

Human Serum Induces Streptococcal C5a Peptidase Expression[∇]

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***Streptococcus agalactiae* is a major pathogen in humans and animals. Virulence factors are often associated with mobile genetic elements, and their expression can be modulated by host factors. *S. agalactiae* harbors the genes for C5a peptidase (*scpB*) and Lmb on a composite transposon structure which is absent in many bovine isolates. To investigate whether these genes participate in the adaptation to human hosts, we determined the influence of human and bovine serum on the promoter activity of *scpB* and *lmb* by using fluorescence-activated cell sorter analysis. Culture in the presence of 1 to 50% human serum resulted in a dose-dependent induction of reporter gene activity for *scpB* but not *lmb*. Reporter gene activity was, however, unchanged following growth in fetal calf serum. Interestingly, a bovine strain did not display any induction of *scpB* by either bovine or human serum. Reverse transcription-PCR analysis was used to confirm differential induction of *scpB* in *S. agalactiae* and showed a similar induction of the *Streptococcus pyogenes* C5a peptidase gene *scpA* by human but not bovine serum. The specific induction of the streptococcal C5a peptidase by human serum corresponds to the absence of *scpB* in many bovine *S. agalactiae* isolates and underlines the importance of this virulence factor for human infections.**

Streptococcus agalactiae (group B streptococci [GBS]) was initially identified as the causative agent of bovine mastitis. It later emerged as the major pathogen of bacterial sepsis and meningitis in neonates and has recently been recognized as an increasingly common cause of invasive disease in immunocompromised patients (16). Like other bacterial pathogens, e.g., *Streptococcus pneumoniae* (27), *Enterococcus faecalis* (33), and *Streptococcus mutans* (38), it has the ability to survive and multiply in various anatomical niches and body fluids of the host, including serum, which requires the expression and coordinate regulation of multiple pathogenicity factors.

Genes encoding virulence factors are often associated with mobile genetic elements and controlled by complex regulatory networks (13). The C5a peptidase of *S. agalactiae* is a surface-associated serine protease that plays an important role in virulence of *S. agalactiae* (6, 11, 12). The structure of this protein has recently been solved (9). It cleaves the chemotactic complement component C5a (4, 20, 39), binds to fibronectin, and contributes to cellular invasion (1, 11). Following the identification of the gene in *Streptococcus pyogenes* (44, 45), a nearly identical gene was found in *S. agalactiae* (12). Together with the detection of flanking mobile genetic elements, the finding suggests horizontal gene transfer of the C5a peptidase gene among pyogenic streptococci (8, 17). Interestingly, all human but only some bovine *S. agalactiae* isolates possess the *scpB* gene (14, 17). Genetic polymorphisms alter the functional activity of C5a peptidase (5) but do not affect its ability to bind fibronectin (40). Overall, its conserved nucleotide sequence in several β -hemolytic streptococcal species, its ubiquitous expression, and its surface localization make it a good vaccine

candidate for *S. agalactiae*, as well as *S. pyogenes*, infections (10, 21, 29, 32).

Immediately downstream of *scpB* lies *lmb*, the gene encoding a surface-associated lipoprotein of *S. agalactiae* that mediates binding to human laminin (37) and is involved in the invasion of brain endothelial cells (41). Similar to the findings for *scpB*, the nucleotide sequences of *lmb* of *S. pyogenes* and *S. agalactiae* are >98% identical (15, 37, 42). Both genes are part of a composite transposon structure which is flanked by insertion sequence (IS) elements (Fig. 1) and appears to build a functional unit that is lost or acquired en bloc (8, 17).

Based on the observation that the entire transposon structure is found in the vast majority of human *S. agalactiae* strains but in only some bovine isolates, we were interested in the question of whether *scpB* and *lmb* specifically contribute to human pathogenicity. For this purpose, we determined if the genes are part of the bacterial system of adaptation to various host compartments by measuring transcription activity in the presence of human or bovine serum.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains and plasmids used in this study are listed in Table 1. The *S. agalactiae* strain O90R (ATCC 12386) was used for amplifying the promoter sequences of *scpB* and *lmb*, for constructing the mutant strains O90R*scpB*Prom and O90R*lmb*Prom, for preparation of RNA for LightCycler experiments, and together with strain O90R*ΔscpBΔlmb*, for growth monitoring. The *S. agalactiae* strains O90R*scpB*Prom and O90R*lmb*Prom were used for fluorescence-activated cell sorter (FACS) analysis. In addition to the human wild-type strain O90R, the bovine *S. agalactiae* strain BSU77, the *rgfC* mutant strain O90R*ΔrgfC*, and the previously described *S. agalactiae* clinical isolates AC475 (35), BSU147, and BSU191 (7) served as hosts for the *scpB* reporter gene plasmid. The *Streptococcus pyogenes* strain BSU316 is a clinical isolate that was used to determine mRNA levels of *scpA* by reverse transcription-PCR (RT-PCR) analysis. *Escherichia coli* strain DH5 α served as a host for recombinant pBSU409 plasmids. Streptococcal strains were cultured in Todd-Hewitt broth (THB) (Oxoid) supplemented with 0.5% yeast extract (THY) or on THY agar at 37°C. Mutant strains harboring pBSU409 plasmids were cultured in medium containing spectinomycin (120 μ g/ml) at a temperature of 37°C. *E. coli* strain DH5 α was cultured in LB (Luria-Bertani) medium or on LB agar at 37°C.

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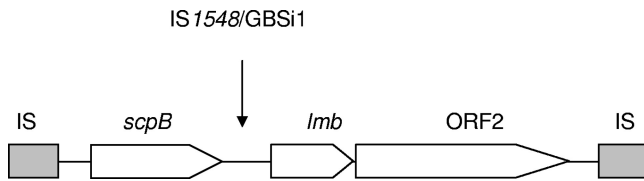


FIG. 1. Genetic structure of the chromosomal region encoding the surface proteins ScpB and Lmb in *S. agalactiae* strain O90R (17). The three genes are located within a putative composite transposon flanked by IS elements. The IS element IS1548 or the intron GBSi1 is inserted between *scpB* and *lmb* in other *S. agalactiae* lineages.

Mutant *E. coli* strains harboring pBSU409 plasmids were cultured in medium containing spectinomycin (100 µg/ml) at a temperature of 37°C.

