Revisiting the regulation of the capsular polysaccharide biosynthesis gene cluster in Staphylococcus aureus

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Summary

Capsular polysaccharide (CP) biosynthesis in Staphylococcus aureus is tightly controlled resulting in a heterogeneous phenotype within a population and CP being mainly detectable in nongrowing cells. Expression of the corresponding biosynthesis gene cluster is driven by one promoter element (Pcap). Here, we demonstrate that Pcap contains a main SigB-dependent promoter. The SigB consensus motif overlaps with a previously described inverted repeat (IR) that is crucial for cap expression. The essentiality of the IR is derived from this region acting as a SigB binding site rather than as an operator site for the proposed cap activators RbsR and MsxB. Furthermore, Pcap contains an extensive upstream region harboring a weak SigA-dependent promoter and binding sites for cap repressors such as SaeR, CodY and Rot. Heterogeneous CP synthesis is determined by SigB activity and repressor binding to the upstream region. SigB dependency and regulation by the upstream repressors are also sufficient to explain the temporal gene expression pattern at the transcriptional level. However, CP synthesis remains growth phase-dependent even when transcription is rendered constitutive, suggesting additional post-transcriptional regulatory circuits. Thus, the interference of multiple repressors with SigB-dependent promoter activity as well as post-transcriptional mechanisms ensure the appropriate regulation of CP synthesis.

Introduction

Staphylococcus aureus is an opportunistic pathogen that asymptomatically colonizes parts of the human population, thereby increasing the risk of subsequent infections. Its capacity to cause a wide variety of diseases depends on secreted virulence factors as well as cell surface-attached proteins and polysaccharides (Gordon and Lowy, 2008; Weidenmaier and Lee, 2017; de Jong et al., 2019). The capsular polysaccharide (CP) is one of these cell surface structures playing an important role in S. aureus pathogenesis and bacterial evasion of the host immune defenses (O’Riordan and Lee, 2004; Weidenmaier and Lee, 2017). Therefore, it is being discussed as a target for immunotherapy and as a vaccine candidate (Missiakas and Schneewind, 2016; Weidenmaier and Lee, 2017; Ansari et al., 2019).

CP serotypes 5 and 8 are the two main CP serotypes produced by S. aureus strains (Arbeit et al., 1984; Hochkeppel et al., 1987; Roghmann et al., 2005; Verdier et al., 2007). Their structure is highly similar due to the closely related cap5 and cap8 gene clusters. These allelic operons consist of 16 genes, cap5/8A to cap5/8P, whose gene products are involved in CP biosynthesis, O-acetylation, transport and regulation (O’Riordan and Lee, 2004; Weidenmaier and Lee, 2017; Rausch et al., 2019). The cap operon is thought to be mainly transcribed as a single large 17 kb transcript driven by one principal promoter element (Pcap), located upstream of capA (Sau et al., 1997; Ouyang et al., 1999). While cap gene clusters are extremely conserved across S. aureus genomes, not all clinically relevant isolates produce CP and acapsular variants may emerge during chronic infections. The loss of CP expression can typically be explained by mutations in any of the cap genes essential for CP synthesis or in the promoter region (Cocchiaro et al., 2006; Tuchscherr et al., 2010). For instance, the acapsular phenotype of strains from the USA300 lineage was attributed 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). In addition to mutations that abolish its production, CP synthesis can also be switched off in response to environmental conditions via a complex regulatory network. Extensive in vitro and in vivo analyses have shown that cap expression is highly sensitive to changes in nutrients, pH, CO₂, and oxygen availability (Sutra et al., 1990; Dassy et al., 1991; Stringfellow et al., 1991; Lee et al., 1993; Poutrel et al., 1995; Herbert et al., 1997; Pohlmann-Dietze et al., 2000; George et al., 2015). Interestingly, CP synthesis was commonly found to be strictly growth phase-dependent and detectable only after post-exponential growth phase (Poutrel et al., 1995; Dassy and Fournier, 1996; Pohlmann-Dietze et al., 2000; Cunnil et al., 2001; George et al., 2015). In addition, not all bacteria in a population are CP-positive as revealed by flow cytometry and immunofluorescence (IF) of in vitro- and in vivo-grown bacteria (Poutrel et al., 1997; Pohlmann-Dietze et al., 2000; George et al., 2015; Conlon et al., 2016). As only nonencapsulated cells are able to adhere to endothelial cells (Pohlmann-Dietze et al., 2000), while CP protects bacterial cells from phagocytosis (Karaka et al., 1988; Thakker et al., 1998; Portoles et al., 2001), it is likely that CP heterogeneity provides better adaptability of the population as a whole. So far, the underlying regulatory mechanisms of this particular expression pattern (early-Off/late-Heterogeneous) are only partially understood.

In general, Pcap activity correlates with CP synthesis, indicating that regulation occurs predominantly at the transcriptional level (Ouyang et al., 1999; Meier et al., 2007; Jansen et al., 2013; Hartmann et al., 2014; George et al., 2015). Yet, the data to explain the molecular mechanisms of cap regulation are puzzling. The identified transcriptional start site (TSS) is not preceded by a classical sigma factor consensus sequence (Ouyang et al., 1999); instead, several inverted and direct repeats were identified further upstream, among which a 10 bp inverted repeat (IR) was shown to be crucial for promoter activity (Ouyang et al., 1999). It has been proposed that this IR functions as an operator site for the cap activators RbsR and MsaB (Lei and Lee, 2015; Batte et al., 2016). RbsR also functions as a repressor of the rbsUDK operon involved in ribose uptake. While the presence of ribose relieves repression of the rbsUDK operon by RbsR, the presence and absence of ribose had no effect on cap expression (Lei and Lee, 2015). MsaB is described as a transcriptional factor with DNA binding capacity (Batte et al., 2016) but is also annotated as cold-shock protein CspA, which exerts regulatory effects via RNA binding (Caballero et al., 2018). In addition to RbsR and MsaB/CspA, there are several other transcription factors (MgrA, CcpA, RpiR, SpoVG, CcpE, XdrA, CodY, Rot), two-component regulatory systems (Agr, ArlRS, KdpDE, AirRS, SaeRS) and the alternative sigma factor B (SigB) known to be involved in the regulation of cap expression (O’Riordan and Lee, 2004; Weidenmaier and Lee, 2017). The role of these regulators was mainly deduced from the characterization of single regulatory mutants and, in most cases, it remains unclear how they affect cap expression. In particular, SigB and Agr are believed to act indirectly via other regulatory systems. For instance, the absence of a SigB consensus motif in front of the proposed TSS led to the hypothesis that SigB acts through the SigB-dependent cap regulators SpoVG, ArlRS and RbsR (Bischoff et al., 2001; 2004; Meier et al., 2007; Schulthess et al., 2009; Lei and Lee, 2015). SigB is a central part of the general stress response, and is activated upon environmental stresses and toward stationary growth phase (Pane-Farre et al., 2006). Its activity is regulated mainly at the post-translational level by a complex regulatory cascade involving RsbW, RsbV and RsbU, encoded within the rsbUVWsigB operon (Senn et al., 2005).

