Adhesion to cellulose of the Gram-positive bacterium *Ruminococcus albus* involves type IV pili

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This study was aimed at characterizing a cell-surface 25 kDa glycoprotein (GP25) that was previously shown to be underproduced by a spontaneous adhesion-defective mutant D5 of *Ruminococcus albus* 20. An antiserum against wild-type strain 20 was adsorbed with the mutant D5 to enrich it in antibodies ‘specific’ to adhesion structures of *R. albus* 20. The resulting antiserum, called anti-Adh serum, blocked adhesion of *R. albus* 20 and reacted mainly with GP25 in bacterial and extracellular protein fractions of *R. albus* 20. The N-terminal sequence of purified GP25 was identical to that of CbpC, a 21 kDa cellulose-binding protein (CBP) of *R. albus* 8. The nucleotide sequence of the *gp25* gene was determined by PCR and genomic walking procedures. The *gp25* gene encoded a protein of 165 aa with a calculated molecular mass of 16940 Da that showed 72.9% identity with CbpC and presented homologies with type IV pilins of Gram-negative pathogenic bacteria. Negative-staining electron microscopy revealed fine and flexible pili surrounding *R. albus* 20 cells while mutant cells were not piliated. In addition, immunoelectron microscopy showed that the anti-Adh serum probing mainly GP25, completely decorated the pili surrounding *R. albus* 20, thereby showing that GP25 was a major pilus subunit. This study shows for the first time the presence of pili at the surface of *R. albus* and identifies GP25 as their major protein subunit. Though GP25 was not identified as a CBP, isolated pili were shown to bind cellulose. In conclusion, these pili, which belong to the family of type IV pili, mediate adhesion of *R. albus* 20 to cellulose.

Keywords: adhesion-defective mutant, cell-surface glycoprotein, cloning of type IV pilin, immunoelectron microscopy

INTRODUCTION

*Ruminococcus albus*, *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* are the three cellulolytic bacterial species considered today to play a major role, with rumen fungi, in fibre breakdown in the rumen of herbivores (Lee *et al.*, 2000). They have been shown to adhere to cellulose and to possess cellulolytic and xylanolytic enzymes that enable them to efficiently degrade plant cell wall polysaccharides (Forsberg *et al.*, 2000). *R. albus* was shown to be far more abundant than either *R. flavefaciens* and *F. succinogenes* in the bovine rumen whatever diet the animals were fed (Weimer *et al.*, 1999). *In vitro* studies confirmed that *R. albus* out-competed the two other species and became predominant in cultures where the three species were grown in the presence of cellulose as a growth substrate, alone or associated with non-cellulolytic species (Chen & Weimer, 2001). These studies tend to show that *R. albus*, *R. flavefaciens* and *F. succinogenes* do not act synergistically, but rather compete for their substrate, and that *R. albus* displays several ecological advantages that allow it to compete successfully. We previously showed that *R. albus* surpassed *R. flavefaciens* and

Abbreviations: CBP, cellulose-binding protein; GP25, cell-surface 25 kDa glycoprotein.
The EMBL accession number for the sequence reported in this paper is AJ416469.
F. succinogenes competitively in initial adhesion to cellulose (Mosoni et al., 1997) and this could contribute to the overall success of R. albus in both in vivo and in vitro studies.

Recent studies have started to unravel some features of rumen ruminoococi that may be valuable in the understanding of the adhesion mechanisms of R. albus to cellulose. First, R. albus and R. flavefaciens were shown to possess a cellulosome-like structure (Ohara et al., 2000; Ding et al., 2001). Such a structure might, like in Clostridium thermocellum, mediate adhesion of the bacteria to cellulose (Bayer et al., 1998a, b). In addition to this cellulyotic complex, R. albus strain 8 was shown to possess a cellulose-binding protein (CBP), called CbpC, that may be involved in adhesion to cellulose and that presented homology with type IV pilins of Gram-negative bacteria (Pegden et al., 1998). It was then suggested that adhesion to cellulose by R. albus might be due to a combination of cellulosomes and Pil-proteins (Morrison & Miron, 2000; Miron et al., 2001a).

