chains made of polymeric disaccharides (N-acetylmuramic acid- (β, 1-4) -N-acetylglucosamine (MurNAc - GlcNAc)) residues that are connected by short peptides (Weidel and Pelzer, 1964; Lohmann *et al.*, 2012). The D-lactyl moiety of MurNAc is amide-linked to a pentapeptide that is composed of L-alanine, D-glutamic acid, L-lysine and D-alanine-D-alanine. There are several possible modifications like the amidation (Figueiredo *et al.*, 2012; Münch, D. *et al.*, 2012) of the D-glutamic acid or *O*-acetylation of MurNAc in pathogens (Endl *et al.*, 1983; Bera *et al.*, 2006).

Two or more peptides protruding from neighboring glycan chains can be connected to form dimeric or multimeric crosslinks. These peptides are crosslinked by interpeptide bridges, which consist predominantly of pentaglycine ($Gly_5$), and interlink between L-lysine at position 3 and D-alanine at position four of the neighboring peptides of another glycan strand. This generates a three dimensional network that surrounds the cell (Groppa *et al.*, 2012; Kengara *et al.*, 2013). In *S. aureus* crosslinking occurs at about 80-95% of PG subunits.

Furthermore proteins are covalently anchored to the pentaglycine bridge by a special sorting system known as the sortase (Weidenmaier and Peschel, 2008). In *S. aureus* two sortases are present. The constitutively produced staphylococcal surface protein sortase A (SrtA) anchors all proteins containing the “LPXTG“ motif (Schleifer *et al.*, 1983; Mazmanian *et al.*, 1999), whereas SrtB recognizes the cell wall sorting sequence “NPQTN“ and is only expressed under iron depleting conditions, *e. g.* during host infection (Marraffini *et al.*, 2006; Yokoe *et al.*, 2014).

Another component of the cell wall is the peptidoglycan anchored wall teichoic acid (WTA). It ts covalently bound by a phosphodiester bound to the OH group at position

C6 of MurNAc by a GlcNAc residue (Begg and Donachie, 1985; Weidenmaier *et al.*, 2008; Gibson, 2009). The anchor structure consists of N-acetyl D-mannosamine (ManNAc), GlcNAc and two molecules of glycerol phosphate (GP). The main constituent of *S. aureus* WTA is ribitol phosphate (RboP), which consists of about 40 repeating units. This zwitterionic polymer has negatively charged phosphate groups and is later D-alanylated introducing positively charged amino groups (Endl *et al.*, 1983; Weidenmaier *et al.*, 2008) whereas *S. carnosus* WTA probably is composed of polyglycerol phosphate (GroP) repeating units (Kengara *et al.*, 2013). Nevertheless, only 7.7% of MurNAc is phosphorylated suggesting that every thirteenth MurNAc is substituted by WTA.

In pathogenic staphylococci, such as *S. aureus*, every second MurNAc is modified by O-acetylation at C-6 atom, producing the 2,6-NO diacetylmuramic acid derivative (Bera *et al.*, 2006). The modification is carried out by an O-acetyl transferase (OatA) leading to a lysozyme resistant phenotype (Bera *et al.*, 2005).

Other components that interact but are not covalently bound to the PG are cell wall associated proteins, such as cell wall hydrolases and major autolysins. Furthermore, cell wall spanning molecules, such as lipoproteins (LPPs) and lipoteichoic acids (LTA) are attached to the cytoplasmic membrane (Heilmann *et al.*, 1997; Stoll *et al.*, 2005; Biswas *et al.*, 2006; F. Götz, 2006).

### *3.3.1 PEPTIDOGLYCAN BIOSYNTHESIS*

The biosynthesis of peptidoglycan occurs in four steps. An overview is given in Figure 1. It starts in the cytoplasm with the synthesis of the nucleotide-activated precursors UDP-Glc*N*Ac and UDP-Mur*N*Ac. The latter is synthesized from UDPGlc*N*Ac by the enzymes MurA and MurB. The stepwise ligation of the amino acid L-Ala, D-Glu, L-Lys, and D-Ala-D-Ala to UDP-Mur*N*Ac is catalyzed by the enzymes MurC, MurD, MurE, and MurF, respectivley (Barreteau *et al.*, 2008). D-amino acids are converted from the L-enantiomers by racemases. During the ligation reaction the D-Ala-D-Ala dipeptide is formed by Ddl A and B. The 2nd steps are located at the cytoplasmic membrane or more precise at its inner leaflet. MraY forms the so called Lipid I by transferation of Mur*N*Ac pentapeptide to undecaprenylpyrophosphate and then the glycosyltransferase MurG transfers a Glc*N*Ac residue from UDP-Glc*N*Ac to lipid I, gaining undecaprenyl-pyrophosphoryl- Mur*N*Ac pentapeptide-Glc*N*Ac, the precursor Lipid II. The sequential synthesis of the interpeptide bridge is mediated by the non-ribosomal peptidyltransferases of the Fem (factors essential for methicillin resistance) AB family (Schneider *et al.*, 2004). Their substrate is either Lipid II or the soluble UDP-MurNAc pentapeptide.

Lipid II is then translocated from the inner to the outer leaflet of the cytoplasmic membrane by the proposed flippases FtsW or RodA (3.4). On the outside Lipid II is accessible by the glycosyltransferases (GTase) and transpeptidases (TPase) in form of various Penicillin binding proteins (PBPs) or monofunctional GTases. The GTases use Lipid II as substrate of the glycosyltransferase reactions to polymerize new glycan strands (Step III). Biochemical data and crystal structures indicate that GTases act processively. Lipid II is the acceptor and the glycan strand is the donor in this reaction, which releases the undecaprenol pyrophosphate moiety from the growing glycan strand (Perlstein *et al.*, 2010). The peptide moieties from different glycan strands are crosslinked by transpeptidase reactions to produce the peptidoglycan polymer (Sauvage *et al.*, 2008). The pentapeptide is the donor and a tri-, tetra-, or pentapeptide is the acceptor for this transpeptidase reaction. Finally this results in an indirect crosslinking between Lys of the acceptor and D-Ala at position 4 of the donor mediated by the interpeptide bridge. The energy used to from this crosslink comes from the solved peptide bond of the terminal D-Ala which is then released.

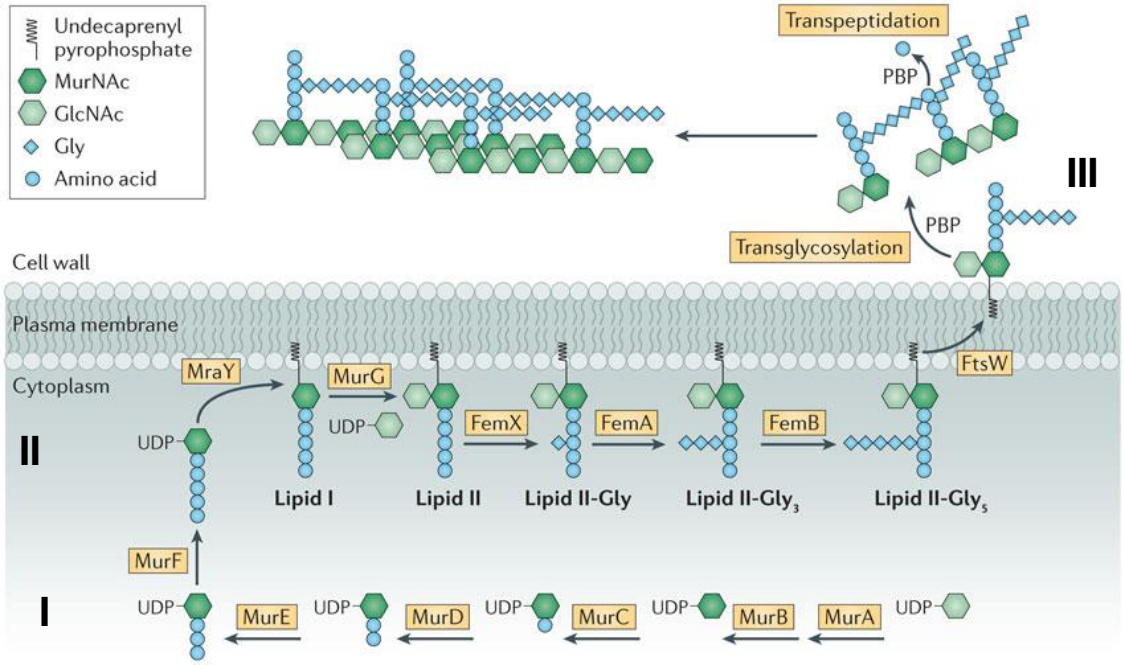


**Figure 1: Pathway of peptidoglycan synthesis in *S. aureus***
(I) The assembly of the lipid II precursor is universal for eubacteria; the MurA reaction is the first step committed to peptidoglycan synthesis. (II) The interpeptide is synthesized on the membrane-bound precursor. (III) Extracellular steps catalyzed by transglycosylases and transpeptidases (PBPs) lead to mature peptidoglycan (Pinho *et al.*, 2013).

### *3.3.2 PEPTIDOGLYCAN HYDROLYSATION*

Peptidoglycan hydrolases (Mur*N*Ac-l-alanine amidases, DD-endopeptidases and lytic transglycosylases), play important roles during growth, cell separation, and cell wall turnover (Gilpin *et al.*, 1974; Wong *et al.*, 1974). In *E. coli* the soluble peptidoglycan turnover products are transported into the cytoplasm where they are recycled for *de novo* peptidoglycan synthesis (Park and Uehara, 2008). Not much is known in Gram positive bacteria, but recycling is under investigation (Reith and Mayer, 2011). The hydrolases target different structures of the cell wall. Cleavage of the sugar backbone occurs specifically by muramidases, such as lysozyme, mutanolysin, or glucosaminidases. Within the stempeptide amidases cleave between N-acetylmuramic acid and L-alanine. The interpeptide bridge is targeted by endopeptidases such as lysostaphin, which cleaves mainly between the glycines on position 2 and 3 as well as position 3 and 4 (Thumm and Götz, 1997; Kull, 2009).

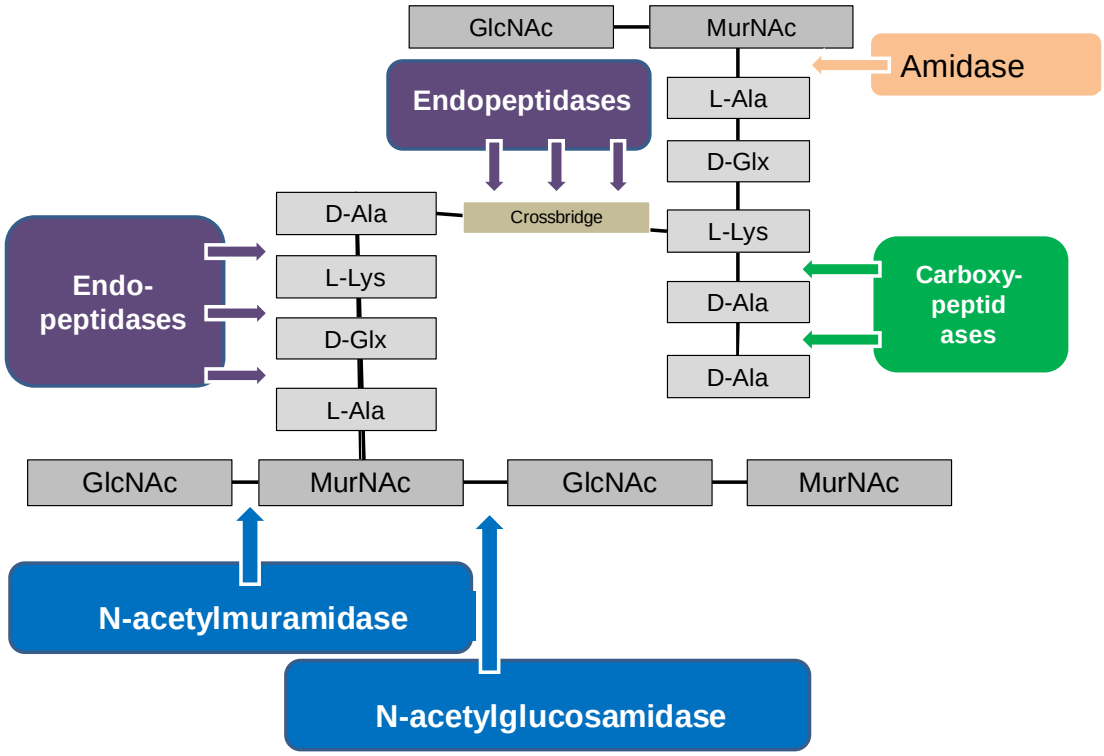


**Figure 2: Peptidoglycan hydrolases and their site of cleavage.**
Different muropeptide cleaving enzymes and their target sites are shown in this picture.

The muramidase lysozyme plays an important role in the host defense by innate immunity. It is a component of neutrophile granules and the major secretory product of macrophages. However, the earlier mentioned O-acytylation works as a resistance mechanism for *S. aureus* (Bera *et al.*, 2005; Bera *et al.*, 2006). The muramidases mutanolysin or chalaropsis muramidase derived from *Streptomyces spec*. can access and cleave the peptidoglycan sugar backbone of *S. aureus* (Hash, 1967; Yokogawa, 1974).The species *S. carnosus* TM300 has no *oatA* homolog and the MurNAc is not O-acetylated, hence the peptidoglycan is lysozyme susceptible (Bera *et al.*, 2006).
The major autolysin (Atl) plays an important role during cell separation in staphylococci. This is a bifunctional protein, which contains an N-terminal N-acetylmuramyl-L-alanine amidase (AM) domain and a C-terminal N-acetylglucosaminidase (GL) domain. AtlE was the first major autolysin described in *S. epidermidis* (Oshida *et al.*, 1995; Heilmann *et al.*, 1997). In *S. aureus* (Baba and Schneewind, 1998; Biswas *et al.*, 2006; Schlag, M. *et al.*, 2010) and in *S. carnosus* (Schumacher, 2008; Costa, 2009) AtlA and Bph are homologous proteins respectively. *S. aureus* cells lacking *atl* form big clusters and were unable to divide properly (Sugai *et al.*, 1995; Heilmann *et al.*, 1997; Biswas *et al.*, 2006).

## 3.4 *SEDS PROTEINS*

SEDS stands for shape, elongation, division, and sporulation. These proteins belong to a family of integral membrane proteins that are present in all cell wall-containing bacteria (Ikeda *et al.*, 1989) and are highly conserved. In many organisms these proteins are essential and the elucidation of their mode of action by loss of function analysis cannot be done. RodA and FtsW are generally regarded as involved in the translocation of lipid II during cell elongation and cell division, respectively (Mohammadi *et al.*, 2011). In *Bacillus subtilis* the depletion of *rodA* leads to a block in elongation cell growth (Henriques *et al.*, 1998) but in *E. coli* RodA is not strictly essential for cell viability. Mutants lacking this SEDS protein show a slow growth and small cell diameters but are viable in minimal medium (Begg and Donachie, 1985; Cava *et al.*, 2013). In addition, temperature-sensitive *E. coli ftsW* mutations lead to blocks at both early and late stages of cell division, suggesting that FtsW acts during both initiation and septum maturation (Perez-Nunez *et al.*, 2011). For *E. coli* it is proposed that RodA is part of the elongation complex and FtsW functions as part of essential division complexes for PG synthesis. Each complex is thought to include one protein from the SEDS family and at least one PBP although several PBPs may associate with a single SEDS protein (Typas *et al.*, 2012). Mutations in *E. coli ftsI* (encoding PBP3) that reduce the ability to divide can be suppressed by *rodA* mutations that, by themselves, interfere with normal cell growth (Begg *et al.*, 1986). There exists a subset of SEDS proteins that are not essential: SpoVE is responsible for heat resistance of spores in *B. subtilis* (Real *et al.*, 2008) and its loss does not influence the viability of the vegetative cells. FtsW of *Steptomyces coelicolor* is only needed during nonessential sporulation for septation and Z-ring stabilization but its deletion does not affect cross wall formation in vegetative hyphae (Mistry *et al.*, 2008). Its gene *ftsW* was just recently shown to be activated by the transcriptional regulator WhiA (Matthew J. Busha *et al.*, 2013). In *Corynebacterium glutamicum* RodA is solely localized to the apical pole, where cell growth takes place. Interestingly, deletion of *rodA* is tolerated but the cells grow slower and have a smaller and shorter morphology than the wild type cells (Sieger 2013). However, all these data come from rod-shaped bacteria, but so far there are no SEDS deletion mutants in bacteria that do not perform elongation or apical extension.

*S. carnosus* is the only sequenced Staphylococcus strain that possess three genes annotated to encode proteins of the SEDS family: *ftsW1* (Sca_0739), *ftsW2*

(Sca_1886), and *rodA* (Sca_1584). All other strains contain only one copy of the *ftsW* gene and one of *rodA*. Since it has never been observed that bacteria of this genus grow in other shapes then cocci, they do not perform elongational growth and therefore there is no discrimination between an elongasome and a divisiome. In rod shaped bacteria the SEDS proteins FtsW and RodA play their distinct role in either one of the peptidoglycan biosynthesizing machineries. The question occurs why two or even three SEDS proteins are needed in staphylococcal bacteria. The divison pattern of dividing in a 90° angle might be the answer (Turner *et al.*, 2010). Somehow the localization of the division plane has to be regulated and at least one flippase has to be present at the new division sites. One could envision that the three SEDS proteins take turns to define the new division plane.

### *3.4.1 RodA*

SEDS proteins and PBPs are co-localized in the genome of several bacteria but not in staphylococci. The *rod*A gene of *S. carnosus* is an orphan and annotated as Sca_1584. It is 1226 bp in size and neighbors on the upstream side a two gene operon of the D-alanine-D-alanine ligase and the UDP-N-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase Sca_1583 and Sca_1582 respectively (Figure 9). On the downstream side is a putative copper-transporting P-type ATPase located. *Rod*A´s orientation is contrary to almost all genes in this region with the exception of Sca_1589 and Sca_1590 a putative cardiolipin synthetase and a hypothetical protein respectively, which build an operon downstream of *rod*A.

The gene product of *rodA* is a membrane protein with 405 amino acids. They form a structure of ten transmembrane helices (TMH). Computal analysis revealed that the C as well as the N-terminus is located in the cytoplasma. For the Bacillus homologue this was confirmed experimentally (Real *et al.*, 2008). An over 60 amino acids long extracellular loop forms between TMH 7 and TMH 8. Smaller loops with approximately 20 animo acids in size are located between TMH 3 and TMH 4 on the outside and TMH 8 and TMH 9 on the inside of the cytoplasmic membrane as predicted by SOSUI and confirmded by Quick 2D. Special motives other than the high similarity to proteins of the SEDS family could not be recognized.

Even though membrane topology was determined for SpoVE of *B. subtilis* it was not possible to identify functional region within this protein (Real *et al.*, 2008). Recently it was shown that the predicted transmembrane helix 4 is required for the flipping activity especially the two charged residues R145 and K153. Furthermore there is size exclusion for the transport which can be hindered by a Lipid II variant increased by 420 Da. This leads to the assumption of a pore like mechanism for the FstW of *E. coli* (Mohammadi *et al.*, 2014).

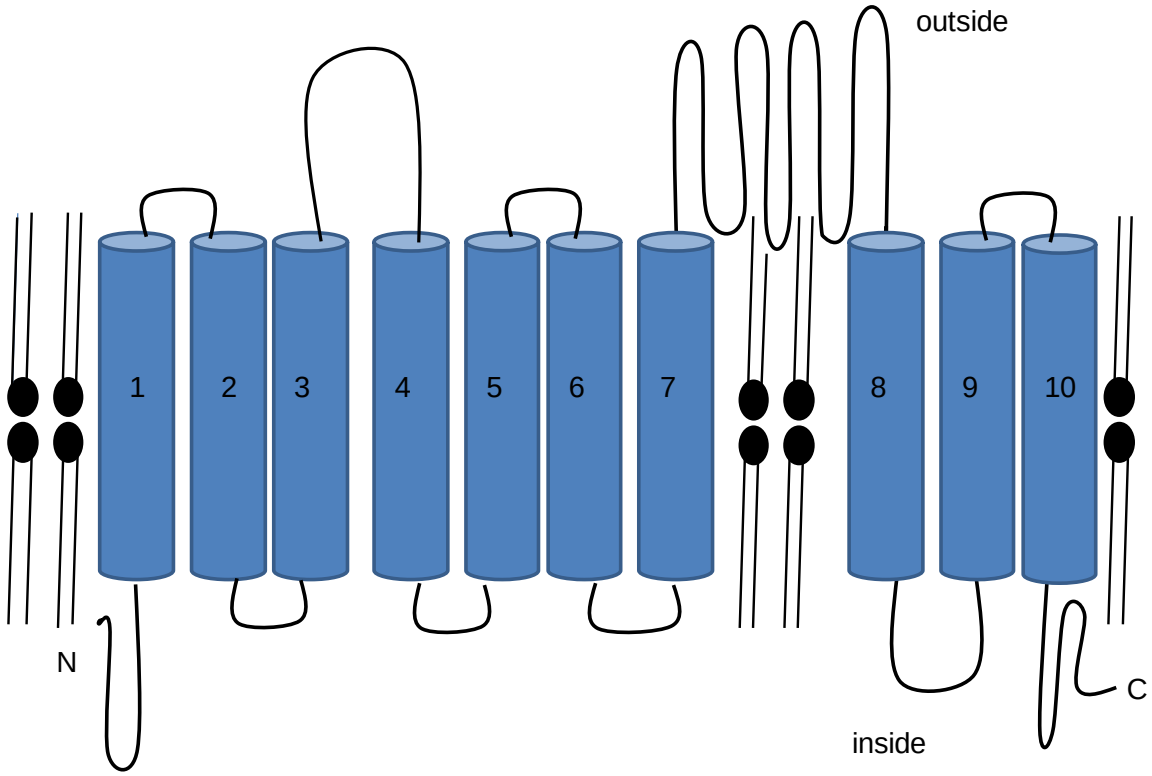


**Figure 3: Topology model of RodA**
Model created with predictions of SOSUI.

# 4 METHODS AND MATERIALS

## 4.1 *ABBREVIATIONS*

| | |
|---|---|
| A | Ampere |
| Ala | Alanine |
| Ami | Amidase |
| APS | Ammoniumpersulfate |
| *B.* | *Bacillus* |
| BHI | Brain Heart Infusion |
| bidest | double destilled |
| BM | Basic Medium |
| bp | Basepairs |
| BSA | bovine serumalbumine |
| C-terminal | Carboxy terminal |
| C-Terminus | Carboxy terminus |
| cat | Chloramphenicolacetyltransferase |
| CHAP | cystein-,histidindependent amidohydrolases/peptidases |
| CW | Cell wall |
| DA | Diaminoacids |
| DAPI | 4',6-diamidino-2-phenylindole |
| DNA | Desoxyribonucleine acid |
| *E. coli* | *Escherichia coli* |
| EDTA | Ethylendiamintetraacetat |
| EtOH | Ethanol |
| ERT | Eppendorf reaction tube |
| g | Gramm |
| gen. | genomic |
| GFP | green *fluorescence* protein |
| Glc*N*Ac | *N*-Acetylglucosamine |
| Glu | Glutamic acid |
| Gly | Glycine |

| | |
|---|---|
| His | Histidine |
| HPLC | high performance liquid chromatography |
| k | Kilo- |
| kan | Kanamycin |
| kb | Kilobasepairs |
| kDa | Kilodalton |
| KO | Knock-out |
| l | Liter |
| LB | Luria Bertani |
| Lys | Lysine |
| LysM | Lysine motif |
| m | Milli- |
| M | Molar (= mol per liter) |
| mA | Milliampere |
| min | Minute |
| ml | Milliliter |
| mM | Millimolar |
| MRSA | Methicillin/Multi-resistent *Staphylococcus aureus* |
| MS | Mass spectrometry |
| Mur*N*Ac | *N*-Acetylmuramic acid |
| Mut | Mutanolysine |
| Ni | Nickel |
| N-terminal | Amino terminal |
| N-Terminus | Amino terminus |
| nm | Nanometer |
| OD | Optical density |
| ONC | Overnight culture |
| p | Pico- |
| PALM | Photoactivated Localization Microscopy |
| PAGE | Polyacrylamidgelelectrophoresis |
| PBP | Penicillinbinding protein |
| PCR | Polymerase chain reaction |
| PG | Peptidoglycan |
| Ppm | Parts per million |

| | |
|---|---|
| rpm | rounds per minute |
| REM | Raster electron microscopy |
| *S.* | *Staphylococcus* |
| *Sca* | *Staphylococcus carnosus* |
| SDS | Sodiumdodecylsulfat |
| SEDS | shape, elongation, division and sporulation |
| SfGFP | superfolder green fluorescent protein |
| STROM | Stochastic Optical Reconstruction Microscopy |
| SP | Signalpeptide |
| TAT | Twin-Arginin Transport |
| TEMED | Tetramethylethylenediamine |
| $T_M$° | melting temperature |
| Tris | Tris(hydroxymethyl)aminomethan |
| u | units |
| UDP | Uridindyl phosphate |
| V | Volt |
| VRSA | Vancomycin-resistenter *Staphylococcus aureus* |

## 4.2 *AMINO ACID CODE*

| | | | | | |
|---|---|---|---|---|---|
| A | Ala | alanine | I | Ile | isoleucine |
| R | Arg | arginine | C | Cys | cysteine |
| K | Lys | lysine | S | Ser | serine |
| D | Asp | aspartic acid | L | Leu | leucine |
| T | Thr | threonine | E | Glu | glutamic acid |
| M | Met | methionine | V | Val | valine |
| F | Phe | phenylalanine | N | Asn | asparagine |
| W | Trp | tryptophan | G | Gly | glycine |
| P | Pro | proline | Y | Tyr | tyrosin |
| H | His | histidine | Q | Gln | glutamine |

## 4.3 *BACTERIAL STRAINS*

**Tab. 1: Used bacterial strains**

| Species | Strain | Genotype | Reference |
|---|---|---|---|
| ***Escherichia coli*** | DC10B | dam$^+$ ∆dcm ∆hsdRMS endA1 recA1 | (Monk *et al.*, 2012) |
| ***Escherichia coli*** | DH5α | *endA1 hsdR17* (rK - mK+) *supE44 thi-1 recA1 gyrA* (Nalr) *relA1*Δ(*lacZYAargF*) *U169 deoR* (Φ80*dlacZ*ΔM15) | (Hanahan 1983) |
| ***Escherichia coli*** | XL-1 blue | *endA1 gyrA96* (nal$^R$) thi-1 recA1 relA1 lac glnV44 F´[::Tn10 proAB$^+$ lacI$^q$ ∆ (lacZ)M15] hsdR17 ($r_{K^-}$ $m_{K^+}$) | (Bullock *et al.,* 1987) |
| ***Escherichia coli*** | BL21 (DE3)-RIL | (*E. coli* B) F− *ompT hsdS* (rB− mB −) *dcm* + Tetr *gal* λ (DE3) *endA* Hte (*argU ileY leuW* Camr) | (Greener *et al.* 1997) |
| ***Staphylococcus aureus*** | 8325 NCTC 8325 (RN1) | | (Novick and Richmond 1965) |
| ***Staphylococcus aureus*** | RN42220 | derivative of NCTC 8325 | (Iordanescu and Surdeanu 1976) |
| ***Staphylococcus carnosus*** | TM300 | | (Schleifer and Fischer, 1982) (Rosenstein *et al.*, 2009) |
| ***Staphylococcus carnosus*** | TM300 ∆ *rod*A | TM300 Δ*rodA*::*lox72* | This study |
| ***Staphylococcus carnosus*** | TM300 ∆ *fts*W1 | TM300 Δ*ftsW1*::*lox72* | This study |
| ***Staphylococcus carnosus*** | TM300 ∆ *fts*W2 | TM300 Δ*ftsW2*::*lox72* | This study |
| ***Staphylococcus warneri*** | SG1 | | (Kloos & Schleifer 1975) |

## 4.4 *MEDIA*

The culture media were autoclaved for 20 min at 121 °C and 2 bar. For the preparation of plates 15 g agar were added.

