Glycosylation of the flagellin of the polar flagellum of *Azospirillum brasilense*, a Gram-negative nitrogen-fixing bacterium

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The glycosylation of the flagellin of the polar flagellum of *Azospirillum brasilense* Sp7 is demonstrated in several ways: (1) by a decrease in apparent *M_r* after chemical deglycosylation; (2) by sugar staining after SDS-PAGE; (3) by use of a sugar-specific monoclonal antibody in immunogold labelling coupled with transmission electron microscopy. In addition, two mutant flagellins are described; they differ in glycan composition when compared with the wild-type. The glycosylation of a bacterial flagellin is unusual and has to be taken into account in the proposed model for the assembly of the flagellar filament.

Keywords: *Azospirillum brasilense*, glycosylation, bacterial glycoprotein, polar flagellin, glycosylation mutants

INTRODUCTION

Azospirilla are plant-root-associated nitrogen-fixing bacteria able to promote plant growth (Okon, 1994). In view of their possible application as a biofertilizer, the molecular mechanisms that govern the association of these bacteria with plant roots are of interest.

An *Azospirillum brasilense* cell can display two types of flagella: one polar flagellum that is present under all growth conditions and, in addition, several lateral flagella that are only synthesized on (semi-)solid surfaces (Tarrand *et al.*, 1978). The polar flagellum is responsible for the locomotion in liquid medium (swimming), while the lateral flagella are believed to be involved in swarming over solid surfaces (Hall & Krieg, 1983). It has been suggested that, in natural environments, free swimming through the water space, rather than swarming, is needed for migration to plant roots because of the dependence of this migration on soil moisture (Bashan, 1986; Zhulin & Armitage, 1993).

Previous work in our laboratory revealed the presence of several loci involved in motility on the chromosome and the 90 MDa plasmid (p90) of *A. brasilense* Sp7 (van Rhijn *et al.*, 1990; Croes *et al.*, 1991). Croes *et al.* (1991) replaced large parts of the p90 plasmid DNA with a kanamycin-resistance-encoding gene cassette. In mutant $\Delta 071$, approximately 14 kb is deleted and this resulted in an

altered motility. Replacing another part of p90 (mutant $\Delta 084$) resulted in a non-flagellate phenotype and a reduced capacity to adsorb to plant roots. This result, together with several additional lines of evidence, demonstrated unequivocally that the polar flagellum promotes adsorption of *A. brasilense* to plant roots (Croes *et al.*, 1993). Bacterial adsorption is believed to be a key step in plant root colonization, which in turn is essential for plant growth promotion.

Glycosylation of proteins in prokaryotes is a rather recent research topic but seems to be widespread in both the domain of the Archaea and the domain of the Bacteria (Woese *et al.*, 1990). The first chemically characterized example is the cell surface protein of the archaeobacterium *Halobacterium halobium* (Mescher & Strominger, 1976; Wieland *et al.*, 1982). More recently, evidence has also been provided for the occurrence of glycoproteins in Bacteria, most of them Gram-positive. The best studied examples of bacterial glycoproteins are surface layer (S-layer) proteins (Messner & Sleytr, 1991, 1992). Non-S-layer glycoproteins have been reported but only in a few cases have structural studies been performed (for a review, see Sandercock *et al.*, 1994).

Glycosylation of flagellin has to our knowledge only been reported for some Archaea (Lechner & Wieland, 1989; Wieland *et al.*, 1985; Southam *et al.*, 1990; Kalmokoff *et al.*, 1992; Yao *et al.*, 1992) and is possibly also present in minor flagellar polypeptides of *Spirochaeta aurantia* (Brahamsha & Greenberg, 1988). Several other post-translational modifications of flagellin such as phosphorylation (Kelly-Wintenberg *et al.*, 1993; Logan *et*

Abbreviations: mAb2, class 2 monoclonal antibody; p90, 90 MDa plasmid of *A. brasilense*; PAS staining, periodic acid/Schiff staining; TEM, transmission electron microscopy; TFMS, trifluoromethanesulfonic acid.

al., 1989), methylation (Ambler & Rees, 1959) and sulfatation (Wieland *et al.*, 1985) have been reported.