In a recent study, we showed that a spontaneous adhesion-defective mutant (D5) of R. albus 20 was affected on different features associated to cell-surface components. In comparison to the parent, the mutant presented a reduced cellulase activity, a low production of a cell-surface 25 kDa glycoprotein (GP25) and an altered glycoconalx (Mosoni & Gaillard-Martine, 2001). We proposed the extracellular glycoconalx as a third adhesion mechanism, in addition to Pil proteins and cellulosome-like structures. GP25 was present in large quantities in the membrane fraction and extracellular medium of R. albus strain 20 cultivated on cellobiose, while it was not produced or was underproduced by the adhesion-defective mutant cultivated in rumen fluid or chemically defined medium, respectively. GP25 was also shown to be neither a CBP nor an endoglucanase, suggesting that it does not play a direct role in the adhesion of R. albus strain 20 to cellulose.

In this study, we made further comparisons of R. albus 20 and its adhesion-defective mutant D5 to characterize GP25 and better understand its role in the adhesion of R. albus 20 to cellulose.

METHODS

Bacterial strains and culture media. Ruminococcus albus 20 (ATCC 27211), its adhesion-defective mutant R. albus D5 (Mosoni & Gaillard-Martine, 2001) and R. albus 8 (provided by M. Cotta, University of Urbana Champain, IL, USA) were anaerobically maintained at 39°C in a chemically defined (CD) medium (Rasmussen et al., 1988) supplemented with 100 μM 3-phenylpropionic (PPA) and phenylacetic acids (PAA), 80 mg methionine L-1 and 20 μg arginine L-1, with 0.3% (w/v) cellobiose as sole source of carbohydrate. Cultures were also performed in a medium containing 40% (v/v) rumen fluid (RF) (Halliwell & Bryant, 1963). Escherichia coli strains used for cloning (DH5α) and for subsequent expression of a pET construct [BL21 (DE3)] were obtained from Life Technologies and Stratagene, respectively. The vector pET28a was obtained from Novagen. E. coli strains were routinely grown on Luria–Bertani (LB) medium with appropriate antibiotic selection at 37°C; conditions used for expression of cloned products are given below.

Preparation of antisera. Polyclonal antibodies were produced against R. albus 20 by injecting adult New Zealand white rabbits subcutaneously with a suspension of 10⁷ cells of R. albus 20 in 1 ml of a sterile solution of 9 g NaCl L-1 that was emulsified in Freund’s incomplete adjuvant. Injections were performed three times at 3 weeks intervals and the rabbits were bled 2 weeks after the last immunization. The blood was incubated without agitation for 3 h at 37°C and centrifuged at 10000 g for 10 min. The upper serum phase was stored at −20°C. This antiserum (i.e. anti-R. albus 20 serum) was repeatedly adsorbed with R. albus D5 to remove the antibodies recognizing common antigens between the parent and the mutant, thus enriching the antiserum in antibodies specific to the adhesion structure lost in the mutant. The resulting antiserum was called anti-Adh serum. Dot blot analysis showed that the anti-Adh serum reacted differentially between the parent and the mutant and that the slight reaction with the mutant was not abolished by additional adsorption steps with the mutant. Another antiserum obtained by immunization of a rabbit with the adjuvant mixture devoid of bacteria was used as a control (i.e. control antiserum).

Inhibition of bacterial adhesion. To evaluate the effect of the anti-Adh serum, anti-R. albus 20 serum and the control antiserum on blocking adhesion of R. albus 20 to cellulose, 200 ml of an overnight culture of R. albus 20 on CD cellobiose medium was centrifuged at 12500 g for 10 min and the cell pellet was washed three times with an anaerobic buffered mineral solution (Bryant & Burkey, 1953) before resuspending in the same volume of buffer. This cell suspension (OD600 0.94) was distributed in different tubes to which were added the different antisera at final concentrations of 1, 2 and 5% (v/v). The mixture was gently agitated for 1 h at room temperature and the percentage of adhering cells was determined as described previously after addition of crystalline cellulose to the cell suspension (Mosoni & Gaillard-Martine, 2001). Each assay was performed in triplicate.

