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Analysis of RogB-Controlled Virulence Mechanisms and Gene Expression in *Streptococcus agalactiae*

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Streptococcus agalactiae **is the leading cause of bacterial sepsis and meningitis in neonates and also the causative agent of different serious infections in immunocompromised adults. The wide range of diseases that are caused by *S. agalactiae* suggests regulatory mechanisms that control the formation of specific virulence factors in these bacteria. The present study describes a gene from *S. agalactiae*, designated *rogB*, encoding a protein with significant similarity to members of the RofA-like protein (RALP) family of transcriptional regulators. Disruption of the *rogB* gene in the genome of *S. agalactiae* resulted in mutant strain RGB1, which was impaired in its ability to bind to fibrinogen and fibronectin. Mutant RGB1 also exhibited a reduced adherence to human epithelial cells but did not show an altered invasion of eukaryotic cells. By real-time PCR analysis, mutant RGB1 revealed an increased expression of the *cpsA* gene, encoding a regulator of capsule gene expression. However, strain RGB1 exhibited a reduced expression of the *rogB* gene and of two adjacent genes, encoding putative virulence factors in *S. agalactiae*. Furthermore, mutant RGB1 was impaired in the expression of the *fbsA* gene, coding for a fibrinogen receptor from *S. agalactiae*. The altered gene expression in mutant RGB1 could be restored by plasmid-mediated expression of *rogB*, confirming a RogB deficiency as the cause for the observed changes in virulence gene expression in *S. agalactiae*. Reporter gene studies with a promotorless luciferase gene fused to *fbsA* allowed a growth-dependent analysis of *fbsA* expression in *S. agalactiae*. These reporter gene studies also suggest that RogB exerts a positive effect on *fbsA* expression in *S. agalactiae*.**

Streptococcus agalactiae, also named group B streptococcus (GBS), is the leading cause of bacterial sepsis and meningitis in neonates in many industrialized countries (1). In addition, it is the cause of substantial pregnancy-related morbidity and has emerged as an increasingly common cause of invasive disease in the elderly and in immunocompromised persons (51, 53). The spectrum of diseases caused by GBS in adults ranges from urinary tract and soft tissue infections to life-threatening sepsis and meningitis (40). Besides being the causative agent of many different types of infections, GBS can persistently colonize the human skin and mucous membranes without inducing clinical symptoms (41).

Like many other pathogens, GBS can attach to epithelial surfaces by binding to different host cell proteins. Binding of GBS to human laminin is mediated by the lipoprotein Lmb, which has been studied on the molecular level (43). Although GBS does not bind soluble fibronectin on its surface (4), adherence of the bacteria to immobilized fibronectin has been convincingly demonstrated (45). In a recent study, Beckmann et al. (3) identified C5a peptidase from GBS to mediate binding of the bacteria to fibronectin. In addition, fibronectin binding of GBS has been shown to mediate the invasion of the bacteria into host cells (7). Binding of GBS to human fibrinogen is brought about by the fibrinogen receptor FbsA, which interacts with fibrinogen by repeptive units and which is widely distributed in different GBS strains (39).

Recently, the genomic sequences of the serotype III GBS strain NEM316 (17) and of the serotype V strain 2603 V/R (47) were published. Analysis of the obtained sequence data revealed the presence of several putative virulence genes, including bacterial surface proteins and virulence regulators. Although a few regulatory systems from GBS have been studied on the molecular level (10, 34, 42), the targets and stimuli of most transcriptional regulators from GBS are still unknown.

Pathogenic bacteria often use global regulatory networks to control the expression of different virulence factors in response to changing environmental cues throughout the infection process. In *Streptococcus pyogenes* several regulatory proteins are involved in the transcriptional control of virulence factors. Besides two-component signal transduction systems (14, 21–23, 26) and the multiple gene activator Mga (32), the two regulatory proteins RofA and Nra have been shown to exert a significant effect on the expression of various virulence genes in *S. pyogenes* (2, 15, 16, 19, 25, 28, 31). RofA and Nra exhibit 62% identity to each other and comprise a novel family of transcriptional regulators (16, 19). Analysis of the genome database identified two further RofA homologous proteins in the chromosome of *S. pyogenes* and one in the genome of *Streptococcus pneumoniae* (19). These new members of the RofA-like protein family were named, accordingly, RALPs and are suggested to play a role in the regulatory network of virulence in the two pathogens.

The present study describes a new member of the RALP family of transcriptional regulators from GBS, designated RogB. By insertional inactivation of the *rogB* gene in the chromosome of GBS the importance of RogB for the binding of the bacteria to host cell proteins and the adherence to and invasion into eukaryotic cells was addressed. Using real-time PCR, the expression of known and putative virulence genes was compared between the *rogB* mutant and its parental strain. Finally,

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reporter gene studies addressed the role of RogB for the expression of the *fbsA* gene, encoding a fibrinogen receptor from GBS.

MATERIALS AND METHODS

Bacterial strains, culture conditions, plasmids, and proteins. GBS strain 6313 is a serotype III clinical isolate obtained from an infected neonate and has been described previously (49). GBS 6313 *fbsA-luc* harbors a promotorless luciferase gene, transcriptionally fused to the *fbsA* gene in the chromosome of this strain. The GBS mutants RGB1 and RGB1 *fbsA-luc* are *rogB*::pG$^+$host6 derivates of the GBS strains 6313 and 6313 *fbsA-luc*, respectively, carrying an insertionally inactivated *rogB* gene. *Escherichia coli* DH5α (20) served as host for the GBS pTEX5236 cosmid gene library (35) and for the recombinant pG$^+$host6 and pAT28 plasmids.

The *rogB* gene was isolated from a pTEX5236-based (46) gene bank of GBS 6313 constructed in *E. coli* (35). Plasmid pCR-TOPO (Invitrogen) served for cloning and sequencing of the 350-bp *rogB*-specific PCR product, plasmid pUC18 (50) was used for subcloning the *rogB* encoding region in *E. coli*, and the vector pG$^+$host6 (Appligene) served for the disruption of *rogB* in the genome of GBS 6313. Plasmid pAT28 is an *E. coli-Streptococcus* shuttle vector (48) and served for the construction of the *rogB*-carrying plasmid pAT*rogB* that was used for complementation analysis of GBS strain RGB1. For the construction of plasmid pAT*rogB*, the *rogB* gene was amplified from chromosomal DNA of GBS 6313 by PCR with the primers CCGCGGATCCCAACTCCTATTGTGCCG and CGG CACGAGCTCGTCACTCCATGAATCTCTTG. The *Bam*HI and *Sac*I restriction sites used for cloning are underlined. The *rogB*-containing PCR product and plasmid pAT28 were digested with *Bam*HI and *Sac*I, ligated, and transformed into *E. coli* DH5α. The plasmids pAT28 and pAT*rogB* were subsequently transformed into the GBS strains 6313, RGB1, 6313 *fbsA-luc*, and RGB1 *fbsA-luc*.