Here we provide evidence that the flagellin of the polar flagellum of the Gram-negative bacterium *A. brasilense* Sp7 is a glycoprotein. This flagellin is designated Fla1 to discriminate it from the flagellin, Laf1, of the lateral flagella. We also describe glycosylated flagellins of two previously described p90 mutants that are affected in their glycan moiety when compared with the wild-type. Mutant Δ 071 is one of the p90 deletion mutants (Croes *et al.*, 1991); the *exoC* mutant has a Tn5 transposon insertion in a gene encoding a phosphomannomutase (Michiels *et al.*, 1988; Petersen & Vanderleyden, 1994). To our knowledge, such glycosylation mutants have never been reported.

METHODS

Bacterial strains and growth conditions. The *Azospirillum brasilense* strains used are listed in Table 1. Cells were grown at 30 °C on Luria-Bertani medium (Sambrook *et al.*, 1989), supplemented with 2.5 mM CaCl₂ and 2.5 mM MgSO₄. For the mutant strains, kanamycin was added at a concentration of 25 µg ml⁻¹.

Isolation of *A. brasilense* polar and lateral flagella. This procedure was as described by Schlöter *et al.* (1994). Briefly, cells grown on agar plates were collected in a small volume of a Tris buffer. Flagella were sheared in a blender and the cell bodies were separated from the flagella using differential centrifugation. The two types of flagella were separated using a CsCl density gradient.

Chemical deglycosylation of flagellin. Sugar residues were removed from the flagellins using the GlycoFree Chemical Deglycosylation kit of Oxford Glycosystems.

One-dimensional gel electrophoresis (SDS-PAGE) and Western blotting. The discontinuous SDS gel system described by Laemmli (1970) was used with a 5% (w/v) stacking gel and a 12% (w/v) resolving gel. The samples were boiled for 5 min in sample buffer. The resulting gels were stained with Coomassie brilliant blue R250 or electroblotted onto an Immobilon-P membrane (Millipore).

Sugar staining of blotted proteins. Periodic acid/Schiff (PA/S) staining was done according to the method of Strömqvist & Grufmann (1992). Vicinal diols in the sugar residues of blotted proteins were oxidized with periodic acid and subsequently treated with Schiff's reagent. The Glycan Detection kit was purchased from Boehringer Mannheim. This kit also uses periodic acid oxidation of blotted proteins. Subsequently a digoxigenin group is coupled to the aldehydes formed. The digoxigenin is then detected using an alkaline-phosphatase-coupled anti-digoxigenin antiserum. The latter

method proved in this case to be more sensitive than the detection with Schiff's reagent.

Immunodetection of non-treated and periodic-acid-oxidized flagellin. We adopted the procedure of Woodward *et al.* (1985) with some modifications. All incubations were done at room temperature. The blots were blocked for 30 min in 0.1% BSA/PBS (g l⁻¹: NaCl, 8.0; KCl, 0.2; Na₂HPO₄, 1.44; KH₂PO₄, 0.24; pH 7.2) and subsequently rinsed with a 50 mM sodium acetate buffer (pH 4.5). Oxidation of the blots took place during a 1 h incubation in the dark in a freshly made solution of 10 mM periodic acid in the sodium acetate buffer. In the meantime, the blots for immunodetection of non-oxidized flagellin were incubated in buffer without periodic acid. After a rinse in sodium acetate buffer, aldehyde groups were reduced with a fresh solution of 50 mM sodium borohydride in PBS for 30 min. At this stage, the blots are ready for immunodetection. This was done according to the method of Harlow & Lane (1988). The blots were rinsed twice in 0.1% BSA/PBS, incubated for 1 h with the class 2 monoclonal antibody mAb2 (Schlöter *et al.*, 1994), washed once with PBS and twice with TBS (g l⁻¹: NaCl, 8.0; KCl, 0.2; Tris, 3.0; pH 8.0), incubated for 1 h with secondary antibody [goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate, Bio-Rad] diluted 1:3000 in 0.1% BSA/TBS, washed again with TBS and incubated with the bromochloroindolyl phosphate/nitro blue tetrazolium substrate, which forms a black-purple precipitate at the site of enzyme binding.

Immunogold labelling of whole cells and transmission electron microscopy (TEM). Cells were taken from plates, resuspended in 0.85% NaCl and treated further essentially as described by Levanony & Bashan (1989). Briefly, cells were fixed on Formvar- and carbon-coated grids with 2% (v/v) glutaraldehyde in a 0.1 M cacodylate buffer. After rinsing the grids with double-distilled water, non-specific binding was blocked with 1% (w/v) BSA in PBS. The grids were transferred to the monoclonal antibody solution and incubated for 90 min. After rinsing, blocking was repeated. The grids were incubated in the gold-labelled secondary antibody [anti-mouse IgG (whole molecule) gold conjugate 5 nm, Sigma ImmunoChemicals] diluted 1:10 in TBS. After rinsing in TBS and rinsing with double-distilled water, the grids were examined by TEM (Philips EM 400).