**Tab. 2: Used media and chemical composition**

| Type | Chemical composition |
|---|---|
| **B2-Medium** | 10 g/l Trypton |
| | 25 g/l Yeast extract |
| | 25 g/l NaCl |
| | 5 g/l Glucose |
| | 1 g/l $K_2HPO_4 * 3H_2O$ |
| | pH 7,5 |
| **Basic (B)-Medium** | 10 g/l Pepton |
| | 5 g/l Yeast extract |
| | 5 g/l NaCl |
| | 1 g/l Glucose |
| | 1 g/l $K_2HPO_4 * 3H_2O$ |
| | pH 7.2 |
| **Brain Heart Infusion (BHI)-Medium** | 37 g/l Brain Heart Broth |
| | pH 7.2 |
| **Luria Bertani (LB)-Medium** | 10 g/l Trypton |
| | 5 g/l Yeast extract |
| | 10 g/l NaCl |
| | pH 7.0 |
| **Super Optimal Broth (SOB) Medium** | 2% w/v Tryptone (20 g/l) |
| | 0.5% w/v Yeast extract (5 g/l) |
| | 8.56 mM NaCl (0.5 g/l) |
| | 2.5 mM KCl (0.186 g/l) |
| | 10 mM $MgCl_2$ (anhydrous 0.952 g/l) or |
| | 10 mM $MgSO_4$ (heptahydrate 2.408 g/l) |
| | pH7.0 |

| | |
|---|---|
| SOC Medium | As above<br>Additional 20mM glucose (3.603 g/l) |

**Tab. 3: Antibiotic solutions**

| Antibiotics | Stock solution | Solving solution | Selection-concentration | Purchased by |
|---|---|---|---|---|
| **Ampicillin** | 100 mg/ml | $H_2O_{bidest}$ | 100 µg/ml | Carl Roth GmbH, Karlsruhe |
| **Chloramphenicol** | 10 mg/ml | EtOH (70 %) | 10 µg/ml | Carl Roth GmbH, Karlsruhe |
| **Kanamycin** | 30 mg/ml | $H_2O_{bidest}$ | 30 µg/ml | Carl Roth GmbH, Karlsruhe |
| **Tetracycline** | 25 mg/ml | EtOH (70 %) | 25 µg/ml | Sigma-Aldrich Chemie GmbH, Taufkirchen |

All solutions were filter sterilized and stored at -20 °C. In terms of thermo stability autoclaved media were complemented at < 50 °C with its respective antibiotic.

**Tab. 4: Used plasmids**

| Plasmid | Size | Host | Selection | Reference |
|---|---|---|---|---|
| **pCX30Δ82cw** | 6.5 | *S. carnosus* | $Cm_r$ (10 µg/ml) | (Strauß and Götz 1996) |
| **pTX30Δ82cw** | 9.0 | *S. carnosus* | $Tc_r$ (12.5 µg/ml) | (Strauß and Götz 1996) |
| **pCX31H** | 4.8 | *S. carnosus* | $Cm_r$ (10 µg/ml) | Albrecht 2012 |
| **pCX33TLCH** | 6.0 | *S. carnosus* | $Cm_r$ (10 µg/ml) | Albrecht 2012 |
| **pBT2** | 6.3 | *S. carnosus*<br>*E. coli* | $Cm_r$ (10 µg/ml)<br>$Am_r$ (100 µg/ml) | (Brückner 1997) |
| **pSG1** | 8.3 | *S. carnosus*<br>*E. coli* | $Cm_r$ (10 µg/ml)<br>$Am_r$ (100 µg/ml) | Krismer 2012 |
| **pRAB1** | 8.3 | *S. carnosus*<br>*E. coli* | $Cm_r$ (10 µg/ml)<br>$Am_r$ (100 µg/ml) | (Leibig *et al.* 2008) |
| **pJet** | 2.9 | *E. coli* | $Am_r$ (100 µg/ml) | |

## 4.5 *OLIGO NUCLEOTIDES*

**Tab. 5: Used oligonucleotides**

| Amplicon | Primer fw<br>with enzymes sites and enzyme | Primer rv<br>with enzymes sites and enzyme | Size Amplicon [bp] |
|---|---|---|---|
| **RodA upstream** | attagttatagctagcacttcagaatctaactctgc<br>**NheI** | tacatgatatctgcagaagttccccttttaaattgc<br>**PstI** | 1039 |
| **RodA downstream** | tacagctagcctgcagtagaatgttttctgataacc<br>**PstI** | tatatataatgtcgacatccactttctcaagc<br>**SalI** | 1494 |
| **RodA** | ttaattggatcctaaattaggaggtattaatt<br>**BamHI** | atatcccgggcgctcgataacctg<br>**XmaI/SmaI** | 1225 |
| **FtsW1 upstream** | tatgagctcgcgatatctatttagaatgatattagg<br>**EcoRV** | tacagtgtctctgcagtctaatatttgaagaaatcc<br>**PstI** | 1364 |
| **FtsW1 downstream** | tatctatgtactgcagtaaagacgtattccataacg<br>**PstI** | acatcatgtgtcgaccttatgataatcttcaatcg<br>**SalI** | 830 |
| **FtsW1** | ttaattggatcctaaattaggaggtattaatt<br>**BamHI** | tattaacccgggtaatgatgaacg<br>**XmaI/SmaI** | 1228 |
| **FtsW2 upstream** | tatgtatatagatatctgcaacagaatcagcaatcg<br>**EcoRV** | taatctacatctgcaggagatatggaatataaatgc<br>**PstI** | 1105 |
| **FtsW2 downstream** | tatatagatactgcaggtttcaactatctcacaacg<br>**PstI** | atcgagcgcggtcgactatttaactaatataattgg<br>**SalI** | 1486 |
| **FtsW2** | ttaattggatcctaattaggaggtattaatt<br>**BamHI** | aataacccgggtaatgatgaacg<br>**XmaI/SmaI** | 1207 |
| **universal primer** | agagtttgatcmtggctcag | aaggaggtgwtccarcc | 1500 bp |

## 4.6 *ENZYMES*

**Tab. 6: Used enzymes and supplier**

| Enzym | Supplier |
|---|---|
| **BamHI** | Thermo Scientific, Karlsruhe |
| **High Fidelity PCR Enzyme Mix** | Thermo Scientific, Karlsruhe |
| **KpnI** | Thermo Scientific, Karlsruhe |
| **Mutanolysine** | Sigma-Aldrich Chemie GmbH, Taufkirchen |
| **NheI** | Thermo Scientific, Karlsruhe |
| **RNase** | Sigma-Aldrich Chemie GmbH, Taufkirchen |
| **SalI** | Thermo Scientific, Karlsruhe |
| **T4-DNA-Ligase** | Thermo Scientific, Karlsruhe |
| **HindIII** | Thermo Scientific, Karlsruhe |
| **PstI** | Thermo Scientific, Karlsruhe |
| **EcoRI** | Thermo Scientific, Karlsruhe |
| **Phusion® hot start high-fidelity DNA polymerase** | Finnzymes Oy, Espoo (Finland) |
| **High fidelity polymerase, Taq polymerase** | Genaxxon BioScience GmbH, Ulm |
| **Lysostaphin** | Genmedics GmbH, Reutlingen |
| **Restriction endonucleases, alkaline phosphatase(CIAP),T4 polynucleotide kinase (PNK), T4-DNA ligase, high fidelity PCR enzyme Mix, Taq DNApolymerase** | Fermentas GmbH, St. Leon-Rot |
| **Restriction endonucleases** | New England Biolabs, Schwalbach |
| **Hen egg white lysozyme** | Serva, Heidelberg |
| **Trypsin, thrombin from bovine plasma, mutanolysin, desoxyribonuclease 1, ribonuclease** | Sigma, Deisenhofen |

**Tab. 7: Used molecular biological kits and their source of supply**

| Source | Kit |
|---|---|
| **Bio-Rad Laboratories, München** | InstaGene™ Matrix |
| **Fermentas GmbH, St. Leon-Rot** | Rapid Ligation Kit |
| | CloneJet™ PCR cloning Kit |
| **Lonza** | QCL-1000 Kit (Endotoxin detection) |
| | Endo Trap® Red Column (Endotoxin removal) |
| **QIAGEN GmbH, Hilden** | QIAex II Gel extraction Kit, QIAquick-Kit |
| | QIAGEN-Plasmid-Midi-Kit |
| **Stratagene GmbH, Heidelberg** | StrataClean®-Resin |

**Tab. 8: Used molecular weight markers and their source of supply**

| Supplier | Product |
|---|---|
| **Fermentas, St. Leon-Rot** | GeneRuler™ 1 kb DNA ladder |
| | GeneRuler™ DNA ladder Mix |
| | PageRuler™ prestained protein ladder |
| **PEQLAB, Erlangen** | peqGOLD protein-Marker IV (prestained) |

## 4.7 *OTHERS*

**Tab. 9: Other materials and solutions and their supplier**

| Label | Supplier |
|---|---|
| **10 x High Fidelity PCR Buffer inkl. $MgCl_2$** | Thermo Scientific, Karlsruhe |
| **10 x Buffer Tango (with BSA)** | Thermo Scientific, Karlsruhe |
| **10 x Buffer $O^+$ (with BSA)** | Thermo Scientific, Karlsruhe |
| **DNA Loading Dye (6x)** | Thermo Scientific, Karlsruhe |
| **dNTP Mix** | Thermo Scientific, Karlsruhe |
| **GeneRuler™ 1 kb DNA Ladder** | Thermo Scientific, Karlsruhe |
| **$MgCl_2$ (25 mM)** | Thermo Scientific, Karlsruhe |

## 4.8 *CHEMICAL SOLUTIONS*

**Tab. 10: Chemicals solutions**

| Chemicals | Source |
|---|---|
| **Acrylamid (Rotiphorese® Gel 30)** | Carl Roth GmbH, Karlsruhe |
| **Agar-Agar** | Carl Roth GmbH, Karlsruhe |
| **Agarose** | Biozym Scientific GmbH, Hessisch Oldendorf |
| **APS (Ammoniumpersulfate)** | SERVA Electrophoresis GmbH, Heidelberg |
| **Brain Heart Broth** | Sigma-Aldrich Chemie GmbH, Taufkirchen |
| **Bromphenolblue** | Sigma-Aldrich Chemie GmbH, Taufkirchen |
| **BSA (Bovines Serumalbumin)** | Sigma-Aldrich Chemie GmbH, Taufkirchen |
| **$CaCl_2$ * $2H_2O$** | Carl Roth GmbH, Karlsruhe |
| **Chisom** | Fisher Scientific UK, Loughborough |
| **Coomassie Brilliantblau R-250** | AppliChem GmbH, Darmstadt |
| **DAPI** | AppliChem GmbH, Darmstadt |
| **EDTA** | Merck KGaA, Darmstadt |
| **Acetic acid 100 % p.a.** | VWR International GmbH, Darmstadt |
| **Ethanol absolut p.a.** | Sigma-Aldrich Chemie GmbH, Taufkirchen |
| **Ethidiumbromide** | Carl Roth GmbH, Karlsruhe |
| **Glucose** | SERVA Electrophoresis GmbH, Heidelberg |
| **Glycerol** | Sigma-Aldrich Chemie GmbH, Taufkirchen |
| **Yeast extract** | Carl Roth GmbH, Karlsruhe |
| **HCl** | Carl Roth GmbH, Karlsruhe |
| **Imidazol** | Sigma-Aldrich Chemie GmbH, Taufkirchen |

| | |
|---|---|
| **Isopropanol** | Fisher Scientific UK, Loughborough |
| **KCl** | Merck KGaA, Darmstadt |
| **$K_2HPO_4$ * $3H_2O$** | Carl Roth GmbH, Karlsruhe |
| **KOH** | Merck KGaA, Darmstadt |
| **Methanol** | Sigma-Aldrich Chemie GmbH, Taufkirchen |
| **Methylenblue** | Merck KGaA, Darmstadt |
| **$MgCl_2$** | Carl Roth GmbH, Karlsruhe |
| **$NaH_2PO_4$ * $H_2O$** | Merck KGaA, Darmstadt |
| **$Na_2HPO_4$ * $2H_2O$** | Merck KGaA, Darmstadt |
| **NaCl** | Merck KGaA, Darmstadt |
| **$NaClO_4$** | Sigma-Aldrich Chemie GmbH, Taufkirchen |
| **NaOH** | Merck KGaA, Darmstadt |
| **Pepton** | Carl Roth GmbH, Karlsruhe |
| **Phenol** | Carl Roth GmbH, Karlsruhe |
| **Pregallidermin** | B. Krismer, Tübingen |
| **Saccharose** | Carl Roth GmbH, Karlsruhe |
| **SDS (Sodiumdodecylsulfat)** | Carl Roth GmbH, Karlsruhe |
| **Temed (Tetramethylethylendiamin)** | Sigma-Aldrich Chemie GmbH, Taufkirchen |
| **Tris** | AppliChem GmbH, Darmstadt |
| **Trypton** | Oxoid Ltd., Hampshire |
| **Xylose** | Sigma-Aldrich Chemie GmbH, Taufkirchen |
| **β-Mercaptoethanol** | Merck KGaA, Darmstadt |

## 4.9 *COMPUTAL PROGRAMS AND DATABASES*

**Tab. 11: Used computal programs and Databases**

| Programs | Reference |
|---|---|
| **Clone Manager 9** | Scientific & Educational Software, Cary |
| **i-control 1.8 SP1** | Tecan Group Ltd., Männedorf |
| **Kyoto Encyclopedia of Genes and Genomes** | http://www.genome.jp/kegg/kegg2.html |
| **LAS AF** | Leica Microsystems CMS GmbH, Wetzlar |
| **NCBI** | U.S. National Library of Medicine, Bethesda http://www.ncbi.nlm.nih.gov/ |
| **TMHMM 2.0** | Krogh, et al., 2001 http://www.cbs.dtu.dk/services/TMHMM/ |
| **TMRPres2D** | Spyropoulos, et al., 2004 |
| **Phyre2** | © Structural Bioinformatics Group Imperial College, London www.sbg.bio.ic.ac.uk/phyre2/ |
| **HHprep** | http://toolkit.tuebingen.mpg.de/hhrep |
| **SOSUI engine ver. 1.11** | http://bp.nuap.nagoya-u.ac.jp/sosui/ |
| **Quick 2D** | http://toolkit.tuebingen.mpg.de/quick2_D |
| **Image J 1.45s** | National Institutes of Health |
| **Zeiss LSM Image Browser Version 4.2.0.121** | Carl Zeiss MicroImaging GmbH |
| **Endnote X6** | Thomson Reuters, Carlsbad |
| **Microsoft Office 2007** | Microsoft Dtld. GmbH, Unterschleißheim |
| **NEBuilder™** | http://nebuilder.neb.com/ |
| **Chem Biodraw 13** | Perkin Elmer Cambridge Soft. |

## 4.10 *DEVICES*

**Tab. 12: Used technical devices and their source of supply**

| Device | Model | Supplier |
|---|---|---|
| **Incubation chamber** | B5042E | Heraeus Holding GmbH, Hanau |
| **Bunsenburner** | Phoenix II eco | Schuett-biotec GmbH, Göttingen |
| **Ice supply** | AF 100 | Scotsman Ice Systems, Mailand |
| **Elektroporator** | Multiporator® | Eppendorf AG, Hamburg |
| **Gel documentation system** | | LTF Labortechnik GmbH & Co. KG, Wasserburg/Bodensee |
| **Gel documentation system** | | Vilber Lourmat, Eberhardzell |
| **Heating block** | | Bachofer Laboratoriumsgeräte, Reutlingen |
| **HPLC** | 1200 series | Agilent Technologies Inc., Santa Clara |
| **Mikroscope** | DM5500 B | Leica Microsystems GmbH, Wetzlar |
| **Mikrowave** | NN-SD456W | Panasonic Corporation, Osaka |
| **Magnet stirrer** | RCT basics | IKA®-Werke GmbH & CO. KG, Staufen IKA RCT IKAMAG |
| **PCR-Cycler** | Primus 96 plus | MWG-Biotech AG, Penzberg |
| **pH-Meter** | Hydrus 300 | Fisher Scientific UK, Loughborough |
| **Photometer** | Helios alpha | Thermo Fisher Scientific Inc. Waltham |
| **Plate Reader** | Infinite M200 | Tecan Group Ltd., Männedorf |
| **Ultrapurewater apparatus** | Milli-Q Plus PF | EMD Millipore Corporation, Billerica |
| **Shaking incubator** | KF-4<br>Innova 44 | Infors AG, Bottmingen |

| | | |
|---|---|---|
| | | New Brunswick Scientific, Edison |
| **Power supply** | LKB-GPS 200/400 | Pharmacia, Ratingen |
| | 2297 Macrodrive 5 | LKB Bromma |
| **Laminar Workflow** | SterilGARD VBM 400 | Baker Company Inc., Sanford |
| **DNA-Sequencer** | LI-COR 400L | MWG Biotech, Ebersberg |
| **Camera** | Camera DC3 | Leica Microsystems GmbH, Wetzlar |
| **Drying incubator** | T 6120 | Heraeus Holding GmbH, Hanau |
| **Vortex** | Vortex Genie 2 | Bender & Hobein GmbH, Zürich |
| **Scale** | Weighing machine BP 61-OCE | Sartorius AG, Göttingen |
| | special accuracy weighing machine Kern EW 4200-2NM | KERN & SOHN GmbH, Balingen |
| **Waterbath** | | Bachofer Laboratoriumsgeräte, Reutlingen |
| **Centrifuges** | Centrifuge Z 216 MK | Hermle Labortechnik, Wehingen |
| | Centrifuge Multifuge X3R | Thermo Scientific, Karlsruhe |
| | Table centrifuge Z 233 M-2 | Hermle Labortechnik, Wehingen |
| | Vakuumzentrifuge Univapo 100 H | UniEquip Laborgerätebau- und Vertriebs GmbH, Planegg |

## 4.11 *CONSUMABLE MATERIALS AND PLASTIC ARTICLES*

**Tab. 13: Used consumable material and plastic articles and their source of supply**

| Supplier | Product |
|---|---|
| **Eppendorf, Hamburg** | 1.5 ml & 2 ml Eppendorf reaction tubes (ERTs) |
| **GE Healthcare Europe, München (Whatman, Dassel)** | Whatman® Protran® nitrocellulose, filter sterilizer (0.2 µm), |
| **Greiner Bio-One, Frickenhausen** | FalconTM tube PS 12 ml,<br>FalconTM tube PS 50 ml,<br>single-use inoculating loop |
| **Henke Sass Wolf, Tuttlingen** | Injection Norm-Ject (1 ml, 5 ml, 20 ml) |
| **Millipore, Schwalbach** | Filter Millipak Express 20 (0.22 µm),<br>StericupTM<br>PES membrane (0.22 µm) |
| **Molecular Bioproducts, San Diego** | 200 µl reaction tubes |
| **Roth, Karlsruhe** | glass beads (0.25-0.5 mm) |
| **Thermo Fisher Scientific** | 96 well plate NunclonTM surface<br>96 well plate Nunc black U96 PP-0.5 ml |

## 4.12 *METHODS FOR WORKING WITH BACTERIA*

### *4.12.1 CULTIVATION OF BACTERIA*

Cultures of staphylococci were cultivated in B-medium on a shaker at 37 °C and 150 rpm. When xylose inducible gene expression was carried out, cells were cultivated without glucose (catabolite repression). The bacterial growth was observed at an optical density $OD_{578}$ nm in a photometer. Usually, cells were grown until an $OD_{578}$ = 0.6 and the gene expression was induced by adding in total 0.5% xylose. *E. coli* strains were cultivated with B-medium, too.

### *4.12.2 STORAGE OF BACTERIA*

For medium-term storage bacterial strains were plated on BM plates and incubated overnight at 37 °C. The plates were stored at 4 °C in case of E. coli for 2-3 weeks and for *S. carnosus* for 6-8 weeks. For long-term storage of bacteria one volume of overnight culture was mixed with one volume of autoclaved freeze-medium (65% glycerol, 0.1 M $MgSO_4$, 25 mM Tris/HCl, pH 8.0) and stored at -70 °C. Bacteria were harvested by centrifugation for 10-20 min at 4 °C and 4’500 rpm.

## 4.13 *METHODS FOR WORKING WITH NUCLEIC ACIDS*

### *4.13.1 ISOLATION AND PURIFICATION OF NUCLEIC ACIDS*

Solutions and buffers were assembled using fine chemicals and deionized purified Milli-Q water. The DNA was kept at 4 °C for short-term storage whilst at -20 °C long term storage.

### *4.13.2 ISOLATION OF PLASMID-DNA FROM E. COLI*

High amounts of plasmid-DNA were isolated from 50-100 ml culture using the "QIAGENPlasmid- Midi-Kits" (QIAGEN GmbH, Hilden). Herewith, DNA was obtained by the principle of alkaline lysis (Birnboim and Doly 1979) and is further purified by anion-exchange chromatography. The isolation was carried out using the producer‘s manual. Small amount of ultrapure DNA was isolated from 4 ml culture by "QIAprep-spin columns" (QIAGEN GmbH, Hilden), which works in a similar way but with a different neutralization buffer.