General DNA techniques. Standard recombinant DNA techniques were employed for nucleic acid preparation and analysis. Genomic streptococcal DNA was isolated as described previously (30). Plasmid DNA was isolated and purified by using a QIAprep spin miniprep kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR was carried out with *Taq* polymerase according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany) with 35 cycles of amplification steps of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. PCR products were sequenced on an ABI Prism 310 genetic analyzer using an ABI Prism BigDye Terminator cycle sequencing kit, version 1.1 (Applied Biosystems, Weiterstadt, Germany). *S. agalactiae* strain O90R was transformed according to an established protocol (31).

Construction of pBSU409. To investigate the promoter activity of *scpB* and *lmb*, a novel enhanced green fluorescent protein (EGFP) reporter plasmid for streptococci was constructed. Vector pAT28 (43) and cloning vector pEGFP (Clontech Laboratories, Mountain View, CA) were used for the construction of pBSU409. Both plasmids were digested with the restriction enzymes BamHI and XbaI. The DNA fragment carrying the *egfp* sequence was isolated from DNA agarose gel by using a QIAquick gel extraction kit (Qiagen, Hilden, Germany). After insertion of *egfp* into pAT28, the multiple cloning site (MCS) was modified by using a DNA fragment consisting of the oligonucleotides pAT28-EGFP5 and pAT28-EGFP6 (Table 2). Annealing of both oligonucleotides generates an artificial DNA fragment with ends compatible with the restriction enzymes BamHI and SacI. Following the digestion of the first pAT28-EGFP construct with the two enzymes, the novel artificial MCS fragment was inserted into the vector. Correct construction of the novel MCS site was determined by PCR using primers flanking the MCS site (pAT28-3 and pAT28-EGFP4) (Table 2) and sequencing of the resulting PCR product.

Generation of *scpB* and *lmb* reporter gene constructs. The promoter regions of *scpB* and *lmb* were amplified with the primers scpBProm1 and scpBProm2 (Table 2) and lmbProm1 and lmbProm2 (Table 2), respectively. The resulting PCR products and the vector pBSU409 were digested with the enzymes BamHI and EcoRI, ligated, and transformed into *E. coli* cells. Correct construction of the plasmids was controlled through PCR with primers flanking the insertion site (pAT28-3 and pAT28-EGFP4) (Table 2) and sequencing of the PCR products. Recombinant plasmids harboring the *scpB* or *lmb* promoter sequences upstream of the *egfp* gene were transformed into the *S. agalactiae* wild-type strain O90R.

Construction of the *rgfC* deletion mutant. For construction of the *rgfC* deletion mutant, the plasmid pGhost5 (2) was used. DNA regions upstream and downstream of the intended deletion site were amplified by PCR with the primer combination rgfCdel1 and rgfCdel2 and the primer combination rgfCdel3 and rgfCdel4 (Table 2). The resulting PCR products were cloned into pGhost5, transferred into the chromosome of strain O90R, and subsequently mobilized as described in reference 18. The resulting mutant strain, O90RΔ*rgfC*, harbors a deletion of nucleotides 32 to 1276 of the 1,335 nucleotides of *rgfC*. Correct deletion of the targeted *rgfC* region was confirmed by DNA sequencing.

FACS analysis. The recombinant *S. agalactiae* strains carrying the *scpB* reporter gene construct were incubated in 5 ml of THY medium containing spectinomycin (120 µg/ml) and in 5 ml of spectinomycin-containing THY medium supplemented with increasing amounts (1 to 50%) of serum (human serum, heat-inactivated human serum, fetal calf serum, and heat-inactivated fetal calf serum). Human serum was obtained from PAA Laboratories GmbH in Pasching, Austria, and fetal calf serum from Biochrom, AG, in Berlin, Germany. Blood was subjected to a natural clotting process and centrifuged to remove cells and fibrin. According to company information, serum was collected from healthy donors and represents pools from more than 50 individuals with endotoxin levels of ≤20 endotoxin units/ml. In addition, two different pools of human serum collected and pooled at our institute from 10 donors for each pool were used to study the induction of *scpB* transcription. In these pools, blood was subjected to a natural clotting process and centrifuged to remove cells and fibrin. The cultures were grown to stationary phase overnight, pelleted, washed, and resuspended in phosphate-buffered saline (PBS). Aliquots were analyzed by FACS (FACSCalibur; Becton Dickinson Immunocytometry Systems, San Jose, CA) until 10,000 fluorescent events were counted. Side and forward scatter data were collected using linear amplifiers (forward scatter, EOO, and side scatter, 400), and fluorescence data were collected using logarithmic amplifiers (fluorescence channel 1, 700). For assays testing the influence of fibronectin and C5a, the *S. agalactiae* strain O90RscpBProm was incubated in spectinomycin containing THY medium supplemented with 140 µg/ml of fibronectin (Sigma-Aldrich GmbH, Steinheim, Germany) or 10 to 200 ng/ml of C5a (Sigma-Aldrich). The cultures were grown to stationary phase overnight, pelleted, washed, and resuspended in PBS. Aliquots were analyzed by FACS as described above.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description ^a	Source or reference
<i>E. coli</i> DH5α	<i>endA1 hsdR17 supE44 ΔlacU169 (φ80lacZΔM15) recA1 gyrA96 thi-1 relA1</i>	Boehringer Ingelheim
<i>S. agalactiae</i> strains		
O90R (ATCC 12386)	R. Lancefield grouping strain, human isolate	American Type Culture Collection
AC475	Clinical isolate	35
BSU147	Clinical isolate	7
BSU191	Clinical isolate	7
BSU77	Bovine isolate (Gi11d)	Gießen collection (C. Lämmler)
O90RΔ <i>scpB</i> Δ <i>lmb</i>	O90R derivative, <i>scpB</i> - <i>lmb</i> deletion mutant	17
O90RΔ <i>rgfC</i>	O90R derivative, <i>rgfC</i> deletion mutant	This study
O90RpBSU409	O90R derivative harboring pBSU409 without insert	This study
O90R <i>scpB</i> Prom	O90R derivative harboring pBSU409 <i>scpB</i> Prom	This study
O90R <i>lmb</i> Prom	O90R derivative harboring pBSU409 <i>lmb</i> Prom	This study
<i>S. pyogenes</i> BSU316	Clinical isolate	Ulm collection
Plasmids		
pAT28	Sp ^r <i>ori</i> pUC <i>ori</i> pAmβ1	43
pEGFP	Am ^r , source of <i>egfp</i>	Clontech Laboratories
pBSU409	pAT28 derivative, novel MCS, carrying a promoterless <i>egfp</i> gene	This study
pBSU409 <i>scpB</i> Prom	pBSU409 derivative carrying the promoter region of <i>scpB</i>	This study
pBSU409 <i>lmb</i> Prom	pBSU409 derivative carrying the promoter region of <i>lmb</i>	This study

^a Sp, spectinomycin; Am, ampicillin.

