Universidade Estadual de Campinas
Faculdade de Odontologia de Piracicaba

LÍVIA ARAUJO ALVES

Estudos de fatores regulados por CovR e VicRK envolvidos na susceptibilidade à opsonização pelo sistema complemento e na virulência sistêmica de *Streptococcus mutans*

Analysis of factors regulated by CovR and VicRK involved in the susceptibility to opsonization by the complement system and systemic virulence in *Streptococcus mutans*

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Orientadora: Profª. Drª. Renata de Oliveira Mattos Graner

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A Ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

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Por ter me dado forças e me conduzido até aqui.
"Eu posso ir, muito além de onde estou.
O teu amor é o que me conduz.
Pois confio no Senhor".
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Não é sobre chegar no topo do mundo
E saber que venceu
É sobre escalar e sentir
Que o caminho te fortaleceu.
É saber se sentir infinito
Num universo tão vasto e bonito
É saber sonhar
E, então, fazer valer a pena cada verso
Daquele poema
Sobre acreditar.

Trecho da Canção "Trem Bala", de autoria de Ana Vilela

RESUMO

Streptococcus mutans (SM) é uma espécie bacteriana comum da cavidade bucal de humanos envolvida na patogênese da cárie dental, a qual pode promover infecções sistêmicas ao atingir e persistir na corrente sanguínea (Nomura *et al.*, 2006). Para persistir nos nichos do hospedeiro, SM utiliza sistemas reguladores transcricionais de dois componentes (SDCs). Os SDCs CovR e VicRK de SM regulam genes de virulência associados à ligação a polissacarídeos extracelulares (PEC) produzidos a partir da sacarose (g*bpB*, *gbpC* e *epsC*) e/ou na interação da parede celular com a matriz de biofilmes (*wapE, lysM, 2146c, smaA*). A inativação destes SDCs promove resistência à fagocitose por PMN na presença de sangue (Negrini *et al.,*2012), indicando papel da regulação de funções de escape a opsoninas do sangue, como o sistema complemento. O objetivo deste trabalho foi identificar os mecanismos moleculares através dos quais CovR e VicRK modulam a susceptibilidade de SM à imunidade mediada pelo sistema complemento e a sobrevivência em sangue. Os resultados obtidos foram incluídos em três artigos científicos apresentados em capítulos. No capítulo I, caracterizamos o perfil de susceptibilidade ao complemento na espécie SM. Análises de citometria de fluxo e de RT-PCRq revelaram que cepas isoladas de infecções sistêmicas têm menor susceptibilidade à opsonização pelo complemento e expressão reduzida de *covR* comparadas a isolados bucais. A inativação de *covR* em SM UA159 aumentou a resistência à opsonofagocitose por PMN mediada pelo complemento, de forma dependente da ligação a PEC, e aumentou a sobrevivência em sangue humano *ex vivo* e na corrente sanguínea de ratos. Isolados de sangue mostraram expressão significativamente maior de genes reprimidos por CovR (*gbpC* e *epsC*) e a maior ligação a PEC. Consistentemente, a inativação de *gbpC* e *epsC* reduziu a ligação a PEC e aumentou a susceptibilidade ao complemento, estabelecendo a função destes genes no escape à opsonofagocitose. No capítulo II, mostramos que VicRK regula a susceptibilidade de SM ao complemento de forma independente de PECs. A inativação de *vicK* promoveu resistência à opsonização pelo complemento. Análises transcricionais indicaram que VicR reprime a expressão de proteases Smu.399 e PepO e de proteínas enolase e gliceraldeído-3-fosfato desidrogenase de superfície (GAPDH), implicadas na virulência de estreptococos. A inativação de *smu.399* e *pepO* aumentou a susceptibilidade ao complemento, revelando papel na virulência. No capítulo III, demonstramos que CovR e VicRK regulam a proteína ligadora de colágeno Cnm, associada com à virulência por mecanismos não completamente conhecidos. Análises transcricionais e de interação entre proteínas recombinantes rVicR e rCovR com a região promotora de *cnm* demonstraram que CovR e VicR atuam respectivamente, como indutor e repressor de *cnm*. Os mutantes *covR* e *vicK* demonstraram diversos

fenótipos compatíveis com as alterações na expressão de *cnm*. Assim, estabelecemos que os SDCs CovR e VicRK regulam múltiplos fatores de escape à opsonização pelo sistema complemento e/ou na sobrevivência em sangue. Estes resultados contribuem para a definição de alvos terapêuticos para controle das infecções sistêmicas por esta espécie.

Palavras-chave: *Streptococcus mutans*, Endocardite bacteriana, Proteínas do Sistema Complemento.

ABSTRACT

Streptococcus mutans (SM) is a common bacterial species of the oral cavity of humans involved in the pathogenesis of dental caries, which can promote systemic infections once reaching and persisting in the bloodstream (Nomura *et al.*, 2006). To persist in the host niches, SM applies two-component transcriptional regulatory systems (TCS). The TCS CovR and VicRK of SM regulate virulence genes associated with SM-binding to extracellular polysaccharides (EPS) produced from sucrose (*gpbB, gbpC* and *epsC*) and /or involved in the interaction of the cell wall with the biofilm matrix (*wapE, lysM, 2146c, smaA*). Inactivation of this TCS promotes resistance to phagocytosis by PMN when in the presence of blood (Negrini *et al.,* 2012), indicating that these systems regulate genes for evasion to blood opsonins, such as the complement system. The aim of this study was to identify the molecular mechanisms through CovR and VicRK modulate the susceptibility of *S. mutans* to complement mediated immunity and survival in the bloodstream. The results obtained were included in three scientific articles which are presented as chapters. In chapter I, we characterize the profile of SM strains susceptibilities to the complement. Flow cytometry and RT-PCR analysis revealed that strains isolated from systemic infections show lower susceptibility to complement deposition and reduced expression of *covR* compared to oral isolates. Inactivation of *covR* in SM UA159 increased resistance to complement mediated opsonophagocytosis by PMN in a way dependent on binding to EPS, *ex vivo* survival in human blood and in the bloodstream of rats. Blood isolates showed significantly higher expression of CovR-repressed genes (*gbpC* and *epsC*) and, consistently, increased binding to EPS. Consistently, inactivation of *gbpC* or *epsC* reduced binding to EPS and increased susceptibility to complement. In Chapter II, we show that VicRK regulates SM susceptibility to complement-mediated immunity, in a EPS-independent way. Inactivation of *vicK* increased resistance to complement opsonization. VicRK was shown to directly or indirectly repress the expression of Smu.399 and PepO complement proteases and the enolase and surface glyceraldehyde-3-phosphate dehydrogenase (GAPDH) proteins implicated in systemic virulence in other streptococci. Finally, in Chapter III, we established that CovR and VicRK directly regulate the Cnm protein, a which is important for systemic virulence in a sub-set of strains harboring *cnm* gene. Transcriptional analyses and assessment of interaction of recombinant proteins rCovR and rVicR with the *cnm* promoter region established that CovR acts as an inducer of *cnm*, whereas *vicR* acts as a repressor. Cnm-mediated virulence phenotypes were significantly associated with lower and higher *cnm* expression in the *covR* and *vicK* mutants, respectively. Thus, we showed that the TCSs CovR and VicRK regulate several systemic virulence factors dependent and independent of the interaction with

EPS, involved in evasion to complement-mediated immunity and survival in blood. These results further confirm the importance of SM in systemic infections and contribute to the definition of therapeutic targets for controlling these infections.

Keywords: *Streptococcus mutans*, Bacterial endocarditis, Complement system proteins.

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1 INTROODUÇÃO

Streptococcus mutans é uma espécie comum da microbiota bucal envolvida na patogenia da cárie dentária (Mattos-Graner *et al*., 2014; Klein *et al.,* 2015). Esta espécie expressa diversos genes de virulência envolvidos no aumento da biomassa do biofilme dentário (a placa dental), através da síntese de polissacarídeos extracelulares (PEC) altamente estáveis a partir da sacarose e por promover quedas duradouras de pH local. Estas funções favorecem a emergência de microrganismos acidogênicos e ácido-tolerantes no biofilme, cuja capacidade de produzir a desmineralização dos dentes é aumentada (Koo *et al.,* 2013).

Além de seu importante papel na patogênese da cárie, *S. mutans* também são reconhecidos como importantes patógenos oportunistas de infecções sistêmicas, incluindo-se bacteremias, ateromatose e endocardites infecciosas (EI) (Nakano *et al*., 2009). Embora diversos mecanismos de virulência implicados na cariogenicidade de *S. mutans* tenham sido identificados (Banas *et al*., 2011; Lemos *et al.,* 2013; Klein *et al.,* 2015), pouco se sabe ainda sobre os mecanismos implicados na capacidade destes microrganismos causar infecções sistêmicas (Nakano *et al*., 2009; Abranches *et al*., 2011). Entretanto, estudos em diferentes populações indicam que *S. mutans* é uma das espécies bucais mais prevalentemente encontradas em espécimes de válvulas cardíacas de pacientes com endocardite infecciosa e em ateromas (Nakano *et al.,* 2006; Fernandes *et al.,* 2014). O DNA de *S. mutans* foi detectado em mais de 68,6% de espécimes de válvula cardíaca de pacientes com EI e em 74,1% de ateromas removidos em cirurgias cardiovasculares, sendo estes porcentuais significativamente superiores aos de outras espécies de estreptococos comuns da cavidade bucal e de periodontopatógenos, como *Porphyromonas gingivalis* e *Treponema denticola* (Nakano *et al.,* 2006). Mais recentemente no Brasil, *S. mutans* foi detectado em 100% das amostras de ateromas obtidas de 13 pacientes submetidos a cirurgias cardiovasculares, prevalência significativamente superior às de *P. gingivalis* e *T. denticola* (Fernandes *et al*., 2014). Estes dados indicam que *S. mutans* não apenas tem frequente acesso à corrente sanguínea a partir de nichos bucais, mas é capaz de resistir a mecanismos de defesa presentes na corrente sanguínea, para se estabelecer nos tecidos cardiovasculares.

S. mutans são classificados nos sorotipos *c, e, f,* e *k*, os quais refletem a composição dos polissacarídeos de ramnose-glicose ancorados à parede celular (Nakano & Ooshima, 2009). A maioria dos isolados de *S. mutans* da cavidade bucal é do sorotipo *c* (70-80%), sendo os sorotipos

e, f e *k* encontrados em menor frequência (20, 5 e 2-5%, respectivamente). O sorotipo *k* e outros sorotipos diferentes de *c* aumentam em proporção entre isolados de tecido cardíaco, mas o sorotipo *c* é ainda predominante em amostras de EI (Nakano & Ooshima, 2009). Cepas de *S. mutans* do sorotipo *c* foram detectadas em cerca de 30,3 e 65,6% dos espécimes de válvula cardíaca e ateromas de pacientes submetidos a cirurgias cardiovasculares. Entre os isolados do sorotipo *k*, estas prevalências foram 9,1 e 25%, respectivamente (Nakano & Ooshima, 2009). Além disto, cepas de *S. mutans* apresentam a proteína Cnm (detectada em 20% de 102 isolados clínicos analisados), a qual é encontrada com maior frequência entre os sorotipos *f* e *k* (81,3 e 41,7%, respectivamente) e em apenas 7% no sorotipo *c* (Nakano *et al*., 2007). Cnm (c*ollagen binding protein of S. mutans)* é reconhecida como importante fator de virulência sistêmica em *S. mutans* e medeia a ligação a componentes da matriz extracelular, como colágeno e laminina. Ainda, Cnm está envolvida na invasão de células endoteliais (Abranches *et al*., 2011; Nomura *et al*., 2014), na agregação plaquetária e formação de vegetações em válvulas cardíacas em modelo animal (Nakano *et al.,* 2007). Cnm é, portanto, um fator de virulência cepa- e sorotipo-específico na espécie *S. mutans*. Embora a frequência de *cnm* seja rara no sorotipo *c* (Nakano *et al*., 2007), este é frequentemente isolados de amostras cardíacas (Nakano & Ooshima, 2009), sugerindo que *S. mutans* apresentam proteínas adicionais de virulência sistêmicas e independentes de Cnm. Assim, identificamos no genoma de *S. mutans* UA159 (sorotipo *c*) os genes *smu.399* e *pepO* (*smu.2036*) e, as proteínas *moonlight* enolase (*smu.1247*) e a gliceraldeído-3 fosfato desidrogenase (GAPDH) de superfície (*smu.360*). Em outras espécies de estreptococos, com exceção da Smu.399, as proteínas PepO, enolase e GAPDH, quando presentes na superfície celular, estão envolvidas na interação com componentes do hospedeiro, como plasminogênio e fibronectina (Pancholi, 2001; Bergmann *et al*., 2004; Madureira *et al*., 2007; Agarwal e*t al*., 2013). Estes genes também foram identificados no genoma de todas as cepas *S. mutans* com genoma conhecido (Cornejo *et al*., 2013), indicando importância biológica. Os mecanismos e sistemas envolvidos na regulação de fatores dependentes e independentes de Cnm ainda não foram investigados em *S. mutans*.

Durante o processo de invasão e colonização do hospedeiro, as bactérias se adaptam rapidamente a estresses ambientais, os quais incluem inúmeros fatores de defesa do hospedeiro (Stephenson & Hoch, 2002). Para isto, empregam sistemas de transdução de sinal designados Sistemas de Dois Componentes (SDCs) (Raghavan & Groisman, 2010; Capra & Laub, 2012). Um SDC típico é formado por uma proteína sensora de membrana histidina quinase (K) e uma proteína

intracelular cognata capaz de se ligar a regiões reguladoras dos genes alvo (regulador de resposta, R). Ao detectar um estímulo ambiental, comumente localizado no meio externo ou na membrana citoplasmática, a proteína sensora sofre auto-fosforilação em um resíduo conservado de histidina localizada na porção intracelular. O grupo fosfato adquirido é subsequentemente transferido ao regulador de resposta cognato, o qual sofre alterações na sua conformação, ligando-se a sequências consenso das regiões promotoras dos genes alvo, os quais têm sua transcrição induzida ou reprimida (Capra & Laub, 2012).

Os genomas de cepas *S. mutans* apresentam entre 13 e 14 SDC e pelo menos um R órfão, designado CovR, cuja proteína sensora cognata não foi identificada nesta espécie. Há grande interesse no estudo dos SDCs VicRK e CovR em *S. mutans*, porque estes sistemas regulam diretamente genes de virulência associados à formação de biofilmes cariogênicos (Mattos-Graner & Duncan, 2017). Dentre estes, VicRK induz os genes que codificam enzimas glucosiltranferases B (*gtfB*) e C (*gtfC*), frutosiltransferase (*ftf*), assim como a proteína ligadora de glucano B (*gbpB*), a qual está envolvida na capacidade de *S. mutans* se ligar à matriz extracelular de glucano (Senadheera *et al.,* 2005; Duque *et al.,* 2011; Stipp *et al.*, 2013). O SDC VicRK regula ainda diveras hidrolases de peptideoglicano (mureína), as quais aparentemente estão envolvidas em diversos processos fisiológicos importantes para o crescimento em fase planctônica e em biofilmes, como a divisão da parede celular, liberação de DNA genômico para o meio externo e a manutenção da homeostase de parede celular (Senadheera *et al.,* 2012; Stipp *et al.,* 2013). Uma vez que a parede celular de bactérias Gram-positivas é o principal alvo dos mecanismos de defesa inato e adaptativo do hospedeiro, é possível que o SDC VicRK desempenhe papel importante para a sobrevivência de *S. mutans* nos nichos do hospedeiro. Estudos do nosso grupo demonstraram ainda que a proteína reguladora VicR deste sistema é capaz de se ligar à sequências promotoras dos seus genes alvo juntamente com o regulador órfão CovR (Stipp *et al*., 2013).

CovR é um regulador conservado em diversas espécies de patógenos do gênero estreptococos como parte do SDC CovRS. Entretanto em *S. mutans* CovR é um regulador órfão pois não foi identificada a proteína sensora cognata CovS (Mattos-Graner & Duncan, 2017). CovR atua como um regulador atípico, pois diferente das maioria dos R que induzem a transcrição dos genes alvo, CovR funciona como uma regulador negativo (repressor). A proteína reguladora CovR foi originalmente identificada em *Streptococcus pyogenes* como um repressor de genes para a síntese de cápsula da ácido hialurônico (Levin & Wessels, 1998) cuja composição é

molecularmente idêntica ao ácido hialurônico dos tecidos do hospedeiro, e a qual tem função crucial no escape de *S. pyogenes* à fagocitose por neutrófilos mediada por opsoninas da imunidade inata (por exemplo o sistema complemento) e adapativa (anticorpos IgG e IgM). A designação CovR (cov de *control of virulence*) foi proposta com base em dados posteriores de que CovR reprime diretamente diversos genes de virulência de *S. pyogenes* envolvidos no escape ao sistema complemento, à fagocitose e na conversão do plasminogênio em plasmina (Federle *et al.,* 1999). Em *S. mutans*, CovR foi originalmente identificado como um repressor do gene que codifica a proteína ligadora de glucano C (GbpC), uma proteína ancorada à parede celular requerida para a ligação de *S. mutans* a glucanos (Sato *et al*., 2000). Posteriormente, estabeleceu-se que CovR reprime diretamente os genes *gtfB/C, ftf, gpbB* assim como os genes *epsC* e *lysM* envolvidos na interação de *S. mutans* à matriz extracelular polissacarídica de biofilmes e na homeostase da parede celular (Biswas *et al.*, 2006; Dimitriev *et al.*, 2011; Stipp *et al*., 2013).

Portanto, ambos os SDCs VicRK e CovR parecem atuar conjuntamente na regulação da transcrição de diversos genes de *S. mutans* envolvidos na síntese e interação com polissacarídeos extracelulares sintetizados a partir da sacarose (*gtfB*/*C*, *ftf*, *gbpB*), o primeiro atuando como indutor e outro como repressor destes genes. Além disto, ambos os sistemas regulam diversos genes envolvidos na biogênese e homeostase da parede celular, os quais codificam enzimas com atividades líticas de parede celular incluindo-se os genes que codificam as proteínas WapE, SmaA, Smu.2146c, LysM e/ou EpsC (Stipp *et al.,* 2013). A inativação de cada um destes genes em *S. mutans* promoveu defeitos na formação de biofilmes na presença de sacarose e/ou prejudicou a integridade da parede celular durante o crescimento planctônico, com liberação de DNA para o meio extracelular (Stipp *et al.,* 2013). Assim, iremos investigar também neste trabalho, a hipótese de que estes genes estejam envolvidos no escape de *S. mutans* a fatores de defesa do hospedeiro que interagem com a parede celular.

Em *S. mutans*, a inativação dos sistemas CovR e VicRK promove resistência à fagocitose por PMN de sangue periférico humano, de forma dependente da presença de sangue (Negrini *et al.,* 2012), indicando que estes sistemas regulam genes de escape a opsoninas presentes no sangue. O sistema complemento consiste numa das principais defesas da imunidade inata presente nos fluidos corpóreos para a rápida eliminação de microrganismos e potencializa também funções efetoras mediadas por anticorpos (Walport, 2001). Este sistema é composto por aproximadamente 30 proteínas, incluindo proteínas solúveis e de membrana. As proteínas solúveis são

imediatamente ativadas após o contato com a superfície bacteriana, gerando uma cascata proteolítica de ativação, que culmina nas suas funções efetoras, as quais incluem opsonização e fagocitose, citólise mediada pelo complemento (formação do complexo de ataque à membrana – CAM) e produção de mediadores inflamatórios (Merle *et al*., 2015). As proteínas solúveis do complemento compõem as diferentes cascatas proteolíticas que ativam as proteínas do CAM (C5 a C9), os fatores opsonizantes (C3b/iC3b, C4b) e fatores quimiotáticos/inflamatórios (C3a, C4a, C5a). Além disto, o sistema complemento inclui receptores expressos por fagócitos e outras células (incluíndo-se CR1 e CR3 em PMN) e proteínas reguladoras (inibidoras) solúveis de fase fluída (por exemplo, Fator H e C4BP) ou de membrana (por exemplo, DAF e MCP), as quais impedem a ativação e/ou funções efetoras do complemento sobre células próprias (Holers *et al.*, 2014). Há três principais formas de ativação da cascata proteolítica do complemento, as quais envolvem diferentes proteínas iniciais, a via clássica (proteínas C1q (C1r/C1s) e/ou CRP/petraxinas, C4, C2), a via das lectinas (MBL, Ficolinas (FCN): FCN1 (Ficolina-M), FCN2 (Ficolina-L) e FCN3 (Ficolina-H), C4, C2) e a via alternativa (C3, fator B e D) (Walport, 2001; Ricklin & Lambris, 2007).

A ativação da via clássica ocorre pela ligação da proteína C1q às regiões Fc dos anticorpos IgM ou IgG (Kang *et al.,* 2006), sendo esta via denominada com frequência como depedente de anticorpos. As vias alternativas e da lectina são ativadas na ausência de anticorpos, fazendo parte da imunidade inespecífica (inata) do organismo. Na via alternativa, basta a presença no patógeno e de determinadas características químicas, como a ausência de ácido siálico e de proteínas reguladoras de superfície, para que a via alternativa seja desencadeada. Um dos resultados mais importantes da ativação destas cascatas para a eliminação de bactérias Gram-positivas, como *S. mutans*, é a geração das enzimas convertases de C3 das vias clássica/lectina (convertase C4b2b) ou alternativa (convertase C3bBb), as quais amplificam a clivagem de C3 para ligação covalente do fragmento C3b (ativo ou inativo: iC3b) à parede celular bacteriana. C3b/iC3b atuam como opsoninas, favorecendo o rápido reconhecimento e eliminação destas bactérias por fagócitos, através dos seus receptores CR1/CR3 de C3b/iC3b (processo designado opsonofagocitose).

Tipicamente, espécies bacterianas patogênicas expressam um conjunto de fatores de escape ao sistema complemento (Lambris *et al*., 2008; Zipfel & Skerka, 2009). Estudos em *Streptococcus pneumoniae*, indicam que hidrolases de mureína estão envolvidas no escape ao sistema complemento e à opsonofagocitose através de diversos mecanismos, como a interferência

na ligação de fatores ativadores do sistema complemento à superfície bacteriana (por exemplo, proteína C-reativa), clivagem de C3b do complemento ou ainda ligação a proteínas reguladoras do complemento de fase fluída (Ramos-Sevillano *et al*., 2011; Ramos-Sevillano *et al.,* 2015). A síntese de cápsula é outro importante fator inibidor da opsonização por C3b (Yuste *et al*., 2008; Hyams *et al*., 2013). Em *S. mutans*, os SDCs VicRK e CovR regulam a síntese e interação com polissacarídeos extracelulares a partir da sacarose (glucanos) durante a formação de biofilme (Duque *et al*., 2011; Stipp *et al*., 2013). Outras proteínas de superfície que interagem com componentes do hospedeiro ou com polissacarídeos extracelulares podem ainda participar do escape ao complemento, como as proteínas PAc (proteína antígeno c: *protein antigen c*) e Gbps (proteínas ligadoras de glucano: *Glucan-binding proteins*), as quais são consideradas fatores potenciais de resistência à fagocitose por PMNs, por mecanismos não conhecidos (Nomura *et al*., 2004; Nakano *et al*., 2006). Como mencionado anteriormente, os SDCs CovR e VicRK também estão envolvidos na regulação das Gbps (Stipp *et al.,* 2013).

Como primeira etapa para investigar a capacidade de *S. mutans* em escapar do sistema complemento, verificamos que a inativação de *covR* e *vicK* reduz a deposição de C3b sobre a superfície de *S. mutans* (Alves, 2014 – dissertação de mestrado). Estes dados indicaram que genes regulados por estes sistemas devem ser importantes para o escape de *S. mutans* à opsonofagocitose e consequentemente para sua sobrevivência na corrente sanguínea e/o estabelecimento em tecidos internos. Assim, o objetivo geral deste trabalho de doutorado foi identificar os mecanismos moleculares através dos quais VicRK e CovR modulam a susceptibilidade de *S. mutans* à imunidade mediada pelo sistema complemento, opsonofagocitose e a sobrevivência na corrente sanguínea.

Resultados deste trabalho de doutorado foram publicados em dois artigos científicos (Alves *et al*., 2016; Alves *et al.,* 2017) e mais um artigo submetido (Alves *et al.,* 2017b). Os três primeiros artigos serão apresentados em capítulos. No capítulo I, caracterizamos os fatores regulados por CovR que interferem na susceptibilidade de *S. mutans* ao sistema complemento e sobrevivência em sangue humano (Alves *et al*, 2016). No capítulo II, os fatores regulados pelo sistema VicRK que afetam a susceptibilidade de *S. mutans* à imunidade mediada pelo sistema complemento (Alves *et al.,* 2017). Por fim, no capítulo III, mostramos que os SDCs CovR e VicRK regulam também a proteína Cnm, associada à virulência sistêmica de *S. mutans*.

2 ARTIGOS

2.1 Artigo 1: CovR regulates *Streptococcus mutans* susceptibility to complement immunity and survival in blood.

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Running title: *S. mutans* susceptibility to complement immunity

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CovR Regulates *Streptococcus mutans* Susceptibility To Complement Immunity and Survival in Blood

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Streptococcus mutans*, a major pathogen of dental caries, may promote systemic infections after accessing the bloodstream from oral niches. In this study, we investigate pathways of complement immunity against *S. mutans* and show that the orphan regulator CovR ($CovR_{Sm}$) modulates susceptibility to complement opsonization and survival in blood. *S. mutans* blood isolates showed reduced susceptibility to C3b deposition compared to oral isolates. Reduced expression of $covR_{Sm}$ in blood strains was associated with increased transcription of $CovR_{Sm}$-repressed genes required for *S. mutans* interactions with glucans (*gbpC*, *gbpB*, and *epsC*), sucrose-derived exopolysaccharides (EPS). Consistently, blood strains showed an increased capacity to bind glucan *in vitro*. Deletion of $covR_{Sm}$ in strain UA159 (UAcov) impaired C3b deposition and binding to serum IgG and C-reactive protein (CRP) as well as phagocytosis through C3b/iC3b receptors and killing by neutrophils. Opposite effects were observed in mutants of *gbpC*, *epsC*, or *gtfBCD* (required for glucan synthesis). C3b deposition on UA159 was abolished in C1q-depleted serum, implying that the classical pathway is essential for complement activation on *S. mutans*. Growth in sucrose-containing medium impaired the binding of C3b and IgG to UA159, UAcov, and blood isolates but had absent or reduced effects on C3b deposition in *gtfBCD*, *gbpC*, and *epsC* mutants. UAcov further showed increased *ex vivo* survival in human blood in an EPS-dependent way. Consistently, reduced survival was observed for the *gbpC* and *epsC* mutants. Finally, UAcov showed an increased ability to cause bacteremia in a rat model. These results reveal that $CovR_{Sm}$ modulates systemic virulence by regulating functions affecting *S. mutans* susceptibility to complement opsonization.

Streptococcus mutans is a common species of the oral cavity of humans involved in the pathogenesis of dental caries, which can promote infective endocarditis and other systemic infections after gaining access to the bloodstream (1–4). However, factors involved in *S. mutans* survival in the bloodstream are unknown but likely include mechanisms to evade host immunity. *S. mutans* expresses the orphan response regulator CovR ($CovR_{Sm}$) (also known as GcrR) (5–8), which is an orthologue of the CovR protein of the two-component regulatory system (TCS) CovRS (also known as CsrRS) of the pathogenic species *Streptococcus pyogenes* (group A *Streptococcus* [GAS]) and *Streptococcus agalactiae* (group B *Streptococcus* [GBS]). In GAS, *S. pyogenes* CovR ($CovR_{Spy}$) typically functions as a repressor of a panel of virulence genes involved in the evasion of host immunity and tissue invasiveness (9). In *S. mutans*, $CovR_{Sm}$ represses virulence factors involved in the establishment of *S. mutans* in dental biofilms (7, 8, 10, 11), but its role in systemic virulence is unknown. Genes directly repressed by $CovR_{Sm}$ include *gtfB* and *gtfC*, which encode glucosyltransferases B and C, respectively, required for the extracellular synthesis of glucans from sucrose (7), major structural exopolysaccharides (EPS) of cariogenic biofilms (1, 2). $CovR_{Sm}$ also inhibits the expression of several genes involved in cell wall biogenesis and surface interactions with EPS, including GbpB (glucan-binding protein B), GbpC (glucan-binding protein C), EpsC (enzyme for exopolysaccharide synthesis [UDP-*N*-acetylglucosamine 2-epimerase]), LysM (lysine motif protein), and WapE (cell wall protein E) (8, 10–12).