GBS was cultivated at 37°C in Todd-Hewitt yeast broth (THY) consisting of Todd-Hewitt broth (Oxoid) supplemented with 1% of yeast extract. GBS strains carrying the pG$^+$host6 plasmid inserted into the chromosomal copy of *rogB* were selected on THY medium containing erythromycin (5 μg/ml). GBS strains carrying the plasmids pAT28 or pAT*rogB* were grown in the presence of spectinomycin (200 μg/ml). *E. coli* was grown at 37°C in Luria broth. Recombinant *E. coli* clones carrying pTEX5236-, pG$^+$host6-, pAT28-, or pUC18-based plasmids were selected in the presence of chloramphenicol (15 μg/ml), ampicillin (50 μg/ml), spectinomycin (100 μg/ml), or ampicillin (100 μg/ml).

Fibrinogen, fibronectin, laminin, collagen I, and collagen IV were purchased from Sigma-Aldrich. Fibrinogen was passed through a gelatin-Sepharose column to remove contaminating fibronectin.

Construction of an *fbsA-luc* transcriptional fusion in GBS. A promotorless luciferase gene (*luc*) was isolated from plasmid pFW11-luc (31) by *Bam*HI/*Hin*dIII digestion. The *luc* box was subsequently ligated into the *Bam*HI/*Hin*dIII-digested vector pG$^+$host6, resulting in plasmid pGluc, in which the luciferase gene is flanked by two multiple cloning sites. Subsequently, the 3′ end of *fbsA* was amplified by PCR from chromosomal GBS DNA by PCR with the primers 5′-CCGCGGATCCGTAGGTCAACTTATAGGG and 5′-CCGCGGATCCAT TATACTTAATTTTCATTGCG. The *Bam*HI restriction sites used for cloning are underlined. After digestion of the obtained PCR fragment and of plasmid pGluc with *Bam*HI, the 3′ *fbsA* fragment was ligated into pGluc and transformed in *E. coli* DH5α. Insert-carrying clones were subsequently sequenced to identify clones with the correct orientation of the 3′ end of *fbsA* in pGluc. The resultant plasmid was termed pGluc*fbsA*3′. The downstream region of *fbsA* was amplified by PCR from chromosomal GBS DNA with the primers 5′-TGGCACAAGCT TCAATCATTTAGTAACTATATATAATG and 5′-GAGCGGGGTACCGTT TCACTTGTTCTATTGG. The *Hin*dIII and *Kpn*I restriction sites used for cloning are underlined. The PCR product and plasmid pGluc*fbsA*3′ were digested with *Hin*dIII and *Kpn*I, ligated, and transformed in *E. coli* DH5α. The resultant plasmid, pGluc-*fbsA*, was transformed in GBS 6313 with subsequent erythromycin selection at 30°C. Cells in which pGluc-*fbsA* had integrated into the chromosome were selected by growth of the transformants at 37°C with erythromycin selection as described previously (27). Four of such integrant strains were serially passaged for 3 days in liquid medium at 30°C without erythromycin selection to facilitate the excision of plasmid pGluc-*fbsA*, leaving the desired promotorless luciferase box in the chromosome. Dilutions of the serially passaged cultures were plated onto agar plates and single colonies were tested for erythromycin sensitivity to identify pGluc-*fbsA* excisants. Chromosomal DNA of GBS 6313 and of 24 erythromycin-sensitive GBS excisants was tested by Southern blotting after *Hin*dIII digestion by using a digoxigenin-labeled *fbsA* gene probe obtained with the primers 5′-GTCCTGTATCTGCTATGGATAGTGTTGG and 5′-ACATTT TGATCATCACCTG.

TABLE 1. Oligonucleotide primers for quantitative reverse transcription-PCR by using a LightCycler

Gene	Sequence (5′-3′)	
	Forward primer	Reverse primer
gyrA	GACGTTCAGGTATTCAC	TCAAACTGAGGTACGACG
cfb	TGAGGCTATTACTAGCGTGG	AAGTCGACAGCATCACACG
lmb	ATGGAAGTCACACAAGGC	ATAGCAGCAACTGAGCCG
sodA	CATCATGATAAGCACCATGC	TGGAGTATCTTGATTGGCAG
cpsA	GGTGATAGTCAAGCTATGG	TCTATCGTTATCGCCTCC
hylA	CCTATTATCCAACGTACCG	GAACCTGTAACTGATAACGG
scpB	AACAGTAGCAGATGACGC	AGCTAGTGCAGCATTACC
lytR	GATGATGAACCAGTTGCACG	TGCCATTGTTGTAGTGAGCC
rogB	GCAGTTGCACAAGATAGTC	TTTGAGAGAGAGTTTCTG
fbsA	GTAGGTCAACTTATAGGG	ATACTTAATTTTCATTGCG
sag1408	TTCGGCACAATAGGAGTTG	CTTAACTTGCCAAGTCTGG
sag1407	TGGTGACTTATGGACG	TGTACCAATACCACCTG

Construction of the GBS *rogB* mutants RGB1 and RGB1 *fbsA-luc*. The thermosensitive plasmid pG$^+$host6 (Appligene) was used for targeted disruption of *rogB* in GBS strains 6313 and 6313 *fbsA-luc* to construct mutants RGB1 and RGB1 *fbsA-luc*, respectively. An internal *rogB* fragment was amplified by PCR with the primers 5′CGCGGATCCATGATTCAGGCAGGTTACC and 5′TGG CACAAGCTTGGAAGTAAGGTAAGCAAG. The *Bam*HI and *Hin*dIII restriction sites used for cloning are underlined. The resulting PCR product and plasmid pG$^+$host6 were digested with *Bam*HI and *Hin*dIII, ligated, and transformed into *E. coli* DH5α. Plasmid pG*rogB* was subsequently transformed into the GBS stains 6313 and 6313 *fbsA-luc* by using the method of Ricci et al. (36), and transformants were selected by growth on erythromycin agar at 30°C. Cells in which the plasmid had integrated into the GBS chromosome were identified by growth at 37°C with erythromycin selection as described previously (27). Successful disruption of *rogB* was confirmed by Southern blotting with *Acc*I-digested chromosomal DNA of the GBS parental strains 6313 and 6313 *fbsA-luc* and their mutants, RGB1 and RGB1 *fbsA-luc*, by using a digoxigenin-labeled *rogB* fragment obtained with the primers 5′-CACTTGGTTGCAATGTTTG and 5′-CTTACTGATAAGCCCGAGG.

