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Development and Application of Pathovar-Specific Monoclonal Antibodies That Recognize the Lipopolysaccharide O Antigen and the Type IV Fimbriae of *Xanthomonas hyacinthi*

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The objective of this study was to develop a specific immunological diagnostic assay for yellow disease in hyacinths, using monoclonal antibodies (MAbs). Mice were immunized with a crude cell wall preparation (shear fraction) from *Xanthomonas hyacinthi* and with purified type IV fimbriae. Hybridomas were screened for a positive reaction with *X. hyacinthi* cells or fimbriae and for a negative reaction with *X. translucens* pv. graminis or *Erwinia carotovora* subsp. *carotovora*. Nine MAbs recognized fimbrial epitopes, as shown by immunoblotting, immunofluorescence, enzyme-linked immunosorbent assay (ELISA), and immunoelectron microscopy; however, three of these MAbs had weak cross-reactions with two *X. translucens* pathovars in immunoblotting experiments. Seven MAbs reacted with lipopolysaccharides and yielded a low-mobility ladder pattern on immunoblots. Subsequent analysis of MAb 2E5 showed that it specifically recognized an epitope on the O antigen, which was found to consist of rhamnose and fucose in a 2:1 molar ratio. The cross-reaction of MAb 2E5 with all *X. hyacinthi* strains tested showed that this O antigen is highly conserved within this species. MAb 1B10 also reacted with lipopolysaccharides. MAbs 2E5 and 1B10 were further tested in ELISA and immunoblotting experiments with cells and extracts from other pathogens. No cross-reaction was found with 27 other *Xanthomonas* pathovars tested or with 14 other bacterial species from other genera, such as *Erwinia* and *Pseudomonas*, indicating the high specificity of these antibodies. MAbs 2E5 and 1B10 were shown to be useful in ELISA for the detection of *X. hyacinthi* in infected hyacinths.

Xanthomonas hyacinthi (ex Wakker 1883) sp. nov., nom. rev. from *Xanthomonas campestris* pv. hyacinthi (36) infects hyacinths and some related ornamental bulbous crops (14). The spread of an *X. hyacinthi* infection (yellow disease) can be a fast process (35) and can cause considerable economic loss for the hyacinth growers; for example, 710,000 hyacinth bulbs were condemned because of yellow disease in 1997. Consequently, a rapid diagnosis for the presence of *X. hyacinthi* in plant material is a prerequisite for taking decisive actions to prevent further spread of disease. *Xanthomonas* pathovars are difficult to distinguish, as they are almost identical in bacteriological and biochemical traits (7). Pathovars of *Xanthomonas* can be differentiated by their ability to infect certain host plants, often economically important plant crops. A reclassification study of the genus *Xanthomonas*, using phenotypic, chemotaxonomic, and genotypic approaches, showed that *X. hyacinthi* strains constitute a homogenous group with a genotype distinct from that of *X. campestris* (36).

Various reports describe the identification and detection of *Xanthomonas* species and pathovars by serological (2, 4, 5) and DNA-based (17, 18, 30) methods. The detection of plant pathogens with antisera is still the method of choice for many plant inspection services because of the relatively low costs and the presence of technical infrastructure based on automated enzyme-linked immunosorbent assays (ELISA). Therefore, we initiated a study of the surface antigens of *X. hyacinthi* for the development of a serological detection assay. There are several reports describing the production of monoclonal antibodies (MAbs) specific for *Xanthomonas* pathovars (2, 4, 5). Most of the strategies used involved immunization of mice with whole-cell preparations; however, raising antibodies to well-characterized antigens would have obvious advantages.

Recently, the existence of type IV fimbriae among xanthomonads has been reported (24, 34). These filamentous protein polymers have been shown to have antigenic properties as good as those of type IV fimbriae of other bacterial species, such as *Neisseria gonorrhoeae* and *Pseudomonas aeruginosa* (22, 31). Immunoblot experiments indicated that *Xanthomonas* pathovars showed variation in molecular mass of the fimbrial subunit (34). These findings suggested that *Xanthomonas* type IV fimbriae may contain unique determinants, as found for other type IV fimbriae, and that this multimeric surface antigen from *X. hyacinthi* might be suitable for MAb production. Other components of *Xanthomonas* known to have antigenic properties include the outer membrane proteins and lipopolysaccharide (LPS) (1–5), and it has been shown that variation in outer membrane proteins and LPS is correlated with the pathovar grouping of *X. campestris* (21, 23).

In this report, we obtained MAbs by immunizing mice with purified fimbriae and shear fractions of *X. hyacinthi* and analyzed them for application to phytodiagnostic purposes. The antifimbria MAbs reacted with different fimbrial epitopes. We found that the anti-LPS MAbs recognized the O antigen of

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X. hyacinthi S148. This antibody was found to be *X. hyacinthi* specific. The O antigen of S148 was partially characterized and shown to consist of rhamnose (Rha) and fucose (Fuc). The cross-reaction of these MAbs with all strains of *X. hyacinthi* tested showed that this Rha-Fuc O antigen is conserved within the species.

MATERIALS AND METHODS

Cultures and media. The bacterial strains used in this study are listed in Table 1. Bacterial cells were cultured on nutrient yeast agar (NYA; Difco Laboratories, Detroit, Mich.). Some *Xanthomonas* species and pathovars were grown on different media as prescribed by the LMG Culture Collection, Ghent, Belgium.

Cultivation and inoculation of hyacinths. The cultivars Anna Marie, Carnegie, Pink Pearl, and King of the Blues were maintained in a greenhouse with a day-night regimen of 12 h of light (25°C; relative humidity, 70%) and 12 h of darkness (10°C; relative humidity, 90%). Leaves were spray inoculated with bacterial suspensions (10^6 to 10^7 CFU/ml) or with phosphate-buffered saline (PBS) as a control. *X. hyacinthi* S148 and TV45 were used for these experiments. After 2 to 3 weeks, lesions were visible and leaf material was collected for experimental use.

Preparation of antigens and immunization of mice. Cell extracts were prepared by ultrasonication (Branson, Danbury, Conn.) of bacterial suspensions (10^9/ml), as previously described (34). Protein-free samples were prepared by incubation with 50 μg of proteinase K (Sigma, St. Louis, Mo.) per ml for 120 min at 60°C. A crude outer bacterial surface preparation was obtained by shearing cells of strain S148 as previously described (34) and concentrating to 0.5 mg of protein/ml in PBS. Two 6-week-old New Zealand mice were injected intraperitoneally with 0.25 ml of this preparation, mixed 1:1 with Freund's complete adjuvant (FCA), three times at 2-week intervals. Native fimbriae were isolated and purified from strain S148 (34) and concentrated to 0.5 and 0.1 mg/ml in PBS, respectively. The fimbrial preparations were mixed 1:1 with FCA, and from each preparation, 0.25 ml was injected intraperitoneally (three times with 2-week intervals) in two 6-week-old BALB/c mice. Two weeks after the third injection, the mice received a final booster injection with the same preparation but without FCA, and after 4 days, the spleens were collected.

Modification of protein antigens. Differential epitope recognition of the antifimbrial MAbs was visualized by immunoblotting proteolytic fragments of the fimbrial 17-kDa protein subunit following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). These fragments were obtained by partial proteolysis of the 17-kDa protein with γ-chymotrypsin (Sigma) using 1 U/μg of protein for 10 min at 25°C.