An isogenic mutant of $covR_{Sm}$ obtained from UA159 (serotype *c*) shows impaired susceptibility to phagocytosis by human polymorphonuclear leukocytes (PMNs) in a blood-dependent manner (13). Among the four known *S. mutans* serotypes (serotypes *c*, *e*, *f*, and *k*), serotype *c* is the most prominent serotype in the oral cavity (~70 to 80% of strains) and is frequently associated with systemic infections, being detected in 30.3 and 65.5% of *S. mutans*-positive specimens of cardiac valves and atheromatous plaques, respectively, from patients subjected to cardiac surgeries (14, 15). Serotype *c* strain MT8148 survives during 1 to 2 days in the bloodstream of rats (16), further suggesting mechanisms of evasion of blood immunity. In this study, we investigated the roles of $CovR_{Sm}$ in the susceptibility of *S. mutans* strains to complement immunity mediated by C3b, a major opsonin present in blood and other host fluids (17, 18). Profiles of C3b deposition on strains isolated from blood of patients with bacteremia and/or infective endocarditis and on strains from the oral cavity were compared to assess diversity in susceptibility to complement immunity. The low susceptibility to C3b deposition observed for blood isolates was then compared to transcript levels of $covR_{Sm}$ and of $CovR_{Sm}$-repressed genes. Effects of $covR_{Sm}$ deletion in strain UA159 (serotype *c*) on the binding of C3b, IgG antibodies, and C-reactive protein (CRP) and on phagocytosis mediated by C3b/iC3b or IgG

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TABLE 1 Strains used in this study

Strain	Relevant characteristic(s)	Source or reference
UA159	Oral isolate, caries-affected child; Erm^s $Spec^s$ Kan^s	ATCC
UAcovR	Δ*covR*::Erm^r	13
UAwapE	Δ*wapE*::Erm^r	11
UAlysM	Δ*lysM*::Erm^r	11
UAepsC	Δ*epsC*::Erm^r	11
UAgbpC	Δ*gbpC*::Erm^r	This study
$UAcovR^+$	Δ*covR*::Erm^r pDL278::*SMU.1924*; $Spec^r$	13
$UAwapE^+$	Δ*wapE*::Erm^r pDL278::*SMU.1091*; $Spec^r$	11
$UAlysM^+$	Δ*lysM*::Erm^r pDL278::*SMU.2147c*; $Spec^r$	11
$UAepsC^+$	Δ*epsC*::Erm^r pDL278::*SMU.1437c*; $Spec^r$	11
$UAgbpC^+$	Δ*gbpC*::Erm^r; pDL278::*SMU.1396*; $Spec^r$	This study
MT8148	Oral isolate, healthy Japanese child	19
C1	Δ*gbpC*::Kan^r; mutant of MT8148	19
S5	Δ*gtfBC*::Erm^r; double mutant of MT8148	20
BC7s	Δ*gtfD*::Erm^r Δ*gtfBC*::Kan^r; triple mutant of MT8148	20
2ST1	Oral isolate, caries-affected child	21
2VS1	Oral isolate, caries-affected child	21
3SN1	Oral isolate, caries-free child	21
4SM1	Oral isolate, caries-free child	21
4VF1	Oral isolate, caries-affected child	21
5SM3	Oral isolate, caries-free child	21
8ID3	Oral isolate, caries-free child	21
11A1	Oral isolate, caries-free child	21
11SSST2	Oral isolate, caries-free child	21
11VS1	Oral isolate, caries-free child	21
15JP3	Oral isolate, caries-free child	21
15VF2	Oral isolate, caries-affected child	21
SA12	Blood, infective endocarditis	22
SA13	Blood, bacteremia	22
SA14	Blood, infective endocarditis	22
SA15	Blood, bacteremia	22
SA16	Blood, infective endocarditis	22
SA17	Blood, bacteremia	22
SA18	Blood, infective endocarditis	22
D39	*Streptococcus pneumoniae* serotype 2 (NCTC 7466)	NCTC
TIGR4	*Streptococcus pneumoniae* serotype 4 (ATCC BAA-334)	ATCC

receptors and killing by human neutrophils (PMNs) were determined. Mechanisms of $CovR_{Sm}$ regulation of *S. mutans* susceptibility to complement immunity were then investigated by assessing the effects of the deletion of $CovR_{Sm}$-regulated genes (*gtfB*, *gtfC*, *gbpC*, *epsC*, *lysM*, and *wapE*) on C3b- and antibody-mediated immunity in the presence or absence of sucrose-derived EPS. Finally, strains were compared regarding *ex vivo* survival in human blood and in a rat model of bacteremia and infective endocarditis.

MATERIALS AND METHODS

Studied strains and culture conditions. Strains used in this study are described in Table 1. Strains were grown (37°C with 10% CO_2) from frozen stocks in brain heart infusion (BHI) agar (Difco). BHI agar or chemically defined medium (CDM) (10) with or without sucrose (0.01 and 0.1%) was used in the experiments. Erythromycin (10 μg/ml), spectinomycin (200 μg/ml), or kanamycin (500 μg/ml) (Merck Labs, Germany) was added to media for cultivation of deletion and complemented mutants.

Construction of *gbpC* deletion and complemented mutants. The nonpolar *gbpC* deletion mutant was obtained from strain UA159 (UAgbpC) by double-crossover recombination with a null allele (of 2,315 bp) constructed by PCR ligation (23). In the recombinant allele, an internal sequence of 1,455 bp of the encoding region of *gbpC* was replaced by an erythromycin resistance cassette (Erm^r) obtained from plasmid pVA838. The complemented *gbpC* mutant ($UAgbpC^+$) was obtained by transforming UAgbpC with plasmid pDL278 containing an intact copy of *gbpC* and the spectinomycin resistance gene. Primers used for the construction of mutants are shown in Table 2.

RNA isolation, reverse transcription, and qPCR. RNA was purified from strains at the mid-log phase of growth (A_{550} of 0.3) by using an RNeasy kit (Qiagen, Germany) and treated with Turbo DNase (Ambion, USA), as described previously (11). The cDNA was obtained from 1 μg of RNA by using random primers (24) and SuperScript III (Life Technologies, USA), according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed with a StepOne real-time PCR system (Life Technologies) with cDNA (10 ng), 10 μM each primer, and 1× Power SYBR green PCR master mix (Lifetech) in a total volume of 10 μl. The cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, the optimal temperature for primer annealing (Table 2) for 15 s, and 72°C for 30 s. Tenfold serial dilutions of genomic DNA (300 ng to 0.003 ng) were used to generate standard curves for the absolute quantification of RNA expression levels. Melting curves were obtained for each primer set. Results were normalized against *S. mutans* 16S rRNA gene expression values (24). Assays were performed in duplicate with at least two independent RNA samples.

***S. mutans* interaction with EPS.** Cell aggregation mediated by sucrose-derived EPS was assessed as described previously (25). Briefly, strains were grown in BHI medium (37°C with 10% CO_2 for 18 h), and an equal number of cells was transferred to fresh BHI medium supplemented with 0.1% sucrose and incubated for 24 h (37°C with 10% CO_2). Cell aggregation was then visually inspected.

Surface-associated EPS was analyzed by scanning electron microscopy (SEM) in strains grown in BHI medium or CDM with or without 0.1% sucrose. Briefly, cultures grown during 18 h in BHI medium or CDM were 100-fold diluted with fresh medium containing or not containing 0.1%

TABLE 2 Oligonucleotides used in this study

Oligonucleotide	Sequence (5′–3′)[a]	Product size; positions or relevant characteristic[b]
ermE1-AscI	TTGGCGCGCCTGGCGGAAACGTAAAAGAAG	998 bp; amplicon containing the Erm^r gene from pVA838
ermE2-XhoI	TTCTCGAGGGCTCCTTGGAAGCTGTCAGT	
gbpCP1	CCCTCAACACACTCTGCTAA	473 bp; 323 bp upstream to 150 bp downstream of the *gbpC* ORF
gbpCP2-AscI	TTGGCGCGCCCGGTTCTGATGCTTGTGTAT	
gbpCP3-XhoI	TTCTCGAGGGAGAAATGCGTGTTAGAGA	387 bp; 1,605 bp upstream to 240 bp downstream of the encoding region of *gbpC*
gbpCP4	CTTACCCATCACAAAAACCA	
C1-SacI	GGGAGCTCCCCTCAACACACTCTGCTAA	2,139 bp; amplicon containing the encoding region of *gbpC* for mutant complementation
C2-SphI	GGGCATGCAACAAGAACTGCTGCTCAAG	

[a] Underlined sequences indicate restriction enzyme linkers.
[b] ORF, open reading frame.

sucrose and incubated to reach an A_{550} of 0.3. Cells from volumes of 500 μl were then harvested by centrifugation, washed with phosphate-buffered saline (PBS), and processed for SEM analysis, as previously described (12). Samples were analyzed with a scanning electron microscope (JSM 5600LV; JEOL, Japan).

Volunteers, sera, and blood samples. Blood samples from six healthy subjects (three males and three females; mean age, 30 years [range, 25 to 45 years]) were collected by venipuncture in heparin vacuum tubes (BD Vacutainer), according to standard protocols previously approved by the Ethical Committee of the Piracicaba Dental School, State University of Campinas (protocol number 031/2012). Serum samples were stored in aliquots at −70°C until use. Levels of C3 in serum samples were determined as described below and were within normal levels in all volunteers (mean, 1.91 mg/ml; standard deviation [SD], 0.68 mg/ml; range, 1.18 to 2.88 mg/ml) (26). Mean levels of IgG and IgM in the same samples were also determined and were, respectively, 11.68 (±1.82) mg/ml and 1.35 (±0.64) mg/ml. Serum samples from one volunteer, which were representative of C3, IgG, and IgM levels, were used as controls. Commercial human serum depleted of C1q was obtained from Calbiochem (MA, USA). The Calbiochem C1q-depleted serum is free of EDTA and retains alternative pathway activity (27). As a control for integrity, C1q-depleted serum was supplemented with purified human C1q (Calbiochem) to a physiological concentration range (75 μg/ml in 100% serum). Heat-inactivated sera (56°C for 20 min) were also used as negative controls in preliminary experiments and showed minimal effects on comparative analyses of C3b deposition between strains.

Determination of total levels of C3, IgG, and IgM antibodies in serum. The serum concentrations of C3, IgG, and IgM antibodies were determined by enzyme-linked immunosorbent assays (ELISAs) using commercial systems for the quantification of human complement C3 (Molecular Innovations, MI, USA), human IgG, and human IgM (Bethyl Laboratories, Inc., TX, USA), respectively. Briefly, 100 μl of serum samples diluted in dilution buffer (1:100,000, 1:500,000, and 1:10,000, respectively) was added to 96-well plates coated with anti-C3, anti-IgG, or anti-IgM and then incubated for 30 min at room temperature (RT). After a series of three washes with wash buffer, 100-μl aliquots of antibodies specific to C3, human IgG, or human IgM were added per well, and plates were incubated (RT) for 1 h. After a new series of washes, 100 μl per well of secondary horseradish peroxidase (HRP)-conjugated antibodies (1:50,000) was added, and incubation continued for 30 min. After a new series of washes, 100 μl per well of a chromogenic HRP substrate (3,3′,5,5′-tetramethylbenzidine) was added, and plates were incubated for 30 min. Reactions were stopped by the addition of 1 N H_2SO_4 to the mixture. Absorbances (A_{450}) were measured in a microtiter plate reader (Versa Max) and converted to micrograms per milliliter using standard curves for C3 (0.02 to 10 ng/ml), IgG (0.69 to 167 ng/ml), or IgM (1.03 to 250 ng/ml) antibodies.

C3b deposition on *S. mutans*. Deposition of C3b on the surface of serum-treated *S. mutans* strains was determined as described previously (27, 28), with some modifications. Briefly, ~10^7 CFU of strains at the mid-log phase of growth (A_{550} of 0.3) were harvested by centrifugation (10,000 × *g* at 4°C), washed two times with PBS (pH 7.4), and suspended in 20 μl of 20% serum (diluted in PBS). Samples were then incubated (37°C for 30 min) and washed twice with PBS–0.05% Tween (PBST). Cells were then incubated on ice (40 min) with fluorescein isothiocyanate (FITC)-conjugated polyclonal goat anti-human C3 IgG antibody (ICN, CA, USA) (1:300 in PBST). After two washes with PBST, bacterial cells were fixed in 3% paraformaldehyde in PBS and analyzed on a FACSCalibur flow cytometer (BD Biosciences) using forward- and side-scatter parameters to gate at least 25,000 bacteria. Results were expressed as the geometric mean fluorescence intensity (MFI) of C3b-positive cells or as the mean fluorescence index (FI) (percentage of positive cells multiplied by the MFI) (29, 30). Control samples included bacteria treated only with PBS instead of serum.

PMN isolation, opsonophagocytosis, and killing assays. Human PMNs were isolated from fresh heparinized blood samples from one reference volunteer by centrifugation over a double gradient composed of Histopaque-1119 and Histopaque-1083 (Sigma-Aldrich), as previously described, with modifications (31). Red blood cells were eliminated by hypotonic lysis. Isolated PMNs were suspended in RPMI 1640 medium (Gibco, Life Technologies, NY, USA) supplemented with inactivated 10% fetal bovine serum. Cell viability (>98%) was monitored by trypan blue exclusion. Cell purity (>95%) was monitored by May-Grunwald-Giemsa staining.

For opsonophagocytosis assays, bacteria were previously labeled with FITC as described previously (32), with some modifications. Briefly, 500 μl of bacterial strains (A_{550} of 0.3) was washed two times with PBS, suspended in 600 μl of carbonate buffer (0.15 M Na_2CO_3, 0.9% NaCl [pH 9]) with 1.7 mg/ml of FITC (Sigma), and incubated for 1 h (with shaking at RT in the dark). Cells were then harvested and washed three times with PBST, and aliquots were stored overnight in 10% glycerol at −70°C. Bacterial labeling was analyzed with a fluorescence microscope (Leica) and by flow cytometry (FACSCalibur; BD).

For C3b deposition, aliquots containing 10^7 CFU of FITC-labeled bacteria were incubated with 20% serum and added to wells of 96-well plates containing 2 × 10^5 PMNs in 50 μl of RPMI medium to a multiplicity of infection (MOI) of 200 bacteria per PMN. After incubation (37°C and 10% CO_2 with gentle shaking) for 5 or 30 min, reaction mixtures were fixed by the addition of 100 μl of 3% of paraformaldehyde. PMNs were then analyzed by using a FACSCalibur instrument (BD Biosciences), and the frequency of phagocytosis was expressed as the number of PMN cells with intracellular bacteria, within a total of 10,000 PMNs analyzed (33). The MOI was determined in preliminary experiments testing MOIs of 20 to 200 bacteria per PMN, after 5 to 60 min of incubation. Data from flow cytometry analysis was compared to data from light microscopy analysis of samples stained by using May-Grunwald-Giemsa stain, as previously described (13). These comparisons confirmed that most of the PMN-associated bacteria were internalized.

To assess phagocytosis by PMNs through C3b/iC3b receptors, similar assays were performed with PMNs previously incubated (37°C for 30 min) with mouse anti-CD35 monoclonal antibodies (MAbs) (BioLegend, CA, USA) or anti-CD11b/CD18 MAbs to block CR1 or CR3 receptors (34, 35), respectively. As a reference, PMNs incubated with anti-CD32 (FcγRII) (eBioscience, CA, USA) or anti-C16 (FcγRIII) (BioLegend, CA, USA) MAbs were also tested.

Opsonophagocytic killing was assessed as previously described (36, 37), with modifications. Briefly, preopsonized bacteria (20% human serum for 30 min at 37°C) were added to samples of human PMNs in RPMI with 10% human serum at an MOI of 200:1. After incubation (37°C for 10 and 30 min) with shaking, reactions were stopped at 4°C. PMNs were harvested by centrifugation (500 × *g* for 8 min at 4°C), washed twice with PBS (pH 7.2), and lysed with 2% saponin (12 min at 37°C), and viable counts of intracellular bacteria were determined by plating serial dilutions onto BHI agar. Bacterial counts were also determined in control wells with identically treated samples without PMNs. Viable bacteria were counted in culture supernatants of PMN samples to monitor the number of extracellular bacteria. Percent intracellular survival was calculated as follows: (CFU ml^{-1} test well)/(CFU ml^{-1} control well) × 100.

Determination of binding of serum IgG, IgM, and CRP to *S. mutans*. Binding of serum IgG, IgM, or CRP to *S. mutans* was determined as previously described (27, 38), with some modifications. Briefly, bacterial strains were harvested from 500 μl of cultures of UA159 (A_{550} of 0.3), washed twice with PBS (pH 7.0), incubated with 20% serum, and washed with PBST. To assess surface levels of IgG, IgM, or CRP, cells were then incubated with polyclonal goat IgG anti-human IgG Fc conjugated with FITC (Novus Biological, USA) (1:900), polyclonal mouse IgG anti-human IgM Fc conjugated with allophycocyanin (APC) (1:1,000) (BioLegend, USA), or goat IgG anti-human CRP conjugated with FITC (GeneTex, USA) (1:100), respectively. *Streptococcus pneumoniae*

strains D39 and TIGR4 were used as controls for CRP binding, because the *S. pneumoniae* cell wall contains known CRP ligands (phosphorylcholine [PCho]) (39).

After 40 min of incubation on ice, bacterial cells were washed twice with 300 μl of PBST, harvested (16,000 × *g* for 2 min), and suspended in 300 μl of 3% paraformaldehyde. Flow cytometry analyses were performed as described above, using forward- and side-scatter parameters to gate at least 25,000 bacteria. Bacterial samples treated with PBS instead of serum were used as negative controls.

***Ex vivo* survival of *S. mutans* strains in human blood.** Bacteria from cultures grown in BHI medium (A_{550} of 0.3) were harvested (11,000 × *g* for 2 min), washed twice in PBS, and resuspended in 1 ml of fresh whole human blood. Samples were then incubated (37°C and 5% CO_2 with gentle agitation), and aliquots were collected at different time points (5, 30, 60, 120, and 240 min), serially diluted, and plated onto BHI agar for determination of bacterial counts. Aliquots collected just after bacterial suspension in blood were used for initial measurements of CFU per milliliter (time zero). Changes in numbers of viable bacteria were then calculated in relation to counts at time zero to reduce the influence of variations in blood-mediated aggregation between strains on the numbers of CFU per milliliter. Three independent experiments were performed in duplicate with blood samples from one volunteer with reference levels of C3, IgG, and IgM (2.8, 14.6, and 1.5 μg/ml of serum, respectively).

Survival of *S. mutans* strains in a rat model of bacteremia and infective endocarditis. Protocols for the animal experiments were approved by the institutional animal care and use committee of the Osaka University Graduate School of Dentistry (approval number 24-019). All rats were treated humanely in accordance with the National Institute of Health and AERI-BBRI Animal Care and Use Committee guidelines. The rat infective endocarditis model was used according to methods described previously, with some modifications (40). In brief, 21 Sprague-Dawley male rats (400 to 500 g each) were anesthetized with a mixture of xylazine and midazolam (0.1 ml–100 g). A sterile polyethylene catheter with a guide wire was surgically placed across the aortic valve of each animal via the right carotid artery, and the tip was positioned and placed at the aortic valve in the left ventricle. A bacterial suspension (10^9 cells per body, from cultures in BHI medium) in PBS was intravenously administered through the jugular vein. Bacterial clearance was examined by measuring the numbers of bacteria in blood samples from the jugular veins, which were taken at 1, 3, 6, and 24 h and 7 days after the initial infection. The blood samples were placed onto Mitis-Salivarius agar (Difco Laboratories, Detroit, MI, USA) plates containing bacitracin (0.2 U/ml; Sigma Chemical Co., St. Louis, MO, USA) and 15% (wt/vol) sucrose (MSB agar) and incubated at 37°C for 48 h. Seven days after bacterial infection, the rats were sacrificed by an overdose of anesthesia, and the aortic valves were extirpated, transversely sectioned, and subjected to Gram staining and to bacterial recovery for microbial counting.

Statistical analyses. Flow cytometry data (percentages of positive bacteria or PMNs and MFI or FI values) were analyzed by comparing means of data from at least three independent experiments using a nonparametric Kruskal-Wallis test with Dunn's *post hoc* test. Comparisons of the mean MFI or FI values for surface-bound C3b between oral and blood isolates were performed by using a Mann-Whitney U test. Spearman's rank correlation was applied to analyze associations between MFI values of surface C3b and those of surface IgG. *Ex vivo* survival in blood was compared between strains by testing differences in relative bacterial counts (log CFU per milliliter) at each time point of incubation in relation to initial counts in blood suspensions. Bacterial counts in the rat bloodstream were also compared between strains. Relative or absolute bacterial counts were compared between strains at each time point by using a Kruskal-Wallis test with Dunn's *post hoc* test, using correction for repeated measures (30).

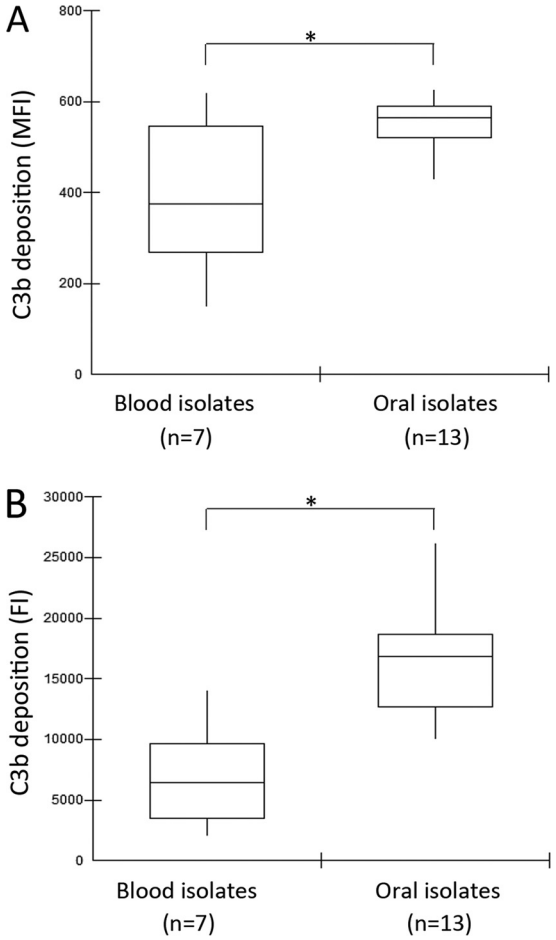

FIG 1 Box plot comparisons of C3b depositions between *S. mutans* strains isolated from blood samples and those of strains isolated from the oral cavity. C3b bound to serum-treated strains was probed with anti-C3 antibody conjugated with FITC for flow cytometry analysis. (A) Levels of C3b bound to *S. mutans* strains are expressed as geometric mean fluorescence intensity (MFI) values. (B) Fluorescence index (FI) values were obtained by multiplying the percentage of C3b-positive cells by MFI values for C3b. MFI and FI data are means of results from three independent experiments. Asterisks indicate significant differences between groups ($P < 0.05$ as determined by a Mann-Whitney U test).

RESULTS

***S. mutans* strains isolated from blood show reduced susceptibility to C3b deposition compared to oral isolates.** Although complement immunity is recognized as an important blood defense against streptococcal species (18, 28, 41), the profiles of *S. mutans* susceptibility to complement-mediated opsonization were unknown. Thus, we compared patterns of C3b deposition on strains isolated from the bloodstream of patients with bacteremia associated or not with infective endocarditis ($n = 7$) and isolates of the oral cavity ($n = 13$), including reference strain UA159. As shown in Fig. 1, blood isolates showed reduced levels of C3b deposition compared to oral isolates. Two blood serotype *c* strains (SA13 and SA18) showed the lowest MFI values for C3b (mean MFI values, 148.9 ± 65.8 and 215.7 ± 112.0, respectively) and low FI values (mean FI values, 2,069.0 ± 802.8 and 3,019.9 ± 1,056.4, respectively). Mean MFI and FI values of strain UA159 were 623.2 (±100.3) and 17,678.4 (±2,908.7), respectively.

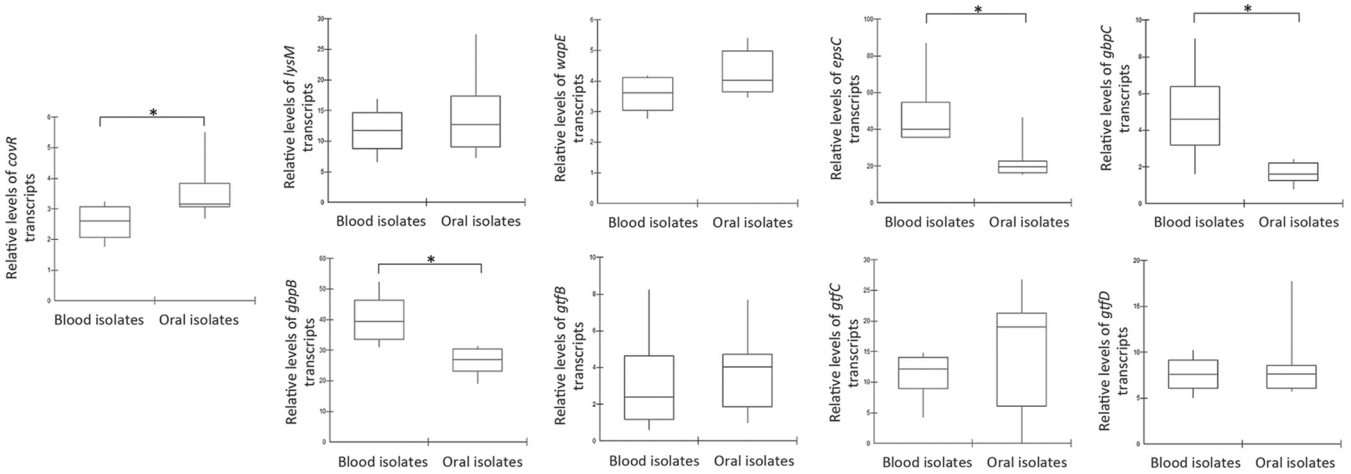

FIG 2 Reverse transcription-qPCR comparisons of transcript levels of $covR_{Sm}$ and $CovR_{Sm}$-regulated genes (*lysM*, *wapE*, *epsC*, *gbpC*, *gbpB*, *gtfB*, and *gtfC*) in blood ($n = 4$) and oral ($n = 5$) strains of *S. mutans*. The *gtfD* gene, which is not regulated by $CovR_{Sm}$, was tested as a control. Asterisks indicate significant differences in mean levels of transcripts between groups ($P < 0.05$ as determined by analysis of variance with Tukey's *post hoc* test).

***S. mutans* blood strains show reduced activity of $covR_{Sm}$ and increased transcription of $CovR_{Sm}$ target genes required for surface interaction with EPS.** To investigate the role of $CovR_{Sm}$ in strain susceptibilities to C3b deposition, transcript levels of $covR_{Sm}$ and downstream genes of four blood strains showing the lowest levels of C3b deposition (SA13, SA15, SA16, and SA18) were compared with those of four oral isolates with the highest levels of binding to C3b (2VS1, 11A1, 11SSST2, and 8ID3) and reference strain UA159. The $CovR_{Sm}$-repressed genes selected were those affecting *S. mutans* cell surface properties, including *lysM*, *wapE*, *epsC*, *gbpB*, *gbpC*, *gtfB*, *gtfC*, and *gtfD* (glucosyltransferase D-encoding gene) (7, 11, 42). As shown in Fig. 2, blood strains showed lower levels of $covR_{Sm}$ transcripts than did the oral isolates. Consistently, blood isolates showed increased transcription of *epsC*, *gbpC*, and *gbpB*, which are genes involved in *S. mutans* surface interactions with EPS (11, 12, 43). No significant differences in transcript levels of *lysM*, *wapE*, *gtfB*, *gtfC*, or *gtfD* were detected (Fig. 2). Levels of C3b deposition in the analyzed strains negatively correlated with transcript levels of *epsC* (Spearman correlation [r], -0.45; $P < 0.05$), *gbpB* (r, -0.21; $P < 0.05$), and *gbpC* (r, -0.35; $P < 0.05$). Thus, diversity in the transcriptional activities of $covR_{Sm}$ and of $CovR_{Sm}$-repressed genes is associated with differences in levels of C3b deposition in *S. mutans* strains.

