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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).

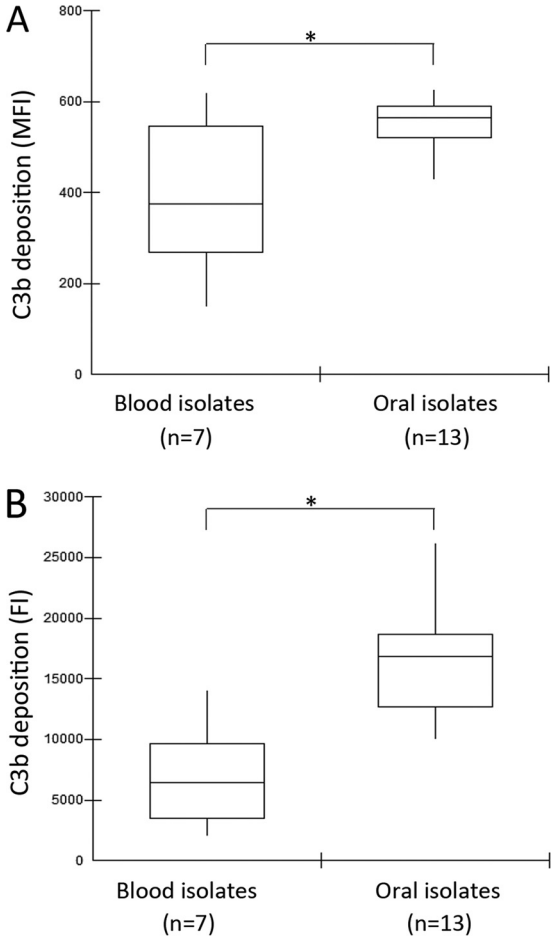

FIG 1 Box plot comparisons of C3b depositions between *S. mutans* strains isolated from blood samples and those of strains isolated from the oral cavity. C3b bound to serum-treated strains was probed with anti-C3 antibody conjugated with FITC for flow cytometry analysis. (A) Levels of C3b bound to *S. 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.

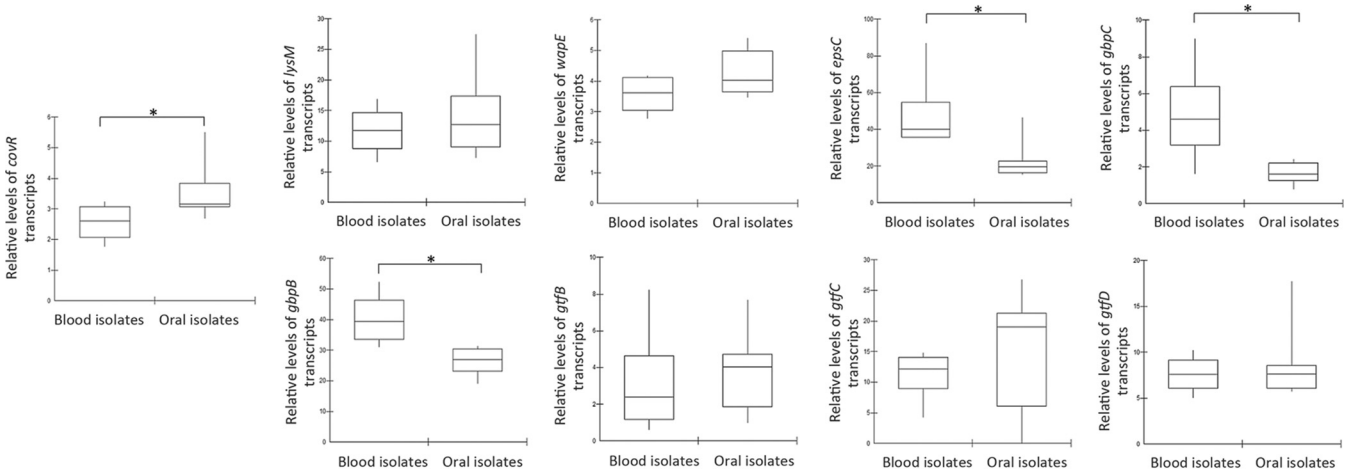

FIG 2 Reverse transcription-qPCR comparisons of transcript levels of $covR_{Sm}$ and $CovR_{Sm}$-regulated genes (*lysM*, *wapE*, *epsC*, *gbpC*, *gbpB*, *gtfB*, and *gtfC*) in blood ($n = 4$) and oral ($n = 5$) strains of *S. mutans*. The *gtfD* gene, which is not regulated by $CovR_{Sm}$, was tested 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).