For the quorum sensing system Agr, it was shown that Agr-mediated cap activation occurs via inactivation of the repressor Rot (George et al., 2015). Rot is known to bind to several target genes; however, its binding motif is ill-defined (Killikelly et al., 2015). Nevertheless, Rot as well as the DNA binding proteins CodY and SaeR are likely candidates for direct interference with the Pcap promoter element. CodY represses many metabolic and virulence genes, including the cap operon (Pohl et al., 2009; Majerczyk et al., 2010), and binding of CodY to the Pcap region has been demonstrated (Majerczyk et al., 2010; Batte et al., 2018; Lei and Lee, 2018). The two-component system (TCS) SaeRS regulates a number of virulence factors, with cap being one of the few genes repressed (Steinhuber et al., 2003; Luong et al., 2011; George et al., 2015). With only a poorly conserved SaeR consensus sequence in Pcap, it remains unclear whether SaeR exerts its effect on cap expression indirectly or via direct promoter interaction.

All in all, despite a plethora of work published on cap regulation, results are yet inconclusive regarding Pcap architecture and the molecular interference of the different regulatory elements. Here, we redefined the Pcap promoter structure and reinvestigated the main players of cap expression. We identified a SigB consensus motif overlapping with the IR structure. In addition, we found a second weak SigA-dependent promoter in the Pcap upstream region, as well as binding sites for three cap repressors, CodY, Rot and SaeR, which interfere with SigB-dependent promoter activity. Thus, the early-Off/late-Heterogeneous cap expression pattern is a consequence of SigB activity together with repression mediated through the Pcap upstream region. However, negative regulation of CP synthesis in early growth phase is maintained through additional post-transcriptional mechanisms.
Results

$P_{\text{cap}}$ contains SigA- and SigB-dependent promoters

Previous primer extension analysis of the 344 bp comprising $P_{\text{cap}}$ revealed a TSS −17 bp upstream of the ATG start codon of $\text{capA}$ (Ouyang et al., 1999). However, RNA-Seq data indicate an alternative TSS further upstream, at position −41 from the $\text{capA}$-coding region (data not shown), consistent with recent whole genome analyses of TSSs in $S. aureus$ (Mäder et al., 2016; Prados et al., 2016). To resolve this ambiguity, we employed 5′ rapid amplification of cDNA endings (5′ RACE) (Fig. 1A). Most of the clones (6/10) revealed a TSS at position −41 bp from the $\text{capA}$ start codon, which is preceded by a putative SigB consensus sequence. This SigB promoter consists of a conserved SigB −35 motif (GTTTTAA) and a −10 region harboring three mismatches (ATGTAA versus GGGTAT) (Homerova et al., 2004). Remarkably,

![Diagram](image_url)

**Fig. 1.** $P_{\text{cap}}$ promoter architecture and $P_{\text{cap}}$ variants employed in this study.
A. $P_{\text{cap}}$ (344 bp) in front of $\text{capA}$ with magnified SigA and SigB promoters. Black dots represent the putative TSSs suggested by 10 analyzed 5′ RACE clones. Sigma factor −35 and −10 motifs are underlined, bent arrows indicate the corresponding TSSs. The TSS and inverted repeat (IR) structure proposed by Ouyang et al. (1999) are marked as black triangle and opposing arrows, respectively. Vertical dashed lines indicate sites of promoter truncation. Numbers mark positions with reference to the ATG of $\text{capA}$. The native Shine Dalgano (SD) sequence is labeled and indicated by a box.

B. $P_{\text{cap}}$ promoter fusion variants in front of an artificial ribosomal binding site (sod) and $gpreneur$ gene. Numbers show truncation sites and asterisks indicate point mutations (* −56:G → T; ** −58:A → G, −57:T → G, −52:A → T).

C. Genomic $P_{\text{cap}}$ variants in front of $\text{capA}$. Numbers show truncation sites and asterisks indicate point mutations (** −58:A → G, −57:T → G, −52:A → T).
the SigB −35 consensus sequence is located within the IR that is crucial for \( P_{\text{cap}} \) activity (Ouyang et al., 1999). The identified TSS confirmed our RNA-Seq data and the TSS prediction of Prados et al. (2016). In addition, 5′ RACE revealed one clone with a putative TSS at position −128 bp upstream of the \( \text{capA} \) start codon, which was also predicted by Prados et al. (2016). A conserved SigA consensus sequence was identified in front of this TSS containing canonical −35 and −10 regions. Interestingly, the TSS −17 bp upstream of the \( \text{capA} \) start codon proposed by Ouyang et al. (1999) was not detected. Though one 5′ RACE clone suggested a potential TSS in close proximity, at position −21, this was not preceded by a sigma factor consensus sequence, challenging the presence of a functional promoter. Using 5′ RACE and sequence analysis, we provide evidence for a dominant SigB-dependent promoter and an additional SigA-dependent promoter further upstream in the \( P_{\text{cap}} \) promoter element.

