Development of Specific Recombinant Monoclonal Antibodies Against the Lipopolysaccharide of *Ralstonia solanacearum* Race 3

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ABSTRACT

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Recombinant single-chain antibodies (scFvs) against the lipopolysaccharide of *Ralstonia solanacearum* (biovar 2, race 3) were successfully selected by phage display from a large combinatorial antibody library. Characterization with regard to cross-reaction and use in routine immunoassays showed that the selected antibodies had improved characteristics when compared with the polyclonal antiserum that is currently used for brown rot

diagnosis of potato in the Netherlands. The isolated monoclonal scFvs reacted in both enzyme-linked immunosorbent assay (ELISA) and immunofluorescence cell staining with all race 3 strains tested, but with only some strains belonging to other races. Furthermore, only a few cross-reactions with saprophytic bacteria, which also cross-reacted with polyclonal antisera, were observed. Using ELISA, one of the recombinant antibodies detected as few as 5×10^3 bacteria in potato tuber extracts. Therefore, this antibody is potentially useful for detection of *R. solanacearum* race 3.

Additional keywords: bacterial wilt, *Burkholderia solanacearum*, *Pseudomonas solanacearum*.

Ralstonia solanacearum is the causal organism of bacterial wilt and can infect over 450 different plant species including many economically important crops (7). The genus *R. solanacearum* (formerly *Pseudomonas solanacearum*) can be classified into three races (designated according to host-range specificity) or grouped into five biovars on the basis of utilization of disaccharides and hexose alcohols. Race 3, nearly synonymous with biovar 2, forms a homogeneous group (26), and its host range is restricted to potato, eggplant, and tomato. Race 3 is more adapted to temperate regions and is responsible for recent outbreaks of potato brown rot disease in different countries in Europe (27).

Quarantine measures have been taken in many countries to minimize the risk of introduction and spreading of the potato brown rot pathogen. Serological techniques such as enzyme-linked immunosorbent assay (ELISA), immunofluorescence cell staining (IF), and immunofluorescence colony staining (IFC) are commonly used to monitor the presence of the pathogen in potatoes and in the environment. In general, they are considered to be a good compromise between sensitivity and specificity of detection, and between ease and expense of application (5,22,25).

So far, only polyclonal antibodies (PAb) against whole or glutaraldehyde-fixed cells of *R. solanacearum* race 3 have been used in certification and risk assessment programs (5,11). However, the occurrence of false-positive reactions due to the presence of cross-reacting saprophytic bacteria in the extracts is considered a serious drawback to the use of PABs. In IF, up to 3% false-positive reactions were recorded (11), while in ELISA, the frequency of false-positive reactions can sometimes exceed this level (J. G. Elphinstone, *personal communication*). Consequently, positive reactions need to be followed by confirmation assays, for which often tedious and time-consuming bioassays have to be used.

In contrast to PABs, hybridoma-derived monoclonal antibodies (MAbs) recognize only one epitope and, thus, a higher level of specificity is feasible. Attempts to produce MAbs against *R. solanacearum* were successful (1,2,8,22). However, these MAbs were not developed for brown rot diagnosis in potatoes and have limited usefulness in this regard. The species-specific immunoglobulin M MAb Ps1, raised by Alvarez et al. (1,2), reacted with extracellular polysaccharide-I in a very sensitive immunoassay (17), but failed to react with nonmucoid mutants (2) that can still be pathogenic (31). Moreover, MAb Ps1 is not useful for IFC in agarose plates, because its large molecular mass (900 kDa) reduces gel diffusion and slows down staining and washing procedures. The MAbs developed by He (8) reacted with races 1 and 2, but not with race 3 strains (8). Strain-specific MAbs that do not cross-react with the most closely related species (*R. picketti*, *R. syzygii*, *Burkholderia cepacia*, or the banana blood disease bacterium [BBD]) were developed successfully by Robinson-Smith et al. (22). However, these MAbs were not useful for certification of potato propagation material, because they did not react with 11 of 23 *R. solanacearum* strains tested, including two race 3 strains. In addition, they did not react in IF techniques, and a 100-fold decrease in sensitivity was observed in ELISA when PABs were replaced by MAbs.

The difficulties mentioned above in developing a *R. solanacearum* race 3-specific MAb that is suited for potato brown rot diagnosis using ELISA, IF, and IFC techniques might be caused by inefficient exploitation of the immune repertoire in the hybridoma technique. Although the available (mouse) antibody repertoire is estimated to include over 10^8 different antibodies, usually only a few thousand different hybridoma clones are obtained, of which, on the average, less than 1% produce antigen-binding antibodies. Thus, the probability of selecting MAbs of sufficient specificity and affinity for use in a diagnostic assay is low.