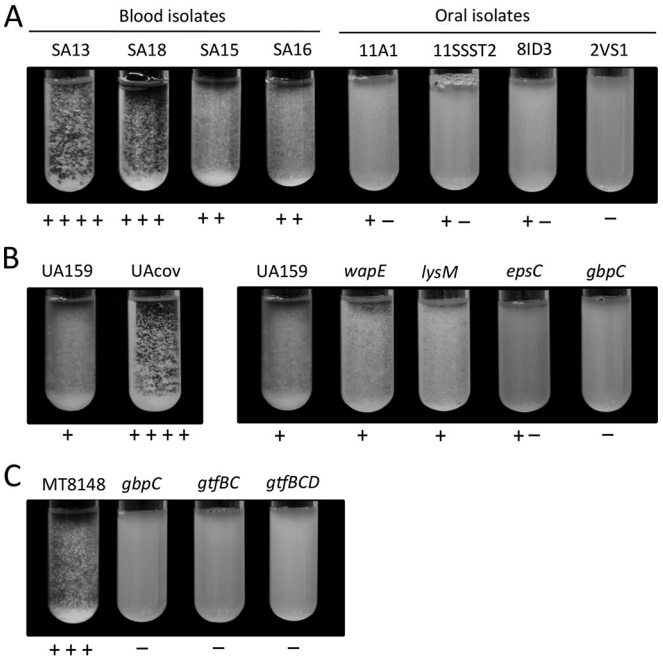

FIG 3 Comparisons of *S. mutans* capacities to aggregate in the presence of sucrose-derived EPS. Strains were incubated in BHI medium supplemented with 0.1% sucrose during 24 h for visual analysis of clump formation. Intensities of cell aggregation were determined by a blind examiner and are indicated 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.

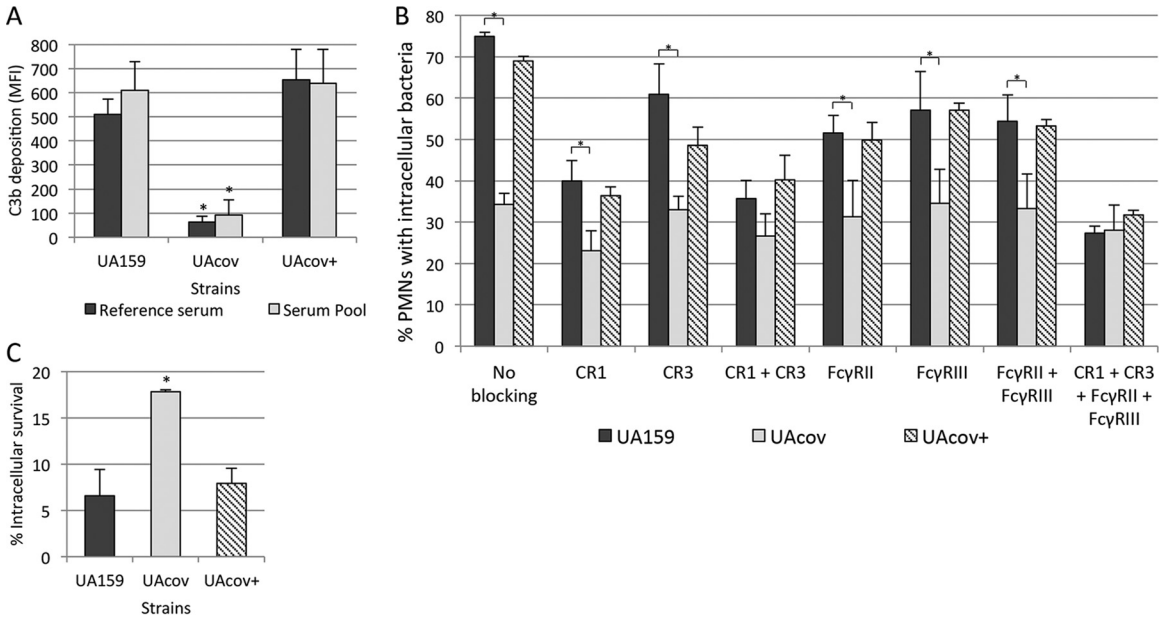

FIG 4 Comparisons of C3b deposition, opsonophagocytosis, and killing by PMNs between *covR* mutant (UAcov), parent (UA159), and complemented (+) strains. (A) Intensities of C3b deposition (MFI) in strains treated with a reference serum or pools of sera obtained from six volunteers were determined by 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