Quantification of specific transcripts with LightCycler real-time PCR. GBS strains were grown in 50 ml of THY broth to exponential growth phase (optical density at 600 nm [OD_{600}] = 0.30), and RNA was isolated by using the RNeasy kit (Qiagen) as described previously (35). Contaminating DNA was degraded by digestion with DNase as described elsewhere (18). To exclude the possibility of DNA contamination during RNA preparation, RNA samples were subjected to PCR amplification without prior reverse transcription. However, no amplificates were obtained. Reverse transcription of RNA was performed with random hexanucleotides and the RevertAid First strand cDNA synthesis kit (MBI Fermentas) according to the instructions of the manufacturer. For expression analysis of the genes *cfb*, *lmb*, *sodA*, *cpsA*, *hylB*, *lytR*, *gyrA*, *rogB*, *fbsA*, *sag1478*/*gbs1408*, and *sag1477*/*gbs1407*, the primers listed in Table 1 were used. The temperature profile for template amplification was essentially as described elsewhere (18). In brief, the sample was initially denatured for 1 cycle at 95°C for 30 s, following 44 cycles of denaturation at 95°C for 1 s, annealing at 50°C for 15 s, and extension with fluorescence acquisition at 72°C for 30 s. The temperature transition during the amplification was set to 20°C/s. Melting-curve analysis was performed at between 65 and 95°C with stepwise fluorescence acquisition and a temperature transition of 0.1°C/s. Sequence-specific standard curves were generated by using 10-fold serial dilutions (10^5 to 10^8 copies) of genomic DNA. The quantitiy of cDNA for the investigated genes was normalized to the quantity of *gyrA* cDNA in each sample. The *gyrA* gene was chosen as an internal standard since gyrase genes in streptococci and staphylococci represent ubiquitously expressed housekeeping genes that are frequently used for the normalization of gene expression in quantitative reverse transcription-PCR experiments (6, 18). Each experiment was performed at least four times with two independent RNA preparations.

General DNA techniques. Chromosomal GBS DNA was isolated according to the method of Pospiech and Neumann (33). Conventional techniques for DNA manipulation, such as restriction enzyme digests, PCR, ligation, transformation by electroporation, and Southern blotting, were performed as described by Sambrook et al. (38).

Determination of luciferase activity. For assessment of luciferase activity from *fbsA-luc* transcriptional fusions in GBS, the bacteria were grown aerobically in THY broth at 37°C with shaking. For determining the luminescence of the bacterial culture, 1-ml samples were taken at different time points to determine the OD_{600} of the culture and to measure the luciferase activity. Luciferase activity was measured essentially as described by Podbielski et al. (31). Briefly, 150 μl of bacterial cell suspension was transferred in a sample tube of the Flash'n'Glow luminometer (Berthold). The reservoirs of the luminometer were filled with 2.5× assay buffer (62.5 mM glycyl-glycin [pH 7.8], 25 mM $MgCl_2$) and 330 μM D-luciferin. Then, 200 μl of 2.5× assay buffer and 200 μl of luciferin solution were automatically added to the bacterial suspension in the sample tube, and the luminescence was immediately measured for 15 s at 22°C. The relative light units (RLU) at the different time points were obtained by subtraction of the luminescence at the beginning of the experiment from the luminescence at later time points.

Binding of FITC-labeled GBS to immobilized human matrix proteins. Terasaki microtiter plates were coated with fibronectin, fibrinogen, laminin, or collagens I or IV, and the binding of fluorescein isothiocyanate (FITC)-labeled GBS to the immobilized proteins was measured essentially as described by Podbielski et al. (31). In brief, 10 μl of a 100-μg/ml stock solution of human fibronectin, fibrinogen, laminin, or collagen I or IV was added to each well, followed by incubation overnight at room temperature in a moist chamber. Subsequently, the microtiter plates were washed with phosphate-buffered saline (PBS), and residual buffer was carefully removed. FITC-labeling of GBS was performed with cultures in the exponential (OD_{600} = 0.5) and in the stationary (OD_{600} = 1.5) growth phases. A total of 12 ml of bacterial culture was pelleted by centrifugation, washed with 12 ml of PBS, and resuspended in 2 ml of FITC-solution (1 mg of FITC/ml in 50 mM sodium carbonate buffer [pH 9.2]). After a 20-min incubation in the dark, the cells were pelleted by centrifugation, washed twice with PBS, and sonicated for 20 s to disrupt bacterial chains. The bacterial suspension was adjusted to an OD_{600} of 1.0 with PBS to ensure an equal number of bacteria per volume for the different strains. Subsequently, the suspension was vortexed vigurously and kept in the dark until use. Then, 10 μl of FITC-labeled streptococci were added to each Terasaki well coated with different human proteins. After a 60-min incubation at 37°C, unbound bacteria were removed by five washes with PBS, and bound bacteria were fixed with 0.5% glutaraldehyde for 5 min. The plates were finally washed twice with PBS, and the fluorescence of each well was determined in an automated Cyto-Fluor II fluorescence reader (PerSeptive Biosystems) at excitation and detection wavelengths of 485 and 530 nm, respectively. The efficiency of FITC labeling of the bacteria was determined by incubating 500 μl of the FITC-labeled bacteria for 60 min at 37°C, followed by three washes of the bacteria with PBS, resuspension of the cells in 500 μl of PBS, and measurement of the fluorescence of 10-μl aliquots of the suspension in uncoated Terasaki mitrotiter plates. The amount of bound bacteria per well was calculated as the percentage of total labeled bacteria added to each well. Each assay was measured in triplicate and repeated at least four times.

Binding of soluble ^{125}I-labeled human proteins to GBS. The human proteins fibrinogen, fibronectin, laminin, and collagens I and IV were radiolabeled with ^{125}I by using the chloramine T method (24). Binding of the labeled proteins to GBS was performed essentially as described by Chhatwal et al. (9). Briefly, cultures in the exponential (OD_{600} = 0.5) or stationary (OD_{600} = 1.5) growth phase were pelleted by centrifugation, washed twice with PBS supplemented with 0.02% Tween 20 (PBST), and adjusted photometrically to a transmission of 10% at 600 nm to ensure an equal number of bacteria per volume for the different strains. A total of 0.2 ml of the bacterial suspension was added to 20 μl of ^{125}I-labeled protein (50,000 cpm). After an incubation for 1 h at room temperature, the streptococci were sedimented by centrifugation and washed with 1 ml of PBST. The radioactivity of the pellet was finally measured in a gamma counter (Packard Instruments). The amount of bacterium-bound protein was calculated as the percentage of total radiolabeled protein added to the bacteria. Each experiment was repeated at least three times in triplicate.

Epithelial cell adherence and internalization assay. Adherence of GBS to epithelial cells and invasion into epithelial cells was assayed essentially as described elsewhere (5, 37). Since GBS reveals growth in tissue culture medium, thereby influencing the number of bacteria that can adhere to and invade the host cells, the number of bacteria after growth for 2 h in tissue culture medium was set as the input inoculum as described elsewhere (13). To determine the input inoculum of each strain, 1 ml of RPMI tissue culture medium was inoculated with 5×10^6 bacteria of the different strains, and the total number of bacteria after growth for 2 h was quantitated by plating serial dilutions onto THY agar plates. Since the different strains revealed identical growth in RPMI medium ($\nu = 1.75\ h^{-1}$), the total number of bacteria after growth for 2 h was $5.5 \pm 0.2 \times 10^7$. This value was taken as input inoculum for the different GBS strains and used to determine the multiplicity of infection in the adherence assays.

