Rga is a regulator of adherence and pilus formation in *Streptococcus agalactiae*

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Streptococcus agalactiae is the leading cause of bacterial sepsis and meningitis in neonates and is also the causative agent of several serious infections in immunocompromised adults. *S. agalactiae* encounters multiple niches during an infection, suggesting that regulatory mechanisms control the expression of specific virulence factors in this bacterium. The present study describes the functional characterization of a gene from *S. agalactiae*, designated *rga*, which encodes a protein with significant similarity to members of the RofA-like protein (RALP) family of transcriptional regulators. After deletion of the *rga* gene in the genome of *S. agalactiae*, the mutant strain exhibited significantly reduced expression of the genes *srr-1* and *pilA*, which encode a serine-rich repeat surface glycoprotein and a pilus protein, respectively, and moderately increased expression of the *fbsA* gene, which encodes a fibrinogen-binding protein. Electrophoretic mobility shift assays demonstrated specific DNA binding of purified Rga to the promoter regions of *pilA* and *fbsA*, suggesting that Rga directly controls *pilA* and *fbsA*. Adherence assays revealed significantly reduced binding of the Δ *rga* mutant to epithelial HEP-2 cells and to immobilized human keratin 4, respectively. In contrast, the adherence of the Δ *rga* mutant to A549 cells and its binding to human fibrinogen was significantly increased. Immunoblot and immunoelectron microscopy revealed that the quantity of pilus structures was significantly reduced in the Δ *rga* mutant compared with the parental strain. The wild-type phenotype could be restored by plasmid-mediated expression of *rga*, demonstrating that the mutant phenotypes resulted from a loss of Rga function.

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INTRODUCTION

Streptococcus agalactiae, also named group B *Streptococcus*, is the major cause of bacterial sepsis and meningitis in neonates and has emerged as an increasingly common cause of invasive diseases in immunocompromised people (Balter *et al.*, 2000). It is also a commensal bacterium that asymptotically colonizes the gastrointestinal and urogenital tracts of women (Balter *et al.*, 2000). Neonates acquire *S. agalactiae* from colonized mothers by aspiration of infected amniotic fluid or vaginal secretions during birth (Spellerberg, 2000). Furthermore, *S. agalactiae* colonizes the mammary glands of ruminants where it can trigger an inflammation of the gland, thereby causing mastitis (Keefe, 1997).

S. agalactiae is thus able to colonize multiple hosts and to adapt within the host to different body sites, including

those that are typically sterile. The ability to colonize multiple and diverse environments requires the bacterium to express multiple sets of adaptation genes, which are involved in virulence and thereby cause disease. Several regulators of expression of virulence genes have been identified in *S. agalactiae*. The two-component system CovS/CovR is a global regulatory system that controls the expression of up to 139 genes (Jiang *et al.*, 2005; Lamy *et al.*, 2004). It also modulates the activity of multiple factors that contribute to virulence such as haemolysis, the CAMP reaction and adherence to epithelial cells. In addition, deletion of the *covSR* locus leads to a diminished virulence in an animal model (Jiang *et al.*, 2005; Lamy *et al.*, 2004). The regulatory activity of CovR is modulated not only by its cognate sensor CovS but also by the target of the kinase Stk1, which forms a eukaryotic-type serine/threonine kinase together with Stp1 (Rajagopal *et al.*, 2003, 2006). Another global regulator in *S. agalactiae* is RovS, which is a member of the Rgg family of regulatory proteins (Samen *et al.*, 2006). This protein regulates, in addition to its own expression, the expression of the fibrinogen

Abbreviations: EMSA, electrophoretic mobility shift assay; RALP, RofA-like protein; RT-PCR, real-time PCR.

The GenBank/EMBL/DDBJ accession number of the genomic DNA sequence of *S. agalactiae* NEM316 is AL732656.

receptor and adhesin FbsA, the superoxide dismutase and the β -haemolysin/cytolysin (Samen *et al.*, 2006). Furthermore, RovS was also shown to regulate the expression of RogB (Samen *et al.*, 2006), another regulator of virulence traits in *S. agalactiae* (Gutekunst *et al.*, 2003). The regulator RogB controls the expression of *cpsA*, a regulator of capsule synthesis, *pilA* (*gbs1478*) and *pilB* (*gbs1477*), encoding pilus proteins, and *fbsA* (Drams *et al.*, 2006; Gutekunst *et al.*, 2003). Moreover, its regulatory activity influences bacterial attachment to fibronectin (Gutekunst *et al.*, 2003). RogB is a member of the RofA-like protein (RALP) family of transcriptional regulators. Members of this family were intensively investigated in *Streptococcus pyogenes* and were demonstrated to control bacterial interactions with host cells, avoidance of host cell damage and balanced virulence factor expression during the stationary phase of growth (Beckert *et al.*, 2001; Kreikemeyer *et al.*, 2003; Kwinn *et al.*, 2007; Molinari *et al.*, 2001; Podbielski *et al.*, 1999). RALP proteins typically bear two helix–turn–helix DNA-binding motifs at their N terminus, which is in line with data showing the ability of RofA and of a further RALP protein (RlrA) to bind to the promoter region of their target genes (Granok *et al.*, 2000; Hava *et al.*, 2003). Regulators of the RALP family have been described to act as activators and/or repressors of gene expression (Kreikemeyer *et al.*, 2003).