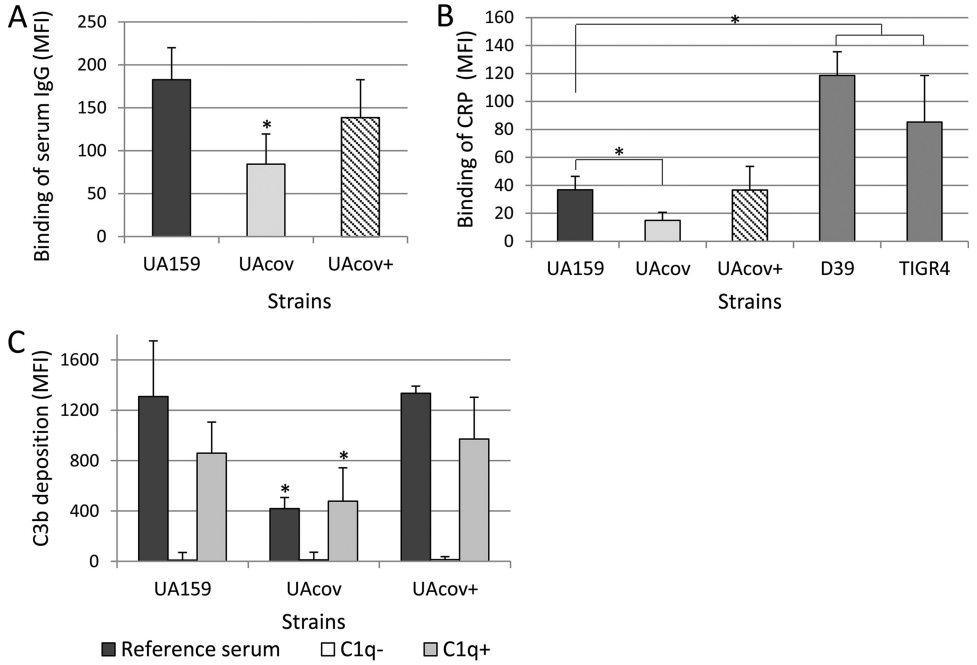

FIG 5 Binding of serum IgG, CRP, and C3b to *S. mutans* strains in the presence of serum. (A and B) Strains were treated with 20% human serum, and levels of surface IgG (A) and CRP (B) were determined by flow cytometry (MFI). *S. pneumoniae* strains D39 and TIGR4 were used as controls for CRP binding. (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-