## 4.14 *BUFFERS AND SOLUTIONS*

**Tab. 14: Buffers and solutions**

| Buffer Name | Contents |
|---|---|
| **Phenol/"Chisom" (Roti® phenol)** | 25 parts neutral phenol, 24 parts $CHCl_3$, 1 part isoamyl alcohol<br>buffered with 20 mM Tris/HCl pH 8.0 |
| **Chisom** | 24 parts $CHCl_3$, 1 part isoamyl alcohol |
| **3M Na-acetate** | 3 M NaAc, pH 5.2 (adjusted with acetic acid) |
| **Milli-Q water** | Water was purified using the Milli-Q apparatus and autoclaved for later usage with nucleic acid |
| **50x TAE-buffer** | 2 M Tris/HCl, 0.5 M NaAc, 50 mM EDTA, pH 7.8 |
| **E1 solution** | 25% sucrose, 10% Ficoll, 0.4 mg/ml RNase, 1 mg/ml lysozyme,<br>filled up to 2 ml with 1x TAE-buffer. |

## 4.15 *ISOLATION OF PLASMID-DNA FROM S. CARNOSUS*

High amounts of plasmid-DNA were isolated from 50-100 ml culture using the "QIAGENPlasmid-Midi-Kits" (QIAGEN GmbH, Hilden). Cells from an overnight culture were resuspended in P1 buffer (up to 100 ml culture in 4 ml buffer). Per 4 ml buffer 30-45 µl Lysostaphin (0.5 mg/ml) were added. The cell solution was incubated in the water bath for 20 min at 37 °C for cell wall degradation. The additional steps were carried out according to the manufacturer‘s protocol. Small amounts of DNA were isolated with the "QIAprep-spin columns" (QIAGEN GmbH, Hilden). From 4 ml overnight culture plasmid DNA was isolated. After resuspension of cells in 250 µl buffer P1, 20 µl Lysostaphin (0.5 mg/ml) was added for cell wall degradation. The incubation took place in a water bath over 20 min at 37°C. The additional steps were carried out according to the manufacturer‘s protocol.

## 4.16 *SEPARATION OF PLASMID PROFILES ACCORDING TO ECKHARDT*

The lysis according to Eckhardt (Eckhardt 1978) was carried out to quickly check whether or not plasmids contain inserts or not judged by a shift in plasmid size. Originally set up for *E. coli* only, cells were lysed by lysozyme and SDS during the gel forerun avoiding shear forces. For working with staphylococcal cells 20µg/ml Lysostaphin were added to the lysis buffer E1 (25% Saccharose, 10% Ficoll, 0,4 mg/ml RNase, 1mg/ml Lysozym solved in TAE-Puffer) The gel was composed of 0.8% - 1.5% (w/v) agarose in 1x TAE-buffer. After boiling, 2 ml of 10% SDS-solution were added per 100 ml agarose solution at ca. 60°C. The solidified gel was covered with 1x TAE buffer straightaway before starting the gel run. A single colony was picked with a tooth pick and resuspended in 10 µl 1x TAE buffer. Afterwards, 20 µl of E1 solution was added and carefully mixed. Cell suspension of 20 µl was loaded on the agarose gel and left for 5 min. The gel was pre-run for 30 min at 20 V and further for 1-3 hrs at 120 V. The gel was watered subsequently for 30 min and stained for 20 min in ethidium bromide.

## 4.17 *ISOLATION OF CHROMOSOMAL DNA OF S. CARNOSUS*

### *4.17.1 PHENOL CHLOROFORM EXTRACTION*

The isolation of chromosomal *S. carnosus*-DNA was carried out after a modified protocol according to Marmur (Marmur 1961). The cells were lysed almost totally and in the adjacent steps contaminants, such as cell debris and proteins were removed. Cells of an overnight culture were harvested for 3 min at 13'000 rpm by centrifugation in 2x 2 ml ERTs. Cell sediments were resuspended in 400 µl buffer P1 and 40 µl Lysostaphin (0.5 mg/ml) were added. The mixture was thoroughly vortexed and incubated for 30 min at 37°C in a water bath, until the cell solution became quite viscous. Afterwards, 40 µl of a saturated SDS solution (45%) was added, vortexed for 1 min, and incubated for another 5 min at 37 °C in the water bath. Subsequently, 100 µl 5 M $NaClO_4$ (dissolved in water, sterile filterized) were added and vortexed for 1 min Afterwards, 500 µl Roti® Phenol was added, vortexed for 1 min and centrifuged for 30 min at 13'000 rpm. The upper phase was transferred to a new 1.5 ml ERT, using a cropped pipette tip. Subsequently, 500 µl Chisom was added and the solution was

vortexed for 1 min the phases were separated by centrifugation for 30 min at 13'000 rpm. The upper phase was transferred to a new 1.5 ml ERT, 0.7 volumes of isopropanol (= 350 µl) were subsequently added and the solution was mixed thoroughly on a vortex. The DNA was fished using a yellow pipette tip, transferred to 1 ml 70% EtOH and centrifuged for 30 min at 13'000 rpm. The DNA sediment was washed once again with 0.5 ml 70% EtOH. Finally, the DNA was vacuum dried by a Univapo 100 H vacuum centrifuge and dissolved in 50-100 µl Milli-Q-water. The DNA was stored overnight at 4 °C and later at -20 °C for long term storage.

### *4.17.2 DETERMINATION OF DNA CONCENTRATION*

The concentration of DNA was determined photometrically at an extinction of 260 nm. Absorption of 1 refers to a concentration of 50 µg/ml double-stranded DNA. Low DNA concentrations were estimated in comparison with standard-DNAs of known concentration in an EtBr stained agarose gel. The extinction ratio of 260/280 is used to determine the purity of the DNA with 1.8 for DNA without RNA or protein contaminations.

## 4.18 *ANALYSIS OF DNA BY GEL ELECTROPHORESIS*

### *4.18.1 AGAROSE GEL ELECTROPHORESIS OF DNA FRAGMENTS*

DNA fragments of different size were separated by electrophoresis in TAE-agarose gels (14x 11 cm, 0.8-2.0% agarose in TAE buffer) at 120 V. Before the gel run, DNA was mixed with ¼ volumes of 5x DNA sample buffer (25% Ficoll® 400,
50 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol). The sample volume for analytical gels was 20 µl and was up to 100 µl for preparative gels. The DNA-standards GeneRuler 1 kb DNA ladder or DNA ladder mix were used as the marker. DNA of analytical gels was stained in an EtBr staining solution (2 µg/ml) for 10 min, decolorized in water for 5-10 min and photographed under the UV-light. Preparative gels were stained in methylene blue (0.1% in $dH_2O$) for 5-10 min and decolorized for 30-60 min in water. Thereby, DNA bands became visible and were excised from the agarose gel using a scalpel.

### 4.18.2 *ISOLATION OF DNA FROM AGAROSE GELS*

The to-be-recovered DNA fragment was excised using a scalpel and was collected in a 2 ml ERT. The DNA was recovered using the "QIAquick Gel Extraction-Kit“. The agarose was dispersed at 50 °C in a heat block, adsorbed to glass particles packed in columns and eluted after a wash step with TE-buffer. This system is very suitable for DNA fragments above 4000 nt. The isolation was carried out according to the manufacturer‘s protocol.

## 4.19 *MODIFICATION OF DNA IN VITRO*

### 4.19.1 *CLEAVAGE, DEPHOSPHORYLATION AND LIGATION OF DNA*

DNA cleavage with restriction endonucleases (RENs) was carried out according to the manufacturer‘s protocol. For the analytical DNA cleavage 0.05-0.5 µg of DNA were applied, and for preparative use 0.5-5 µg of DNA was sufficient. The minimum and maximum reaction volume was set to 20 µl and 60 µl, respectively. All reactions with RENs were carried out at 37°C, but *Sma*I at 30°C. For the analytical purpose a reaction time of 1.5 hrs and for the preparative usage a reaction time of 2.5-3 hrs were applied. The RENs with their appropriate buffers were obtained from New England Biolabs GmbH and Fermentas GmbH.

The 5‘-phosphate group of the free DNA-ends was enzymatically removed by calf Intestine alkaline phosphatase (CIAP), after the hydrolytic cleavage of a vector by a REN. This dephosphorylation prevents the linearized vector from religation. The CIAP was applied either directly to the enzyme reaction buffer, or CIAP reaction buffer was applied to the purified and in Tris-buffered vector fragment. Per µg DNA 1-10 U of CIAP were added, and the reaction batch was incubated for 1 h at 37°C. Later, the CIAP was heat-inactivated for 15 min at 65°C.

The ligation of free DNA ends was carried out with T4-DNA ligase. Therefore, DNA was prepared using preparative agarose electrophoresis. The vector–insert ratio was 1:4 or 1:8. The ligation was carried out either with the 5x rapid ligation buffer and 2 µl ligase over 45 min or with the 10x ligation buffer and 1µl ligase at 8-14°C overnight.

### *4.19.2 POLYMERASE CHAIN REACTION*

The polymerase chain reaction (PCR) makes it possible to amplify specific DNA fragments *in vitro* (Mullis and Faloona 1987; Bej *et al.* 1991). Synthetic oligonucleotides (Primer) complementary to the target sequence and flanking the to-be-amplifying region are used as start points for a thermostable DNA-polymerase. Primer ends often were modified to include recognition sites for RENs or an optimized ribosomal binding site. The basis of this technique is a DNA-Polymerase, of which the optimized temperature is *ca*. 75°C. In general, the Taq polymerase derived from *Thermoproteus aquaticus* was used for analytical and colony PCRs. The high-fidelity polymerase, a combination of Taq and *pfu* DNApolymerase of *Pyrococcus furiosus*, which possesses 3' to 5' exonuclease proofreading activity, was used for preparative approaches to amplify particular DNA fragments. The "Phusion® Hot Start High-Fidelity DNA Polymerase" was used for Fusion-PCR. Common PCR was carried out in 50 µl reaction volume with 1x reaction buffer containing $MgCl_2$, 25 µM dNTP mix (dATP, dCTP, dGTP, dTTP), 0,5 µl primer (100 pmol/µl), 2 U polymerase and 100 ng DNA template or 1-2 µl chromosomal DNA. A standard PCR-protocol is as follow

**Tab. 15: Standard PCR protocol**

| | Denaturation | Annealing | Elongation |
|---|---|---|---|
| **Start** | 94 °C / 5.00 min | | |
| **Cycle (1-25)** | 94 °C / 0.50 min | estimated / 1.50 min | 72 °C / estimated |
| **End (Cycle 26)** | 94 °C / 0.50 min | estimated / 1.50 min | 72 °C / 12 min |
| **Store at 8°** | | | |

Primer-annealing temperatures were estimated from the effective binding nucleotides to the target sequence using GCG Wisconsin Package 10 or the information provided by biomers.net but can also be estimated by using the formula:

$$(A + T) * 2 + (G + C) * 4 = Annealing\ temperature$$

Unless specifically described by the Kits manufacturer, 1.000 nt are elongated in 1 min by DNA polymerase. The last cycle includes a doubled elongation time. Afterwards, 10% of the PCR-reaction was analyzed on an analytical agarose gel.

#### 4.19.2.1 Purification of DNA products amplified by PCR

Two methods for the purification of the PCR products were used. Either the DNA was purified according to the protocol of the QIAquick PCR Purification Kit when the PCR products were pure and visible as single bands on the analytical agarose gel, or the particular DNA fragment was cut out of a methylene blue stained preparative agarose gel and further purified according to the protocol of the QIAquick Gel Extraction Kit.

### *4.19.3 COLONY PCR*

(Tested for *E. coli* strains & *S. carnosus* TM300)

Colony-PCR is a rapid analytical method to screen for positive clones after plasmid-delivery. In this approach the whole bacterial colony was taken and its DNA served as a template of the PCR reaction instead of a purified DNA. To verify a particular DNA fragment the adjacent primers were chosen and the annealing temperatures was calculated. Using specific primers complementary to the vector and the insert made it possible to control the orientation of the inserted DNA, which was easily shown without any further purification and cleavage of the DNA by RENs. The analytical PCR was carried out in 20 µl total reaction volume by the *Taq* DNA polymerase for 20-25 cycles. A single colony was picked with a sterile pipette tip or a toothpick and transferred first into the prepared PCR-reaction solution and afterwards to a freshly prepared solid BM-agar plate complemented with the respective antibiotic. Afterwards, 5-10 µl of the PCR reaction were analyzed on an analytical agarose gel.

### *4.19.4 FUSION PCR (OVERLAP PCR)*

Great efforts are required to construct gene cassettes flanked by promoter and protein-tag areas and additional recognition sites. By PCR fragments are generated, which have to be cloned step by step into the respective plasmid. The difficulties are further increased the longer the generated PCR product has become. In this case, fusion PCR or overlap PCR is a favorable method to construct a fragment directly in separate PCR reactions. In the last PCR-reaction the two generated fragments are fused. The two separated PCR reactions were carried out using the High-Fidelity DNA-polymerase. After purification of the PCR products the filling reaction was done using Phusion® High-Fidelity DNA Polymerase.

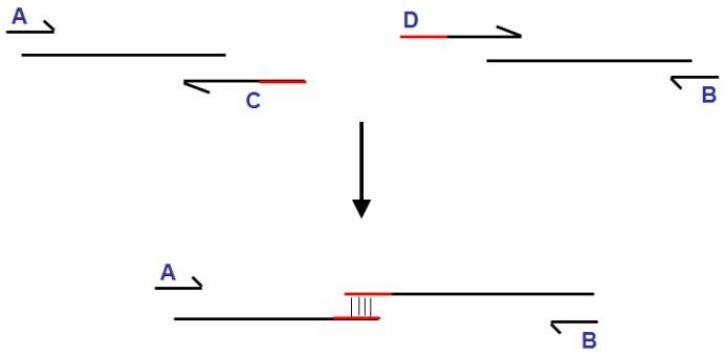


**Figure 4: Principle of fusion-PCR.**
In two separated PCR-reactions the fragments to be fused are generated. In the last step the fusion product is generated by a polymerase producing less mistakes as i.e. Phusion® High-Fidelity DNA Polymerase

## 4.20 *GIBSON ASSEMBLY*

Gibson assembly is an isothermal, single-reaction method for assembling multiple overlapping DNA molecules by the concerted action of a 5´ exonuclease, a DNA polymerase and a DNA ligase. First, DNA fragments were recessed, yielding single-stranded DNA overhangs that could be specifically annealed. T5 exonucleases removed nucleotides from the 5´ends of double-stranded DNA molecules. Complementary single-stranded DNA overhangs annealed, Phusion DNA polymerase filled the gaps and Taq DNA ligase sealed the nicks. T5 exonuclease is heat-liable and it inactivated during the 50°C incubation (Gibson, 2009). The Primer were designed using the NEBuilder™ computal program provided by NEB.

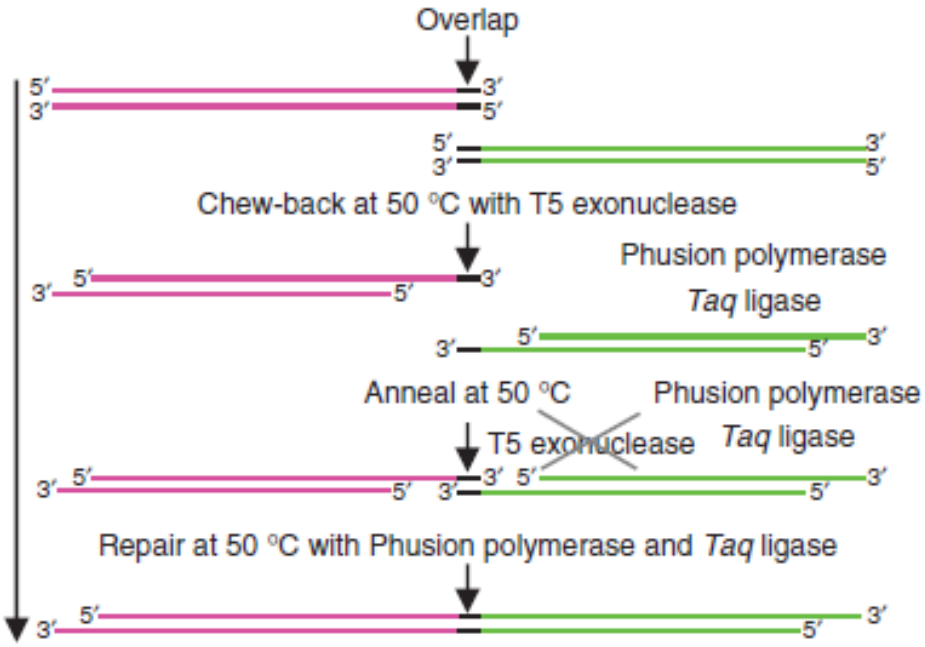


**Figure 5: Principle of Gibson Assembly:**
Two adjacent DNA fragments (magenta and green) sharing terminal sequence overlaps (black) were joined into a covalently sealed molecule in a one step isothermal reaction.

### *4.20.1 SEQUENCE VERIFICATION BY DNA SEQUENCING*

DNA sequencing was carried out by the research assistants of the laboratory according to the chain termination method using an automatic Li-COR sequencer with fluorescent labeled sequence primers (Sanger *et al.,* 1977). For sequencing of chromosomal DNA 20-25 µg DNA were applied. The sequence reaction was carried out according to the cycle sequencing principle, with the "Thermo Sequenase fluorescent labeled prime cycle sequencing kit (Sears *et al.* 1992). One reaction contained 13.5 µl DNA, 0.5 µl DMSO and 1 µl sequencing primer (2 pmol). The analysis of DNA sequences was carried out by the LI-COR software "Data Collection" and "Image Analysis" (Li-COR Inc.). In one sequence-run 500-750 nucleotides could be identified. The sequencing reaction of plasmid DNA contained 0.5-1.0 µg DNA and up to 900 nucleotides could be identified. Alternatively, samples were sent to the sequencing company GATC in Konstanz. PCR products of the to-be-sequenced DNA region were amplified from genomic DNA by High-Fidelity DNA-Polymerase or isolated purified plasmid-DNA was sent directly.

In general, DNA sequences were analyzed by VectorNTI suite 10.3, BioEdit and the GCG Wisconsin Package 10. The BLAST program was used for the comparison of DNA and amino acid sequences, over the internet at "National Center for Biotechnology Information"(Altschul *et al.* 1990; Altschul *et al.* 1997).

## 4.21 *TRANSFORMATION OF BACTERIA*

### *4.21.1 TRANSFORMATION OF E. COLI BY HEAT SHOCK*

#### 4.21.1.1 Preparation of competent *E. coli* cells

40 ml Basic-medium were inoculated 1:200 with ONC of *E. coli* strain DH5α, DC10b or XL1-blue respectively. Cells were grown until an $OD_{578}$ = 0.4 was reached. Afterwards, they were cooled down on ice for 10 min before harvesting by centrifugation at 5'000 rpm for 10 min at 4°C. The cell sediment was resuspended in 20 ml ice-cold 50 mM $CaCl_2$ and was incubated for 25 min on ice. Afterwards, cells were spun down by centrifugation at 5'000 rpm for 10 min at 4°C. The cell sediment was resuspended in the heat shock buffer (2 ml of 50 mM $CaCl_2$ and 500 µl 50% glycerol), separated into aliquots of 100 µl each, and stored at -20°C (Dagert and Ehrlich 1979).

#### 4.21.1.2 Transformation of competent *E. coli* cells

Cells were thawed on ice for 5 min and 10 µl ligated plasmid or 1 µl isolated plasmid was added. The cell solution was incubated at 43°C for 90 sec in a water bath and cooled on ice again. Subsequently, 1 ml B-medium was added and the cell solution was incubated for 1-1.5 hrs shaking at 37°C. Afterwards, 100 µl cell cultures were spread each on two BM agar plates, complemented with the respective antibiotics, and incubated overnight at 37°C. Grown colonies were either transferred to another plate or directly tested whether they included the correct plasmids ( 4.16).

### *4.21.2 TRANSFORMATION OF S. CARNOSUS*

#### 4.21.2.1 Preparation of protoplasts

(Götz, F. *et al.*, 1983; Götz, F. and Schumacher, 1987)

300 ml B-media (1 l flask with baffle) were inoculated with 1 ml ONC of *S. carnosus* TM300. Cells were grown shaking until $OD_{578}$ = 0,35-0,45 at 37°C. After cooling down on ice (10 min) cells were transferred to sterilized centrifuge tubes and spun down for 20 min by centrifugation at 4.500 rpm and 4°C. The sedimented cells were resuspended in 30 ml SMMP-75 media and transferred to sterile 50 ml falcon tubes. Afterwards 10 µl of a filter sterilized Lysostaphin solution (0.5 mg/ml) was added and the whole mixture was incubated overnight for 12-16 hours at 30°C without shaking. The formation of protoplasts was monitored by light microscopy. When the cells were singularized and spheroblasts were visible lysis was stopped. Therefore, the protoplasts were spun down by centrifugation for 20 min at 4.500 rpm and RT. After the supernatant was discarded, residues of lysostaphin were removed by washing with 5 ml SMMP-75 media. The protoplast sediment was resuspended carefully in 2 ml SMMP-75 media and aliquoted in 300 µl portions in 12 ml Greiner tubes. The protoplasts were stored at -70°C.

#### 4.21.2.2 Transformation of *S. carnosus* TM300 protoplasts

300 µl protoplasts were slowly thawn on ice (10 min). The DNA (5-70 µl, 0.5-1.0 µg) was pipetted on the edge of the 12 ml Greiner tube. Adjacent, the DNA was embedded by 2 ml Fusogen (40 g polyethylene glycol 6000, 50 ml 2x SMM, filled up with 100 ml $dH_2O$; filter-sterilized) and flushed into the protoplast solution. During the transformation the tube was carefully rotated and inverted for exactly 2 min (PEG 6000 can damage the protoplasts when extending the transformation time). Addition of 7 ml SMMP-75 media stopped the transformation. Cells were spun down for 20 min by centrifugation (4.500 rpm, RT). The supernatant was discarded and the protoplast sediment was resuspended in the media reflux and plated on two DM3 agar plates (Chang and Cohen, 1979; Götz, F. and Schumacher, 1987), respectively. The DM3 agar-plates were incubated for 3-4.5 h at 37°C for regeneration of the cell walls. 3 ml soft agar (CY3-soft agar, Na-succinate solution, Mix-solution in the proportion of 5:5:1) including the relevant antibiotics in a 10 fold concentration were poured on the pre-incubated DM3 agar-plates. The DM3 agar-plates were incubated for 2-4 days at 37°C until colonies were formed.

#### 4.21.2.3 Transformation of *S. carnosus* by electroporation

The transformation of staphylococcal cells with DNA by electroporation is performed according to the protocol of Löfblom (Löfblom *et al.*, 2007).

### *4.21.3 PREPARATION OF ELECTRO-COMPETENT S. CARNOSUS CELLS*

For the preparation of electro-competent staphylococci the cells were grown overnight in B-Medium. 100 ml of B2 Medium were then inoculated with a 1:200 dilution of the overnight culture and grown to an $OD_{578}$ of 0,7 (37°C, 150 rpm). Growth was stopped by incubating the cells on ice for 15 min The suspension was centrifuged (10 min, 5000 rpm), and the cells were washed three times with $H_2O_{bidest}$ (100 ml, 50 ml und 20 ml), lowering the volume stepwise and again two times with cold 10% glycerol (10 ml and 5 ml). Finally the cells were pelleted in 2 ml 10% glycerol and 100 µl aliquots were kept at -80°C.

### 4.21.4 *ELECTROPORATION BUFFER*

For transformation the cells were thawn on ice and then incubated for 30 min at room temperature. Centrifugation for 2 min at 12.000 rpm and a washing step with 500 µl electroporation buffer (EC; 0,5 M saccharose, 10% glycerol) were performed afterwards. The pellet was washed again and solved in 85 µl EC. 4-6 mg plasmid DNA was added. The cells were transferred into a cuvette (1 mm gap) and the electroporation was performed with the settings 2500 V and 5 ms. Immediately 1 ml B2-Medium was added and the cells were incubated for at 30°C or 37°C for two hours (150 rpm). 200 µl and 800 µl of the transformed cells were plate on agar-plates (selection medium) and incubated at 30°C or 37°C for 24-48 h.

A slight modification of this protocol was that, electro competent cells were spun down immediately at 5000 rpm and 4°C for 15 min the cells were then resuspended in 85 µl EC buffer, containing of 0.5 M sucrose in 10% glycerol, before the transforming plasmid was added in a maximal volume of 15 µl (Yu *et al.*, 2010).