Besides RogB, the genome of *S. agalactiae* NEM316 encodes two members of the RALP family: GBS1426 and Rga (regulation of glycosylated adhesin) (Glaser *et al.*, 2002). In a previous study, a Δ rga deletion mutant was shown to exhibit a decrease in *srrI* transcription and SrrI display on the surface of *S. agalactiae* cells (Mistou *et al.*, 2009). In the present study we present evidence for a regulatory function of Rga in adherence and in pilus formation.

METHODS

Bacterial strains, eukaryotic cells and growth conditions.

S. agalactiae NEM316 (ATCC12403) is a serotype III strain responsible for a fatal case of septicaemia (Glaser *et al.*, 2002). *Escherichia coli* DH5 α was used for cloning purposes, and *E. coli* BL21(λ DE3) served as host for the production of fusion proteins. *S. agalactiae* was cultivated at 37 °C in Todd–Hewitt broth (THB) or Todd–Hewitt yeast broth (THY) containing 1% yeast extract. *S. agalactiae* clones carrying the plasmid pG⁺host6 (Appligene) or pAT18 (Trieu-Cuot *et al.*, 1991) were selected with erythromycin (5 μ g ml⁻¹). *E. coli* was grown at 37 °C in Luria–Bertani (LB) broth, and strains carrying the plasmids pG⁺host6, pAT18 or pET28a were selected by supplementing the media with ampicillin (100 μ g ml⁻¹), erythromycin (300 μ g ml⁻¹) or kanamycin (50 μ g ml⁻¹), respectively.

The cell line HEP-2 (ATCC CCL-23) was obtained from the American type Culture Collection (ATCC). HEP-2 is a human epidermoid carcinoma cell line of the larynx. HEP-2 cells were propagated in minimal essential medium (MEM; Gibco-BRL) supplemented with 10% fetal calf serum (FCS). The cell line A549 (ATCC CCL-185) was obtained from the ATCC. This human lung carcinoma cell line exhibits characteristics typical of type II alveolar pneumocytes. A549

cells were propagated in RPMI medium (Gibco-BRL) supplemented with 10% FCS. Tissue cultures were incubated in a humid atmosphere at 37 °C with 5% CO₂.

Antibodies and human proteins. For the generation of anti-PilB antibodies, affinity-purified hexahistidyl-tagged PilB fusion protein was size-separated by SDS-PAGE and blotted onto nitrocellulose. After staining with Ponceau S, the PilB-containing band was cut out, the nitrocellulose membrane was dissolved in DMSO, and the solution was used for the immunization of mice. Immunization consisted of two subcutaneous applications of the purified protein within 2 weeks. Serum was collected 4 weeks after immunization. Animal experiments were performed according to the procedures approved by Regierungspräsidium Tübingen, Referat 35, Konrad-Adenauer-Straße 20, D-72072 Tübingen, Germany. Goat anti-mouse IgG Fab fragments coupled with 10 nm gold particles, and human fibrinogen were purchased from Sigma Aldrich.

General DNA techniques. Chromosomal DNA of *S. agalactiae* was isolated according to the method described by Pospiech & Neumann (1995). DNA manipulations were performed according to standard protocols (Sambrook & Russell, 2001). Oligonucleotides used in this study are listed in Table 1 and were purchased from MWG Biotech.

Construction of an rga deletion mutant. The *rga* gene was deleted from the chromosome of *S. agalactiae* NEM316 according to a method described previously (Samen *et al.*, 2004). In brief, two DNA fragments flanking the region to be deleted were amplified by PCR from the genome of *S. agalactiae* NEM316. The resulting PCR products were mixed in equal amounts with each other and subjected to a crossover PCR, resulting in one PCR product. The crossover PCR product and the thermosensitive vector pG⁺host6 were digested with appropriate enzymes, ligated and used to transform *E. coli* DH5 α . The resulting plasmid, pG⁺ Δ rga, was introduced into *S. agalactiae* NEM316 by electroporation, and two recombination events flanking the region of the chromosome to be deleted were subsequently selected. Successful deletion was confirmed by Southern blotting with *Pvu*II-digested chromosomal DNA of the *S. agalactiae* parental strain and the deletion mutant.

