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Function and regulation of *Staphylococcus aureus* wall teichoic acids and capsular polysaccharides

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ABSTRACT

Staphylococcus aureus produces different secondary cell wall glycopolymers such as wall teichoic acids (WTA) and capsular polysaccharides (CP). These structures play an important role in *S. aureus* colonization, pathogenesis and bacterial evasion of the host immune defences. To fulfil their diverse functions, biosynthesis of both glycopolymers has to be tightly controlled. Regulation of WTA biosynthesis and modification is only partially understood. The transcription factor MgrA and the two-component systems (TCS) Agr, GraRS, and ArlRS control WTA export, chain-length and modification. CP synthesis is determined by transcriptional and post-transcriptional regulatory circuits. On the transcriptional level expression of the *capA-P* operon is mainly driven by the alternative Sigma factor B and modulated by several transcriptional factors and TCS. Post-transcriptional mechanisms are in place to avoid conflict between precursor usage by the CP synthesis machinery and the synthesis machinery of other cell wall glycopolymers. The complex interplay of these regulatory systems determines the peculiar, strictly temporal expression of CP in the late growth phase and the high degree of phenotypic heterogeneity. Differential expression of CP, WTA and its modification systems during infection and colonisation are likely important for disease development, immune escape and survival within the host.

1. Introduction

The cell envelope of *S. aureus* consists of peptidoglycan, secondary cell wall glycopolymers and proteins. This multicomponent composition affords protection against cellular immunity and antibiotics and is involved in staphylococcal virulence. Two major secondary cell wall glycopolymers are wall teichoic acids (WTA) and capsular polysaccharides (CP). Their synthesis and modification is highly variable, tightly regulated and plays a key role in host-cell interactions. Thus, they are considered promising targets for anti-infective therapies and vaccines (Weidenmaier and Lee, 2017; Ansari et al., 2019). Structure and function of WTA and CP were recently reviewed (O'Riordan and Lee, 2004; Xia et al., 2010; Brown et al., 2013; Winstel et al., 2014; Schade and Weidenmaier, 2016; Weidenmaier and Lee, 2017). Here, we briefly summarize the function and molecular make-up of the glycopolymers. We will mainly focus on the regulatory system controlling WTA and CP biosynthesis and implicated functional consequences.

2. Function and regulation of WTA

2.1. Structure and function

WTA is unique with respect to its multiple functions in cell wall biosynthesis and maintenance, cellular physiology, phage interaction, host cell adhesion, antibiotic resistance as well as immune regulation (Weidenmaier and Peschel, 2008; Weidenmaier and Lee, 2017; van Dalen et al., 2019). WTA is present in all *S. aureus* strains analysed so far where it is mostly composed of 30–50 ribitol-phosphate (RboP) repeating units (Brown et al., 2013; Weidenmaier and Lee, 2017). However, certain strains produce poly-glycerol-phosphate (GroP) WTA instead (Winstel et al., 2014). The RboP backbone is connected to the peptidoglycan via a disaccharide linkage unit that contains N-acetylglucosamine (GlcNAC) linked to N-acetylmannosamine followed by two glycerol-3-phosphates (GroP) (Brown et al., 2013; Weidenmaier and Lee, 2017). The RboP is attached to the last GroP of the linkage unit and the RboP repeating units are connected to each other via phophodiester linkages (Weidenmaier and Lee, 2017). In addition, the repeating units are modified with D-alanine and GlcNAc.

The D-alanyl modifications are connected via an ester linkage to position C2 of RboP by the DltABCD enzymes (Peschel et al., 1999;

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Neuhaus and Baddiley, 2003). The repeating units exhibit a zwitterionic charge due to the presence of negatively charged phosphodiesters and positively charged D-alanine ester modifications (Weidenmaier et al., 2010). WTA D-alanylation leads to a more cationic charge of the bacterial surface which affects the interaction of cationic antimicrobial peptides (CAMP) with the bacterial surface and renders *S. aureus* non-susceptible to relatively high CAMP concentrations (Peschel et al., 1999).

Glycosylation modification occurs on position C4 of RboP via the predominant GlcNAc transferases TarM and TarS exhibiting α- and β-(1–4) glycosyltransferase activities, respectively (Brown et al., 2012; Koc et al., 2015; Sobhanifar et al., 2015, 2016). Depending on the *S. aureus* strain, the anomeric configuration of the glycosidic linkage of GlcNAc to RboP can either be α or β or a mixture of both anomers (Endl et al., 1983; Winstel et al., 2015). In addition to the C4 glycosylation, a recent study reported the presence of GlcNAc modifications on the C3 position of RboP units (Gerlach et al., 2018). This modification was detected in a number of CC5 and CC398 strains that harbour a prophage encoding an alternative WTA glycosyltransferase (TarP). Interestingly, the C3 GlcNAc modification leads to a less immunogenic WTA polymer. Strains with C3 modifications cannot be inactivated by dominant antibodies against the C4 modified WTA which allows such strains to escape the immune response of the host more efficiently (Gerlach et al., 2018).