Preparation of protein samples from R. albus. After growth on RF cellobiose medium, R. albus cells were separated from the extracellular culture fluid by centrifugation at 10000 g for 20 min at 4°C. The cell pellets were washed three times in 50 mM sodium phosphate buffer (pH 6.9), resuspended in 1/10 volume of the same buffer, aliquoted and frozen. The supernatants containing extracellular proteins were dialysed against distilled water (cut off at 10 kDa) to remove undesirable rumen fluid components, lyophilized, resuspended in 1/10 volume of phosphate buffer, aliquoted and frozen. The protein concentration in bacterial and extracellular culture fluid samples was determined by the Bradford (1976) method with BSA as standard.

GP25 microsequencing. Extracellular proteins (100 μg) of R. albus 20 and D5 were separated by two-dimensional electrophoresis (2-DE) and stained with Coomassie blue R-250 as described previously (Michel et al., 1997). The comparison of the 2-DE profile of strain 20 producing GP25 with that of the mutant not producing GP25 allowed GP25 to be located on the gel. The protein spot was then electroblotted onto a PVDF membrane, excised and stored at −20°C. The N terminus was directly sequenced from the spot by Edman degradation with an automated microsequenator (Applied Biosystems). Protein sequence analysis was performed with the Internet facility site available at http://www.infobiogen.fr.

Amplification procedure. Genomic DNA was isolated from cultures of R. albus 20, D5 and 8 grown on CD cellobiose
medium as described by Wilson (1987). The oligonucleotide primers were purchased from Eurogentec. Considering the perfect identity between the N terminus of GP25 (this study) and CbpC of R. albus 8, two primers defined from the cbpC gene (accession no. AF089753-1) were used to amplify a DNA fragment encoding GP25 in R. albus 20 and D5. The forward primer was 5'-GAACGGCGGTATTACAATTAGA-3' and the reverse primer was 5'-GCCGCCGCAAACCTTATCATTTG-3'. PCR was carried out with 1 U AmpliTaq DNA polymerase (Applied) and was subjected to 25 cycles of amplification consisting of 90 s denaturation at 94 °C, 1 min annealing of primers at 55 °C, and 1 min elongation at 72 °C. A final elongation of 7 min at 72 °C was added. A negative control (without DNA) was included in every PCR procedure. The genomic DNA of R. albus 8 was used as a positive control.

DNA sequencing and sequence analysis. The PCR products from genomic DNA of R. albus 20 and D5 were sequenced in both directions using an ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) with the same cbpC primers as those used for amplification. Genomic walking procedures (Universal GenomeWalker Kit; Clontech) were used to sequence upstream and downstream of the DNA fragment obtained by PCR. DNA sequence analyses were performed with the Internet facility site available at http://www.infobiogen.fr.

Cloning and expression of GP25. The ORF region (i.e. putative gp25 gene) identified in the PCR product obtained above was amplified, without the first 24 nt encoding the signal sequence of the predicted protein, using primers designed to contain Nhel and XhoI sites (forward primer: 5'-CTAGCTAGCTTTCAACATCTGTGAGCTGCTTAGT-3'; reverse primer: 5'-CCGCCTGAGCGGCGGCAAACCTTATCATTTG-3'). The product was cloned into an Nhel/XhoI-treated pET28a(+) vector such that a 6-His stretch was fused to the N terminus of the recombinant protein. Clones were expressed in E. coli BL21 (DE3) as follows. Single colonies were transferred into 10 ml LB medium supplemented with 1 % (w/v) glucose and 25 µg kanamycin ml⁻¹ and were allowed to grow at 37 °C with agitation until an OD₆₀₀ of 0.8–1 was reached. IPTG was then added and the culture was incubated for a further 17 h at 37 °C.

Cultures were centrifuged (10000 g at 4 °C for 15 min) and the cell pellet was resuspended in 250 µl PBS and incubated with 1 mg lysozyme ml⁻¹ (Sigma) at 4 °C for 30 min. To avoid protein aggregation after cell lysis, Triton X-100 at 1% (v/v) was added and the mixture was agitated at 4 °C for 10 min.

After centrifugation (10000 g, 4 °C, 30 min) the supernatants containing cytoplasmic proteins were collected and stored at −20 °C.

SDS-PAGE and Western blotting. Bacterial and extracellular proteins of R. albus strains were subjected to Tricine-SDS-PAGE (16.5 %, w/v, acrylamide) (Schagger & von Jagow, 1987). Recombinant E. coli cytoplasmic proteins were analysed by SDS-PAGE (15 % acrylamide) according to Laemmli (1970). Protein bands were visualized by Coomassie staining following the instructions given for the Gelcode blue stain reagent (Pierce).