Hybridoma production. Hybridoma cells were produced by using equal amounts of the myeloma cell line Sp2/0-Ag-14 and spleen cells from the immunized mice which showed the highest titer in ELISA against fimbrial preparations or *X. hyacinthi* whole-cell preparations. Fusion and culturing of hybridoma cell lines were performed by the method of Boonekamp et al. (6). To prevent growth of yeast, 5 μg of amphotericin B (Fungizone; Imperial Laboratories Ltd., Andover, United Kingdom) was added per ml of medium. Selected hybridoma cultures were subcloned by the limiting dilution technique.

MAb production and isotyping. Hybridoma fluid samples were centrifuged for 10 min at 1,000 × *g* (Sigma 2-15 centrifuge, 4×25 ml rotor, 4°C) to remove cellular debris. Culture supernatants were used directly for immunological experiments or stored at −20°C. Immunoglobulin isotyping was performed with the Sigma Mouse Monoclonal Antibody Isotyping kit.

ELISA. The antigen-coated plate (ACP)-ELISA was used for the screening of positively reacting hybridoma supernatants. Polyvinyl chloride 96-well plates (Costar, Cambridge, Mass., and Greiner, Frickenhausen, Germany) were coated with 100-μl portions of bacterial suspension (approximately 10^8 bacterial cells/ml) in coating buffer (0.05 M carbonate-bicarbonate buffer [pH 9.6]) by drying in a 37°C ventilated incubator and stored at −20°C until used. In some experiments, microtiter plates were coated with purified fimbriae (3 μg/well). For direct antibody sandwich (DAS)-ELISA, plates were coated with gamma globulin, isolated from polyclonal rabbit antisera raised against whole cells of *X. hyacinthi* S148, with 0.1 μg of gamma globulin/well. Subsequently, bacterial dilutions or infected-hyacinth extracts were added and incubated at 20°C. ACP-ELISA and DAS-ELISA plates were blocked with 0.5% skim milk powder (Oxoid, Basingstoke, United Kingdom) in PBS with 0.5% Tween 20 (PBST), washed three times with PBST, and hybridoma supernatant (100 μl/well) was added. Following incubation for 2 h at 37°C, plates were washed, and goat anti-mouse alkaline phosphatase (American Qualex, La Miranda, Calif.) diluted 1:10,000 was added for 1 h at 37°C. Plates were washed with PBST and developed by adding substrate, consisting of *p*-nitrophenyl-phosphate in 10% (vol/vol) diethanolamine buffer, pH 9.8. Absorbance at 405 nm was measured with an Anthos Labtec ELISA reader after 1 and 2 h of incubation at 37°C. The positive-negative threshold of the ELISA was determined as three times the mean of the absorbance of PBS control samples or healthy hyacinth samples. In practice, this meant that absorbance values above 0.08 were considered positive.

LPS isolation. For preparation of LPS, the cell pellet obtained from a 5-liter culture of strain S148 was extracted by a modified hot phenol-water method (10) and the aqueous-phase material was fractionated by size exclusion chromatography on a Sephadex G-150 superfine column (Pharmacia, Uppsala, Sweden), as

TABLE 1. Bacterial strains used in this study

Species	Strain designation	Source[a]
Xanthomonas albilineans	887	LMG
Xanthomonas arboricola pv. pruni	852	LMG
Xanthomonas axonopodis pv. begoniae	241, 1925	NCPPB
Xanthomonas axonopodis pv. citri	682, 990	LMG
Xanthomonas axonopodis pv. dieffenbachiae	1104	IPO-DLO
Xanthomonas axonopodis pv. malvacearum	761, 996	LMG
Xanthomonas axonopodis pv. manihotis	784	LMG
Xanthomonas axonopodis pv. phaseoli	7488, 7455	LMG
Xanthomonas axonopodis pv. vasculorum	901	LMG
Xanthomonas axonopodis pv. vesicatoria	512	IPO-DLO
	901, 905, 910, 929	LMG
Xanthomonas axonopodis pv. vignicola	381	IPO-DLO
Xanthomonas campestris pv. campestris	43, 367, 372	IPO-DLO
	10, 11	NAKB
	173	LBO
	568	LMG
Xanthomonas campestris pv. fici	701	LMG
Xanthomonas campestris pv. gummisudans	732	LMG
Xanthomonas fragariae	708	LMG
Xanthomonas hortorum pv. pelargonii	7314	LMG
Xanthomonas hyacinthi	S148, S133, S171, S172, NN1, NAD55, TV43, HK60	LBO
Xanthomonas oryzae	630	LMG
Xanthomonas oryzicola	797	LMG
Xanthomonas populi	889	PD
	730	LMG
Xanthomonas translucens pv. cerealis	3213, 890	LMG
Xanthomonas translucens pv. graminis	726	LMG
	2700, 3041	NCPPB
Xanthomonas translucens pv. hordei	737	LMG
Xanthomonas translucens pv. phlei	3231	NCPPB
Xanthomonas translucens pv. poae	3230	NCPPB
	728	LMG
Xanthomonas translucens pv. translucens	2904	NCPPB
	876, 5263	LMG
Xanthomonas vasicola pv. holcicola	736	LMG
Xanthomonas vesicatoria	911, 920	LMG
	512	IPO-DLO
Agrobacterium tumefaciens	85	LMG
Bacillus subtilis	7135	LMG
Erwinia amylovora	2024	LMG
Erwinia carotovora subsp. *carotovora*	550	LBO
	2417	LMG
Erwinia chrysanthemi	108	LBO
	2488	LMG
Erwinia cytolytica	75	LBO
Erwinia rhapontici	164	LBO
Klebsiella oxytoca	3055	LMG
Pseudomonas aeruginosa	1242	LMG
Pseudomonas fluorescens	2434	PD
Pseudomonas marginata	570	LBO
Pseudomonas syringae pv. syringae	1247	LMG
Stenotrophomonas maltophilia	958	LMG
Xylophilus ampelinus	5856, 523	LMG

[a] Abbreviations: LMG, Bacteria Collection Laboratorium Microbiologie, Universiteit Gent, Ghent, Belgium; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden Laboratory, Harpenden, Hertsfordshire, United Kingdom; IPO-DLO, Research Institute for Plant Protection, Wageningen, The Netherlands; NAKB, Inspection Service for Floriculture and Arboculture, Roelofarendsveen, The Netherlands; LBO, Bulb Research Centre, Lisse, The Netherlands; PD, Plant Protection Service, Wageningen, The Netherlands.