2.2. Regulation of WTA synthesis and modification

Although WTA is a dominant surface epitope with multiple roles in host infection, regulation of WTA biosynthesis and modification remains poorly understood. This might be attributed to the fact that the genes involved in WTA biosynthesis (*tarO, tarAHGBXD, tarI'J'KFIJL, mnaA*) and modification (*dltXABCD, tarM, tarS, tarP*) are scattered throughout the genome (Fig. 1). With respect to the regulation of the WTA main chain biosynthesis, so far only a few reports shed light on possible mechanisms that affect WTA chain length and WTA amounts in the cell wall. Chain length is determined by the two RboP polymerases TarK and TarL, which produce WTA of different size with TarK producing up to 50% shorter polymers than TarL (K-WTA versus L-WTA). The Agr quorum sensing system was shown to modulate the ratios of K-WTA and L-WTA via repression of *tarK*. Thus, while under low Agr activity K-WTA and L-WTA are produced, L-WTA is the dominant form under high Agr activity (Meredith et al., 2008). Furthermore, a recent report indicated that altered Agr activity affects WTA biosynthesis and leads to strain specific changes in the amounts of WTA in the cell wall (Wanner et al., 2017). Highly virulent strains exhibit a higher Agr activity and show a consistently higher amount of WTA in their cell walls. This WTA_{high} phenotype is dependent on an increased expression of the *tarH* gene, which encodes for the energizing ATPase subunit of the TarG/H ABC transporter (Wanner et al., 2017). TarG/H is responsible for the transport of WTA over the cytoplasmic membrane. In line with prior reports (Swoboda et al., 2009; Campbell et al., 2012; Brown et al., 2013) it is postulated that the transport constitutes a rate limiting step of WTA biosynthesis. Expression of *tarH* is controlled by direct binding of the repressor Rot to the *tarH* promoter (Wanner et al., 2017). The intracellular effector of the Agr system, RNAIII anneals to target mRNAs including *rot* resulting in repression of translation initiation followed by de-repression of Rot-regulated target genes (Boisset et al., 2007). Additional evidence for a differential regulation of WTA biosynthesis under certain physiological conditions came from studies that implicated WTA in antibiotic resistance (Bertsche et al., 2011, 2013). These studies reported that cell wall stress mediated by different antibiotics can lead to a phenotype that is characterized by a significant increase in WTA amounts in the cell walls of antibiotic resistant strains. The mechanism leading to altered WTA synthesis remains to be elucidated. Regulation of WTA biosynthesis gene clusters *in vivo* has been sparsely analysed. Analyses of transcription during nasal colonisation