TABLE 2. Oligonucleotide primers and LightCycler hybridization probes

Primer	Description ^a	Sequence ^b
pAT28-3	Control of recombination	5' GTTGTGTGGAATTGTGAGCGG 3'
pAT28-EGFP4	Control of recombination	5' CCTTGAAGAAGATGGTGCCG 3'
pAT28-EGFP5	Construction of the novel MCS	5' CAGGCCTACTAGTCGTACG 3'
pAT28-EGFP6	Construction of the novel MCS	5' GATCCGTACGACTAGTAGGCTGAGCT 3'
scpBProm1	Construction of O90RscpBProm	5' GCGCCGGAATTCGGAGGTACAAAATAAGCAACTC 3'
scpBProm2	Construction of O90RscpBProm	5' GCGCGGATCCGTGTCGTCCTTTTCATGATG 3'
lmbProm1	Construction of O90RlmbProm	5' GCGCCGGAATTCGTATTACTTAAACTGATAAGTAGC 3'
lmbProm2	Construction of O90RlmbProm	5' GCGCGGATCCGTACCTCCTCAATTATAATTTAACC 3'
rgfCdel1	Construction of <i>rgfC</i> deletion	5' TGGCACAAGCTTGAAGCATTTAGCAGAAGG 3'
rgfCdel2	Construction of <i>rgfC</i> deletion	5' CCCATCCACTAAACTTAAACAAAGCTATCTCTAAGATGG 3'
rgfCdel3	Construction of <i>rgfC</i> deletion	5' TGTTTAAAGTTTGTGATGGGCTATTGCAACAAGTAGCC 3'
rgfCdel4	Construction of <i>rgfC</i> deletion	5' CCGCGGATCTAGAAACCATTGGACGCG 3'
scpBLC1for	RT-PCR experiments, <i>scpB</i>	5' ACCAGCCATATCTTCAACAACA 3'
scpBLC1rev	RT-PCR experiments, <i>scpB</i>	5' AACATCGGCAACATTTCTCAAC 3'
lmbLC1for	RT-PCR experiments, <i>lmb</i>	5' TTCGAATAGCATGAGCAA 3'
lmbLC1rev	RT-PCR experiments, <i>lmb</i>	5' AGAAGCAGAGCAACTAAGTGAAGA 3'
gyrA-F	LightCycler primer, reference gene, <i>gyrA</i> in GBS	5' CTGGTCCAGATTTTCTAC 3'
gyrA-R	LightCycler primer, reference gene, <i>gyrA</i> in GBS	5' TGCGCTTTTCTGTGCTA 3'
gyrA-FL	LightCycler hybridization probe, reference gene, <i>gyrA</i> in GBS	CCAGTTTCATAGGCACGGTAA-FL
gyrA-LC	LightCycler hybridization probe, reference gene, <i>gyrA</i> in GBS	R640-ACCTGAACGTCCCATCACCAG-PH
gyrA-R2	LightCycler primer, reference gene, <i>gyrA</i> in GAS	5' CACTCCTTCACGGTAGATT 3'
gyrA-S	LightCycler primer, reference gene, <i>gyrA</i> in GAS	5' CTGGACCTGACTTTCCG 3'
gyrA-FL2	LightCycler hybridization probe, reference gene, <i>gyrA</i> in GAS	ATTGACGCCATAGGAAATTCAGTAACC-FL
gyrA-LC2	LightCycler hybridization probe, reference gene, <i>gyrA</i> in GAS	R640-CAATACGTTCCCGACCTGTTTGAGITG-PH
scpB-se	LightCycler primer, <i>scpB</i>	5' CAGCTGAGGTCACAATGCTAAC 3'
scpB-as	LightCycler primer, <i>scpB</i>	5' GCTCAAGCTATCAGAGATGCTGTC 3'
scpB-FL	LightCycler hybridization probe, <i>scpB</i>	GGTTGGCGTAAGCTAGTGACGCA-FL
scpB-LC	LightCycler hybridization probe, <i>scpB</i>	R640-TACCAAAGCTCATATTAATCACCTTAGCTCCC-PH
lmb-fw	LightCycler primer, <i>lmb</i>	5' GCCTTGTGTGACTTCCATATCTT 3'
lmb-as	LightCycler primer, <i>lmb</i>	5' TGATGTGAGGATGATCCATCAG 3'
lmb-FL	LightCycler hybridization probe, <i>lmb</i>	CGCGTCATAAATAGCTGCCACATCATTTACA-FL
lmb-LC	LightCycler hybridization probe, <i>lmb</i>	R640-ACGGTTCAAAGGAATGAATGCCTGCA-PH

^a GAS, group A streptococci.

^b FL, fluorescein; R640, LightCycler Red640 *N*-succinimide ester; PH, 3' phosphate.

RNA preparation and analysis. Total RNA was prepared from *S. agalactiae* strain O90R and *S. pyogenes* strain BSU316 grown to an optical density at 600 nm (OD₆₀₀) of 1.0 in 100 ml of THY medium and in 100 ml of THY medium supplemented with 10% serum (human serum, heat-inactivated human serum, fetal calf serum, or heat-inactivated fetal calf serum). Cells were lysed mechanically with glass beads in a cell disrupter (Bio 101, Vista, CA). Purification of RNA was achieved with an RNeasy mini kit (Qiagen, Hilden, Germany) as instructed by the manufacturer. For RT-PCR experiments, 4 ng of DNase-treated RNA was used as a template (OneStep RT-PCR kit; Qiagen, Hilden, Germany). The RT reaction was carried out at 55°C for 30 min. After PCR activation at 95°C for 15 min, PCR was carried out with 28 cycles of amplification steps of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. The RT-PCR kit was used according to the manufacturer's instructions; RT-PCR was performed with 30 pmol of each primer. To control for DNA contamination of RNA samples, 4 ng of DNase-treated RNA was used as a template in a PCR without the RT step under the above-described conditions. PCRs were carried out with the same primers used for the LightCycler experiments (Table 2). RNase-free water served as a negative and DNA as a positive control for the RT-PCR.