For adherence and invasion assays, A549 cells were transferred to 24-well tissue culture plates at 4×10^5 cells per well and cultivated overnight in RPMI tissue culture medium (Gibco-BRL) supplemented with 10% of fetal calf serum. The medium was subsequently replaced with 1 ml of fresh RPMI medium.

For adherence assays, the A549 cells were infected with 5×10^6 bacteria, and the infected cells were incubated in RPMI tissue culture medium for 2 h at 37°C, resulting in an input inoculum of $(5.5 \pm 0.2) \times 10^7$ bacteria and a multiplicity of infection of 138:1 (see above). The epithelial cells were subsequently detached from the well by the addition of trypsin-EDTA and lysed by the addition of 300 μl of distilled water. The number of cell-adherent bacteria was determined by plating appropriate dilutions of the lysate onto THY agar plates. Due to the lysis of the eukaryotic cells in this approach, the calculation of cell-adherent bacteria also included bacteria that had invaded the host cells. Therefore, the number of invasive bacteria was subtracted from the obtained numbers of cell-adherent bacteria to calculate the actual number of adherent bacteria. To determine the number of adherent bacteria per eukaryotic cell, the number of cell-adherent bacteria was divided by the number of A549 cells per assay.

For invasion assays, the epithelial cells were infected with 5×10^6 streptococci, incubated for 2 h at 37°C, and washed three times with PBS. Subsequently, the infected cells were incubated for 2 h in tissue culture medium supplemented with penicilling G (10 U) and streptomycin (0.01 mg) to kill extracellular bacteria. After three washes with PBS, the epithelial cells were detached by the addition of trypsin-EDTA and lysed in 300 μl of distilled water. The amount of invasive bacteria was quantitated by plating serial dilutions of the lysate onto THY agar plates. The invasion index (13) was calculated as follows: (number of invasive/number of adherent bacteria) × 100%. Each experiment was repeated at least three times in triplicate.

Nucleotide sequence accession number. The nucleotide sequence of the *rogB* encoding region from *S. agalactiae* was submitted to the EMBL nucleotide sequence database and was assigned accession no. AJ279088.

RESULTS

Isolation and characterization of the *rogB* gene from GBS. In an approach to identify virulence-associated transcriptional regulators in GBS before the genome sequence of GBS had been published, RofA-like protein (RALP) sequences from different origins, including two newly identified RALPs in the unfinished genomic sequence of *Streptococcus equi* (http://www.sanger.ac.uk/) were aligned, resulting in the identification of two regions with high similarity, i.e., the region from amino acids 146 to 154 and from amino acids 256 to 260 in the RofA sequence from *S. pyogenes* (Fig. 1). Based on the two conserved peptide sequences, the degenerate primers rog1 (GAG TATCGWATWCGWTWYYT) and rog2 (ATAWATWARA AAWADRTARTC) were synthesized and used for PCR with chromosomal DNA from GBS, resulting in the amplification of a fragment of 350 bp. The PCR product was cloned in the pCR-TOPO vector, and the insert from one clone was subsequently sequenced. Analysis of the obtained sequence revealed an incomplete open reading frame (ORF); the deduced amino acid sequence of this ORF exhibited significant similarity to RALP sequences (not shown). This result indicated that GBS possesses a RALP that was termed regulator of group B streptococcus (RogB). To isolate the entire *rogB* gene from GBS, the 350-bp PCR product was used as a digoxigenin-labeled probe to screen a GBS cosmid library in *E. coli*. Of 723 cosmid clones, 6 hybridized to the 350-bp *rogB* probe. One of the cosmids was partially digested, and fragments ranging in size between 2 to 4 kb were ligated in the *E. coli* plasmid pUC18. The digoxigenin-labeled *rogB* probe was subsequently used to identify clones carrying the *rogB* gene in pUC18, and the 2.4-kb insert of one subclone was finally sequenced. Analysis of the obtained sequence revealed one complete ORF and one in-