## 4.22 *CONSTRUCTION OF THE KNOCK OUT VECTORS*

In *S. carnosus* TM300 the genes *ftsW1*, *ftsW2*, and *rodA* are encoded by Sca_0739 (1228bp), Sca_1886 (1207bp), and Sca_1584 (1217bp) respectively. For gene deletion, an approximately 1000 bp region up- and downstream of each gene was amplified by PCR. Both DNA fragments were cloned into the pJet vector using a CloneJet® kit from Fermentas resulting in pJet *rodA*up and pJet *rodA*down which were used to transform chemo-competent *E. coli* Xl1-blue cells. Each insert was cut again from the particular vector by using the restriction enzyme site introduced by the amplification primers. We used SalI and PstI for the downstream regions and NheI and PstI for the up-stream regions. The knock-out (KO) vector pBT2 (Brückner, 1997) was cut with the enzymes SalI and NheI resulting in a linear vector. A triple ligation of the up and down-stream fragments and the cut pBT2 lead to a full circular vector (pBT2up-down) containing a PstI enzyme site between the up and down-stream fragments. The *aphAIII* resistance cassette including the lox66 and the lox71 recognition site for the cre-recombinase and the PstI restriction sites (Leibig, Martina *et al.*, 2008) was amplified and cloned into the PstI digested pBT2up-down vector resulting in the knock-out vector pBT2rodA. This vector first transformed chemically E*. coli* XL1-blue and was then isolated again to be sequenced. A plasmid containing the correct insert was then

electroporated into *S. carnosus* TM300 as described (Lofblom *et al.*, 2007)(4.21.2.3) with slight modifications. For FtsW1 and FtsW2 the procedure was according to that listed above with the exception that the forward primer for the up-stream region does not contain a NheI site but an EcoRV site.

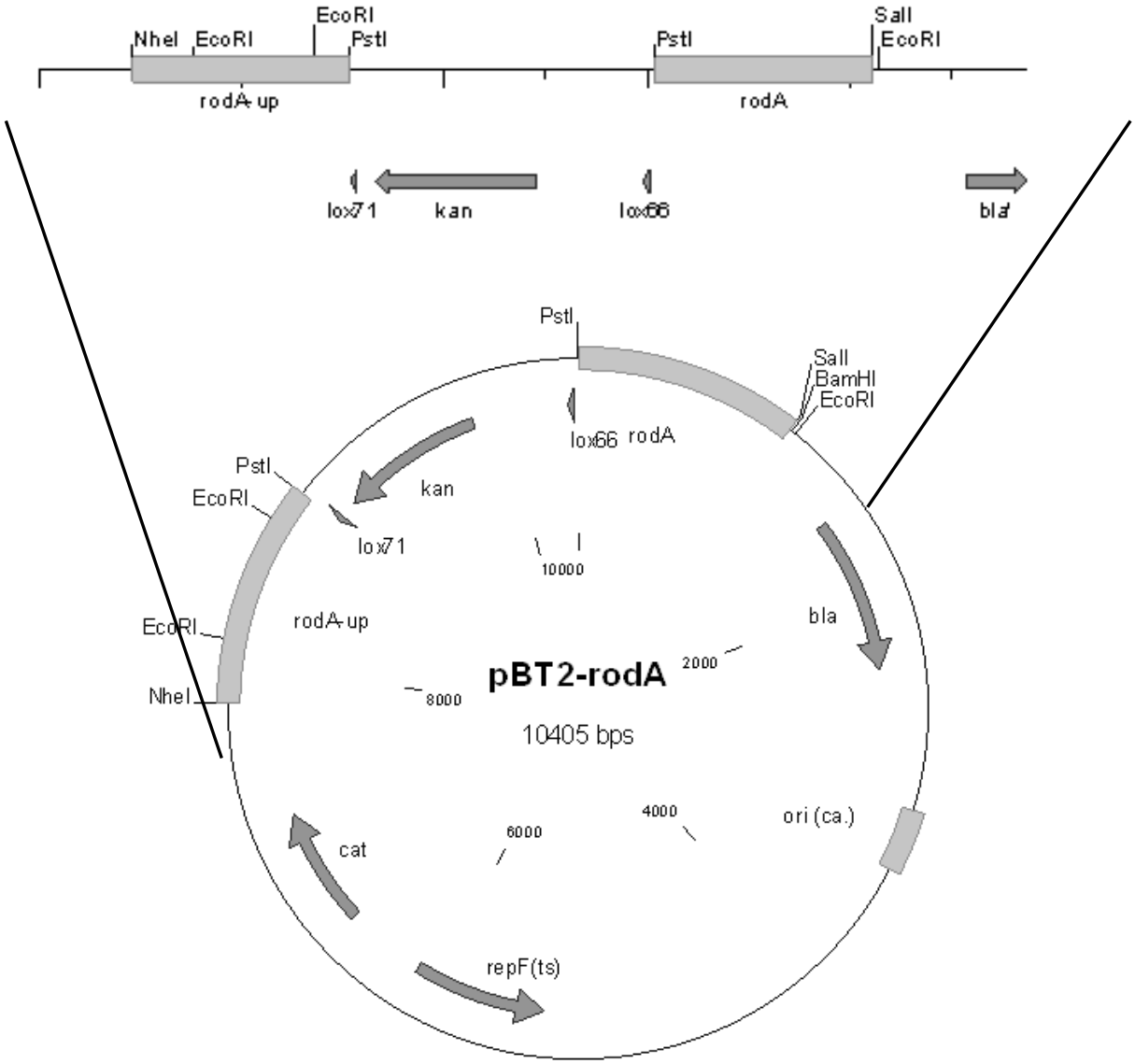


**Figure 6: pBT2 *rod*A knock out vector.**
Up- and Down-stream region of *rod*A were cloned in the vector with the restriction enzymes NheI/PstI and SalI/PstI respectively. The kanamycin resistance cassette flanked by the lox-sites was cloned in with the PstI restriction enzyme. The KO vectors for *fts*W1 and *fts*W2 were constructed accordingly.

## 4.23 *INACTIVATION OF CHROMOSOMAL GENES IN S. CARNOSUS TM300*

The method is based on the exchange of genes in the genome of *S. carnosus* via the homologous recombination. Herewith, the chromosomal encoded gene of interest can be exchanged by an antibiotic resistance marker. This method is called “gene knock-out“. The shuttle vector pBT2 was used, which has a temperature sensitive replicon for staphylococci (Brückner, R., 1997(Brückner, 1997). No plasmid replication takes place, when cultivated above 30°C. As selection marker, the kanamycin resistance cassette derived from plasmid pBT2 *srtA* was used and the inactivation vector was cloned in *E. coli (Leibig, M. et al., 2008)*. The flanking regions of the target sequence were cloned into pBT2 (optimal size: each >1kb length and the to-be-deleted region was replaced by the resistance cassette (4.22). The constructed plasmid was verified by sequencing, and inserted into *S. carnosus* TM300 by protoplast transformation (4.21.2.2). The selection occurred by the vector mediated resistance to chloramphenicol (10 µg/ml Cm) on BM agar plates. Plasmid DNA was isolated and the inserted genes were controlled by restriction enzyme analysis. Subsequently, an overnight culture was inoculated (50 ml) and cells were grown at 30°C for the plasmid replication in the presence of chloramphenicol. Afterwards, 50 ml preheated medium containing 7.5 µg/ml Kanamycin were inoculated with the overnight culture (1:1000) and incubated at 40°C in a water bath overnight. Now, the cells were cured of the pBT2 knock out plasmid, as its replication stopped at 40°C and, consequently, the plasmid was diluted. 50 ml preheated medium without antibiotics was inoculated 1:1000 with the overnight culture and incubated overnight at 40°C. From a dilution series ($1x\ 10^{-5}$ -$1x\ 10^{6}$) of overnight grown *S. carnosus* colonies were picked on kanamycin and Cm10 agar plates and incubated overnight at 37°C. *S. carnosus* clones growing on kanamycin but not on chloramphenicol lost their plasmid, but, integrated the resistance cassette into the chromosome. Contrary, *S. carnosus* colonies, which were able to grow on both kanamycin and chloramphenicol still carried the plasmid. Putative KO clones were tested by PCR and sequencing of the respective region.

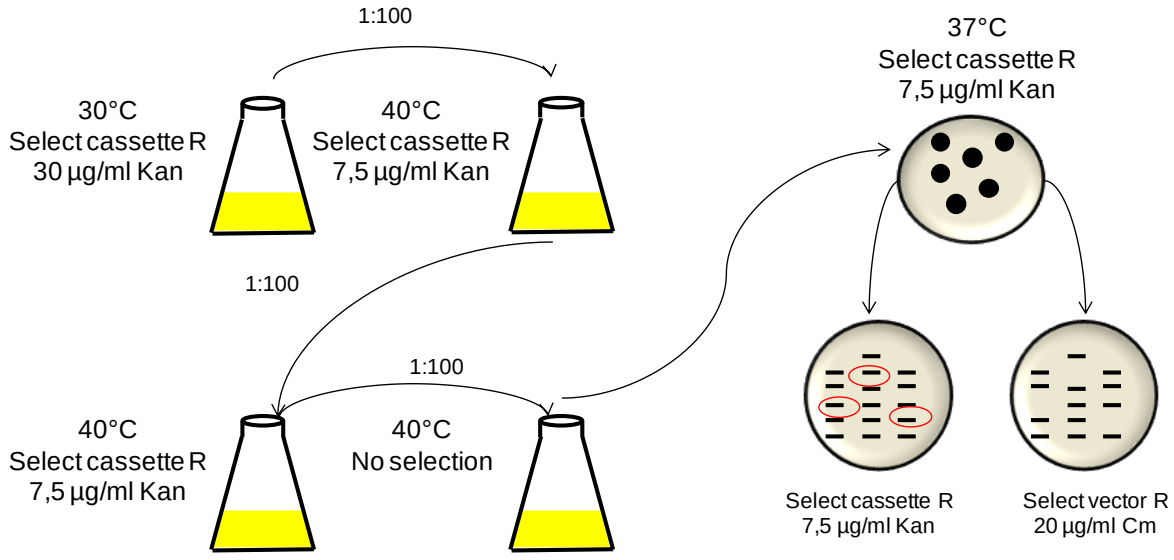


**Figure 7: Knock out procedure**
Procedure for homologous recombination using the plasmid pBT2 according to (Brückner, 1997). Different temperature steps exploiting the temperature sensitive origin of replication for staphylococci of the pBT2 plasmid leads to a dilution of the plasmid and homologues recombination between the plasmid and the respective region in the chromosome.

## 4.24 *REMOVAL OF THE RESISTANCE MARKER*

Once an antibiotic resistance marker is integrated into the genome, it cannot be reused for another gene knock out. Therefore, the kanamycin resistance cassette *aphIII* was flanked by the so-called *lox* (locus of x-over P1)-sites, *lox66* and *lox71*. After the integrated antibiotic cassette was confirmed by sequencing, it was excised using Cre-recombinase, which specifically recognizes *lox66* and *lox71* and removes the space in-between, generating a *lox72*-site (Leibig, M. *et al.*, 2008). The latter is poorly recognized by the Cre-recombinase. Consequently, another gene knock out can be carried out in this strain with a *kanR* cassette, which might be used again with *lox*-sites. The constructed *S. carnosus* mutant strain was protoplast transformed with the *cre*-recombinase gene encoded on pRAB1 (Leibig, M. *et al.*, 2008) and selected for Cm10 resistance at 30°C. Colonies were picked on Cm10 and Kan 7.5 agar plates, and incubated at 30°C overnight. The colonies, which were able to grow on both selection media, were streaked on BM and Cm10 agar plates and incubated overnight at 37°C. Strains without plasmid were not able to grow on Cm10 plates anymore.

## 4.25 *CONSTRUCTION OF THE COMPLEMENTATION VECTORS*

Complementation of the RodA-mutant was achieved with the pCX33rodA vector. Therefore the *rodA* gene was amplified by PCR with primers containing restriction site for BamHI (forward), including the native SD sequence, and SmaI (reverse) resulting in a 1229 bp fragment. This gene region was inserted into a derivate of the pCX31 vector (Wieland *et al.*, 1995) under the control of a xylose inducible promoter resulting in the pCX31*rod*A. The *S. carnosus* Δ*rodA* strain was transformed with this plasmid gaining a complemented mutant strain (4.21.2.3). Plasmid isolation and sequencing of the cloned region confirmed the complementation. According to this procedure the *fts*W1 and *fts*W2 mutants were also complemented.

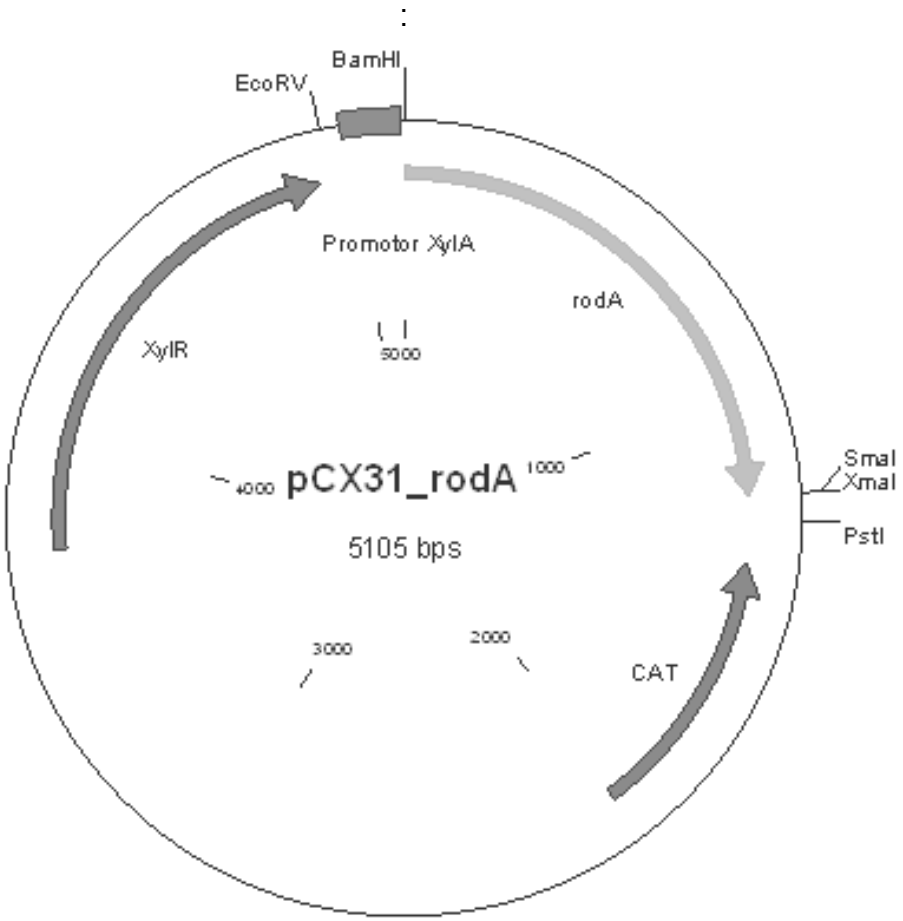


**Figure 8: Plasmid map of pCX31_*rodA***
The xylose inducible complementation vector is displayed with all improtant features like the inducible promoter XylA, the xylose regulator XylR, the chloramphenicol resistance gene *cat* and the complementary gene are displayed. The construction of the other vectors was performed according to this one.

## 4.26 *MUROPEPTIDE ANALYSIS BY UHPLC*

All strains were grown in B-Medium up to an $OD_{578}$ = 0.7. The peptidoglycan of the study strains was isolated, then digested with a muramidase, and analyzed by HPLC essentially as described before (Bertsche, U. *et al.*, 2013) (Cecolabs; Tuebingen, Germany). The analyses were done on a Waters Acquity H-Class (UPLC) with a Prontosil C18-(120-3-C18) 150*4.6 mm AQ 3.0 µm column (Bishoff, Leonberg, Germany), but a protocol adjusted for HPLC or an Agilent 1200, using a Prontosil C18-(120-3-C18) 250*4.6 mm AQ 3.0 µm from Bishoff (Leonberg, Germany). The gradient of 150 min started with 100 mM sodium phosphate + 5% methanol (buffer A) and ran to 100mM sodium phosphate + 30% methanol (buffer B).

## 4.27 *MASS SPECTROMETRY (MS) ANALYSIS.*

HPLC peaks-of-interest were collected and analyzed by MS. The liquid chromatography system used was an Agilent HPLC-MS-System Modell 1200 connected to an Ultra Trap System. Muropeptides were separated on a Prontosil C18-(120-3-C18) 250*4.6 mm AQ 3.0 µm from Bishoff (Leonberg, Germany). The gradient started with solvent A (0.1% formic acid) for 5 min, followed by a linear gradient in 60 min to 20% Solvent B (0.06% formic acid in methanol) with a flow rate of 0.5 ml/min. The sample volume was 100 µl. For ionisation ESI positive and negative was alternated. The mode was set on Ultra Scan Capillary with a current of 3.5 kV. The nebulizer was set on 40 psi and dry gas was 12.0 l/min. The temperature was 350°C The software used was the 6300 Series Trap Control Version 6.1, of Bruker Daltonik.

The high-resolution LC-ESI-MS and MS/MS measurements were carried out on a Bruker Daltonics MaXis 4G connected to a Dionex Ultimate 3000 system using a reversed-phase Prontosil C18-(120-3-C18) 250*4.6 mm AQ 3.0 µm column from Bishoff (Leonberg, Germany) and a linear gradient elution with solvents A (0.1% formic acid) and B (0.06 % formic acid in acetonitrile starting at 10 % B to 100 % B in 140 min at a flow rate von 0.3 ml/min. The acquisition parameters for the positive ion polarity were a capillary voltage of 4.5 kV and an end plate offset of -500 V. The nebulizer pressure was set to 2.0 bar and dry gas flow to 8.0 L $min^{-1}$ at a dry heater temperature of 200°C. The measurements were internally calibrated using sodium formate as a reference. Peaks had been desalted before analysis.

## 4.28 *AMINO ACID ANALYSIS*

Amino acid analysis was performed by an adjusted protocol which depends on the protocols of GRACE and Agilent. The cell wall of the *rodA*-mutant the *S. carnosus* wild type TM300 and the *S. aureus* SA113 wild type was isolated after 24 h (Bertsche, U. *et al.*, 2013). The lyophilized cell wall was hydrolyzed by adding 600 µl 6 N HCl to 45 mg substrate and incubated by 110°C for at least 18 h. The released amino acids were then neutralized for 72 h in NaOH atmosphere under vacuum conditions. OPA derivatisation was performed in the injection-needle of the HPLC (Agilent 1200) as pre-column derivatisation. Ortho-Phthaldialdehyde (OPA) was purchased from GRACE Davison. The stock solution of 10 mg /ml was diluted 1:10 in 1 M Borate-buffer (61.8 g borate in 1 liter of HPLC-grade-water). 6 µl OPA and 1.5 µl substrate were mixed for 90 sec in the injection needle and then separated via HPLC with an Agilent 1200 series HPLC-system using a Grom-SIL OPA-3 (5µm) 4.0 x 150 mm column. The gradient was run in 24 min from 100% buffer A (25 mM Sodium-phosphate buffer with pH =7.2) to 100% buffer B (50% 25 mM sodium-phosphate buffer, pH =7.2, 35% methanol, and 15% acetonitrile) in a stepwise manner. The column temperature was 25°C and the flow rate was 1.1 ml $min^{-1}$. Absorption at 340 nm was detected and the data was analyzed with the ChemStation software.

## 4.29 *MICROSCOPY*

### *4.29.1 LABELLING OF ACTIVE PG SYNTHESIS SITES.*

(Turner *et al.*, 2010)

To analyze the sites of active peptidoglycan biosynthesis the cells were grown over night in 10 ml BHI-Medium (Brain-Heart-Infusion) containing 0.125 M D-serine. Then the cells were diluted into the same medium to an $OD_{578}$ 0.01 and grow to an $OD_{578}$ between 0.3 and 0.4. Cells were centrifuged at 5000 rpm for 10 minutes and resuspended in 50 ml BHI (without D-serine) and afterwards grown for 25 min (pulse) before D-serine was added (chase). 1 ml Samples were taken every 15 min, washed with 0.5 ml PBS pH 7.8 and labelled with a mixture of fluorescent vancomycin (Van-FL) and vancomycin 1:1 to 1 µg/ml. By this method only newly synthesized peptidoglycan should be labeled, because of the old one being protected against vancomycin by the incorporation of D-Serine instead of D-Alanine.

### 4.29.2 *DELTA-VISION AND FLUORESCENCE MICROSCOPY*

Cells were incubated first with D-Serine, washed and incubated without D-Serine and finally labeled with vancomycin FITC as fluorescence dye (4.29.1). Subsequently, 200 µl cells were spun down by centrifugation and were washed three times in 1 ml PBS. All centrifugation steps were carried out in 1.5 ml ERT's at 6'000 rpm for 5 min at RT From the 200 µl PBS dissolved cell solution 10 µl were transferred to agarose coated slides (1% (w/v) of agarose was dissolved in 1x TAE and 1 ml was applied to dust-free glass slides). The location of the peptidoglycan biosynthesis of *S. carnosus* was determined by fluorescence microscopy. The slides were analyzed by fluorescence microscopy (Leica Dm 5500 B, Camera DFC 360 Fx). Alternatively, the samples were analyzed by a delta-vision microscope. The advantage of a deltavision microscope is the possibility of the deconvolution. Deconvolution is a software-based process by which one can "re-focus" an out of focus image. Deconvolution occurs after image acquisition, and uses nearest-neighbor algorithms to extract information out of blurred regions of an image to clean up these regions - they then appear to be closer to, if not actually in the same plane of focus as the rest of the image.

### 4.29.3 *TRANSMISSION ELECTRON MICROSCOPY*

For closer examination of the cell surface and the division planes TEM was used. Exponentially grown cells were resuspended at 37°C in 6 ml PBS at $OD_{578}$ of 0.5 at 578 nm where cells gradually start to autolyse; 2 ml samples were taken at time points of 0, 2 and 4 h. Samples were processed for TEM as described previously (Biswas et al., 2006). Briefly, bacterial cells in the stationary phase were harvested and washed twice with 100 mM phosphate buffer (pH 7.0). Cells were fixed using the glutaraldehyde/$OsO_4$ method. Fixed cells were covered with 2% agarose and blocks were cut out. After washing, agar blocks were dehydrated in an ethanol series beginning with 50% ethanol and finally placed in water-free acetone. Samples were then embedded in Spurr's resin and polymerized at 60°C for 2 days. Ultrathin sections were cut with an ultramicrotome with a diamond knife.

Samples were poststained with 1% uranyl acetate for 1 h and Reynold's lead citrate for 20 min and examined with a Zeiss EM 109 transmission or a Philips CM10 electron microscope electron microscope at 80 kV.

## 4.30 *STROM*

#### 4.30.1.1 STROM working principle

STORM is a super-resolution imaging technique that utilize sequential activation and time-resolved localization of photoswitchable fluorophores to create high resolution images. The fundamental principle behind stochastic optical reconstruction microscopy (STORM) is that the activated state of a photoswitchable molecule must lead to the consecutive emission of sufficient photons to enable precise localization before it enters a dark state or becomes deactivated by photobleaching. Additionally, the sparsely activated fluorescent molecules must be separated by a distance that exceeds the Abbe diffraction limit (in effect, greater than approximately 250 nanometers) to enable the parallel recording of many individual emitters, each having a distinct set of coordinates in the lateral image plane.

#### 4.30.1.2 STORM sample preparation

For preparation of the samples with Gold Fiducial Particles 103 nm gold nanoparticle were vortexed until particles were fully suspended. 5 µl of 103 nm gold nanoparticle suspension were added to 95 µl water (HPLC grade). The suspension was briefly vortexed again. 5 µl of this suspension were put onto a Poly-L-Lysine coated slide and dried with nitrogen. Bacterial cells were added from the suspension samples after Van-FL labeling (4.29.1) in the same way. No washing step is required. The samples were transferred to the STROM and analyzed for 1 h. Gold particles are fixing points for the imagine software and work as drift correction during the STORM observation time

# 5 AIM

In the coccal organism *S. carnosus* there are genes annotated for three SEDS-family proteins (*rodA*, *ftsW1*, and *ftsW2*). RodA is responsible for cell elongation in rod-shaped bacteria while FtsW is part of the divisome. The elucidation of the role of these proteins in a coccal organism has been the major goal of this study; especially because *S. carnosus* cells are true cocci lacking an elongation growth phase. Therefore, construction of the deletion mutants for all three genes was planned and performed. These mutants should be characterized regarding obvious phenotypes which can give insights in the protein/gene function. In the beginning growth experiments were performed to decide if the deletion had a general effect on the fitness of the cells. To test an effect on the cell shape simple microscopy studies followed. Detailed insights were gained by intensifying these studies with fluorescence techniques, super resolution microscopy and electron microscopy.

The cell wall was the focus of the biochemical characterization of the mutants. Therefore HPLC analysis of the isolated muropeptides was performed. The general pattern was compared and divergent peaks were further analyzed by mass spectrometry.

# 6 RESULTS

## 6.1 *CONSTRUCTION OF THE KNOCK-OUT MUTANTS.*

To investigate the role of a gene with unknown function the common approach is to create a loss of function mutant and to analyze the phenotype of this mutant. Therefore, a gene replacement for all 3 annotated SEDS proteins in *S. carnosus* was planned. An overview of the gene organization is given in Fig. 9.