***S. mutans* blood strains show an increased capacity to bind EPS produced in the presence of sucrose, similarly to the $covR_{Sm}$ isogenic mutant.** Because *gbpB*, *gbpC*, and *epsC* encode proteins for *S. mutans* binding to sucrose-derived EPS (glucan) (11, 12, 43), we compared the capacities of aggregation in the presence of sucrose of blood and oral isolates. Isogenic mutants of $covR_{Sm}$ and of $CovR_{Sm}$-repressed genes (*gbpC*, *epsC*, *gtfB*, *gtfC*, *lysM*, and *wapE*) were also tested, except for *gbpB*, which is essential for *S. mutans* viability (12). As shown in Fig. 3A, blood isolates showed a higher capacity to aggregate in BHI medium containing 0.1% sucrose than did oral isolates. Blood strains SA13 and SA18 showed aggregation phenotypes similar to that of the UAcov strain (Fig. 3B). As anticipated, the *gbpC* isogenic mutant did not aggregate, while only weak aggregation was detected in the *epsC* mutant (Fig. 3C). In addition, because *gtfB*, *gtfC*, and *gtfD* are required for the synthesis of glucan from sucrose, mutants of these genes obtained from strain MT8148 did not aggregate (Fig. 3C). The aggregation phenotypes of the *gbpC* mutants obtained from MT8148 (Fig. 3C) and UA159 (Fig. 3B) were similar. SEM analysis supported data from a previous report (11) on the increased interaction of UAcov with sucrose-derived EPS in biofilms and confirmed the strain capacities to bind sucrose-derived EPS under the growth conditions applied in the C3b binding assays (data not shown).

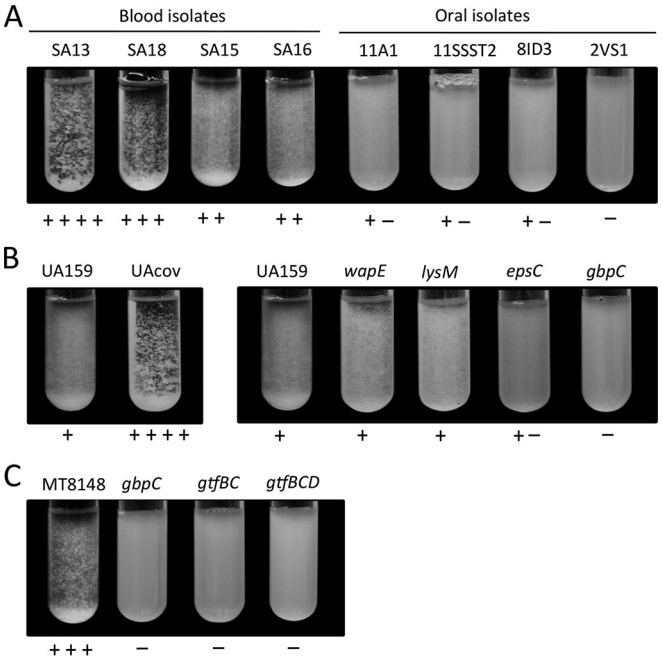

FIG 3 Comparisons of *S. mutans* capacities to aggregate in the presence of sucrose-derived EPS. Strains were incubated in BHI medium supplemented with 0.1% sucrose during 24 h for visual analysis of clump formation. Intensities of cell aggregation were determined by a blind examiner and are indicated below the respective images. (A) Comparisons between blood and oral strains. (B) Comparisons between parent strain UA159 and the respective knockout mutants. (C) Comparisons of parent strain MT8148 with the respective knockout mutants.

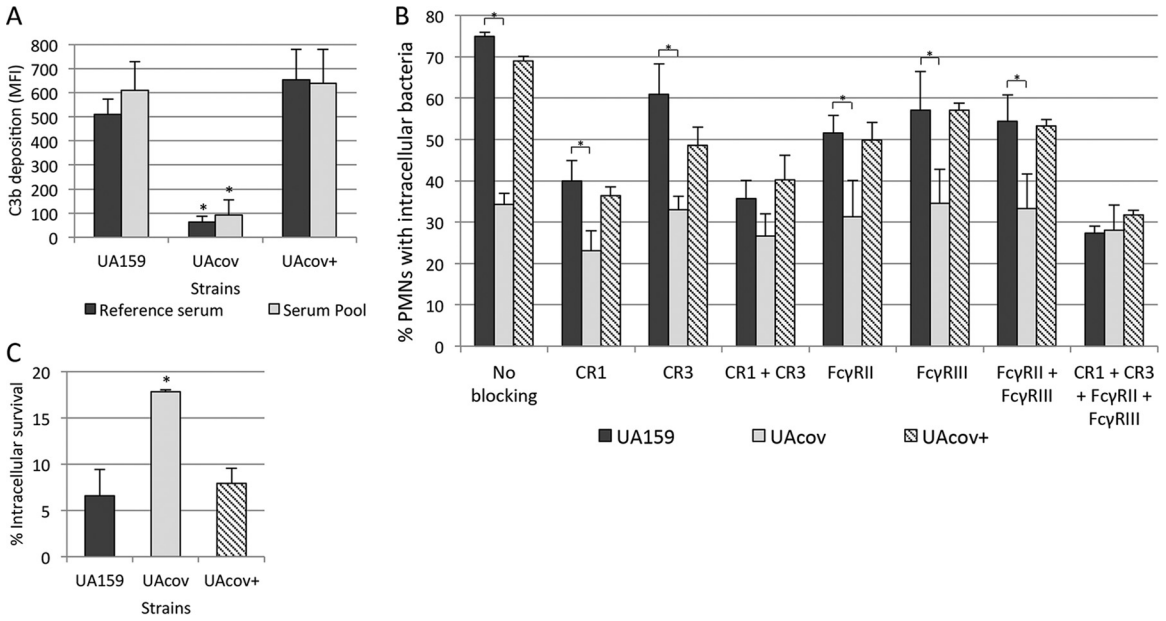

FIG 4 Comparisons of C3b deposition, opsonophagocytosis, and killing by PMNs between *covR* mutant (UAcov), parent (UA159), and complemented (+) strains. (A) Intensities of C3b deposition (MFI) in strains treated with a reference serum or pools of sera obtained from six volunteers were determined by flow cytometry. (B) Percentages of PMNs with associated bacteria were assessed after 5 min of exposure of PMNs to FITC-labeled bacteria in the presence of 20% serum. Untreated PMNs and PMNs treated with MAbs to block CR1 (CD35), CR3 (CD11b/CD18), FcγRII (CD32), and/or FcγRIII (CD16) receptors were tested. (C) Percentages of intracellular survival in PMNs after 10 min of incubation with preopsonized bacteria were calculated in relation to bacterial counts of no-PMN control samples. Columns represent means of data from three independent experiments. Bars indicate standard deviations. Asterisks indicate significant differences in relation to UA159 under the same conditions ($P < 0.05$ as determined by a Kruskal-Wallis test with Dunn's *post hoc* test).

Inactivation of $covR_{Sm}$ impairs deposition of C3b, phagocytosis mediated by C3b/iC3b receptors, and opsonophagocytic killing by human PMNs. UAcov shows low susceptibility to phagocytosis by human PMNs in a serum-dependent way (13), suggesting that $CovR_{Sm}$ regulates surface components affecting serum opsonization. As shown in Fig. 4A, deposition of C3b was impaired in UAcov. This phenotype was completely restored in the complemented $UAcov^+$ mutant. Significant reductions in the frequencies of phagocytosis of UAcov in the presence of 20% serum were also observed in comparison to parental strain UA159 or $UAcov^+$ (Fig. 4B). Importantly, blocking of CR1 and/or CR3 receptors of PMNs with anti-CD35 (CR1) or anti-CD11b/CD18 (CR3) antibodies reduced differences in the frequencies of phagocytosis between UA159 and UAcov (Fig. 4B). Additionally, the simultaneous blockage of CR1 and CR3 receptors abolished differences in the frequencies of phagocytosis between these strains (Fig. 4B), reflecting the multiple and cooperative functions of CR1 and CR3 in bacterial phagocytosis mediated by C3b/iC3b (44). Treatment of PMNs with anti-CD32 (FcγRII) or anti-CD16 (FcγRIII) antibodies also reduced the phagocytosis of UA159, although blockage of both Fcγ receptors did not eliminate differences in the frequencies of phagocytosis between UA159 and UAcov (Fig. 4B).

To examine if the reduced phagocytosis of UAcov was associated with reduced killing by PMNs, strains were compared in opsonophagocytic killing assays. As shown in Fig. 4C, UAcov showed increased survival to PMN during 10 min of incubation. Similar results were obtained after exposure of PMNs to the tested strains during 30 min (data not shown). Viable bacteria in PMN culture supernatants were monitored, confirming the reduced phagocytosis of UAcov compared to UA159; mean counts of UAcov bacteria in culture fluids were significantly higher than UA159 counts ($P < 0.05$). These data establish the strong influence of C3b/iC3b deposition on *S. mutans* phagocytosis and imply that reduced deposition of C3b/iC3b on UAcov not only impairs phagocytosis mediated by C3b/iC3b receptors but also is associated with reduced killing by PMNs.

C3b deposition on *S. mutans* is strongly dependent on C1q of the classical pathway of complement activation. We hypothesized that EPS bound to the *S. mutans* surface could compromise antibody recognition of immunogenic surface proteins, thus affecting the classical pathway of complement activation. In this pathway, the proteolytic cascade initiates with C1q binding to different host components bound to the bacterial surface, most prominently IgG or IgM antibodies but also acute-phase proteins of innate immunity, e.g., CRP (17, 18). Therefore, we analyzed the effect of $covR_{Sm}$ inactivation on levels of antibodies bound to *S. mutans* (from pools of sera from six volunteers) and investigated the effect of the classical pathway on binding of C3b to *S. mutans*. Because the classical pathway can also be activated by CRP bound to the surface of streptococcal species containing CRP ligands (27, 45), we additionally assessed the binding of this acute-phase protein to *S. mutans*. As shown in Fig. 5A, significant reductions in the binding of IgG antibodies were observed for UAcov compared to the parent strain. In addition, although levels of CRP bound to *S. mutans* UA159 were low compared to those for the *S. pneumoniae* control strains, UAcov showed reduced binding to CRP compared to UA159 (Fig. 5B). Importantly, levels of surface-bound IgG and CRP were restored in complemented mutant strain $UAcov^+$ (Fig. 5A and B). No significant changes in the binding of IgM antibodies to *S. mutans* were observed (data not shown).

C3b deposition was minimal when strains were treated with

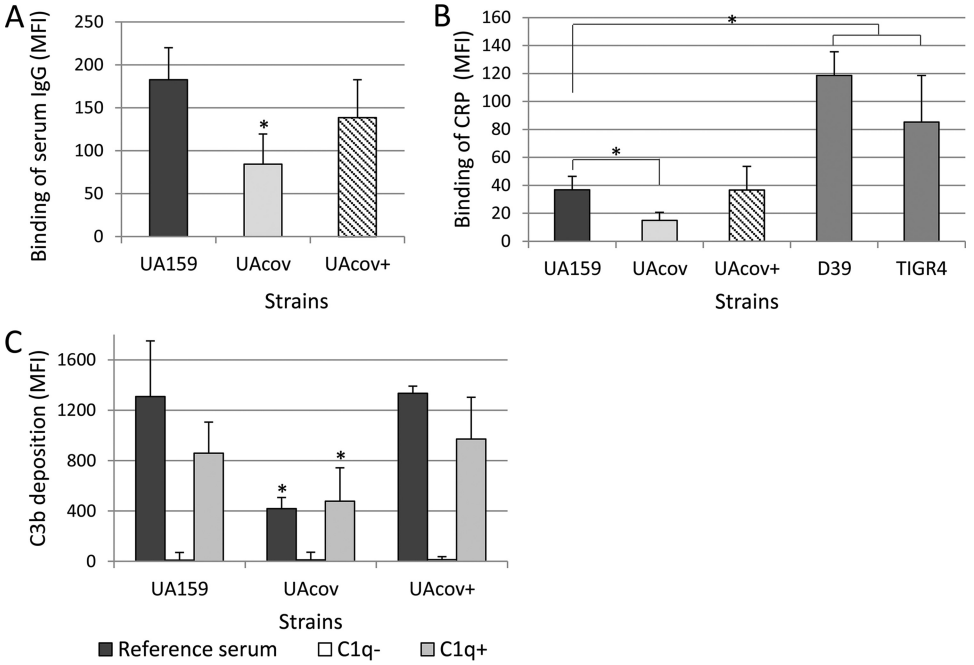

FIG 5 Binding of serum IgG, CRP, and C3b to *S. mutans* strains in the presence of serum. (A and B) Strains were treated with 20% human serum, and levels of surface IgG (A) and CRP (B) were determined by flow cytometry (MFI). *S. pneumoniae* strains D39 and TIGR4 were used as controls for CRP binding. (C) Levels of C3b binding were measured after treatment of bacteria with reference serum, serum depleted of C1q (C1q−), or serum depleted of C1q and supplemented with purified C1q (C1q+). Columns represent means of results from three independent experiments; bars represent standard deviations. Strains were compared by using a Kruskal-Wallis test with Dunn's *post hoc* test. Asterisks indicate significant differences in relation to UA159 under the same conditions ($P < 0.05$).

C1q-depleted serum compared to normal serum. Supplementation of C1q-depleted serum with purified C1q (to physiological levels) restored C3b deposition (Fig. 5C). These data indicate that most of the C3b bound to the *S. mutans* surface resulted from the activation of the C1 component of the classical pathway. Thus, $covR_{Sm}$ inactivation impairs *S. mutans* surface binding of serum IgG antibodies and CRP, serum components that trigger the classical pathway of complement activation, a major pathway involved in C3b deposition on *S. mutans*.

C3b deposition on *S. mutans* is affected by interaction with sucrose-derived EPS. *S. mutans* accesses the bloodstream from oral niches, where it is exposed to dietary sucrose to synthesize EPS, including glucan. $covR_{Sm}$ inactivation in serotype *c* strains upregulates not only genes for the synthesis of sucrose-derived glucan (*gtfB*, *gtfC*, and *gtfD*) but also genes encoding glucan-binding proteins (*gbpB* and *gbpC*) and EpsC (*epsC*), which are involved in surface binding to these polymers (7, 8, 10, 11, 43). Therefore, to address whether sucrose-derived EPS on the *S. mutans* surface influences C3b deposition, we compared levels of C3b binding of the parent strain to those of isogenic $covR_{Sm}$, *gtfBCD*, *gbpC*, and *epsC* mutants previously grown in medium with different concentrations of sucrose, which were then harvested, washed, and exposed to serum. As shown in Fig. 6A, the parent and $covR_{Sm}$ strains grown in the presence of sucrose showed reduced levels of C3b compared to those of the same strain grown in medium without sucrose. C3b deposition was more intensely reduced in UAcov in the presence of sucrose. When grown in CDM supplemented with 0.1% sucrose, mean levels of C3b (MFI) in UAcov were 4-fold lower than those in the parent strain UA159 (82.3 versus 326.4). In sucrose-free CDM, levels of C3b in UAcov were only 1.9-fold lower than C3b levels in the parent strain UA159 (507.4 versus 991.5). In BHI medium, UAcov showed a 4-fold reduction in C3b deposition, but in BHI medium with 0.1% sucrose, UAcov showed a 9.4-fold reduction in C3b deposition compared to that of the parent strain (33.4 versus 315.4). Although the level of C3b binding was higher in strains grown in sucrose-free CDM than in strains grown in BHI medium (Fig. 6A), the addition of 0.01% sucrose to CDM was sufficient to eliminate these differences. This result suggests that there may be trace amounts of sucrose in complex BHI medium, although we can not rule out that unknown BHI medium components adsorbed to the *S. mutans* surface could also influence C3b deposition.

Because sucrose-derived glucans are synthesized by GtfB, GtfC, and GtfD, we further confirmed if deletion of multiple *gtf* genes (double *gtfBC* and triple *gtfBCD* mutants) would eliminate the effect of previous exposure to sucrose on *S. mutans* susceptibility to C3b deposition. GtfB synthesizes insoluble glucan (rich in α1-3 linkages), while GtfC synthesizes a mixture of insoluble and soluble (rich in α1-6 linkages) glucans, and GtfD synthesizes only soluble glucan (1). As expected, significant increases in C3b deposition were observed for the *gtfBC* and *gtfBCD* mutants compared to parent strain MT8148 under all culture conditions ($P < 0.05$ as determined by a Kruskal-Wallis test with Dunn's *post hoc* test). Supplementation of growth medium with sucrose did not significantly affect C3b deposition on these mutants (Fig. 6B). Therefore, sucrose-derived EPS impacts C3b deposition on the *S. mutans* surface. Of note, analyses of the effects of *gtfBCD* on C3b opsonization were performed with mutants previously obtained from strain MT8148, because *gbpC* inactivation affected C3b deposition on MT8148 in a fashion similar to that observed for UA159 (see below).

Because levels of sucrose in the bloodstream seem to be mini-

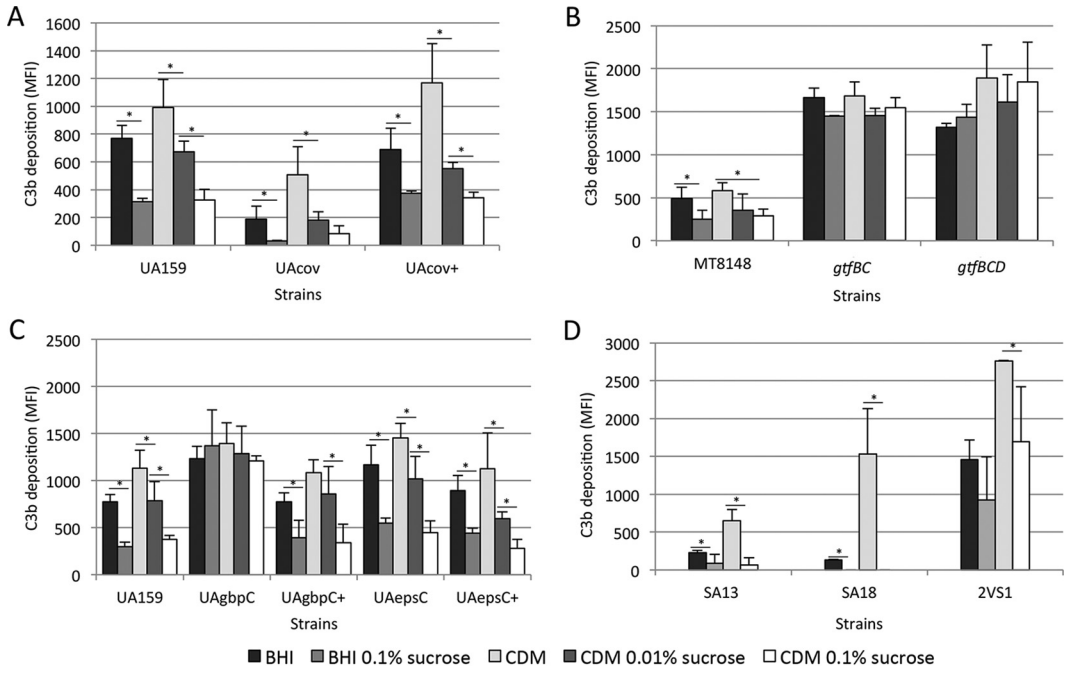

FIG 6 Effects of previous growth in medium supplemented with sucrose on strain susceptibilities to C3b deposition. Strains grown in BHI medium or CDM supplemented or not with 0.01 or 0.1% sucrose were harvested, washed with PBS, and treated with human serum for C3b deposition. Columns represent mean MFI values for C3b determined by flow cytometry in three independent experiments. Bars represent standard deviations. Asterisks indicate significant differences between groups ($P < 0.05$ as determined by a Kruskal-Wallis test with Dunn's *post hoc* test).

mal (46), we assessed whether the capacity of *S. mutans* to bind sucrose-derived EPS influences C3b deposition. Thus, levels of C3b in the *gbpC* mutant (UAgbpC) were compared to those in parent strain UA159 previously grown in the presence or absence of sucrose. As shown in Fig. 6C, deletion of *gbpC* promoted a significant increase in C3b deposition compared to the parent strain, especially when strains were recovered from sucrose-containing medium: BHI medium containing 0.1% sucrose (mean 4.6-fold increase in the C3b MFI) and CDM containing 0.1% sucrose (mean 3.2-fold increase in the C3b MFI) ($P < 0.05$ as determined by a Kruskal-Wallis test with a *post hoc* test). These results are compatible with the inability of UAgbpC to bind EPS produced from sucrose (Fig. 3B and SEM analysis data not shown). Similar results were observed for the *gbpC* mutant obtained from strain MT8148 (data not shown). The influence of sucrose-derived products on C3b deposition was further analyzed in the *epsC* mutant. Previous growth of the *epsC* mutant in the presence of sucrose reduced C3b deposition (Fig. 6C), which is compatible with the finding that UAepsC retained some capacity to bind EPS (Fig. 3B). Thus, the expression of proteins that bind sucrose-derived EPS significantly affects *S. mutans* susceptibility to C3b deposition. Of note, wild-type strain MT8148 showed lower levels of binding to C3b than did the UA159 strain (Fig. 6A and B), consistent with its higher capacity to interact with sucrose-derived EPS than UA159 (Fig. 3C).

Finally, to confirm that the reduced susceptibility of blood isolates to C3b deposition was promoted by sucrose-derived EPS, we compared levels of C3b deposition on blood strains SA13 and SA18 grown in the four growth media. As a reference, oral strain 2VS1 (with reduced binding to sucrose-derived EPS) (Fig. 3A) was also tested. As expected, levels of C3b deposition on SA13 and SA18 were increased when the strains were grown in sucrose-free CDM (Fig. 6D). Levels of C3b deposition on 2VS1 were significantly higher than those observed for the blood strains under all growth conditions ($P < 0.05$ as determined by a Kruskal-Wallis test), but the addition of sucrose to media did not significantly affect C3b binding to this strain (Fig. 6D). Thus, the low susceptibility of blood strains to C3b deposition is influenced by sucrose-derived EPS in a fashion similar to that observed for the *covR* mutant.

Influence of sucrose-derived EPS on binding of serum IgG to the *S. mutans* surface and on frequencies of opsonophagocytosis and killing by human PMNs. Because complement activation on *S. mutans* was found to be strongly dependent on the classical pathway, we investigated whether changes in C3b deposition in *S. mutans* promoted by previous growth in the presence of sucrose could be associated with reduced binding to serum IgG. As shown in Fig. 7A, levels of IgG bound to UA159 and UAcov were impaired when these strains were grown in medium with added sucrose. In addition, exposure to sucrose significantly reduced phagocytosis and killing by PMNs, especially in UAcov (Fig. 7B and C). Consistent with data from flow cytometry analyses of phagocytosis (Fig. 7B), mean counts of extracellular UAcov bacteria in the supernatants of PMNs analyzed in killing assays were 1.6- and 6.3-fold higher than those of extracellular UA159 bacteria when strains were respectively grown in CDM and CDM with 0.1% sucrose (data not shown). In addition, similarly to UAcov, the growth of blood strains SA13 and SA18 in medium supplemented with sucrose impaired the binding of serum IgG (Fig. 7D), reduced the frequency of phagocytosis (Fig. 7E), and increased resistance to killing by PMNs (Fig. 7F). In contrast, medium supplementation with sucrose promoted limited effects on IgG

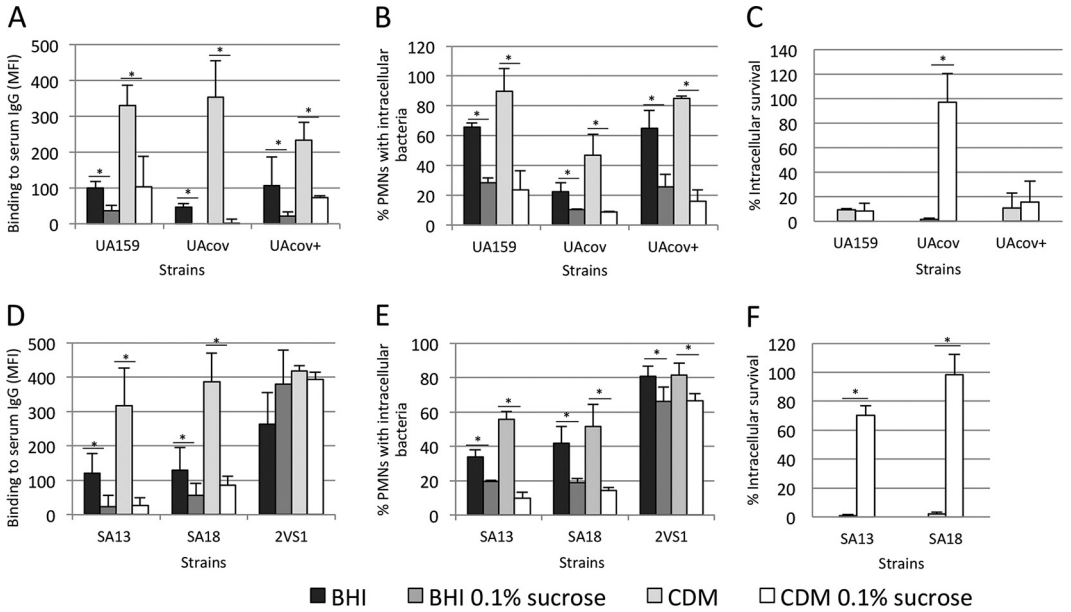

FIG 7 Effects of previous growth of *S. mutans* strains in medium supplemented with sucrose on binding to serum IgG, susceptibility to phagocytosis by PMNs, and killing by PMNs. (A and D) Strains grown in BHI medium or CDM supplemented or not with 0.01 or 0.1% sucrose were harvested, washed with PBS, and treated with human serum for IgG binding. Levels of IgG binding were determined by flow cytometry and are expressed as MFI values. (B and E) FITC-labeled strains grown in different media were incubated with PMNs isolated from human peripheral blood in the presence of 20% serum during 5 min. (C and F) Strains grown in CDM supplemented or not with 0.1% sucrose were preopsonized and incubated with PMNs (10 min). Percentages of intracellular survival were calculated in relation to viable counts determined for the no-PMN control samples. Columns represent means of data from three independent experiments. Bars indicate standard deviations. Asterisks indicate significant differences in comparison to the parent strain ($P < 0.05$ as determined by a Kruskal-Wallis test with Dunn's *post hoc* test).

binding and phagocytosis in 2VS1 (Fig. 7D and E). These results support the role of surface-associated EPS in the evasion of opsonophagocytosis and killing by PMNs.

Binding of serum IgG to the *S. mutans* surface and rates of serum-mediated phagocytosis were also assessed in mutants of $CovR_{Sm}$-repressed genes involved in the synthesis of and/or interaction with EPS (Fig. 8). Significant increases in IgG binding to the *S. mutans* surface were promoted by the inactivation of *epsC*, *gbpC* (Fig. 8A), and *gtfBCD* (Fig. 8C). The inactivation of *lysM* and *wapE* had modest effects on IgG binding (Fig. 8A), compatible with the limited effects of these genes on binding to sucrose-derived EPS (Fig. 3B). Increases in phagocytosis were also observed for the *epsC*, *gbpC*, and *gtfBCD* mutants (Fig. 8B and D). As observed for C3b deposition, complementation of the *gbpC* and *epsC* mutants restored levels of IgG binding and phagocytosis (Fig. 8A and B). These findings strengthen the influence of *S. mutans* interactions with sucrose-derived EPS on strain susceptibilities to opsonic phagocytosis by human PMNs.