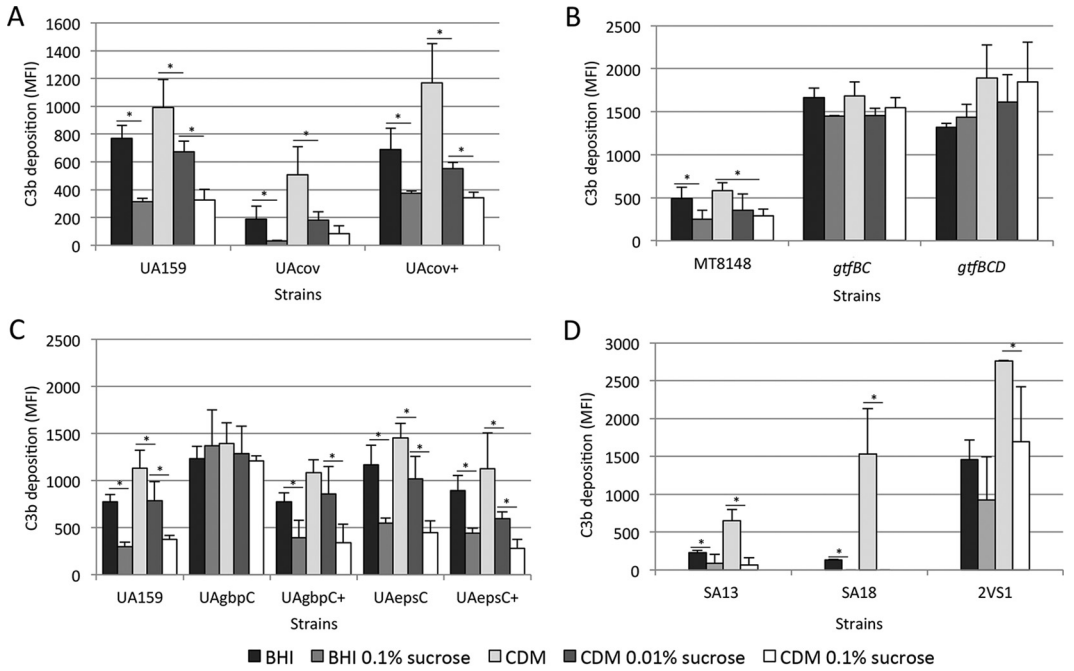

FIG 6 Effects of previous growth in medium supplemented with sucrose on strain susceptibilities to C3b deposition. Strains grown in BHI medium or CDM supplemented or not with 0.01 or 0.1% sucrose were harvested, washed with PBS, and treated with human serum for C3b deposition. Columns represent mean 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

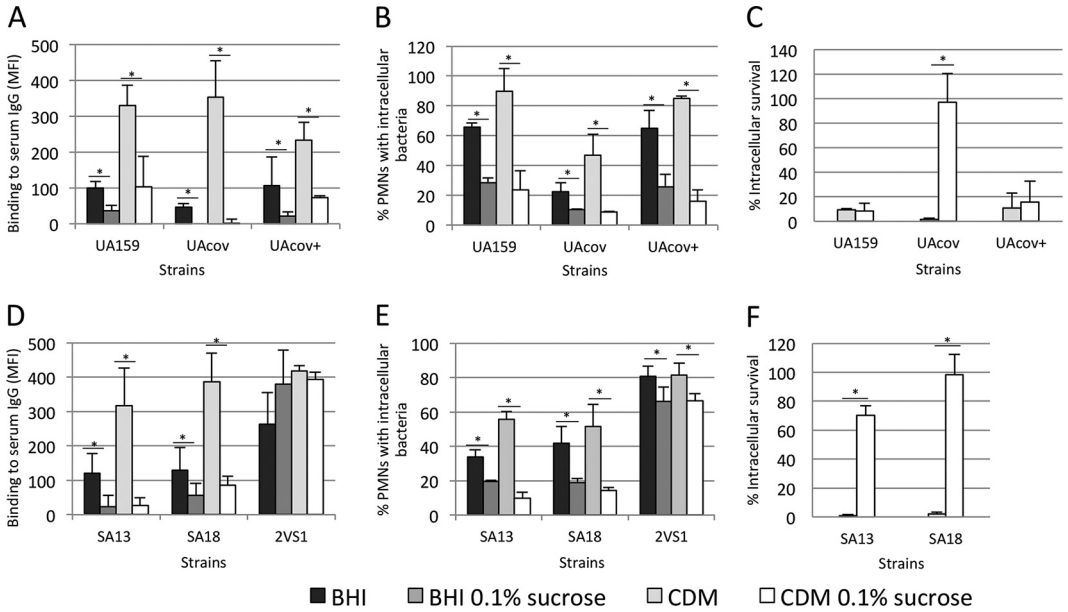

FIG 7 Effects of previous growth of *S. mutans* strains in medium supplemented with sucrose on binding to serum IgG, susceptibility to phagocytosis by PMNs, and killing by PMNs. (A and D) Strains grown in BHI medium or CDM supplemented or not with 0.01 or 0.1% sucrose were harvested, washed with PBS, 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-