**cap expression is mainly driven by direct SigB regulation**

To analyze promoter activities, we constructed various \( P_{\text{cap}}-\text{gpven} \) promoter fusions (Fig. 1B), including deletions and variations of the putative SigA- and SigB-dependent promoters. Cloning the full-length \( P_{\text{cap}} \) in front of \( \text{gpven} \) and an artificial ribosomal binding site (Liese et al., 2013) (pCG717) resulted in detectable \( \text{gpven} \) expression and was used as reference for all further experiments (Fig. 2A). Deletion of the downstream element containing the putative TSSs at position −17 (Ouyang et al., 1999) and −21 in pCG720 did not influence \( \text{gpven} \) expression. This supports that there is no active promoter located in this region and that these putative TSSs may have been derived from RNA processing. A construct containing only the SigA-dependent promoter (pCG722) resulted in a low but detectable fluorescence signal, suggesting weak promoter activity. In contrast, a construct containing only the SigB-dependent promoter (pCG719) resulted in a strong promoter activity. This indicates dual promoter activity driving \( P_{\text{cap}} \) expression: a weak SigA-dependent promoter located in the upstream region plus a main SigB-dependent promoter further downstream. The deletion of the upstream region containing the SigA consensus sequence (pCG719) resulted in promoter activity higher than that of the full-length construct (pCG717). Taken together, these results suggest that despite containing a functional SigA-dependent promoter, the upstream region mainly functions as a repressive element.

To further prove that SigB is directly involved in \( \text{cap} \) activation, the expression of the full-length \( P_{\text{cap}} \) promoter fusion was measured in wild-type and a \( \text{sigB} \) mutant over time. In addition, constructs where the SigB −10 consensus sequence was mutated to either abolish (pCG723) or enhance (pCG724) SigB affinity were included (Fig. 2B). Deletion of \( \text{sigB} \) (data not shown) or a loss-of-function mutation in the SigB consensus sequence eliminated \( P_{\text{cap}} \) activity. In contrast, the strong SigB consensus motif further enhanced promoter activity in comparison to the native promoter. These results reveal that \( \text{cap} \) expression is mainly and directly driven by SigB activity.

**MsAB/CspA activates cap expression by modulating SigB activity**

Interestingly, the crucial IR structure that in fact constitutes the SigB −35 motif was shown to be the binding site for two \( \text{cap} \) activators, RbsR and MsA (Lei and Lee, 2015; Batte et al., 2016). To elucidate whether and how these regulators interfere with SigB-dependent promoter activity, full-length \( P_{\text{cap}} \) promoter fusions were introduced.
into msa and rbsR knockout mutants. Under our growth conditions, deletion of rbsR showed no effect on P\textsubscript{cap} activity (Fig. S1).

In the msa mutant, P\textsubscript{cap} promoter activity was lower than in the wild-type, supporting the finding that MsaB/CspA contributes to cap activation (Fig. 3A). We hypothesized that MsaB/CspA exerted its effect by modulating SigB activity. To test this hypothesis, we used the dual promoter fusion construct pCG742 to simultaneously measure P\textsubscript{cap} and P\textsubscript{asp23} activities. The P\textsubscript{asp23} promoter is widely used as marker for SigB activity (Gertz et al., 1999; Giachino et al., 2001; Homerova et al., 2004) and was cloned in front of gpcer (Liese et al., 2013). We found P\textsubscript{cap} and P\textsubscript{asp23} activities to be highly correlated, in line with the assumption that both are controlled directly by SigB. The activity of both promoters was lower in a msa mutant (Fig. 3A and B). It was previously shown that MsaB/CspA could bind to rsbVWsigB transcript, likely leading to its stabilization (Caballero et al., 2018). In this case, expression of sigB alone should lead to MsaB/CspA-independent regulation. Therefore, we expressed sigB from a constitutive promoter in a rsbUVWsigB mutant (const. sigB). By this means, we also circumvented post-transcriptional regulation of SigB by the RsbUVW phosphorelay. As expected, neither P\textsubscript{cap} activity (Fig. 3A) nor P\textsubscript{asp23} activity (Fig. 3B) was affected by msa deletion in this background. To exclude additional regulation by direct binding of MsaB/CspA to P\textsubscript{cap}, we performed electrophoretic mobility shift assays (EMSAs) with purified MsaB/CspA protein. Even using high amounts of MsaB/CspA protein, no band shift was observed (Fig. S2). Thus, MsaB/CspA promotes cap expression via modulation of SigB activity and not by direct interaction with P\textsubscript{cap}.

**Upstream promoter region leads to P\textsubscript{cap} repression**

To follow up on the observation that the P\textsubscript{cap} upstream region is of repressive function (Fig. 2A), we investigated the role of the known cap repressors CodY, Rot and Sae. Full-length (pCG717) and truncated (pCG719 and pCG722) P\textsubscript{cap} promoter fusions were introduced in codY-, rot- or sae-negative background. Mutation of any of the three regulators resulted in a significant increase of full-length P\textsubscript{cap} activity (Fig. 4A). The effect of the individual regulators is additive, since in the sae codY rot triple mutant, the promoter activity is further enhanced compared to the single mutants. If these repressors target the P\textsubscript{cap} upstream region, their deletion should not affect promoter activity of a construct lacking this part of the promoter. Indeed, repressor mutations have no or only minor effects on promoter activity of construct pCG719 (Fig. 4B). Interestingly, promoter activity of the full-length construct in the triple mutant remained significantly below the level of the upstream-truncated construct (Fig. 4A and B). This indicates that, besides CodY, Sae and Rot, additional repressive factors are acting on the P\textsubscript{cap} upstream region.

We further analyzed whether the repressors also affect the activity of the weak SigA-dependent promoter located in this region (Fig. 4C). Remarkably, neither Rot...
nor CodY showed any influence on promoter activity in a construct only containing the upstream part of the promoter (pCG722). However, mutation of sae resulted in increased SigA-dependent promoter activity, even though it remained weak in comparison to the full-length promoter (pCG717). Hence, while all three repressors target the upstream region and affect SigB-dependent promoter activity, only Sae additionally represses the weak SigA-dependent promoter.

**Sae, Rot and CodY repress Pcap by direct binding**

We have demonstrated that Sae, Rot and CodY repress Pcap, but it remains unclear whether repression occurs through direct DNA–protein interaction or rather indirectly through the complex regulatory network. To elucidate the nature of the repressors, we performed EMSAs with purified SaeR, Rot and CodY proteins. As SaeR only binds DNA in its phosphorylated state (Sun et al., 2010), we created a phosphomimetic SaeR with a D51E substitution. Incubation of increasing amounts of SaeR D51E with fluorescence-labeled Pcap upstream fragment (−78 to −344 from capA, see Fig. 1A) resulted in a retarded protein–DNA complex (Fig. 5A), which was not observed using the unphosphorylated native SaeR (Fig. 5B). Binding is consistent with a putative SaeR binding motif located between −79 bp and −94 bp upstreams of the capA start codon (Liu et al., 2016). Specific binding to the Pcap upstream region was also found for Rot and CodY (Fig. 5C and D). These findings are in line with the promoter activities described above showing that Sae, Rot and CodY target the Pcap upstream region (Fig. 4A and B). Binding of CodY to the downstream fragment (+10 to −77 from capA, see Fig. 1A) is unspecific as band shifts are eliminated by specific and unspecific unlabeled competitors (Fig. S3D).