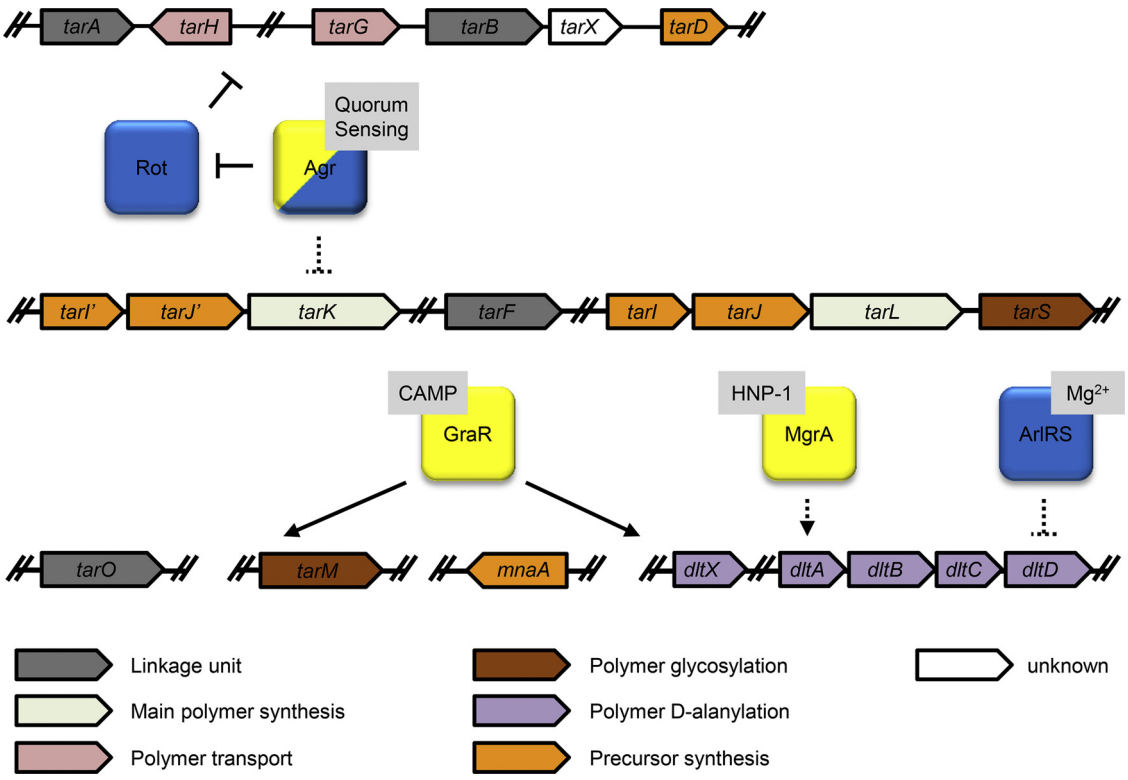

Fig. 1. Genetic organisation, function and regulation of genes involved in WTA biosynthesis and modification. Regulators in yellow indicate up-regulation, in blue down-regulation. Solid lines show direct interaction of the regulators with target genes, dashed lines mark unknown mechanisms of regulation. Signals controlling the activity of the regulators are shown in boxes. CAMP: cationic antimicrobial peptides, HNP: human neutrophil peptide.