TABLE 2. MAbs obtained after immunization with purified fimbriae (15) and partly purified LPS (8) from *X. hyacinthi* S148

MAb	Isotype	Reactivity of MAbs in[a]:			
		Immunoblot	ELISA	IF	Immuno-EM
Group 15					
12G9	IgG1	+	−	−	−
8C11	IgG2a	++	−	ND	−
6A3	IgG1	+	−	ND	−
5D12	IgG3	+	−	+	−
3B7	?[b]	+	+/−	ND	−
1A7[c]	IgG1	+	−	+	−
6C9[c]	IgM	+	−	+	+
5G8[c]	IgG1	+	−	ND	+
9A2	?	+/−	−	+	+
Group 8					
2E5	IgM	+	++	+	+
1B10	IgG1	+	++	+	−
3E10	IgG3	+	++	+	−
6A1	IgG1	+	++	+	−
6A12	IgG2b	+	++	+	−
9H3	IgG1	+	++	+	−
12G7	IgG3	+	++	+	−
2D10	IgG1	+	++	+	−

[a] IF, immunofluorescence labeling experiments; immuno-EM, immunoelectron microscopy. Intensity of the reaction: ++, strong reaction; +, medium reaction; +/−, weak reaction; −, no reaction; ND, not determined.
[b] ?, no distinct isotype.
[c] Weak cross-section with *X. translucens* pv. hordei and pv. translucens.

previously described (26). The eluted fractions were assayed colorimetrically for 3-deoxy-D-manno-2-octulosonic acid (Kdo) by the thiobarbituric acid assay (38) and for hexose with phenol-sulfuric acid (40); LPS-containing fractions were identified by PAGE analysis.

PAGE and immunoblot analyses. LPS samples were analyzed by deoxycholic acid PAGE, using 18% acrylamide gels. The gels were either silver stained for LPS (32) or Alcian blue-silver stained for possible K antigens (26). For immunological analysis of LPS samples, polyacrylamide gels were blotted to Nytran+ membranes (Schleicher and Schuell, Keene, N.H.) with a Trans-Blot SD apparatus (Bio-Rad). Fimbriae or other protein samples were analyzed by SDS-PAGE, using 12% Laemmli gels (15). Immunoblotting of separated protein samples was performed as previously described (34). In some experiments, a slot blot apparatus (Miniblotter 16; Immunetics, Cambridge, Mass.) was used for simultaneous testing of several antisera for recognition of blotted antigens.

Processing of leaf tissue samples of hyacinths. Inoculated areas of hyacinth leaves were excised (1 cm^2; average wet weight of 60 mg) and immersed in 1-ml portions of PBST in small plastic bags. Homogenization of the samples was performed with a hand homogenizer (Bioreba AG, Basel, Switzerland). Subsequently, the plant extracts were serially diluted in antibody-coated microtiter plates and processed as described above.

Immunofluorescence experiments. Samples of hyacinths infected with *X. hyacinthi* and samples of bacteria were incubated on microscope slides and examined as previously described (34). For fluorescence labeling, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse polyvalent immunoglobulins (immunoglobulin A [IgA], IgG, and IgM) and goat anti-rabbit IgG (Sigma) were used.

Glycosyl residue analysis. Sugar composition analysis was performed by gas chromatography-mass spectrometry of the trimethylsilyl methyl glycoside derivatives (40), using a 30-m DB1 fused silica column (J&W Scientific, Folsum, Calif.) on a 5890A GC-MSD (Hewlett-Packard, Palo Alto, Calif.). Inositol was used as an internal standard, and retention times were compared to those of authentic monosaccharide standards.

^{1}H NMR analyses. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was performed with a Brüker AM 250 apparatus. The samples were dissolved in ^2H_2O, and the spectra were obtained at 308 K. Chemical shifts were established relative to acetone.

Immunoelectron microscopy. Incubation of *X. hyacinthi* cells on Formvar-carbon-coated nickel grids with antisera was performed as previously described (34). MAbs were used in dilutions of 1:5 or 1:10 in PBS. Goat anti-mouse IgG and IgM, conjugated with 10-nm-diameter gold particles (Biocell Laboratories International, Cardiff, United Kingdom), was used as the secondary antibody. After counterstaining with phosphotungstic acid and air drying, grids were examined with a Philips transmission electron microscope type 201 or CM100, each operated at 60 kV.

RESULTS

Selection of MAbs specific for *X. hyacinthi*. Immunizations with LPS and fimbrial fractions yielded numerous hybridoma cell lines. About 60 clones reacted positively with *X. hyacinthi* samples in ELISA or immunoblot analyses. Subsequent screening by ELISA and in immunoblotting experiments of the hybridoma clones for negative reactions with *Erwinia carotovora* subsp. *carotovora* 550 and *X. campestris* pv. graminis LMG 726 identified 15 clones which recognized exclusively *X. hyacinthi* antigens (Table 2).

Specificities of the MAbs. Two distinct groups of MAbs were identified: group 8 MAbs, resulting from immunization with a shear fraction of *X. hyacinthi* S148, reacted strongly in ACP-ELISA with *X. hyacinthi* isolates. Group 15 MAbs, resulting from immunization with fimbriae, reacted strongly with a protein band in immunoblotting but only weakly with whole-cell antigens in ACP-ELISA. The MAbs belonged to the immunoglobulin classes IgG1, IgG3, IgG2a, IgG2b, and IgM (Table 2).

To assess whether the MAbs reacted with a polysaccharide component or a protein epitope, *X. hyacinthi* cells were sonicated and incubated with or without proteinase K. Next, these extracts were separated by SDS-PAGE and blotted onto nitro-

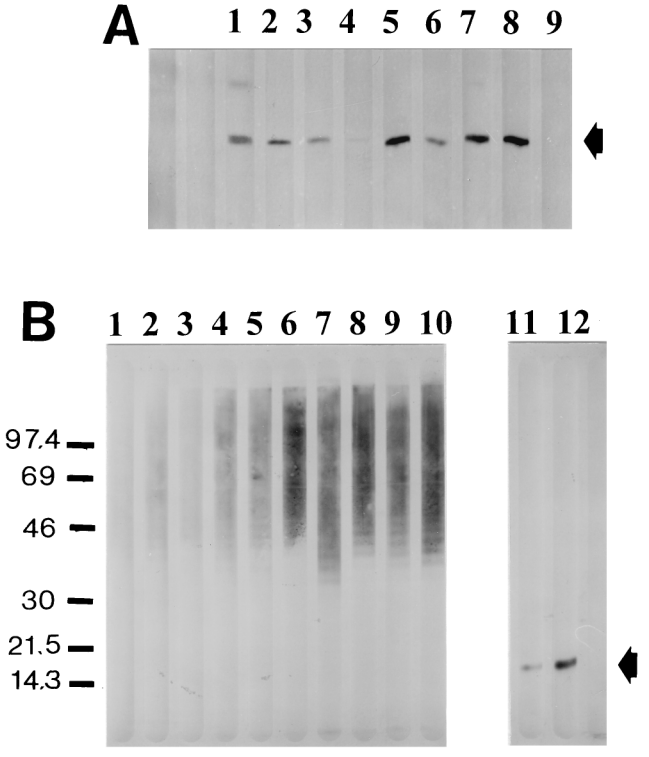

FIG. 1. Recognition of fimbriae from *X. hyacinthi* S148 after separation of sonicated cells by SDS-PAGE and immunoslot blotting by group 15 MAbs. MAbs are 3B7 (lane 1), 1A7 (lane 2), 6A3 (lane 3), 5G8 (lane 4), 6C9 (lane 5), 5D12 (lane 6), 8C11 (lane 7), 12G9 (lane 8), and normal (preimmune) mouse serum (lane 9). The position of the 17-kDa fimbrial subunit is indicated by the arrow. (B) Reactions of group 8 and 15 MAbs on immunoslot blots with sonicated *X. hyacinthi* S148 cells after incubation with protease K (lanes 1 to 10) or without protease K treatment (lanes 11 and 12) and subsequent separation by SDS-PAGE. Antibodies are MAb 6C9 (lane 1), polyclonal rabbit IgG raised against the 17-kDa fimbrial subunit (lane 2), MAb 12G9 (lane 3), MAb 9H3 (lane 4), MAb 3E10 (lane 5), MAb 2D10 (lane 6), MAb 6A12 (lane 7), MAb 12G7 (lane 8), MAb 1B10 (lane 9), MAb 2E5 (lane 10), MAb 6C9 (lane 11), and MAb 12G9 (lane 12). The position of the fimbrial subunit is indicated by the arrow. The positions of molecular size standards (in kilodaltons) are shown to the left of the blot.