For Western blot analysis, proteins were transblotted onto nitrocellulose membranes (Trans-Blot; Bio-Rad). The membranes were then blocked by soaking for 1 h at 20 °C in 10 % (w/v) dried milk in PBS. The membranes were then transferred into the same PBS solution containing the appropriate antiserum and incubated for 2 h. For R. albus protein samples, the antiserum used was either the anti-Adh serum (dilution 1:2000) or an anti-CbpC serum (dilution 1:400), kindly provided by Dr Morrison (University of Columbus, Ohio, USA) and corresponding to polyclonal antibodies specific to the 21 kDa pil-like protein, called CbpC, of R. albus 8. After two washes of 5 min in PBS containing 0.1 % (v/v) Tween 20 and one wash of 15 min in PBS, the membranes were incubated for at least 1 h with a 1:7000 dilution of peroxidase-conjugated goat anti-rabbit IgG (Nordic). The membranes were washed as described above and bound antibodies were detected by ECL (Amersham), according to the supplier’s indications. For recombinant E. coli protein samples, the antisera used were either the anti-Adh serum adsorbed with E. coli BL21 (DE3) to limit non-specific recognition of E. coli proteins by R. albus antibodies or a peroxidase-conjugated anti-His antibody (Sigma; dilution 1:2000) allowing identification of the recombinant His-tagged protein. With the latter antiserum, bound antibodies were detected after the antiserum was applied and the membrane washed.

Electron microscopy. Bacterial cells, obtained from R. albus 20 and D5 colonies after growth on CD cellobiose agar plate in an anaerobic chamber, were placed onto collodion-coated 300 mesh copper grids, negatively stained with 1% phosphotungstic acid for 1 min and dried prior to examination in an EM400 transmission electron microscope (Philips Electron Optics) at an accelerating voltage of 80 kV. Immunogold labelling of R. albus 20 and D5 cells was performed onto collodion-coated nickel grids with the anti-Adh serum diluted 1/8 in PBS containing 0.1% (v/v) Tween 20 and 10 nm colloidal gold anti-rabbit immunogoldin G (Sigma) diluted 1/50 in PBS. After several washes in PBS, the grids were stained with 1% (v/v) uranyl acetate and rinsed in distilled water before examination.

Adhesion assay to cellulose of native pili of R. albus 20. The extracellular culture fluid of R. albus 20 was shown by electron microscopy to contain pili released from bacteria and was used as a crude preparation of native pili. The adhesion assay was performed by adding 5 ml of extracellular culture fluid of R. albus 20 to tubes containing 100 mg microcrystalline cellulose Sigmacell 20. After gentle shaking for 1 h at room temperature, cellulose was sedimented by centrifugation at 500 g for 2 min at 4 °C. The supernatant, corresponding to the extracellular culture fluid after contact with cellulose, was transferred into another tube. The cellulose pellet was washed three times with distilled water to elute components non-specifically bound. The composition of the extracellular culture fluid before and after contact with cellulose was compared by Western blotting using the anti-Adh serum.

![Fig. 1. Adhesion of R. albus 20 to Sigmacell 50 cellulose after incubation without antiserum (○) or with various concentrations of control antiserum (□), anti-Adh serum (●) or anti-R. albus 20 serum (■). Each value is the mean of three determinations. Error bars indicate standard deviation.](image-url)
Components of the extracellular culture fluid that remained linked to the cellulose pellet after the washings were eluted by adding 100 µl SDS-PAGE denaturing loading buffer and analysed by Western blotting as described above. This adhesion assay of native pili to cellulose was performed in triplicate. As a negative control, the experiment was performed with phenyl-Sepharose instead of cellulose, because *R. albus* 20 does not adhere to this substratum.