These diversity and efficiency problems were solved by recent advances in molecular immunology (Fig. 1). Forced cloning of antibody variable heavy (V_H) and light (V_L) chain genes by reverse-transcription polymerase chain reaction (RT-PCR) (20,24) allowed

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amplification of the DNA encoding the antibody repertoire. Expression of variable antibody fragments (Fv) in *Escherichia coli* as single-chain Fv (scFv) molecules was enabled by coupling V_H and V_L domains with a flexible peptide linker (10). Cloning of a pool of scFv-encoding genes, in which the V_H and V_L domains were randomly combined, allowed the generation of large combinatorial antibody libraries. Today combinatorial libraries of over 10¹⁰ different antibodies have been constructed (6,30). This, in combination with the display of functional antigen-binding fragments on the tips of filamentous phage (9,16), created a powerful system to select specific MAbs. It was shown that this phage-display system (Fig. 2) allowed direct selection (panning) of rare specificities from combinatorial antibody libraries through successive rounds of phage growth and selection for antigen binding without the need for immunization (6,30), i.e., without the use of experimental animals.

In this study, the versatility of the phage-display system was challenged for the selection of *R. solanacearum*-specific MAbs. To achieve this, phages derived from a large naive human combinatorial antibody library (30) were panned against purified lipopolysaccharides (LPS) of *R. solanacearum*, and the expressed scFvs were characterized by ELISA and IF.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* strains used for the isolation of recombinant antibodies were TG1 (K12, $\Delta(lac-pro)$, *supE*, *thi*, *hsdD5* / F' *traD36*, *proA*⁺*B*⁺, *lacI*^f, *lacZ* Δ M15) for selection of specific phage antibodies and HB2151 (K12, *ara*, $\Delta(lac-pro)$, *thi*/F' / *proA*⁺*B*⁺, *lacI*^f Δ M15) for production of soluble scFvs.

Strains of *R. solanacearum* and strains of bacteria that cross-reacted with polyclonal antisera to *R. solanacearum* were pre-

served at -80°C on beads (Protect Bacterial Preservers; Biotrading Benelux, Wilnis, the Netherlands) in 8 mg of 15% glycerin per ml of LabLemco broth (CM15; Oxoid, Hampshire, United Kingdom). For each experiment, bacteria were grown for 48 to 72 h at 27°C on plates of Trypticase soy broth (30 g/liter; BBL Microbiology Systems, Becton Dickinson, Cockeysville, MD), and prior to use, were transferred to slopes of growth factor agar (2.3 mM K₂HPO₄, 0.2 mM MgSO₄·7H₂O, 1.7 mM NaCl, 4.3 mM NH₄H₂PO₄, 5 mM glucose, 0.5% [wt/vol] yeast, and 1.5% [wt/vol] agar, pH 7.2) and grown for 24 h at 27°C. Characterization of cross-reacting bacteria by fatty acid analysis using the Microbial Identification System (Microbial ID, Newark, DE) was done by J. G. Elphinstone (Central Science Laboratory, York, United Kingdom).

Purification of LPS. The purification of LPS from *R. solanacearum* (strain 1609 race 3, biovar 2) was performed according to De Weger et al. (4).

Panning procedure. Selection of phage antibodies (PhAbs, phages expressing functional scFvs on their surface) from the human combinatorial antibody library was performed according to Vaughan et al. (30) with a few modifications. Immunosorbent tubes (Maxi-sorb; Nunc, Roskilde, Denmark) were coated with 500 µg of LPS (125 µg/ml in 50 mM NaHCO₃, pH 9.8) for 18 h at 4°C. The tubes were washed twice with phosphate-buffered saline (PBS) and blocked with PBM-2% (PBS containing 2% skimmed milk powder) for 30 min at room temperature. Simultaneously, 2 ml of a stock derived from the Vaughan library (30) containing 2.5 × 10¹³ PhAbs was mixed with 2 ml of PBM-4% and preincubated for 30 min. After removing the blocking solution from the tubes and washing with PBS, 4 ml of PhAbs was added to the tubes. PhAbs were allowed to bind to LPS for 30 min on a roller bench and for another 90 min without rotation. Free PhAbs were removed by wash-