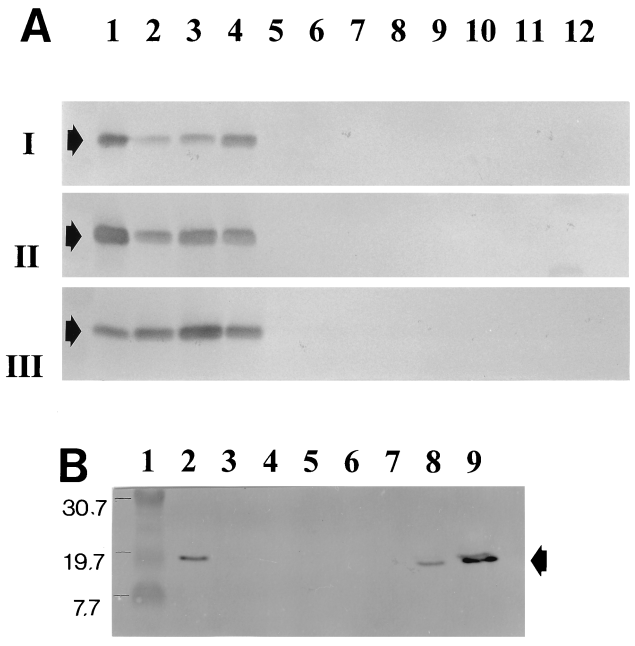

FIG. 2. Immunoblotting analysis of sonicated bacterial cell fractions with group 15 antifimbrial MAbs. (A) Lanes 1 to 4, *X. hyacinthi* S148, TV43, NAD55, and S133, respectively; lane 5, *X. fragariae*; lane 6, *X. vasicola* pv. holcicola; lane 7, *X. populi*; lane 8, *X. axonopodis* pv. citri; lane 9, *X. hortorum* pv. pelargonii; lane 10, *X. axonopodis* pv. phaseoli; lane 11, *X. oryzae*; lane 12, *X. vesicatoria*. The immunoblots were developed after incubation with MAb 8C11 (I), 12G9 (II), or 5D12 (III). The position of the 17-kDa fimbrial subunit in each blot is indicated by an arrow. (B) Immunoblot of *X. translucens* pathovars, developed with MAb 6C9. Lane 1, marker proteins (positions [in kilodaltons] shown to the left of the blot]); lane 2, *X. translucens* pv. translucens LMG 876; lane 3, *X. translucens* pv. phlei LMG 730; lane 4, *X. translucens* pv. poae NCPPB 3230; lane 5, *X. translucens* pv. translucens LMG 5263; lane 6, *X. translucens* pv. cerealis LMG 679; lane 7, *X. translucens* pv. graminis LMG 726; lane 8, *X. translucens* pv. hordei LMG 737; lane 9, *X. hyacinthi* S148. The position of the fimbrial subunit is indicated by the arrow.

cellulose membranes. Analyses of the immunoblots showed that the group 15 MAbs reacted with the 17-kDa fimbrial subunit protein (Fig. 1A). The group 8 MAbs yielded a ladder-like pattern, suggesting that the reaction was with a polysaccharide component of *X. hyacinthi* (Fig. 1B).

A large group of *Xanthomonas* pathovars and other bacterial species (Table 1) were analyzed with the MAbs in ACP-ELISA and in immunoblotting experiments to test the specificity of the two panels of MAbs. No cross-reaction of the group 8 MAbs was found with any other bacterial isolates; they reacted solely with the *X. hyacinthi* isolates in ELISA. Importantly, all strains of *X. hyacinthi* were recognized by the group 8 MAbs. The 17-kDa fimbrial subunit of the *X. hyacinthi* strains was recognized by the group 15 antibodies in immunoblotting experiments (Fig. 2 and Table 2). However, only *X. translucens* pv. hordei (LMG 737) and *X. translucens* pv. translucens (LMG 876) reacted in immunoblots with MAb 6C9 (Fig. 2B). These proteins also reacted weakly with MAbs 1A7 and 5G8 (not shown), but the other MAbs showed no visible reaction with the 17-kDa fimbrial subunit protein of these pathovars.

Recognition of antigens by group 8 and group 15 MAbs. The group 15 MAbs were tested with different immunological techniques (Table 2). All nine MAbs reacted with the fimbrial subunit of *X. hyacinthi* isolates in immunoblotting experiments, but not with *X. translucens* pv. graminis. MAb 12G9 showed a relatively weak reaction, suggesting that it recognized a different subunit epitope than MAb 8C11. The group 15 MAbs reacted weakly in a DAS-ELISA with *X. hyacinthi* cells. When applied to immunofluorescence labeling, the group 15 MAbs 5D12, 1A7, 9A2, and especially MAb 6C9 showed polar labeling of individual cells; fluorescent fimbrial filaments could be visualized under the microscope (Fig. 3A). The group 15 MAbs were also used for immunogold localization studies using electron microscopy (Fig. 4B to D). A distinct tagging of the fimbriae from strain S148 with gold particles was found for MAbs 6C9, 5G8, and 9A2. Some unlabeled fimbriae were also visible in Fig. 4C. The fimbriae of the other strains of *X. hyacinthi* (Table 1) were labeled with gold as well (not shown).