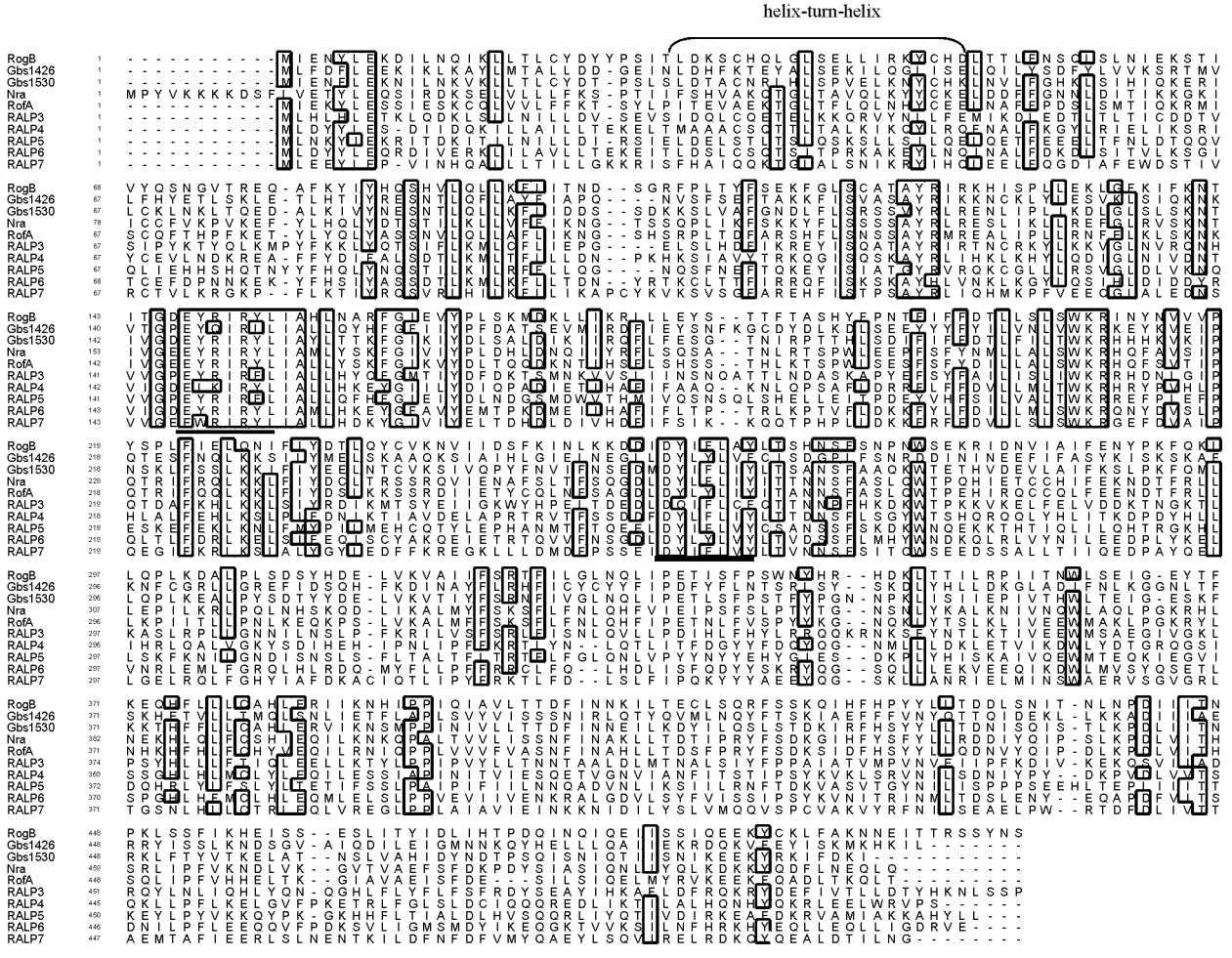

FIG. 1. Alignment of the amino acid sequence of RogB from GBS with RofA, Nra, RALP3, and RALP4 from *S. pyogenes*, RALP5 from *S. pneumoniae*, RALP6 and RALP7 from *S. equi*, and the RALP encoding ORFs Gbs1426 and Gbs1530 in the genome of GBS NEM316. Identical amino acid residues between at least 7 of the 10 aligned proteins are boxed. The putative helix-turn-helix motif is indicated by a bracket. Underlined are two highly conserved regions within the aligned proteins that were used for the design of degenerate primers to isolate the *rogB* gene from GBS 6313.

complete ORF (Fig. 2). Within the complete ORF the entire sequence of the *rogB*-specific 350-bp PCR product could be identified. This ORF was thus designated *rogB*. Inspection of the *rogB* gene identified two potential start codons: one at bp 373 (ATG) and the other at bp 398 (TTG). The translational start of the *rogB* gene is postulated to be located at bp 398 because of the presence of a reasonable ribosome-binding site (GAGAGAGA) in front of this start codon. Using bp 398 as the translational start, the *rogB* gene has a size of 1,427 bp and encodes a protein of 509 amino acids, with a predicted molecular mass of 60,261 Da. In front of the *rogB* gene, several A- and T-containing stretches could be identified that are typical for noncoding regions in GBS. The incomplete ORF extends from the end of the sequenced fragment at bp 2398 to bp 1980 and codes for a polypeptide of 137 amino acids. Computer-assisted database analysis of its deduced polypeptide revealed significant similarity to galactosyltransferases from other organisms. The respective ORF was therefore designated *galT*. Between the two ORFs, at positions 1926 to 1987, a potential rho-independent terminator structure ($\Delta G = -42.2$ kcal) could be identified, indicating transcriptional termination of the two ORFs at this site.

Analysis of the genome sequences of the meanwhile sequenced GBS strains NEM316 and 2603 R/V identified gene *gbs1479* in the genome of strain NEM316 and gene *sag1409* in the genome of strain 2603 V/R to encode the RogB protein. In both genomes the *rogB* gene is followed by an antiparallel-oriented gene, encoding a putative galactosyltransferase. Interestingly, the *rogB* gene is preceded in both genome sequences by two genes encoding putative cell surface proteins (Fig. 2). In the genome of GBS strain NEM316 these ORFs were designated Gbs1478 and Gbs1477, and in the genome of strain 2603 V/R these ORFs were termed Sag1408 and Sag1407. Although the exact function of these ORFs is unknown, their deduced polypeptides reveal similarity to fibronectin- and collagen-binding proteins from streptococci and staphylococci and, therefore, these ORFs represent putative virulence factors from GBS. For further analysis, we adapted the gene designation of GBS strain NEM316 for these genes, i.e., they were termed *gbs1478* and *gbs1477*.

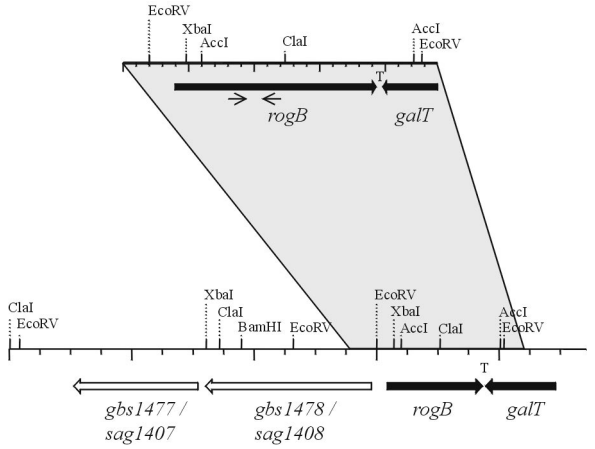

FIG. 2. Restriction map of the *rogB*-encoding region in GBS. The upper restriction map displays the RogB-encoding region in GBS 6313, while the lower restriction map shows the respective region in the genomes of GBS NEM316 and GBS 2603 V/R, respectively. The shaded area marks identical regions in the genome of the different strains. Black arrows indicate the positions of the *rogB* (starting at bp 398) and the *galT* gene, and a "T" represents the proposed transcriptional terminator between the two genes. The light arrows below the *rogB* gene indicate the location of the primers used for amplification of the partial *rogB* sequence from GBS 6313. Open arrows represent the genes *gbs1477*/*sag1407* and *gbs1478*/*sag1408* in the genomes of GBS NEM316 and GBS 2603 V/R, respectively.

RogB is similar to other RALPs. Basic local alignment search tool (BLAST) databank analysis of the deduced RogB protein revealed 50.2, 49.9, 36.2, and 24.8% identities to the RALP transcriptional regulators RofA, Nra, RALP3, and RALP4, respectively, from *S. pyogenes*; 25.0 and 24.9% identities to RALP6 and RALP7, respectively, from *S. equi*; and 22.9% identity to RALP5 from *S. pneumoniae* (Fig. 1). In the genome sequence of GBS NEM316 and GBS 2603 R/V, two additional putative RALP-encoding ORFs were identified. In the genome of GBS NEM316 the deduced amino acid sequence from the genes *gbs1426* and *gbs1530* exhibited 36.6 and 52.0% identities, respectively, to RogB. The deduced polypeptide from gene *sag1356* in GBS 2603 R/V reveals 36.6% identity to RogB. In GBS 2603 R/V, the *sag1463* gene is highly identical to gene *gbs1530* from NEM316; however, *sag1463* from 2603 R/V carries a nonsense mutation at bp 621, resulting in the permature termination of protein synthesis. The similarity of RogB to the above-listed proteins ranged from 42 to 84%. Using the method of Dodd and Egan (12), a putative helix-turn-helix DNA-binding motif could be identified in the N-terminal region of RogB (Fig. 1). The location of the helix-turn-helix motif in RogB corresponds to the respective motifs in the other RALPs (16, 19, 31). The homology data and the putative DNA-binding motif suggest that RogB is a transcriptional regulator in GBS.