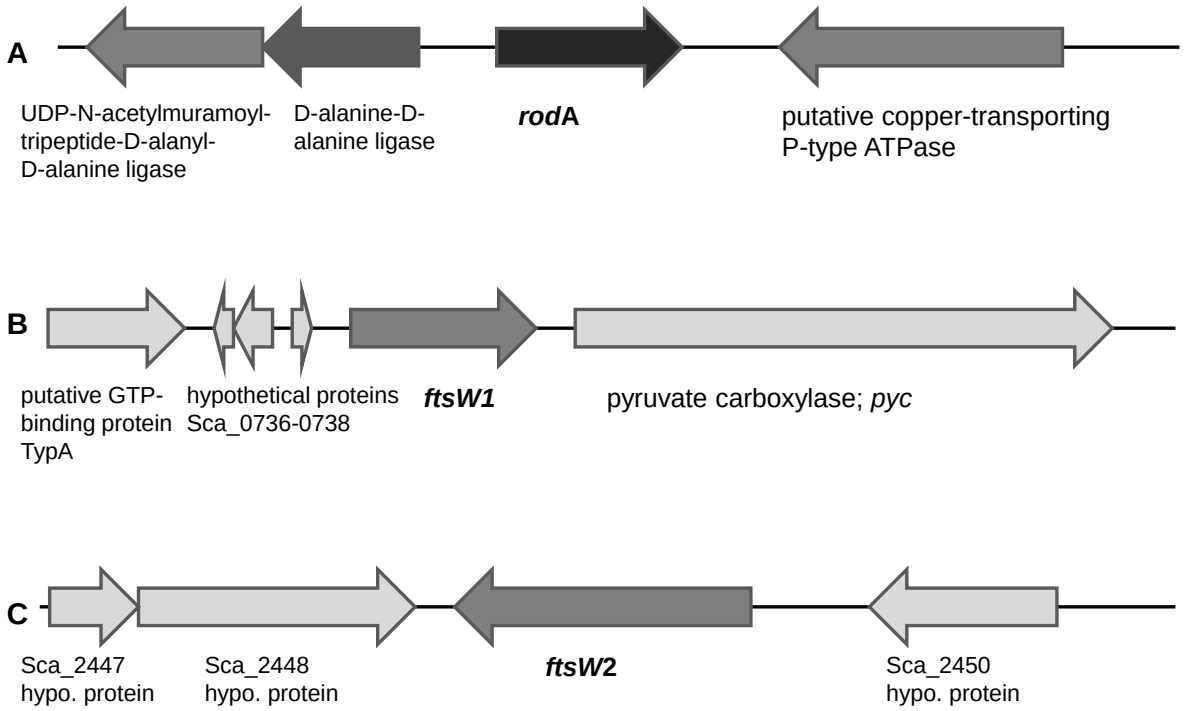


**Figure 9: Location of the three SEDS proteins in *S. carnosus* TM300**
This is an overview of the three SEDS gene containing regions of *S. carnosus* TM300. The genes Sca_1584 (*rod*A) (A), Sca_0739 (*fts*W1) (B) and Sca_2449 (*fts*W2) (C) are marked and their neighboring genes are displayed.

The knock-out procedure itself was performed following the protocol for allelic replacement (Brückner, 1997). Thereby the *rodA* gene was replaced by an *aphAIII*-cassette resulting in Kanamycin resistance of the mutant. To receive a clear knock-out mutant the kanamycin resistance cassette was excised by the cre-recombinase (Leibig, M. *et al.*, 2008), which was introduced into *S. carnosus* Δ*rodA*. After recognition of the lox 66 and lox 71 sites, the recombinase cuts out the DNA region between these two sites resulting in an marker less mutant and a lox 72 site, which itself is a poor substrate for the cre-recombinase enabling construction of a double mutant by the

same technique (Leibig, Martina *et al.*, 2008). All three genes could be successfully deleted as single mutants. However, a double mutant could not be created independent of the combination tested.

## 6.2 *VERIFICATION OF GENE DELETIONS BY PCR*

Growth on kanamycin containing plates indicates that the *aphA*III cassette is working while growth on chlorampenicol indicates that the pBT2 plasmid containing the chloramphenicol resistance is still present in the cells. Therefore a clone that grows on kanamycin but does not grow on chlorampenicol should have integrated the *aphA*III cassette into the genome and lost the plasmid. To verify the deletions the genomic DNA of one or more clones was isolated. The verification was done before the cre-recombinase was introduced to create marker less mutations. To test the genomic DNA PCR was performed. Primers to amplify the gene of interest (which should have been replaced by the *aphAIII* cassette), primers for the *aphAIII* cassette as well as primer for a third gene locus at a different and distant site were used. For control issues TM300 WT DNA and different plasmids were used as template.

**RodA**

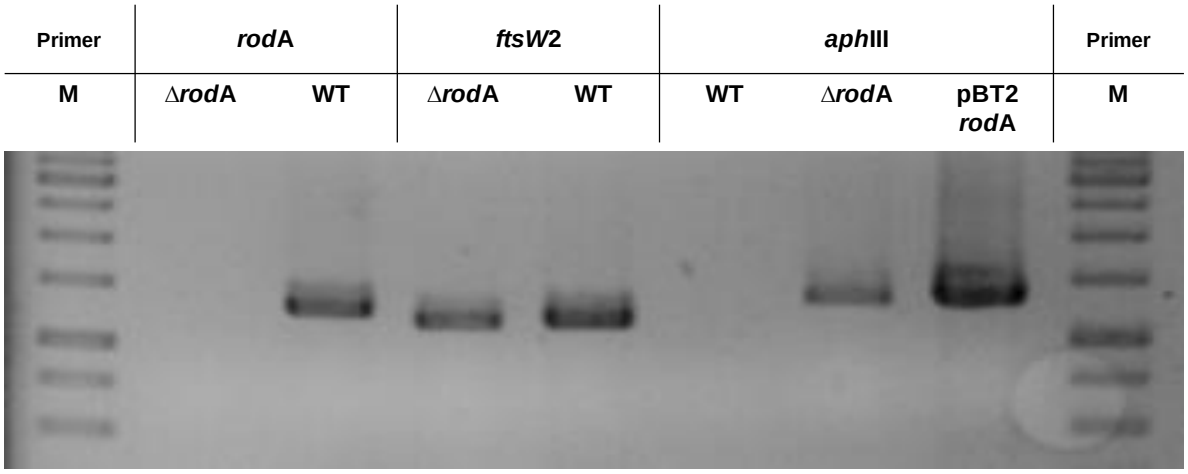


**Figure 10: Verification of the Δ*rod*A::*aph*III deletion mutant in *S. carnosus*:**
Deletion of *rod*A was verified by PCR using the respective genomic DNAs or plasmid pBT2 *rod*A as template. The *rod*A gene was lost in the mutant. Instead the *aph*A III cassette could be amplified from this genomic DNA. The upper row indicates the amplicons which were expected by using the respective primers. The second row displays the template DNA used. The marker is a 1Kb letter from Fermentas.

In the case of the Δ*rodA* mutant primers for *rodA*, *ftsW2* and the *aph*-cassette were used. The genomic DNA of the mutant clone (Δ*rodA)* resulted in no amplification of

*rodA* but with the wild type DNA there was an amplicon with the expected size of approximately 1400 bp. With the primers for *ftsW2* an amplicon of the correct size from both templates was achieved. As expected the *aphAIII*-cassette could be amplified from the *rodA* mutant but not from the wild type. To add an additional control here the pBT2 knock out vector was also used as a template for the amplification of the *aph*-cassette, which gave a positive result. Additionally, plasmid isolation was performed with the selected clones but no plasmid could be obtained, proofing that the plasmid was lost.

**FtsW1**

In the case of *fts*W1 the mutant was also verified by PCR using the genomic DNA of a mutant and a wild type clone as well as plasmid DNA to control the reaction. Here it was renounced to use primers for a third distant locus. The PCR for *fts*W1 gave an amplicon with the wild type DNA as template and none for the mutant DNA, while the control with a pJet plasmid containing *fst*W1 was also positive. *aph*III could be amplified from the mutant DNA and the pBT2 *fts*W1 knock vector as template, while there was no amplicon with the genomic wild type DNA

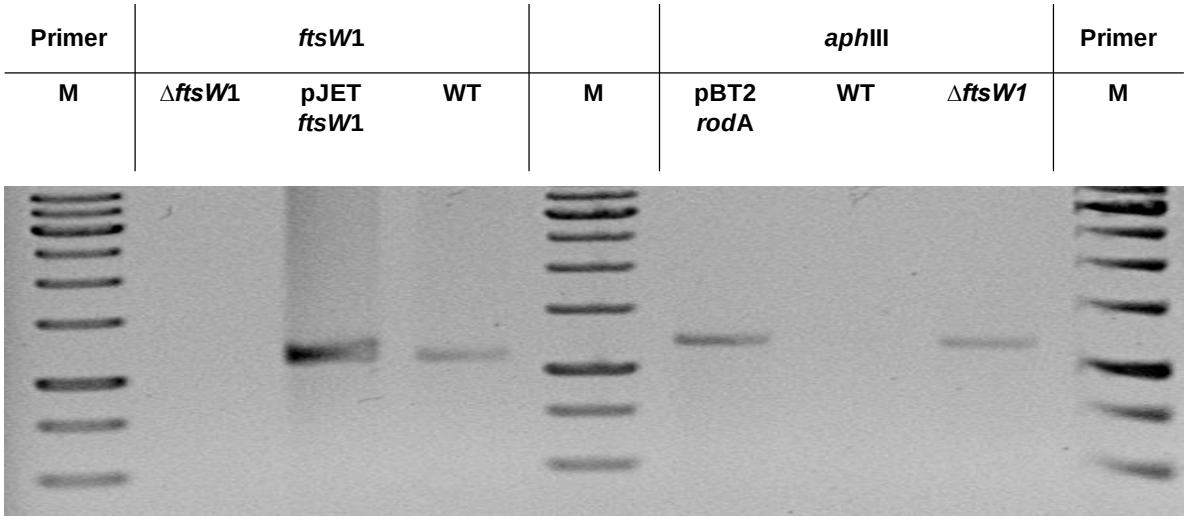


**Figure 11: Verification of the Δ*fts*W1::*aph*III deletion mutant in *S. carnosus*:**
EtBr stained 1% agarose gel demonstrating the deletion of *fts*W1. The upper lane indicates the amplicons which were expected by using the respective primers. The second lane displays the template DNA used. The marker is a 1Kb letter from Fermentas.

**FtsW2**

The DNA was tested with primers for *fts*W2 and *aph*III respectively. The PCR for *fts*W2 gave an amplicon with the wild type DNA as template and none for the mutant DNA while the control with a pJet plasmid containing *fst*W2 was also positive. *aph*III could be amplified from the mutant DNA and the pBT2 *fts*W2 knock vector as template while there was no amplicon with the genomic wild type DNA

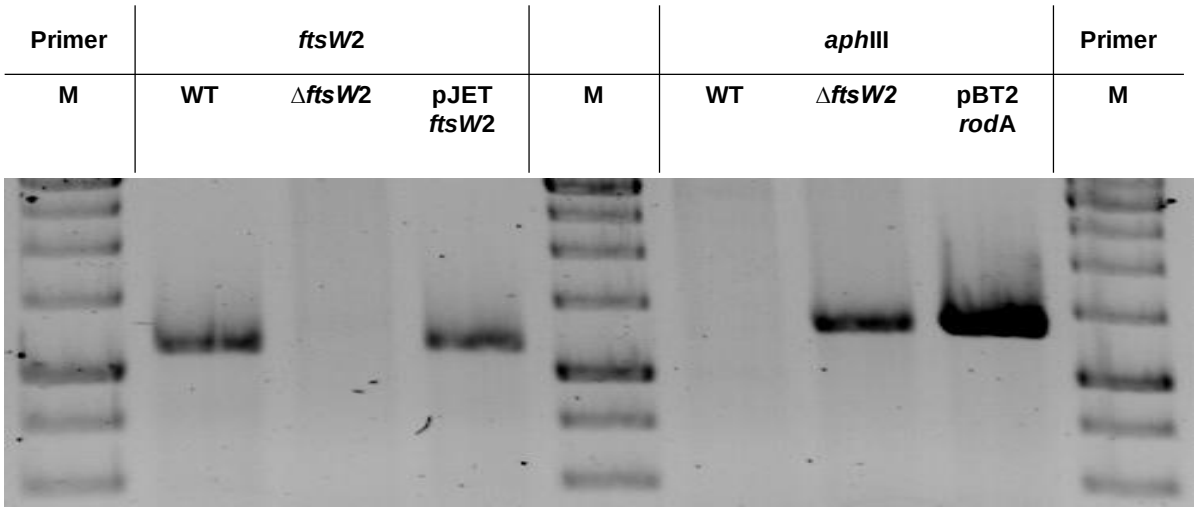


**Figure 12: Verification of ∆*fts*W2::*aph*III deletion mutant in *S. carnosus*:**
EtBr stained 1% agarose gel demonstrating the deletion of *fts*W2. The upper lane indicates the amplicons which were expected by using the respective primers. The second lane displays the template DNA used. The marker is a 1Kb letter from Fermentas.

As only ∆*rod*A gave an obvious phenotype all further experiments described were performed with this mutant. A short summary on ∆*fts*W1 and ∆*fts*W2 is given in section 6.9 on page 78.

## 6.3 *VERIFICATION OF THE RODA MUTANT*

### *6.3.1 STRAIN TYPING BY PCR*

To further verify the Δ*rodA* mutant as an *S. carnosus* TM300 PCR was performed with various primer pairs (Kull, 2009) to discriminate different staphylococcal strains from one another. The selected genes are listed in Tab. 16. Only when *S. carnosus* subsp. *carnosus* or *S. carnosus* subsp. *utilis* have been used as template all of the primer pairs could produce an amplicon. In different staphylococcal species only some amplicons would be detected. For example Sca_2051 is also detectable in *S. gallinarum* DSM 20610T and *S. sciuri* subsp. *sciuri* DSM 20345T or Sca_0076 in *S. haemolyticus* CCM 2737T and *S. lugdunensis* ATCC 43809T. The results of *S. carnosus* TM300 Wt and *S. carnosus* TM300 Δ*rodA* were compared. In the both cases all 5 primer pairs produced an amplicon with the specific size (Figure 13) and therefore the *rod*A deletion mutant was identified as derived from *S. carnosus* TM300.

**Tab. 16: Target genes picked for verification of a strain as *S. carnosus* TM300**

| Gene | Size (bp) | Annotation |
|---|---|---|
| **Sca_0076** | 1146 | putative oxidoreductase |
| **Sca_0079** | 843 | hypothetical protein |
| **Sca_0909** | 1311 | putative membrane protein |
| **Sca_2051** | 1266 | conserved hypothetical protein |
| **Sca_2158** | 912 | putative UDP-Glucose 4-Epimerase |

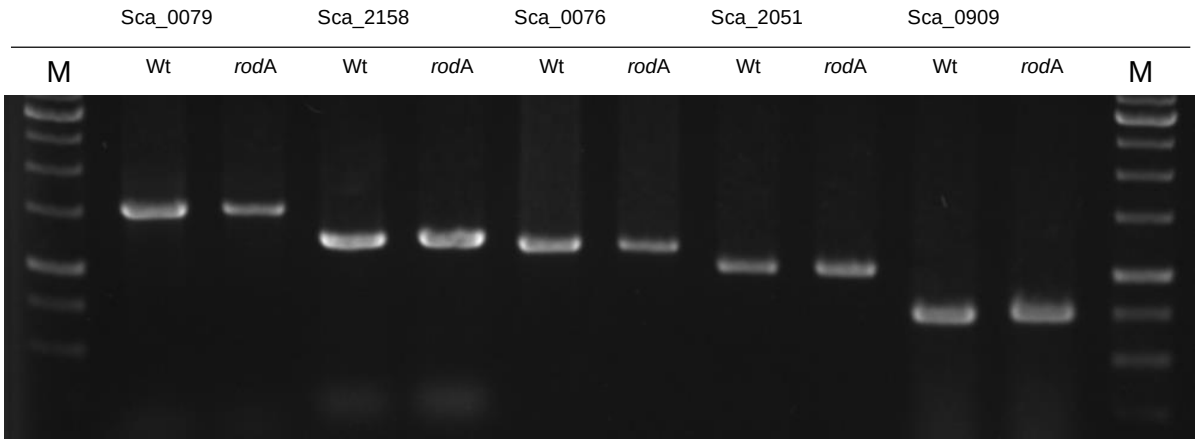


**Figure 13: Specific PCR products to verify *S. carnosus* species:**
Comparison of *S. carnosus* TM300 and *S. carnosus* TM300 Δ*rod*A::lox72. PCR products with same size in both strains show that *S. carnosus* TM300 Δ*rod*A::lox72 is indeed derived from *S. carnosus* TM300.

## 6.4 *SEQUENCING OF 16S RRNA LOCUS*

To further identify the mutant strain as a deletion of *rodA* in the *S. carnosus* TM300 the genetical background was verified by an analysis of the 16S ribosomal RNA. The 16S rDNA sequence is a gene encoding small subunit ribosomal RNA. This gene contains conserved sequences of DNA common to all bacteria and divergent sequences unique to each species of bacteria. Primer were constructed that could amplify the DNA locus of the 16S ribosomal RNA of various bacterial species such as *Listeria, Bacteroides, Mycobacteria, Clostridia, Corynebacteria, Pseudomonas, Staphylococcus, Escherichia, Streptococcus* and more. These so called Universal Primers (Tab. 5) were used to amplify these approximately 1500 bp from the WT and the Δ*rod*A mutant, which were then cloned into the pJet cloning vector and finally sequenced. The revealed gene sequences were blasted. First hit in both cases was *S. carnosus* TM300 16S rRNA. The next possible candidate already contained 3 nucleotide exchanges within the first 1000 bp which could not be found in the amplified regions. Therefore the Δ*rod*A mutant is definitely an offspring of *S. carnosus* TM300.

## 6.5 *CHARACTERIZATION OF THE RODA DELETION MUTANT*

### *6.5.1 DELETION OF RODA FROM S. CARNOSUS TM300 AFFECTS GROWTH*

To study the role of the SEDS proteins in *S. carnosus* growth experiments were performed. As the SEDS proteins are thought to be part of the cell division machinery one would expect the deletions to influence growth.

Deletion of *rodA* generated a viable *S. carnosus* strain with a reduced growth rate compared to the wild type *S. carnosus* TM300 (Figure 14). This indicated that RodA plays indeed a role during cell proliferation of staphylococci. Complementation (compl.) of this mutant by the expression of the *rodA* gene from the xylose (xyl) inducible plasmid pCX31 (Wieland *et al.*, 1995) demonstrated that *rodA* deletion was causative for the observed growth defect. The Δ*rodA* mutant strain had a shortened lag phase compared to the wild type strain, but then growth slowed down after about 4 hours. This could also be seen when the cells were dilution plated on agar. After 3 hours the Δ*rodA* mutant grew in thick patches up to a dilution of $10^{-5}$ while the wild type strain and the complemented mutant grew in single colonies already at the $10^{-4}$ dilution. At time point 5 hours, the picture was conversed. The Δ*rodA* mutant only grew until $10^{-4}$

while the other two strains formed colonies up to $10^{-6}$. This reduction in cell amount indicates that part of the Δ*rodA* mutant cells died. After 8 hours, there were still about ten times more viable cells of the wild type strain and of the complemented mutant than of the Δ*rodA* mutant, which reflected the situation in liquid culture. In contrast to the mutant the wild type strain had a real lag-phase and therefore began to grow slower than the mutant. After the transition to the exponential phase the wild type grew stronger until both strains reached an equal according to the OD at about 5 hours from start. From this time point on the wild type surpassed the mutant in OD and reached a higher maximum OD value than the mutant. The CFU determination showed that there was no detectable lysis during the growth experiment for the wild type.

Repression of the complementation plasmid by 0.5% glucose resulted in a strain mimicking the growth of the mutant strain, albeit with a higher OD value at the end. When complementation was induced by 0.5% xylose, the mutant strain grew like the wildtype parent. Induction of the RodA protein to complement the mutant restored the growth to wild type level also for the early stages. In the exponential phase the induced complementation ascended as steeply as the wild type but the maximum OD value was slightly lower than that of the wild type. This was also observed in the CFU determination in the late stages of the growth experiment, where the CFU is not quite on wild type level.

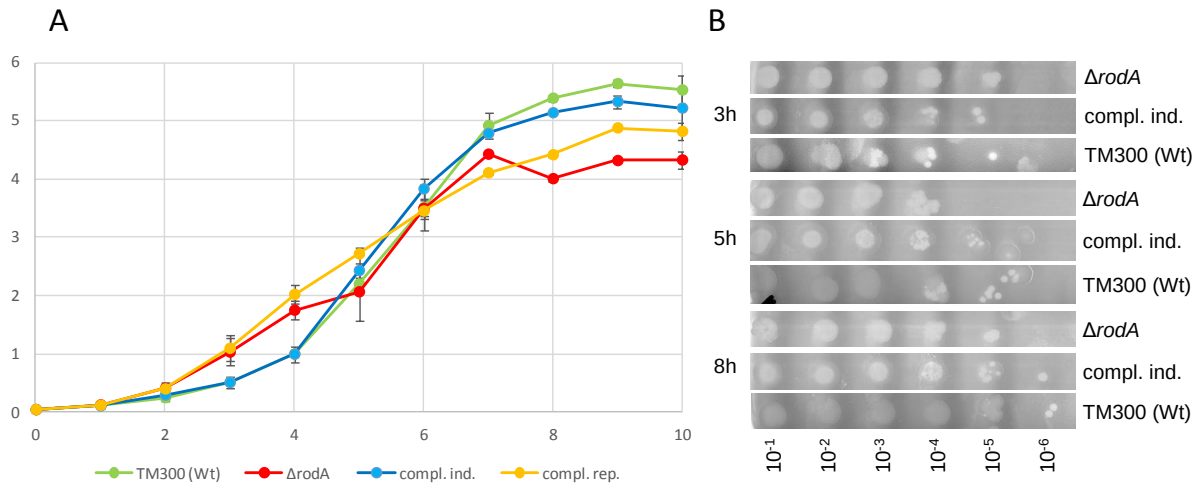


**Figure 14: Growth curve and CFU-determination**
(A) *S. carnosus* TM300 and Δ*rod*A mutant cells were grown in liquid B-medium without glucose. The $OD_{578}$ was measured every 60 minutes. (B) After 3, 5 and 8 hours the CFU was determined on solid B-medium.

### 6.5.2 THE RODA MUTANT IS MORE PRONE TO LYSIS

By chance I observed that the Δ*rod*A mutant formed clusters when incubated without shaking. To test this under defined conditions, cells were grown over night in 50 ml flasks filled with BM at 37°C shaking at 130 rpm. After the first incubation the cell suspensions were further incubated at room temperature on a bench for another night without shaking. After 48 h of incubation the Δ*rod*A mutant cells tended to clustering whereas in the wild type strain and in the complemented mutant this was deferred until 72 h. Resuspending by hand would lead to a sticky slimy cell suspension of Δ*rod*A mutant cells in comparison to the wild type that would dissolved as clouds in the suspension. In this experiment the induced complementation did actually fully restore the mutant phenotype to wild type level. Addition of 10 µg/ml DNase and incubation for another 2 h with a short resuspension at the end disintegrated the cluster of the Δ*rod*A mutant cells again.