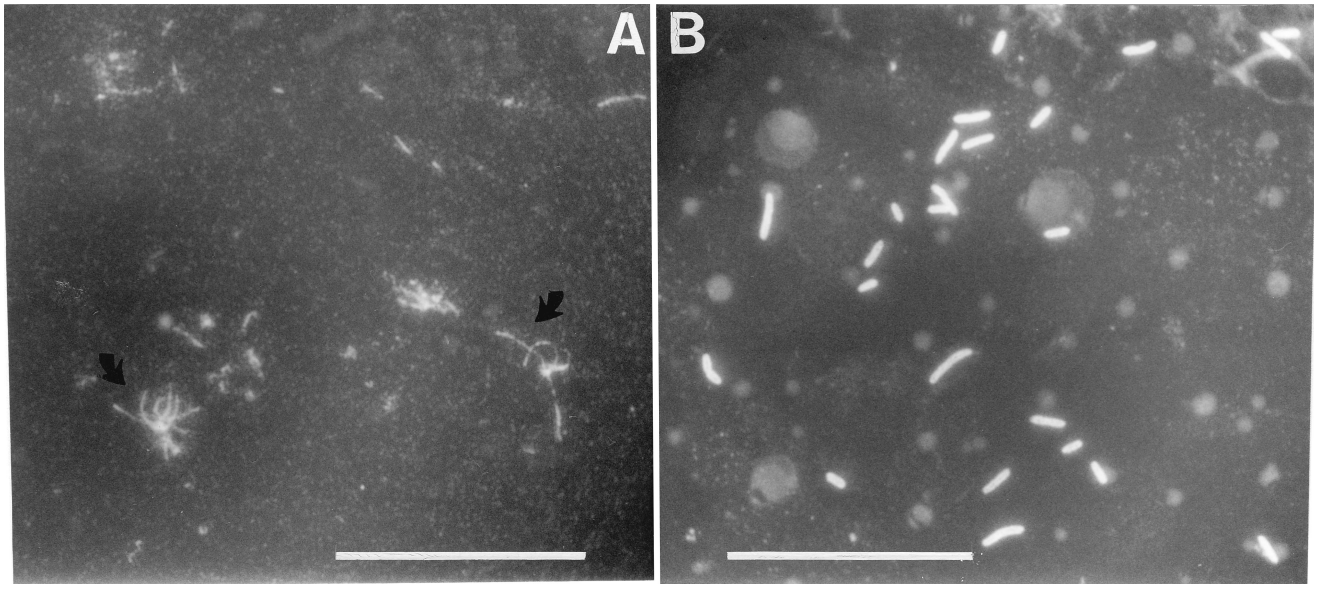

FIG. 3. Fluorescent *X. hyacinthi* cells resulting from indirect FITC labeling after incubation with MAbs. (A) For cells incubated with MAb 6C9, fluorescent fimbrial strands are visible (arrows). (B) Cells incubated with MAb 2E5 are labeled all around. Bars, 15 μm.

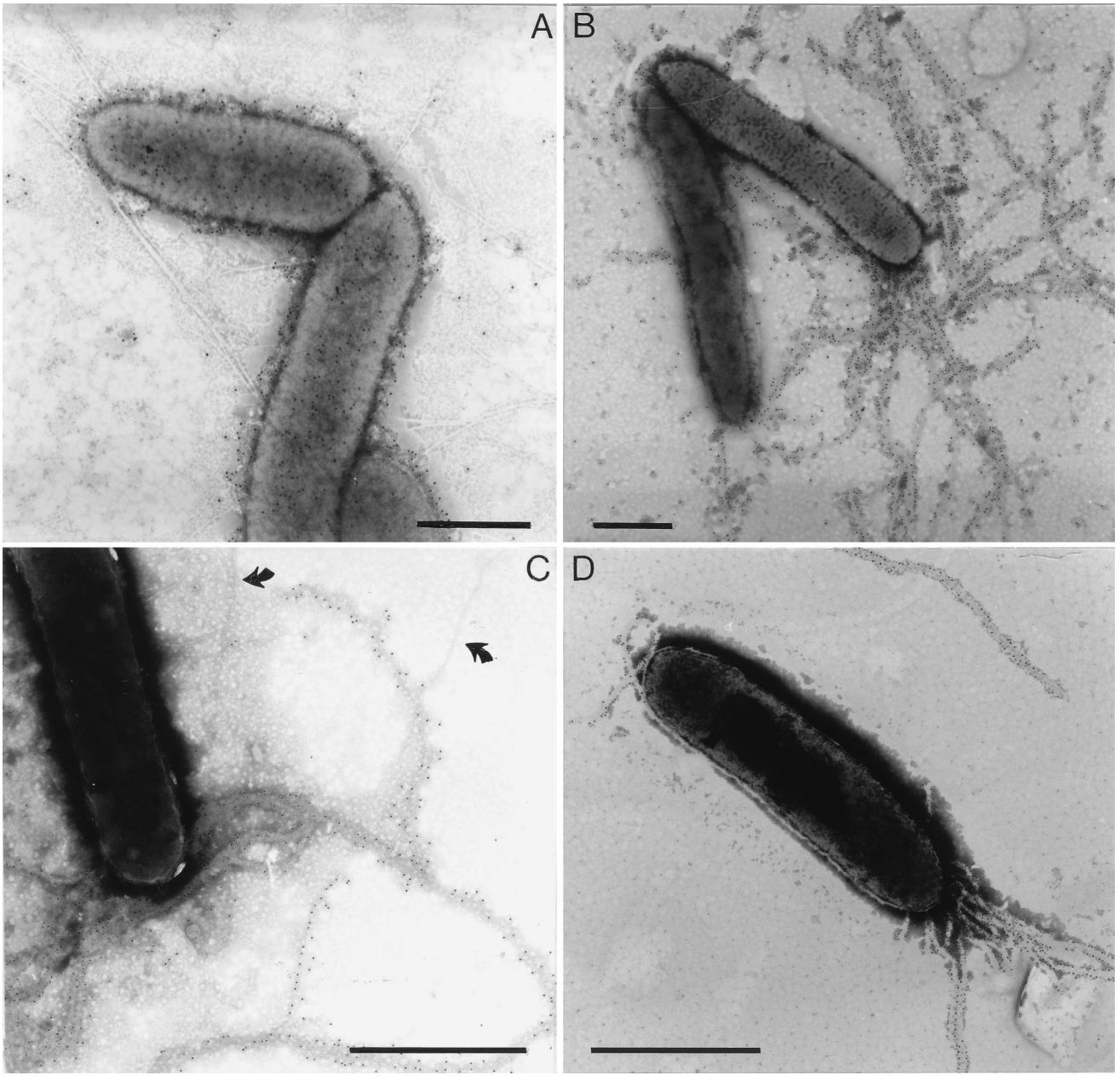

FIG. 4. Electron microscopy of immunogold-labeled cells of *X. hyacinthi* S148. The bacterial surface structures were labeled with 10-nm-diameter gold-conjugated anti-mouse IgG and IgM after incubation with MAbs 2E5 (A), 6C9 (B), 9A2 (C), and 5G8 (D). Panels B to D show fimbrial structures that are gold tagged; arrows indicate unlabeled fimbriae (C). In panel A, the cell surface LPS is labeled. Bars, 1 μm.

MAb 5D12 labeled only certain parts of the fimbriae but not the tips (not shown).

For further assessment of the differences in recognition sites of group 15 MAbs, attempts were made to localize the epitopes on the 17-kDa fimbrial subunit, by using partial proteolysis with γ-chymotrypsin (Fig. 5). MAbs 12G9 and 1A7 recognized only the intact 17-kDa fimbrial subunit, whereas MAbs 6C9, 8C11, and 5D12 recognized other, smaller peptide fragments as well. Polyclonal rabbit antiserum against the 17-kDa fimbrial subunit recognized multimers of the fimbrial subunit in immunoblotting experiments (Fig. 5, lane 9).

Compared to group 15 MAbs, the group 8 MAbs reacted differently in the immunological tests. Using ELISA, relatively high titers were found with most group 8 MAbs, especially with MAbs 2E5 and 1B10 (Fig. 6); however, only MAb 2E5 labeled the cell surface in immunoelectron microscopy of the *X. hyacinthi* cells (Fig. 4A), and the gold particles appeared to bind closely to the surface (the fimbriae were not recognized). When applied to immunofluorescence labeling experiments, the group 8 MAbs labeled *X. hyacinthi* cells, resulting in a halo of green light around the cell (Fig. 3B). Most members of the group 8 MAbs reacted on immunoblotting (Table 2 and Fig. 1B); the low-mobility ladder-like signal suggested the recognition of an LPS component of *X. hyacinthi*.