**RESULTS**

**Effect of antibodies on adhesion of *R. albus* 20 to cellulose**

Antibodies ‘specific’ to adhesion structures of *R. albus* 20 were produced by adsorption of the anti-*R. albus* 20 serum with the adhesion-defective mutant D5. The antiserum obtained, called anti-Adh serum, was tested for blocking adhesion to cellulose of the parent *R. albus* 20 cells and it was compared with the blocking effect of the anti-*R. albus* 20 serum and that of the control antiserum (Fig. 1). Without antibodies, the adhesion of *R. albus* 20 was very high, since a mean of 94% of the cells bound to crystalline cellulose. Adhesion was significantly reduced (47% adhering cells) in the presence of the control antiserum, showing that this serum was not neutral and that non-specific recognition occurred between the antibodies present in this serum and *R. albus*. Adhesion of strain 20 decreased strongly with the anti-Adh serum since at 1% concentration, only 8% of the cells adhered. Higher concentrations of this antiserum did not block adhesion to a significantly greater degree (4% adhering cells at 5% concentration). With the anti- *R. albus* 20 serum at 1% concentration, the same result as with the anti-Adh serum was observed. At higher concentrations, this serum completely blocked adhesion. Consequently, the anti-Adh serum blocked adhesion of *R. albus* 20, and this was achieved moreover with the same efficiency as the anti-
**Ruminococcus albus** 20 serum when the sera were used at the lowest concentration.

**Western blotting analysis of bacterial and extracellular proteins of *R. albus***

The anti-Adh serum was used to identify the proteins involved in the adhesion of *R. albus* 20 by Western blotting, but also to underline some homologous proteins in strain 8 of *R. albus*. Bacterial and extracellular proteins were analysed separately and are presented in Fig. 2. Fig. 2(a) shows that among the bacterial proteins of strain 20 (lane 1), the anti-Adh serum reacted mainly with a protein of 25 kDa (arrow a), and at a lower intensity with several proteins ranging from 40 to at least 115 kDa. The same Western blot profile was obtained with mutant D5 (Fig. 2a, lane 2), except that the 25 kDa protein was probed with a much lower intensity than in the parent. This protein probably corresponded to the 25 kDa glycoprotein that we showed in a previous study to be underproduced by the mutant and that we called GP25. With strain 8 (Fig. 2a, lane 3), the anti-Adh serum did not react with a 25 kDa protein but with a 21 kDa protein (arrow b) that might correspond to CbpC, a CBP probably involved in adhesion of *R. albus* 8 to cellulose (Pegden et al., 1998). This reaction could be visualized only if the amount of strain 8 proteins loaded on the gel was twice that of strains 20 and D5. To explain this cross-reaction between GP25 and CbpC by a possible homology between the two proteins, another Western blot was performed with the same samples as in Fig. 2(a) except that an anti-CbpC antiserum was used. Fig. 2(b) shows that this serum reacted mainly with the 21 kDa protein of strain 8 corresponding to CbpC (lane 3), but also reacted with GP25 of strain 20 (lane 1). GP25 was probably not produced in large enough quantity by the mutant D5 to be recognized by this serum (lane 2). In addition, the anti-CbpC antiserum bound to high-molecular-mass proteins (≥66 kDa) in strains 20 and D5 and to a low-molecular-mass protein of approximately 13 kDa in strains 20 and D5, and 11 kDa in strain 8.

Similar results were obtained with extracellular proteins of *R. albus* 20, D5 and 8. In Fig. 2(c), GP25 was the major protein recognized by the anti-Adh serum in the extracellular proteins of strain 20 (lane 1), while it was hardly detected in the extracellular proteins of mutant D5 (lane 2). The cross-reaction of the anti-Adh serum and the anti-CbpC serum with CbpC and GP25, respectively, was observed as in the bacterial protein samples (Fig. 2c, d). Comparison of the results obtained with bacterial and extracellular proteins shows that GP25 was abundant in both bacteria (Fig. 2a, lane 1) and extracellular fluid (Fig. 2c, lane 1), as shown by the high intensity of the reaction between GP25 and the anti-Adh serum. The reaction between the anti-CbpC antiserum and CbpC was not as intense in the extracellular fluid of strain 8 (Fig. 2d, lane 3) as in the bacteria (Fig. 2b, lane 3), indicating that CbpC was more associated with the bacteria than released in the extracellular medium.