LightCycler experiments. To quantify RT-PCR results, LightCycler experiments were performed. Real-time RT-PCRs were carried out with RNA as a template and hybridization probes for detection. The LightCycler RNA amplification kit hybridization probes (Roche Diagnostics, Mannheim, Germany) were used as instructed by the manufacturer. Primers and hybridization probes (Table 2) were present in a final concentration of 0.5 and 0.2 μM, respectively. Magnesium chloride was added to a final concentration of 7 mM. Amplification was carried out using a LightCycler program with an elongation time of 10 s and an annealing temperature of 61°C for *scpB* and 59°C for *lmb*. Standard curves were generated by using 40, 8, 4, 2, and 1 ng of RNA as the template in the RT-PCR. Two samples (4 ng) were prepared, and the mean of both values was used in subsequent calculations. The efficiency (*E*) of the reaction was determined with the equation $E = 10^{-1/\text{slope}}$. Subsequently, the mRNA ratio was calculated using the following formula: $E(\text{target})^{\Delta\text{cp}(\text{target})}/E(\text{ref})^{\Delta\text{cp}(\text{ref})}$. $E(\text{target})$ represents the efficiency of the gene of interest (*scpB* or *lmb*), whereas $E(\text{ref})$ represents the efficiency of the reference gene. $\Delta\text{cp}(\text{target})$ refers to the difference between two measurements of the crossing points of the gene of interest, whereas $\Delta\text{cp}(\text{ref})$ stands for the difference between the crossing points of the reference gene for two RNA samples. The gyrase A gene *gyrA* was chosen as the reference gene, due to its stable expression under different environmental con-

ditions and growth phases. The quantification results are expressed as the number of mRNA copies of the gene of interest per mRNA copy of *gyrA*.

Growth curves. To determine if the C5a peptidase gene is required for survival of *S. agalactiae* in human serum, *S. agalactiae* strain O90R and strain O90RΔ*scpB*Δ*lmb*, in which the entire composite transposon structure is deleted, were used. Both strains were grown at 37°C in THY or THY medium supplemented with 10% and 50% of human serum and 10% and 50% of fetal calf serum. Cultures were monitored for 8 h by measuring the OD₆₀₀ every hour.

RESULTS AND DISCUSSION

Construction of pBSU409. A novel EGFP reporter gene construct for use in streptococci was constructed to test the influence of serum on *scpB* and *lmb*. The promoterless *egfp* gene was cloned downstream of the MCS of pAT28 (43). To eliminate complementary sequences within the vector that caused difficulties in initial cloning experiments, the MCS of the construct was modified. The MCS of pAT28 (EcoRI, SacI, KpnI, SmaI, and BamHI) was replaced by a novel MCS site consisting of the following restriction enzyme sites: EcoRI, SacI, StuI, SpeI, BsiWI, and BamHI (Fig. 2). The novel vector pBSU409 was used as a reporter plasmid for subsequent FACS analysis.

Promoter activity of *scpB* and *lmb* in the presence of serum. Experiments to study promoter activity of *scpB* and *lmb* in the presence of serum were carried out with the reporter plasmid pBSU409. The promoter sequences of *scpB* and *lmb* were inserted upstream of the promoterless copy of the *egfp* gene (Fig. 2). Recombinant plasmids were transformed into the *S. agalactiae* wild-type strain O90R, resulting in the two strains O90R*scpB*Prom and O90R*lmb*Prom. To determine the influence of human serum on the expression of *scpB* and *lmb*,

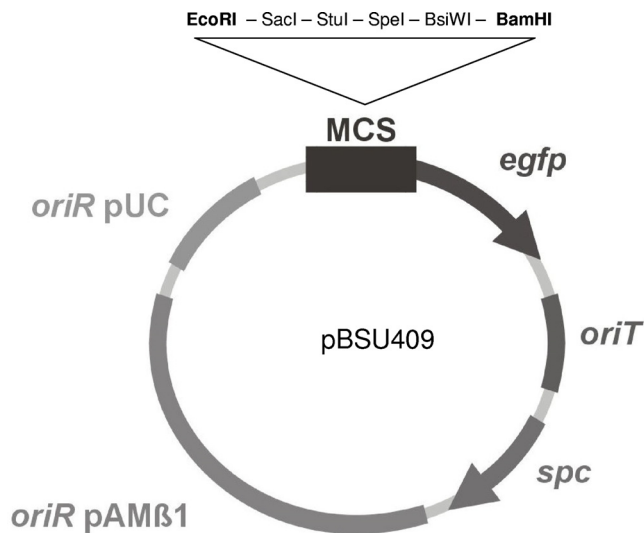


FIG. 2. Structure of the novel EGFP reporter plasmid pBSU409. Arrows indicate the direction of transcription. *oriR*, origin of replication; *oriT*, origin of transfer; *spc*, spectinomycin resistance gene.

strains O90RscpBProm and O90RlmbProm were cultured in THY medium supplemented with increasing amounts of human serum. Cultures were grown to stationary phase overnight and analyzed by FACS. A clear induction by human serum could be observed for *scpB* (Fig. 3), whereas no influence on the expression of *lmb* was detected (Fig. 3).

To investigate if the results were specific for human serum, the experiments were repeated using fetal calf serum as a stimulus. Compared with the reporter gene activity observed following the exposure to human serum, fetal calf serum did not show any influence on the expression of *scpB* or *lmb*. The values were indistinguishable from those for controls. Experi-

ments using heat-inactivated human serum were performed with the *scpB* reporter construct in strain O90R and the *rgfC* deletion mutant, to further analyze the inducing factor in human serum. Heat inactivation of the serum samples did not destroy its ability to induce the expression of *scpB* (Fig. 4 and 5). However, in some preparations using the wild-type strain O90R, a reduced activity was noted (Fig. 5).

To see if the results obtained for strain O90R using a commercial preparation of human serum could also be observed in clinical strains of *S. agalactiae* with different serum preparations, we transformed the *scpB* reporter gene construct into the clinical strains AC475, BSU147, and BSU191. All of these strains were then grown in the presence of 10% of the commercial serum preparation and two independent pools of human serum collected at our institute. Growth in the presence of 10% fetal calf serum served as a control. Induction of *scpB* transcription was present in all of these strains (Fig. 6); while some variation in the amount of induction could be observed, depending on the strain and the source of serum used, it was clearly shown that induction is present in different human *S. agalactiae* strains stimulated with different sources of human serum. Whether the observed induction of *scpB* is dose dependent was investigated by exposing strain AC475 harboring the *scpB* reporter gene construct to human serum from our commercial supplier in concentrations ranging from 1 to 10%. Stimulation with fetal calf serum served as a control in this case. In contrast to the results with serum concentrations above 10%, a clear dose-dependent induction could be observed (Fig. 7), starting with concentrations of serum supplementation as low as 1%.