RESULTS

Characterization of the flagellation of wild-type Sp7 and mutants Δ 071, *exoC* and Δ 084 by SDS-PAGE patterns

Isolation of flagella and separation of polar and lateral flagella of *A. brasilense* are described elsewhere (Schlöter *et al.*, 1994). Wild-type polar flagellin, Fla1, is a protein with an apparent *M_r* of approximately 100 000. Repeatedly we observed at least a double band on SDS-PAGE, indicating

Table 1. *A. brasilense* strains used in this study

Strain	Properties	Reference
Sp7	Wild-type, ATCC 29145	Tarrand <i>et al.</i> (1978)
Sp7 <i>exoC</i>	Tn5-insertion mutant of Sp7	Michiels <i>et al.</i> (1988)
Sp7 p90D071 or Δ 071	Deletion mutant of Sp7	Croes <i>et al.</i> (1991)
Sp7 p90D084 or Δ 084	Deletion mutant of Sp7	Croes <i>et al.</i> (1991, 1993)

Fig. 1. (a) One-dimensional SDS-PAGE patterns (Coomassie blue staining) of the flagellins of *A. brasilense* strains Sp7, $\Delta 071$ and *exoC*. After TFMS deglycosylation a clear decrease in apparent M_r of the polar flagellins is visible (b) resulting in an apparent M_r of 70000 for the three strains. The broad band of the TFMS-treated flagellins is probably due to incomplete deglycosylation. The migration distances of the M_r markers are indicated on the left. The identity of the contaminating protein bands with an apparent M_r around 45000 is not known. Some of these might represent contaminating lateral flagellin. It has been noticed that they do not change in size after deglycosylation.

that there might be multiple flagellins differing in size of the protein core or the extent of post-translational modifications.

One-dimensional SDS-PAGE patterns of flagellar preparations for wild-type Sp7 and two different p90 mutants are compared in Fig. 1(a).

The polar flagellin of the Tn5-induced *exoC* mutant has an apparent M_r of 87000 instead of 100000. It also has a less acidic pI than Sp7 flagellin (De Mot & Vanderleyden, 1989). A two-dimensional PAGE preparation of *exoC* proteins is shown by De Mot & Vanderleyden (1989). They designated the polar flagellin spot as a truncated protein.

Deletion mutant $\Delta 071$ has a polar flagellin with approximately the same apparent M_r as the polar flagellin of *exoC*. This flagellin also has a less acidic pI than wild-type Fla1 (data based on two-dimensional PAGE, not shown).

As already shown by Croes *et al.* (1993), the p90 deletion mutant $\Delta 084$ is non-flagellated. Consequently a 'flagellar preparation' of this bacterium does not show the Fla1 protein on SDS-PAGE gels (data not shown).

Chemical deglycosylation of the Fla1 protein of strains Sp7, $\Delta 071$ and *exoC*

The Glycofree Chemical Deglycosylation kit was used to attempt to remove glycosyl residues on the polar flagellins. The kit uses treatment with anhydrous

trifluoromethanesulfonic acid (TFMS) and removes both N- and O-glycosylation. The conditions used have been proven to cause no significant cleavage of peptide bonds. An SDS-PAGE gel of non-treated and TFMS-treated flagellins of both wild-type and mutants is shown in Fig. 1(a, b). This shows clearly the decrease in apparent M_r after TFMS treatment. The apparent M_r of the TFMS-treated flagellins of all tested samples is the same, approximately 70000.

Sugar staining (PA/S) of deglycosylated flagellins was negative (data not shown) indicating the absence of carbohydrate modification, at least within the detection limits of the test system. Interestingly, the polar flagellins that are normally water soluble start to precipitate after the TFMS treatment. A possible explanation is that the sugars are responsible for a hydrophilic surface of the flagellin so that it remains in solution when the sugars are present.