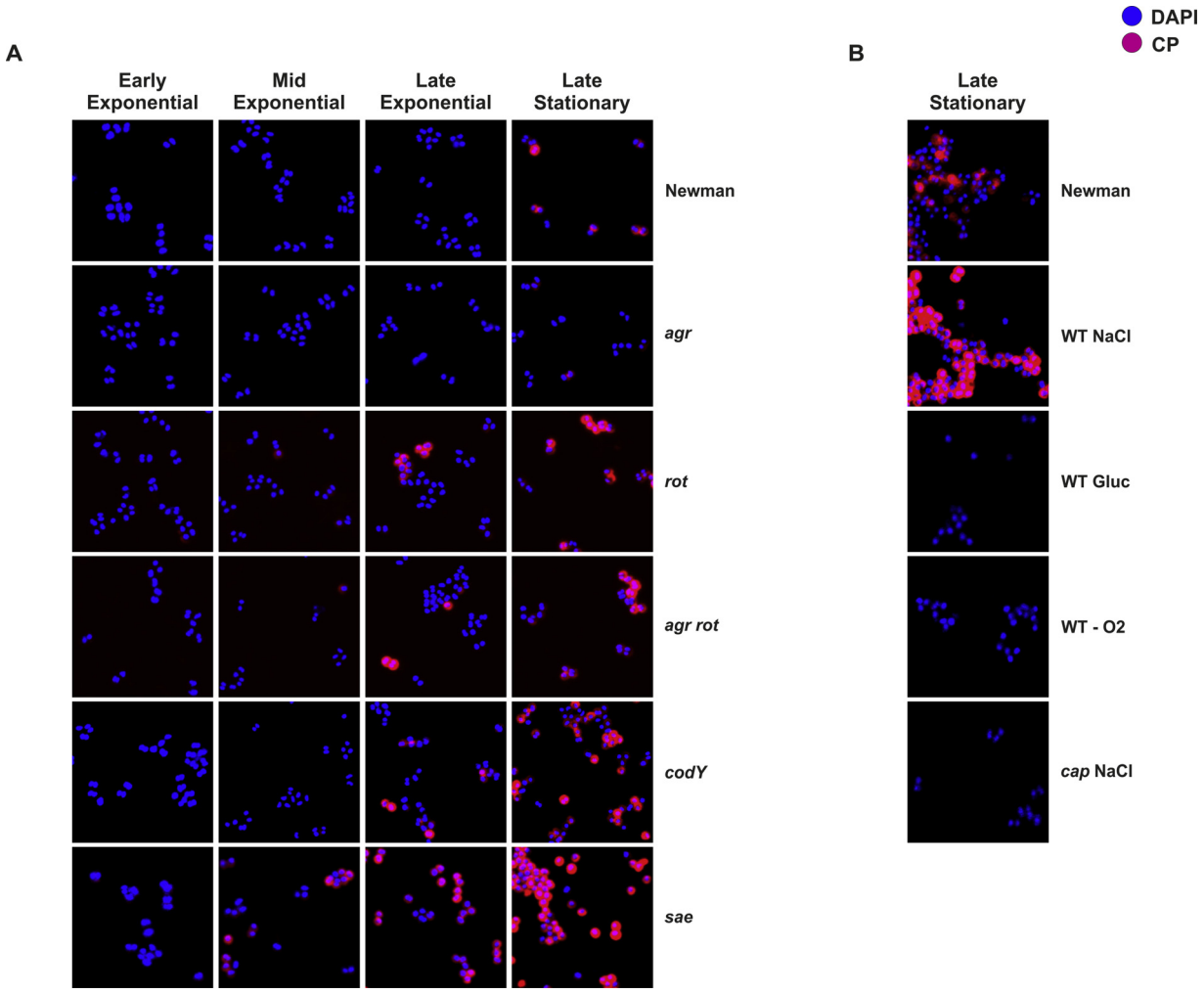

Fig. 2. Temporal and heterogeneous synthesis of CP influenced by (A) different regulators and (B) environmental conditions. CP was detected by immunofluorescence. (A) *S. aureus* strain Newman and isogenic mutants grown in LB medium to the indicated growth phase (modified from George et al., 2015). (B) *S. aureus* strain Newman grown in LB supplemented without or with 2 M NaCl, 1% glucose or grown anaerobically. A *cap* mutant strain was included as negative control (modified from George et al., 2015).

showed increased *tarO* expression at the onset of colonisation in an animal model (Burian et al., 2010).

Modification of WTA is sensitive to various environmental conditions. D-alanine ester content is regulated in response to physiological factors like salt concentration, temperature and pH (Neuhaus and Baddiley, 2003; Koprivnjak et al., 2006). For instance, Mg^{2+} induced *dlt* repression was shown to depend on the two-component system (TCS) ArlRS (Koprivnjak et al., 2006). In addition, the TCS GraRS is thought to directly up-regulate the expression of the *dlt*-operon and putative binding sites of the response regulator GraR have been identified upstream of the operon (Falord et al., 2011). GraS is involved in sensing of host-derived CAMP, but may also respond to other signals like oxidative stress (Falord et al., 2011). Recently, also the transcription factor MgrA was shown to positively regulate *dltA* expression, especially in response to human defense peptides (Li et al., 2019).

Regulation of the genes encoding for the GlcNAc glycosyltransferases TarM, TarS and TarP is only poorly studied. Recently, it was demonstrated that *S. aureus* is able to modify the phenotype of its WTA glycosylation pattern dependent on environmental conditions. The β-GlcNAc anomer was preferentially expressed at the expense of the α-GlcNAc anomer when grown on stress-inducing culture medium containing high NaCl concentration (Mistretta et al., 2019). Furthermore, while the inoculum used to infect animals produced almost exclusively α-GlcNAc WTA, bacteria recovered from infected organs produced only β-glycosylated WTA (Mistretta et al., 2019). The underlying molecular mechanisms remain elusive but may involve regulation by GraRS. A putative GraR binding site was predicted upstream of *tarM* and *tarM* expression was decreased in a *graRS* mutant (Falord et al., 2011). In another study TarS dependent GlcNAc modification of WTA was shown to play an important role in oxacillin resistance and interestingly only *tarS* but not *tarM* expression was strongly up-regulated by oxacillin (β-lactam) treatment (Brown et al., 2012). Again, the molecular mechanisms that confer this differential regulation remain unknown.

3. Function and regulation of CP

3.1. Structure and function

CP serves as essential virulence factor due to its anti-phagocytic properties which are important for immune evasion (Thakker et al., 1998; Nanra et al., 2013). However, depending on the infection setting either the presence or absence of CP has been reported to be advantageous for *S. aureus* (O'Riordan and Lee, 2004; Tuchscherr et al., 2010). CP enhances virulence in murine models of bacteraemia (Thakker et al., 1998; Watts et al., 2005), septic arthritis (Nilsson et al., 1997), abscess formation (Portoles et al., 2001), and surgical wound infection (McLoughlin et al., 2006). In contrast, in mammary gland infections

(Tuchscherr et al., 2005) and in catheter-induced endocarditis (Baddour et al., 1992; Nemeth and Lee, 1995) CP mutants are more virulent. This is likely because CP also inhibits the adherence of the underlying adhesins to their specific target molecule (Pohlmann-Dietze et al., 2000; Risley et al., 2007). CP-negative *S. aureus* strains are frequently isolated from patients with osteomyelitis, mastitis or cystic fibrosis, providing evidence that the loss of CP expression may be advantageous for *S. aureus* during chronic infection (Herbert et al., 1997; Lattar et al., 2009; Tuchscherr et al., 2010). The loss of CP expression can typically be explained by mutations in any of the genes essential for CP synthesis or in the promoter region (Cocchiaro et al., 2006; Tuchscherr et al., 2010). For instance, strains from the USA300 lineage are non-encapsulated due to conserved mutations in the *cap5* locus (Boyle-Vavra et al., 2015). However, this assumption has been recently challenged by the finding that USA300 strains might indeed produce CP during infection (Mohamed et al., 2019).