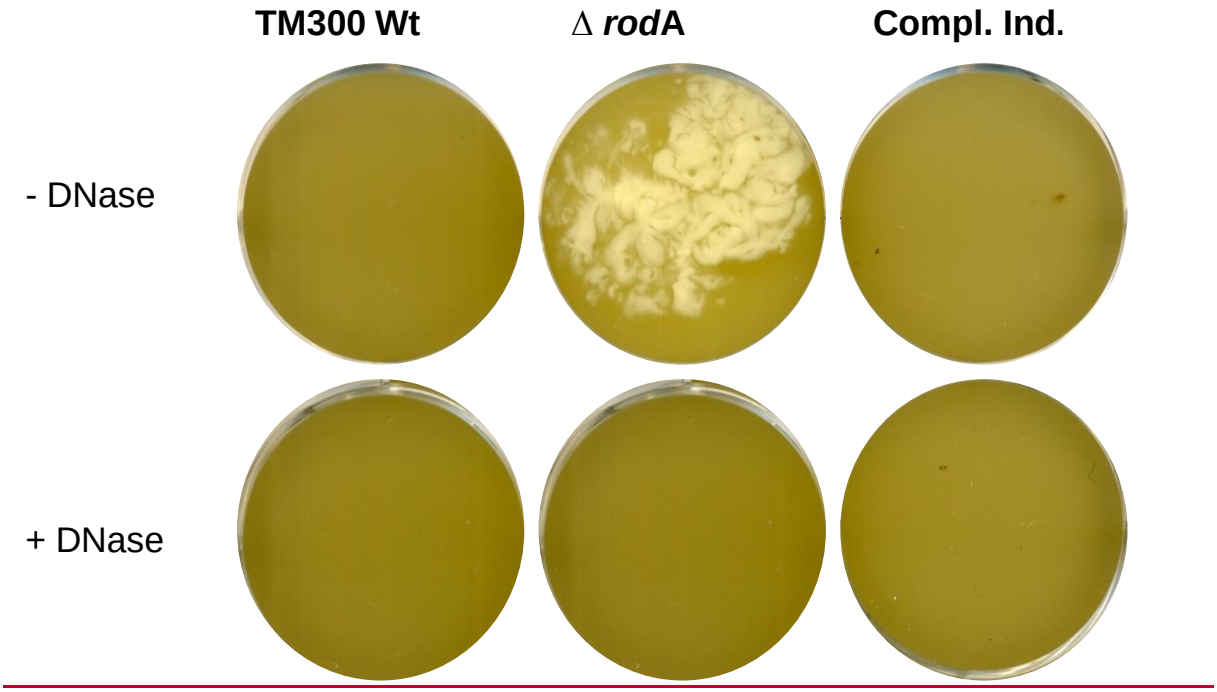


**Figure 15: Lysis assay**
Response of *S. carnosus* TM300 and Δ*rod*A to DNase: Cultures were grown over night at 37°C and then kept at room temperature for 48 h without shaking. DNase treatment was performed for 2 h at 37°C. Sedimented cells were resuspended before taking the picture. For the sake of a better result during photography cultures were transferred to 6 well plates which did not reflect the light as much as the flask would do

### 6.5.3 *Electron microscopical analysis*

To examine, whether the deletion of *rodA* affects the morphology of the cells several microscopical analysis were performed. By using light microscopy there were no detectable differences in shape or size. Therefore electron microscopy experiments of log cells were performed by York Stierhoff at the ZMBP Tübingen. Again there were no differences in size or shape. Also the division planes did not seemed to be altered in position or form. There were minor differences in the surface but it was not convincing to a point that it could be stated as a new phenotype for the mutant.

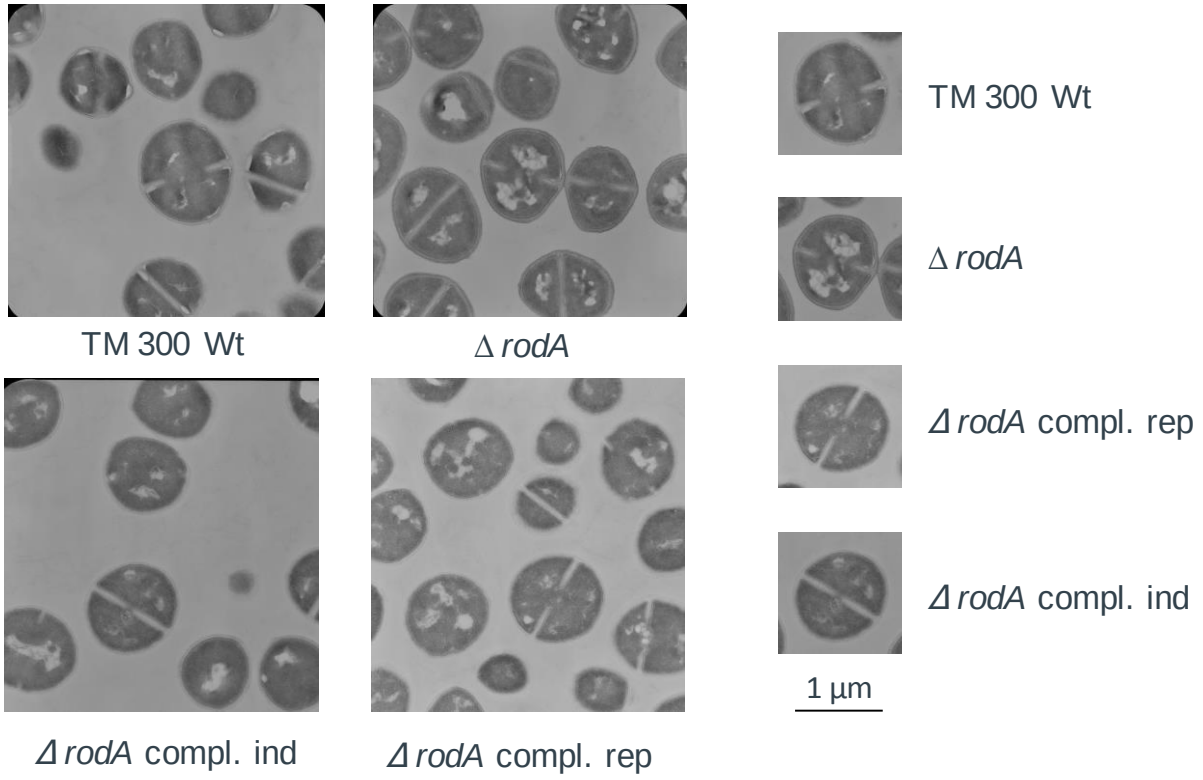


**Figure 16: TEM pictures**
Pictures were taken of *S. carnosus* TM300 WT, Δ*rod*A::lox72 and the complemented mutant in induced as well as in repressed state.

### 6.5.4 *Sites of active peptidoglycan synthesis*

The growth experiments and the observed lysis suggested an effect of the *rod*A deletion on the peptidoglycan (PG) of *S. carnosus*. To determine the sites of active PG synthesis cells were fed with D-Serine to incorporate it into the cell wall precursors at position 5 instead of D-ala. This prevents vancomycin binding to this very sites and allows for a labeling of newly synthesized peptidoglycan by a pulse chase method followed by FITC vancomycin (Van-FL) addition.

While in the wild type strain Van-FL stained the septa and to a lower amount the cell walls, only weak spots of fluorescence and very faint septa were observed in the Δ*rodA* mutant (white arrow Figure 17). As expected also the glucose repressed complemented mutant (compl. + rep.) showed a weaker septal staining and reminded of the Δ*rod*A mutant. In contrast the complemented mutant in which the *rodA* gene was expressed by xylose showed septal staining almost at the wild type level. In all four conditions the cells were viable as judged by DNA labeling with RedDot, but the mutant strain seems to lack fresh D-Ala-D-Ala residues which are required for proper Van-FL staining.

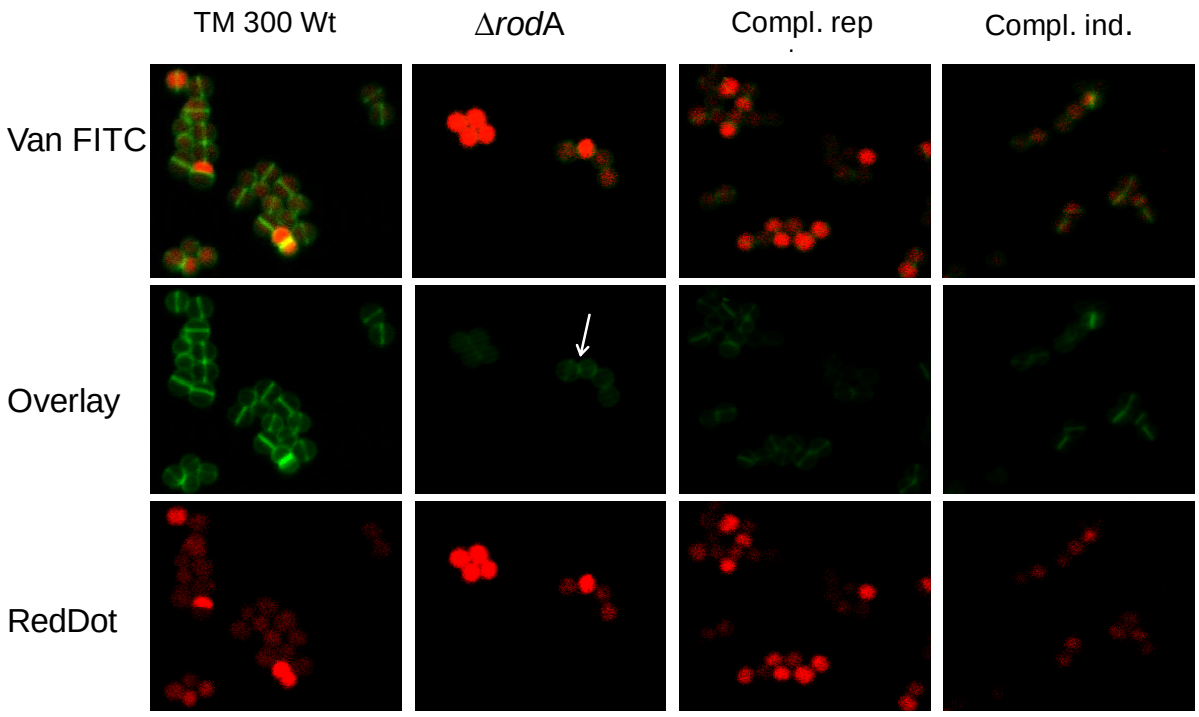


**Figure 17: Confocal laser scanning microscopy (CLSM) pictures**
*S. carnosus* TM300Wt, Δ*rod*A and the complemented mutants (repressed and induced) were incubated in the presence of D-serine and then grown in medium without D-serine for 25 min (pulse) prior to labeling with fluorescent Vancomycin [Van-FL].The samples from time t=0 are shown here. As incorporation of D-serine prevents vancomycin binding, only the peptidoglycan synthesized during the pulse is labeled by Van-FL. In all four cases the cells are viable as judged by DNA labeling with RedDot. The pictures were taken by Christian Liebig from the MPI Tübingen.

Van-FL labeled cells were also investigated by delta vision microscopy under the same experimental conditions. Delta vision can be used for deconvolution which in principle is a mathematical application that is used for image restoration. Literally spoken making straight lines out of blurred ones. With this technique, also the Δ*rodA* mutant showed septal labeling, but the label was uneven, and often formed spots within the whole cell. These spots were also observed in the repressed complementation, but neither in wild type cells nor when complementation was induced. For these images I used the delta vision microscope in the laboratory of Prof. Simon Foster, Sheffield UK.

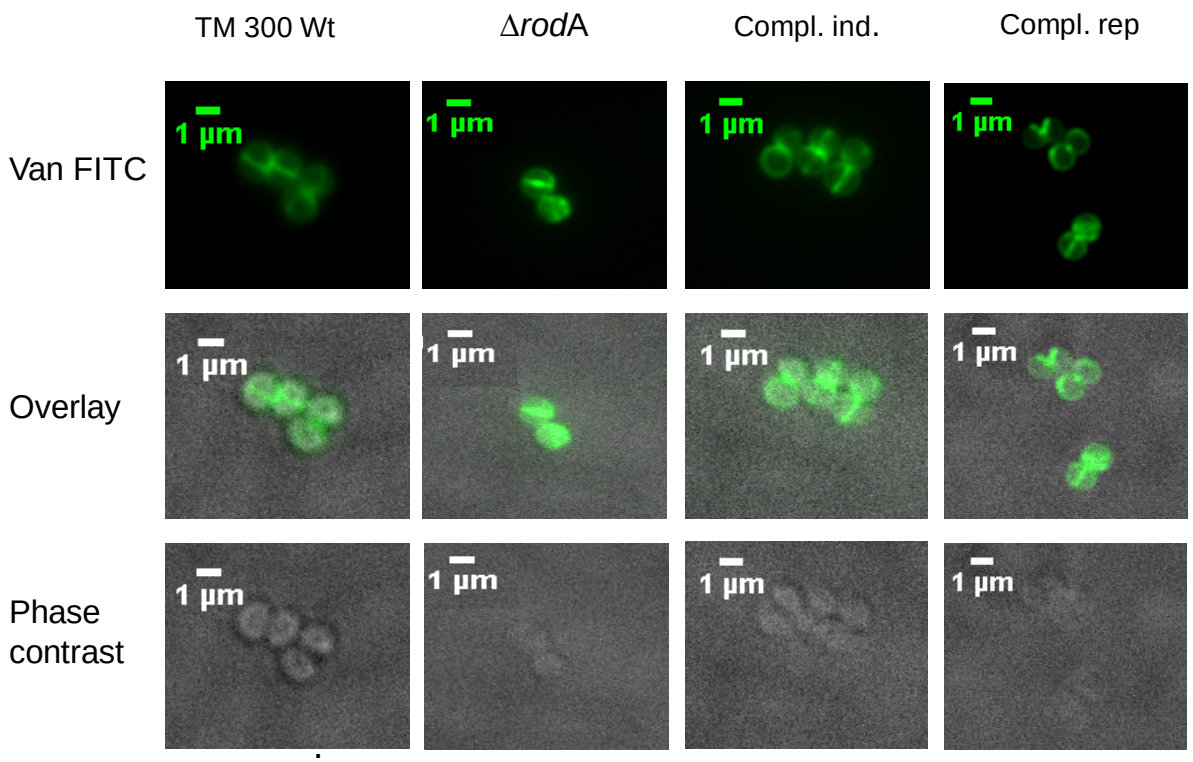


**Figure 18: Deltavision microscopy pictures**
Cells were labeled with VanFL. The pictures in the top row show only the fluorescence channel in the bottom row only the phase contrast channel and in the middle there is a merge of both channels.

### *6.5.5 STROM*

To get even further insights into the localization of the peptidoglycan biosynthesis I was able to perform another form of fluorescence microscopy named STROM (Stochastic Optical Reconstruction Microscopy) in the laboratory of Prof. Simon Foster (Sheffield, UK) together with Dr. Robert Turner. With this technique it is possible to gain an even higher resolution than normal light microscopy and still having the advantage of

fluorescence. The STORM utilizes sequential activation and time resolved localization of photoswitchable fluorophores to create high resolution images. The goal was to narrow down the localization of the newly synthesized peptidoglycan and the altered localization in the *rodA* mutant as shown above. The cells were grown overnight then diluted in BHI medium containing 0.125 M Serine as described in the labeling method. Poly-lysine slides were precovered with nano-gold particles and dried with nitrogen gas. The cells were incubated with a Vancomycin which was linked to a specific fluorophore suitable for the STORM. After washing the cells were attached to poly-lysine covered slides and dried with nitrogen.

Unfortunately no further insights were gained by this method. The pictures confirmed more or less the observations from the other fluorescence microscopy experiments with labeled division planes in the wild type cells and accumulations of labeling at unspecific points of the division plane in the Δ*rodA* mutant cells.

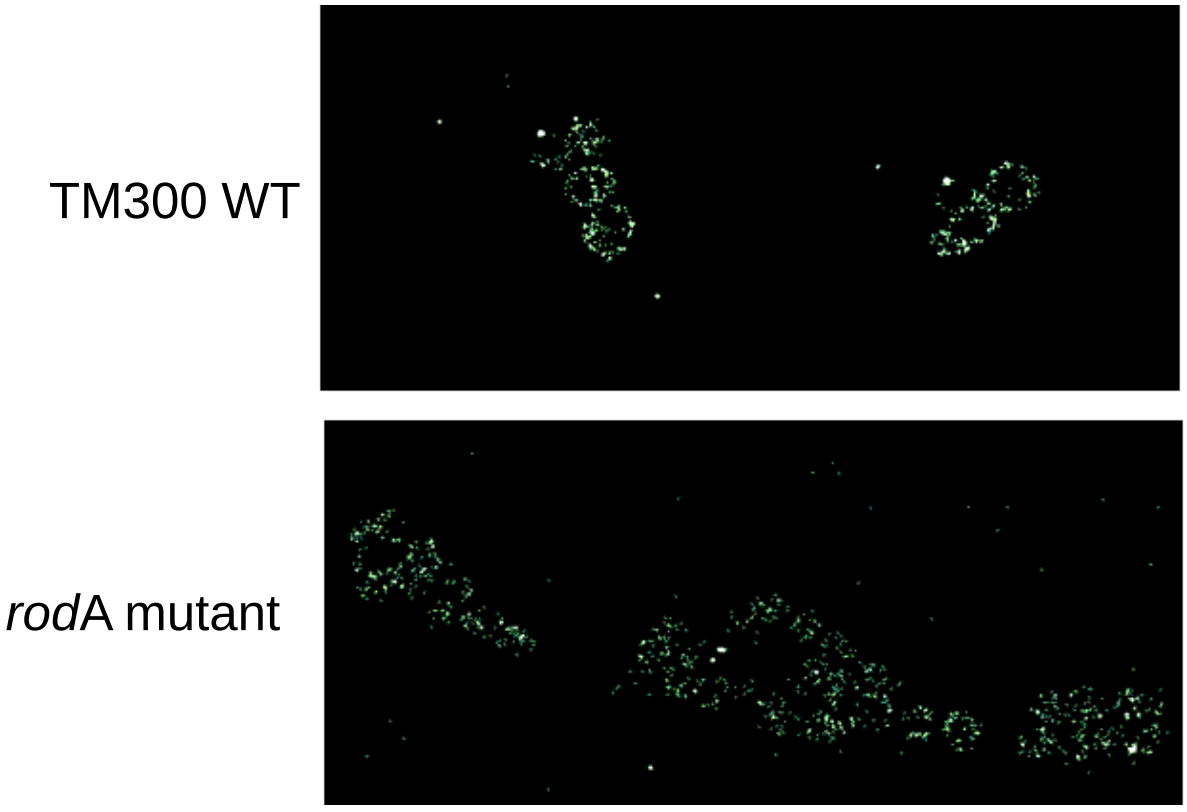


**Figure 19: STORM (Stochastic Optical Reconstruction Microscopy)**
Pictures of *S. carnosus* TM300 Wt and Δ*rod*A::*lox72*: Computal reconstruction of STROM pictures taking of two strains labeled with VanFL according to the protocol listed above.

### *6.5.6 PEPTIDOGLYCAN ANALYSIS BY HPLC*

#### 6.5.6.1 Muropeptide composition

As SEDS proteins are potential flippases for the peptidoglycan precursor lipid II (Mohammadi *et al.*, 2011) the muropeptide pattern of the wild type *S. carnosus* TM300 strain, the isogenic Δ*rodA* mutant as well as the complemented strain were investigated. The muropeptide pattern of the Δ*rod*A mutant (red) differed from the one of *S. carnosus* TM300 WT (green). On first sight the pattern was shifted to longer retention times in the mutant. Additionally, peaks were not that sharp but appeared broad and blurred. Repression of the expression of *rod*A in the complementation strain (orange) lead to the exact same pattern as in the mutant. The complemented mutant (blue) is intermediate between *S. carnosus* TM300 and the Δ*rodA* mutant muropeptide pattern. This showed that the differences in the muropeptide pattern were indeed caused by the absence of RodA.

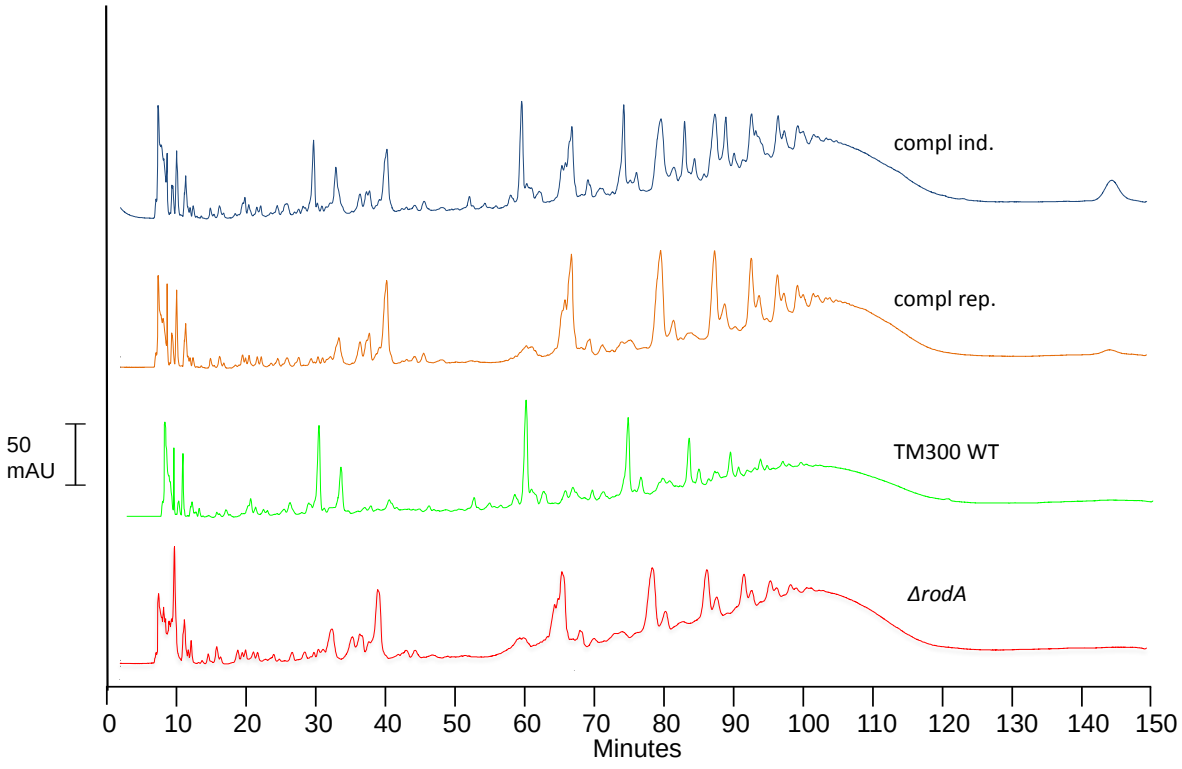


**Figure 20: HPLC analysis of peptidoglycan**
Peptidoglycan was isolated, digested by mutanolysin into muropeptides, and separated by HPLC. The muropeptide pattern of the Δ*rod*A mutant (red, Δ*rodA*) was altered compared to the wild type TM300 strain (green, TM300 WT). The complemented strain (blue, Compl. ind) showed an intermediate state. The repressed complementation (orange; Compl. rep) was the same as the mutant pattern.

As the muropeptide pattern of *S. carnosus* TM300 (WT) was different from the muropeptide patterns published for *S. aureus* strains (de Jonge *et al.*, 1992; Bertsche, U. *et al.*, 2011; Bertsche, U. *et al.*, 2013) *S. carnosus* was directly compared with S. aureus SA113. Indeed it was observed that the muropeptide pattern of *S. aureus* (black) differed from the one of *S. carnosus* (green). On first sight, the Δ*rod*A mutant pattern (red) appeared to resemble *S. aureus* more than the one of *S. carnosus*. Therefore muropeptide peaks indicated by numbers were collected and further analyzed by mass spectrometry (MS).

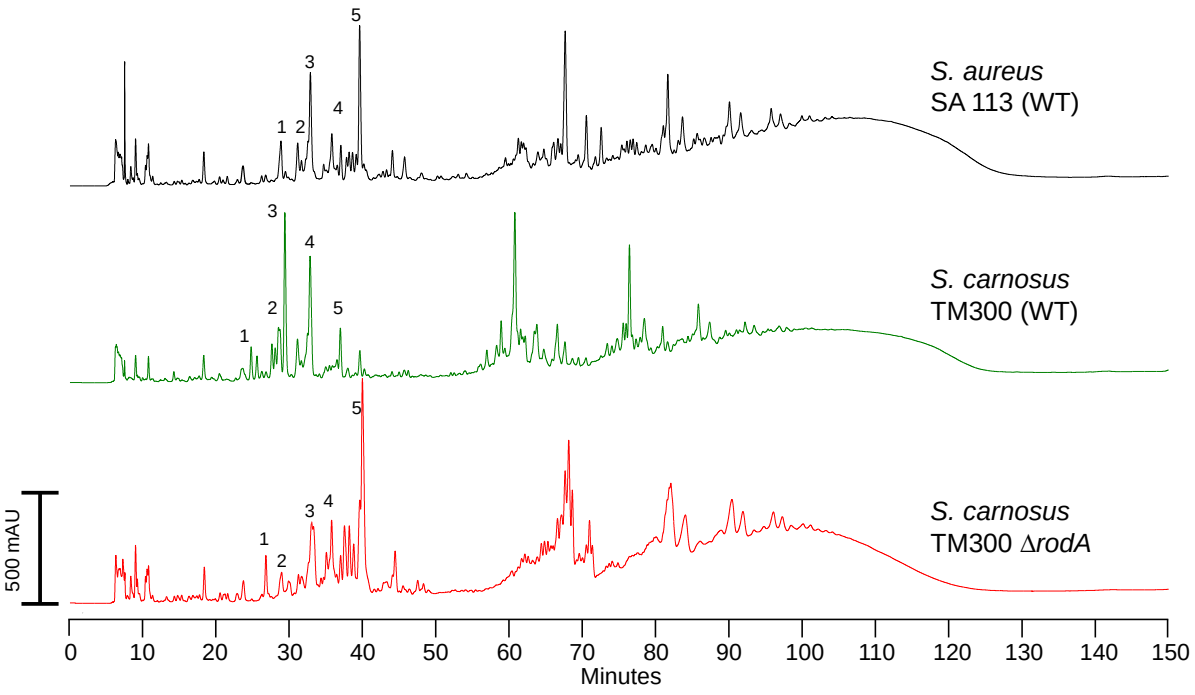


**Figure 21: Comparison between PG of *S. aureus* SA113 and *S. carnosus* TM300.**
The patterns of *S. aureus* SA 113 WT, *S. carnosus* TM300 WT and *S. carnosus* TM300 Δ*rod*A were compared. The indicated peaks that were collected to be analyzed further.