Inactivation of $covR_{Sm}$ and of the $CovR_{Sm}$-repressed genes *gbpC* and *epsC* affects survival of *S. mutans* in human blood and systemic virulence. Because complement immunity is an important mechanism of blood clearance of streptococcal pathogens (28, 30, 47), we investigated the effects of the inactivation of $covR_{Sm}$ and downstream genes on the *ex vivo* survival of *S. mutans* in human blood. The UAcov mutant showed an increased capacity to survive in human blood, which was completely restored in the $UAcov^{+}$ complemented mutant (Fig. 9A). Consistent with the role of $CovR_{Sm}$ as a direct repressor of *gbpC* and *epsC*, the UAgbpC and UAepsC mutants showed reduced survival in blood compared to the parent strain and the respective complemented mutants (Fig. 9B and C). To further confirm the effects of EPS on the increased *ex vivo* survival of UAcov in blood, assays were performed with strains grown in sucrose-free CDM and in CDM with 0.1% sucrose. As shown in Fig. 9D, differences in survival in blood between UAcov and UA159 were eliminated when strains were grown in CDM, whereas CDM supplementation with sucrose increased differences between UAcov and parent strains (Fig. 9E). Thus, sucrose-derived EPS are involved in the increased survival of UAcov in human blood.

Comparisons of bacterial counts in the bloodstream of rats confirmed the findings of *ex vivo* survival in human blood for UAcov. As shown in Table 3, the UAcov mutant survives for longer periods and at higher counts in the rat bloodstream than the parent or complemented strains. Two rats infected with UAcov died during the experiment, while no deaths occurred in the UA159-infected group. Higher *S. mutans* counts were found in valves of UAcov-infected rats (mean, 20,090 ± 44,467 CFU/ml; median, 40 CFU/ml) than in valves of UA159-infected animals (mean, 1,352 ± 2,723 CFU/ml; median, 0 CFU/ml), although differences between strains did not reach statistical significance ($P > 0.05$ as determined by a Kruskal-Wallis test with Dunn's *post hoc* test).

DISCUSSION

The complement system plays multiple roles in the elimination of microorganisms, both as part of the innate immune system and by augmenting antibody-mediated immunity (17, 18). The reduced susceptibilities to C3b deposition found in *S. mutans* blood strains (Fig. 1) indicate that evasion of complement immunity is important for the systemic virulence of *S. mutans*. In GAS, natural mu-

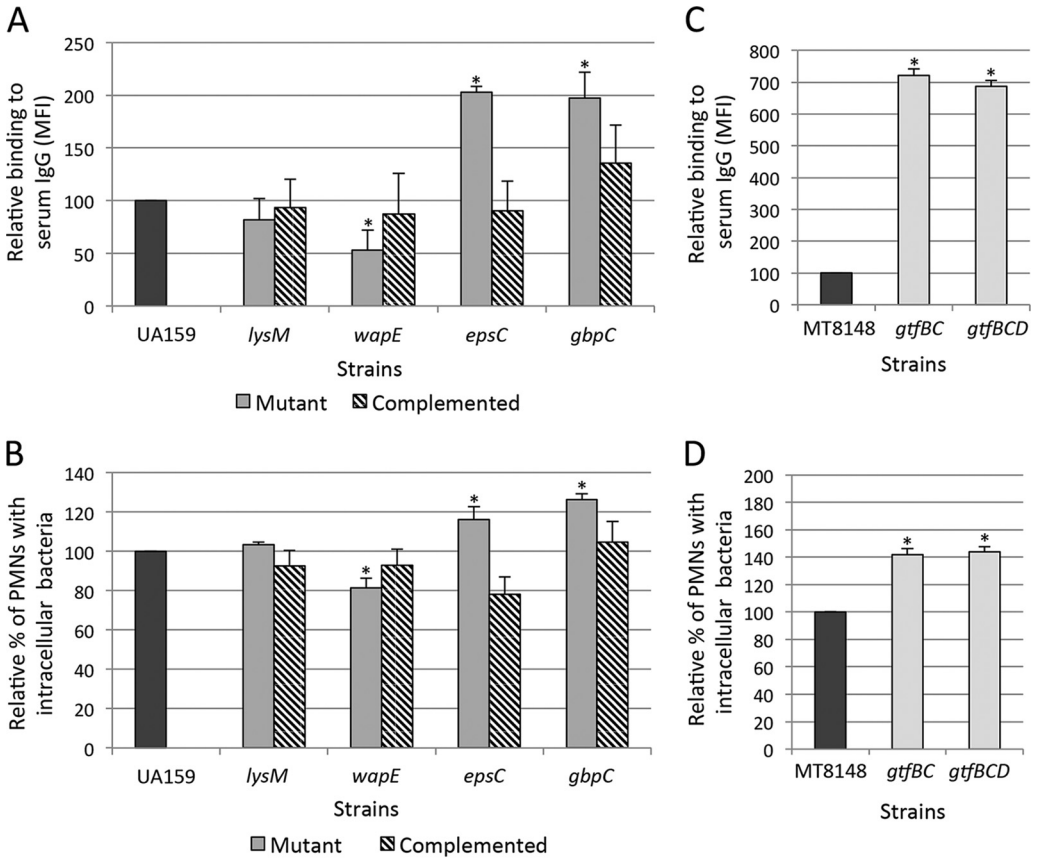

FIG 8 Comparisons of binding to serum IgG (A and C) and of phagocytosis by human PMNs (B and D) between mutants of genes regulated by $CovR_{Sm}$. Mutant or complemented strains were compared with the respective parent strains (UA159 or MT8148). Columns represent means of data from three independent experiments; bars indicate standard deviations. Asterisks indicate significant differences in relation to the parent strain ($P < 0.05$ as determined by a Kruskal-Wallis test with Dunn's *post hoc* test).

tations in $covR_{Spy}$ were detected in strains involved in human infections, and inactivation of the $CovRS_{Spy}$ TCS enhanced strain virulence in murine models (48–50). Virulence genes repressed by $CovR_{Spy}$ include genes involved in complement evasion (e.g., *has* operon for hyaluronic acid capsule synthesis) (50–52), which are not present in *S. mutans* genomes (53, 54). Reduced transcript levels of $covR_{Sm}$ in blood isolates associated with increased transcription of $CovR_{Sm}$-repressed genes (*gbpC*, *gbpB*, and *epsC*) suggest that the diversity in $covR_{Sm}$ activities influences the capacities of *S. mutans* strains to survive in the bloodstream.

In GAS and GBS, the $CovRS_{Spy}$/*S. agalactiae* CovRS ($CovRS_{Sag}$) regulons show strain specificity (55, 56). The $CovR_{Sm}$ regulon was assessed in serotype *c* strain UA159 (8, 11), but its diversity remains to be investigated in strains associated with systemic infections. *S. mutans* serotype *c* was detected with higher frequencies in *S. mutans*-positive specimens of heart valves and atheromatous plaques from patients subjected to cardiovascular surgeries (30.3 and 65.5% of specimens, respectively) than serotype *k* (detected in 9.1 and 25% of these specimens, respectively) (14), which was previously implicated in systemic infections (57). Interestingly, 77% of serotype *k*-positive specimens were also positive for serotype *c* (41), suggesting synergy of *S. mutans* serotypes for systemic virulence. The systemic virulence of serotype *k* is associated with the expression of the collagen-binding proteins Cnm and Cbm, which are involved in the capacity of *S. mutans* to invade endothelial cells *in vitro* (40, 58–60) and to form vegetations on injured heart valves in a rat model of infective endocarditis (40). However, serotype *c* strains rarely harbor these genes (22), and there is no report that $CovR_{Sm}$ regulates *cnm* or *cbm*.

A major function of complement immunity against Gram-positive bacteria is to covalently bind C3b/iC3b opsonins on the bacterial surface through the activity of C3 convertases on C3 (17, 18). C3 convertases result from proteolytic cascades initiated by different mechanisms, known as the classical, mannan-binding lectin, and alternative pathways (17). Functions of each pathway in complement immunity against streptococci seem to be species specific (27, 28, 30, 45) and are usually circumvented by multiple evasion mechanisms (41, 61, 62). Here, we show that the classical pathway plays a major role in complement deposition on *S. mutans* (Fig. 5C), which is consistent with the reduced binding of IgG antibodies to UAcov (Fig. 5A). Because C1q is activated through its binding to IgG on the bacterial surface, assessing individual roles of complement and IgG in *S. mutans* opsonization is difficult. In addition, although *S. mutans* seems to not have prototypical CRP ligands (63), $covR_{Sm}$ inactivation also reduced binding to CRP (Fig. 5B). CRP levels are increased in the bloodstream of subjects with biofilm-dependent oral diseases, e.g., gingivitis and periodontitis (64); thus, the role of acute-phase proteins in *S. mutans* blood clearance needs to be investigated.

The major known role of $CovR_{Sm}$ in *S. mutans* biology is to

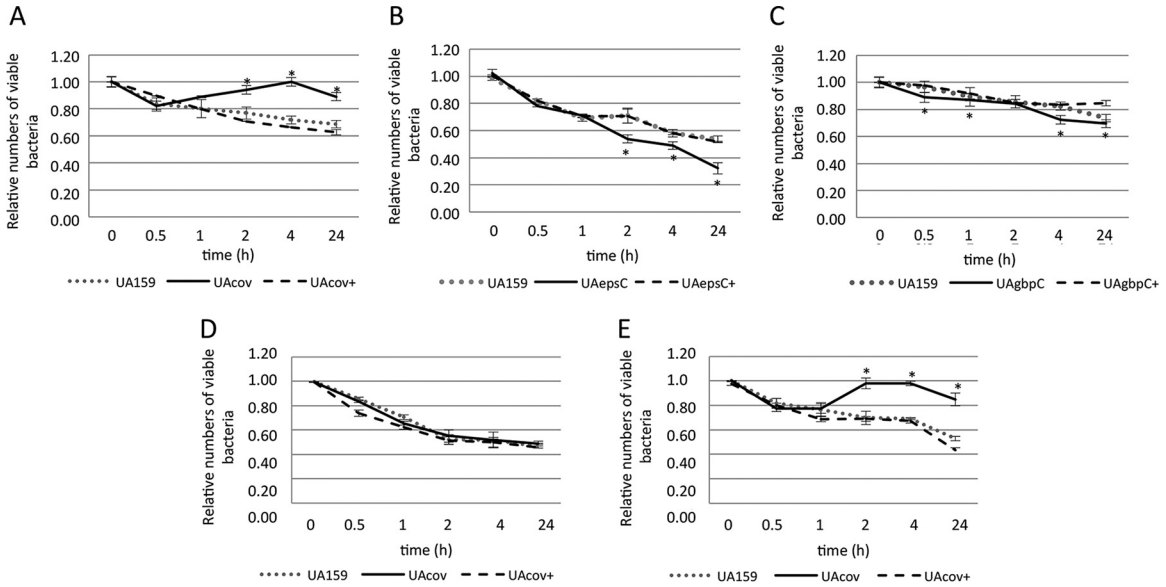

FIG 9 *Ex vivo* viability in human blood. Numbers of viable bacteria (log CFU per milliliter) were expressed in relation to initial counts in blood suspensions (time zero). Strains were grown in BHI medium (A to C), CDM (D), or CDM supplemented with 0.1% sucrose (E). Data represent means of results from triplicates of one representative experiment. Bars indicate standard deviations. Differences in relation to the parent strain at each time point were tested by a Kruskal-Wallis test with Dunn's *post hoc* test (*, $P < 0.05$).

regulate the expression of secreted enzymes for the synthesis of EPS from sucrose and cell surface components involved in interactions with EPS (5, 6, 10, 11). Some of these genes, e.g., *gtfBC*, *ftf*, and *wapE*, are controlled by a complex regulatory circuit (11, 65–67), which might explain the lack of associations between *gtfBC* transcription and profiles of $covR_{Sm}$ expression among the tested strains (Fig. 2). Because the secreted GtfBC enzymes are stable in saliva and bind to several oral bacteria (2), strains with increased binding to EPS could benefit from Gtf-producing members of the same ecological niche. Thus, an increased capacity to bind EPS would be more significant for systemic virulence than the ability to produce Gtfs itself. In serotype *c* strain V403, the deletion of multiple genes required for the synthesis of EPS from sucrose (*gtfB*, *gtfC*, and *ftf*) increased *S. mutans* phagocytosis by human granulocytes and reduced virulence in an animal model of infectious endocarditis (68). Consistently, our *gtfBC* and *gtfBCD* mutants showed high susceptibility to C3b opsonization even when grown in the presence of sucrose (Fig. 6). However, the production of sucrose-derived EPS impaired C3b deposition only in strains that were able to bind these polymers; the *gbpC* mutant was susceptible to C3b deposition even when grown in the presence of sucrose (Fig. 6). Thus, the expression of *gbpC*, and perhaps other glucan-binding proteins upregulated in blood isolates, e.g., GbpB (Fig. 2), might be critical for EPS-mediated complement evasion.

The increased binding of the *gbpC* mutant to serum IgG (Fig. 6) further indicates that surface EPS may impair antibody-mediated activation of complement in a way analogous to that of capsules of *S. pneumoniae* (45, 47, 69). Besides GbpC, EpsC showed a prominent influence on complement opsonization (Fig. 6 and 8). In Gram-positive bacteria, EpsC is required for the production of UDP-ManNAc, an intermediate for the synthesis of EPS which is also required for the attachment of teichoic acids to the cell wall (70–73). The *epsC* mutant retained some degree of binding to sucrose-derived EPS (Fig. 3B), which might explain, at least in part, the remaining influence of sucrose on the binding of C3b to this mutant (Fig. 6). Alternatively, EpsC could also affect sucrose-independent mechanisms of *S. mutans* evasion of complement immunity. Functional analyses of EpsC might shed new light on its roles in complement susceptibility.

Although the effects of the *covR* deletion on C3b opsonization were more clearly observed when strains were grown in sucrose-containing media, reductions in levels of C3b binding to UAcov were still observed in sucrose-free CDM (Fig. 6), which suggests that $CovR_{Sm}$ regulates additional functions of complement evasion. The lower levels of C3b binding to blood strains grown in sucrose-free CDM (especially in SA13) (Fig. 6) account for the hypothesis that *S. mutans* strains apply multiple mechanisms of complement evasion. In GAS strains, $CovR_{Spy}$ plays multiple roles in complement evasion besides regulating capsule production (50,

TABLE 3 Bacterial counts in blood of rats ($n = 7$)

Infecting strain	Mean CFU/ml of blood ± SD (no. of rats with bacteria recovered)[a]				
	1 h	3 h	6 h	24 h	7 days
UA159	1,627 ± 1,029 (7)	247 ± 135 (7)	19 ± 19 (4)	0 (0)	0 (0)
UAcov	1,767 ± 1,053 (7)	194 ± 147 (7)	119 ± 98* (6)	0 (0)	16 ± 36* (5)
UAcov$^+$	2,501 ± 2,309 (7)	104 ± 64* (7)	16 ± 15 (4)	3 ± 5 (2)	0 (0)

[a] Asterisks indicate significant differences in relation to the parent strain at the same time period ($P < 0.05$ as determined by a Kruskal-Wallis test with Dunn's *post hoc* test).

52, 74). Studies are under way to identify additional factors affecting *S. mutans* susceptibility to complement immunity.

The increased persistence of UAcov in human blood mediated by sucrose-derived EPS (Fig. 9) and its ability to cause bacteremia in rats (Table 3) further strengthen the role of $CovR_{Sm}$ in systemic virulence. Reduced C3b/IgG opsonization of UAcov is, at least in part, explained by the upregulation of *epsC* and *gbpC*, because the inactivation of these genes reduced survival in human blood (Fig. 9A and B). Different from this study, no reduction in survival in the bloodstream of rats was observed for a *gbpC* mutant (C1) obtained from MT8148 compared to a streptomycin-resistant MT8148 variant (MT8148R) (75). Although we found that C1 has an increased susceptibility to C3b deposition compared to the MT8148 parent strain (data not shown), C3b deposition in the MT8148R variant is unknown. As shown in this study, differences in growth media can affect *S. mutans* susceptibility to complement opsonization. Furthermore, bacterial aggregation mediated by blood components could affect bacterial counts in blood suspensions. UAcov shows increased aggregation in blood compared to UA159 (data not shown), which could explain the initial reductions in UAcov counts in the *ex vivo* assays, although bacterial loads were normalized by initial blood counts (Fig. 9). Increased aggregation of UAcov could occur in the rat bloodstream; thus, survival rates of UAcov (Table 3) might be underestimated.

There may also be differences between human blood and rat blood in complement activation on *S. mutans*. *S. mutans* is an exclusive species of humans; thus, levels and epitope specificities of IgG antibodies to *S. mutans* may differ in human and rat sera. There are further differences in the production and structure of CRP between rats and humans (76). In addition, CR1, shown to be important for *S. mutans* opsonophagocytosis, is also involved in blood clearance by human erythrocytes through immune adherence (77). Because rodent erythrocytes do not express CR1 (77), a more complete analysis of the influence of C3b deposition on blood clearance of *S. mutans* would require animal models designed to assess CR1-mediated immune adherence (78). Apart from the limitations of our model, significant increases in viable counts of UAcov bacteria in the rat bloodstream compared to those of UA159 bacteria were detected (Table 3). At 6 h postinfection, the counts of UAcov mutant bacteria were 6.3-fold higher (detected in 85.7% of the animals) than those of the parent strain (detected in 57.1% of animals). Although UAcov counts in heart valves were higher than UA159 counts, these differences did not reach significance. Because only the numbers of viable bacteria were assessed, we cannot exclude the possibility that increased differences in tissue infection between strains might have been observed if total levels of bacteria in heart valve specimens were measured by using culture-independent methods. In addition, survival of UAcov in the rat bloodstream would likely increase if strains were previously grown in medium with 0.1% sucrose added. Therefore, studies are required to improve *in vivo* models for assessing the influence of complement evasion on the systemic virulence of *S. mutans*.

In summary, this study provides evidence that systemic virulence of *S. mutans* strains involves reduced susceptibility to complement-mediated opsonization. Roles of $CovR_{Sm}$ in resistance to complement immunity involves regulation of the capacity of *S. mutans* to interact with EPS, which in turn affects complement activation. Two $CovR_{Sm}$-repressed genes, *gbpC* and *epsC*, were identified as playing important roles in resistance to complement immunity and survival in blood, as revealed by transcriptional profiles of these genes in isolates from systemic infections and by molecular analyses of isogenic mutants.

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2.2 Artigo 2: The two-component system VicRK regulates functions associated with *Streptococcus mutans* resistance to complement immunity.

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Running title: VicRK on *S. mutans* evasion to complement

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ORIGINAL ARTICLE

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The two-component system VicRK regulates functions associated with *Streptococcus mutans* resistance to complement immunity

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Summary

Streptococcus mutans, a dental caries pathogen, can promote systemic infections upon reaching the bloodstream. The two-component system (TCS) VicRK$_{Sm}$ of *S. mutans* regulates the synthesis of and interaction with sucrose-derived exopolysaccharides (EPS), processes associated with oral and systemic virulence. In this study, we investigated the mechanisms by which VicRK$_{Sm}$ affects *S. mutans* susceptibility to blood-mediated immunity. Compared with parent strain UA159, the *vicK*$_{Sm}$ isogenic mutant (UAvic) showed reduced susceptibility to deposition of C3b of complement, low binding to serum immunoglobulin G (IgG), and low frequency of C3b/IgG-mediated opsonophagocytosis by polymorphonuclear cells in a sucrose-independent way ($P<.05$). Reverse transcriptase quantitative polymerase chain reaction analysis comparing gene expression in UA159 and UAvic revealed that genes encoding putative peptidases of the complement (*pepO* and *smu.399*) were upregulated in UAvic in the presence of serum, although genes encoding murein hydrolases (SmaA and Smu.2146c) or metabolic/surface proteins involved in bacterial interactions with host components (enolase, GAPDH) were mostly affected in a serum-independent way. Among *vicK*$_{Sm}$-downstream genes (*smaA*, *smu.2146c*, *lysM*, *atlA*, *pepO*, *smu.399*), only p*epO* and *smu.399* were associated with UAvic phenotypes; deletion of both genes in UA159 significantly enhanced levels of C3b deposition and opsonophagocytosis ($P<.05$). Moreover, consistent with the fibronectin-binding function of PepO orthologues, UAvic showed increased binding to fibronectin. Reduced susceptibility to opsonophagocytosis was insufficient to enhance *ex vivo* persistence of UAvic in blood, which was associated with growth defects of this mutant under limited nutrient conditions. Our findings revealed that *S. mutans* employs mechanisms of complement evasion through peptidases, which are controlled by VicRK$_{Sm.}$

KEYWORDS
bacteremia, complement system, *Streptococcus mutans*, two-component system

1 | INTRODUCTION

Streptococcus mutans plays important functions in the assembly of cariogenic biofilms, which include secretion of glucosyltransferases required for the synthesis of insoluble exopolysaccharides (EPS) from sucrose.[1] Sucrose-derived EPS bound to glucan-binding proteins expressed on the *S. mutans* surface further reduces bacterial susceptibility to blood immunity, so accounting for the capacity of this microorganism to promote bacteremia.[2,3] During the processes of host colonization or infection, *S. mutans* uses two-component systems (TCS) to sense and respond to environmental challenges. These transductional systems are typically composed by a sensor histidine kinase membrane protein and an intracellular response regulator. Thirteen or 14 TCS as well as the orphan response regulator called CovR (also known as GcrR) were identified in the available genomes of *S. mutans*.[4-6] The TCS VicRK$_{Sm}$ is of special interest because it regulates functions required for *S. mutans* cariogenicity and cell wall integrity. VicRK$_{Sm}$-induced genes include those encoding the glucosyltransferases B (GtfB) and C (GtfC) (*gtfB* and *gtfC*, respectively) for the synthesis of glucan EPS from sucrose, the glucan-binding protein B (GbpB) (*gbpB*) and the murein hydrolases LysM and SMU.2146c (*lysM* and *smu2146c*, respectively), which are involved in *S. mutans* interactions with EPS.[7-10] On the other hand, *VicRK$_{Sm}$* also strongly represses *smaA*, which encodes the murein hydrolase SmaA.[10]

The general role of VicRK$_{Sm}$ as a modulator of cell division, cell wall biogenesis and interaction with EPS might explain the essentiality of this TCS for *S. mutans* viability,[8,9,11] increasing the interest in VicRK$_{Sm}$ as a therapeutic target to control *S. mutans* infections.[12,13] However, we have previously observed that deletion of the gene encoding the VicK$_{Sm}$ sensor protein (*vicK$_{Sm}$*) in *S. mutans* strains impaired bacterial phagocytosis by polymorphonuclear cells (PMN) in samples of human blood.[14] These findings indicate that VicRK$_{Sm}$ downstream genes could be involved in *S. mutans* evasion of blood-mediated opsonophagocytosis and hence, in systemic virulence. Therefore, it is important to identify gene functions that account for the resistance of *vicK$_{Sm}$* mutants to opsophagocytosis by PMN.

The aim of this study was to investigate the molecular mechanisms by which deletion of *vicK$_{Sm}$* affects *S. mutans* susceptibility to blood-mediated immunity. Here, we assessed the effects of *vicK* deletion on *S. mutans* binding to major blood opsonins [C3b of the complement system and immunoglobulin G (IgG) antibodies], and on its susceptibility to opsonophagocytosis by PMN isolated from peripheral blood. Also, interactions with plasma fibronectin, and *ex vivo* persistence in human blood were investigated. We then determined the contribution of VicRK$_{sm}$ downstream genes (*smaA*, *smu.2146c*, *smu.399*, and *pepO*), known to be involved in functions previously associated with complement evasion in other streptococci, to the *vicK* mutant phenotypes.

2 | METHODS

2.1 | Studied strains, culture conditions, oligonucleotides, and construction of mutants

The studied strains are depicted in Table 1. Strains were grown from frozen stocks in brain–heart infusion (BHI) agar (Difco) (37°C; 10% CO_2, 24 hours). Colonies were then inoculated in BHI, and incubated for 18 hours. Inocula of BHI cultures with adjusted absorbance (A_{550nm}) were then transferred to fresh BHI, chemically defined medium (CDM)[15] supplemented or not with sucrose (0.01% or 0.1%), or RPMI-1640 (Gibco, Life Technologies, Grand Island, NY). The non-polar isogenic mutants of *pepO* (UApepO) and *smu.399* (UA399) were obtained in strain UA159 by double cross-over recombination with null alleles, which were constructed using a polymerase chain reaction (PCR) ligation strategy as previously described.[9,14] Briefly, *pepO* and *smu.399* mutants were obtained by replacing the internal sequences of the encoding regions of *pepO* [1432 base pairs (bp)] or *smu.399* (405 bp) with an erythromycin-resistance gene (amplified from plasmid pVA838). To obtain the complemented strains (+), UApepO and UA399 mutants were transformed with plasmid pDL278 (which harbors a spectinomycin-resistance gene) containing the intact copy of the respective deleted gene. Erythromycin (10 μg mL^{-1}) and spectinomycin (200 μg mL^{-1}) (Merck Labs, Darmstadt, Germany) were added to growth medium for the maintenance of mutant and complemented strains, respectively. Oligonucleotides used for construction of mutants and transcriptional analyses are shown in Table 2.

TABLE 1 Strains used in this study

Strains	Relevant characteristics	Source or reference
UA159	Erm^s, Spec^s	ATCC
UAvic	Δ*vicK*::Erm^r	[9]
UAsmaA	Δ*smaA*::Erm^r	[10]
UA2146c	Δ*smu.2146c*::Erm^r	[10]
UA399	Δ*smu.399*::Erm^r	This study
UApepO	ΔpepO::Erm^r	This study
UAvic+	UAvic with pDL278::*SMU.1516*; Spec^r	[9]
UAsmaA+	UAsmaA with pDL278::*smu.609*; Spec^r	[10]
UA2146c+	UA2146c with pDL278::*smu.2146c*; Spec^r	[10]
UA399+	UA399 with pDL278::*smu.399*; Spec^r	This study
UApepO+	UA2146c with pDL278::*smu.2146c*; Spec^r	This study

2.2 | Volunteers, sera, and blood samples

Blood samples were collected by venepuncture in heparin vacuum tubes (BD Vacutainer®) from six healthy subjects (three men, three women; mean age 30 years, range 25-45 years), who were enrolled in a previous study,[3] according to standard protocols previously approved by the Ethics Committee of the Piracicaba Dental School, State University of Campinas (proc. no. 031/2012). Serum samples were stored in aliquots at −70°C until use. Serum samples from one volunteer were used as reference. Commercial human serum depleted of C1q and purified human C1q were obtained from Calbiochem (San Diego, CA). Heat-inactivated sera (56°C during 20 minutes) were also

TABLE 2 Oligonucleotides used in this study

Primer name	Sequence 5′-3′	Product size (bp)	Source
16SRNAF 16SRNAR	CGGCAAGCTAATCTCTGAAA GCCCCTAAAAGGTTACCTCA	190	[17]
smu360F smu360R	CCTAACTCAACTGGTGCTGCT CAGCATTCACTTCATCAACAG	161	This study
smu630F smu630R	AGAATGGATGCTCTTGGCTTA GCTGTCATAGGCTGTGTTTCA	170	This study
smu676F smu676R	TCGTATGGAAGGTGAAGTC GTAAGAGCCCTGAGATTGAT	218	This study
smu2036F smu2036R	TACCCATAGCTTGAGGTGT ACACCAGAACTGCCTTTAG	253	This study
smu399F smu399R	GATTGAAGAGTCACCGGATA CCGCTTGTTTAGTCTCTTGA	242	This study
smu1247F smu1247R	GACTTCTTCACCTGGTTTG CTCACTCAGATGCTCCAAT	251	This study
smu609F smu609R	GGCACAAGGAACCTATCACTTT GCTTTCCAATAACAACATAACGAC	191	[10]
smu2146F smu2146R	AATCTGTTCTTGCTCACACTGC ACATTATCAGTTGGTTCAGTTGCT	145	[10]
smu2147cF smu2147cR	TTATCAGAGATTGCTTCAACACA CTGAGGTTTCTGCTTCATTTATC	175	[10]
ermE1-AscI ermE2-XhoI	TTGGCGCGCCTGGCGGAAACGTAAAAGAAG TTCTCGAGGGCTCCTTGGAAGCTGTCAGT	998	[10]
smu.399P1 smu.399P2-AscI	TCTTCTTCACCATTTCTTGC TTGGCGCGCCCGGTGACTCTTCAATCAAAA	496	This study
smu.399P3-XhoI smu.399P4	TTCTCGAGTGTTGAGAGTCATGGAGAGG AAAGCTGCCTGATGGTTACT	505	This study
smu.2036P1 smu.2036P2-AscI	TTTACTATCGGCGCTAAGGT TTGGCGCGCCACTGTTTCGGAAAATGTGG	501	This study
smu.2036P3-XhoI smu.2036P4	TTCTCGAGGACGATGGAACTTCACAAAA GATCAAAGGCAATTTACGGG	490	This study
smu.399C1-PvuI smu.399C2-BamHI	GGCGATCGTGGATGTTACGTGGACTGT GGGGATCCGGCCATCATAAAGTGCTAAA	1883	This study
smu.2036C1-BamHI smu.2036C2-SphI	GGGGATCCATGCCGCTAATTGCTCAAG GGGCATGCAGTCAATGAAAAAACGCTTGA	2750	This study

[a]Underlined sequences indicate restriction enzyme linkers.

used as negative control in preliminary experiments, and showed minimal effects on comparative analyses of C3b deposition between mutant and parent strains.