**SigB-dependent regulation and various repressors targeting the upstream region contribute to temporal and heterogeneous CP synthesis**

So far, we analyzed Pcap promoter activity using artificial promoter fusion constructs. To confirm that our findings translate into CP production, we used IF for CP detection. This also allows monitoring the onset of CP production and CP heterogeneity on the single cell level. Cultures were diluted thrice to ensure that all bacteria are actively dividing after inoculation and do not carry residual CP from stationary phase. Bacteria were analyzed throughout growth at different time points (T0–T4) (Fig. 6 and Table S1). On exponentially growing bacteria, no CP was detectable, but upon reaching stationary phase (T3) (Fig. 6 and Table S1). On exponentially growing bacteria, no CP was detectable, but upon reaching stationary phase approximately 40% of the population became CP-positive, which is consistent with previous results (George et al., 2015). With SaeR D51E...
being a regulator of late genes, we first investigated the effect of constitutive sigB expression on CP synthesis during growth. Constitutive sigB expression in a rsbUWSigB-negative background resulted in earlier onset of CP production and 50% CP-positive bacteria in stationary phase. In addition, we generated a Pcap mutant in which the upstream promoter region containing repressor binding sites was chromosomally deleted (ΔPcap upstream, Fig. 1C). Also in this strain, CP production started earlier and 83% CP-positive cells could be detected in the late growth phase. Of note, the effect of the Pcap upstream region deletion was more profound than that of constitutive sigB expression.

Furthermore, IF revealed that in bacteria from stationary phase, much of the heterogeneity is reduced in the ΔPcap upstream strain and omitted in combination with constitutive sigB expression. Thus, it seems to be the combination of repression via transcriptional regulators and SigB-dependent promoter activity that is responsible for the heterogeneous CP expression pattern in stationary phase.

However, throughout all experiments, CP expression remained growth phase-dependent. We thought that this could be due to the weak SigB promoter of Pcap. Therefore, we additionally altered the SigB −10 region to the conserved SigB −10 motif on the chromosome in the Pcap upstream-truncated strain (ΔPcap upstream, strong SigB, Fig. 1C). Together with constitutive sigB expression, this shifted the onset of CP even further toward early growth phase. However, the majority of the bacterial population still remained CP-negative in early logarithmic growth phase.

The bacteria analyzed by IF also contained the dual promoter fusion construct pCG742 (Pasp23-gpcer, Pcap-gpven) which allowed us to simultaneously analyze SigB activity throughout growth on the single cell level (Fig. 7A). As expected, Pasp23 and Pcap activities reached their maximum in stationary phase cells, confirming growth phase-dependent SigB activity. However, in contrast to Pcap activity (yellow), Pasp23 (blue) activity was already detectable in some bacteria from the exponential growth phase. This indicates that SigB activity itself is also heterogeneous but is not sufficient to activate Pcap in the early growth phase.

CP synthesis is also controlled on the post-transcriptional level

To analyze whether growth phase dependency on the CP level correlates to capA transcript levels, capA mRNA was quantified by qRT-PCR at T0–T2 (Fig. 7B). As expected, in the wild-type strain, capA expression was strongly repressed in early growth phase. In contrast, constitutive capA expression was achieved in a strain with upstream-deleted Pcap promoter, strong SigB consensus sequence and constitutive sigB expression (ΔPcap upstream, strong SigB, const. sigB), exceeding that of the wild-type. Of note, CP production is still growth phase-dependent under these conditions (Fig. 6), suggesting the existence of post-transcriptional mechanisms regulating CP synthesis.

Pcap regulation is conserved in different S. aureus strains

So far, all experiments have been performed in strain Newman which is special due to its hyperactive SaeRS system (Mainiero et al., 2010). To validate our findings in a different S. aureus background, we chose the
widely studied community-acquired methicillin-resistant *S. aureus* strain USA300 JE2. This strain has an acapsular phenotype due to three crucial mutations in the P<sub>cap</sub> promoter region and in the coding regions of cap5D and cap5E (Boyle-Vavra et al., 2015). Therefore, we first generated derivatives in which we either only repaired the mutation in P<sub>cap</sub> (P<sub>cap</sub> repaired) or all three mutations (cap repaired) (Fig. 8 and Table S2). In line with previous observations, the USA300 JE2 wild type shows an acapsular phenotype and the repair of the mutation in P<sub>cap</sub> alone is not sufficient to enable CP production in vitro (Boyle-Vavra et al., 2015). Only when all three mutations were repaired, USA300 JE2 was capable of producing CP, following the same peculiar expression pattern as strain Newman: CP-positive cells were observed toward late growth phase and CP expression was highly heterogeneous. Upon deletion of the P<sub>cap</sub> upstream region and introduction of the fully conserved SigB consensus sequence (cap repaired, ΔP<sub>cap</sub> upstream, strong SigB), CP-positive cells were detected earlier and, in late growth phase, all cells were CP-positive. Therefore, the CP expression pattern of USA300 cap repair and its regulation closely resembles that of Newman.

**Discussion**

CP protects *S. aureus* against phagocytosis, but also hampers adherence to endothelial cells and matrix proteins. It is believed that heterogeneity of CP expression has evolved to provide better adaptability of the bacterial population during infection and colonization (George et al., 2015). Apart from this heterogeneous phenotype, CP production is strongly growth phase-dependent, with encapsulated cells found only toward stationary growth phase (George et al., 2015). During the last decades, many regulatory proteins have been shown to have an impact on CP production, forming
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a complex regulatory network. However, to date, it is not known which regulator is mainly responsible for this peculiar expression pattern. Here, we revisited the role of important molecular determinants of P<sub>cap</sub> activity, summarized in Fig. 9.