Disruption of *rogB* impairs the binding of GBS to host proteins. In *S. pyogenes*, the transcriptional regulators RofA and Nra control the interaction of the bacteria with extracellular matrix (ECM) proteins. To analyze the importance of RogB for the binding of GBS to human proteins, the *rogB* gene was insertionally inactivated in GBS 6313, resulting in GBS mutant RGB1. Southern blot analysis confirmed the successful disruption of the *rogB* gene in the chromosome of RGB1 (data not shown). Since the *rogB* gene is followed by a strong rho-independent terminator and the *galT* gene, which is oriented in opposite direction to *rogB*, disruption of *rogB* in mutant RGB1 does not exert a polar effect on the expression of downstream genes. No difference in the growth rate and final optical cell density between GBS RGB1 and its parental strain was observed (data not shown). To address the influence of RogB on the formation of putative adhesins in GBS, the ability of the GBS strains 6313 and RGB1 to bind to soluble or immobilized ECM proteins was quantitated. By using cells from the exponential (OD_{600} = 0.5) and the stationary (OD_{600} = 1.5) growth phases, the importance of the growth phase on the adhesive properties of GBS was analyzed. Both GBS strains revealed no binding of soluble or immobilized collagen I, collagen IV, or laminin and no binding of soluble fibronectin (not shown). However, GBS 6313 and GBS RGB1 accumulated soluble fibrinogen on their surface and bound to immobilized fibrinogen and fibronectin, respectively (Fig. 3). As shown in Fig. 3, both strains revealed a growth-phase dependency in their binding to fibrinogen and fibronectin, i.e., in exponentially growing cells the interaction with fibrinogen or fibronectin was increased by ca. 30 and 20%, respectively. However, compared to the parental strain, the GBS mutant RGB1 exhibited a 45% reduced binding to soluble and immobilized fibrinogen (Fig. 3A and B). Similarly, the binding of GBS mutant RGB1 to immobilized fibronectin was reduced by ca. 25% (Fig. 3C). The introduction of the vector pAT28 into the GBS strains 6313 or RGB1 had no influence on the different binding of the two strains to fibrinogen and fibronectin (data not shown). However, pAT28-mediated expression of *rogB* in strain RGB1 pAT*rogB* restored its growth-phase-dependent binding to fibrinogen and fibronectin to the wild-type level (Fig. 3). These data indicate that binding of GBS to human fibrinogen and fibronectin is regulated by RogB and the growth phase of the bacteria.

RogB is required for efficient eukaryotic cell adherence. A decreased interaction of a microorganism with ECM proteins often impairs its capability to adhere to and to invade host cells. Therefore, the GBS strains 6313, RGB1, and RGB1 pAT*rogB* were tested for their adherence to and invasion of the human epithelial cell line A549. Adherent and invasive bacteria were quantitated by plate viability counts. As depicted in Fig. 4A, the GBS wild-type strain 6313 revealed an adherence of 14.8 ± 1.1 bacteria per A549 cell. In contrast, the adherence of mutant RGB1 was reduced by 46% compared to the wild-type strain. However, plasmid-mediated expression of *rogB* in GBS strain RGB1 pAT*rogB* restored its adherence to A549 cells to the wild-type level (Fig. 4A). These data suggest that the *rogB* gene is involved in the binding of GBS to epithelial cells.

To compare the invasion of the different GBS strains into A549 cells, the number of invasive bacteria of each strain was related to the number of adherent bacteria, resulting in the calculation of the invasion index (13). As depicted in Fig. 4B, GBS strains 6313, RGB1, and RGB1 pAT*rogB* revealed similar invasion indices, indicating that *rogB* is not involved in the invasion of epithelial cells by GBS.

Expression profiling of virulence genes in GBS 6313 and GBS RGB1. The previous results suggested a significant effect

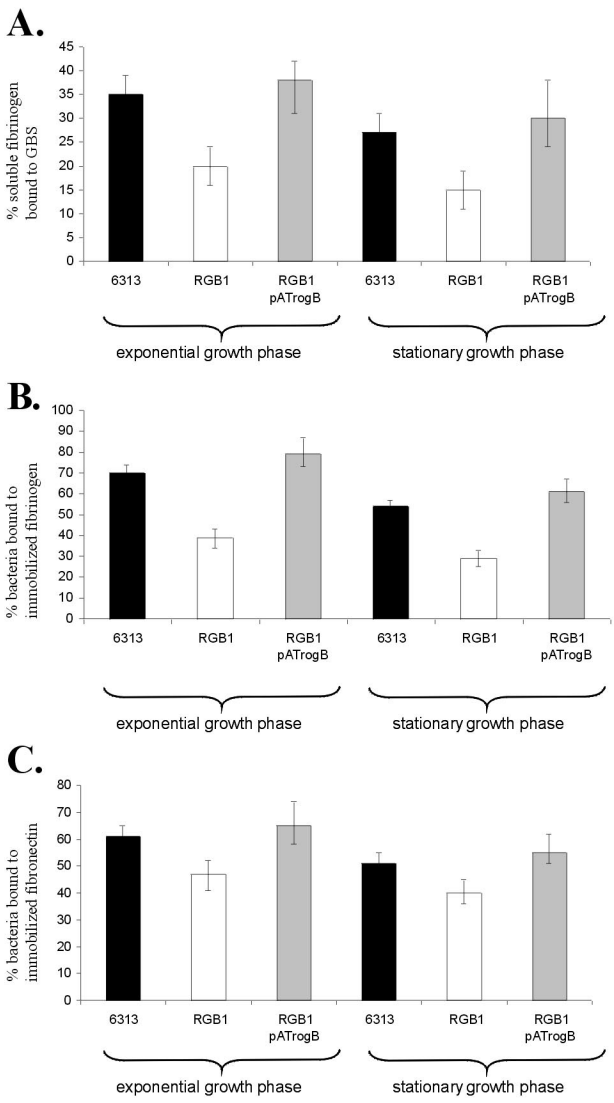

FIG. 3. Binding of soluble fibrinogen to GBS and attachment of GBS to immobilized fibrinogen and fibronectin, respectively. The assays were performed with GBS cells from the exponential and from the stationary growth phase. The ordinate represents the mean binding of total ^{125}I-labeled soluble fibrinogen to the GBS strains 6313, RGB1, and RGB1 pAT*rogB*, respectively (A), or the mean binding of the total bacteria of the GBS strains 6313, RGB1, or RGB1 pAT*rogB* to immobilized fibrinogen (B) or fibronectin (C). Each assay was performed at least four times in triplicate.