2.3 | C3b deposition on *S. mutans* strains

Deposition of C3b on the surface of serum-treated strains was determined as previously described[16] with some modifications.[3] Briefly, approximately 10^7 colony-forming units (CFU) of *S. mutans* strains harvested (10 000 ***g***, 4°C) from BHI cultures at mid-log growth phase (A_{550nm} 0.3) were washed twice with phosphate-buffered saline (PBS) (pH 7.4) and suspended in 20% of serum in PBS. After 30-min incubation (37°C), cells were washed twice with PBS-Tween 0.05% (PBST), and incubated on ice (40 minutes) with fluorescein isothiocyanate (FITC) -conjugated polyclonal goat IgG anti-human C3 antibody (ICN, Irvine, CA) (1 : 300 in PBST). Cells were then washed twice with PBST, fixed in 3% paraformaldehyde in PBS and analyzed on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) using forward and side scatter parameters to gate at least 25 000 bacteria. Levels of surface-bound C3b were expressed as the geometric mean fluorescence intensity (MFI) of C3b-positive cells. Control samples included bacteria treated only with PBS instead of serum.

2.4 | Binding of serum IgG antibodies to *S. mutans* strains

Levels of serum IgG reactive with *S. mutans* were determined as previously described.[3] Briefly, bacterial strains at mid-log phase of growth

(A_{550nm} 0.3) were harvested from BHI cultures (500 μL), washed twice with PBS (pH 7.0), and incubated with 20% serum in PBS. Cells were then washed with PBST and incubated (on ice) for 40 minutes with polyclonal goat IgG anti-human IgG conjugated with FITC (Novus Biologicals, Littleton, CO) (1:900). After two washes with PBST, cells were then harvested by centrifugation and suspended in 3% paraformaldehyde. Flow cytometry analyses were performed as described before, using forward and side scatter parameters to gate at least 25,000 bacteria. Bacterial samples treated with PBS instead of serum were used as negative controls.

2.5 | PMN isolation and opsonophagocytic assays

Isolation of human PMN from samples of fresh heparinized blood collected from a reference volunteer were performed as described elsewhere.[3] Cell viability (>98%) was monitored by trypan blue exclusion and cell purity (>95%) by May–Grunwald Giemsa staining. Bacteria applied in phagocytosis assays were labeled with FITC as previously described,[3] and aliquots were stored overnight in 10% glycerol at −70°C. Bacterial labeling was monitored in a fluorescent microscope (Leica DM LD), and by flow cytometry (FACSCalibur; BD Biosciences). Aliquots containing 10^7 CFU of FITC-labeled bacteria were incubated with 20% serum and exposed to 2×10^5 PMN to a multiplicity of infection of 200 bacteria per PMN.[3] After incubation (37°C, 10% CO_2, gentle shaking) for 5 or 30 minutes, reactions were fixed by addition of 100 μL of 3% paraformaldehyde. PMN were then analyzed using FACSCalibur (BD Biosciences), and the frequency of phagocytosis was expressed as the number of PMN cells with intracellular bacteria, within a total of 10,000 PMN analyzed. To confirm that most PMN-associated bacteria were internalized, flow cytometry results were compared using light microscopy analysis of samples stained using May–Grunwald–Giemsa, as previously described.[14] In addition, to confirm that phagocytosis by PMN involved binding to surface C3b or IgG, similar assays were performed with PMN previously incubated (37°C, 30 minutes) with mouse monoclonal antibodies (mAbs) anti-CD35 (Biolegend, San Diego, CA) to block CR1 receptors, or with mAbs anti-CD32 to block IgG2 Fc receptors (eBioscience, San Diego, CA).[3]

2.6 | RNA isolation and transcriptional analysis of strains exposed to human serum

Transcriptional analysis of *smaA*, *smu.2146c*, *lysM*, *atlA*, *gapN*, *gapC*, *smu.1247* (*eno*), *smu.399*, and *pepO* was performed in strains exposed or not to human serum. Briefly, strains at mid-log phase (A_{550nm} 0.3) in BHI were harvested (6000 ***g***, 5 minutes, 4°C), resuspended in BHI or BHI supplemented with 20% human serum and incubated (37°C; 5% CO_2) for 30 minutes. Then, cells were harvested, washed with cold saline, and total RNA was purified using RNeasy kit (Qiagen, Hilden, Germany). Samples were then treated with Turbo DNase (Ambion, Austin, TX), as previously described.[10] The cDNA was obtained from 1 μg of RNA using random primers[17] and SuperScript III (Life Technologies), according to the manufacturer's instructions. Quantitative PCR was performed in a StepOne™ Real-Time PCR System (Life Technologies) with cDNA (10 ng), 10 μmol L^{-1} of each primer, and 1× Power SYBR® Green PCR Master Mix (Life Technologies) in a total volume of 10 μL. The cycling conditions included incubation at 95°C (10 minutes), followed by 40 cycles of 95°C (15 seconds), optimal temperature for primer annealing (55-60°C, 15 seconds) (Table 2), and 72°C (30 seconds). Ten-fold serial dilutions of genomic DNA (0.003-300 ng) were used to generate standard curves for absolute quantification of transcript levels. Melting curves were obtained for each primer set. Results were normalized against *S. mutans 16SrRNA* gene expression.[17] Assays were performed in triplicate with three independent RNA samples.

2.7 | Binding of *S. mutans* strains to fibronectin

Fibronectin-binding assays were performed as previously described,[18] with modifications.[19] Briefly, human plasma fibronectin (Sigma-Aldrich) (50 μg mL^{-1}) was immobilized in 96-well plates for 18 hours at 4°C, washed with PBS and blocked with 5% bovine serum albumin for 1 hours at 37°C. Strains from 18-h cultures in BHI were washed twice in PBS (pH 7.2), and resuspended in the same buffer to 1×10^9 CFU mL^{-1}. Volumes of 100 μL of these suspensions were transferred to fibronectin-coated wells and incubated (37°C) for 3 hours. Afterwards, unbound cells were removed by a series of three washes with PBS. Fibronectin-adherent cells were stained with 0.05% crystal violet, and intensities of staining were measured by spectrophotometry (A_{575nm}) in 7% acetic acid eluates. Wells treated similarly but without fibronectin or bacterial suspensions added were used as negative controls.

2.8 | Analysis of the production of surface enolase and glyceraldehyde-3-phosphate dehydrogenase

Protein extracts from whole cells were obtained as previously described[10] with minimal modifications. Briefly, strains at mid-log phase of growth in BHI, were harvested (10 000***g***, 4°C, 3 minutes), washed twice with cold saline, and 125 μL of ultrapure water with 0.16 g of 0.1-mm Zirconium Beads (Biospec, Bartlesville, OK) were added for cell disruption on a Mini-beadbeater (Biospec) (maximum power; two cycles of 45 seconds with 1 minutes rest on ice). The culture supernatants were also collected, 0.1 mmol L^{-1} of phenylmethylsulfonyl fluoride was added for inhibition of serine proteases, and dialyzed at 4°C against 0.02 mol L^{-1} PBS (pH 6.5) followed by 0.125 mol L^{-1} Tris–HCl. Afterwards, these samples were 100-fold concentrated by lyophilization.[20] Protein concentration was determined using a Bradford assay kit (BioRad, Hercules, CA), according to the manufacturer's protocol.

α-Enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured in whole cell extracts and culture supernatants using immuno-dot blot and/or Western blot assays performed with polyclonal rabbit IgG antibodies to surface enolase or to extracellular GAPDH of *S. pyogenes*, which were kindly provided by Dr. Vijay Pancholi (The Ohio State University College of Medicine, Columbus, OH).

Antibody titers were determined in preliminary Western blot assays, using the *S. pyogenes* strain SF130 (ATCC12344) as control. Equivalent amounts of protein (20 μg) were used in dot blot assays, which were performed as described elsewhere[21] with some modifications. Briefly, samples were blotted onto nitrocellulose membranes (BioRad), and blocked (2 hours) with PBS with 5% skimmed milk. After a series of three washes with PBS, the membranes were probed with rabbit anti-serum to enolase (1:2000) or to extracellular GAPDH (1:40 000) for 90 minutes. Membranes were then washed and incubated (room temperature, 60 minutes) with goat IgG anti-rabbit IgG conjugated with horseradish peroxidase (Thermo Fisher Scientific, Waltham, MA). Immune reactions were detected using the SuperSignal West Dura system (Thermo Fisher Scientific). Serially diluted samples of *S. pyogenes* extracts were used as standard. Autographs were scanned using the ALLIANCE 9.7 documentation system (Uvitec Cambridge) and intensities of the reactions measured using the IMAGEJ software (http://imagej.nih.gov/ij/). Densitometric measures within a linear range of the *S. pyogenes* standard curves were expressed as arbitrary units. Experiments were performed in triplicate. For the Western blot assays, samples were resolved in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels, transferred to PVDF membranes (Millipore, Billerica, MA), and immune reactions were performed as described before. Protein profiles were monitored in duplicate gels stained with Coomassie blue. As a control for *S. mutans*-secreted proteins, samples of culture supernatants were also probed with anti-GbpB antibody.[9]

2.9 | *Ex vivo* survival of *S. mutans* strains in human blood

Bacterial survival in human blood was analyzed as previously described.[3] Briefly, cells from BHI cultures (A_{550nm} 0.3) were harvested (11 000 ***g***, 2 minutes), washed twice in PBS, and resuspended in fresh whole human blood (1 mL) collected from the reference volunteer. Samples were then incubated (37°C, 5% CO_2, gentle agitation), and aliquots were collected at different time-points (from 0.5 to 24 hours), serially diluted and plated on BHI agar for determination of bacterial counts (CFU mL^{-1}). Aliquots collected just after bacterial suspension in blood (time 0) were used as initial blood counts. Changes in bacterial counts were then calculated in relation to initial counts, to reduce the influence of variations in blood-mediated aggregation between strains in the numbers of CFU mL^{-1}. Three independent experiments were performed in triplicate.

2.10 | Statistical analyses

Flow cytometry data (MFI values for C3b and IgG, percentages of PMN with FITC-labeled bacteria) and relative measures of α-enolase and GAPDH were analyzed by comparing means of three independent experiments, using nonparametric Kruskal-Wallis analysis with Dunns's *post hoc* test. Transcriptional data were analyzed using analysis of variance with *post hoc* Dunnett's test. For growth curve experiments and for *ex vivo* survival in blood, Kruskal-Wallis with Dunns's *post hoc* test and correction for repeated measures were used.

3 | RESULTS

3.1 | Deletion of *vicK* impairs deposition of C3b, binding to serum IgG and opsonophagocytosis by human PMN from peripheral blood

Previously, we observed that deletion of *vicK* in UA159 (UAvic) and in LT11 impaired phagocytosis by PMN in samples of human blood.[14] Because complement-mediated opsonization is crucial for efficient phagocytosis of *S. mutans* by PMN,[3] we compared the susceptibility of UA159, UAvic and UAvic+ to C3b deposition. As shown in Figure 1A, deposition of C3b was significantly impaired in UAvic when strains were treated with a reference serum, or with pooled sera, whereas C3b deposition was completely restored in the complemented mutant UAvic+. Minimal C3b deposition on UA159, UAvic, and UAvic+ was observed in C1q-depleted serum, compatible with previous findings that C3b deposition on *S. mutans* is dependent on the classical pathway of complement activation. Because specific binding of IgG to the bacterial surface activates the C1 complex of the classical pathway, we also compared levels of IgG binding to the tested strains. As shown in Figure 1B, the UAvic mutant showed reduced binding to serum IgG whereas the levels of IgG binding to UAvic+ did not significantly differ from the parent strain.

Consistently with low levels of C3b and IgG binding, the UAvic showed impaired opsonophagocytosis (Figure 1C). To confirm that the frequencies of phagocytosis measured in our assays involved PMN interactions with surface-bound C3b and/or IgG opsonins, we assessed the influence of blocking PMN receptors for C3b (CR1) and Fc portions of IgG antibodies (FcγRII) on phagocytosis efficiencies. As shown in Figure 1C, blocking of either CR1 (CD35) or FcγRII (CD32) receptors strongly reduced phagocytosis of UA159 (and of UAvic+), and so reduced differences in the frequencies of phagocytosis between the three tested strains.

3.2 | Deposition of C3b on UAvic is not influenced by sucrose-derived EPS

The major role of the TCS $VicRK_{Sm}$ in cariogenicity of *S. mutans* seems to be associated with transcriptional activation of genes required for the synthesis of and interaction with EPS derived from sucrose.[7,10] Because the UAvic mutant has reduced production of sucrose-derived EPS and defects in biofilm formation,[9,10] it seems likely that reduced susceptibilities of this mutant to C3b deposition and IgG binding were not due to surface-associated EPS or to altered expression of surface components in response to sucrose. To address this issue, we compared levels of C3b deposition between strains grown in sucrose-free CDM and in complex BHI with strains grown in media (CDM and BHI) supplemented with increasing amounts of sucrose. Because sucrose-derived EPS bound to *S. mutans* blocks C3b deposition,[3] previous exposure of UA159 and UAvic+ to sucrose reduced levels of C3b deposition on these strains (Figure 1D). However, the UAvic mutant showed low levels of C3b deposition even when grown in sucrose-free CDM (Figure 1D). Hence, reduced susceptibility of UAvic to

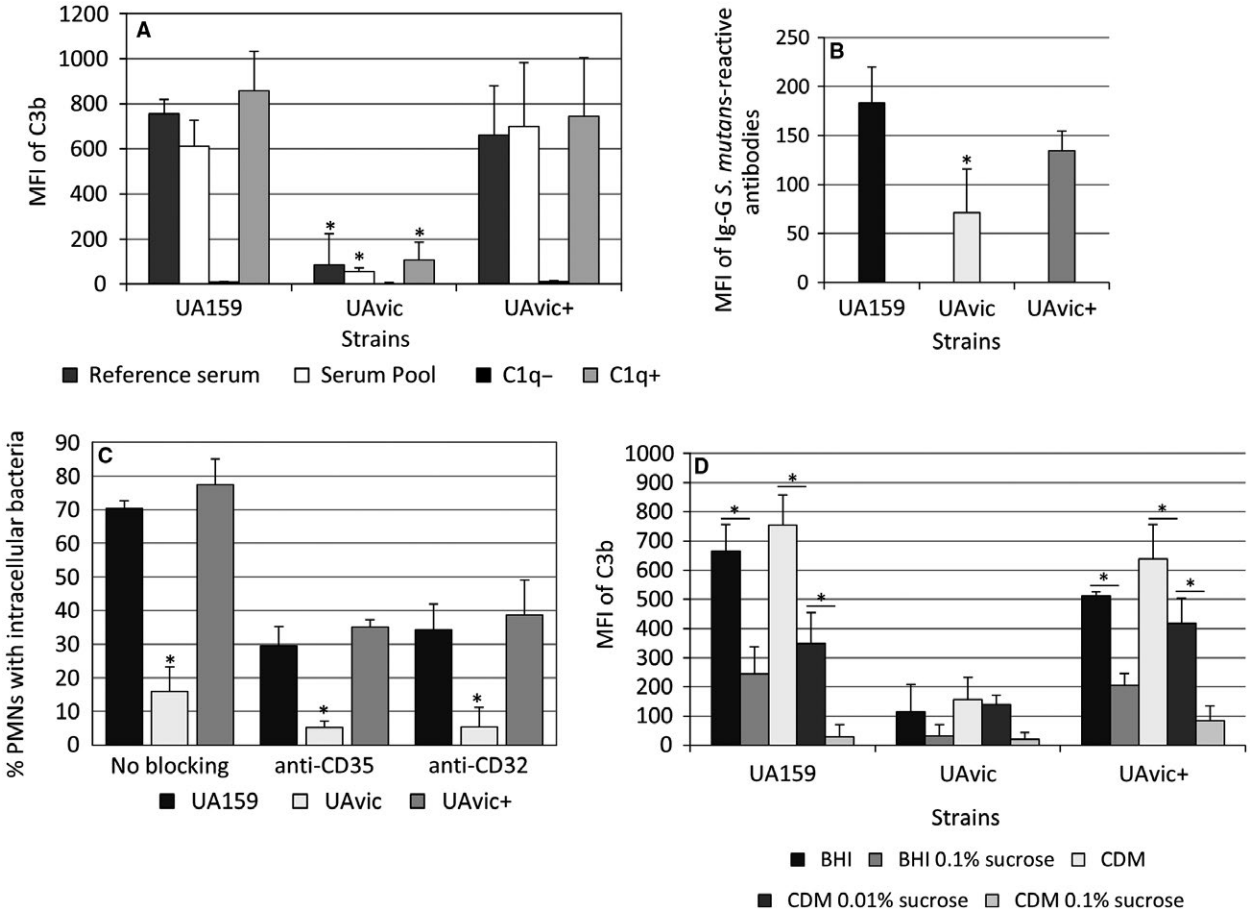

FIGURE 1 Comparisons of C3b deposition, IgG binding and opsonophagocytosis by polymorphonuclear cells (PMN) between the *vicK* mutant (UAvic) and parent (UA159) or complemented strain (UAvic+). Intensities of C3b deposition (A) or binding to serum IgG antibodies (B) were determined by flow cytometry (MFI) in strains treated with 20% human serum. (A) Levels of surface C3b were measured after bacterial treatment with a reference serum, pools of sera obtained from six volunteers, commercial serum depleted of C1q (C1q−) and C1q− serum supplemented with purified C1q (C1q+). (B) Binding to IgG was measured in strains treated with a reference serum. (C) Percentages of PMN with internalized bacteria were determined after exposure of PMN isolated from peripheral blood to fluorescein isothiocyanate-labeled bacteria in the presence of 20% serum. PMN treated with monoclonal antibodies to block CR1 (CD35) or FcγRIIa (CD32) receptors were used as control. (D) Levels of C3b deposition were measured in strains grown in brain–heart infusion (BHI) or chemically defined medium (CDM) supplemented or not with 0.01%-0.1% sucrose. Columns represent means of three independent experiments. Bars indicate standard deviations. Asterisks indicate significant differences in relation to UA159 within the same condition (A-C) or between conditions under horizontal lines (D) (Kruskal-Wallis with *post hoc* Dunn's test; $P<.05$)

C3b deposition does not rely on sucrose-derived EPS or on altered responses to sucrose.

3.3 | VicRK$_{Sm}$ regulates cell surface biogenesis and complement evasion in the presence of serum

The VicRK$_{Sm}$ seems to be activated in response to cell wall stress,[9,22] which might include interactions with host immune components, e.g. pentraxins and complement proteins.[23,24] Therefore, we investigated the effects of *vicK*$_{Sm}$ inactivation on the transcription of downstream cell wall homeostasis genes (*smaA*, *smu.2146c*, *lysM*, and *atlA*)[10,25] on cells exposed to BHI supplemented with 20% serum and to unsupplemented BHI at mid- and late-exponential growth. Genes encoding the anchorless surface proteins α-enolase (*eno*: *smu.1247*) and the extracellular GAPDH (*gapC*), were also tested because these proteins are involved in binding to serum or extracellular matrix components.[26-28] Interestingly, an *in silico* screening of the genomes of 22 *S. mutans* strains available in the NCBI database, revealed two genes (*smu.399* and *pepO*) potentially involved in evasion to complement immunity. *Smu.399* and *pepO* were found in all *S. mutans* strains analyzed, and the promoter regions of both genes include putative VicR consensus motifs,[29,30] which are shown in Table 3. BLAST analyses of another 20 genes previously implicated in evasion to complement system in other species of streptococci and in *Staphylococcus aureus*,[31] did not reveal orthologues in *S. mutans* strains (data not shown).

As shown in Figure 2A, at mid-exponential growth, *smaA* was significantly upregulated in UAvic compared with UA159 in both conditions, absence (12.9-fold increase; analysis of variance with *post hoc*

TABLE 3 Putative binding motifs of $VicR_{Sm}$

NCBI number	Putative VicR binding motif [a]	Strand	Position (bp)[b]
SMU_2036 (*pepO*)	TGTAAATGATATGaAgC	–	136
	TGTGAAGGCATTGgTtAg	–	109
SMU_399	TGTTAAAAAAATaTTAA	+	82
	TGTTATGGCACTGgTAgC	+	391

[a]VicR box: TGTWAHNNNNNTGTWAH; where W is A or T and H is A, T or C; mismatches are indicated by lower cases.
[b]Position from translation start site.

Dunnett's test, $P<.01$) and presence of serum (3.6-fold increase, $P<.01$). At late-log phase, *smaA* upregulation achieved significance only in the absence of serum (3.6-fold increase; $P<.05$). Consistent with the role of $VicRK_{Sm}$ as inducer of *smu.2146c*,[10] this gene was significantly down-regulated in the UAvic mutant either in the presence or absence of serum at mid- (13.7- and 10.9-fold decreases respectively in the absence and presence of serum; $P<.01$) and late-log (13.0- and 12.2-fold decrease, respectively, in the absence and presence of serum; $P<.01$) growth phases. No significant alterations in transcript levels of the autolysin-encoding genes *lysM* and *altA* were detected (data not shown). On the other hand, the UAvic mutant showed increased expression of the metabolic genes only in cells at mid-log phase exposed to BHI (2.6-, 2.4-, and 2.2-fold increase for *eno*, *gapC*, and *gapN*, respectively; $P<.05$), suggesting that the presence of serum inhibited transcription of these genes in UAvic (Figure 2B). Compared with the other functional gene classes (Figure 2A,B), at mid-log growth phase, genes encoding peptidases of the complement (*smu.399* and *pepO*) were more clearly upregulated in the presence of serum (2.9- and 2.7-fold increase for *smu.399* and *pepO*, respectively), proportionally to their upregulation in the absence of serum (3.6- and 3.8-fold increase, respectively, for *smu.399* and *pepO*) (Figure 2C). At late-log growth phase, *pepO* upregulation was observed only in the presence of serum (3.9-fold upregulation; $P<.05$) (Figure 2C). Therefore, deletion of *vicK* in UA159 transcriptionally affects genes involved in cell wall biogenesis (*smaA*, *smu.2146c*), metabolic enzymes (*eno*, *gapN*, *gapC*) and putative complement proteases (*smu.399* and *pepO*), but these genes are differently affected by the presence of human serum.

3.4 | SmaA, Smu.399, and PepO affect C3b deposition and opsonophagocytosis by human PMN

In *S. pneumoniae*, murein hydrolases significantly affect bacterial susceptibility to complement immunity and opsonophagocytosis through multiple mechanisms including reduction of cell surface interaction with complement activators (e.g. C-reactive proteins), degradation of C3b linked to the cell wall, and recruitment of fluid phase inhibitors of complement.[32,33] Hence, reduced susceptibility of UAvic to complement-mediated opsonization could be, at least in part, due to the altered activities of genes *smaA* and *smu2146c*. To address this issue, we measured levels of opsonin binding and opsonophagocytosis in the *smaA* and *smu.2146c* isogenic mutants. Unexpectedly, although *smaA* is over-expressed in the UAvic mutant, deletion of *smaA* significantly reduced *S. mutans* binding to C3b (52.5% reduction; $P<.05$) (Figure 3A), which was associated with reductions in IgG and opsonophagocytosis (Figure 3B,C). These phenotypes were restored in the complemented mutant. No significant changes in C3b deposition and phagocytosis were observed in the *smu.2146c* mutant, although these phenotypes were affected in the complemented mutant UA2146c+ (Figure 3A,C). Therefore, reduced susceptibility of UAvic to opsonophagocytosis does not involve altered expression of cell wall hydrolases SmaA and SMU.2146c, although SmaA influences *S. mutans* susceptibilities to C3b deposition and opsonophagocytosis.

On the other hand, deletion of *smu.399* and *pepO* increased deposition of C3b on *S. mutans* in 27.5 and 62%, respectively ($P<.05$) (Figure 3A). Compared with UA159, the *smu.399* and *pepO* mutants had increased susceptibility to opsonophagocytosis (Figure 3C), although binding to IgG was significantly increased only in the *pepO* mutant ($P<.05$) (Figure 3B). Importantly, the phenotypes of *pepO* and *smu.399* mutants were restored in the complemented strains (Figure 3). Hence, *pepO* and *smu.399* account for UAvic resistance to C3b-mediated opsonization.

3.5 | The *vicK* mutant has increased binding to human fibronectin

The presence of fibronectin in serum might affect the course of the classical pathway of complement activation because it binds to the collagen-like domain of C1q.[34] Additionally, PepO of *S. pneumoniae* functions as a fibronectin-binding protein[35] and the gene encoding the PepO orthologue of *S. mutans* was upregulated in UAvic (Figure 2C). Therefore, we compared the ability of UAvic, UA159, and UAvic+ to bind to human plasma fibronectin. As expected, the UAvic showed 5.4-fold increase ($P<.01$) in binding to fibronectin (Figure 4A) whereas the complemented strain displayed wild-type binding levels (Figure 4A). Hence, our findings show that the TCS $VicRK_{Sm}$ modulates the ability of *S. mutans* to bind fibronectin.