cap expression is mainly driven by SigB activity

We show that the main P<sub>cap</sub> promoter is SigB-dependent. This is based on (i) 5' RACE results revealing a new TSS downstream of a conserved SigB consensus sequence with minor mismatches in the −10 region; (ii) only very weak promoter activity in a sigB mutant or upon mutation of the −10 consensus sequence; (iii) higher P<sub>cap</sub> activity when the SigB −10 region matches the proposed SigB consensus sequence; and (iv) correlation of P<sub>asp23</sub> and P<sub>cap</sub> promoter activity. Of note, the SigB −35 region is directly located within the IR which was shown to be crucial for cap expression (Ouyang et al., 1999). Thus, the effects of previously reported mutations in this region can be simply explained by its function as a SigB binding site. A positive effect of SigB on cap expression was previously shown. However, it was believed to be mediated via the SigB-dependent cap activators SpoVG, ArlRS-MgrA and RsbR (Bischoff et al., 2001; 2004; Meier et al., 2007; Schulthess et al., 2009; Lei and Lee, 2015). Our results highlight the importance of SigB for cap expression and clearly demonstrate that its main impact is through direct SigB-dependent promoter activation. Nevertheless, other SigB-dependent cap regulators may contribute to the fine tuning of cap expression and amplify SigB dependency.

MsaB/CspA activates cap expression by modulating SigB activity

It was previously shown that the SigB −35 consensus sequence functions as binding site for two cap activators RbsR and MsaB (Lei and Lee, 2015; Batte et al., 2016). We did not observe an effect of RbsR on P<sub>cap</sub> activity, neither in wild-type nor in a sae-negative...
background. RbsR likely functions as metabolic sensor, and thus the discrepancy with the results of Lei and Lee (Lei and Lee, 2015) could be due to differences in growth conditions.

We reproduced the activating effect of MsaB/CspA on cap expression, but demonstrated that it is mediated by modulation of SigB activity: msa deletion negatively affected P_{cap} and P_{cap23} activities and constitutive expression of sigB rendered both promoters insensitive to MsaB/CspA. Moreover, in contrast to Batte et al. (2016), no detectable MsaB/CspA binding to P_{cap} was observed, challenging the role of MsaB/CspA as a classical transcription factor. Indeed, it was recently shown that MsaB/CspA binds rsbVWsigB mRNA, thereby increasing transcript stability (Caballero et al., 2018). Strong influence of MsaB/CspA on SigB activity is consistent with previous findings that MsaB/CspA increases expression of sigB and its target genes (Katzip et al., 2005; Sahukhal and Elasri, 2014; Donegan et al., 2019). With respect to direct P_{cap} regulation, our results indicate that under the conditions employed, the region containing the previously described IR motif functions instead as a promoter for the SigB holoenzyme.

**Cap expression is modified by upstream SigA promoter and repressor binding sites**

Upstream of the main SigB-dependent promoter we identified an additional weak SigA-dependent promoter with conserved SigA −35 and −10 consensus sequences. However, the SigA-dependent promoter seems to play a minor role in cap expression, with its activity being mainly detectable upon deletion of sae. The reason for such low promoter activity is unclear and might be due to suboptimal structure and spacing of the SigA consensus in P_{cap} (Ruff et al., 2015; Browning and Busby, 2016). Furthermore, the average activity of SigA promoters has been found to be generally lower than that of SigB promoters (Mäder et al., 2016). However, for any given promoter sequence, changes in temperature, salt and solute...
Fig. 9. Transcriptional regulation of cap expression. Cap expression is mainly driven from a SigB-dependent promoter. However, there is a second weak SigA-dependent promoter further upstream. In addition, the P\textsubscript{cap} upstream region is targeted by the cap repressors SaeR, CodY, Rot and others. Of note, Rot and CodY only interfere with SigB-dependent promoter activity, whereas Sae is able to repress both. A predicted SaeR binding motif is located between the SigA- and SigB-dependent promoters. P\textsubscript{cap} contains a second CodY binding site reaching into the coding region of capA. The depicted binding sites for the upstream regulators (relative to each other and the SigA promoter) are arbitrary and further studies are needed to localize these site(s) and their mechanisms of action. SigB activity is modulated by MsaB/CspA, promoting sigB transcript stability. Consequently, MsaB/CspA likely also affects indirect SigB-dependent P\textsubscript{cap} activation through ArIR-MgrA, SpoVG and RbsR. However, for RbsR we were unable to prove an activating effect on cap expression. [Colour figure can be viewed at wileyonlinelibrary.com]

concentrations, as well as protein factors and ligands can affect its kinetics by 10–1000-fold or more (Ruff et al., 2019). Therefore, we cannot rule out that under certain conditions the SigA-dependent promoter gets activated. This may be the case during infections with strains from the USA300 lineage. Among others, USA300 strains carry a point mutation within the SigB consensus motif of P\textsubscript{cap}. Interestingly, strains harboring only this mutation seem to produce CP under infectious conditions (Mohamed et al., 2019), which might be attributed to activation of the weak SigA-dependent promoter.

Besides containing a SigA-dependent promoter, we observed that the P\textsubscript{cap} upstream region is of repressive function. cap repression was confirmed by promoter activity assays, suggesting that the main function of the upstream promoter region is to reduce SigB-dependent promoter activity. Indeed, there are many transcription factors and TCS known to repress cap indirectly via other regulators or directly through direct DNA binding. It is likely that the P\textsubscript{cap} upstream region is targeted by many of these repressors. Here, we focused on the cap repressors SaeR, CodY and Rot for which a direct interaction with the P\textsubscript{cap} upstream region was demonstrated by EMSAs. While Sae, CodY and Rot all interfered with SigB-dependent promoter activity, only Sae additionally repressed activity of the SigA-dependent promoter. This is in line with a predicted SaeR binding site located between the two promoters (Liu et al., 2016). We hypothesize that the repression of SigB-dependent promoter activity occurs via steric interference, whereas the SigA-dependent promoter activity might be repressed via a roadblock mechanism. Nonetheless, the molecular mechanism for the long-distance effect of Rot and CodY on the SigB-dependent promoter activity remains to be elucidated. One may speculate that secondary structures of the promoter bring these two regulators in close proximity to the SigB consensus motif, allowing them to interfere with SigB binding. It is well known that DNA structural elements like supercoiling are involved in the control of bacterial gene expression (Dorman and Dorman, 2016), and cap expression was indeed shown to be supercoiling sensitive (Schröder et al., 2014).

Of note, there are two CodY binding sites within the cap locus. One reaching into the coding region of capA, consistent with previous findings (Lei and Lee, 2018), and one in the P\textsubscript{cap} upstream region. Functional assays using truncated promoter fusion constructs indicate that the upstream binding site alone is sufficient for cap repression.