The gene *scpB* is generally present in human strains but often absent in bovine strains. To determine whether induction of *scpB* by human serum can be seen in bovine strains carrying the gene *scpB*, the *scpB* promoter construct was transferred into the bovine *S. agalactiae* strain 11d. Measurement of EGFP

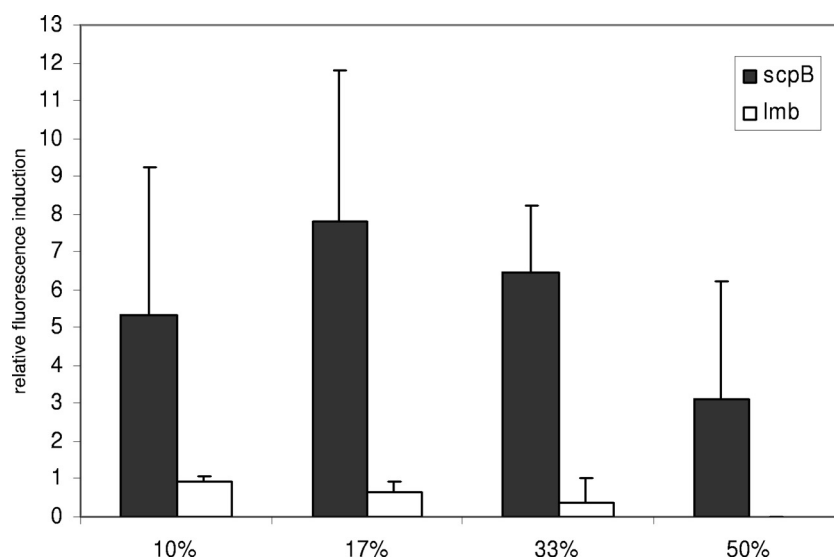


FIG. 3. Induction of *scpB* and *lmb* reporter gene activity in *S. agalactiae* strains O90RscpBProm and O90RlmbProm following culture in THY medium supplemented with various concentrations of pooled human serum. Shown are mean values above baseline (controls were set to 1) and standard deviations from three independent experiments. The amount of serum supplementation is indicated. EGFP fluorescence was measured by FACS analysis.

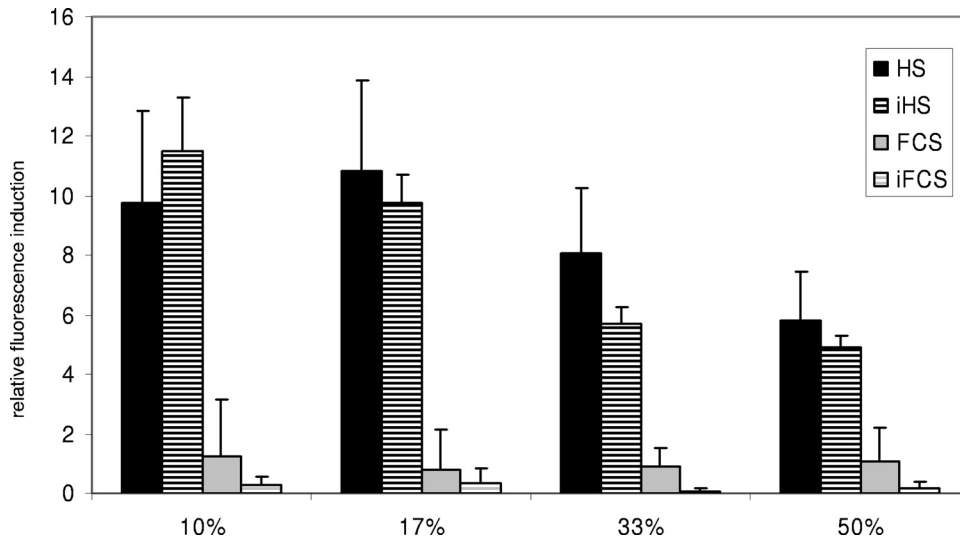


FIG. 4. Induction of *scpB* reporter gene activity in strain O90RΔrgfC, which harbors a deletion of the *rgfC* histidine kinase, following culture in THY medium supplemented with different serum stimuli. The amount of serum supplementation is indicated. Shown are mean values above baseline (controls were set to 1) and standard deviations from three independent experiments. HS, pooled human serum; iHS, heat-inactivated pooled human serum; FCS, fetal calf serum; iFCS, heat-inactivated fetal calf serum.

fluorescence after exposure of this construct to human serum, inactivated human serum, fetal calf serum, and inactivated fetal calf serum in various concentrations did not reveal any induction of the gene in the bovine background (Fig. 5).

Our studies were carried out to further investigate the role of the composite transposon structure encoding *lmb* and the C5a peptidase gene in *S. agalactiae* in human infections. *S. agalactiae* strains lacking the composite transposon structure are almost exclusively of bovine origin (14, 17), indicating a specific importance of the genes carried on this element for

human infections. Lmb is a surface lipoprotein mediating attachment to immobilized human laminin (37), while the C5a peptidase of *S. agalactiae* is anchored to the cell surface via the LPXTG motif (25). The C5a peptidase is a well-characterized virulence factor of pyogenic streptococci, with crucial roles in the inhibition of host defenses, the binding of fibronectin, and the invasion of streptococci into eukaryotic cells (4, 5, 6, 11, 12, 20, 39, 40; for a comprehensive review of the topic, see reference 26). Its role as a virulence factor in *S. pyogenes* has been examined by showing that *scpA*-negative mutants are cleared