3.6 | The *vicK* mutant has increased production of α-enolase, but not of extracellular GAPDH

Surface-associated GAPDH and α-enolase promote bacterial binding to fibronectin, plasminogen and/or other host glycoproteins of the extracellular matrix, including collagens and laminin.[36,37] Although the mechanisms by which these glycolytic enzymes can be associated with

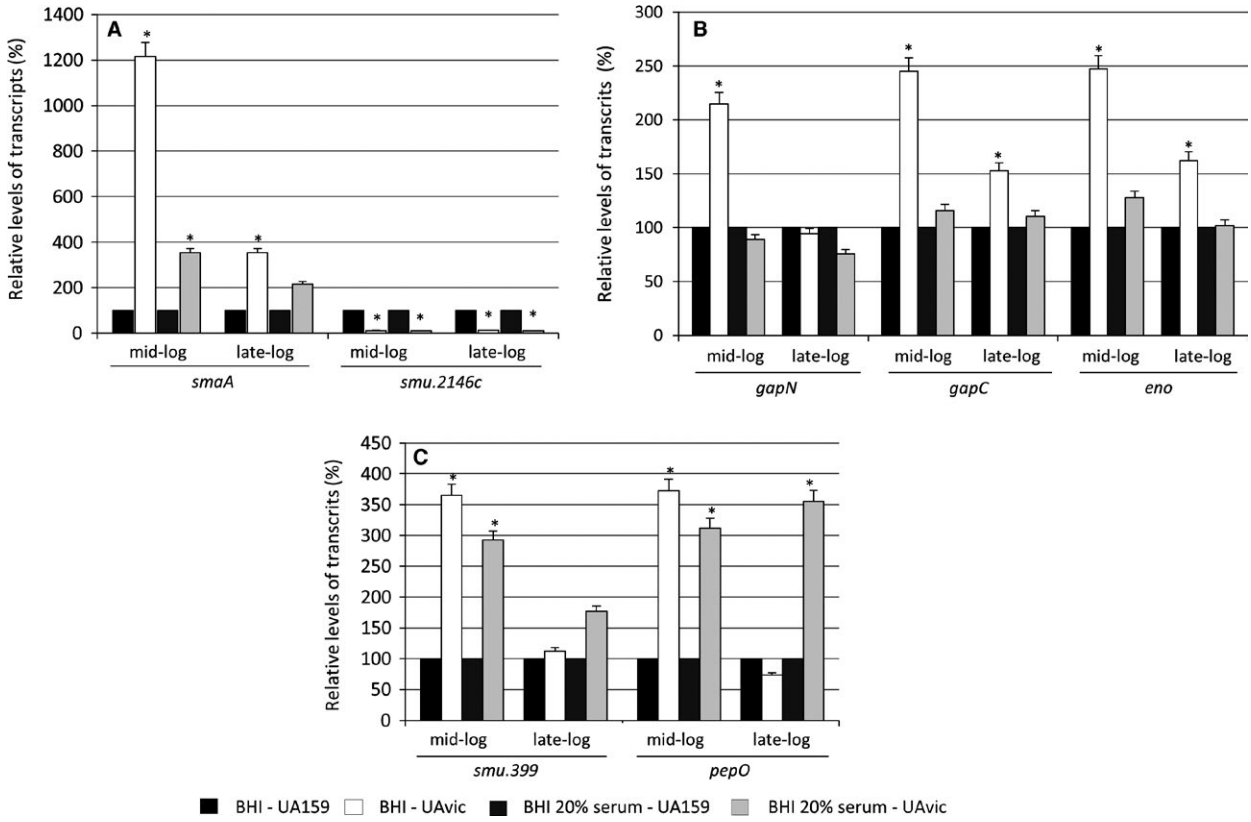

FIGURE 2 Comparative analysis of transcriptional profiles of *vicK* mutant (UAvic) with parent strain UA159 in the presence and absence of serum. Strains at mid- and late-log phases of growth were harvested and exposed to brain–heart infusion (BHI) both or BHI supplemented with 20% human serum before RNA isolation. Relative amounts of transcripts of UA159 at each condition were set to 100% to calculate relative transcript levels obtained with UAvic at the same condition. VicRK$_{Sm}$-downstream genes encoding murein hydrolases (A), metabolic enzymes (B) and peptidases of the complement (C) were analyzed. Columns represent means of three independent experiments; bars represent standard deviations. Asterisks indicate significant differences in relation to parent strain (analysis of variance with *post hoc* Dunnett's test; *P<.05)

the bacterial surface are still unknown,[28,36] studies in *S. pneumoniae* revealed that enolase is released in strains with defects in septum formation promoted by altered activities of the VicRK$_{Sp}$-regulated gene *pcsB*, a *gbpB* orthologue of *S. mutans*.[30,38] Therefore, we compared levels of surface GAPDH and α-enolase in cell lysates and in culture supernatants of strains UAvic, UAsmaA, and UA2146c with parent and respective complemented strains. As shown in Figure 4B and C, when compared with UA159, whole cell extracts of the UAvic mutant at mid-log phase have increased levels of α-enolase (4.8-fold increase; P<.05), which were restored to parental levels in the UAvic+ complemented strain. Levels of cell-associated α-enolase were not altered in the *smaA* and *smu2146c* mutants (Figure 4C). α-Enolase could not be detected in 100-fold concentrated culture supernatants of the strains analyzed, although high levels of GbpB (a secreted and surface-associated protein)[21] could be detected in the same samples in parallel immunoassays (data not shown). No significant changes in production of GAPDH were observed between the tested strains (data not shown). Hence, the *vicK* mutant shows overall increased production of α-enolase, although no significant levels of enolase and GAPDH could be detected in its culture supernatants.

3.7 | Deletion of *vicK* result in diminished survival of *S. mutans* in human blood, and reduced growth in poor nutrient medium

To persist in the bloodstream, bacteria must survive the host defenses and undergo physiological changes to adapt to nutrient limitations present in blood.[39] Therefore, we assessed the capacities of the UAvic mutant to persist in human blood and to grow in poor nutrient medium (RPMI). As shown in Figure 5A, over time, the numbers of viable UAvic recovered from blood in the survival assays were overall lower than those observed for UA159 and UAvic+, with 2 and 24 hours incubation periods showing statistically significant reductions compared with parent and complemented strains (P<.05). Survival of UAvic+ in blood was very similar to the parent strain. Comparisons of the growth curves of the studied strains in rich medium (BHI) revealed no alterations in growth rate in UAvic compared with UA159 (Figure 5B). However, the UAvic showed significantly reduced growth rate in RPMI, when compared with UA159 and UAvic+ (Figure 5C). The growth rate of UAvic+ was slower in both media when compared with UA159, probably because when growing UAvic+, the media were

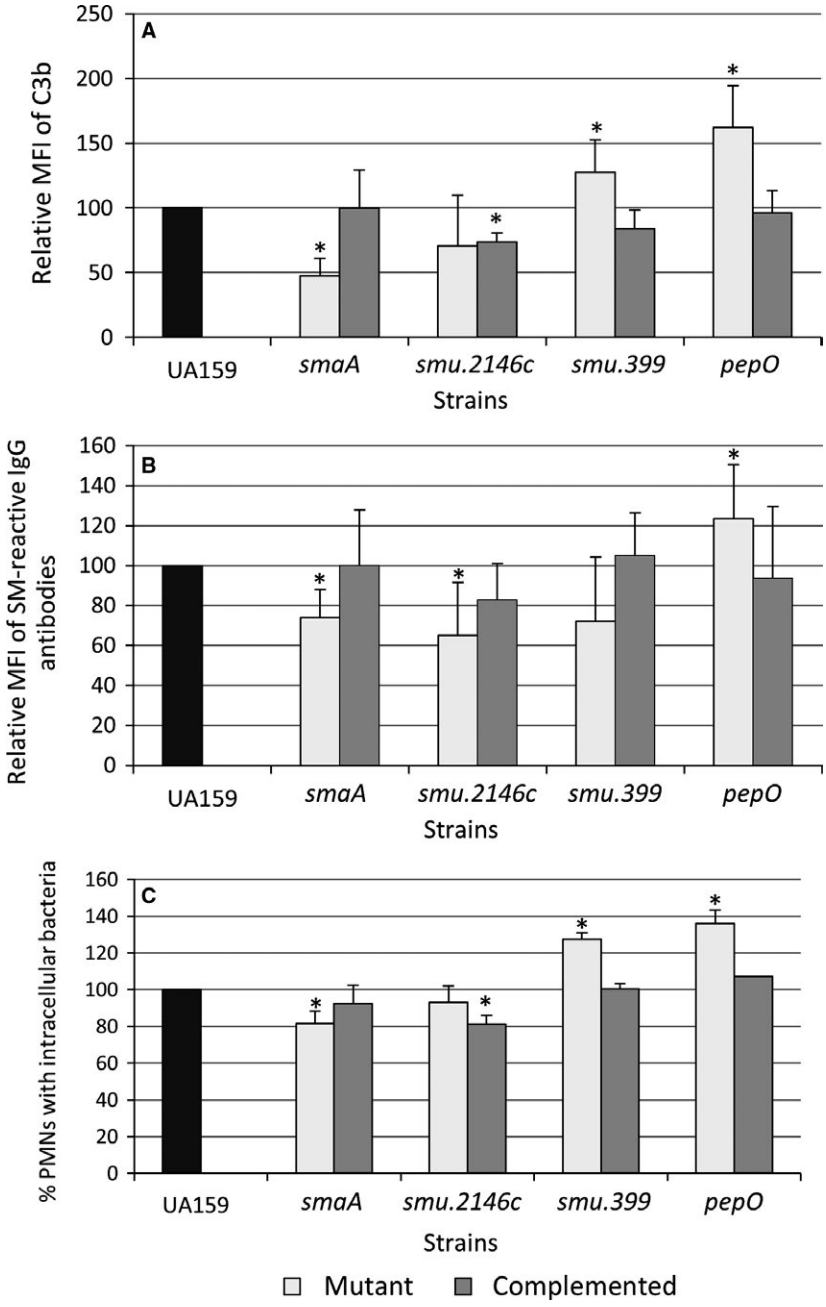

FIGURE 3 Effects of deletion of $VicR_{Sm}$ downstream genes (*smaA*, *smu2146*, *smu.399*, and *pepO*) on C3b deposition, binding to serum IgG and opsonophagocytosis by polymorphonuclear cells. Measures obtained for mutant and complemented strains were expressed in relation to UA159 (set to 100%). Asterisks indicate significant differences in relation to parent strain (Kruskal-Wallis with *post hoc* Dunn' test; $P<.05$)

supplemented with spectinomycin to maintain the plasmid expressing $vicK_{Sm}$. Hence, the *vicK* mutant has a diminished capacity to persist in human blood compared with parent and complemented strains, which is associated with its defects in adaptation to limited nutrient conditions.

4 | DISCUSSION

Streptococcus mutans is an important species of viridans streptococci involved in cardiovascular diseases.[40,41] Mechanisms of systemic virulence of *S. mutans* remain to be elucidated, but seem to involve expression of different genes in serotype- and strain-specific ways.[3,40-43] Previously, we observed that deletion of *vicK* and *covR*, known to coordinate the expression of virulence genes involved in *S. mutans* cariogenicity,[7,10,15] reduced *S. mutans* phagocytosis by PMN in samples of human blood in the serotype *c* strain UA159.[14] Inactivation of *covR* was later shown to promote *S. mutans* resistance to complement opsonization and survival in blood through upregulation of *gbpC* and *epsC* (CovR-repressed genes), genes required for *S. mutans* binding to sucrose-derived EPS.[3] Strains isolated from systemic infections express reduced levels of *covR* and stably bind to EPS, which in turn, functions as an anti-opsonic capsule.[3] However, binding to EPS does not entirely explain diversity in susceptibility to complement immunity among strains.[3] In addition, the mechanisms involved in the reduced susceptibility of the *vicK* mutant to opsonophagocytosis by PMN remained unknown, because this mutant is defective in the synthesis of EPS and in binding to these polymers,[7,9,10] which should increase its

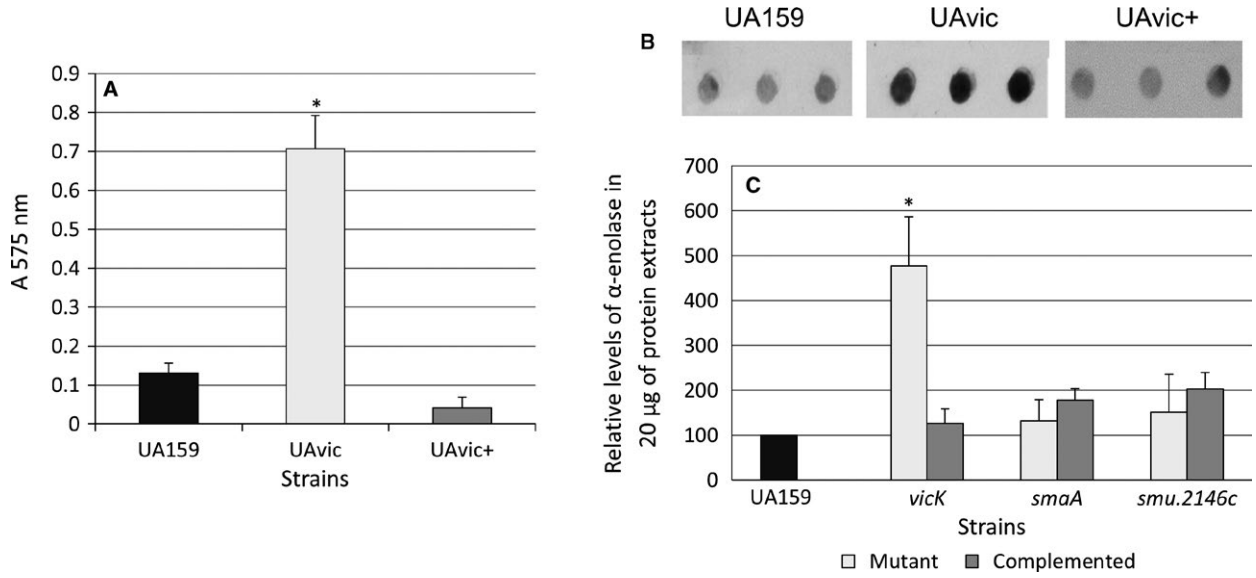

FIGURE 4 Comparisons of binding to human plasma fibronectin and production of α-enolase between UAvic and parent or complemented strains. (A) Fibronectin binding was expressed as the absorbance of crystal violet stain (A_{575nm}) eluted from bacteria bound to immobilized fibronectin. (B) Levels of α-enolase produced by strains were measured in whole cell extracts using immuno dot blot assays with polyclonal antibody anti-*Streptococcus pyogenes* α-enolase. (C) Densitometric measures of immune reactions were expressed as the relative levels of α-enolase in relation to parent strain (set as 100%). Columns represent means of three independent experiments; bars represent standard deviations. Asterisks indicate significant differences in relation to parent strain (Kruskal-Wallis with *post hoc* Dunn's test; $P<.05$)

susceptibility to complement-mediated opsonization. In the present study, we show that the *vicK* mutant is resistant to C3b deposition and has reduced binding to serum IgG antibodies, explaining its low susceptibility to phagocytosis in blood. Low levels of C3b deposition in UAvic were observed even when this strain was grown in sucrose-free medium (Figure 1D), implying that *S. mutans* expresses additional proteins to evade complement deposition, besides proteins involved in binding to EPS.

Streptococcal pathogens, e.g. *S. pyogenes* and *S. pneumoniae*, typically apply a diverse array of mechanisms to evade complement immunity, including the production of EPS capsule, secretion of proteases for degradation of complement components, and surface binding to fluid phase inhibitors of the complement system or to other host proteins, which indirectly inhibit complement activation.[31,44,45] Because the *vicK* mutant in UA159 is defective in binding to sucrose-derived EPS,[9,10] but resistant to C3b deposition, analysis of this strain was important for the screening of EPS-independent mechanisms of complement evasion. Hence, we identified four genes (*smaA*, *smu2146c*, *smu.399*, and *pepO*) that were transcriptionally affected in the UAvic mutant in the presence of serum. The genes encoding peptidases of the complement (*smu.399* and *pepO*) were more clearly altered in response to serum in relation to unsupplemented BHI, when compared with genes encoding murein hydrolases (*smaA* and *smu.2146c*) and to the metabolic genes (*eno*, *gapC*, and *gapN*). Similarly to our results, peptidases of complement are also upregulated in *Streptococcus agalactiae* exposed to 10% of serum.[46] It is worth noting that BHI might include stimulatory components also found in serum, because this complex medium is rich in tissue and immune host factors. It could explain, at least in part, the effects of BHI on transcription of *smu.399* and *pepO* observed in cells at mid-exponential growth. Transcriptional changes in *smaA* and *smu.2146c* in either the presence or absence of serum might also reflect multiple biological functions of these genes in response to different stimuli.

Phenotypic analyses of *smaA*- and *smu.2146c*-defective strains did not explain the resistance of UAvic to C3b deposition. Moreover, *S. mutans* expresses other murein hydrolases, which are downregulated at transcriptional and/or post-transcriptional levels in $vicK_{Sm}$ mutants, including LysM[10] and AtlA.[25] However, deletion of *lysM* does not affect complement opsonization,[3] and deletion of *atlA* increases *S. mutans* susceptibility to phagocytosis by PMN.[47] These findings suggest that increased resistance of the UAvic strain to C3b deposition and opsonophagocytosis is not associated with altered functions of the murein hydrolases investigated so far. However, here we show that *S. mutans* expresses peptidases of the complement (Smu.399 and PepO) that are negatively regulated by $VicRK_{Sm}$. Deletion of *smu.399* and *pepO* significantly increases C3b deposition and opsonophagocytosis of *S. mutans* (Figure 3), suggesting that these peptidases may be potential targets to control systemic infections by this species.

In *S. pneumoniae*, PepO ($PepO_{Sp}$) binds to C4b-binding protein (C4BP), a fluid-phase inhibitor of the classical pathway of the complement system,[48] the major pathway of complement activation on *S. mutans*.[3] The amino acid sequence of $PepO_{Sm}$ shows 79 and 88% similarity with PepO orthologues expressed by *S. pneumoniae*[35] and *S. pyogenes*,[49] respectively. Secreted $PepO_{Sp}$ further promotes degradation of C3b through binding to plasminogen and its activation to plasmin. $PepO_{Sp}$ also binds to fibronectin, which is present in soluble form in serum and saliva, and is involved in a large number of physiological processes, including formation of vegetations on injured

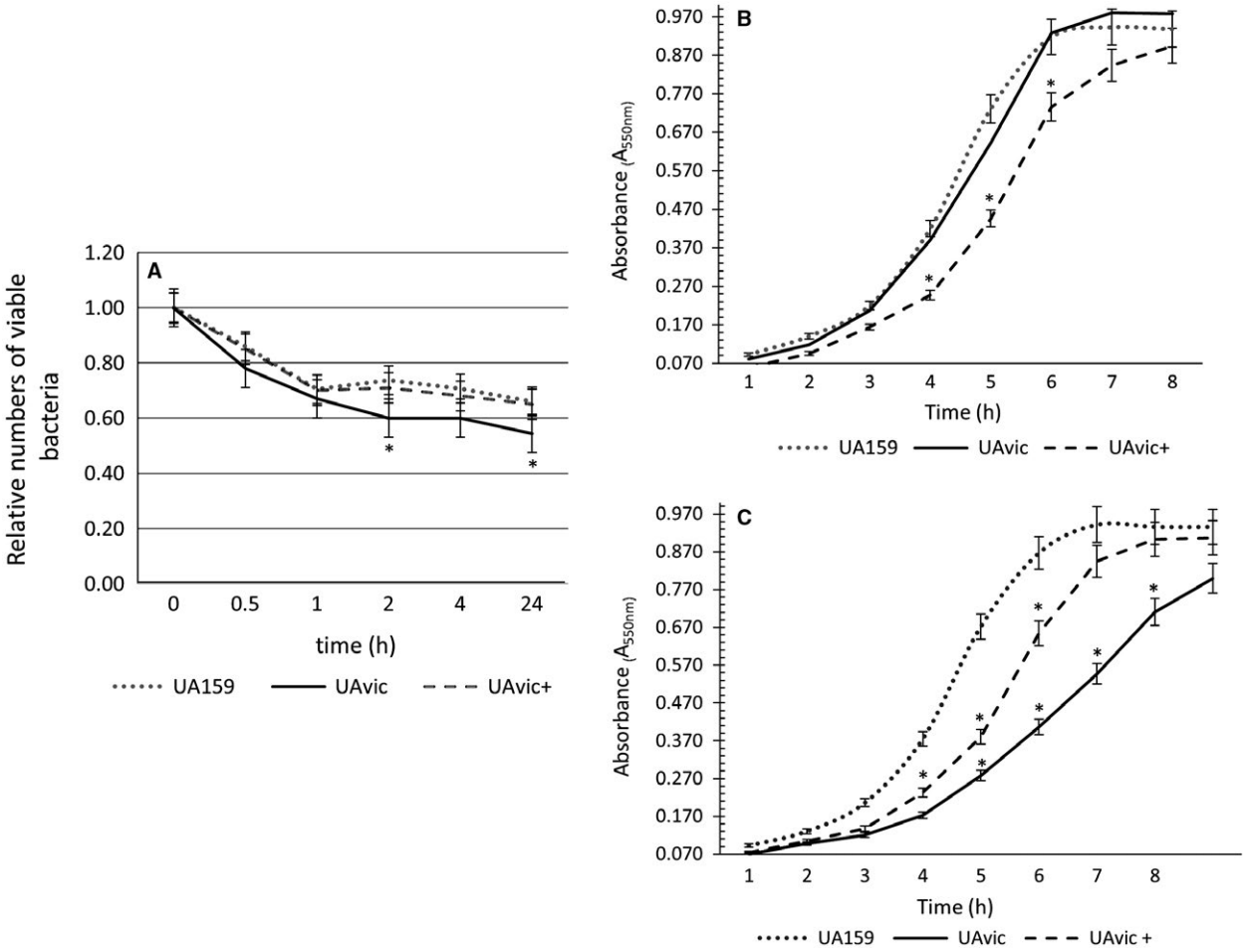

FIGURE 5 Comparisons of *ex vivo* survival in human blood and growth in different nutritional conditions. (A) Numbers of viable bacteria (log CFU mL^{-1}) were expressed in relation to initial counts in blood suspension (time 0). Growth curves in brain–heart infusion (B) and in poor nutrient RPMI (C). Dots represent means of triplicate of one representative experiment. Bars indicate standard deviations. Differences in relation to parent strain at each time-point were tested using Kruskal-Wallis with *post hoc* Dunn's test with correction for repeated measures (*P<.05)

cardiac endothelium and atheromatoses.[50,51] The enhanced capacity of UAvic to bind to fibronectin is therefore compatible with the upregulation of $pepO_{Sm}$ in UAvic, and with resistance of this mutant to opsonophagocytosis. In *S. mutans* strain GS5, binding to fibronectin was associated with resistance to opsonophagocytosis and increased survival in the bloodstream, but this property was associated with the expression of *atlA*,[47] whose transcription was not significantly altered in UAvic (data not shown). In addition, *S. mutans* expresses other surface proteins that could contribute to fibronectin binding,[37] but their role in the UAvic phenotype remains to be elucidated. Studies are under way to define the role of $PepO_{Sm}$ in systemic infections by *S. mutans*.

UAvic shows defects in septum division associated with reduced expression of GbpB,[9] an essential protein involved in *S. mutans* binding to EPS and in cell wall division.[9,52] Downregulation of the GbpB orthologue (PcsB) in *S. pneumoniae* affects cell wall division[30] and promotes the release of enolase.[38] Enolase on *S. pneumoniae* surface binds to C4BP to inhibit complement activation.[53] Therefore, upregulation of enolase in UAvic could contribute to UAvic resistance to C3b deposition. Although we could not detect enolase in the culture supernatants of the studied strains, it is possible that extracellular enolase could be associated with the *S. mutans* cell wall.

The essentiality of $VicR_{Sm}$ regulator for *S. mutans* viability is not entirely understood.[7,9,10] In addition, $vicK_{Sm}$-defective strains show increased sensitivity to oxidative, pH, and osmotic stresses.[9,11,25,54] $VicRK_{Sm}$ is also responsive to nutritional changes,[11] and here we show that the $vicK_{Sm}$ mutant is defective in metabolic adaptation to limited nutrient conditions. All of these defects should reduce the capacity of $vicK_{Sm}$-defective strains to persist in blood, because metabolic adaptation to limited nutrient conditions and resistance to oxidative and pH stresses present in blood are crucial for bacterial survival in the bloodstream.[39,55] It is therefore possible that the increased resistance of UAvic to opsonophagocytosis may have counterbalanced the UAvic deficiencies in stress response and/or adaptation to blood nutrient limitations, resulting in limited changes in curves of UAvic persistence in blood.

In summary, in this study we showed that deletion of *vicK* reduces *S. mutans* susceptibility to phagocytosis by PMN by impairing C3b deposition and surface binding to serum IgG, in a way that is independent of the production of sucrose-derived EPS or of the expression of

murein hydrolases encoded by *smaA* and *smu2146c*. Two novel genes expressed by *S. mutans* under regulation of $VicRK_{Sm}$ were identified and were shown to contribute to *S. mutans* resistance to C3b deposition (*pepO* and *smu.399*), establishing that this bacterium expresses multiple factors associated with complement immunity evasion. Although deletion of $vicK_{Sm}$ results in increased resistance to opsonophagocytosis, it does not contribute to *S. mutans* survival in human blood, which is, at least in part, associated with defects in adaption to nutrient limitations.

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2.3 Artigo 3: CovR and VicRK regulate transcription of the collagen binding protein Cnm of *Streptococcus mutans.*

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Running title: CovR and VicRK regulate Cnm in S. mutans

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CovR and VicRK regulate transcription of the collagen binding protein Cnm of *Streptococcus mutans*

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Abstract

Cnm is a surface-associated virulence factor present in a subset of *Streptococcus mutans* strains that mediates binding to extracellular matrices (ECM) and intracellular invasion. Despite the association of Cnm with both oral and non-oral infections, the regulatory mechanisms governing its expression are poorly understood. Here, we showed that transcription of *cnm* is directly and independently controlled by the orphan response regulator CovR and the two-component system (TCS) VicRK. *In silico* analysis identified CovR- and VicR-binding motifs in the regulatory region of *cnm* as well as *pgfS*, a gene encoding for a glycosyltransferase responsible for Cnm glycosylation. Quantitative real-time PCR and Western blot analyses of Δ*covR* and Δ*vicK* strains revelated that CovR is a positive regulator of *cnm* whereas the VicRK TCS acts as a negative regulator. Electrophoretic mobility shift assays confirmed that CovR and VicR directly bind to the *cnm* and *pgfS* promoter regions. In agreement with the role of VicRK as a negative regulator, the Δ*vicK* strain showed enhanced binding to collagen and laminin, higher intracellular invasion rates and increased virulence in the *Galleria mellonella* invertebrate model. The Δ*covR* strain showed decreased intracellular invasion rates but loss of CovR did not affect virulence in *G. mellonella* and, unexpectedly, increased collagen and laminin binding activities. Collectively, our results expand the repertoire of virulence-related genes regulated by CovR and VicRK to include a core (*pgfS*) as well as a non-core (*cnm*) gene. In addition, our findings further underscore the importance of Cnm in *S. mutans*-host interactions.

Importance. *Streptococcus mutans* is the main etiological agent of dental caries, the most prevalent infectious disease in the world. Also, *S. mutans* can cause systemic infections such as infective endocarditis resulting in high mortality and morbidity rates. The Cnm adhesin of *S. mutans* is an important virulence factor that is associated with systemic infections and more recently was demonstrated to contribute to caries. Despite the association of Cnm with both oral and non-oral infections, the regulatory mechanisms governing its expression are poorly

understood. Here, we identify two independent regulatory systems that control Cnm production. A better understanding of the mechanisms controlling the expression of virulence factors like Cnm can facilitate the development of new strategies for combating bacterial infections.

Introduction

Streptococcus mutans is a major pathogen associated with dental caries and also implicated in extra-oral infections, in particular infective endocarditis (IE) (1, 2). Once in the bloodstream, *S. mutans* must first escape host surveillance mechanisms and then rely on its ability to interact with components of the extracellular matrix (ECM) in order to adhere to and colonize non-oral tissues (3). In *S. mutans*, Cnm (collagen-binding protein of *S. mutans*) is a surface adhesin that mediates binding to both collagen and laminin (4, 5). Interestingly, the expression of Cnm has been associated with a variety of systemic infections such as IE, hemorrhagic stroke, cerebral microbleeds and IgA nephropathy, among others (6-8). The *cnm* gene is found in approximately 15% of clinical isolates and is particularly prevalent in strains isolated from blood and specimens of heart valves (2, 9). Although Cnm can be found in the four *S. mutans* serotypes (*c, e, f* and *k*), it is more commonly present in strains of the less prevalent serotypes *e, f,* and *k*, and rarely in strains of the more prevalent serotype *c* (4, 5). Studies from our group revealed that Cnm mediates *S. mutans* binding to collagen and laminin, and is responsible for intracellular invasion of human coronary artery endothelial cells (HCAEC) and virulence in *Galleria mellonella*, an invertebrate model of systemic infection (5, 10). We have also showed that Cnm is a glycoprotein that is post-tranlationally modified by at least one glycosyltransferase, *pgfS*, located immediately downstream the *cnm* gene (11).