SigB activity and upstream repressors determine temporal/heterogeneous CP synthesis

We showed that SigB-dependent regulation and the regulators targeting the upstream region contribute to the temporal pattern of CP production and to its phenotypic heterogeneity. Congruent with SigB being a known activator of late genes (Bischoff et al., 2001; 2004; Pane-Farre et al., 2006; Mäder et al., 2016), we observed earlier onset of CP production and more CP-positive cells in stationary phase upon constitutive sigB expression in a rsbUVWsibB-negative background. However, the greater impact on temporal and heterogeneous CP production was observed upon deletion of the upstream promoter region resulting in single CP-positive cells already in early exponential growth phase and almost all cells being CP-positive in later growth. The effects of constitutive sigB expression and P\textsubscript{cap} upstream deletion were additive, and only in combination the phenotypic heterogeneity could be completely abolished in stationary growth phase. This suggests that the regulators targeting the upstream region of P\textsubscript{cap} are mainly responsible for heterogeneous CP production, with the residual heterogeneity resulting from variable SigB activity within cells. These data support the prediction by Sharon et al. stating that more transcription factor binding sites result in noisier promoters (Sharon et al., 2014). A similar pattern of CP production was shown in the USA300 background, indicating a conserved regulatory mechanism.

Of note, even upon P\textsubscript{cap} upstream deletion and constitutive sigB expression, CP synthesis remains growth
phase-dependent which is not reflected at the transcriptional level, indicating that further post-transcriptional levels of regulation are in place. These might be required as CP synthesis is linked to the metabolic status of the cell. For instance, UDP N-acetylglucosamine used for CP biosynthesis is mainly derived from gluconeogenesis, which naturally occurs when glucose becomes limited toward later growth phases (Sadykov et al., 2010). In addition, CP, peptidoglycan and wall teichoic acids synthesis make use of the universal bactoprenol carrier lipid, which could become limited in earlier growth phases (Campbell et al., 2012). This coordination of the different cell wall polymers synthesis was recently shown to involve reversible protein phosphorylation of the capsular biosynthesis gene products CapM and CapE (Rausch et al., 2019).

In summary, our results show that CP synthesis is tightly controlled at the transcriptional level. However, post-transcriptional mechanisms are also in place to avoid conflict between precursor usage by the CP synthesis machinery and the synthesis machinery of other cell wall glycolipopolymers in growing bacterial cells. Further in-depth studies are required to fully understand this regulation and to increase the potential of CP as prospective target for novel anti-infective strategies.

**Experimental procedures**

**Bacterial strains and growth conditions**

Strains and plasmids are listed in Tables S3 and S4. For overnight culture, strains were grown in lysogeny broth (LB) medium (low salt) with appropriate antibiotics (10 μg ml⁻¹ chloramphenicol (Cm10), 10 μg ml⁻¹ erythromycin, 50 μg ml⁻¹ kanamycin, 3 μg ml⁻¹ tetracycline) at 37°C and 200 rpm. Day cultures were inoculated from overnight cultures to an OD₆₀₀ of 0.05 and were grown without antibiotics.

**Growth curves and fluorescence measurements**

Overnight cultures were diluted into an OD₆₀₀ of 0.05 and 200 μl were loaded onto a 96-well U-bottom plate (Greiner Bio-One). Continuous absorbance and fluorescence were monitored with a TECAN Infinite 200 microplate reader (Bio-One). Continuous absorbance and fluorescence were measured in the microplate reader. Absorbance was calculated and used to correct the optical densities measured in the microplate reader.

**Strain construction**

All plasmids and oligonucleotides are listed in Tables S4 and S5, respectively. The transposon mutants Newman rbsR and Newman sae rbsR were constructed by phage transduction of the transposon insertions from the Nebraska transposon mutant NE425 to Newman and Newman-29 and then verified by PCR.

Strains Newman msa, Newman ΔPcap upstream, Newman ΔPcap upstream, strong SigB, USA300 Pcap_repaired, USA300 cap repaired and USA300 ΔPcap upstream, strong SigB were created using the temperature-sensitive pMAY plasmid for allelic exchange (Monk et al., 2012). Corresponding homologous regions were PCR amplified as indicated in Table S5 and inserted into the EcoRI-linearized pMAY backbone via Gibson assembly. The resulting plasmids were verified by PCR using primers pMAYcontrolfor and pMAYcontrolrev and sequencing, and then electroporated into strain RN4220 and transduced into the target strains. Allelic exchange was performed as described before (Monk et al., 2012) with few alterations. Briefly, a single colony was homogenized in 200 μl of TSB and 50 μl of serial dilutions (10⁻¹–10⁻⁵) were plated on TSA-Cm10 and incubated overnight at 37°C. Large colonies were picked and subcultured on TSA-Cm10 at 37°C. Integrants were confirmed via PCR using primers pMAYcontrolfor and pMAYcontrolrev as well as a primer pair flanking the individual homologous regions (Table S5). One integrant colony was used to inoculate an overnight culture in 10 ml TSB grown at 28°C without chloramphenicol, and later plated on TSA containing 0.7–1 μg ml⁻¹ anhydrotetracycline. After 48 h of growth at 28°C, colonies were picked on both blood and TSA-Cm10 plates and incubated at 37°C. Cm10-sensitive colonies were screened by PCR with the oligonucleotides mentioned above and by sequencing to identify clones containing the desired mutation.

The rbsUVWsigB mutants were obtained using the temperature-sensitive shuttle vector pBASE6 (Geiger et al., 2012). Replacement was introduced by creating PCR products using RN6390 as a template and primer pairs MazSIG-for and Hybrid-MazSIG-rev, as well as MazSIGrev and Hybrid-MazSIG-for for the homologous regions. The tetracycline resistance cassette was amplified from pCG75 using primers TetMfor and TetMrrev. PCR products were linked using primers BglIIMazSIGfor and SalIMazSIGrev and inserted into Gibson assembly. The resulting plasmids were verified by PCR with the oligonucleotides mentioned above and by sequencing to identify clones containing the desired mutation.

The rbsUVWsigB strains were constructed by phage transduction of the transposon insertions from the Nebraska transposon mutant NE425 to Newman and Newman-29 and then verified by PCR.