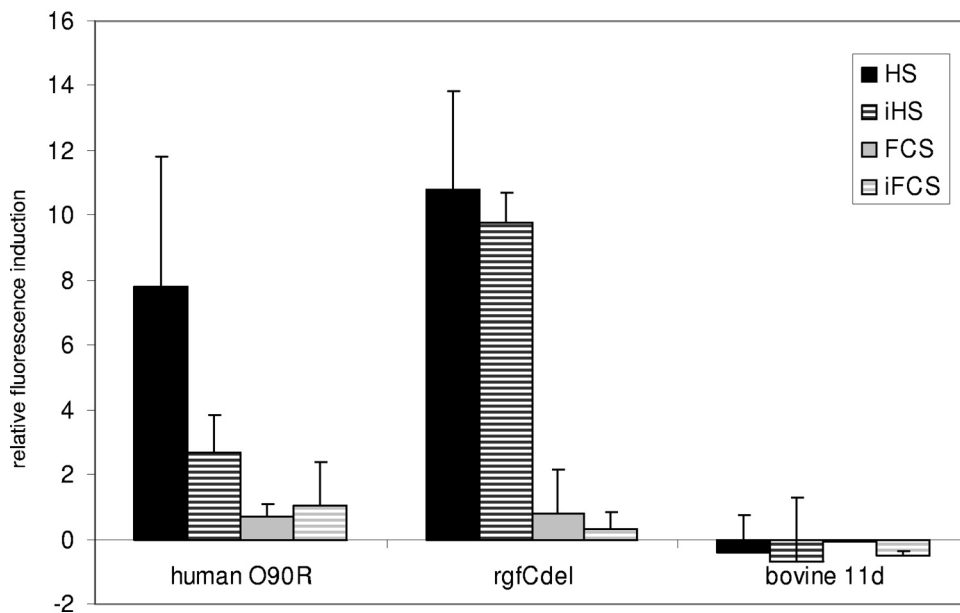


FIG. 5. Induction of *scpB* reporter gene activity in the human *S. agalactiae* strains O90R and O90RΔrgfC (*rgfCdel*) and the bovine strain Gi11d following culture in THY medium supplemented with different serum stimuli at a concentration of 17%. Shown are mean values above baseline (controls were set to 1) and standard deviations from three independent experiments. HS, pooled human serum; iHS, heat-inactivated pooled human serum; FCS, fetal calf serum; iFCS, heat-inactivated fetal calf serum.

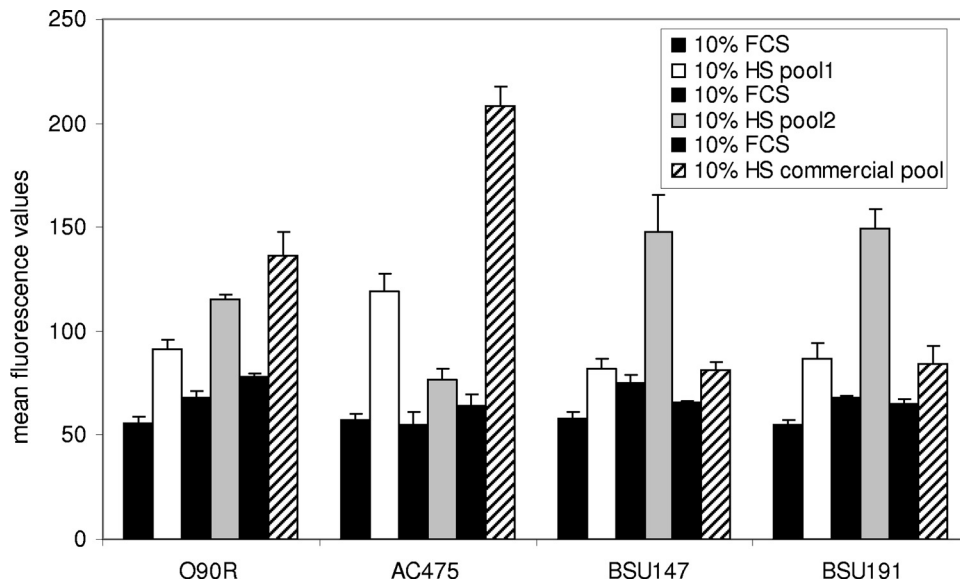


FIG. 6. Induction of C5a peptidase transcription in different *S. agalactiae* strains using various pools and sources of human serum. All strains shown were transformed by plasmid pBSU409 harboring the *scpB* reporter gene construct (pBSU409scpBProm). Strains were grown at 37°C overnight in THY broth supplemented with 10% human serum as indicated or fetal calf serum as a control. Mean fluorescence values were measured by FACS analysis. Shown are means and standard deviations from five independent experiments. HS, human serum; FCS, fetal calf serum.

more rapidly from infected tissue and from the nasopharynx in *S. pyogenes* mouse models (21, 23, 22). But its role as a virulence factor has been challenged recently (19), which may be connected to an ongoing controversy regarding the species specificity of C5a peptidase cleavage. For the C5a peptidase of

S. agalactiae, it has been shown that it cleaves human C5a but has no activity on the C5a of other species, including mice (3), while a broader range of activity on the C5a of different species has been demonstrated for the *S. pyogenes* C5a peptidase (22). Our finding of a species-specific induction of the *scpB* gene by

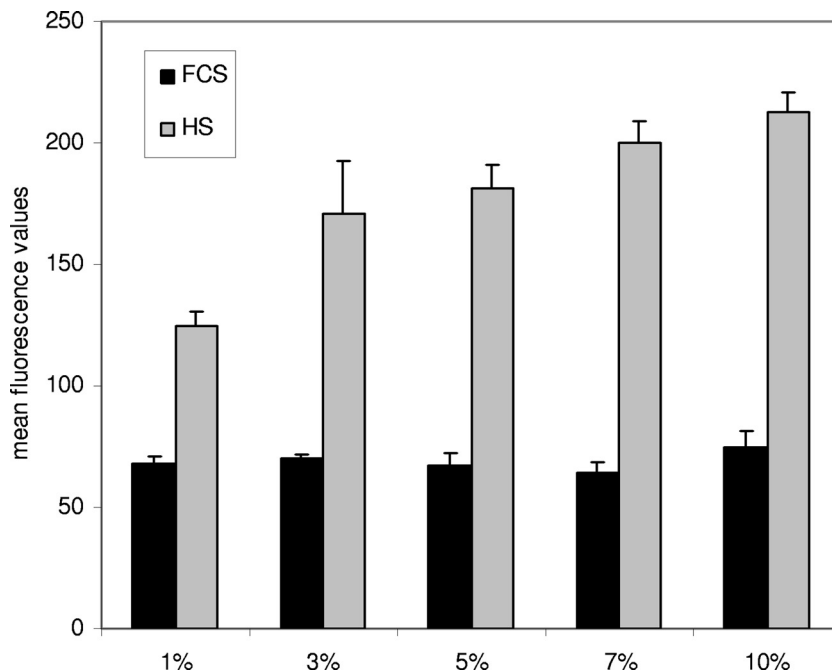


FIG. 7. Dose-dependent induction of C5a peptidase transcription in *S. agalactiae* strain AC475 harboring plasmid pBSU409scpBProm. Bacteria were cultivated overnight at 37°C in THY broth supplemented with human serum in concentrations as indicated. Fetal calf serum served as a control. Shown are mean fluorescence values and standard deviations from five independent experiments as measured by FACS analysis. HS, human serum; FCS, fetal calf serum.