Antigen characterization. Polysaccharides were extracted from S148 cells and analyzed by SDS-PAGE and Alcian blue-silver staining to characterize the antigen recognized by group 8 MAbs (Fig. 7A, lane 1). Two major components were found

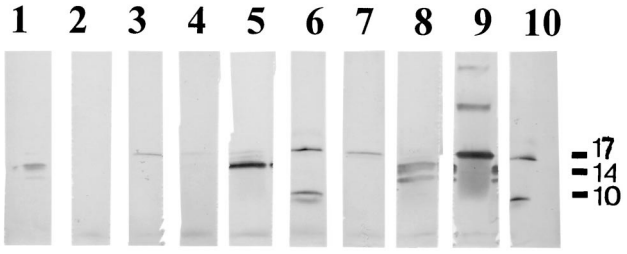

FIG. 5. Immunoblotting analysis of protease-treated fimbrial subunits. Purified fimbriae of *X. hyacinthi* S148 were treated with γ-chymotrypsin. Subsequently, SDS-PAGE, immunoblotting, and incubation were performed. The following antifimbrial antibodies were used: MAb 9A3 (lane 1), MAb 2E5 (lane 2), MAb 3B7 (lane 3), MAb 1A7 (lane 4), MAb 6A3 (lane 5), MAb 5D12 (lane 6), MAb 12G9 (lane 7), MAb 6C9 (lane 8), polyclonal rabbit antiserum against the 17-kDa fimbrial subunit (lane 9), and MAb 8C11 (lane 10). The positions of markers are depicted in kilodaltons to the right of the blot.

in the extract. The high-mobility banding component is probably rough LPS (R-LPS); the low-mobility component showing a ladder pattern (region I) is probably the result of a sequential degree of polymerization of structurally constant repeating units, which could be from capsular antigens or smooth LPS (S-LPS) (9, 25). The fact that both components were visible without the Alcian blue prestain (not shown) suggested that the ladder pattern was the result of the presence of S-LPS (26). A portion of the preparation was subjected to polymyxin chromatography, which specifically binds LPS. The ladder pattern material was bound by the polymyxin, which also indicated that region I is due to S-LPS (data not shown).

Immunoblotting analyses confirmed that MAb 2E5 recognized only the region I material, and not the R-LPS (Fig. 7B, lane 1). To demonstrate that there was no physical impediment to the binding of the low-molecular-weight R-LPS, a polyclonal antiserum that recognizes common epitopes in *Xanthomonas* spp. was employed (Fig. 7C). A distinct binding of the R-LPS was demonstrated. We concluded that MAb 2E5 was specific for the region I component.

Purification and structural analyses of the LPS component, recognized by MAb 2E5. A crude polysaccharide preparation was fractionated by size exclusion chromatography which resulted in two major pools that were analyzed by SDS-PAGE (Fig. 7A, lanes 2 and 3). This showed that the chromatography

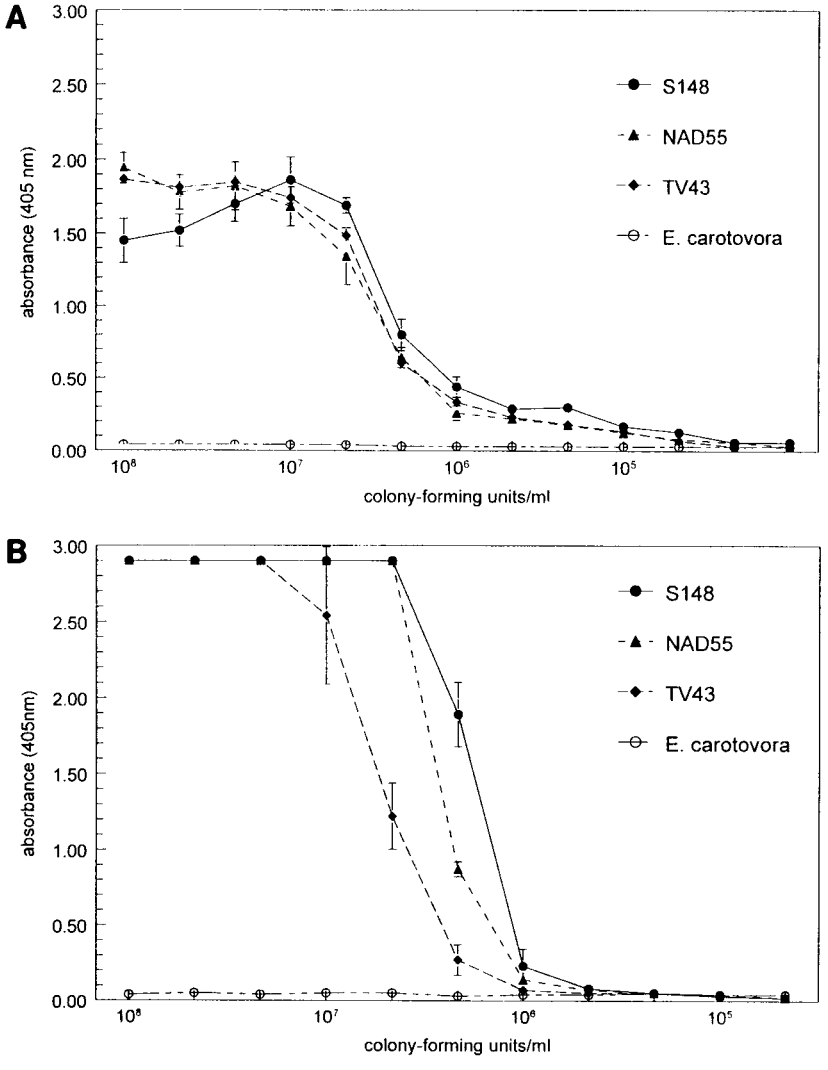

FIG. 6. Titration of MAbs 2E5 (A) and 1B10 (B) in ELISA, using total cell preparations of *X. hyacinthi* S148, NAD55, and TV43 and *Erwinia carotovora* subsp. *carotovora* as the target. All MAb dilutions were tested in triplicate. The error bars represent the standard errors of the means.

completely separated the R-LPS from the region I material. Both were analyzed by immunoblotting (Fig. 7B, lanes 2 and 3). As expected, a ladder pattern in region I was found. In contrast, there was no recognition of the highly mobile R-LPS. Both components were then analyzed for glycosyl residue composition and by ^{1}H NMR spectroscopy (Fig. 8).