**Nucleotide and amino acid sequence analysis of GP25**

GP25 was separated from the extracellular proteins of strain 20 by 2-DE, electroblotted onto a PVDF membrane and microsequenced. The 10-residue N-terminal sequence of GP25 was XTLVELLVVI, where X represents an unidentified amino acid residue. This sequence is 100% identical to the N-terminal sequence of CbpC in which X corresponds to a phenylalanine that is subsequently methylated, according to the amino acid sequence predicted from the *cbpC* gene (Pegden et al., 1998). The homology between GP25 and CbpC, suggested by the results obtained by Western blots, was therefore confirmed by the N-terminal sequence. Consequently, primers based on the oligonucleotide sequence of the *cbpC* gene were used to amplify a DNA fragment of approx. 0.5 kb that was subsequently sequenced. The regions upstream and downstream from this fragment were sequenced using genomic walking procedures. Using these methods, we identified an ORF, preceded by a putative promoter and RBS and followed by a transcriptional terminator. The ORF would encode a protein of 163 aa with a calculated molecular mass of 16940 Da. Amino acids 10–18 in the predicted amino acid sequence were identical to those obtained by N-terminus sequencing of GP25, suggesting that the DNA fragment obtained by PCR contains the *gp25* gene. To confirm that this gene actually encodes GP25, the ORF region without the 24 first nt encoding the signal sequence of the predicted protein was cloned in *E. coli* and the proteins expressed by the recombinant *E. coli* were analysed by Western blotting with the anti-Adh serum. Comparison of the proteins produced by the induced and non-induced recombinant *E. coli* (lanes 1 and 2, respectively) shows that one protein of approximately 22 kDa was differentially expressed (Fig. 3a). Though its molecular mass is slightly higher than that of the predicted protein (18.5 kDa), the fact that it is the only protein recognized by the anti-His antiserum (Fig. 3b) shows that it is indeed the recombinant protein. In addition, this protein was recognized by the *E. coli*...
adsorbed anti-Adh serum (Fig. 3c). This result, in addition to the sequence data, allows the conclusion that the DNA sequence obtained by PCR and genomic walking procedures contains the entire gp25 gene. This gene and the region immediately upstream (containing a putative promoter) was sequenced from mutant D5 and the nucleotide sequence was found to be identical to that obtained from strain 20, showing that no mutation was present in this gene in the adhesion-defective mutant.

The nucleotide sequence of gp25 (498 nt) and cbpC (510 nt) have 79.5% identity and the predicted amino acid sequences of both genes showed 72.9% identity. The 64-residue N-terminal sequences of GP25 and CbpC are identical. As already shown for CbpC (Pegden et al., 1998), the N-terminal third of GP25 shows significant sequence identity with N-terminal sequences of type IV pilins from Gram-negative pathogenic bacteria (Vibrio cholerae, Moraxella bovis, Neisseria gonorrhoeae, Pseu-
was not piliated (Fig. 4c) and, very rarely, a very few pili could be seen extending from D5 cells. Under the same growth conditions as those used for strain 20, *R. albus* 8 morphology was more comparable to mutant D5 (scarce pili on the cells and no pili in the extracellular culture fluid) than strain 20 (not shown). Immunoelectron microscopy showed that the anti-Adh serum, probing mainly GP25, completely decorated the pili surrounding *R. albus* 20 (Fig. 4d), thereby showing that GP25 was a major pilus subunit. No immunolabelling was observed with mutant D5, probably because of the absence of pili (not shown).

**Adhesion assay to cellulose of native pili of *R. albus* 20**

Considering that GP25 was not identified as a CBP (Mosoni & Gaillard-Martinie, 2001), it was important to show whether the pili, essentially made of GP25, were part of the adhesion machinery of *R. albus* 20. To determine if pili were able to bind cellulose, an adhesion assay to cellulose was performed using the extracellular culture fluid of *R. albus* 20 as a crude preparation of isolated pili. The binding of pili to cellulose was visualized by Western blotting using the anti-Adh serum recognizing mainly GP25. Fig. 5(a) shows that the extracellular culture fluid contains GP25 (arrow a, lane 1) as already shown in Fig. 2(c), indicating the presence of pili. After the extracellular culture fluid was put in contact with cellulose, it no longer contained GP25 (lane 2) and GP25 was the most abundant protein bound to cellulose (lane 3). Fig. 5(b) shows the results obtained with phenyl-Sepharose used as a substratum and to which *R. albus* 20 does not bind. After contact with phenyl-Sepharose, the extracellular culture fluid still contained GP25 (Fig. 5b, lane 2) and no protein recognized by the anti-Adh serum was bound to this substratum. Binding of the pili to cellulose was therefore clearly shown by the association of GP25 to cellulose, while the pili did not bind at all to phenyl-Sepharose.