human but not bovine serum seems to support the results of studies showing a very restricted species-specific activity of the C5a peptidase, but we looked at induction of the gene and not the enzymatic activity of the C5a peptidase. Species-specific induction of *scpB* does not, however, extend to the *lmb* gene present on the transposon structure. The expression of *lmb* was not affected by exposure to sera from different hosts, indicating that *scpB* and *lmb* are regulated by different mechanisms, a finding that is consistent with the presence of a putative promoter region directly upstream of *lmb* (37).

Induction of C5a peptidase expression in an *S. agalactiae* *rgfC* knockout mutant. The two-component regulator system *rgf* has been shown to inhibit transcription of C5a peptidase in *S. agalactiae* (36). To investigate whether the induction of the gene by serum is regulated by this system, we transferred the plasmid construct into the *rgfC* mutant strain O90R Δ *rgfC* and tested the resulting strain for *scpB* promoter activity in the presence of serum. The histidine kinase of the *rgf* regulator system is encoded by *rgfC*. As shown by the results in Fig. 4 and 5, the strain displayed an induction of *scpB* in FACS analysis following exposure to human serum that was greater than the induction observed in the wild-type strain. In accordance with the results for the wild-type strain, the induction of *scpB* by human serum was still present after heat inactivation of the serum sample and could be demonstrated for a wide range of different serum concentrations (Fig. 4). These results are consistent with the previously described increased transcription of *scpB* in *rgf* mutants (36), but since the inducing effect of human serum is clearly present in the regulator mutant, *rgf* is most likely not responsible for the regulation of increased *scpB* transcription following exposure to serum.

Promoter activity of *scpB* in the presence of fibronectin and C5a. The C5a peptidase encoded by *scpB* specifically cleaves the human complement component C5a and mediates binding to fibronectin. To test if one or both of these molecules represent the inducing factor in human serum, FACS analysis with the EGFP reporter gene construct was carried out. Strain O90R*scpB*Prom was incubated overnight in regular growth medium (THY) supplemented with 140 μ g/ml fibronectin. The amount of fibronectin added is consistent with the physiological concentration of fibronectin in human serum (28). The reporter gene activity was compared to the activity of the construct after growth in medium without fibronectin supplementation. Strain O90R*scpB*Prom cultured in THY medium supplemented with 33% human serum served as a positive control. The reporter gene activities of the strain grown with or without fibronectin supplementation were, however, indistinguishable (data not shown). In similar experiments, we tested whether C5a stimulates *scpB*. FACS analysis was carried out following supplementation of the culture medium with 10 to 200 ng of C5a per ml. As a positive control, O90R*scpB*Prom was incubated in THY medium supplemented with 33% human serum. Based on the physiological concentrations of C5a in serum (34), this medium contains approximately 4 ng of C5a per ml. Cultures were grown to stationary phase overnight, and the whole experiment was done in triplicate. No induction of *scpB* by C5a supplementation was noted (data not shown), indicating that the *scpB*-inducing factor in human serum is neither fibronectin nor C5a. Together with the experiments using heat-inactivated serum, these experiments were per-

formed to characterize the unknown factor in human serum that is responsible for the induction of *scpB* transcription. C5a and fibronectin both represent interaction partners of the C5a peptidase (1, 36, 20, 39). In particular, the results for C5a are consistent with the fact that the inducing factor is not destroyed by heat. The elucidation of the structure of C5a peptidase led to the hypothesis that a eukaryotic molecule binds to the RGD sites present in C5a as a conformational trigger that is required for efficient binding of *S. agalactiae* to eukaryotic cells (9). It is possible that the putative conformational trigger is present in human serum and represents the same molecule that induces the transcription of the C5a peptidase. In any case, the data from our experiments and the data from the C5a peptidase structure are consistent with the hypothesis that the C5a peptidase is a key molecule in the complex interactions of streptococci with their host. Further biochemical characterization of the signal that is recognized by *S. agalactiae* will be carried out in a separate investigation.

Determination of mRNA levels of *scpB* and *lmb* by real-time RT-PCR. Quantitative real-time RT-PCR experiments were carried out in order to confirm the results of FACS analysis. Whereas FACS analysis was based on the expression of the reporter gene *egfp* in the recombinant *S. agalactiae* strains O90R*scpB*Prom and O90R*lmb*Prom, the LightCycler experiments were based on transcript levels to quantify the mRNA of streptococcal wild-type strain O90R. We investigated the influence of human serum, heat-inactivated human serum, fetal calf serum, and heat-inactivated fetal calf serum on the expression of *scpB* (Fig. 8A) and the influence of human serum and fetal calf serum on the expression of *lmb* (Fig. 8C). Streptococcal wild-type strain O90R was incubated in THY medium and in THY medium supplemented with 10% serum. The cultures were grown to an OD₆₀₀ of 1.0. Total RNA was prepared, and RT-PCRs were carried out as detailed in Materials and Methods. Using the LightCycler, we quantified the amounts of *scpB* and *lmb* mRNA in samples without stimulus and samples that were grown in the presence of different serum stimuli. Each experiment was done in triplicate. Quantitative real-time RT-PCRs revealed an approximately threefold-greater amount of *scpB* mRNA in the presence of human serum and an approximately two- to threefold-greater induction of *scpB* following exposure to heat-inactivated human serum. With fetal calf serum and heat-inactivated fetal calf serum, no differences in the amount of *scpB* mRNA could be detected. Consistent with results from the FACS analysis, no influence of human serum and fetal calf serum on the amount of *lmb* mRNA was found. Taken together, we were able to verify the results of FACS analysis for *scpB* and *lmb* with quantitative real-time RT-PCRs.