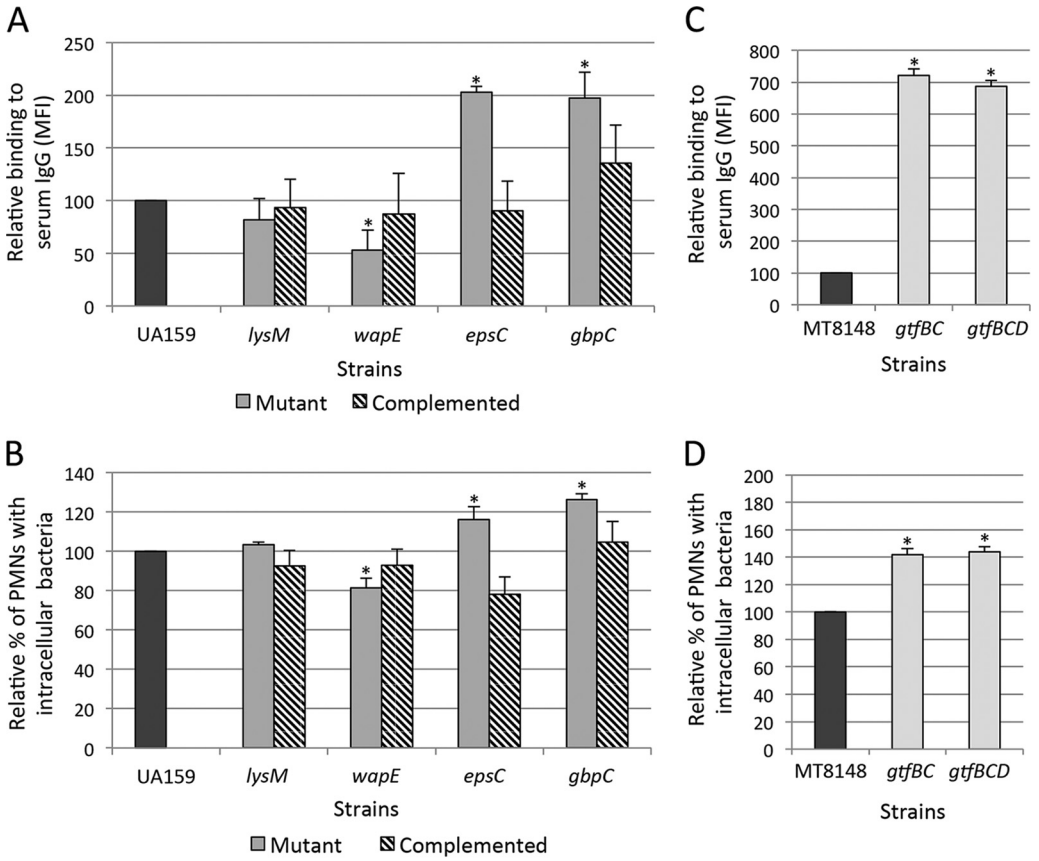

FIG 8 Comparisons of binding to serum IgG (A and C) and of phagocytosis by human PMNs (B and D) between mutants of genes regulated by $CovR_{Sm}$. Mutant or complemented strains were compared with the respective parent strains (UA159 or MT8148). Columns represent means of data from three independent 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

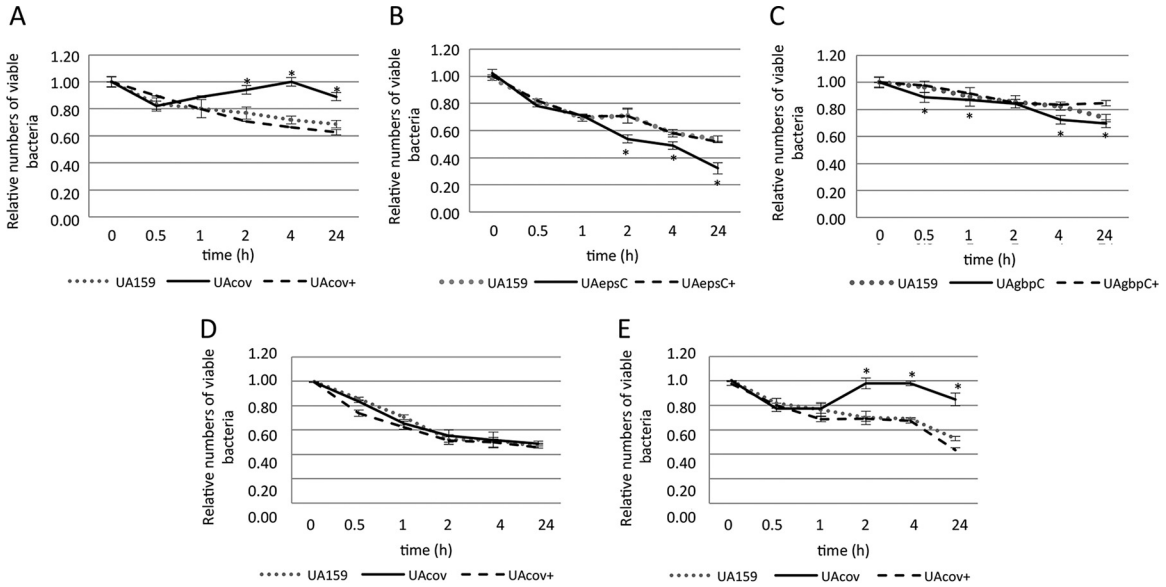

FIG 9 *Ex vivo* viability in human blood. Numbers of viable bacteria (log CFU per milliliter) were expressed in relation to initial counts in blood suspensions (time zero). Strains were grown in BHI medium (A to C), CDM (D), or CDM supplemented with 0.1% sucrose (E). Data represent means of results from 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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