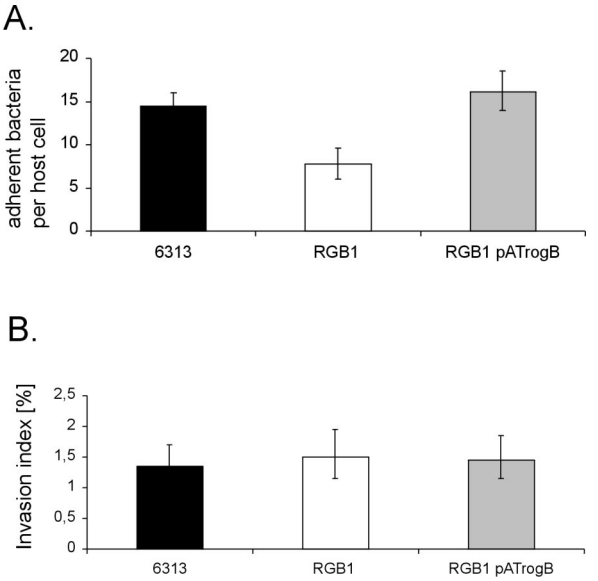

FIG. 4. Eukaryotic cell adherence of the GBS strains 6313, RGB1, and RGB1 pAT*rogB* and determination of the invasion indices of these strains. (A) The adherence of the different strains to A549 cells is given as number of adherent bacteria per eukaryotic cell. (B) The invasion index was obtained by relating the number of invasive bacteria to the number of adherent bacteria. Each experiment was performed at least three times in triplicate.

of RogB on virulence mechanisms in GBS. Since in *S. pyogenes* members of the RALP family control a variety of virulence genes at the transcriptional level, the expression profile of important virulence genes was analyzed by real-time PCR in GBS RGB1 and its parental strain 6313. Expression profiling was performed in a LightCycler with the *cfb*, *lmb*, *sodA*, *cpsA*, *hylB*, *scpB*, *lytR*, *gyrA*, *rogB*, and *fbsA* genes encoding CAMP factor, the laminin-binding protein Lmb, superoxide dismutase, an activator of capsule gene expression, hyaluronate lyase, C5a peptidase, an autolysin response regulator, gyrase subunit A, RogB regulator protein and fibrinogen receptor FbsA, respectively. Also compared in the two GBS strains was the expression profile of the genes *gbs1478* and *gbs1477*, preceding *rogB*, and encoding putative virulence factors from GBS. Equal amounts of total RNA from exponential-phase (OD_{600} = 0.3) cultures of GBS 6313 and GBS RGB1 were reverse transcribed and used to quantitate the transcript levels of the above-mentioned genes by real-time PCR. The obtained data were normalized to the expression of the *gyrA* gene in the two strains. A twofold difference in transcription was interpreted as a significant difference in expression between the two strains. Relative to GBS 6313, transcription of the genes *cfb*, *lmb*, *sodA*, *hylB*, *scpB*, and *lytR* was unaltered in the *rogB* mutant RGB1. However, expression of the *cpsA* gene was (4.78 ± 0.55)-fold increased in mutant RGB1 compared to its parental strain. In contrast, the transcription levels of the genes *rogB*, *fbsA*, *gbs1478*, and *gbs1477* were (3.29 ± 0.51)-, (3.13 ± 0.34)-, (3.35 ± 0.47)-, and (2.74 ± 0.49)-fold higher in GBS 6313 than in GBS RGB1. This result suggests that RogB exerts a negative effect on the transcription of *cpsA* in GBS and that it activates the expression of the adjacent genes *gbs1478* and *gbs1477*, and that of *fbsA*, encoding a fibrinogen receptor from GBS. In addition, the *rogB* gene appears to be autoregulated by the RogB protein. However, disruption of *rogB* may also decrease the stability of the truncated *rogB* transcript.

Plasmid-mediated expression of *rogB* in strain RGB1 pAT*rogB* resulted in (0.76 ± 0.44)-, (0.18 ± 0.36)-, and (3.23 ± 0.59)-fold-increased expression of the genes *rogB*, *fbsA*, and *gbs1477*, respectively, compared to the GBS wild-type strain 6313. The expression of the genes *gbs1478* and *cpsA* was reduced (0.62 ± 21) and (0.13 ± 0.67)-fold, respectively, in strain RGB1 pAT*rogB* compared to the wild-type strain. This finding shows that in strain RGB1 pAT*rogB* the expression of the genes *rogB*, *fbsA*, *gbs1478*, and *cpsA* is restored to about the

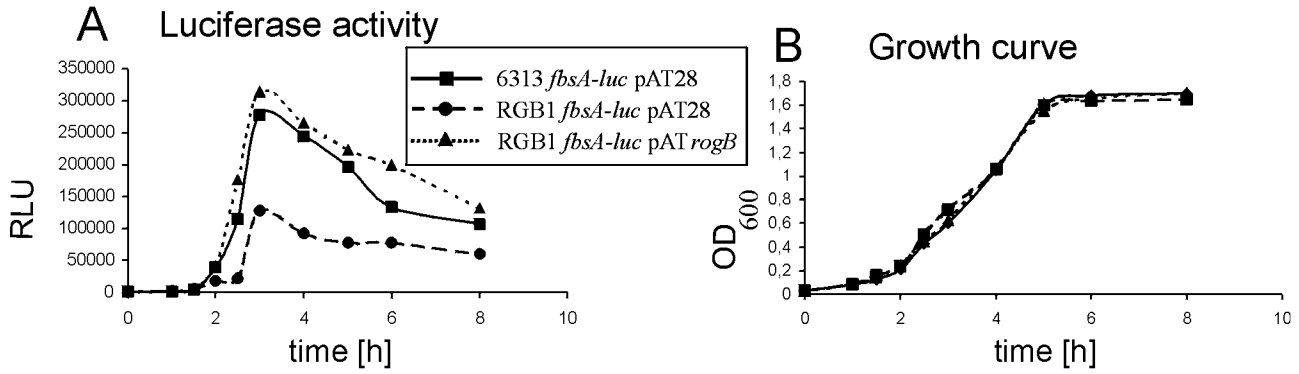

FIG. 5. Growth curve and expression profile of an *fbsA*-luciferase transcriptional fusion in the GBS strains 6313 *fbsA-luc* pAT28, RGB1 *fbsA-luc* pAT28, and RGB1 *fbsA-luc* pAT*rogB*. The bacteria were grown aerobically in THY liquid medium, and at different time points samples were withdrawn for the determination of the luciferase activity (A) and the OD_{600} (B) of the culture. Luciferase activity is expressed in RLU.

wild-type level. However, plasmid-mediated expression of *rogB* appears to result in an elevated expression of the gene *gbs1477*.

Expression of *fbsA* is controlled by RogB and the growth phase of GBS. Among the genes that were shown to be under transcriptional control of RogB, the *fbsA* gene represents the best-studied virulence factor from GBS. Therefore, the influence of RogB on *fbsA* expression was characterized in more detail by transcriptionally fusing the *fbsA* gene in the chromosome of GBS with a promotorless luciferase gene. After two recombination events, a luciferase cassette without vector sequences was placed behind the *fbsA* gene in the chromosome of GBS 6313. In the resultant GBS strain, 6313 *fbsA-luc*, expression of *fbsA* can be directly quantitated by measuring its luciferase activity. Correct insertion of the reporter gene into the genome of GBS 6313 was confirmed by Southern blot hybridization (data not shown). To analyze the importance of *rogB* in controlling the expression of *fbsA* in GBS, the *rogB* gene was insertionally inactivated in the chromosome of GBS 6313 *fbsA-luc*, and the mutant strain was termed RGB1 *fbsA-luc*. Southern blot analysis confirmed the successful disruption of the *rogB* gene in the chromosome of mutant RGB1 *fbsA-luc* (results not shown). For complementation studies, the GBS strains 6313 *fbsA-luc* and RGB1 *fbsA-luc* were transformed with the vector pAT28, and strain RGB1 *fbsA-luc* was transformed with the *rogB*-carrying plasmid pAT*rogB*. The synthesis of luciferase was subsequently measured in the resultant GBS strains during growth of the bacteria in complex media, and the results were plotted as RLU and OD_{600}, respectively, against time. As shown in Fig. 5B, the three strains exhibited identical growth behavior in complex medium. In all three strains, transcription of *fbsA* increased significantly during the early exponential growth phase, peaked in the middle of exponential growth, and decreased moderately at the transition from the exponential to the stationary growth phase (Fig. 5A). However, expression of *fbsA* in mutant RGB1 *fbsA-luc* pAT28 was on average 50% lower than in strain 6313 *fbsA-luc* pAT28. Plasmid-mediated expression of *rogB* in strain RGB1 *fbsA-luc* pAT*rogB* increased the *fbsA* transcription to values comparable to those of the wild-type strain 6313 *fbsA-luc* pAT28. These findings confirm our results obtained by real-time PCR and show that the presence of RogB stimulates the transcription of the *fbsA* gene in GBS.