To succeed as a pathogen, bacteria must sense and rapidly adapt to many adverse conditions encountered during the invasion and colonization process. This adaptation commonly depend on signal transduction two-component systems (TCS) that are comprised of an environmental sensing membrane-bound histidine kinase (HK) that activates a response regulator

(RR), which is a DNA binding protein that modulates expression of target genes when phosphorylated by the HK. In the *cnm*-negative *S. mutans* strain UA159, 14 complete TCS have been identified (12, 13) including a TCS designated VicRK (Vic, for virulence control) as well as an orphan RR, CovR (control of virulence; also known as GcrR). In UA159, VicR and CovR directly regulate genes implicated in the synthesis of and interaction with extracellular polysaccharides (14-17), which are major components of the dental biofilm matrix and directly associated with *S. mutans* pathogenicity (18, 19). For example, *gbpB* (glucan binding protein B) was found to be positively regulated by VicR (17, 20), while *gtfB, gtfC* (glucosyltransferase B and C) and *gbpC* (glucan binding protein C) are repressed by CovR (16). More recently, CovR and VicRK were also shown to contribute to *S. mutans* ability to interactact with components of the immune system (21-23). Specifically, CovR was shown to regulate susceptibility to complement immunity and survival in blood which was strongly associated with increased expression of genes involved with production of or interaction with extracellular polysaccharides (*gtfB, gtfC, gbpC, gbpB and epsC*) (22). On the other hand, a *vicK* mutant strain showed reduced susceptibility to deposition of C3b of complement, low binding to serum immunoglobulin G (IgG), and low frequency of opsonophagocytosis by polymorphonuclear (PMN) in a sucrose-independent fashion (21). In addition, the Δ*vicK* strain showed strong interaction with human fibronectin, another important component of the host ECM (21).

Because Cnm is an important virulence factor of *S. mutans* responsible for tight interactions with ECM components and shown to interfere with complement activation (5, 24) and both CovR and VicRK are critical regulators of virulence genes in *S. mutans*, we investigated wether *cnm* was regulated by CovR, VicRK, or both. Through *in silico* analysis, we identified CovR and VicR consensus motifs in the regulatory regions located upstream *cnm* and *pgfS,* a gene encoding a glycosyltransferase involved in Cnm glycosylation. Using mutational and *in vitro* approaches, we demonstrated that CovR and VicRK are directly and specifically involved in the transcriptional regulation of *cnm* and *pgfS* in the Cnm$^+$ strain OMZ175. CovR was shown to function as a positive

regulator of *cnm* and *pgfS* while the VicRK system functioned as a repressor of both genes. In agreement with the role of VicRK as a negative regulator of *cnm* and *pgfS*, inactivation of *vicK* in OMZ175 resulted in increased binding to collagen and laminin, increased invasion of HCAEC, and increased virulence in *G. mellonella*. Inactivation of *covR* in OMZ175 decreased HCAEC invasion rates as compared to the parent strain but did not affect virulence in *G. mellonella*. Unexpectedly, the *covR* mutant strain showed increased binding to collagen and laminin. Collectively, our results expand the repertoire of virulence-related genes regulated by CovR and VicRK to include a core (*pgfS*) as well as a non-core (*cnm*) gene. In addition, our findings further underscore the importance of Cnm in *S. mutans*-host interactions.

Results

CovR and VicRK are involved in regulation of *cnm* and *pgfS*

In silico analysis identified consensus sequences for CovR and VicR binding in the promoter regions of *cnm* and *pgfS* (Table 1). Putative CovR- and VicR-binding sites were located 29 bp (CovR) and 5 bp (VicR) from the predicted -10 region of the *cnm* promoter. The CovR motif had 4 mismatches whereas the VicR motif displayed two mismatches from their respective consensus motifs (17). In addition, overlapping CovR-binding motif (3 mismatches, 131 bp from the predicted -10 region of *pgfS* promoter) and VicR-binding motif (2 mismatches, 125 bp from from the predicted -10 region of *pgfS* promoter) were identified in the intergenic region upstream *pgfS*, a gene previously shown to be involved in the posttranslational modification of Cnm (11).

To determine whether CovR and VicRK regulate transcription of *cnm* and *pgfS*, we inactivated the *covR* and *vicK* genes from the Cnm$^+$ strain OMZ175 to generate, respectively, the OMZ175Δ*covR* and OMZ175Δ*vicK* strains. Of note, *vicR* is an essential gene in *S. mutans* (15) and studies on the role of the VicRK TCS in this organism have resorted to phenotypic characterizations of *vicK* mutans and *in vitro* promoter binding assays using recombinant VicR protein (15, 17, 25, 26). When compared to the parent strain OMZ175, the relative levels of of *cnm*

and *pgfS* transcripts were significantly lower ($p<0.05$) in OMZ175Δ*covR* by 1.5- and 2-fold, respectively (**Fig. 1A-B**). On the other hand, expression of *cnm* and *pgfS* was increased by 3- and 2.5-fold ($p<0.01$), respectively, in OMZ175Δ*vicK* when compared to the parent strain OMZ175. We also assessed the transcriptional levels of *pgfS* in the Δ*covR* and Δ*vicK* mutants of UA159 and observed similar patterns of regulation, i.e. downregulated in Δ*covR* and upregulated in Δ*vicK* (**Fig. 1C**), suggesting that regulation of the core gene *pgfS* is conserved among *S. mutans* strains.

Next, we performed Western blot analysis to determine the levels of Cnm in the OMZ175, OMZ175Δ*covR* and OMZ175Δ*vicK* strains. In agreement with the qRT-PCR analysis, the levels of Cnm were reduced by ~50% in OMZ175Δ*covR* and elevated (~2-fold) in OMZ175Δ*vicK* as compared to OMZ175 (**Fig. 1D**); Cnm levels were restored to wild-type levels in the complemented *covR* (Δ*covR*$^+$) and *vicK (*Δ*vicK*$^+$*)* strains.

CovR and VicR directly bind to the promoter regions of *cnm* and *pgfS in vitro*

Next, we used electrophoretic mobility shift assays (EMSAs) with rCovR and rVicR proteins to determine whether the regulatory effects in *cnm* and *pgfS* expression observed in the Δ*covR* and Δ*vicK* strains were direct or indirect. The results indicate that both CovR and VicR specifically bind to the promoter regions of *cnm* and *pgfS*, an effect that was reversed by addition of unlabeled (cold) DNA probe 200-fold in excess (**Fig. 2**). To further demonstrate specificity of the assays, we used *covR* (CovR autoregulated) and *gbpB* (a direct VicR target) DNA probes as positive controls and *gtfD* and *covR* DNA as negative controls for rCovR and rVicR, respectively. As expected, rCovR interacted with the *covR* probe but not with the *gtfD* probe whereas rVicR interacted with the *gbpB* probe but not with *covR* DNA (data not shown).

Inactivation of *covR* and *vicK* affects the expression of Cnm-dependent phenotypes

The results described above revealed that CovR and VicR directly control *cnm* expression in *S. mutans*. Here, we tested whether the different levels of Cnm in the Δ*covR* and Δ*vicK* strains

would reflect in phenotypic changes that relate to Cnm expression. Specifically, we assessed the capacity of the Δ*covR* and Δ*vicK* mutant strains to bind to collagen or lamimin *in vitro*, invade HCAEC and kill *G. mellonella*. As expected, considering the negative role of the VicRK system in *cnm* expression (**Fig. 1**), when compared to the parent OMZ175 strain, the OMZ175Δ*vicK* mutant showed increased binding to either collagen or laminin (>250-fold), increased HCAEC invasion (2.5-fold) rates and slightly enhanced (albeit not statistically significant) virulence in *G. mellonella* (**Fig. 3**). Unexpectedly, the OMZ175Δ*covR* also showed enhanced ability to bind to collagen or laminin (20- and 10-fold, respectively) whereas HCAEC invasion rates were reduced by approximately 50% and virulence in *G. mellonella* was not altered (**Fig. 3**). Given the unexpected finding that *covR* inactivation, a negative regulator of *cnm*, led to increases in collagen/lamining binding activities, we investigated the effect of *covR* inactivation to collagen and laminin binding efficiency in *S. mutans* B14, a serotype *e* Cnm^+ strain also shown to avidly bind to collagen or laminin in a Cnm-dependent manner (5). We found that the B14Δ*covR* strain displayed collagen- and laminin- binding profiles similar (i.e., enhanced binding when compared to the parent B14 strain) to those observed for the OMZ175Δ*covR* strain (data not shown).

Discussion

In *S. mutans*, Cnm is a surface-associated glycoprotein shown to mediate adhesion to collagen and laminin and invasion of endothelial cells (4, 5). In addition, we and others have shown that Cnm is a virulence factor that contributes to systemic virulence of *S. mutans* in the *G. mellonella* invertebrate model (5), to *S. mutans* infectivity in a rabbit model of infective endocarditis (27, 28), and to caries severity in a rat model (29). However, the mechanisms regulating expression of *cnm* remain largely unknown. More recently, we found that *cnm* is genetically-linked to *pgfS*, which encodes for a glycosyltransferase involved in the O-glycosylation of Cnm (11). Because glycosylation confers increased stability to Cnm, the inactivation of *pgfS* led to a decrease in several Cnm-mediated phenotypes (11). In the present study, we show that both *cnm* and *pgfS*

are directly regulated by CovR and VicRK providing the first evidence of direct regulation of a non-core gene (*cnm*) and flanking core gene (*pgfS*) by these important virulence gene regulators.

In *Staphylococcus aureus*, WalR, a VicR homolog, was shown to bind to a conserved DNA motif, consisting of two hexanucleotide direct repeats, separated by five nucleotides [5'-TGT(A/T)A(A/T/C)-N(5)-TGT(A/T)A(A/T/C)-3'] (30). More recently, a VicR consensus binding site almost identical to that found in *S. aureus* was described in *S. mutans* [5'-TGT(A/T)(A/T)(T/A)A(A/T)(T/A)(T/A)(T/C)(A/G)(T/A)N(A/T) (17). VicR controls, directly or indirectly, the expression of genes involved in the synthesis of expolysaccharides (*gtfBC*, *gtfD*), glucan-binding proteins (*gbpB*), proteins associated with cell wall biogenesis (*smaA, lysM, wapE*), competence (*comC*, *comDE*, *comX*) and mutacins (*nlmAB*, *nlmC*, *nlmD*) (15, 17, 25, 26). While the VicRK TCS typically acts as a direct activator of genes involved in biofilm formation (15, 17), VicRK was also shown to function as a transcriptional repressor (17, 21). In the serotype *c* strain UA159, CovR was shown to regulate the expression of at least 128 genes (6.5% of the entire genome) (31). Previous studies have indicated a significant overlap between the CovR and VicRK regulons in the UA159 strain (17, 26). Interestingly, CovR and VicR often regulate the same gene in an opposite manner whereby one regulator functions as an activator while the other serves as a repressor (13, 16, 17). Consistent with the roles of CovR and VicRK in coordinating expression of virulence genes in *S. mutans,* here we identified *cnm* and *pgfS* as additional examples of virulence-related genes that are co-regulated by CovR and VicRK. The location of the CovR- and VicR consensus binding sequences in the *cnm* regulatory region offered clues of the functional roles of CovR and VicR. Usually, transcription activators bind upstream the -35 region such that RNA polymerase interactions with DNA are facilitated by the transcriptional regulator-DNA interaction. On the other hand, repressors generally compete with the RNA polymerase for binding to the promoter region such that consensus binding sequences of repressors are often located very close or overlap with the -35 and -10 sequences. The consensus CovR-binding sequence, shown to function as positive regulator for *cnm*, was located 29 bp upstream of the predicted -10

region. On the other hand, VicR consensus sequence, shown to function as a negative regulator, was located 5 bp downstream of the predicted -10 sequence of the *cnm* promoter; potentially allowing VicR to interfere with the RNA polymerase recognition of the promoter sequence. In the *pgfS* regulatory region, CovR and VicR binding motifs are located in a distal site from the -10 region and overlap by 7 bp. Thus, the overlapping location of both VicR and CovR bindings sites in the *pgfS* promoter suggests that these regulators compete for the same DNA region to exert its function.

In bacteria, protein glycosylation contributes to protein folding and secondary structure formation, cell adhesion, thermodynamic stability, modulation of immune recognition and protection against proteolytic degradation (32, 33). While protein targets of PgfS-mediated glycosylation other than Cnm have not been identified, it is likely that PgfS modifies proteins that are part of the *S. mutans* core genome considering that *pgfS* itself is part of the *S. mutans* core genome. In fact, preliminary two-dimensional (2D) lectin blot analysis identified several protein spots recognizing wheat germ agglutinin (WGA) that are absent in the Δ*pgfS* strain (unpublished data). Thus, the transcriptional regulation of *pgfS* by CovR and VicRK suggests that *S. mutans* protein glycosylation profiles may vary according to environmental cues that trigger CovR and VicRK regulatory activities. The regulation of *pgfS* by CovR and VicRK and the importance of protein glycosylation to *S. mutans* pathophysiology represent new aspects of *S. mutans* research that deserves further scrutiny.

While the negative (repressor) role of VicRK in the transcription of *cnm* was in agreement with the expression of Cnm-related phenotypes in the Δ*vicK* strain, the increased collagen- and laminin-binding activities of the Δ*covR* strain were unexpected given that CovR was shown to function as a positive transcriptional regulator of *cnm*. On the other hand, the differences in Cnm levels in the OMZ175Δ*covR* was consistent with the impaired ability of this mutant to invade HCAECs, a trait that is also linked to Cnm expression (5, 27). There are least two additional surface proteins in *S.*

mutans, namely SpaP and WapA, that have been shown to interact with collagen *in vitro* (34-36). Thus, we wondered if expression of one or both proteins was overexpressed in the OMZ175Δ*covR* strain thereby providing an explanation for the unexpected increase in collagen (and laminin) binding of the Δ*covR* strain. Western blot analysis using anti-WapA and anti-SpaP specific antibodies (a gift from Dr. L. J. Brady, University of Florida) revealed that expression of either SpaP or WapA was not altered in the OMZ175Δ*covR* strain when compared to the parent OMZ175 strain (data not shown). Additional studies to identify, localize and quantify all surface-associated proteins in OMZ175 and OMZ175Δ*covR* will soon be underway as a way to understand the increased ECM-binding activity of the Δ*covR* strain.

While expression of Cnm has been intimately associated with intracellular invasion and increased virulence in *G. mellonella* (5), the Δ*covR* mutant of OMZ175 showed diminished HCAEC invasion rates when compared to the parent strain but no differences in *G. mellonella* killing kinetics. In the *S. mutans* UA159 strain, deletion of *covR* has been associated with increased systemic virulence based on enhanced resistance to complement-mediated opsonophagocytosis by PMNs, increased persistence in human blood *ex vivo* and in the bloodstream of rats (22). While the loss of CovR has a negative effect on *cnm* expression, there may be other virulence factors that are negatively regulated by CovR that contribute to the systemic virulence of OMZ175 that may compensate for the lower expression of Cnm in the OMZ175Δ*covR*.

In summary, the present study identifies CovR and VicRK as direct regulators of *cnm* and *pgfS* in *S. mutans.* The altered transcriptional levels of *cnm* and *pgfS* observed in the *covR* and *vicK* mutants affected phenotypes associated with Cnm expression, including collagen- and -laminin binding, invasion of HCAEC and systemic virulence in *G. mellonella.* These findings open new venues to understand the molecular mechanisms involved in the expression of these important virulence genes of *S. mutans*, which is crucial for defining strategies to control systemic infections by this oral pathogen.

Material and Methods

Bacterial strains and culture conditions. Strains used in this study are listed in Table 2. All *E. coli* strains were routinely grown in Luria-Bertani (LB) media at 37°C. When required, kanamycin (100 ug mL^{-1}) or ampicillin (100 ug mL^{-1}) was added to LB broth or agar plates. Strains of *S. mutans* were routinely cultured in brain heart infusion (BHI) medium at 37°C in a humidified 5% CO_2 atmosphere. When required, kanamycin (1000 ug mL^{-1}), erythromycin (10 ug mL^{-1}) or spectinomycin (1500 ug mL^{-1}) was added to BHI broth or agar plates.

Genetic manipulation of *S. mutans* OMZ175. Mutations of *covR* and *vicK* in OMZ175 were generated by amplifying the flanking and coding regions of *covR* and *vicK* from the *S. mutans* UA159 background strain that had been previously replaced by an erythromycin resistance cassette generating the Δ*covR* and Δ*vicK* mutants in UA159 (20, 23). Then, 100 ng of the PCR product was used to transform OMZ175 in the presence of the ComX-inducing peptide (XIP) as described elsewhere (37). Transformants were selected on plates containing erythromycin and gene inactivations confirmed by PCR and DNA sequencing analysis. To construct a *covR* complemented strain, the full-length *covR* gene was cloned into the integration vector pMC340B (38). The resulting plasmid (pMCcovR) was then tranformed into the *covR* mutant strain (OMZ175Δ*covR*) for integration at the *mtlA1* locus. Complemented strains were selected on plates containing kanamycin and positive clones confirmed by PCR and sequencing of the *mltA1* locus. To complement the *vicK* OMZ175 mutant strain, the plasmid pDL278-*vicK* previously used to complement the *vicK* mutant in UA159 (20) was used to transform the OMZ175Δ*vicK* strain. Transformants were selected on plates containing spectinomycin and screened by PCR. All primers used for the genetic manipulation of *S. mutans* are listed in Table 3.

qRT-PCR analysis. RNA was extracted from cultures grown to mid-exponential phase (OD_{600} ~ 0.3) as previously described (39). Briefly, cDNA from 0.5 μg of RNA was synthesized using a high-

capacity cDNA reverse transcriptase kit containing random primers (Applied Biosystems). Gene-specific primers for the *cnm* and *pgfS* coding sequences (Table 3) were designed using Beacon Designer 2.0 (Premier Biosoft International) to amplify region of each gene 85 to 200 bp in length. Quantitative real-time PCR (qRT-PCR) reactions were performed in an iCycler (Bio-Rad).

Western blot analysis. Whole-cell protein lysates were obtained by homogenization in the presence of 0.1-mm glass beads using a bead beater (Biospec). Protein concentration was determined using the bicinchoninic acid assay (Pierce). Protein lysates were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). Cnm detection was performed using rabbit anti-rCnmA (recombinant A domain of Cnm) polyclonal antibody (10) diluted 1:2000 in phosphate-buffered saline (PBS) plus 0.01% Tween 20 and anti-rabbit horseradish peroxidase (HRP)-coupled antibody (Sigma-Aldrich). Western blots were quantified using ImageJ software.

Production of recombinant rCovR and rVicR protein and Electrophoretic Mobility Shift Assay (EMSA). To obtain His-tagged rCovR and rVicR recombinant proteins, *E. coli* BL21 harboring the expression vectors pET-covR or pET-vicR (17) were grown in LB to an OD_{600} of 0.5 and expression of rCovR or rVicR induced for 3 h with 1 mM isopropyl-β-D-1-thiogalactopyrosinide (IPTG). After cell lysis, recombinant proteins were purified by affinity chromatography with Ni^{2+}-NTA agarose (Qiagen). Eluted recombinant proteins were dialyzed overnight in phosphate buffered saline (PBS) at 4°C. Aliquots of purified proteins were stored at -20°C in 10% glycerol. Protein samples were analyzed by SDS-PAGE followed by Coomassie blue staining. The concentrations of rCovR and rVicR were determined using the bicinconinic acid assay (BCA). To confirm the identity of the purified recombinant proteins, mass-spectometry analysis was performed in the UF ICBR Proteomics and Mass Spectrometry core facility.

For EMSAs, amplicons of the promoter regions of *cnm*, *pgfS*, *gbpB* (VicR positive control), *covR* (CovR positive control and VicR negative control) and *gtfD* (CovR negative control) were obtained with specific primers (Table 3) and biotynilated using the Biotin 3' End DNA Labeling Kit (Thermo Scientific). Binding reactions of labeled DNA (~20 fmoles) with rCovR or rVicR (0, 5, 10 pmol) were carried out in volumes of 20 µL containing 1X Binding Buffer [100 mM Tris, 500 KCl, 10 mM DTT; pH 7.5], poly L-lysine (50 ng μl^{-1}), unspecific competitor poly d(I-C). Samples were incubated at room temperature for 45 min, and DNA-protein complexes separated in non-denaturing 6% acrylamide gels in 0.5 X TBE buffer (pH 8.0). Protein-DNA complexes were electrotransferred to positively charged nylon membranes (Thermo Scientific Fisher) and detected with the LightShift Chemiluminescent EMSA Kit using Stabilized Streptavidin Horseradish Peroxidase conjugate (Thermo Scientific), according to the manufacturer's protocol. To assess the specificity of binding, a 200-fold excess of unlabeled test fragment (cold DNA) was incubated with rCovR or rVicR in each reaction mixture.

ECM binding assay. *In vitro* assays for collagen and laminin binding were performed as described elsewhere (10). Briefly, for ECM binding assays, 100 µl of PBS-washed bacterial suspensions containing approximately $1x10^9$ CFU ml^{-1} was added to each well of a microtiter plate coated for 18 h at 4°C with 40 µg ml^{-1} type I collagen from rat tail (Sigma-Aldrich) or 50 µg ml^{-1} mouse laminin (Becton-Dickinson). Adherent cells were stained with 0.05% crystal violet (CV) solution, and detected by the absorbance at 575nm.

Human Coronary Artery Endothelial Cell (HCAEC) invasion assay. Antibiotic protection assays were performed to assess the capacity of the mutant strains to invade HCAEC (5, 40). Briefly, primary HCAEC (Lonza) suspensions containing 0.5 $x10^5$ endothelial cells were seeded into the wells of 24-well flat-bottomed tissue culture plates and incubated in the presence of gentamicin and endothelial growth factor supplements (Lonza) at 37°C in a 5% CO_2 atmosphere

until they reached 80-90% confluence. Overnight bacterial cultures were washed twice in PBS (pH 7.2) and resuspended in endothelial cell basal medium (EBM-2) (Lonza) containing 2% fetal bovine serum (FBS) without antibiotics. One milliliter of 2% FBS-EBM-2 medium containing $1x10^7$ CFU ml^{-1} of *S. mutans* was used to infect HCAEC-containing wells at a multiplicity of infection (MOI) of 100:1 for 2 h in the absence of antibiotics followed by 3 h incubation in 1 ml of 2%FBS-EBM-2 medium containing 300 µg ml^{-1} gentamicin and 50 µg ml^{-1} penicillin G to kill extracellular bacteria. After incubation with antibiotics, HCAECs were lysed with 1 ml of sterile water and the mixture of lysed HCAEC and *S. mutans* plated onto TSA agar to determine the number of intracellular bacteria. The percentage of invasion for each strain was calculated based on the initial inoculum and the intracellular bacteria recovered from HCAEC lysates.

***Galleria mellonella* infection**. Cultures of *S. mutans* grown overnight were washed twice in sterile saline and 5 µl aliquots of the resuspended culture containing $1x10^8$ CFU/ml injected into the hemocoel of each larva via the last left proleg of *G. mellonella* larvae (weighing 0.2 to 0.3 g) (39). Larvae injected with heat-inactivated *S. mutans* OMZ175 (30 min at 80°C) or sterile saline were used as controls. After injection, larvae were kept in the dark at 37°C, and survival was recorded at selected intervals.

Statistical Analysis A one-way analysis of variance (ANOVA) was performed to verify the significance of binding and invasion assays. Kaplan-Meier killing curves were plotted for *G. mellenolla* infection assays, and estimations of differences in survival were compared using the log rank test. *P* values ≤ 0.05 were considered significant.

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Figures and Legend

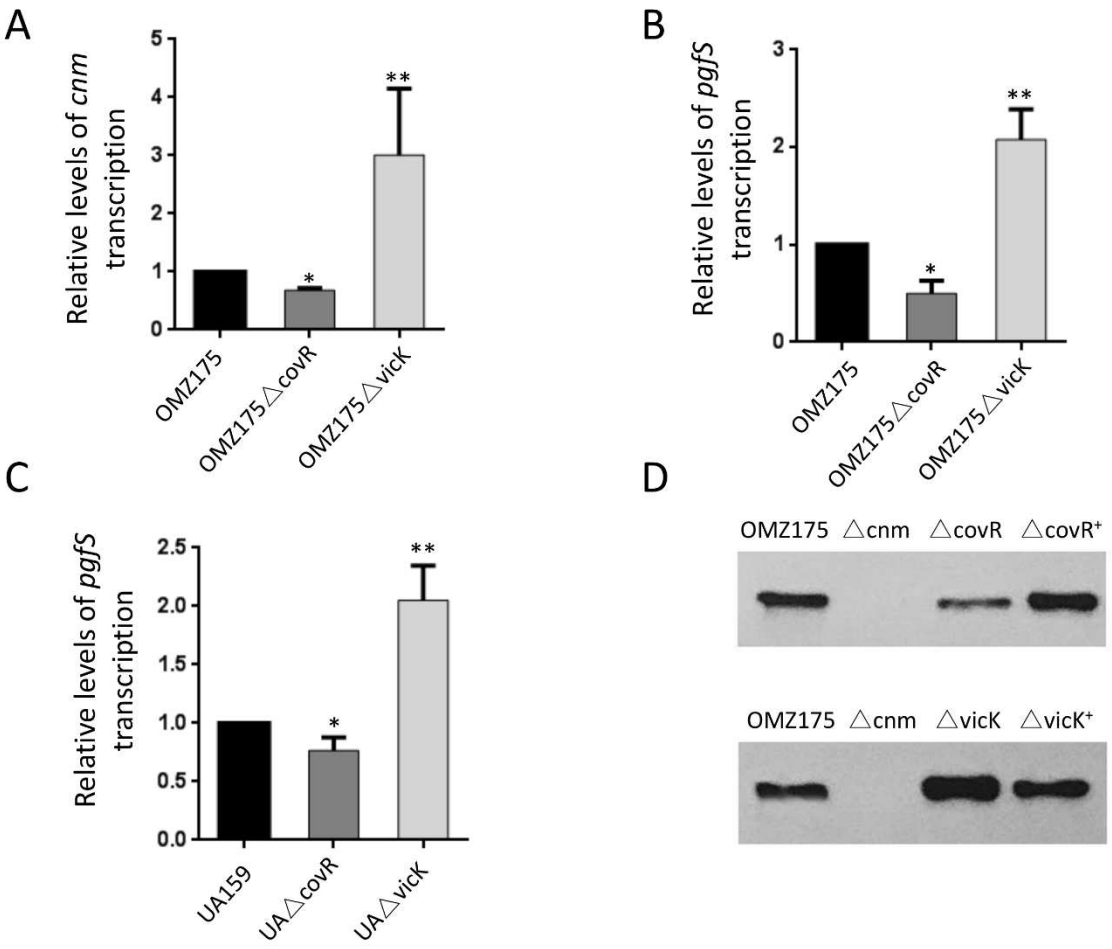

Figure 1. Transcriptional levels of *cnm* and *pgfS* and of abundance of Cnm in *S. mutans* OMZ175 and derivatives. Relative levels of gene transcripts of *cnm* and *pgfS* in mid-exponentially grown cells were determined by qRT-PCR. Levels of gene transcripts of *cnm* (A) and (B) *pgfS* in Δ*covR* and Δ*vicK* mutant strains of OMZ175, and *pgfs* (C) in mutant strains of the *cnm*-negative UA159.

Columns and bars indicate average and standard deviations of at least three independent experiments, respectively. Asterisks indicate significant differences in relation to parent strain (analysis of variance with post hoc Dunnett's test; *p<.05). (D) Detection of Cnm in cell extract of *S. mutans* strains by Western blot analysis. The Δ*cnm* strain was used as a negative control. Blots shown are representatives of three independent experiments.