Plasmid-mediated expression of rga in S. agalactiae. The *rga* structural gene, including its own promoter, was amplified from chromosomal DNA of *S. agalactiae* NEM316 by PCR, and cloned in the *E. coli*/*Streptococcus* shuttle vector pAT18 (Trieu-Cuot *et al.*, 1991). The resulting plasmid pATrga and the vector pAT18 were introduced into *S. agalactiae* by electroporation.

RNA preparation and quantitative real-time (RT)-PCR. The amount of target gene mRNA was quantified as described previously (Samen *et al.*, 2006). Briefly, *S. agalactiae* was grown to mid-exponential phase (OD₆₀₀ 0.3) in THY. Total RNA was prepared from the bacterial culture by using the RNeasy midi extraction kit (Qiagen) and treated with RNase-free DNase (Promega). Quantitative RT-PCR experiments were performed after reverse transcription of RNA with random hexanucleotides and the RevertAid first-strand cDNA synthesis kit (MBI Fermentas) according to the manufacturer's instructions. Expression analysis of target genes was performed with the oligonucleotides listed in Table 1 or described previously (Gutekunst *et al.*, 2003; Samen *et al.*, 2006) with a LightCycler (Gutekunst *et al.*, 2003). The amount of the investigated genes cDNA was normalized to the amount of *gyrA* cDNA in each sample. The expression of the different genes was calculated as the rate of transcript copy compared with the wild-type. Each experiment was performed at least four times with two independent RNA preparations. A twofold difference in mRNA quantity was interpreted as a significant difference in expression between the strains.

Table 1. Oligonucleotides used in this work

Restriction enzyme sites used for cloning are underlined. Complementary DNA sequences between primers are marked in *italics*.

Purpose	Sequence (5'–3')
Genes analysed by quantitative RT-PCR	
<i>gbs1356</i>	GGTTCAAACGACCTCATC
<i>gbs1356</i>	GTGTGTTACGGTCACATG
<i>gbs1143</i>	GTAGCGGTGCATCCAGTAG
<i>gbs1143</i>	CCTGTATATCCATTCCC
<i>gbs2008 (bibA)</i>	GTCCATCAACAGCTCGTG
<i>gbs2008 (bibA)</i>	GTCAGATAGGACACCTGC
<i>gbs1263</i>	CTCCTATCGTACTATCCTTCC
<i>gbs1263</i>	GATAGGTAGTTAGAAGCTCTC
<i>fbsB</i>	GCTTCTGTAGGCTTCAGG
<i>fbsB</i>	CCGTATTGAATCTTAGTTCCTC
<i>gbs1919</i>	CGAAAGCAGTCATTCCATTG
<i>gbs1919</i>	TAGCAGAAAGTATGCACC
<i>srr-1</i>	GTGCTTTGCCATGGTTGGCAAGCAGTTAACAG
<i>srr-1</i>	ACACTTCTGAGGCTGACG
Construction of Δrga mutant	
	CCGTGGATCCTGCTCTCCCTTTAATCGC
	CCCAACAGGATAACATAAACAGGTATCTAAGGATAGTGAGG
	TGTTTATGTTATCCTGTTGGGTACACCCTCTCAAATCAG
	TGGCACAAGCTTTATGGATGAAACCGCTCG
Complementation of Δrga mutant	
	CCGTGGATCCTGCTCTCCCTTTAATCGC
	AAACTGCAGTATGGATGAAACCGCTCG
EMSA	
Promoter region of <i>srr-1</i>	GTAACTGCTTGCCAAAA
	GAGGGGGTATCATAACATAA
Promoter region of <i>pilA</i>	GTATTTGCGACAAACAAAA
	TATTGATATCATAATAAGTGA

Heterologous production and purification of PilB, Rga and K4 fusion proteins. The vector pET28a (Novagen) was used for the synthesis of hexahistidyl-tagged Rga and PilB fusion proteins. The Rga- and PilB-encoding regions were amplified from chromosomal DNA of *S. agalactiae* NEM316 by PCR. After digestion of the PCR product and of plasmid pET28a with appropriate restriction enzymes, the resulting DNA fragments were mixed, ligated and subsequently used to transform *E. coli*. Production and purification of hexahistidyl-tagged fusion protein were performed as described previously (Schubert *et al.*, 2002). The hexahistidyl-tagged C terminus of human keratin 4 was produced and purified as described previously (Samen *et al.*, 2007).

DNA electrophoretic mobility shift assay (EMSA). The binding of the His-tagged Rga protein to promoter regions was tested by DNA EMSA according to a method described previously (Samen *et al.*, 2006).

Binding of FITC-labelled *S. agalactiae* to immobilized proteins. Binding of FITC-labelled bacteria to immobilized proteins was quantified as described previously (Gutekunst *et al.*, 2003). The amount of bound bacteria was calculated as the percentage of total labelled bacteria added to each well.