Strains Newman msa, Newman ΔPcap upstream, Newman ΔPcap upstream, strong SigB, USA300 Pcap_repaired, USA300 cap repaired and USA300 ΔPcap upstream, strong SigB were created using the temperature-sensitive pMAY plasmid for allelic exchange (Monk et al., 2012). Corresponding homologous regions were PCR amplified as indicated in Table S5 and inserted into the EcoRI-linearized pMAY backbone via Gibson assembly. The resulting plasmids were verified by PCR using primers pMAYcontrolfor and pMAYcontrolrev and sequencing, and then electroporated into strain RN4220 and transduced into the target strains. Allelic exchange was performed as described before (Monk et al., 2012) with few alterations. Briefly, a single colony was homogenized in 200 μl of TSB and 50 μl of serial dilutions (10⁻¹–10⁻⁵) were plated on TSA-Cm10 and incubated overnight at 37°C. Large colonies were picked and subcultured on TSA-Cm10 at 37°C. Integrants were confirmed via PCR using primers pMAYcontrolfor and pMAYcontrolrev as well as a primer pair flanking the individual homologous regions (Table S5). One integrant colony was used to inoculate an overnight culture in 10 ml TSB grown at 28°C without chloramphenicol, and later plated on TSA containing 0.7–1 μg ml⁻¹ anhydrotetracycline. After 48 h of growth at 28°C, colonies were picked on both blood and TSA-Cm10 plates and incubated at 37°C. Cm10-sensitive colonies were screened by PCR with the oligonucleotides mentioned above and by sequencing to identify clones containing the desired mutation.

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et al., 2009) into Newman-29, followed by transduction of the rot::bursa aurealis transposon insertions from the Nebraska transposon mutant NE386.

For the generation of a cap mutant in USA300 JE2, pCG132 was transduced from RN4220 pCG132 (Jansen et al., 2013) into USA300 JE2 using phage φ11.

**Promoter fusion constructs**

Two tandem sequences, each comprising a promoter of interest (Pgenep) followed by a strong ribosomal binding site (RBS), genes encoding for fluorescent protein gpVenus (gopenv) or gpCerulean (gopenp) (Liese et al., 2013) and a terminator (ter) sequence, were designed in silico. Restriction sites were designed to flank both promoter replacements. The entire cassette encompassing Pcap-RBS-gpVenus-ter and PtagH-RBS-gpCerulean-ter was also flanked by restriction sites. The dual promoter-reporter protein fusion cassette was synthesized by Life Technologies GmbH and provided in a pUC18-like E. coli vector backbone (pMA-RQ, geneart plasmid). The dual promoter-reporter fusion cassette was subcloned into pCG246 (Helle et al., 2011) using restriction sites Sphi and NarI. The resulting plasmid pCG570 was verified by restriction digestion (BamHI) and using the primers pCG246for, pCG246rev, Pcap_rev and PtagHrev for sequencing. The plasmid pCG570 was introduced into RN4220 and then transduced into strain Newman.

Promoter truncations and mutations were generated with the Q5 Site-Directed Mutagenesis Kit from NEB according to the manufacturer's instructions. All primers used are enlisted in Table S5. For plasmids pCG742, pCG815 and pCG816, PtagH was replaced with Pasp23 via Gibson cloning. The asp23 promoter region was amplified from Newman genomic DNA using primers pCG657gibfor and pCG657gibrev and inserted into BamHI and EcoRI digested pCG717, pCG719 and pCG769.

**RNA isolation**

A day culture was inoculated from an overnight culture and grown to an OD600 of 0.3. The day culture was then used to inoculate a second day culture to an OD600 of 0.05, which was grown to an OD600 of 0.3 and used again for subculturing. When the third day culture reached an OD600 of 0.3, 10 ml of cell suspension was harvested (T0). After additional 2 h (T1) and 4 h (T2) of growth, 5 ml of S. aureus cells were harvested. Cells were lysed in 1 ml TRIzol reagent (Life Technologies, Germany) with 0.5 ml of zirconia–silica beads (0.1 mm diameter) in a high-speed homogenizer. RNA was isolated as described in the instructions provided by the manufacturer of TRIzol (Life Technologies).

**5’ Rapid Amplification of cDNA Endings (5’ RACE)**

5’ RACE was performed as described previously (Marincola et al., 2012) using strain Newman pCG717. Briefly, isolated RNA from T2 was treated with MICROBExpress (Ambion) in order to remove rRNAs. After treatment with Cap-Clip Phosphatase (Bionym) to remove pyrophosphate at the 5’ prime end of the native transcripts, a specific RNA 5’ adapter (Table S5) was ligated to the RNA. After phenol/chloroform extraction and ethanol precipitation, the RNA was subjected to reverse transcription using oligonucleotide YFPCCFPpolymerase. Nested PCR was performed using oligonucleotides Race2 and Racecapnestededrev (Table S5). The PCR amplicon was cloned into pCRII-TOPO (Invitrogen) following the manufacturer’s instructions. Single clones were analyzed via PCR using primers Race2 and Racecapnestededrev and the PCR products of 10 clones were sequenced with primer Racecapnestededrev.

**qRT-PCR**

qRT-PCR to quantify cap and gyr mRNA was performed using the QuantiFast SYBR-Green RT-PCR kit (Qiagen). Standard curves were generated using 10-fold serial dilutions (10**5–10**8 copies) of specific in vitro-transcribed RNA standard molecules (Burian et al., 2010). The number of copies of each transcript was determined with the aid of the LightCycler software and cap mRNA was expressed in reference to copies of gyrB.

**Protein expression**

Plasmid pCWsae106 containing SaeR<sup>Δ51E</sup> was created by overlapping PCR employing the oligonucleotides saetetfor1 and 1680I29Gluok as well as 1684U31 and saetetfor1. The amplicon was inserted into pCR2.1. pCWsae106 was then transfected into RN4220 and transduced into strain Newman-29. Coagulase assays were performed to verify the function of the phosphomimetic SaeR<sup>Δ51E</sup> Strain Newman-29 pCWsae106 was used as a template to generate the insert for the SaeR<sup>Δ51E</sup> expression vector with the primers pCG791gibfor and pCG791gibrev. The amplicon was inserted into BamHI-linearized pET15b via Gibson assembly. All other expression vectors were generated accordingly using primers and template DNA as indicated in Table S5. All vectors were verified via PCR and sequencing using primers pET15bfor and pET15brev.