**Cell-surface analysis by electron microscopy**

Negative-staining electron microscopy of whole cells of *R. albus* 20 grown on a CD cellobiose agar plate revealed fine and flexible pili-like filaments (approx. 4 nm diam.) surrounding the cells (Fig. 4a). These pili were abundant in the extracellular culture fluid of strain 20 where they were either broken into short filaments (not shown) or left more or less intact as shown in Fig. 4(b). In that case, their length was at least 3 μm (four times the diameter of the bacteria) and they tended to stack together to form bundles of filaments. With whole-cell preparations of mutant D5, the majority of the cells

**DISCUSSION**

This study shows for the first time the presence of pili at the surface of *R. albus*, a Gram-positive cellulolytic bacterium and identifies GP25 as a protein involved in the adhesion phenotype of *R. albus* 20 and as the major protein subunit of the pili. These pili probably belong to the family of type IV pili since GP25 presents characteristics typical of type IV pilins of many Gram-negative pathogenic bacteria (*V. cholerae*, *M. bovis*, *N. gonorrhoeae*, *P. aeruginosa*, *Dichelobacter nodosus* and *Eikenella corrodens*). In these organisms, type IV pili mediate adhesion to host cells and appear to be largely, if not
completely, composed of a single structural subunit, the pilin, which is of relatively low molecular mass (17–25 kDa) and exhibits a number of distinctive features (Alm & Mattick, 1997). These include a short, positively charged leader sequence, an unusually modified amino acid as the first residue of the mature pilin (N-methylated phenylalanine) and an extremely hydrophobic and highly conserved N-terminal domain. It has been found that interspecies homology between type IV pilins was limited to their highly conserved N-terminal region, while the C-terminal region diverged strongly (Hazes et al., 2000). Thus, it is not surprising that GP25, originating from a Gram-positive bacterium, does not show homology, apart from its N-terminal sequence, with type IV pilins of Gram-negative bacteria. Deinococcus radiodurans is the only Gram-positive bacterium described to date to possess a putative type IV pilin, for which GP25 also showed homology.

In a previous study (Mosoni & Gaillard-Martinie, 2001), we showed that GP25 was glycosylated. This could explain the differences observed between the predicted (16-9 kDa) and SDS-PAGE-determined (approx. 25 kDa) molecular mass of GP25. Type IV pilins of N. gonorrhoeae, N. meningitidis and P. aeruginosa have been shown to be glycosylated (Virji et al., 1993; Castric, 1995; Farge et al., 1995; Power et al., 2000). This post-translational modification occurs on serine 63 of the pilins of both Neisseria species. Considering that GP25 is rich in serines, particularly in the central region of the protein (Ser 57, 62, 63, 65, 67, 72, 75, 81, 83, 88, 89), it is not unreasonable to propose that one or several of these serines might be a site of glycosylation. In this respect, the homologous protein CbpC of R. albus 8, should be less glycosylated or not at all, considering its unique serine (Ser 76) in that central region.

The present results confirmed our previous findings that showed the adhesion-defective mutant produced very low amounts of GP25, in comparison to the parent. This probably resulted in the absence of pili or scarce pili at the surface of the mutant cells. In addition, the anti-Adh serum, reacting mainly with GP25, and hence with the pili, inhibited almost totally the adhesion of the bacteria to cellulose. Furthermore, isolated pili were shown to bind cellulose. Taken together, these results show that pili are implicated in the adhesion of R. albus 20 to cellulose.

If we assume that CbpC is also a pilin subunit in R. albus 8, the fact that R. albus 8 displayed scarce pili could be due to a low production of CbpC. This is in agreement with the Western blotting results using anti-CbpC antiserum and showing a low amount of CbpC in the extracellular medium of R. albus 8. It is also possible that the growth conditions used in this study were not appropriate for R. albus 8 to produce large amounts of CbpC and pili.