Adherence assays. Adherence of *S. agalactiae* to HEp-2 and A549 epithelial cells was assayed as described previously (Gutekunst *et al.*, 2003). The data were expressed as the percentage of adherent bacteria

based on the original inoculum. Each experiment was repeated at least three times in triplicate.

SDS-PAGE and immunoblot. Total cell extracts of *S. agalactiae* were obtained as described previously (Rosini *et al.*, 2006). Cell extracts were separated on 3–8 % NuPage Novex pre-cast gradient gels (Invitrogen). Gels were either silver-stained for direct visualization of pilus structures or wet blotted onto a nitrocellulose membrane for immunoblot analysis as described previously (Rosini *et al.*, 2006). Detection of pilus proteins by immunoblot was performed as described previously (Samen *et al.*, 2006). Densitometry analyses were performed using the Bio-profil software.

Immunoelectron microscopy. Pilus structures were observed by immuno-labelling as described previously (Rosini *et al.*, 2006) with the following modification. After incubation with the secondary antibody, the grids were negatively stained with 2 % uranyl acetate and air-dried. The grids were examined using a Zeiss EM 10 transmission electron microscope.

Sequence analysis. Sequence data were analysed using BLAST and PSI-BLAST (Altschul *et al.*, 1990, 1997). GenBank and CDD (Marchler-Bauer *et al.*, 2005) were consulted.

Statistical analysis. The statistical difference in the adherence of the wild-type and the mutant strain was determined by a two-tailed *t*-test analysis using Microsoft Excel 2002.

RESULTS

Sequence analysis of Rga

BLASTP searches revealed that Rga (GBS1530) from *S. agalactiae* NEM316 had an identity of 53 and 40 %, respectively, to the transcriptional regulators RogB of *S. agalactiae* and RofA of *S. pyogenes*, which both belong to the RALP family of transcriptional regulators. Analysis of the genome sequences of the *S. agalactiae* strains A909 and 2603V/R, identified several nucleotide substitutions and deletions in their *rga* alleles, resulting in a frameshift inactivation of the gene. Whereas in these two strains the *rga* gene appears to be a pseudogene, an intact copy of *rga* can be identified in the genome of the *S. agalactiae* strains H36B (ZP_00782298), 18RS21 (ZP_00779907) and 515 (ZP_00790293), respectively. These findings imply that Rga has a role as a transcriptional regulator in several virulent *S. agalactiae* strains that carry an apparently functional *rga* gene.

Rga regulates the expression of *srr-1*, *pilA* and *fbsA*

Since proteins in the RALP family are reported to be global regulators of virulence factors (Kreikemeyer *et al.*, 2003), we analysed the influence of Rga on the expression of a set of virulence genes. For this purpose, the *rga* gene was deleted in the genome of *S. agalactiae* NEM316, resulting in the mutant Δ *rga*. No difference was observed in the growth rate, final cell density, chain length or clumping between the *S. agalactiae* Δ *rga* mutant and the wild-type strain (data not shown). A complementation strain was constructed by introducing a plasmid-encoded *rga* gene with its own promoter into the mutant Δ *rga*. To compare the wild-type and the mutant strain with the complementation strain, the wild-type and the Δ *rga* mutant were transformed with the empty vector pAT18, resulting in the strains NEM316_pAT18 and Δ *rga*_pAT18, respectively. To investigate the role of Rga for control of virulence gene expression, we quantified in the *S. agalactiae* strains NEM316_pAT18, Δ *rga*_pAT18 and Δ *rga*_pAT*rga* the relative amount of mRNA of 21 genes previously annotated as coding for virulence factors (Table 2), including *rogB* which encodes a RofA-like regulator of the RALP family that is located next to *pilA* in an opposite orientation (Dramsi *et al.*, 2006).

Relative to strain NEM_pAT18, transcription of most of the analysed genes was unaltered in the Δ *rga*_pAT18 mutant (data not shown). However, in the latter, the mRNA level of the *srr-1* gene was fivefold lower, the mRNA level of *pilA* was 24-fold lower, and the mRNA level of the *fbsA* gene was 1.5-fold higher compared with the parental strain NEM_pAT18 (Fig. 1). In the complemented strain Δ *rga*_pAT*rga* the transcript levels of *srr-1*, *pilA* and *fbsA* were similar to that of the parental strain, indicating that the observed effect was caused by the deletion of the *rga* gene. These results indicate that Rga activates the expression of the genes *srr-1* and *pilA*, and that it represses the expression of *fbsA* in *S. agalactiae*.