#### 6.5.6.2 Cross linkage

As the Δ*rod*A mutant had been observed to possess a cell wall that was leakier than the WT, this could be caused by less cross-links formed. Therefore, cross-linkage of both strains was calculated from the muropeptide patterns obtained by HPLC (Figure 21).The amount of cross-linkage in a cell wall is determined by the bonding that each individual muropeptide forms with their neighboring molecules. So the more muropeptides are bond to each other the higher is the cross-linkage. In staphylococci the particular bond that is formed between the non-terminal D-Ala and D-Gly of the adjacent interpeptide bridge. Cross linkage was calculated as follows by summing up ½ dimers + 2/3 trimers+ 9/10 multimers (Stranden *et al.*, 1997). The results given are the mean values of 5 independent experiments.

**Tab. 17: Crosslinkage of *S. carnosus***

| | **TM300 WT** | **Δ*rod*A** |
|---|---|---|
| **Ø** | 80.431 | 79.639 |
| **Standard diviation** | 0.552 | 0.196 |
| **Variance** | 0.406 | 0.051 |

The cross linkage is not different. 80.4 % is an expected value for *S. carnosus* TM300. The mutant value of 79.6 % is a bit lower, but fits quite well into the standard deviation of the WT. While previous experiments had suggested a weaker cell wall there was the possibility that the cross linkage would be lower but only a marginal decrease was observed (
Figure 15: Lysis assay).

## 6.6 *MASS SPECTROMETRY ANALYSIS OF PEPTIDOGLYCAN*

From the HPLC analysis it could be concluded that there is an alteration in the muropeptide composition of the *rod*A knock out strain. To further examine this, the major peaks from wild type *S. carnosus* TM300, the Δ*rod*A mutant and the *S. aureus* SA113 were collected and MS analysis was carried out by Andreas Kulik to determine the masses of the major molecules present in each peak. It turned out, that the muropeptide pattern of *S. carnosus* widely differs from the one of *S. aureus*. Even peaks with similar retention times had different masses, e.g. they are different. The masses were compared to already published masses of *S. aureus* (de Jonge *et al.*, 1992). Indeed, masses were found that indicated an incorporation of serine into the peptidoglycan of the Δ*rod*A mutant. An overview of the found masses is given in Tab. 19. For understanding the nomenclature seeFigure 22.

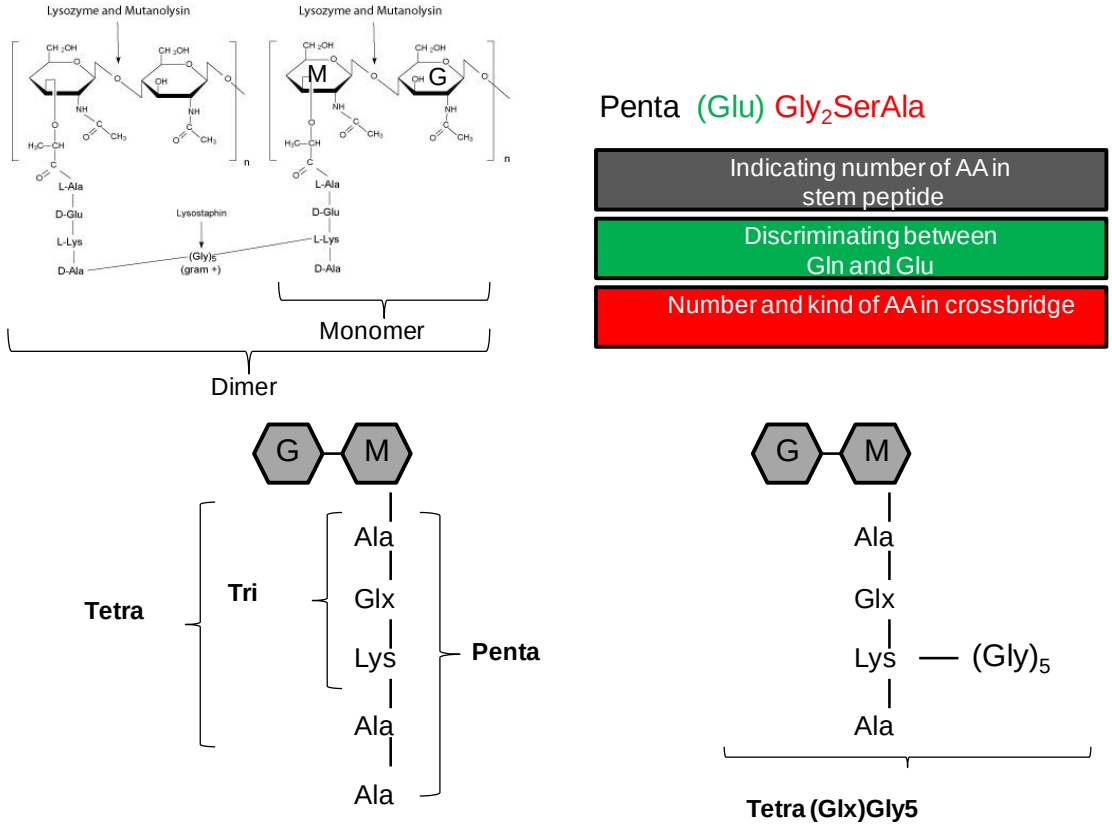


**Figure 22: Nomenclature of muropeptide molecules**

Tab. 18: Masses and molecules calculated by MS analysis

| Δ*rod*A | | Mass determined | Mass calculated | Hypothetical molecule | TM300 WT | | Mass determined | Mass calculated | Hypothetical molecule | SA113 WT | | Mass determined | Mass calculated | Hypothetical molecule |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | | 967,6 | 967.0151 | Tetra(Gln)Ala | **1** | | 882,6 | 882,408 | Tri(Gln)Gly | | | | | |
| | | | 967.0151 | Penta(Gln) | **2** | | 1053,6 | 1053,468 | Tri(Gln)Gly4 | **1** | **a** | 1010,6 | 1010,4783 | Tetra(Gln)Gly2 |
| **2** | **a** | 1010,6 | 1010,4783 | Tetra(Gln)Gly2 | | | 1110,6 | 1110,488 | Tri(Gln)Gly5 | | **b** | 1067,6 | 1067,4983 | Tetra(Gln)Gly3 |
| | **b** | 1067,6 | 1067,4983 | Tetra(Gln)Gly3 | **3** | **a** | 1111,6 | 1111,4723 | Tri(Glu)Gly5 | **2** | **a** | 1238,7 | 1238,5626 | Tetra(Gln)Gly6 |
| | **c** | 1211,7 | 1211,5597 | Tetra(Gln)SerGly4 | | **b** | 1238,7 | 1238,5626 | Tetra(Gln)Gly6 | | | | | |
| **3** | **a** | 1182,7 | 1182,5252 | Tetra(Glu)Gly5 | | **c** | 1295,8 | 1295,5841 | Tetra(Gln)Gly7 | | **b** | 1295,8 | 1295,5841 | Tetra(Gln)Gly7 |
| | **b** | 1325,8 | 1325,600 | Tetra(Gln)SerGly6 | | **d** | 1352,8 | 1352,604 | Tetra(Gln)Gly8 | **3** | | 1352,8 | 1352,604 | Tetra(Gln)Gly8 |
| **4** | | 1024,6 | 1024.0669 | Tetra(Gln)AlaGly | **4** | | 1181,6 | 1181,5412 | Tetra(Gln)Gly5 | | | | | |
| | | | 1024.0669 | Penta(Gln)Gly | | | | | | | | | | |
| **5** | **a** | 1025,6 | 1025.0517 | Penta(Glu)Gly | **5** | | 1252,8 | 1252,5768 | Penta(Gln)Gly5 | | | | | |
| | | | 1025.0517 | Tetra(Glu)AlaGly | | | | | | **4** | | 1024,6 | 1024,4939 | Penta(Gln)Gly |
| | **b** | 1182,6 | 1182,5252 | Tetra(Glu)Gly5 | | | | | | | | | | |
| | **c** | 1081,6 | 1081,5139 | Penta(Gln)Gly2 | | | | | | **5** | | 1252,8 | 1252,5768 | Penta(Gln)Gly5 |
| | **d** | 1168,7 | 1168,554 | Penta(Gln)Ser Gly2 | | | | | | | | | | |
| | **e** | 1211,7 | 1211,5597 | Tetra(Gln)SerGly4 | | | | | | | | | | |
| | **f** | 1282,7 | 1282,594 | Penta(Gln)SerGly 4 | | | | | | | | | | |

## 6.7 *MaXis MS-analysis*

Serine incorporation into the peptidoglycan stem peptide caused by a deletion mutant should be treated carefully, so it was decided to further verify the masses with a more sensitive MS-method which would also gave away the sum formula of the molecule. This analysis was performed by Luise Hoffmann of the AK Grond, Chemistry department.

**Table 20: Mass and sum formula of muropeptides analyzed by MaXis**

| **Monomer TM300 WT pos. mode Rt = 45.0 min, m/z** | **Ion formula Accuracy in ppm** | | **Δ*rod*A mutant pos. mode Rt = 51.6 min, m/z** | **Ion formula Accuracy in ppm** |
|---|---|---|---|---|
| **Tri(Gln)Gly$_5$** | **$[C_{43}H_{75}N_{12}O_{22}]^+$** | | **Penta(Gln)SerGly$_4$** | **$[C_{50}H_{87}N_{14}O_{25}]^+$** |
| 1111.5092 = $[M+H]^+$ cal | 1.9 ppm | | 1283.5955$^+$ = $[M+H]^+$ *cal. 1283.5961* | 0.5 ppm |
| 556.2590 = $[M+2H]^{2+}$ cal | 0.5 ppm | | 642.3025$^{2+}$ $[M+2H]^{2+}$ *cal. 642.3017* | 1.3 ppm |
| 567.2488 = $[M+H+Na]^{2+}$ cal | | | 653.2923 = $[M+H+Na]^{2+}$ *cal. 653.2927* | 0.6 ppm |
| 1133.4889 = $[M+Na]^+$ cal | 3.9 ppm (low intensity) | | 1305.5732 = $[M+Na]^+$ *cal. 1305.5781* | 3.7 ppm (low intensity) |
| | | | 664,2827 = $[M+2\ Na]^{2+}$ *cal. 664.2836* | 1.4 ppm |
| | | | **Penta(Gln)Ser$_2$Gly$_3$** | **$[C_{51}H_{89}N_{14}O_{26}]^+$** |
| | | | 1313.6043 = $[M+H]^+$ *cal. 1313.6067* | 1.8 ppm, |
| | | | 1335.5841 = $[M+Na]^+$ *cal. 1335,5886* | 3.4 ppm (low intensity) |
| | | | 657.3071 = $[M+2H]^{2+}$ cal. 657.3070 | 0.2 ppm |
| | | | 668,2970 = $[M+H+Na]^{2+}$ *cal. 668.2980* | 1.4 ppm |
| | | | 679,2874 = $[M+2\ Na]^{2+}$ *cal*. 679.2889 | 2.3 ppm (low intensity) |

Three different molecules could be identified with great accuracy. Tri(Gln)$Gly_5$ for the major monomer peak of the *S. carnosus* TM300 WT was found and two masses from the major monomer peak of the Δ*rod*A mutant were identified as Penta(Gln)$SerGly_4$ and Penta(Gln)$Ser_2Gly_3$. The identification of the muropeptides that were containing serine was successful for the Δ*rod*A mutant. There was no mass detectable in the WT strain that could possibly contain serine.

## 6.8 *AMINO ACID REACTION*

To determine the amino acid composition of the peptidoglycan the CW of the strains *S. carnosus* TM300 Wt, *S. carnosus* Δ*rod*A and the *S. aureus* SA113 was isolated at OD 0,7. By preparative HPLC the cell wall fractions were divided into three parts; a monomeric, a dimeric and a multimeric fraction. The lyophilized CW was hydrolyzed by adding HCl. For detection OPA (ortho-phthal aldehyde) derivatisation was performed in the injection-needle of the HPLC as pre-column derivatisation. The amino acids of the peptide and the interpeptide bridge could be determined both quantitatively and qualitatively by the height of the peaks and their retention time compared to a standard. A commercial available standard by GRACE containing 18 different amino acids was used and a self-made standard only containing the five amino acids glutamic acid, serine, glycine, alanine and lysine was also prepared.

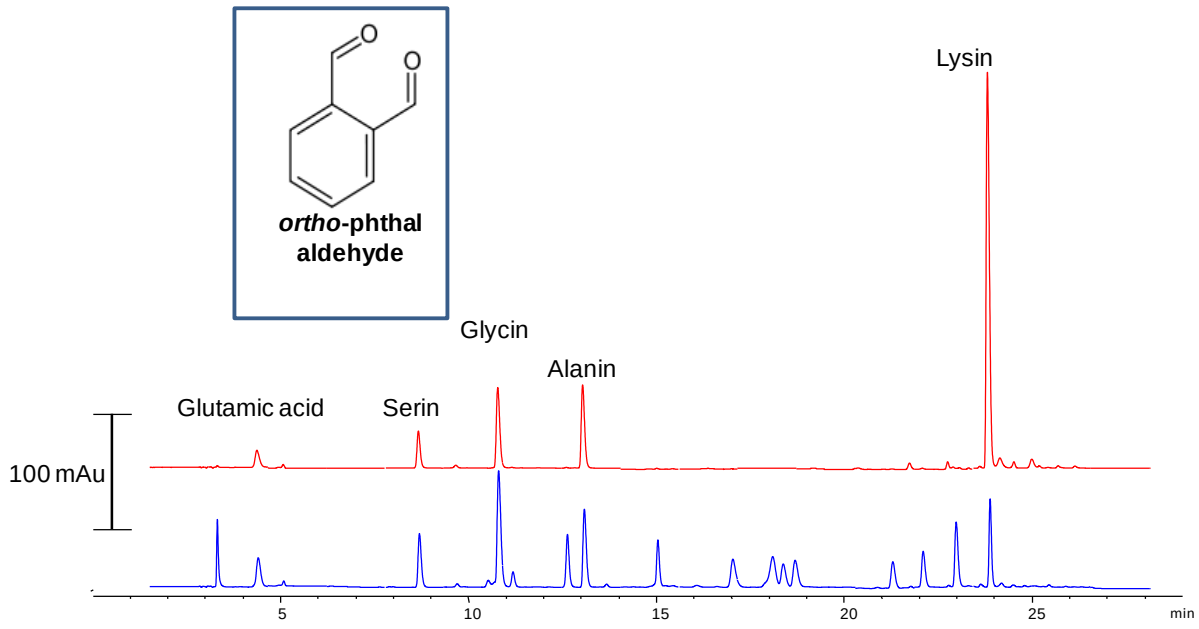


**Figure 23: Standard of the amino acid reaction with OPA:**
Commercial and self-made standard of the amino acid after pre-column derivatisation with ortho-phthalaldehyde and separation via HPLC. The commercial available standard (blue) contained 18 different amino acids while the self-made standard (red) only contained five amino acids which were thought to be present in the peptidoglycan. In the upper left blue box the structure of OPA is shown.

### 6.8.1 *AMINO ACID COMPOSITION OF THE PEPTIDOGLYCAN*

As calculated from the MS analysis data the Δ*rodA* mutant contained muropeptides which carry serine in their peptide moiety and several of them lacked the amidated glutamate at position two of the stem peptide. The peptide moiety of the muropeptides was hydrolysed and the amino acid composition was determined by HPLC using precolumn derivatization by ortho-phthaldialdehyd (OPA). By this method it is not possible to distinguish between glutamate and glutamine, as the latter loses its amino group during hydrolysis. The retention times of the found amino acids were very different, so they could be easily identified by comparison to the self-made standard.

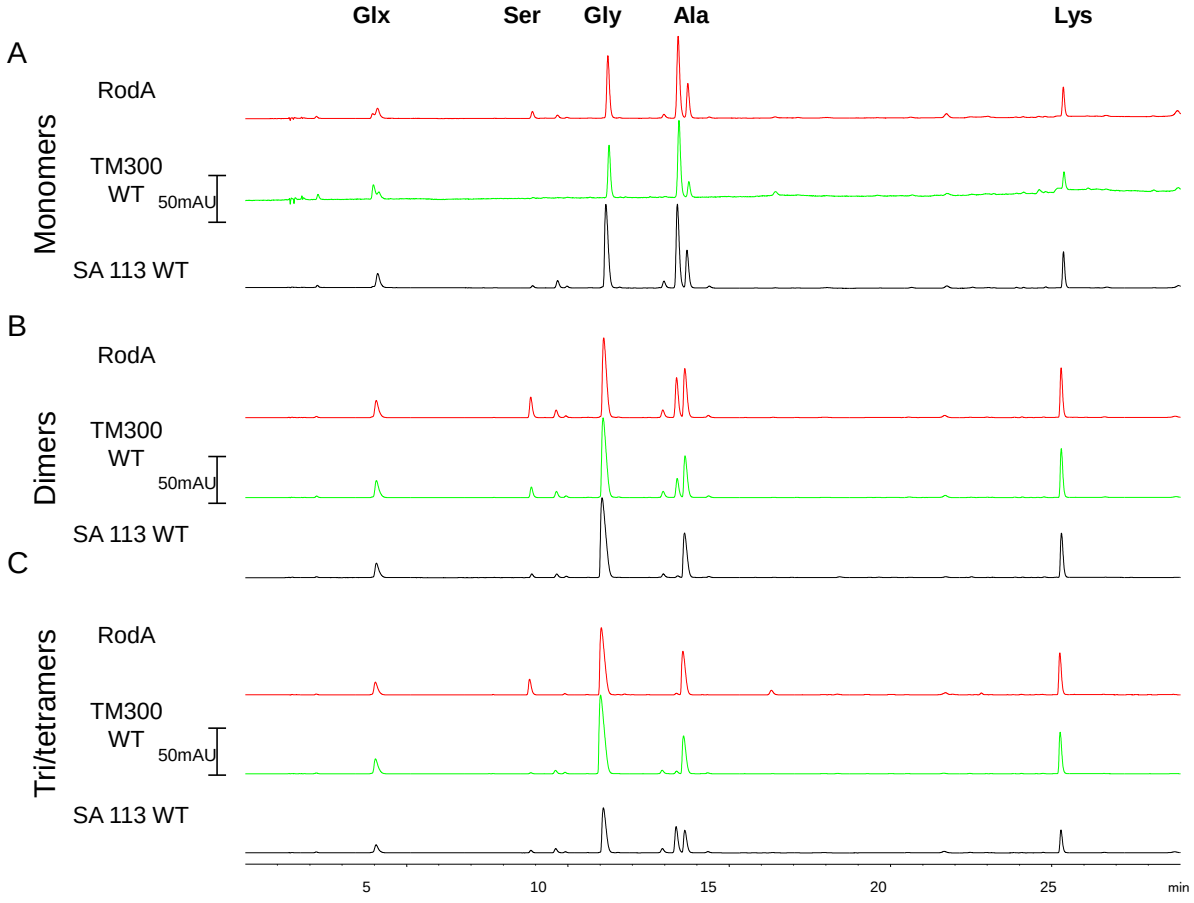


**Figure 24: Amino acid analysis of the different fractions of the mutanolysin digested peptidoglycan :**
*S. carnosus* TM300 WT (green) and Δ*rod*A mutant (red) as well as *S. aureus* SA 113 WT (pink) peptidoglycan was isolated and digested with mutanolysin and separated via HPLC. (A) Monomeric, (B) dimeric and (C) tri/tetrameric fractions were isolated and an amino acid reaction was performed with every single fraction.

The amount of each amino acid was calculated in comparison to glutamate/ine (called Glx), which was set to 1.0 (Tab. 19 ). In *S. aureus* WT serine was not found and *S. carnosus* WT the amino acid serine was found but only in small amount. However, the amount of serine in the Δ*rodA* mutant was increased by 250% compared to *S. carnosus* TM300. This was also reflected by a concomitant decrease of glycine, suggesting that serine is incorporated into the interpeptide bridge.

**Tab. 19: Amino acid distribution per strain and peptidoglycan fraction**
The amount of each amino acid was calculated in comparison to Glx (can stand for glutamic acid or glutamine) which was set to 1. The average amount was calculated from all three fractions.

| | | Glx | | Serine | | Glycine | | L-Alanine | | D-Alanine | | Lysine | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Amount | Ø | Amount | Ø | Amount | Ø | Amount | Ø | Amount | Ø | Amount | Ø |
| | Mono | 1.0 | | 0.1 | | 5.4 | | 4.7 | | 2.0 | | 0.7 | |
| SA113 | Di | 1.0 | 1.0 | 0.2 | 0.2 | 6.8 | 6.0 | 0.1 | 2.4 | 2.9 | 2.4 | 1.0 | 0.9 |
| | Tri-Tet | 1.0 | | 0.2 | | 5.7 | | 2.5 | | 2.3 | | 0.9 | |
| | Mono | 1.0 | | 0.0 | | 5.7 | | 8.9 | | 1.7 | | 1.4 | |
| TM300 | Di | 1.0 | 1.0 | 0.4 | 0.2 | 5.1 | 5.7 | 0.8 | 3.3 | 2.0 | 2.0 | 0.9 | 1.0 |
| | Tri-Tet | 1.0 | | 0.0 | | 6.5 | | 0.1 | | 2.2 | | 0.9 | |
| | Mono | 1.0 | | 0.5 | | 4.4 | | 6.1 | | 2.4 | | 0.9 | |
| Δ*rodA* | Di | 1.0 | 1.0 | 0.8 | 0.7 | 4.6 | 5.0 | 1.7 | 2.6 | 2.4 | 2.7 | 0.9 | 0.9 |
| | Tri-Tet | 1.0 | | 0.9 | | 6.1 | | 0.1 | | 3.2 | | 1.1 | |

## 6.9 *Summary of the ΔFtsW1 and ΔFtsW2 mutants*

The gene replacements of *fts*W1 and *fts*W2 respectively were examined concerning growth (Fig.: 25) and muropeptide pattern (Fig.:26). Differences to the wild-type *S. carnosus* TM300 could not be observed but however to the Δ*rod*A mutant.

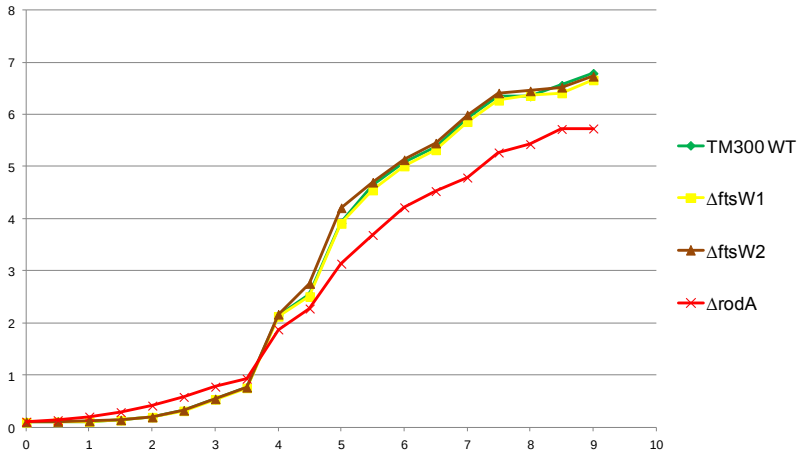


**Figure 25: Growth curve SEDS single mutants and Wild type**
*S. carnosus* TM300 WT and all three single mutants of the SEDS proteins were compared concerning growth. WT (green), Δ*fts*W1 (yellow) and Δ*fst*W2 (brown) showed a very similar growth rate while the Δ*rod*A (red) mutant differed in the beginning as well as at the end.