Amongst clinical *S. aureus* isolates the two serotypes 5 (CP5) and 8 (CP8) are the most prevalent (O'Riordan and Lee, 2004). The structure of CP5 and CP8 is very similar as they both consist of trisaccharide repeating units of D-N-acetyl mannosaminuronic acid, L-N-acetyl fucosamine, and D-N-acetyl fucosamine. The only difference lies in the linkages between the sugars and the site of O-acetylation of the mannosaminuronic acid residues, resulting in the different serotypes (Fournier et al., 1984, 1987; Moreau et al., 1990; Jones, 2005). Polymerized CP is covalently attached to the glycan strands of the peptidoglycan (Chan et al., 2014).

3.2. Influence of environmental signals on CP synthesis in vitro and in vivo

Expression of CP5 and CP8 is strongly dependent on environmental conditions (Sutra et al., 1990; Dassy et al., 1991; Stringfellow et al., 1991; Poutrel et al., 1995). CP production *in vitro* is enhanced under high-salt conditions (Pohlmann-Dietze et al., 2000; George et al., 2015), iron limitation and on solid medium (Lee et al., 1993) but inhibited by yeast extract, alkaline growth conditions, high glucose, low oxygen conditions and high CO_2 (Fig. 2B) (Dassy et al., 1991; Stringfellow et al., 1991; Herbert et al., 1997; George et al., 2015). Most of these environmental cues can be linked to the activity of several regulatory mechanisms described below. Furthermore, *in vitro* CP expression was widely shown to be low during exponential growth phase (Fig. 2A) (Poutrel et al., 1995; Dassy and Fournier, 1996; Pohlmann-Dietze et al., 2000; Cunnion et al., 2001; George et al., 2015; Conlon et al., 2016).

During infection and colonization CP synthesis is also highly variable. CP antigens were detectable in sera and infection sites of infected animals (Arbeit and Dunn, 1987; Arbeit and Nelles, 1987; Lee et al., 1993). However, *ex vivo* analysis of bacteria from cystic fibrosis patient revealed that CP is hardly expressed and only few bacteria are CP-positive. This was linked to the high CO_2 concentrations in the lungs (Herbert et al., 1997). Similar analysis revealed that during nasal colonization only part of the *S. aureus* population is CP-positive (George et al., 2015). Thus, not all bacteria in a population seem to express CP on their surface as confirmed by flow cytometry or immunofluorescence (Poutrel et al., 1997; Pohlmann-Dietze et al., 2000; Seidl et al., 2006; Meier et al., 2007; Jansen et al., 2013; Hartmann et al., 2014; George et al., 2015).

3.3. Regulation of CP

The proteins involved in CP5 or CP8 biosynthesis, O-acetylation, transport and regulation are encoded by 16 genes, *cap5/8A* to *cap5/8 P* (O'Riordan and Lee, 2004; Weidenmaier and Lee, 2017; Rausch et al., 2019). The *cap5* and *cap8* gene clusters are allelic (Goerke et al., 2005) and share a highly similar promoter element (P_{cap}) upstream of *capA* (Herbert et al., 2001). From here, the operon is mainly transcribed as single large 17 kb transcript (Sau et al., 1997; Ouyang et al., 1999). P_{cap} activity generally correlates with CP synthesis indicating that regulation occurs predominantly on the transcriptional level (Ouyang et al., 1999; Meier et al., 2007; Jansen et al., 2013; Hartmann et al., 2014; George et al., 2015).

For a long time the molecular architecture of P_{cap} remained puzzling due to a determined transcriptional start site (TSS) not being preceded by a classical consensus sequence for sigma factor A (SigA) or B (SigB) binding (Ouyang et al., 1999). Instead, within the *cap* promoter region a 10 bp inverted repeat (IR) was shown to be crucial for promoter activity (Ouyang et al., 1999). Recent re-analyses of the P_{cap} promoter structure revealed a different TSS preceded by a canonical SigB consensus motif overlapping with the IR structure (Keinhörster et al., 2019; Mäder et al., 2016; Prados et al., 2016). Thus, the previously observed activating effect of SigB on *cap* expression can be explained by direct SigB-dependent regulation. Other SigB-dependent *cap* activators may contribute to the fine tuning of *cap* expression and amplify SigB-dependency (Keinhörster et al., 2019; Bischoff et al., 2001, 2004; Meier et al., 2007; Schulthess et al., 2009; Lei and Lee, 2015).