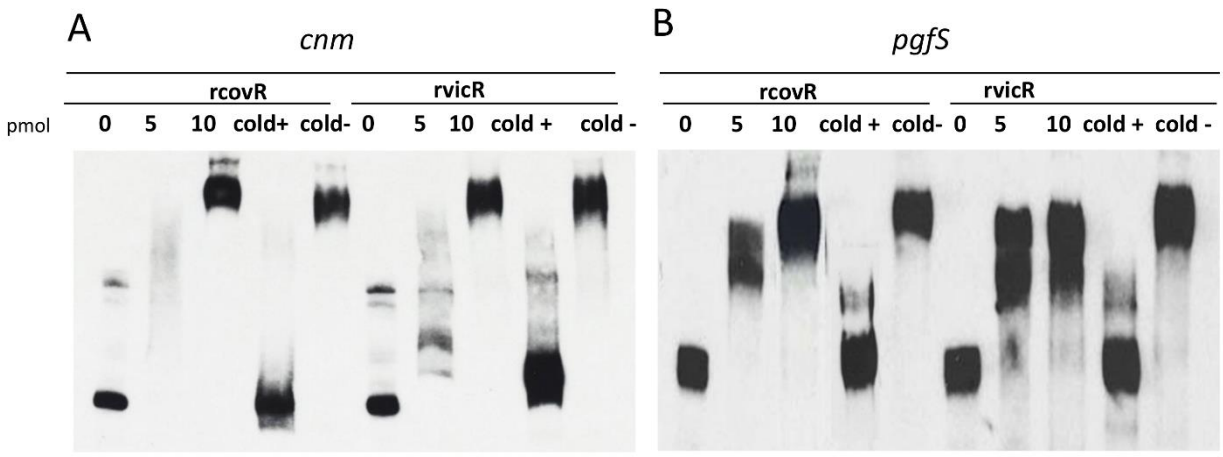

Figure 2. VicR and CovR directly interact with promoter regions of *cnm* and *pgfS*. Recombinant CovR (rCovR) and VicR (rVicR) specifically bound to the promoter regions of *cnm* (A) and (B) *pgfS* as determined by EMSA. Each reaction was performed with 0, 5 or 10 pmol of rCovR or rVicR. Specificity of binding was confirmed in competitive assays with excess of unlabeled specific DNA (cold). Data shown is a representative of three or more independent experiments.

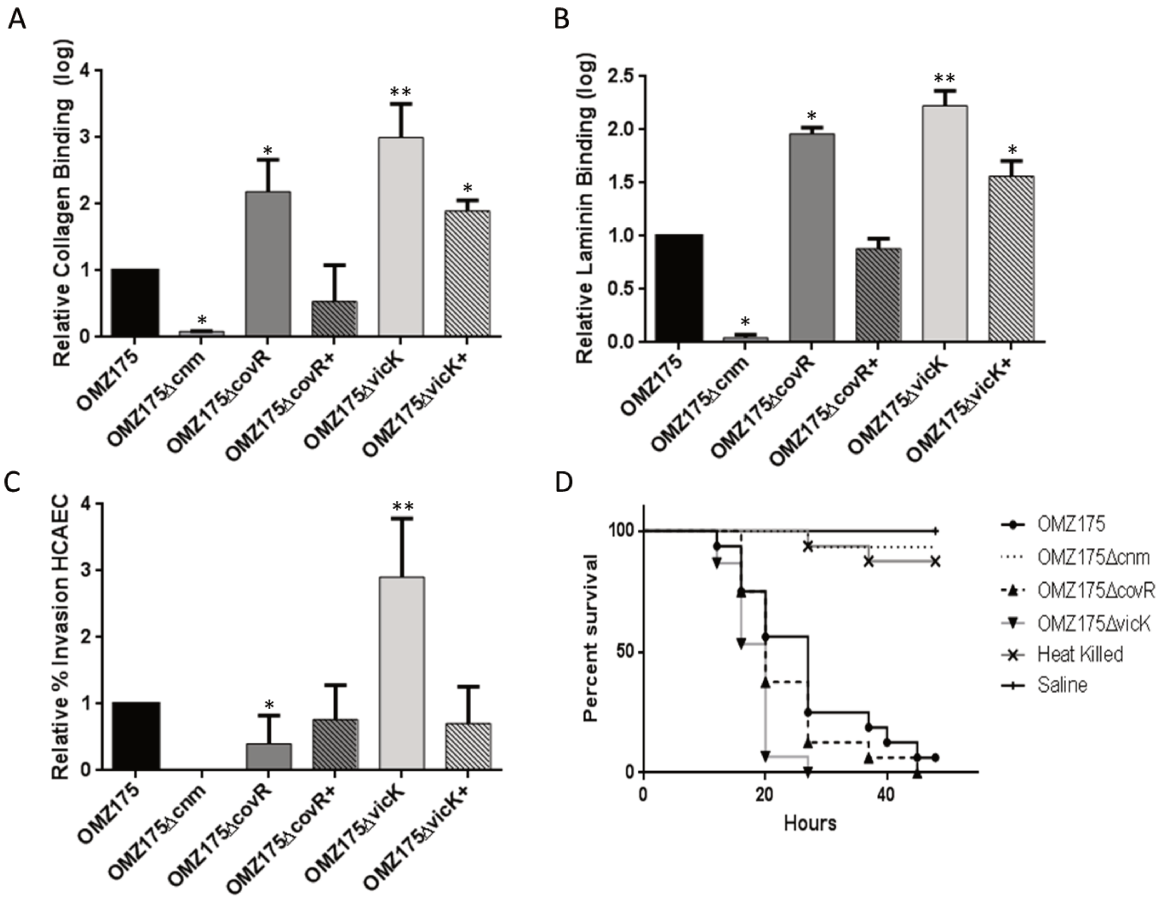

Figure 3. Contribution of CovR and VicRK to Cnm-mediated phenotypes associated to systemic virulence in OMZ175. (A, B) Relative collagen- and laminin- binding of *covR* and *vicK* mutants compared to wild type OMZ175. (C) Percent of HCAEC invasion by *covR* and *vicK* mutants in relation to OMZ175. Bacteria were recovered from the intracellular compartment of HCAEC after 3h of infection. Columns and bars represent means of three independent and standard deviations, respectively. Asterisks indicate significant differences in relation to correspondent wild type (Kruskal-Wallis with post hoc Dunn's test; $p< 0.05$). (D) Percent survival of *G. mellonella* larvae infected with *S. mutans* strains. Injection with heat killed (HK OMZ175) bacteria or sterile saline were used as negative controls. The results are representative of triplicate experiments. Kaplan–Meier killing curves were plotted, and differences in survival were compared using the log rank test; $p< 0.05$.

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Table 1. Sequence and position of the CovR/VicR binding sites in each gene promoter.

NCBI gene number	Gene name	Putative binding motif	Strand	Position (bp)*
CovR consensus motif				
OMZ175	*cnm*	tcgTTTTTAATcAAAt	+	29
OMZ175	*pgfS*	cTATTTTTAAgAAcAC	-	133
VicR consensus motif				
OMZ175	*cnm*	TGTAATATTcTtgTTA	+	5
OMZ175	*pgfS*	TGTTcTAAAcTATTT	-	125

Consensus for CovR AWATTTTTAAWAAAAR and for VicR: TGTWWWAWWNWYRWNW, where W is A or T, R is C or A, Y is C or T, and R is A or G (17). Lower case indicates mismatch. * Distance from putative -10 sequence.

Table 2. Strains used in this study.

Strain	Serotype	Relevant characteristics	Source
S. mutans			
OMZ175	*f*	Dental plaque	B. Guggenheim
OMZ175:Δcnm	*f*	Δ*cnm*::Kan^r	(5)
OMZ175:ΔcovR	*f*	Δ*covR*::Erm^r	This study
OMZ175:ΔvicK	*f*	Δ*vicK*::Erm^r	This study
OMZ175:ΔcovR+	*f*	pMC340B::*SMU_1924*; Kan^r	This study
OMZ175:ΔvicK+	*f*	ΔvicK::Ermr; pDL278::*SMU.1516*; Spec^r	This study
B14	*e*	Dental plaque	A. Bleiweis
B14:ΔcovR	*e*	Δ*covR*::Erm^r	This study
UA159	*c*	Dental plaque	University of Alabama
UAcov	*c*	Δ*covR*::Erm^r	(23)
UAvic	*c*	Δ*vicK*::Erm^r	(20)
E. coli			
BL21-pETrCovRSmu	-	pET22B::covR	(17)
BL21-pETrVicRSmu	-	pET22B::vicR	(17)

Table 3. Oligonucleotides used in this study.

Primer	Sequence [a]	Product size (bp)	Source
Mutant construct			
P1 *covR*	CGTTCTATGAAACCTGTTGA	2,038	(23)
P4 *covR*	CTGCCAACTCATCCATAAC		
P1 *vicK*	TTACCAGATGCTTTTGTTGCT	2,036	(20)
P4 *vicK*	CTCTTGCCGTCTTTCATCAG		
C1 *covR*-SphI	CCTCTACCCAGCATGCCAATGGAAC	1,039	This study
C2 *covR*-XhoI	GTCCAATTTCTCGAGTTATCGCGTG		
qPCR analysis			
covR-RTF	CGAAATATGGCACGAACAC	185	(17)
covR-RTR	AGAGATGGACGGGTATGAA		
vicK-RTF	CGGCGTGATGAATATGATGAA	185	(17)
vicK-RTR	GAGGTTAATGGTGTCCGCAGT		
pgfS-RTF	CACCCTCCTGCTCTCATTCC	166	This study
pgfS-RTR	TGCCATCTGTTAACTGCACAT		
cnm-CF	CTGAGGTTACTGTCGTTAAA	137	(41)
cnm-CR	CAC TGTCTACATAAGCAT TC		
EMSA			
SMU.22-*gbpB*-F	TTGACAGCTTATCCTTTAAATG	300	(17)
SMU.22-gbpB-R	TTTACAGCTGATAATGTTGTCG		
SMU.910-*gtfD*-F	TCTCTCCTGACCACTCCCTTA	324	(17)
SMU.910-gtfD-R	TACCCAGTGCTTTTTAACCTTG		
SMU.1924-*covR*-F	AGATGTCCTCTACCCATTGA	356	(17)
SMU.1924-covR-R	CCTCATATCCTTCATGTTGTA		
Cnm-EMSA-F	CTTCAAGCCAGTCATCTG	340	This study
Cnm-EMSA-R	CAAAATGATGGCAACGGTT		
SMU.2067-*pgfS*-F	CTTGCAGCTGTCTCAATG	350	This study
SMU.2067-*pgfS*-R	TCAATCATTTTTTCTTCATTG		

[a] Underlined sequences indicate restriction enzyme sites.

3 DISCUSSÃO

Embora a imunidade mediada pelo complemento seja reconhecida como um importante fator de defesa do sangue contra *Streptococcus spp.* (Walport, 2001; Brown *et al*., 2002; Lambris *et al*., 2008), o perfil de susceptibilidade à opsonização pelo complemento em *S. mutans* ainda era desconhecido. Assim, neste trabalho caracterizamos e comparamos os padrões de deposição de C3b entre cepas isoladas de sangue de pacientes com bacteremia associadas ou não à endocardite infecciosa, e isolados da cavidade bucal. Ainda, investigamos o papel dos SDCs CovR e VicRK na susceptibilidade de *S. mutans* à deposição de C3b do complemento, à fagocitose mediada por C3b por PMN de sangue periférico, à morte em PMN e persistência em sangue humano e de ratos, utilizando modelos *ex vivo* e *in vivo*, respectivamente.

Demonstramos no Capítulo I, que cepas de *S. mutans* isoladas de sangue apresentam menor susceptibilidade à deposição de C3b comparadas a isolados bucais, sendo essa baixa susceptibilidade associada com menor expressão de *covR* nos isolados de sangue (Alves *et al.,* 2016). A inativação de *covR* em cepas do sorotipo *c* não apenas aumenta a expressão de genes envolvidos com a síntese de glucanos derivados da sacarose (*gtfB, gtfC* e *gtfD*), mas também de genes que codificam proteínas de superfície ligadoras de glucano GbpB, GbpC e EpsC, as quais são envolvidas com a ligação estável destes polímeros à superfície de *S. mutans* durante a formação de biofilmes na presença de sacarose (Biswas & Biswas, 2006; Biswas & Biswas, 2007; Duque *et al*., 2011; Stipp *et al*., 2013). Assim, a baixa deposição de C3b observada nos isolados de sangue foi associada com a menor expressão de *covR* e aumento na transcrição de genes reprimidos por CovR, envolvidos na interação de *S. mutans* a PEC (*gbpB, gbpC* e *epsC*). Ainda neste capítulo, caracterizamos pela primeira vez o papel do regulador CovR na imunidade mediada pelo complemento e na sobrevivência em sangue humano na espécie *S. mutans*. Em *S. pyogenes* o sistema CovRS regula fatores associados no escape ao sistema complemento e de genes envolvidos com virulência sistêmica, incluindo-se o operon para a síntese da cápsula de ácido hialurônico (operon *hasABC*), *ska* (codificador de estreptoquinase, envolvida na degradação de proteínas do complemento através da ativação de plasminogênio em plasmina) e *sagA* (codificador de estreptolisina S, a qual inibe a fagocitose e é citotóxica) (Federle *et al.,* 1999; Agrahari *et al.,* 2013). Entretanto, os genomas de cepas *S. mutans* não albergam nenhum destes genes de virulência (Ajdić *et al.*, 2002; Cornejo *et al*., 2013). Demonstramos que a inativação de *covR* na cepa *S. mutans*

UA159 (sorotipo *c*) reduziu drasticamente a deposição de C3b, a ligação de IgG, como também a fagocitose mediada pelos receptores para C3b/iC3b e morte intracelular em PMNs humanos. Demonstramos ainda que a via clássica (mediada por C1q) é essencial para ativação do complemento em *S. mutans*, uma vez que níveis irrelevantes de C3b se ligam a cepas tratadas com soro humano defectivo em C1q. Mostramos ainda, que a inativação de *covR* afeta a sobrevivência de *S. mutans* em sangue humano utilizando modelo *ex vivo* e aumenta a habilidade de causar bacteremia em modelos de ratos (Alves *et al.,* 2016). No capítulo I, demonstramos também que a interação de *S. mutans* com PEC derivados da sacarose (glucano) afeta significativamente todas as funções mediadas pelo sistema imune investigadas em *S. mutans.* Esses resultados mostram um novo papel dos glucanos na virulência sistêmica desta espécie, o qual tem sido mais associado à virulência em biofilmes cariogênicos (Idone *et al*., 2003; revisado em Koo *et al.,* 2013 e em Klein *et al*., 2015). Assim, os resultados deste capítulo confirmam as primeiras evidências de que os glucanos poderiam contribuir para endocardite bacteriana em *S. mutans* (Munro & Macrina, 1993) e estabelecem mecanismos moleculares pelos quais esta espécie consegue persistir na corrente sanguínea e sobreviver às funções de defesa do hospedeiro. Em resumo, fornecemos dados que suportam um modelo no qual cepas com defeitos na expressão de CovR, tem maior capacidade de se ligar de forma estável a PEC (sintetizados no ambiente bucal na presença de sacarose) promovida pela maior expressão de proteínas ligadoras de glucano (GbpC e EpsC), o que lhes confere uma proteção análoga à de cápsulas polissacarídicas anti-oponizantes após o acesso à corrente sanguínea. A importância de cápsulas polissacarídicas na virulência sistêmica e no escape à imunidade mediada pelo sistema complemento está bem estabelecida em espécies de estreptococos como *S. pneumoniae* (Hyams *et al*., 2010; Hyams *et al.,* 2013) e *S. pyogenes* (Wessels *et al*., 1991; Cole *et al.,* 2010; Falaleeva *et al*., 2014). Nossos dados são ainda compatíveis com achados de que cepas com mutações naturais no sistema CovRS de *S. pyogenes* são hipervirulentas em humanos e em modelos de infecção em animais (Sumby *et al*., 2006). As razões da baixa expressão de *covR* nos isolados de sangue de *S. mutans* estão em estudo. Resultados preliminares revelam um grande número de mutações nas regiões promotoras de *covR* restritas a isolados de sangue (Oliveira *et al*., 2017).

No capítulo II, investigamos as razões pelas quais a inativação do sistema VicRK inesperadamente aumenta a resistência de *S. mutans* à imunidade mediada pelo complemento. O principal papel do SDC VicRK na cariogenicidade de *S. mutans* está associado com a indução de

genes requeridos para síntese (*gtfB/C*) e interação (*gbpB*) com PEC derivado da sacarose (Senadheera *et al*., 2005; Stipp *et al*., 2013). Portanto, uma vez que estes genes têm expressão diminuída no mutante *vicK*, esperávamos que este mutante fosse mais susceptível à deposição de C3b. Como descrito no capítulo I, a interação de SM a PECs é uma importante função de escape ao complemento. Entretanto, demonstramos no Capítulo II, que o SDC VicRK, ao contrário de CovR (Alves *et al*., 2016), regula fatores independentes de interação com PEC (Alves *et al.,* 2017). A susceptibilidade mediada pelo complemento regulada pelo sistema VicRK foi associada com a regulação direta ou indireta dos genes *smu.399* e *pepO*, *enolase e gapdH*. Os genes *smu.399* e *pepO* codificam proteínas com presumível função de degradação de proteínas do complemento. Demonstramos que a inativação de ambos estes genes em *S. mutans* UA159 aumentou significativamente a deposição de C3b e consequentemente, a opsonofagocitose de *S. mutans,* revelando novas proteínas envolvidas no escape ao sistema complemento desta espécie. Este resultado nos direcionou para o estudo mais detalhado das funções de PepO em *S. mutans.* Em *S. pneumoniae,* a endopeptidase O (PepO) é uma proteína de ligação à fibronectina e ao plasminogênio e está envolvida na ligação à proteína reguladora (inibidora) de fase fluída do complemento C4BP (*C4b-binding protein*) e à proteína C1q com inibição da via clássica de ativação do complemento (Agarwal *et al*., 2013; Agarwal *et al*., 2014). Como estabelecido no capítulo I, a via clássica é a principal via de ativação do complemento em *S. mutans*. Verificamos que PepO expressa por *S. mutans* também é capaz de se ligar à fibronectina, ao plasminogênio e à proteína C1q. Além disto, a inativação de *pepO* em *S. mutans* compromete significativamente a virulência sistêmica no modelo *in vivo* de invertebrado *Galleria mellonella* (dados não publicados). Ainda, no capítulo II, demonstramos que a inativação de *vicK* aumentou fortemente a ligação à fibronectina (Alves *et al*., 2017). Uma vez que a adsorção a componentes da matriz extracelular do hospedeiro é reconhecida como importante fator de escape ao sistema complemento utilizado por diversas espécies bacterianas (revisado em Lambris *et al*., 2008), a interação com à fibronectina e ao plasminogênio humano pode ser também um dos mecanismos regulados por VicRK envolvidos no escape ao sistema complemento na espécie *S. mutans.* Os fatores regulados por VicRK que influenciam na susceptibilidade ao sistema complemento e opsonofagocitose, assim como na virulência sistêmica de *S. mutans,* estão sendo estudados em mais detalhes por nosso grupo.

Nossa recente descoberta que os SDCs CovR e VicRK regulam fatores associados à susceptibilidade ao sistema complemento em *S. mutans* UA159 (Alves *et al*., 2016; Alves *et al.,*

2017), evidenciou que esses sistemas poderiam estar envolvidos na regulação de genes adicionais envolvidos na virulência sistêmica desta espécie. A colaboração e oportunidade do doutorado sanduíche na Universidade da Florida (E.U.A.) com o grupo da Dra. Abranches, cuja linha de pesquisa é baseada no estudo da proteína Cnm, nos direcionou a identificar sequências consenso de ligação das proteínas reguladoras rCovR e rVicR na região promotora de *cnm.* Compatível com nossa hipótese, no capítulo III, verificamos que os SDCs CovR e VicRK regulam diretamente a proteína Cnm. Demonstramos que CovR atua como indutor de Cnm, ao contrário do seu papel predominante como repressor dos genes envolvidos na interação com a matriz extracelular polissacarídica de biofilmes (*gtfB/C, ftf, gpbB*, *epsC*) (Biswas *et al.*, 2006; Chong *et al*., 2008; Stipp *et al*., 2013). Análises do regulon de CovR em UA159 também indicam que CovR atua também como um regulador positivo de diversos genes de *S. mutans* (Dmitriev *et al*., 2011). Por outro lado, VicRK atua como repressor de Cnm. A inativação de *covR* e *vicK* na cepa OMZ175 (*cnm* positiva) foi associada com a regulação dos fatores de virulência mediados por Cnm, como a ligação ao colágeno e laminina, invasão às células endoteliais e virulência em modelo de *Galleria mellonella* (Alves *et al*., 2017b). Além disso, em *Staphylococcus aureus,* Cna (proteína ortóloga à Cnm) parece se ligar e sequestrar C1q, prevenindo a ativação da via clássica do sistema complemento (Kang *et al*., 2013), sugerindo que a proteína Cnm de *S. mutans* também contribua para o escape desta espécie ao sistema complemento. Assim, é provável que Cnm participe não somente no processo da adesão de *S. mutans* aos tecidos cardiovasculares, mas também na persistência das cepas *cnm*-positivas na corrente sanguínea.

Portanto, os resultados deste trabalho de doutorado revelam um novo papel dos SDCs CovR e VicRK como reguladores da expressão de diversas funções de virulência sistêmica de *S. mutans*, associadas ao escape ao sistema complemento, sobrevivência na corrente sanguínea e infecção de tecidos cardiovasculares. Através da identificação dos genes regulados por estes SDCs associados a estas funções, estabelecemos que cepas da espécie *S. mutans* podem expressar múltiplos fatores de escape à imunidade do hospedeiro, o que é tipicamente observado em linhagens patogênicas de espécies de estreptococos (Lambris *et al*., 2008). Estes achados justificam, portanto, o frequente envolvimento da espécie *S. mutans* em infecções sistêmicas reveladas em estudos clínicos (Nakano *et al*., 2006; Fernandes *et al*., 2014) e deverão contribuir para a definição de alvos terapêuticos para controle de infecções sistêmicas por estes microrganismos.

4 CONCLUSÃO

Os dados deste trabalho indicam que:

1) Cepas *S. mutans* envolvidas em infecções sistêmicas têm maior resistência ao sistema complemento, quando comparadas a isolados bucais.
2) A diversidade na susceptibilidade de cepas *S. mutans* à deposição de C3b está associada a menor expressão do regulador CovR e consequentemente, ao aumento da expressão de genes envolvidos na ligação a glucanos produzidos a partir da sacarose (*gbpC* e *epsC).*
3) O SDC VicRK influencia na susceptibilidade de *S. mutans* à imunidade mediada pelo sistema complemento de forma independente da síntese e interação com glucano.
4) O sistema VicRK regula as proteases Smu.399 e PepO, as quais estão envolvidas no escape de *S. mutans* à imunidade mediada pelo sistema complemento.
5) Os SDCs CovR e VicRK regulam diretamente o gene que codifica a proteína Cnm (*cnm*). CovR atua como ativador e VicRK como repressor de *cnm.*
6) Os SDCs CovR e VicRK coordenam a expressão de múltiplos fatores de escape à imunidade mediada pelo sistema complemento e virulência sistêmica de *S. mutans.*

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*De acordo com as normas da UNICAMP/FOP, baseadas na padronização do *International Committee of Medical Journal Editors* - Vancouver Group. Abreviatura dos periódicos em conformidade com o PubMed.

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ANEXOS

Anexo 1: Autorização da Editora ASM para Artigo 1 publicado, permitindo sua inclusão na Tese.

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Anexo 2: Autorização da Editora *John Wiley and Sons* do Artigo 2 publicado, permitindo sua inclusão na Tese.

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TERMS AND CONDITIONS

Anexo 3: Carta de submissão do Artigo 3 na *Journal of Bacteriology.*

Dear Ms. Alves,

On November 15, 2017, we received the manuscript "CovR and VicRK regulate transcription of the collagen binding protein Cnm of *Streptococcus mutans*" by Lívia Alves, Renata Mattos-Graner, Tridib Ganguly, Jessica Kajfasz, Erika Harth-Chú, Jose Lemos, and Jacqueline Abranches. The submission form indicates that this paper should be processed as a(n) Full-length text.

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Thank you for submitting your work to Journal of Bacteriology.

Regards,
JB staff

Anexo 4: Certificado de aprovação do Comitê de Ética em Pesquisa com Seres humanos.

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Estudo de fatores e proteínas de superfície de Streptococcus mutans envolvidos na evasão ao sistema complemento"**, protocolo nº 153/2014, dos pesquisadores Lívia Araújo Alves, Natalia Leal Vizoto, Renata de Oliveira Mattos Graner e Thaís Rodrigues de Carli, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 25/03/2015.

The Ethics Committee in Research of the Piracicaba Dental School - University of Campinas, certify that the project **"Analysis of the factors and surface proteins of Streptococcus mutans involved in evasion by the complement system"**, register number 153/2014, of Lívia Araújo Alves, Natalia Leal Vizoto, Renata de Oliveira Mattos Graner and Thaís Rodrigues de Carli, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee on Mar 25, 2015.

Prof. Dr. Jacks Jorge Junior
Secretário
CEP/FOP/UNICAMP

Prof. Dr. Felippe Bevilacqua Prado
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

Anexo 5: Certificado de aprovação para atividades em contenção com organismos geneticamente modificados e seus derivados (CIBio – FOP/

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba
CIBio - Comissão Interna de Biossegurança

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APROVAÇÃO PARA ATIVIDADES EM CONTENÇÃO COM ORGANISMOS GENETICAMENTE MODIFICADOS E SEUS DERIVADOS

Ofício CIBio-FOP n. 002/2015 — Piracicaba, 04 de dezembro de 2015

RESPONSÁVEL PELO PROJETO: Profa. Dra. Renata de Oliveira Mattos-Graner
com cópia para a chefia do departamento

Prezado(a) Professor(a):

O projeto "Identificação de proteínas de superfície de Streptococcus mutans implicadas no escape à opsonização pelo sistema complemento", envolvendo organismos geneticamente modificados (OGM) do tipo I, sob sua responsabilidade, protocolado sob o número de requerimento R002/2015, foi APROVADO pela Comissão Interna de Biossegurança da Faculdade de Odontologia de Piracicaba – UNICAMP para ser desenvolvido nos LABORATÓRIOS CREDENCIADOS DA ÁREA DE MICROBIOLOGIA E IMUNOLOGIA (Nível de Biossegurança NB-1), do Departamento de Diagnóstico Oral da Faculdade de Odontologia de Piracicaba, conforme parecer FAVORÁVEL em anexo. **Aprovação número A002/2015.**

O RESPONSÁVEL PELO PROJETO DEVERÁ:

1) assegurar a plena capacitação da equipe de trabalho e o cumprimento das resoluções normativas (RNs) da CTNBio (disponíveis em www.ctnbio.gov.br). RN 1 [Art. 11 (I, VIII)]

2) manter toda documentação acerca do projeto e da capacitação da equipe de trabalho em arquivo organizado e de pronto acesso, para visitas/inspeções pela CIBio-FOP e/ou órgãos competentes. RN 1 [Art. 11, Art. 18 (IV)]

3) comunicar à CIBio-FOP através de ofício eventuais alterações no projeto e seu local de realização ou na equipe de trabalho. RN 1 [Art.8 (I), Art. 11 (V, IX)]

4) entregar à CIBio-FOP o relatório em formulário padronizado contendo a descrição e o andamento do projeto, impresso em duas vias assinadas/rubricadas pelo responsável pelo projeto e pela chefia do departamento, no primeiro dia útil do mês de fevereiro, anualmente, e quando do encerramento do projeto. RN 1 [Art. 8 (I, IV), Art. 10]

Atenciosamente,

Presidente da CIBio
Prof. Dr. Rafael Nobrega Stipp
Área de Microbiologia e Imunologia - Matrícula: 303502
Departamento de Diagnóstico Oral – FOP/UNICAMP
rafaelns@fop.unicamp.br | (19) 2106-5707

carimbo e rubrica
Responsável pelo Projeto
Profa. Dra. Renata de Oliveira Mattos Granner
Prof. Associado I – Matr. 29.609-8
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Conteúdo: Aprovação para atividades em contenção com OGM	**Emitente:** Prof. Dr. Rafael Nobrega Stipp
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