The glycosyl residue composition of the R-LPS, as determined by gas chromatography-mass spectrometry analysis of the trimethylsilyl methyl glycosides, showed a predominance of mannose (Man), glucose (Glc), galacturonic acid (GalA), and *N*-acetylglucosamine (GlcNAc) in a 1:1:1:1 molar ratio. The latter sugar is probably a component of lipid A, so the core region would consist of the Man, Glc, and GalA. Minor amounts of Rha and Fuc were also detected. There was no Kdo or heptose (Hep) detected by this method. Neither Kdo nor Hep is present in the LPS of this strain, or they are modified in some way that does not allow detection by this method. The R-LPS also contained the β-OH fatty acids that are common components of LPS. Although a lack of standards precludes a molar comparison to the glycosyl components, the fatty acids were abundant and detected in a 1:1 ratio of 3-OH--C-12 and 3-OH--C-13, with minor amounts of 3-OH--C-14. ^{1}H NMR analysis of the R-LPS showed the signals that arise from the fatty acids (Fig. 8A). The signals at 1.2 to 1.5 ppm are due to the various CH_2 protons, and the signal at 0.8 ppm is from the terminal CH_3. The resonances from the carbohydrate protons are found between 3.4 and 4.4 ppm, and the two sharp resonances at 1.95 and 2.05 ppm indicate the presence of acetyl groups on some of the glycosyl residues. Although poorly resolved (due to the poor solubility of the sample), the resonances at 1.8 and 2.4 ppm indicate that there is, in fact, some Kdo associated with the LPS core. In contrast to the R-LPS, the low-mobility ladder pattern material contained predominantly Rha and Fuc (>95% of total carbohydrate) in a 2:1 molar ratio. However, the fatty acids and GlcNAc of lipid A, as well as the core glycosyl components, were also present in the region I preparation. This demonstrated that MAb 2E5 is specific for the O antigen of the LPS. The NMR spectrum (Fig. 8B) showed that the signals associated with the C-6 methyl protons of the Rha and Fuc are found at 1.4 and 1.15 ppm, respectively, and those of the anomeric region are found at 4.6 to 5.4 ppm. A minor resonance at 0.8 ppm is due to the terminal methyl protons of the fatty acids, and the CH_2 resonances are obscured by the Rha and Fuc signals. The 2:1 molar ratio of Rha to Fuc is supported by the relative areas of the C-6 methyl proton resonances; however, it is not clear from the present data if the O antigen is comprised of linear or branched trisaccharide repeats.

In conclusion, all data indicated that the ladder pattern is a result of the sequential degree of polymerization of O antigen in the S-LPS. Due to the fact that MAb 2E5 did not recognize the R-LPS, we can conclude that the epitope is the O antigen, which consists of Rha and Fuc.

Sensitivity of MAbs and application in routine detection of yellow disease. To determine the lowest detection threshold for *X. hyacinthi*, microtiter plates were coated with serially diluted bacterial cells (ACP-ELISA). The range of healthy background was 0.00 to 0.02; which differed significantly (data not shown) from the lower limits of the ELISA. MAb 2E5 could detect as little as 10,000 CFU/100 μl; the detection limit of MAb 1B10 was about 85,000 CFU/100 μl (Fig. 6B). For routine detection of hyacinth plants suspected to be infected, leaf extracts were processed as described above and tested in DAS-ELISA with MAbs 2E5 and 1B10; the detection limits were about 20,000 CFU/100 μl (33×10^5 CFU per g of hyacinth leaf) and 100,000 CFU/100 μl (17×10^6 CFU per g of hyacinth leaf), respectively. When used in immunofluorescence labeling experiments, MAb 2E5 could detect approximately 1,000 cells/ml of hyacinth leaf extract.

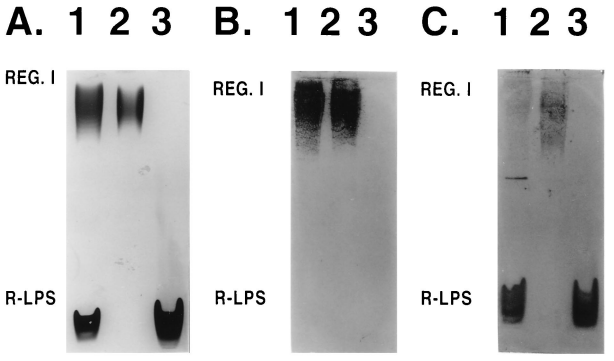

FIG. 7. Alcian blue-silver stained SDS-polyacrylamide gels (A) of crude (lanes 1) and purified (lanes 2 and 3) polysaccharide preparations of *X. hyacinthi* S148 and corresponding immunoblots, developed after incubation with MAb 2E5 (B) and polyclonal rabbit anti-*Xanthomonas* antiserum (C). REG. I, region I.

DISCUSSION

In a previous study of *X. hyacinthi* fimbriae, polyclonal antisera raised against preparations of native or denatured fimbriae of *X. hyacinthi* recognized common epitopes of fimbriae and LPS from other xanthomonads (34). Although the signal in ELISA or immunoblots was considerably weaker than signal obtained with *X. hyacinthi* strains, these polyclonal sera cannot be used for routine detection purposes. The results of this study showed that two panels of anti-*X. hyacinthi* MAbs recognize two defined surface antigens: type IV fimbriae and LPS O antigen. The MAbs recognizing the O antigen of *X. hyacinthi* showed no cross-reaction with any other xanthomonads (Table 1). This was important, as we have occasionally isolated other pathovars from hyacinth plants, including *X. translucens* pv. graminis (33).

Pathovar- or species-specific MAbs have been made against several xanthomonads, including *X. campestris* pv. pelargonii, pv. begoniae, and pv. oryzicola. The antibodies recognizing these pathovars have been shown or suggested to react with LPS or other cell surface polysaccharides (4, 5). We found that the epitope recognized by anti-*X. hyacinthi* MAb 2E5 is the LPS O antigen. Although there was no reaction of MAb 2E5 with the R-LPS, composition analysis showed that the region I material contained typical lipid A fatty acid and the same sugars found in the R-LPS. We also found that the O antigen from *X. hyacinthi* S148 consists of rhamnose and fucose in a 2:1 molar ratio, which is different from the structurally characterized O antigen of *X. campestris* pv. campestris NCPPB 45. The latter has a complex hexasaccharide repeating structure consisting of rhamnose, galactose, and galacturonic acid in a molar ratio of 4:1:1 (8). There are other *Xanthomonas* pathovars (pv. pelargonii, malvacearum, and vasculorum) that produce fucose-containing LPSs (23, 37). However, these bacteria must have epitopes that are immunologically from those of *X. hyacinthi*, since no cross-reaction was found with O-antigen-specific MAbs or antifimbrial MAbs. All strains of *X. hyacinthi* were recognized by MAb 2E5, indicating that the O antigen is not strain specific. This is in contrast to what has been found for enteropathogens, such as *Escherichia coli* or *Salmonella* spp., which produce highly variable O antigens (39). Reports on the occurrence of *X. hyacinthi* in the field are (with a few