Next to the main SigB-dependent promoter a weak SigA-dependent promoter was identified further upstream (Keinhörster et al., 2019). While it seems to play only a minor role for *cap* expression it cannot be ruled out that under certain conditions the SigA-dependent promoter gets activated. This may be the case during infections with CP-positive strains from the USA300 lineage which have a common mutation in the SigB binding motif (Mohamed et al., 2019). Besides containing a SigA-dependent promoter, the extended upstream region of P_{cap} is targeted by many transcriptional factors thereby modulating *cap* expression (see below) (Keinhörster et al., 2019).

The role of regulators affecting *cap* expression was mainly deduced from the characterization of single regulatory mutants and in most cases it remains unclear how *cap* expression is modulated. In particular, the following regulators likely act indirectly via other regulatory systems: Agr (Fig. 2A) (Dassy et al., 1993; Pohlmann-Dietze et al., 2000; Luong et al., 2002; van Wamel et al., 2002; George et al., 2015), SarA (Luong et al., 2002; van Wamel et al., 2002), MgrA (Gupta et al., 2013), ArlRS (Luong and Lee, 2006), CcpA (Seidl et al., 2006, 2009), RpiR (Zhu et al., 2011; Gaupp et al., 2016), ClpC (Luong and Lee, 2006; Graham et al., 2013), RsaA (Romilly et al., 2014), SbcDC (Luong and Lee, 2006; Chen et al., 2007) and SpdC (Poupel et al., 2018). However, there are also many regulators for which a binding to P_{cap} was demonstrated, indicating direct regulation. These are the activators SpoVG, RbsR and KdpE (Fig. 3, yellow) as well as the repressors CcpE, CodY, XdrA, Rot, SaeR and AirR (Fig. 3, blue) which are discussed in the following sections.

The ribose-responsive regulator RbsR (Lei and Lee, 2015) and the putative transcription factor MsaB (Batte et al., 2016) were proposed to activate *cap* expression by directly binding to the 10 bp IR of P_{cap} (now identified as canonical SigB binding motif (Keinhörster et al., 2019)). Interestingly, the presence or absence of ribose had no effect on *cap* expression in the wild type or the *rbsR* mutant (Lei and Lee, 2015). We could not confirm the effect of RbsR on P_{cap} activity (Keinhörster et al., 2019). However, RbsR likely functions as a metabolic sensor, and thus the discrepancy could be due to differences in growth conditions. *rbsR* expression itself is SigB-dependent and accordingly highest towards stationary growth phase (Lei and Lee, 2015). The *msaABCR* operon is involved in the regulation of virulence, biofilm development, antibiotic resistance and persister cell formation in *S. aureus*. Operon deletion and/or the deletion of *msaB* alone reduces *cap* transcription as well as CP production (Batte et al., 2016). However, MsaB is also annotated as cold-shock protein CspA, which exerts regulatory effects via RNA binding. It was shown that MsaB/CspA binds *rsbVWsigB* mRNA, thereby increasing transcript stability (Caballero et al., 2018). This is consistent with the observation that MsaB/CspA increases expression of *sigB* and its target genes (Katzif et al., 2005; Sahukhal and Elasri, 2014; Caballero et al., 2018; Donegan et al., 2019). We were able to reproduce the activating effect of MsaB/CspA on *cap* expression, but demonstrated that this is due to increased SigB activity. Moreover, no