Table 2. Genes analysed by quantitative RT-PCR

Gene name	Product
<i>fbsA</i>	Fibrinogen binding adhesin FbsA
<i>srr-1</i>	Keratin 4 binding adhesin Srr-1
<i>pilA</i>	Pilus protein PilA
<i>cfb</i>	CAMP factor
<i>lmb</i>	Laminin binding protein Lmb
<i>sodA</i>	Superoxide dismutase
<i>cpsA</i>	Activator of capsule gene expression
<i>hylB</i>	Hyaluronate lyase
<i>scpB</i>	C5a peptidase
<i>lytR</i>	Autolysin response regulator
<i>rovS</i>	RovS regulator
<i>cylE</i>	Haemolysin production factor
<i>fbsB</i>	Fibrinogen receptor FbsB
<i>gbs0851</i>	Putative secreted protein
<i>gbs0628</i>	Putative pili protein
<i>bibA</i> (<i>gbs2008</i>)	Adhesin BibA
<i>rogB</i>	RogB regulator
<i>gbs1143</i>	Putative surface protein with LPXTG motif
<i>gbs1919</i>	Putative surface protein with LPXTG motif
<i>gbs1263</i>	Putative surface protein with LPXTG motif
<i>gbs1356</i>	Putative surface protein with LPXTG motif

Binding of Rga to the promoter region of the target genes

To characterize the DNA-binding activity of Rga at the molecular level, EMSAs were performed with purified Rga protein and the promoter regions of the genes *srr-1*, *pilA* and *fbsA*. The DNA fragments were tested for gel retardation with increasing amounts of Rga protein. As a negative control, the promoter region of *cpsA*, which did not reveal transcriptional regulation by Rga in quantitative RT-PCR analysis, was used and was not retarded with the Rga protein (Fig. 1b). As depicted in Fig. 1(c), purified Rga did not specifically retard the migration of a DNA fragment containing the promoter region of *srr-1*, whereas increasing amounts of Rga retarded the migration of both the *pilA* and the *fbsA* promoter region (Fig. 1d and e). Incubation of the tested DNA fragments with BSA did not result in retardation (Fig. 1b–e). These results show that Rga specifically binds to the promoter region of *pilA* and *fbsA*, respectively.

Role of *rga* in adherence to epithelial cells

Srr-1, PilA and FbsA were previously reported to be involved in the bacterial adherence to host cells (Maisey *et al.*, 2007; Samen *et al.*, 2007; Schubert *et al.*, 2004). In particular, it was previously demonstrated that Srr-1 is involved in the adherence of *S. agalactiae* to the human larynx cell line HEP-2, and that FbsA mediates the binding to human pulmonary cell line A549 (Samen *et al.*, 2007; Schubert *et al.*, 2004). Therefore, the ability of the three strains NEM316_pAT18, Δ *rga*_pAT18 and Δ *rga*_pAT*rga* to

Fig. 1. (a) Expression analysis of the genes *fbsA*, *srr-1* and *pilA* in *S. agalactiae* NEM316_pAT18 (black bars), Δ rga_pAT18 (white bars) and Δ rga_pATrga (grey bars). The amount of mRNA of the different genes was measured by quantitative RT-PCR in a LightCycler and normalized to the expression of the *gyrA* gene. (b–e) EMSAs using purified Rga protein and DNA fragments containing the promoter regions of *cpsA* (b), *srr-1* (c), *pilA* (d) and *fbsA* (e).

adhere to these cell lines was investigated. As depicted in Fig. 2(a), Δ rga_pAT18 exhibited a 50 % lower adherence to HEP-2 cells compared with the parental strain. In addition, the adherence of the complemented strain Δ rga_pATrga to HEP-2 cells was comparable to the wild-type strain NEM316_pAT18. These findings demonstrate that Rga has a positive influence on the adherence of *S. agalactiae* to the epithelial cell line HEP-2.

In contrast, adherence of the mutant strain Δ rga_pAT18 to the lung epithelial cell line A549 was increased compared with the wild-type, while the adherence of the complemented strain was similar to that of the wild-type. This shows that Rga has a negative influence on the adherence of *S. agalactiae* to the epithelial cell line A549.

The results obtained demonstrate that Rga enhances the binding of *S. agalactiae* to HEP-2 cells while it inhibits the bacterial binding to A549 cells. These opposite observations imply that Rga activates the expression of adhesin genes involved in the binding to HEP-2 cells, and that it represses the expression of adhesin genes required for the binding to A549 cells.

Deletion of *rga* influences bacterial adherence to immobilized keratin 4 and fibrinogen

The Srr-1 protein has previously been demonstrated to mediate bacterial adherence to HEP-2 cells, and also to bind to the C terminus of human keratin 4 (Samen *et al.*, 2007). Therefore, the importance of Rga for the binding of *S. agalactiae* to the C terminus of human keratin 4 was quantified (Fig. 2b). Compared with the parental strain, the *S. agalactiae* mutant Δ rga_pAT18 revealed a 90 % reduced binding to the immobilized C terminus of human keratin 4. Plasmid-mediated expression of *rga* in strain Δ rga_pATrga restored the binding to the C terminus of human keratin 4 to wild-type level. These data demonstrate that Rga has a positive effect on the attachment of *S. agalactiae* to immobilized human keratin 4.