Sugar detection after SDS-PAGE and electroblotting of 'flagellar preparations' of Sp7, $\Delta 071$, *exoC* and $\Delta 084$ on a polyvinylidene difluoride membrane

Two methods for sugar detection were used, both based on periodate oxidation of the vicinal diols with subsequent detection of the aldehydes formed: PA/S staining (Strömqvist & Grufmann, 1992) and the more sensitive Glycan Detection kit. Both methods gave a good signal for Sp7 polar flagellin and a slightly less intense signal for $\Delta 071$ and *exoC* polar flagellin. No signal was found at the same position for 'flagellar preparations' of $\Delta 084$, the

Fig. 2. Sugar detection on blotted flagellins using the Glycan Detection kit. Sp7 polar flagellin gives a clear reaction. The reaction of $\Delta 071$ and *exoC* polar flagellins is also clear, but is slightly less intense. No reaction is detected for $\Delta 084$. The double band, clearly visible for Sp7 flagellin, is discussed in the first section of Results.

flagella-less mutant (Fig. 2). This indicates that the carbohydrates are associated with the flagellins and are not just comigrating (lipo)polysaccharides. Moreover, one could expect that (lipo)polysaccharides would comigrate with other proteins as well, like the contaminating proteins with apparent M_r around 45 000 that are most likely outer-membrane proteins. This is clearly not the case. On two-dimensional PAGE gels of a total protein extract of *A. brasilense* Sp7, sugar-specific staining was only observed for the Fla1 spot (data not shown).

Since this association of sugars with Fla1 resists boiling in 2% (w/v) SDS (sample buffer), and also boiling in 10 mM EDTA and 8 M urea (data not shown), we conclude that the sugars are tightly associated and most likely covalently bound.

Flagellin-associated carbohydrates differ from other cell surface polysaccharides

Schlöter *et al.* (1994) describe a set of monoclonal antibodies specifically recognizing cell surface components of *A. brasilense* Sp7. mAb2 recognizes the polar flagellin of *A. brasilense* Sp7 (Fig. 3). After periodic acid treatment of blotted flagellin, no binding of mAb2 to flagellin is detected (data not shown), indicating that the mAb2 recognizes carbohydrate epitopes (Woodward *et al.*, 1985).

Immunogold labelling coupled with TEM shows clearly the specificity of mAb2 for polar flagella. No colloidal

Fig. 3. Immunodetection, using mAb2, of polar flagellin blotted on a membrane, before periodic acid treatment. For Sp7, again, a clear double band is visible. This band disappears after periodate oxidation (data not shown). $\Delta 071$ polar flagellin is not recognized by mAb2. *exoC* polar flagellin is also recognized only when it is not oxidized with periodate.

Fig. 4. Immunogold labelling using mAb2 of the polar flagellum of *A. brasilense* Sp7 observed by TEM. A clear specificity for the polar flagellum is visible. Gold particles are distributed evenly over cell surface and background. Bar, 0.1 μ m.

gold particles are observed on lateral flagella (not shown) or on the cell surface (Fig. 4). It can be concluded that the carbohydrate epitope of mAb2 is specific for Fla1 and is not derived from a cell surface polysaccharide contamination.

Interestingly, mAb2 recognizes the *exoC* flagellin, but not the $\Delta 071$ flagellin, suggesting a difference in glycan composition between the two mutant flagellins (Fig. 3).

DISCUSSION

In this paper we provide evidence for the glycoprotein nature of the polar flagellin of *A. brasilense* Sp7.

The reported apparent M_r for the polar flagellin (100 000) is unusually high since the reported apparent M_r of flagellins mainly varies between 30 000 and 70 000. However, the estimated M_r is based on electrophoretic mobility in SDS-PAGE gels and does not necessarily represent the exact size of the polypeptide chain. The protein devoid of sugars, as a result of TFMS treatment, has an apparent M_r of 70 000 and this corresponds more closely to the reported M_r of other flagellins.

For strains Sp7, *exoC* and $\Delta 071$, chemical deglycosylation of the polar flagellum preparation results in a protein with an apparent M_r of 70 000, suggesting that the protein core of the three flagellins is of the same size. Since the *exoC* locus has been shown recently to encode a phosphomannomutase (Petersen & Vanderleyden, 1994) the possibility that the Tn5 has inserted in the structural gene for Fla1 can be excluded. A plausible explanation for the smaller size of the Fla1 in the *exoC* mutant is the absence of a post-translational modification on Fla1, resulting in the less acidic Fla1 protein of the *exoC* mutant. The data could also indicate that the deletion in $\Delta 071$ did not truncate the *fla1* structural gene but that the glycosylation is affected. Moreover, it can be expected that the p90 DNA flanking the deletion is not encoding a part of Fla1 since, at one side, the deletion ends in the previously sequenced *nodPQ* genes (Vieille & Elmerich, 1990) and, at the other side, the DNA sequence, which has also been determined previously, did not reveal an open reading frame nor any similarity to known genes (S. Moens, unpublished; Milcamps, 1993).