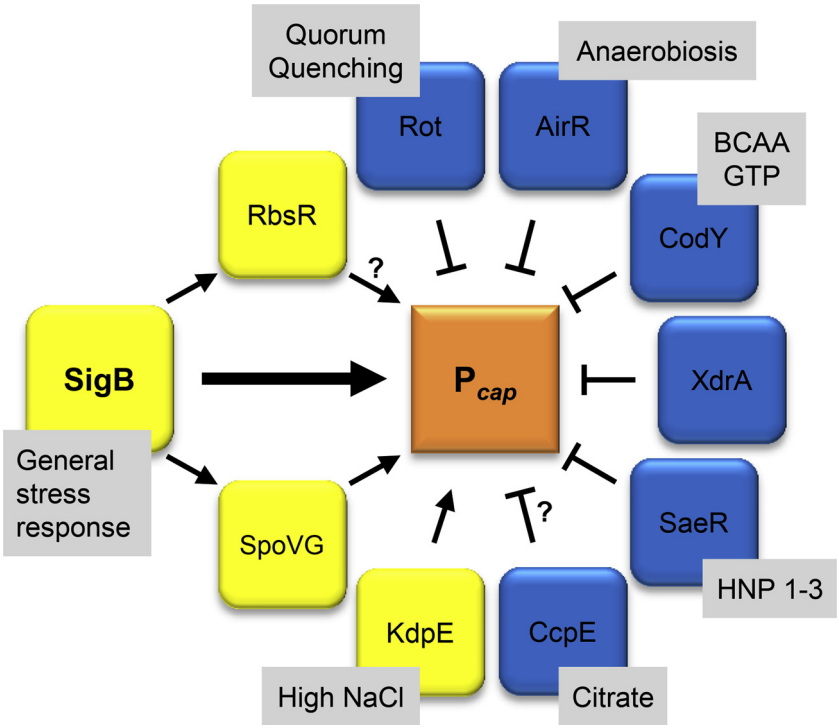

Fig. 3. Regulatory circuits known to directly impact *cap* expression. Regulators in yellow indicate up-regulation, in blue down-regulation of P_{cap} activity. Signals controlling activity of the regulators are shown in boxes. BCAA: branched-chain amino acids, HNP: human neutrophil peptides.

detectable MsaB/CspA binding to P_{cap} was observed (Keinhörster et al., 2019). Taken together, the role of the IR region as a target for other regulators besides SigB has to be questioned.

The transcription factor SpoVG regulates a number of virulence genes including *cap* via direct protein-DNA interaction (Meier et al., 2007; Schulthess et al., 2009, 2011; Jutras et al., 2013). The DNA-binding property of SpoVG is enhanced by phosphorylation via the protein kinase Stk1 (Bischoff et al., 2016). P_{cap} contains a conserved SpoVG binding motif in its extended upstream region and binding of phosphorylated SpoVG to the promoter was demonstrated (Jutras et al., 2013; Bischoff et al., 2016). *spoVG* expression itself is under the control of SigB. P_{cap} activity in a *spoVG* mutant is diminished but still growth phase-dependent (Meier et al., 2007).

KdpDE is a TCS which is inhibited by K^+, c-di-AMP and the auto-inducer AI-2 but activated by high Na^+ concentration (Zhao et al., 2010; Xue et al., 2011; Freeman et al., 2013; Price-Whelan et al., 2013; Moscoso et al., 2016). The direct interaction of the response regulator KdpE and P_{cap} was shown via electrophoretic mobility shift assays (EMSA) (Zhao et al., 2010). Thus, increased *cap* expression in response to high Na^+ is likely due to KdpDE and SigB activity (Pane-Farre et al., 2006; Price-Whelan et al., 2013).

Transcription of tricaboxylic acid cycle genes are controlled by CcpE (Hartmann et al., 2013). Additionally, *capA* levels were found to be decreased in a *ccpE* mutant throughout growth and less CP-positive cells were detected. EMSA failed to show that activation of *cap* is by direct DNA binding of CcpE to P_{cap}, suggesting an indirect regulatory mechanism (Hartmann et al., 2014; Batte et al., 2018). In contrast, in another study *capA* transcription was found up-regulated in a *ccpE* mutant in a citrate-dependent manner and direct CcpE-P_{cap} interaction was shown via EMSA (Ding et al., 2014). The reason for these discrepancies remains to be elucidated.

Regarding *cap* repression many regulators seem to primarily target the extended upstream region of P_{cap} and subsequently modulate the activity of the SigB-dependent promoter (Keinhörster et al., 2019). CodY is a repressor of several metabolic and virulence genes including *cap* (Fig. 2A), where it binds to a conserved consensus sequence (AATTTTCWGAAAATT) (Majerczyk et al., 2010). Binding and thus gene repression is enhanced in the presence of branched chain amino acids and GTP, making CodY most active under conditions of nutrient excess (e.g. early growth phase) (Pohl et al., 2009; Majerczyk et al., 2010). Protein-DNA interaction studies showed that CodY interacts with P_{cap} (Majerczyk et al., 2010; Batte et al., 2018; Lei and Lee, 2018) and footprint analysis revealed that CodY binding reaches into the *capA* coding region (Lei and Lee, 2018). We could show that CodY additionally binds to the upstream region of P_{cap} and that this region is essential and sufficient for the CodY repressive function (Keinhörster et al., 2019). In addition, CodY seems to be required for *cap* repression by XdrA. This transcription factor was shown to bind to P_{cap} with its binding region overlapping with the downstream binding site of CodY, reaching into the *capA* coding region (McCallum et al., 2010; Lei and Lee, 2018). Thus, the two proteins likely interact functionally or physically to repress *cap*. However, the mechanism of the CodY-XdrA interaction remains to be elucidated (Lei and Lee, 2018).