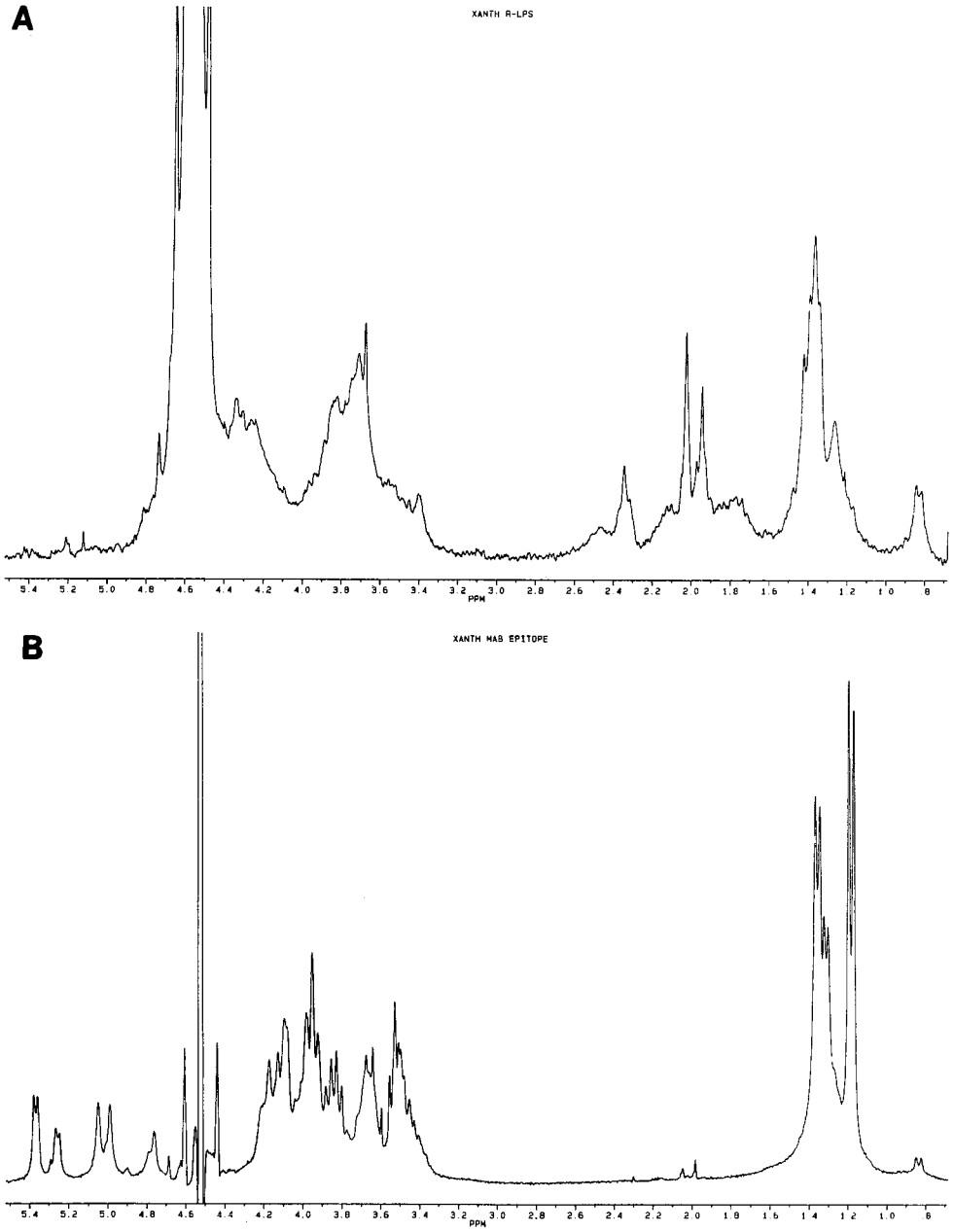

FIG. 8. ^{1}H NMR spectra of the R-LPS (A) and S-LPS (B) of *X. hyacinthi* S148.

exceptions) restricted to The Netherlands, where by far the greater part of hyacinth culture is situated (35). Recently, *X. hyacnthi* was reclassified as a new *Xanthomonas* species, based on biochemical characteristics and its high G+C content (69%) of DNA (36). The collection of *X. hyacinthi* isolates used in this study were isolated from sites separated in time, place, and host plant. The isolates showed differences in growth rate, production of extracellular polysaccharides, and rate of pathogenicity in hyacinths (33).

Analysis of *X. hyacinthi* S148 R-LPS core indicated the presence of Kdo and equimolar amounts of mannose, glucose, and galacturonic acid. The presence of low levels of Kdo is in agreement with the finding that the LPS of *Xanthomonas sinensis* contains one Kdo per LPS molecule (38). In addition, the core composition of *X. hyacinthi* S148 is generally similar to that of *Xanthomonas sinensis* (19) and, interestingly, the R-LPSs of *Sinorhizobium* spp. (27, 28). Future studies may determine whether the LPS core regions of these bacterial species are structurally related.

Fimbriae were only recently described for *Xanthomonas* (24, 34), and this is the first report of MAbs that recognize these filamentous protein structures. Most antifimbria (group 15) MAbs showed good reaction when applied in immunoblotting experiments, in contrast to their low titer in DAS-ELISA. Immunization of mice with purified type IV fimbriae from *X. hyacinthi* S148 resulted in nine MAbs. Three of these MAbs showed only weak cross-reactivity with *X. translucens* pv. hordei and pv. translucens, both of which were isolated from *Hordeum vulgare* (barley). The type IV fimbriae from *X. hyacinthi* and *X. translucens* might have common features and bind

to compatible leaf surface receptors of these monocots. The type IV fimbriae are expressed by a number of bacterial genera, and many aspects of their structure and immunological properties have been described (29). The fimbrial subunit of type IV fimbriae has a conserved N-terminal amino acid sequence and an immunodominant variable central and C-terminal domain. The specificities of the antifimbrial MAbs described here suggest that these parts are recognized by the MAbs. Type IV fimbriae are found and expressed by a number of *Xanthomonas* species and their pathovars (24, 34). A strategy of raising MAbs against type IV fimbriae of other *Xanthomonas* species and their pathovars might be feasible. Another approach is the production of polyclonal sera against synthetic peptides, homologous to the variable and immunodominant domains of the fimbriae (12, 22), resulting in diagnostically applicable antibodies.

A drawback for using type IV fimbriae as the target antigen might be the existence of phase variation or antigenic variation as described for *N. gonorrhoeae* and *Moraxella bovis* (13, 20). However, these phenomena were not found for any of the isolates of *X. hyacinthi* under study. The presence of fimbria-like structures that are not recognized by the antifimbrial MAbs (Fig. 4C) indicates the existence of at least one other type of fimbriae. The expression of different fimbriae in the genus *Xanthomonas* has been detected with *X. campestris* pv. vesicatoria mutants (24). The attachment of *X. hyacinthi* and its type IV fimbriae to stomata of hyacinth leaves (34) suggests a role for these surface antigens in the first stages of yellow disease. Attachment could be inhibited by incubation with antifimbria Fab fragments (34). The adherence of *Pseudomonas aeruginosa* to receptors on human epithelial cells can be blocked by MAbs binding to the C-terminal region of the fimbrial subunit located at the tip (11, 16). As the *X. hyacinthi* antifimbria MAbs showed variation in epitope recognition (Fig. 5), we are currently investigating the application of these MAbs in inhibition experiments of fimbrial attachment.

Importantly, six of the antifimbrial MAbs and in particular the anti-LPS MAbs may be used for the specific detection of *X. hyacinthi*. MAbs 2E5 and 1B10 are currently being tested by the Bulb Inspection Service of The Netherlands. The sensitivity under laboratory conditions of MAb 2E5 is limited to 20,000 CFU/100 μl; under routine conditions, the detection limit in hyacinth leaf extract is close to 50,000 CFU/100 μl. In most cases, this is sufficient to detect the presence of *X. hyacinthi* in the infection sites; the application of these MAbs in immunofluorescence labeling experiments results in higher sensitivity of the detection of this bacterium. However, the use of the MAbs for routine detection with DAS-ELISA is preferred by most inspection services, due to the fast and automated logistics developed over the last decade.

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