Glycosylation of a bacterial flagellin has not previously been reported and raises some intriguing questions about mechanisms of glycosylation and export of the flagellin molecules. Since the glycosylation process was always reported to be membrane located [in eukaryotes, and also apparently in prokaryotes (Hartmann & König, 1989; Lechner & Wieland, 1989; Hartmann *et al.*, 1993), although this is not yet fully proved], the polar flagellin of *A. brasilense* would be expected to cross a membrane before entering the flagellar structure. It is believed that assembly of the extracellular structures of bacterial flagella (filament and hook) involves transport of the component proteins (flagellin and hook protein, respectively) through a central channel in the flagellar basal structure, without crossing the bacterial cell membrane in a signal-peptide-dependent way. An as yet unidentified 'export apparatus' is believed to be located at the flagellar base and to be able to distinguish between the flagellar proteins to be exported and other proteins (Wilson & Beveridge, 1993). Furthermore, it might be expected that there is a kind of barrier in the flagellar basal body in order to maintain the

proton gradient across the cell membrane. This barrier might be the site where the export apparatus and the glycosylation machinery reside. Given the absence of glycosylation on the lateral flagellin (S. Moens, unpublished), the glycosylation mechanism must be localized only at the base of the polar flagellum.

In Archaea, flagellins are also glycosylated, but in this case it is assumed that the transport of flagellin resembles the leader peptide cleavage dependent transport of the type IV pilin-transport superfamily, rather than that of bacterial flagellins (Faguy *et al.*, 1994). The presence of a leader peptide was proven for *Methanococcus voltae* flagellins (Kalmokoff & Jarell, 1991). Alignment of N-terminal sequences of the other methanogen flagellins indicates that it is likely that they are also synthesized with a leader peptide (Kalmokoff *et al.*, 1992).

It was also shown that the *S. aurantia* flagellar filament surface proteins, FlaA, which have no sequence similarity with other bacterial flagellins, have a signal peptide (Brahamsha & Greenberg, 1989). To our knowledge, it is not yet known whether the different flagellins (FlaB) of this organism have signal peptides. However, for another spirochaete, *Treponema pallidum*, the FlaB proteins lack export-related signal peptides, while the FlaA proteins also have a typical export signal peptide (Penn & Luke, 1992).

Not much is known yet about the functional role of the glycosylation of Fla1, as is the case for the other known non-S-layer glycoproteins (Sandercock *et al.*, 1994). Suggestions have been made about the function of the glycosylation on the flagellins of *H. halobium* and *S. aurantia* in the swimming behaviour of these organisms. For most of the peritrichously flagellated bacteria like *Escherichia coli*, counterclockwise (CCW) rotation of the flagella results in a left-handed helical bundle forming of the flagella, and the cell moves forward. Clockwise (CW) rotation disturbs the three-dimensional structure of the flagella, the bundle disassembles, and the cell 'tumbles' and stays at the same site. Halobacterial flagella display other properties: when the right-handed flagellar bundle turns CW, it propels the cell forward, while a CCW-turning bundle pulls the cell (Alam & Oesterhelt, 1984). The glycosylation of the flagellin components could make the different flagella slide more smoothly to one another (Alam & Oesterhelt, 1984) so that the bundle is not disassembling when the turning of the flagella changes direction. Similarly, in *S. aurantia* the glycosylation could make the flagella (located in the periplasmic space) slide more smoothly against the membranes defining the periplasmic space (Southam *et al.*, 1990). However, glycosylation was only reported for flagellar core polypeptides. In liquid medium *A. brasilense*, however, seems to have a normal swimming behaviour for a monotrichously flagellated bacterium: CCW rotation of the filament pushes the cell forward while CW rotation causes a reorientation of the bacterium (Taylor & Koshland, 1974; Zhulin & Armitage, 1993).

Another possible function of the glycosylation could reside in the plant root adsorption of *A. brasilense*. Croes

et al. (1993) demonstrated that the polar flagellum of *A. brasilense* promotes adsorption to plant roots. A plant root lectin could bind the glycan chain(s) on Fla1 or the flagellin itself could act as a lectin recognizing plant sugars.

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