The FbsA protein has been shown to play a major role in the bacterial binding to human A549 cells, and also to bind human fibrinogen (Schubert *et al.*, 2002, 2004). Thus, fibrinogen binding was quantified for the Δ rga mutant. Compared with the parental *S. agalactiae* strain, the mutant Δ rga_pAT18 showed a 40 % increased binding to human fibrinogen. Fibrinogen binding of the complemented strain Δ rga_pATrga was comparable to that of the parental strain. These findings show that Rga has a negative effect on the binding of *S. agalactiae* to human fibrinogen.

Importance of Rga for pili formation

PilA of *S. agalactiae* is a component of pili structures on the surface of the bacteria (Dramsi *et al.*, 2006; Maisey *et al.*, 2007; Rosini *et al.*, 2006). Following SDS-PAGE with gradient polyacrylamide gels, pilus structures form a ladder of bands corresponding to high-molecular-mass proteins (Dramsi *et al.*, 2006; Gianfaldoni *et al.*, 2007; Lauer *et al.*, 2005; Mora *et al.*, 2005; Rosini *et al.*, 2006). Since Rga activates the expression of *pilA*, protein extracts of the three strains NEM316_pAT18, Δ rga_pAT18 and Δ rga_pATrga were size-separated by SDS-PAGE, and subsequently visualized by silver staining. As shown in Fig. 3(a), neither the crude extract of the parental strain NEM316_pAT18 nor that of the mutant Δ rga_pAT18 exhibited a high-molecular mass ladder of bands. Interestingly, the crude extract of the

Fig. 2. Adherence of *S. agalactiae* NEM316_pAT18, Δrga _pAT18 and Δrga _pATrga to the epithelial cell lines Hep-2 and A549 (a) and immobilized human keratin 4 (K4) and human fibrinogen (b). (a) The epithelial cell lines HEp-2 and A549 were infected with equal amounts of each strain, and the number of cell-adherent bacteria was determined relative to the number of input bacteria. Asterisks indicate a significant difference in the adherence abilities of the indicated strains ($P < 0.005$). (b) Bacterial attachment to immobilized human keratin 4 or human fibrinogen was quantified with FITC-labelled bacteria. The y-axes represent the percentage of bacteria bound to immobilized human keratin 4 or human fibrinogen in relation to the number of input bacteria. Asterisks indicate a significant ($P < 0.05$) difference in the binding abilities of the indicated strains. Each assay was performed at least four times in triplicate; bars, SD.

complemented strain Δrga _pATrga reveals a set of high-molecular-mass bands, indicating an increased amount of pilus structures on the surface of this strain, which was probably due to the use of a multi-copy plasmid for complementation. To further investigate the role of Rga in pilus production, the amount of the pilus backbone protein, PilB, was quantified by immunoblotting in the different strains. Prior to this analysis, we determined by reverse-transcription PCR that *pilA* and *pilB* are co-transcribed (data not shown). Subsequently, PilB was overproduced in *E. coli* as a hexahistidyl-tagged protein and, after purification, was used to immunogenize mice. In immunoblots with PilB antibodies, a weak signal was obtained with the extract of the mutant strain Δrga _pAT18 (Fig. 3b). In contrast, an intense ladder of bands was detected in the extracts of both the wild-type and the complemented strain. Moreover, these extracts exhibited an intense band of approximately 80 kDa, which corresponds with the size of the PilB monomer (Dramsı *et al.*, 2006). Bands of higher molecular mass have previously been shown to be covalently linked polymers of PilB (Dramsı *et al.*, 2006). When comparing the band intensities of the different strains, an extract of about 25 μ g of mutant Δrga _pAT18 corresponded in its signal intensity to that of a 1 μ g extract of wild-type NEM316_pAT18. This suggests that the *rga* mutant contains about 25-fold fewer pilus structures on its surface than the wild-type. In contrast, the signal intensity of the 1 μ g extract of the complemented strain Δrga _pATrga corresponded to that of about 5 μ g of the extract from strain NEM316_pAT18. These findings demonstrate that pilus formation was reduced in the Δrga mutant and restored in the complementation strain.

The presence of pili was also investigated by immunoelectron microscopy. Pili on the surface of the different strains

were detected with mouse anti-PilB antibodies, followed by goat anti-mouse IgG coupled to gold particles. As shown in Fig. 3(c), gold particles bound abundantly to pili structures on the surface of the wild-type strain NEM316_pAT18. In contrast, almost no gold particles could be observed on the surface of mutant Δrga _pAT18, indicating severely impaired pilus production in this strain. In the complemented strain Δrga _pATrga, numerous pilus structures could be detected, showing that an extrachromosomal copy of *rga* restores pilus production. Overall, these results demonstrate that Rga is crucial for pilus production in *S. agalactiae*.