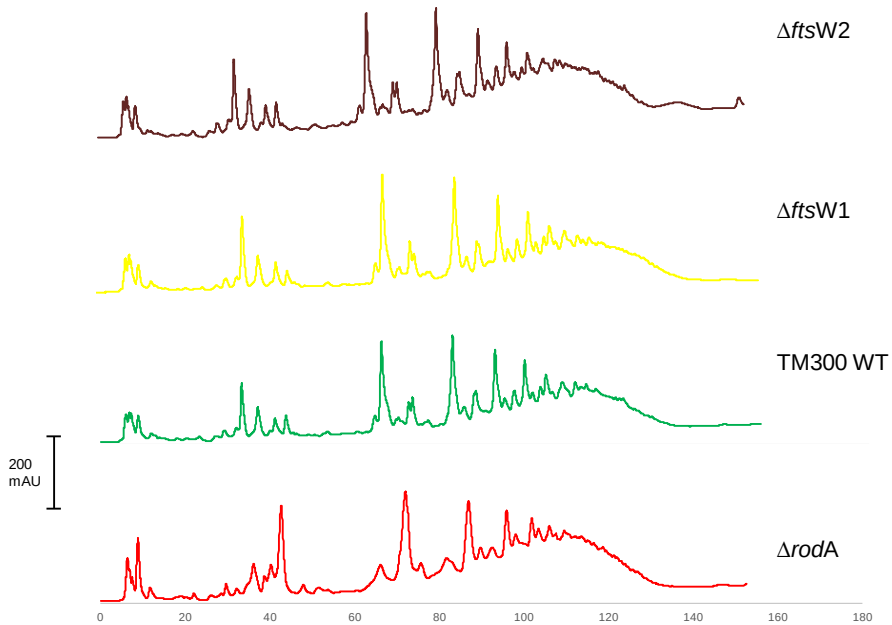


**Figure 26: HPLC analysis of all SEDS mutant strains**
The muropeptide pattern of the three SEDS protein single mutant strains was compared by HPLC. The mutant strains of *fts*W1 (yellow) and *fts*W2 (brown) did not show any differences to the wild type strain (green). As comparison the altered muropeptide pattern of the Δ*rod*A mutant (red) is displayed.

# 7 DISCUSSION

## 7.1 *SEDS PROTEINS*

The abbreviation SEDS stands for Shape Elongation Division and Sporulation. Proteins associated with this family share sequential and functional similarity. They are integral membrane proteins that are present in all cell wall-containing bacteria (Ikeda *et al.*, 1989) and are highly conserved. So far their function was elucidated by mutations affecting cell shape, e.g. turning rods into spheres (*rod* mutations) (Begg and Donachie, 1985) or filament formation.

In several organisms the SEDS protein encoding genes are co-localized on the chromosome with a gene for a monofunctional PBP. Using KEGG (Kyoto Encyclopedia of Genes and Genomes) the chromosomal organization of several frequently used research organisms as well as of all staphylococcal strains sequenced so far was checked. In *Escherichia coli* K-12 MG1655, in *Caulobacter crescentus* NA1000, in *Mycobacterium tuberculosum* H37Rv as well as in *Corynebacterium glutamicum* ATCC 13032 (Bielefeld) there is indeed a co-localization of the gene for the SEDS-protein FtsW with the cell division specific penicillin-binding protein FtsI (PBP3) and the cell wall synthesis proteins MraY and MurG. In addition, in all these strains the RodA gene is a direct neighbor of a PBP gene. However, in *Bacillus subtilis* 168 while there is a co-localization of the gene for the SEDS protein SpoVE with the ones for PBP2B and SpoVD (two PBPs), MraY, and MurG, none of the other seven synthesizing PBP genes is a neighbor of the RodA gene. In *Streptococcus pneumonia* (R6) as well as in all *Staphylococcus* strains whose sequence is available so far, none of the SEDS protein genes is in the vicinity of a PBP gene. But in all staphylococcal genomes checked, *mraY* was always located near a *pbp* gene (one to seven genes apart).

In principle, one SEDS gene should be enough for a coccoidal bacterium to survive, and in *S. aureus rodA* is considered as non-essential (Chaudhuri *et al.*, 2009). However, so far a *rodA* deletion mutant in *S. aureus* SA113 could not be created. *S. warneri* a recently sequenced and annotated staphylococcal strain was checked for *rod*A and only a truncated variant of *rod*A was found (Cheng *et al.*, 2013). But with the first 4 transmembrane domains missing the protein should not be functional, according to (Mohammadi et al., 2014) . With six out of ten transmembrane domains still there

and the big extracellular loop still existing the fundamental function of the protein could very well still be intact but according to recent studies (Mohammadi *et al.*, 2014) it is most likely not, because the missing domains seems to the domains important for the flipping function at least for the FtsW of *E. coli*.

*E. coli* as well as other kinds of bacteria encodes a closely related pair of specialized proteins-PBP3 and FtsW - that function in cell division. In *E. coli* the activities of these two dual-gene systems appear to alternate. The RodA/PBP2 system dominates in case of elongation growth. When cell division appears the FtsW/PBP3 system comes into play (Daniel and Errington, 2003). Growth and division works differently in staphylococci so it is not remarkable that they are no known dual-gene systems. All the more so since not only one but three different SEDS-proteins were found in *S. carnosus* while all other sequenced staphylococcal strains possess only two variants, one copy for *fstW* and one for *rodA*. Bacteria of the genus *Staphyloccus* divide in a 90° angle. They do not undergo elongational growth. They do not sporulate and their shape as cocci cannot be changed as it is the simplest form generally (Lleo *et al.*, 1990). With three out of four aspects of the SEDS proteins not applying for staphylococci we expected an influence on growth by division if we disabled one or more SEDS proteins. Recently FtsW was identified as the Lipid II flippase for *E. coli* (Mohammadi et al., 2011*).* However, the knock-out mutants created in both of the *ftsW* loci of *S. carnosus* did not show any phenotype at all leading to the suggestion that the loss of one copy can be complemented by the other one or by *rod*A. Only deletion of *rod*A resulted in a mutant with an obvious phenotype. As we were unable to generate double mutants of the SEDS proteins in any combination possible it is tempting to conclude that although non-essential on their own at least two functional SEDS proteins are necessary for the viability of *S. carnosus*. One could envision, that the two SEDS proteins take turns in defining the next division plane.

## 7.2 *Effects of the* rod*A deletion on growth and viability*

The deletion of *rodA* in *S. carnosus* created a viable mutant showing a distinct phenotype. First to notice was a growth defect, which was expected from the theory of SEDS proteins. Being a potential flippase for Lipid II it is astonishing that the mutant almost completely skipped the lag phase but started to grow faster in the beginning. With no clear explanation to this observation one could take into consideration that whatever protein it is that takes over the function of RodA during growth is produced differently whether due to an increased expression in the absence of *rodA* or a different pattern in terms of time and growth phase in general. After 4 to 6 hours of growth the OD of both strains was the same. During further growth the wild type strain passed the OD values of the mutant ending up significantly higher. To have a closer look dilution series were plated during the growth which revealed that the declined OD might be due more to dying mutant cells than slowed down growth, because already at a time point when the OD of both strains was about equal (5 h) the wild type formed colonies with a dilution of a factor ten higher than the mutant. This discrepancy even grew as time progressed (Figure 14: Growth curve and CFU-determination). While it is not obvious why the mutant grew stronger than the wild type during the early growth phase the decreased CFU explained the decline during the mid and late exponential phase. The CFU decrease was not seen when the mutant strain was complemented by the expression of *rod*A.

To complement the deletion of *rodA* the low copy vector pCX was used to control the expression of the gene by adding sugars to the medium (Wieland *et al.*, 1995). Xylose induces the expression while glucose blocks it due to the effects of the catabolite repression. Despite all the expression control the complementation did not fully restore the growth to wild type level in stationary phase nor did the repressed *rodA* expression mimic the mutant phenotype exactly, but an effect in both ways was clearly observable. Especially the reduced lag phase of the mutant was also present when *rod*A expression was repressed. In contrary this was not observed anymore when gene expression was induced by xylose. An incomplete repression is not uncommon in these kinds of scenarios and most likely due to the vector still being leaky to some extent. On the other side the induction also does not seems to be complete. The reason could be a simple gene-doses effect because the number of copies is not the same as in a wild type situation. A more complicated regulation mechanism working in *cis* or a regulatory element the plasmid is lacking are further possible explanations.

As it was shown by bacterial-two-hybrid experiments that *rodA* is a part of the peptidoglycan biosynthesis machinery in staphylococci it interacts with PBPs for example. The stability of the cell wall could be influenced by its absence whether RodA is acting one of the Lipid II flippases or interaction partner for other enzymes crucial for peptidoglycan biosynthesis (Deibert, 2011; Steele *et al.*, 2011; Hoovestadt, 2014).

## 7.3 *INSTABLE RODA MUTANT CELLS*

According to the growth curve and CFU experiments it could be shown that the mutant did lyse earlier than the wild type. This was also observed in incubation flasks, when a culture of both strains was held at room temperature for more than 48 h without shaking (

Figure 15). The cells sedimented at the bottom and the medium became clearer. Upon resuspension by hand the wild type cells floated again in the medium. The mutant cells instead formed of viscous phlegm at the bottom of the flasks that could not be resuspended. Once observed by chance it could be repeated sequentially. A DNase treatment with following resuspension resolved the phlegm, indicating that the viscous cell mass was indeed cells clumped together in a DNA mash which leaked out of broken cells. For the wild type strain, this state of degeneration did also appear but with a delay of at least 24 h which speaks in favor of an instable cell wall of the mutant cells and a natural reaction of dying cells instead of an increased autolysis which would be resolved faster (Tobin *et al.*, 1994). Because the DNase treatment was able to dissolve the cell mass it is sure, that the clumps are no kind of biofilm formation (which does not exist in *S. carnosus)* or other sticky meshwork, but are the product of DNA leaking out of the cells.

## 7.4 *LOCALISATION OF PEPTIDOGLYCAN BIOSYNTHESIS*

An instable cell wall could be caused by alterations of peptidoglycan (PG) biosynthesis during cell division. To determine sites of active peptidoglycan biosynthesis in the Δ*rodA* mutant compared to *S. carnosus* TM300 WT, cells were labeled with fluorescent vancomycin [Van-FL]. In order to stain only newly synthesized peptidoglycan, cells were grown in the presence of D-serine, which is incorporated instead of D-alanine on position five of the stem peptide and prevents binding of Van-FL. When the cells were grown again without D-serine, the newly synthesized murein contained D-Ala-D-Ala

residues again, which got labeled by Van-FL and could by visualized by fluorescence microscopy.

While in the wild type strain Van-FL stained the septa and to a lower amount the cell walls, only weak spots of fluorescence and very faint septa in the Δ*rodA* mutant were observed (Figure 17, Figure 18). This showed that peptidoglycan biosynthesis was much slower in the mutant strain than in wild type *S. carnosus* as less newly synthesized cell wall without D-serine was incorporated during the pulse. As expected also the glucose repressed complemented mutant showed only weak septal staining. In contrast the complemented mutant in which the MS analysis *rodA* gene was expressed by xylose showed septal staining almost at the wild type level. So RodA is clearly involved in the peptidoglycan synthesis of *S. carnosus*.

A similar labeling pattern was reported for *Bacillus subtilis*, when grown in the presence of a mixture of D-amino acids, and in *Bacillus subtilis* cells in stationary phase (Lam *et al.*, 2009). This supports the results from the amino acid analysis (see below), where an increase in D-Ala, which indicated stress, was found. This stress indication is also supported by the observed clustering of cells due to DNA webs because of an enhanced lysis. Taken together the Δ*rodA* mutant cells are under stress which affects the bacterial cell wall.

## 7.5 *PEPTIDOGLYCAN COMPOSITION IS ALTERED*

As all the previous experiments hinted for alterations of the cell wall, the muropeptide pattern of the wild type *S. carnosus* TM300 strain, the isogenic Δ*rodA* mutant as well as the complemented strain was investigated and compared in between the *S. carnosus* strains and to the known pattern of *S. aureus* (Figure 20 and Figure 21). It was observed that the muropeptide pattern of *S. aureus* differs from the one of *S. carnosus.* On first sight, the Δ*rodA* mutant pattern with its shifted retention times appeared to resemble *S. aureus* more than the one of *S. carnosus* but the peaks are blurred. However, MS analysis showed that even though peaks had identical retention times, they had different masses, i.e. they are different. The complemented mutant was intermediate between *S. carnosus* TM300 and the Δ*rodA* mutant. This can be explained either by the fact that peptidoglycan was isolated at $OD_{578}$~0.7, which was about the time the complementation effect started and or by a gene-dosage effect that occured after external induction of *rodA*.

In the past the investigation of sets of different strains of cell wall antibiotic resistant mutants and corresponding sensitive parental strains (Bertsche, Ute *et al.*, 2011; Göhring *et al.*, 2011) have never lead to such drastic differences in the muropeptide pattern, indicating that lack of RodA activity (or presence) is not comparable to known antibiotic effects.

## 7.6 *AMINO ACID COMPOSITION OF THE PEPTIDOGLYCAN*

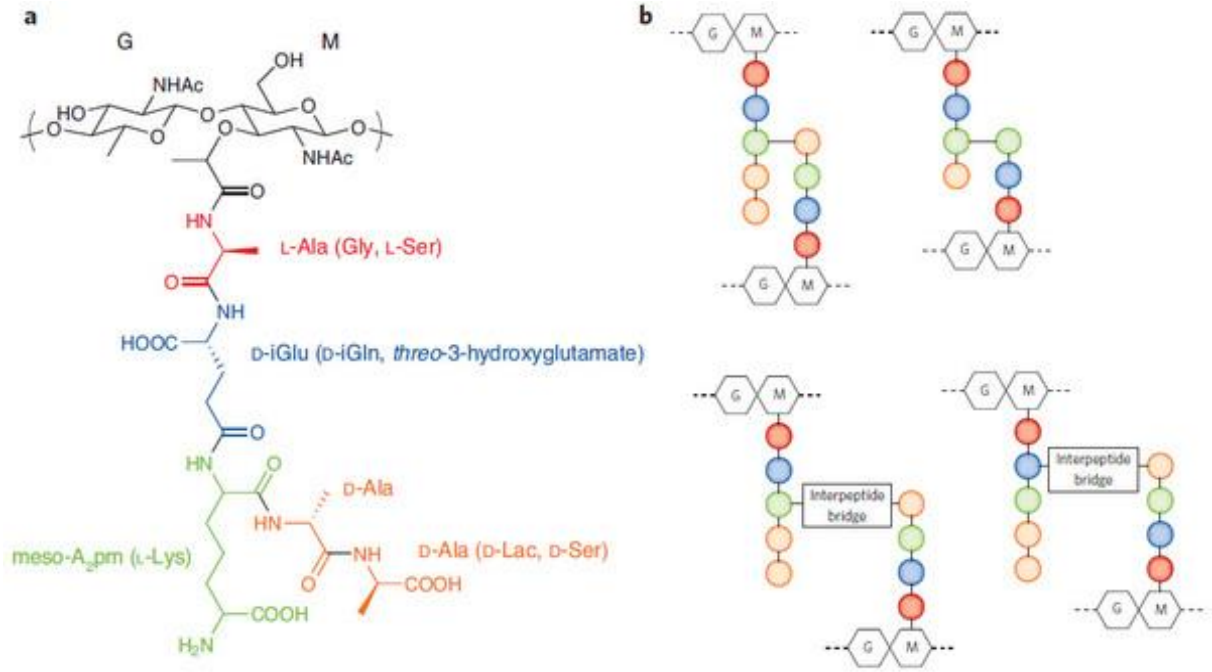


**Figure 27 Variations peptide composition of peptidoglycan (Vollmer, 2012)**

As calculated from the MS analysis data the Δ*rodA* mutant contained muropeptides which carry serine in their peptide moiety and several of them lack the amidated glutamate at position two of the stem peptide. Therefore the amino acid composition of the peptide moieties was determined by HPLC using precolumn derivatization by ortho-phthaldialdehyd (OPA).

Indeed, the amount of serine in the Δ*rodA* mutant increased by 250% compared to *S. carnosus* TM300 WT. This was also reflected by a concomitant decrease of glycine, suggesting that serine is incorporated into the interpeptide bridge. As the amount of serine is usually underestimated (10 to 40%) by the method used this strongly indicates an even higher amount of serine. An amount of 0.09 serine residues per 1 Glx residue has been reported for *S. carnosus* TM300 before, meaning that a small amount of serine is natively incorporated into its interpeptide bridge (Thumm and Götz, 1997).

This indicates that Lipid II with serine is still a substrate for the murein synthesizing penicillin-binding proteins (PBPs), but the PBP activity seems to be slowed down. This could also explain, why the overall cross-linkage of the mutant is not significantly altered, like one would suspect by altering the substrate for the transpeptidases from a D-Ala-D-Ala to a D-Ala-D-Ser. In the VRSA strain Mu50 the amounts of glutamine-non-amidated muropeptides were increased and a decreased cross-linking of peptidoglycan with a greatly decreased dimer/monomer ratio of muropeptides were found (Hanaki *et al.*, 1998) although in this case there is no serine incoorporation.

The amount of glycine and L-alanine also seems to be rather high on the first sight. However, as peptidoglycan is constantly remodeled, glycine residues from former cross-links are often retained. This has already been reported twenty years ago for *S. aureus*. The same paper also shows that there is a certain amount of L-alanine incorporated into the glycine interpeptide bridge of *S. aureus* (de Jonge *et al.*, 1992). In general the stem peptide composition can be quite flexible and must not only consist of L-Ala-D-Glx-L-Lys-D-Ala-D-Ala, indicating that the interpeptide bridge must not strictly consist of five glycine. In addition it was shown for the SEDS protein FtsW of *E. coli* that also the flipping mechanism can tolerate much bigger variations (Mohammadi *et al.*, 2014).

However, the increased incorporation of serine into the interpeptide bridge in the absence of *rod*A was an astonishing observation. This could be a general response to cell wall stress in the *S. carnosus* cells. The wild type cells have a small amount of serine in the cell wall, too. The idea that RodA provides selectivity for the flipped Lipid II may be ruled out on the one hand by the peptidoglycan labeling experiments, where also in wild type cells serine residues in the stem peptide are found only by providing them in the medium (working principle of the vancomycin labeling) and on the other hand from the earlier mentioned tolerance of the flippase activity of the FtsW protein (Mohammadi *et al.*, 2014). The data of this study cannot fully explain where the serine residues are incorporated into the peptidoglycan whether it is instead of alanine in the stem peptide or instead of glycine in the interpeptide bridge. Although muropeptide with a full penta stem-peptide and additional serine see it incorporated in the interpeptide bridge, a well-known resistance mechanism against lysostaphin (Thumm and Götz, 1997). Therefore I propose incorporation into the interpeptide bridge, but I do not know which of the glycine residues are replaced

Regarding the shifted muropeptide pattern in Figure 20 there is a possible explanation given by the in depth MaXis analysis of the single major muropeptide peaks: peak 3 of *S. carnosus* TM300 and peak 5 of the Δ*rodA* mutant. The first mass spectrometric analysis that had been performedby the IonTrap method had already revealed several Tri-peptides in peaks 2 and 3 of wild type strain while the Δ*rodA* mutant peaks consisted of Penta- or Tetra-peptides only. With the TOF analysis of the MaXis instrument Tri(Gln)$Gly_5$ could be detected with an accuracy of 0.5 ppm for peak 3 of the *S. carnosus* TM300 strain. The corresponding muropeptides in the Δ*rodA* mutant peak 5 were Penta(Gln)Ser$Gly_4$ and Penta(Gln)$Ser_2$Gly3, indicating that in the mutant the stem peptide gets not processed, as it was seen in the wild type. The enzyme responsible for this processing must be an L,D-carboxypeptidase. Carboxypeptidases are enzymes that can hydrolyze peptide bonds at the carboxy-terminal end of a protein or a peptide. There are 3 annotated carboxypeptidases in *S. carnosus* TM300 namely both *pbp4* genes (Sca_0291and Sca_2445), which are both D-alanyl-D-alanine carboxypeptidases and therefore ruled out. And the Sca_0214 gene, an L,D-carboxypeptidase which is yet to be examined. The only other staphylococcus known to possess on orthologue of this latter enzyme is *S. pseudointermidius.* One could think of an inhibition of the carboxypeptidase activity by the incorporated serine of the muropeptides maybe due to sterical hindrance of the enzyme substrate complex, resulting in unprocessed Tetra- and Penta-Muropeptides in the Δ*rodA* mutant.

# 8 CONCLUSION

In general *rodA* deletion is possible in *S. carnosus* but causes cell stress, which results in increased lysis. The cell tries to counteract by incorporation of serine into the interpeptide bridge. However, the resulting serine containing muropetides are no longer a substrate for the L,D-carboxypeptidase, which seems to processes the muropeptides of the wild type *S carnosus* TM300 strain. In E*. coli* Tri-muropeptides are proposed to be the initiation point for cell division (Höltje, 1998). One could envision a similar role in *S. carnosus*, which would lead to cell stress when this processing is missing.

With the findings in the CFU/growth and the lysis experiments it is clear that the increased serine incorporation does not increase the cell wall stability. Rather it seems that the serine turns the Lipid II into a poorer substrate for the PBPs which would explain the slower and dispersed biosynthesis of the peptidoglycan with a slower division rate and higher lysis rate and therefore a slower growth rate. While the overall biosynthesis of the cell might suffer from the serine incorporation the turnover processes of the cell are maybe still working properly resulting in a higher lysis rate of the mutant.

It is very fascinating is how bacteria acquire, maintain, and modify their shapes during growth. Especially with the unique divisionpattern of *Staphyloccocci* where a 90° angle to the latest division plane is determined (Wheeler *et al.*, 2011). Maybe they alternate the division planes by an alternating localization of different SEDS proteins, but the fundamental process behind this as well as choosing and maintaining a specific shape is not understood. Of course adaption to the habitat and survival strategies will have a lot of influence but this cannot be the only determination factor since different bacterial species with different shapes are known to in the same environment. Maybe there is something more crucial behind the shape of bacteria.

# 9 OUTLOOK

## 9.1 *FUNCTIONAL ASSAY FOR A FLIPPASE*

It should be testified that this RodA protein of *S. carnosus* is a functional flippase and that its substrate is indeed Lipid II. A biochemical evidence of its functionality for translocation of lipid-linked peptidoglycan precursors would solidify the role of *rodA* during peptidoglycan biosynthesis (Mohammadi *et al.*, 2011).
A possible experiment was performed first by Mohammadi *et al.* in 2014 by using NBD-labelled Lipid II as a donor and tetramethylrhodamine cadaverine (TMR)-labelled vancomycin as an acceptor to generate a strong FRET (fluorescence resonance energy transfer) signal. Here, a potential flippase, FtsW, was overexpressed transgenically in *E. coli* and membrane vesicles were prepared. NBD-labelling on the outer leaflet could be quenched. So FRET signal could only be detected if a translocation from the inner to the outer leaflet has occurred. The labeled vancomycin specifically binds Lipid II (Breukink and de Kruijff, 2006). The overexpression of the target gene increased the FRET signal. The overexpression of control proteins (all of them integral membrane proteins) could not increase the signal.
Using this already established method should allow showing whether or not RodA is a functional flippase in *S. carnosus*. The same test has to be applied to both FtsW proteins.

## 9.2 *SELECTIVE FLIPPING*

With an established assay to test the functionality of flippases it would be a step forward to analyze how specific the flip-flop mechanism works by altering the Lipid II substrate with different amino acids. As it is known that there are a variety of different Lipid II molecules containing different amino acids in the stem-peptide we would see which structures are tolerable and which are not anymore. Also the sugar backbone could be modified as other flippases such as the MPD translocator in eukaryotic cells is able to flip lipids containing Mannose-phosphate-dolichol (Sanyal and Menon, 2010). For this we would need to modify the in vitro biosynthesis of peptidoglycan (Vinatier *et al.*, 2009). As a start the terminal amino acids could be exchanged as these are modifications already found in nature. This assay can also be used to test the substrate

specifity of the different PBPs and maybe antibiotics that target Lipid II or the reaction catalysed by the PBPs transglycolisation and transpeptidation (Bertsche, U. *et al.*, 2005)

## 9.3 *CROSS COMPLEMENTATION*

Complementation of the *rod*A mutant of *S. carnosus* could be tested with the RodA proteins of pathogenic staphylococcal species such as *S. aureus* and *S. epidermidis*. This will help to understand if the mechanism for growth and division is universal in staphylococci or if the there is something special about the *S. carnosus rod*A.

## 9.4 *STRUCTURE DETERMINATION*

The real structure of the muropeptides especially regarding where serine can be incorporated would help to understand the background of some of these missfunctioning processes that occur in the Δ *rod*A mutant. To solve these structures tandem mass spectrometry (MS-MS) analyses have to be performed. With the right set up it is possible to get a mass accuracy necessary to distinguish peptide elemental compositions (Clauser *et al.*, 1999). But so far the set-up was not optimal and until now the performance of a direct LC-MS of the whole muropeptdie patterm was not possible due to unfavorable buffer conditions. High salt buffers will increase the background signal in the MS or the LC separation suffers from the infavorable MS buffer e.g.

## 9.5 *ROLE OF THE L,D-CARBOXYPEPTIDASE*

One point which comes fast to the mind is the deletion of this L,D-carboxypeptidase in *S. carnosus* and the MS-analysis of the *S. pseudointermedies* peptidoglycan regarding an accumulation of muropeptides with only three amino acids in the stem peptide. The same is true for the localization of this enzyme especially if the localization is altered in the Δ*rod*A mutant.

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