DISCUSSION

GBS adapts to and survives at different locations within the human host by sensing the changing surroundings and regulating the expression of virulence genes in response to environmental signals. In the present study, a putative regulatory protein, RogB, was identified and characterized that reveals significant similarity to members of the RALP family of transcriptional regulators (19), among which RofA and Nra from *S. pyogenes* have been extensively studied at the molecular level (2, 15, 16, 25, 28). Insertional inactivation of *rogB* in the chromosome of GBS resulted in a reduced binding of the bacteria to soluble fibrinogen and to immobilized fibrinogen and fibronectin, respectively. This indicates that RogB stimulates the attachment of GBS to ECM and plasma proteins. Also, the regulator RofA from *S. pyogenes* has been shown to enhance the binding of the bacteria to human fibronectin and fibrinogen (15, 16, 25). However, the Nra protein acts predominantly as a repressor of virulence gene transcription and downregulates binding of *S. pyogenes* to type I collagen and fibronectin (31).

The interaction of bacteria with ECM proteins is frequently a prerequisite for the successful colonization of the human host. Interestingly, GBS mutant RGB1 was significantly impaired in its attachment to the human epithelial cell line A549. Also, the disruption of *rofA* in *S. pyogenes* M6 decreased the ability of the bacteria to attach to epithelial cells (2), whereas the inactivation of *nra* in *S. pyogenes* M49 resulted in an increased binding of the mutant to host cells (28). In *S. pyogenes*, binding of the bacteria to fibronectin has been shown to mediate bacterial adherence to and invasion into eukaryotic cells (29, 30, 44). Since RofA stimulates and Nra downregulates fibronectin binding in *S. pyogenes*, the different adherence properties of the *rofA* and *nra* mutants were attributed to alterations in their ability to interact with human fibronectin (2, 28). Recently, fibronectin binding of GBS was shown not to play a role in the adherence of the bacteria to epithelial cells (7). This indicates that the decreased adherence of GBS mu-

tant RGB1 to epithelial cells was not caused by its reduced binding to human fibronectin. Although binding of GBS to fibrinogen has been shown to protect the bacteria against opsonophagocytosis (8, 39), the interaction of GBS with fibrinogen may also play a role in the adherence to epithelial cells. It can therefore be speculated that the impaired binding of GBS RGB1 to human fibrinogen resulted in the reduced adherence to human cells. Alternatively, RogB may stimulate in GBS the synthesis of further adhesins, which have not yet been identified.

Real-time PCR analysis revealed a RogB-dependent effect on the expression of known and putative virulence genes in GBS. The presence of *rogB* was shown to stimulate the expression of the genes *gbs1478* and *gbs1477*, which are located upstream of *rogB* in antiparallel orientation, and to exert a positive effect on transcription or RNA stability of the *rogB* gene. In *S. pyogenes* the RALP-like transcriptional regulators RofA and Nra have been shown to be autoregulated and to control the expression of their upstream located genes *cpa* and *prtF*, respectively (2, 15, 16, 19, 31). The gene products of the genes *prtF* and *cpa*, encoding the fibronectin-binding protein F and the collagen-binding protein Cpa, represent important virulence factors in *S. pyogenes*. In analogy to the similar genetic organization and regulation of the *nra*/*rofA* region in *S. pyogenes* and that of *rogB* in GBS, the genes *gbs1478* and *gbs1477* represent interesting putative GBS virulence genes, whose role for the virulence of the bacteria is currently under investigation.

C5a peptidase is an important virulence factor on the surface of GBS. This protease specifically cleaves the chemotactic complement component C5a, thereby interfering with the recruitment of granulocytes to the site of infection (11, 52). Recently, C5a peptidase from GBS was shown to mediate binding of the bacteria to immobilized fibronectin (3) and to promote internalization of the bacteria into host cells (7). Although disruption of *rogB* resulted in a reduced binding of mutant RGB1 to human fibronectin, real-time PCR revealed no effect of RogB on the expression of the C5a peptidase encoding gene *scpB* in GBS, and there was no difference between the internalization of mutant RGB1 and its parental strain in eukaryotic cells. These findings suggest that RogB controls in GBS 6313 the synthesis of a fibronectin-binding protein that is distinct from C5a peptidase. The presence of several fibronectin-binding proteins has already been suggested by Beckmann et al. (3).

Recently, it was demonstrated that the FbsA protein represents the major fibrinogen receptor in GBS 6313 (39). The reduced binding of mutant RGB1 to human fibrinogen suggests that it is impaired in the synthesis of the FbsA protein. We were able to demonstrate, by real-time PCR and by reporter gene studies, that the disruption of *rogB* indeed decreased the expression of *fbsA* in mutant RGB1 by ca. 50%. These findings indicate that RogB has a prominent effect on the expression of *fbsA* in GBS.

Spellerberg et al. (42) described for the GBS strain O90R a putative quorum-sensing system that consists of the genes *rgfBDAC*. Disruption of *rgfC* in GBS O90R caused an altered fibrinogen binding of the mutant depending on the bacterial cell density. Since the fibrinogen binding of GBS O90R is exclusively mediated by FbsA (39; unpublished results), it can be speculated that in GBS O90R the *fbsA* gene is under the transcriptional control of the *rgfBDAC* quorum-sensing system. Interestingly, neither GBS 6313 (B. Spellerberg, unpublished data) nor GBS NEM316 (17) carry a functional *rgfBDAC* gene locus. This suggests that *fbsA* expression is controlled by several regulatory circuits depending on the genetic background of the GBS strain.

In summary, we have identified and characterized a novel regulatory gene from GBS, termed *rogB*, which is involved in the expresssion control of known and putative virulence genes in these bacteria. Like other members of the RALP family of transcriptional regulators, RogB appears to regulate the interaction of GBS with its human host. Understanding the mode of action of RogB in GBS will contribute significantly to unravel the virulence mechanisms employed by GBS at different sites in the human body.

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