DISCUSSION

S. agalactiae is both a commensal and a pathogenic bacterium in humans and dairy cattle, and is adapted to colonizing various environmental niches. The versatility in adaptation requires genes to be differentially regulated so that the physiological and structural states of the bacterium fit the characteristics of a given environment. This study reports the role of the transcriptional regulator Rga in the expression control of the genes *srr-1*, *pilA* and *fbsA*, encoding a keratin-binding adhesin, a structural subunit of pili and a fibrinogen-binding adhesin, respectively.

Quantitative RT-PCR experiments targeting 21 putative virulence genes revealed that a deletion in the *rga* gene severely impaired the expression of *srr-1*. This is consistent with previous results showing that Rga activates the expression of Srr-1 (Mistou *et al.*, 2009). In addition to Srr-1, we showed that *pilA* expression was decreased in the *rga* deletion mutant. Furthermore, the *rga* mutant was also

Fig. 3. Pilus analysis in *S. agalactiae* NEM316_pAT18, Δrga_pAT18 and Δrga_pATrga. (a) SDS-PAGE of total cell extracts followed by silver staining. Lanes: 1, NEM316_pAT18; 2, Δrga_pAT18; 3, Δrga_pATrga. Approximately 25 μg protein was loaded on the gel. (b) Immunoblot analysis was performed following SDS-PAGE of total cell extracts. Anti-PilB polyclonal mouse antibody was used to probe the pilus structural protein. Densitometry values are given below the Western blot. The arrowheads indicate the bands that were used for the measurements, and the data were calculated by taking the band of the wild-type (lane 25) as 100% (*). The saturated band is indicated by **. (c) Immunogold transmission electron microscopy of pilus structure on the surface of *S. agalactiae* cells. Labelling was performed with anti-PilB polyclonal mouse antibody followed by 10 nm colloidal gold-conjugated anti-mouse IgG antibody.

impaired in adherence to HEp-2 cells, in binding to immobilized human keratin 4, and in the formation of pili on the surface of *S. agalactiae* cells. The *srr-1* gene encodes a glycosylated serine-rich repeat surface protein with an LPXTG motif involved in adherence to epithelial HEp-2 cells, and mediating the bacterial binding to human keratin 4 (Mistou *et al.*, 2009; Samen *et al.*, 2007). Keratin 4 is a constituent of the intermediate filaments, the principal structural elements of the cytoskeleton in eukaryotic cells (Fuchs & Cleveland, 1998). The *pilA* gene is the first gene of the *pil* operon encoding a pilus apparatus, which is a multisubunit hairlike structure that extends from the bacterial cell surface (Telford *et al.*, 2006). In *S. agalactiae*, these structures are involved in adherence to host tissues (Dramsi *et al.*, 2006; Maisey *et al.*, 2007). As Rga activates

the transcription of both *srr-1* and *pilA*, and thereby increases the amount of Srr-1 and pilus proteins on the surface of the bacteria, it is consistent that Rga significantly increases bacterial adherence to some host cells.

The deletion of *rga* also resulted in increased binding of the bacteria to the human lung epithelial cell line A549 and to immobilized fibrinogen. In addition, quantitative RT-PCR showed moderately increased expression of *fbxA* in the *rga* mutant, and EMSA showed specific binding of Rga to the *fbxA* promoter region. Taken together, our findings show that Rga controls *fbxA* expression and FbsA-mediated adherence to fibrinogen and to the human cell line A549. As A549 cells do not possess keratin 4 (Moll *et al.*, 1982), Srr-1-mediated binding does not play a role in the adhesion of *S. agalactiae* to this cell line (unpublished data). This implies that *S. agalactiae* uses a different mechanism to adhere to A549 cells. In fact, both pili and the FbsA protein have been demonstrated to mediate binding to A549 cells (Schubert *et al.*, 2004; Dramsi *et al.*, 2006). While transcription of the *pilA* gene was reduced in the *rga* mutant, that of *fbxA* was increased. Therefore, pilus-mediated adherence should be decreased while FbsA-mediated adherence should be increased. As adherence to A549 cells was significantly increased in the *rga* mutant, it can be speculated that the reduced pilus-mediated adherence in this strain was more than compensated for by the increased expression of *fbxA*.