In an affinity assay intended to identify CBPs from membrane proteins of R. albus 20, we previously showed that GP25 did not bind to cellulose (Mosoni & Gaillard-Martinie, 2001). On the contrary, CbpC was identified, using the same affinity assay, as a major CBP of R. albus 8 (Pegden et al., 1998). It is possible that the conditions used for the affinity assay were inadequate for GP25 to stick to cellulose. Another explanation could be that GP25 takes on a conformation allowing it to bind to cellulose only when it is integrated into the pilus structure. One important feature of type IV pilins of different strains of P. aeruginosa, but also of other pathogenic bacteria, is that they contain two cysteines in the far C terminus of the protein, involved in a disulfide bridge. In P. aeruginosa, the resulting disulfide loop was shown to mediate adhesion (Hahn, 1997). Furthermore, it was recently shown that this short region of the pilin was exposed only at the tip of the pilus (Hazes et al., 2000). GP25, as well as CbpC, contains only one cysteine and therefore cannot have such an adhesion structure.

Though P. aeruginosa was shown to adhere to host cells via the constitutive unit of the type IV pili, this is not the case for N. gonorrhoeae and N. meningitidis for which the adhesin is not the constitutive pilin, but another protein, PilC of 110 kDa, that is only exposed at the tip of the pilus (Rudel et al., 1995). In the adhesion assay of native pili of R. albus 20 to cellulose, we found proteins other than GP25 that were bound to cellulose and recognized by the anti-Adh serum. It is possible that one of these proteins may correspond to the adhesin of the pilus. In addition, we previously showed that R. albus 20 had two major CBPs, CBP1 (115 kDa) and CBP2 (90 kDa) (Mosoni & Gaillard-Martinie, 2001). These proteins may serve as adhesins like in N. gonorrhoeae. We are presently characterizing them to determine if they have a role in the adhesion of R. albus 20 to cellulose.

We previously showed that the spontaneous mutation that impaired R. albus 20 from adhering affected not only the production of GP25 but also other cell-surface components, since the cellulase activity of the mutant was strongly reduced and its glycocalyx was altered (Mosoni & Gaillard-Martinie, 2001). We suggested that the mutation had occurred on a gene controlling either the synthesis or the secretion of surface and extracellular molecules. This assumption is still valid since no mutation was detected in the gp25 gene of mutant D5. In addition, it is in agreement with results obtained with the adhesion-defective mutant of R. albusSY3 that appeared to be blocked in exocellular transport of cellulases (Miron et al., 2001b). Considering that the mechanisms used by R. albus to produce the newly identified type IV pili, and also hydrolytic enzymes or glycocalyx components, are not known, it is rather difficult to explain what mutation may have resulted in the complex phenotype of mutant D5. It is widely asserted that type IV pilus biosynthesis and type II secretion are two linked mechanisms in Gram-negative bacteria (Hobbs & Mattick, 1993; Alm & Mattick, 1997; Sauvonnet et al., 2000; Fernandez & Berenguer, 2000). For instance, in P. aeruginosa, the pilus subunit PilA and the prepilin peptidase PilD, necessary for pilus biogenesis, are also required for efficient protein secretion (Strom et al., 1991; Nunn & Lory, 1992; Lu et
al., 1997). In addition, the biosynthesis of type IV pili and the exopolysaccharide alginate, two virulence determinants of P. aeruginosa, is co-ordinately regulated by a major transcriptional regulator, AlgR (Whitchurch et al., 1996). Consequently, it is possible that the mutation in mutant D5 has affected some transcriptional regulator that controls the expression of gp25 and that this mutation also alters the secretion of glycocalyx components and celluless.

In conclusion, R. albus 20 displays at least two adhesion structures: pili (this study) and glycocalyx (Mosoni & Gaillard-Martine, 2001). The presence of Pil proteins is not limited to strains 20 and 8 and may be widespread throughout R. albus species (Pegden et al., 1998). A cellulose-like structure, to date only identified in R. albus strain F40 (Ohara et al., 2000), might also be involved in adhesion (Morrison & Miron, 2000; Miron et al., 2001a). If the R. albus species actually possesses these three means of adhesion, this might explain why it out-competes the two other rumen cellulosytic species in adhesion to cellulose in competition studies (Mosoni et al., 1997). This would allow R. albus to survive in such a competitive ecosystem as the rumen.

ACKNOWLEDGEMENTS

We are grateful to Dr J. P. Girardeau for his advice throughout this work and to Y. Ribot for technical assistance.

REFERENCES


Received 15 November 2001; revised 1 February 2002; accepted 8 February 2002.