A 10 ml LB culture containing 100 µg ml**−1** ampicillin was inoculated with freshly transformed E. coli BL21 DE3 cells and incubated for 6 h at 37°C and 200 rpm. For the expression culture, 1 L LB medium supplemented with 100 µg ml**−1** ampicillin and IPTG or D(-)-lactose monohydrate (for expression conditions see Table S6) was inoculated with 10 ml day culture and incubated at 16°C and 200 rpm overnight (16–18 h). Cells were harvested (20 min, 2000×g, 4°C) and resuspended in 30 ml ice-cold HisTrap binding buffer (20 mM Na<sub>2</sub>HPO<sub>4</sub>, 500 mM NaCl, 40 mM imidazole, pH 7.4, sterile filtered and degassed) supplemented with 10 µg ml**−1** DNAse (Roth) and cComplete protease inhibitor cocktail (Roche). Cells were lysed using a French press at 1000 psi. The lysate was centrifuged (236 982×g, 45 min, 4°C), and the clear supernatant was filtered (0.22 µm pore size) before being loaded onto a 1 ml HisTrap HP column (GE Healthcare Life Sciences) equilibrated with HisTrap binding buffer. Purification was performed with an ÄKTA purification system (GE Healthcare Life Sciences), and elution was carried out with an imidazole gradient to a final concentration of 500 mM.

For SaeR<sup>Δ51E</sup> batch purification using Ni-NTA agarose resin (Qiagen) equilibrated with HisTrap binding buffer was
performed. After incubation for 1 h at constant overhead rotation at 4°C the Ni-NTA agarose was centrifuged at 500× g for 2 min at 4°C and washed thrice with 37.5 ml HisTrap binding buffer. Elution was performed at increasing imidazole concentrations (250/300/350/500 mM).

Column and batch fractions were analyzed by SDS-PAGE, and the fractions containing the protein of interest were collected, concentrated and rebuffered into EMSA buffer (10 mM Tris-HCl, 50 mM KCl, 5 mM MgCl₂, 10% glycerol, pH 7.4) using Amicon® ultra centrifugal filters (ULTRACEL® 10K/30K) according to the instructions of the manufacturer. To determine the protein concentration of purified proteins, a Pierce™ BCA assay (Thermo Fisher) was performed following the manufacturer’s protocol for a microplate procedure. The absorbance was measured with the TECAN Infinite 200 microplate reader. Purified proteins were stored in aliquots at −20°C.

**Electrophoretic Mobility Shift Assay (EMSA)**

The primers used for EMSAs are listed in Table S5. DNA probes were PCR amplified from strain Newman with fluorescently labeled primers (DY-781, absorption: 783 nm, emission: 800 nm) and purified with the innuPREP PCRpure Kit from Analytik Jena AG according to the manufacturer’s instructions. In a volume of 20 μl, 30 fmol DNA probe were mixed with various amounts of protein. For competition experiments, unlabeled DNA fragment with a sequence identical to the labeled probe was used as a specific competitor and a 161 bp DNA fragment containing the promoter of the 16S rRNA gene was used as an unspecific competitor. Competitors were obtained via ethanol precipitation after PCR and added in 100-fold excess. After incubation for 20 min at room temperature, samples were analyzed by nondenaturing native 6% TBE polyacrylamide gel electrophoresis at 75 V for 90 min in 1x TBE buffer. The fluorescently labeled DNA probes were visualized in the polyacrylamide gel with the Odyssey infrared imaging system (LI-COR) and the Image Studio 4.0 software.

**IF and promoter fusion microscopy**

For synchronization, three subsequent day cultures were inoculated to an OD₆₀₀ of 0.05, grown to an OD₆₀₀ of 0.3 and subcultured twice. When the third subculture reached an OD₆₀₀ of 0.3 (T₀) and after additional 2 h (T₁), 4 h (T₂), 6.5 h (T₃) and overnight growth (T₄) cells were harvested by centrifugation at 3800× g for 10 min at 4°C. Roughly 1.3 × 10⁸ bacteria were suspended in 1 ml fixation solution (3.7% formaldehyde in 1x PBS) and incubated with gentle mixing for 15 min at room temperature. Wells of Ibi-treat µ-slide angiogenesis slides (ibidi®) were loaded with 32 μl of cell suspension and centrifuged at 600× g for 6 min. The cells were washed with 1 x PBS and protein A was blocked by incubation with pre-adsorbed human serum (diluted 1:10 in 1x PBS/0.1% Tween 20) for 1 h. After blocking, the slides were washed thrice with PBS/Tween 20 followed by incubation with rabbit serum raised against CP5 (1 h, diluted 1:200 in PBS/Tween 20). The slides were washed thrice with PBS/Tween 20 followed by incubation with the secondary antibody Cy3-conjugated F(ab)2 goat-anti-rabbit IgG (Dianova, Hamburg) (diluted 1:500 in PBS/Tween 20, 1 h). For generation of pre-adsorbed human serum and antibody generation, see (George et al. 2015). After washing the cells thrice with PBS/Tween 20 each slide was finally mounted using ibidi® fluorescence mounting medium.

Microscopy images were acquired in the confocal mode of an inverted Zeiss LSM 710 NLO microscope equipped with a spectral detector and a Zeiss Plan-Apochromat 63x/1.40 oil DIC M27 objective and ZEN Black software. The following excitation wavelength, laser sources and detection spectra were used for IF: Cy3: Ex: 561 nm/DPSS laser/Em: 566–702 nm and for promoter activity measurement: gpVenus: Ex: 514 nm/argon laser/Em: 519–554 nm, gpCerulan: Ex: 405 nm/diode laser/Em: 454–516 nm. Additionally, a bright-field image was captured. The images were exported in the single channels or as overlays as 16-bit tagged image files after equal adjustment for gain and color intensity within one experiment. For each image about 100 bacteria as detected by bright field were randomly selected and CP-positive bacteria of the corresponding IF image enumerated. Mean percentage of CP-positive bacteria and standard deviations of three biological replicates is given in Tables S1 and S2.

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**Author contributions**

DK, SG and CWo have made major contributions to the conception and design of the study; DK, AS, ADJ, SG, GM, JL, CWe and CWo have made major contributions to the acquisition, analysis or interpretation of the data; DK and CWo have made major contributions to writing the manuscript.

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