Like the other members of the RALP family, Rga is predicted to exhibit two successive helix–turn–helix motifs at its N terminus, which is consistent with a DNA-binding function. EMSAs showed that Rga specifically binds to the promoter region of *pilA* and *fbxA*. This suggests that Rga acts as a transcriptional activator of *pilA* and as a repressor of *fbxA* by interacting physically with their promoter regions. On the other hand, no binding was observed when Rga was incubated with the promoter region of *srr-1*, suggesting that Rga has an indirect effect on *srr-1* expression.

The amino acid sequences of RogB and Rga exhibit 53% identity and 73% similarity. RogB was shown to regulate the expression of *pilA*, *cpsA*, *fbxA* and its own expression (Gutekunst *et al.*, 2003). When testing the wild-type strain and the *rga* deletion mutant by quantitative RT-PCR, the transcript levels of the genes *cpsA* and *rogB* did not differ significantly between the analysed strains. This suggests that despite the high sequence identity of the two regulators, they bind to different targets and consequently control different virulence genes. This would be similar to other RALP regulators such as RofA and Nra which have distinct regulons (Kreikemeyer *et al.*, 2003).

Pilus structures have recently been intensively investigated in streptococci, including *S. agalactiae*. Although important data have been published on their biogenesis and their function (Abbot *et al.*, 2007; Dramsi *et al.*, 2006; Gianfaldoni *et al.*, 2007; Lauer *et al.*, 2005; Maisey *et al.*, 2007; Manetti *et al.*, 2007; Mora *et al.*, 2005; Rosini *et al.*, 2006; Scott & Zähler, 2006; Telford *et al.*, 2006), little is known about the

regulation of their synthesis. Besides RogB (Dramsi *et al.*, 2006; Gutekunst *et al.*, 2003) SrtA has been described as a regulator of pilus biosynthesis (Dramsi *et al.*, 2006). However, the biological significance of pilus biosynthesis regulation in streptococci is only poorly understood. In *Neisseria meningitidis*, adherence to epithelial cells is a multistep mechanism. The early step of adherence involves pilus structures that establish the initial specific physical contact with epithelial cells. Once this contact is established, pilus synthesis is repressed and other genes encoding factors involved in intimate adhesion with epithelial cells are induced (Ofek *et al.*, 2003). A similar mechanism underlying the regulation of pili could be predicted for *S. agalactiae*. Alternatively, or in addition, the pilus synthesis could be induced for adherence to specific tissues in the body. Indeed, in *S. pyogenes*, pili were shown to mediate adherence specifically to human tonsil and skin (Abbot *et al.*, 2007). Supporting this latter hypothesis, adherence to epithelial cells of the *rga* mutant of *S. agalactiae* was different depending on the cell line tested. Indeed, adherence to HEp-2 and A549 cells, which were derived from different sites in the body, was reduced for Hep-2 cells and increased for A549 cells in the mutant strain.

Sequence analysis indicates that *rga* is a pseudogene in *S. agalactiae* A909 and 2603V/R whereas an intact copy was found in *S. agalactiae* NEM316 which was investigated in this work. Similarly, when analysing the other pilus-encoding operon *gbs0628–gbs0634*, a pseudogene encoding a putative transcriptional regulator is located next to this operon. Dramsi *et al.* (2006) reported no labelling when anti-Gbs0628 was used for immunoelectron microscopy. It can be speculated that, similarly to the *pil* operon, these genes encoding pili are expressed to a detectable level in strains where the adjacent regulator gene is functional. Two good candidates are the *S. agalactiae* strains 2603V/R and A909, which seem to carry such a functional regulator gene.

As CovR/S regulates *rga* expression (Lamy *et al.*, 2004), inactivation of *covR/S* should have an indirect effect on pilus production and Srr-1 formation in *S. agalactiae*. However, upon inactivation of *covR/S*, no significant difference in the expression of *srr-1* and *pilA* was reported (Lamy *et al.*, 2004). When testing the effect of *covR/S* inactivation on gene expression in whole-genome microarray experiments, a 2.45-fold increased expression of *rga* was found after *covR/S* inactivation (Lamy *et al.*, 2004). It is possible that this amount of overexpression was not high enough to induce a detectable indirect effect of CovR/S on the expression of *srr-1* and *pilA*. Further experiments are therefore required to unravel the connection between CovR/S- and Rga-mediated regulation of virulence genes in *S. agalactiae*.

Considering the multiple environments *S. agalactiae* is able to colonize within the body, it seems reasonable to speculate that multiple stimuli can trigger adherence through activation of adhesin production, including pilus structures. Both RogB (Gutekunst *et al.*, 2003) and Rga (this work) activate the expression of pili. The fact that two

different regulators can activate the expression of the same pilus structures suggests that these regulators react to different stimuli, and therefore that pilus production is modulated according to different stimuli. Little is known about the stimuli linked to adherence regulation in *S. agalactiae*, and future studies are required to identify these signals and to understand the importance of regulation of adherence traits for this pathogenic bacterium.

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