Transcription analysis of *scpA* in *Streptococcus pyogenes*. The C5a peptidase gene is highly conserved among β -hemolytic streptococci from the pyogenic group, and the respective genes of *S. agalactiae* and the strictly human pathogen *S. pyogenes* are virtually identical (12). To investigate whether similar induction phenomena following exposure to serum could be observed in other streptococci, *scpA* transcription was measured in *S. pyogenes* by real time RT-PCR analysis. Similar to the results for *S. agalactiae*, human but not bovine serum caused an increase in transcription of the gene. Consistent with the results obtained for *S. agalactiae*, this activation was still present

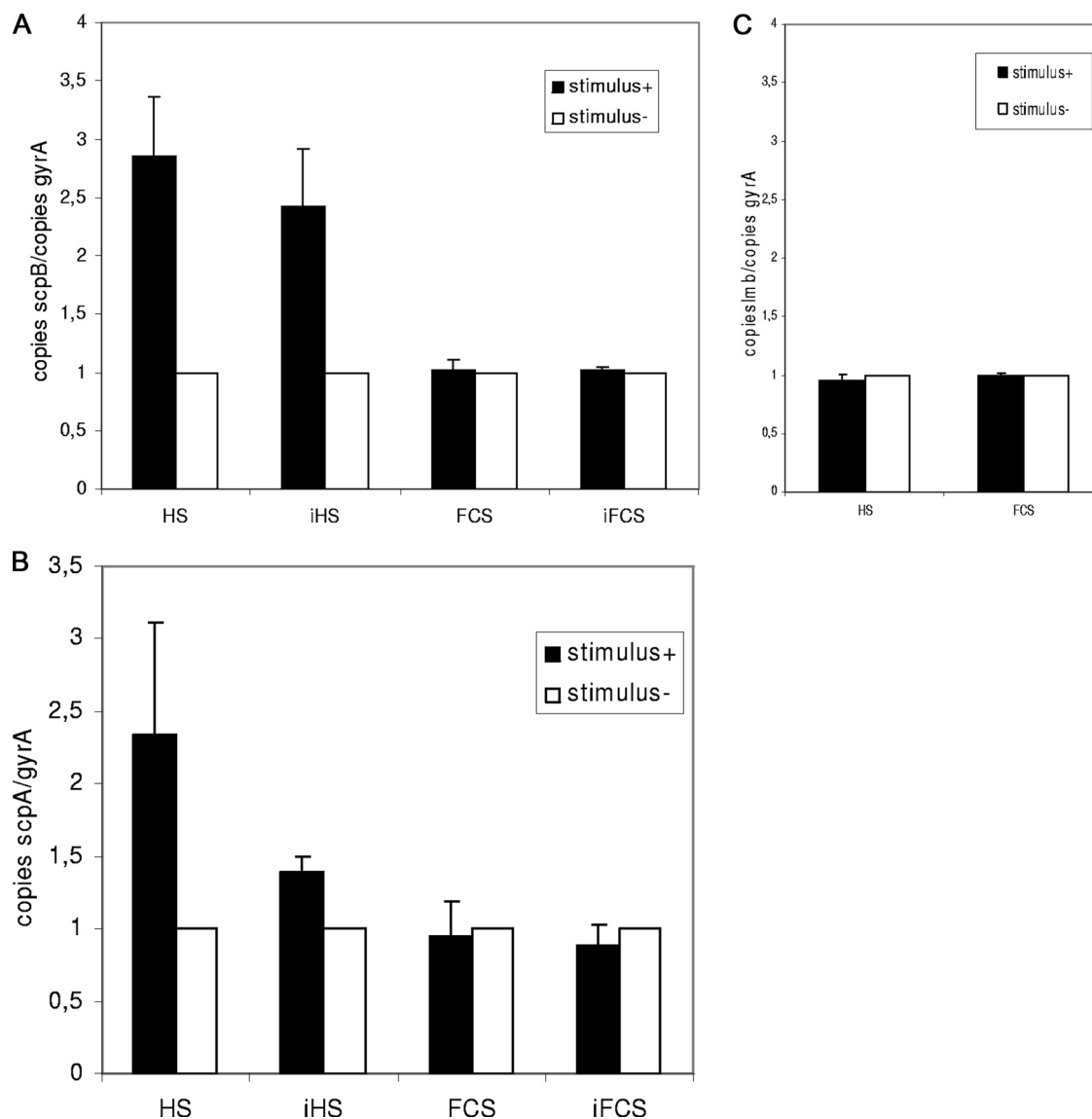


FIG. 8. Quantitative transcript analysis of the *S. agalactiae* virulence genes *scpB* (A) and *lmb* (C) and the *S. pyogenes* gene *scpA* (B) by LightCycler RT-PCR. *ScpB*, *lmb*, and *scpA* transcripts were quantified in relation to the number of *gyrA* transcripts in each sample. Transcription of *scpB*, *lmb*, and *scpA* was determined in samples without stimulus (–) and in samples that were grown in the presence of different serum stimuli (+). Sera were used as indicated. Values from experiments without stimulus were set to 1. Measurements from three separate RNA preparations were used to calculate the means \pm standard deviations. HS, human serum; iHS, heat-inactivated human serum; FCS, fetal calf serum; iFCS, heat-inactivated fetal calf serum.

to some extent after heat inactivation of the pooled human sera that we tested in our experiments (Fig. 8B). In a previous study, proteome analysis of *S. pyogenes* following exposure to human plasma (24) revealed an increase in the expression of C5a peptidase, which is consistent with our data and the hypothesis that the induction of C5a peptidase expression by human serum represents a host-specific adaptation mechanism of human pyogenic streptococcal strains.

Growth of the wild-type strain and *scpB* deletion mutant in human serum. Based on our results showing altered *scpB* expression in the presence of human serum, we were interested in seeing whether the growth of an *scpB* deletion mutant is impaired in THY medium containing human serum. Streptococcal wild-type strain O90R and strain O90R Δ *scpB* Δ *lmb*, with a deletion of the

entire composite transposon structure (17), were used in this experiment. Both strains were cultured in THY medium supplemented with 10% and 50% human serum or fetal calf serum, and growth was monitored over a period of 8 h. No differences between growth kinetics of the two strains could be observed (data not shown). These data suggest that C5a peptidase activity is not necessary for the growth and survival of *S. agalactiae* in the presence of human serum.

In summary, we found a dose-dependent, host-specific induction of the C5a peptidase gene by human but not bovine serum in *S. agalactiae* and *S. pyogenes* bacteria. These results correspond to the absence of *scpB* in many bovine *S. agalactiae* isolates and underline the importance of the gene for human infections.

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