The transcription factor Rot is inactivated by RNAIII of the Agr system (Boisset et al., 2007) thereby being the mediator of the well-known Agr-dependent *cap* expression (Fig. 2A) (George et al., 2015). The DNA binding motif of Rot remains unresolved but was proposed to contain an 18 bp long section of AT-rich DNA (Killikelly et al., 2015). Rot specifically binds to the upstream region of $P_{cap,}$ interfering with SigB-dependent promoter activity (Keinhörster et al., 2019).

The TCS SaeRS is activated by α-defensins and required for the expression of many virulence factors (Geiger et al., 2008). *cap* is one of the few genes which is repressed by SaeRS (Fig. 2A) (Steinhuber et al., 2003; Luong et al., 2011; George et al., 2015). Regulation occurs by binding of phosphorylated response regulator SaeR to the consensus sequence ($GTTAAN_6GTTAA$) (Sun et al., 2010). In P_{cap} the poorly conserved SaeR binding site is located within the extended upstream region. Phosphorylated SaeR specifically binds to P_{cap} thereby repressing both SigB- and SigA-dependent promoter activities (Keinhörster et al., 2019).

The sensor kinase AirS of the TCS AirSR contains a redox active Fe-S cluster which can sense oxygen and redox signals. The oxidized AirS

phosphorylates the response regulator AirR influencing the expression of many regulatory genes as well as inhibiting *cap* under anaerobic conditions. Binding of AirR to P_{cap} was shown (Sun et al., 2012, 2013) but it remains open whether the binding site is also located within the extended upstream region.

On top of all these regulators controlling *cap* expression on the transcriptional level, post-transcriptional regulation is also important for the function of the CP synthesis machinery. The CapAB tyrosine kinase complex controls multiple enzymatic checkpoints through reversible phosphorylation in order to modulate the consumption of essential precursors that are also used in peptidoglycan biosynthesis (Rausch et al., 2019). Furthermore, CP biosynthesis was linked to tyrosine phosphorylation via the Ser/Thr kinase PknB, which can sense cellular lipid II levels (Hardt et al., 2017). PknB signaling serves to reduce CapAB autokinase activity and to reduce the CapM glycosyltransferase activity thereby leading to a shutdown of CP production (Rausch et al., 2019). This likely ensures a sufficient supply of precursors for peptidoglycan synthesis and WTA formation.

3.4. Heterogeneous and temporal CP expression

The most prominent feature of *cap* expression is the strict repression in growing bacteria and the highly heterogeneous expression in bacteria from stationary growth phase (George et al., 2015). In non-growing bacteria only a subpopulation of *S. aureus* is found to express CP. Changing environments or mutations in regulatory genes can shift the ratio of CP-positive bacteria or the onset of *cap* expression during the growth cycle (Fig. 2A and 2B).

On the transcriptional level the temporal and heterogeneous *cap* expression was shown to be determined by direct SigB-dependent regulation and regulators targeting the upstream region of P_{cap} (Keinhörster et al., 2019). Earlier onset of CP production and more CP-positive cells in stationary growth phase could be observed upon constitutive *sigB* expression (Keinhörster et al., 2019), which is consistent with SigB being a known activator of late genes (Bischoff et al., 2001, 2004; Pane-Farre et al., 2006; Mäder et al., 2016). An even more pronounced effect was observed upon chromosomal deletion of the P_{cap} upstream region (Keinhörster et al., 2019). This is in line with the absence of binding sites for the transcriptional repressors Rot and CodY; these being most active during exponential growth phase. In combination, P_{cap} upstream deletion and constitutive *sigB* expression resulted in constitutive *cap* expression and abolished heterogeneity of CP production in stationary phase (Keinhörster et al., 2019). However, CP synthesis remained growth phase-dependent even when *cap* gene expression was rendered constitutive. This is most likely due to the described post-transcriptional levels of regulation (Rausch et al., 2019). Together, the different regulatory circuits ensure that CP is only produced when needed e.g. for phagosomal escape and that a certain subpopulation is CP-negative to allow adherence to host cells. Furthermore they help to avoid conflict in precursor usage by machineries involved in either synthesis of CP or other glycopolymers in growing bacterial cells.

4. Conclusions

Biosynthesis of secondary cell wall glycopolymers has to be tightly controlled to ensure their diverse functions during infection. Further studies are required to unravel the regulatory network that governs WTA biosynthesis and modification. It can be anticipated that many regulatory pathways are involved, possibly resulting in a high degree of heterogeneity with regard to WTA quantity and modification at the single-cell level. So far there are limited tools available to study such variation. In contrast, regulation of CP is much more studied and several involved regulators were identified. CP synthesis is tightly controlled at the transcriptional level, and together with post-transcriptional mechanisms these regulatory circuits determine the peculiar temporal and heterogeneous CP expression. One can assume that such non-genetic variability contributes significantly to the evolutionary success of the species by increasing fitness of the bacterial population.

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