Fig. 1. Outline of antibody engineering. **A**, The minimal requirements of an antibody for antigen recognition are located in the antigen-binding site (variable antibody fragment [Fv]), which is formed by the V_H and V_L domains of the heavy chain and light chain, respectively. **B**, Forced cloning of the V_H and V_L genes is achieved through amplification of synthesized cDNA by reverse-transcription polymerase chain reaction using sets of specific primers. The variability is determined by the source of the mRNA, which can vary from a hybridoma to blood- or spleen-derived B-lymphocytes. **C**, The V_H and V_L domain DNAs, joined by a linker peptide encoding a DNA fragment (L), are inserted into a phagemid vector as a single-chain Fv segment (scFv). In this phagemid, the scFv-encoding DNA is preceded by an inducible promoter (P), a ribosomal binding site (RBS), and a signal peptide (Sp), which directs the produced scFvs towards the periplasm. At the C-terminus, the scFv protein is genetically fused to a His(6)-tag, which can be used for purification, and to a c-myc-tag, which is used for detection in immunoassays. **D**, In the scFv-encoding vector, the location of the ampicillin resistance gene (AMP), the origin of replication for *Escherichia coli* (*E. coli*-ori), and the packaging signal (M13-ori), which is required for phage assembly, are indicated. Depending on the *E. coli* strain used, the scFvs can be expressed as soluble proteins in HB2151 bacteria, wherein translation halts at the amber stop codon. Fusion proteins of scFv with the minor coat protein (G3p) of filamentous phage fd are obtained from TG1 bacteria in which translation proceeds through the amber codon.

ing the tubes 10 times with PBS containing 0.1% Tween-20 (PBST) and another 10 times with PBS to remove the detergent. Bound PhAbs were eluted by adding 1 ml of 0.1 M triethylamine (TEA) and incubation for 10 min on a roller bench. After collecting the TEA eluent, the pH was neutralized by the addition of 0.5 ml of 1 M Tris-HCl, pH 7.4. One milliliter of the eluted PhAbs was used to infect 5 ml of *E. coli* TG1 bacteria (or HB2151 in the final panning round), which were freshly grown to an optical density at 600 nm (OD_{600}) of 0.5 in 2 $\times$ tryptone yeast (2TY) broth (23). After infection for 30 min in a water bath at 37°C without shaking to allow optimal infection, the *E. coli* cells were pelleted (3,000 $\times$ g for 10 min) and resuspended in 1 ml of 2TY broth containing 100 μ g of ampicillin and 2% glucose (2TYB-AMP-2%Glu) per ml. To establish the number of eluted PhAbs, 50 μ l was taken from this suspension, and serial dilutions were plated on 2TY agar plates containing 100 μ g of ampicillin and 2% glucose (2TYA-AMP-2%Glu) per ml. The remaining 950 μ l was plated separately on 225 $\times$ 225-mm 2TYA-AMP-2%Glu plates, and the bacteria were grown for 18 h at 30°C.

This procedure was carried out four times in succession using PhAbs prepared as described below for the second, third, and fourth panning round. To increase the specificity and reduce the background, the stringency was increased after the second panning round. The amount of antigen that was used for coating was then decreased from 125 to 10 μ g/ml in the third round and to 1 μ g/ml in the final panning round. Simultaneously, the stringency was further enhanced by increasing the number of washings from 20 times after the second panning round to 40 times in the subsequent rounds of selection.

Preparation of PhAbs for subsequent panning rounds. The bacteria derived from a previous panning round were scraped from the plate (225 $\times$ 225 mm) and resuspended in 15 ml of 2TYB-

AMP-2%Glu. From these bacteria, 14.5 ml was used to prepare a freezer stock (22), 500 μ l was used to inoculate 50 ml of 2TYB-AMP-1%Glu, and the bacteria were grown at 37°C with shaking (250 rpm). When an OD_{600} of 0.5 was reached, 10^{11} helper phages (M13K07; Pharmacia Biotechnology, Inc., Uppsala, Sweden) were added (multiplicity of infection = 20). The 15-ml tube containing the mixture was put in a water bath at 37°C without shaking to allow for optimal infection. After 30 min, the bacteria were pelleted (2,100 $\times$ g for 10 min) and resuspended in 25 ml of 2TYB-AMP and 25 μ g of kanamycin per ml. The bacteria were transferred to a 250-ml Erlenmeyer flask and grown for 18 h at 30°C with shaking (250 rpm). The 25-ml overnight culture was harvested, and the bacteria were removed by centrifugation (2,100 $\times$ g for 20 min). The PhAbs in the supernatant were precipitated by adding 5 ml of 20% polyethylene glycol 6000 and 2.5 M NaCl and thoroughly mixed for 1 h at 4°C. The precipitated PhAbs were pelleted (2,100 $\times$ g for 20 min) and resuspended in 1 ml of sterile PBS. Usually 5×10^{12} PhAbs per ml were produced, as assayed by infection of strain TG1 and plating for ampicillin resistance. The derived PhAbs were stored at 4°C until use in the next panning round or in phage ELISA.

MvaI fingerprinting. To analyze the diversity of the selected PhAbs, restriction fragment length polymorphism (RFLP) fingerprinting was performed on PCR-amplified scFv DNA. Single colonies were picked and grown for 4 h in 2TYB-AMP-2%Glu. From these suspensions, 2 μ l was taken and added to a 48- μ l PCR mix containing 2.5 μ M dNTPs, 0.25 U of Super Taq DNA polymerase (HT Biotechnology, Cambridge, United Kingdom), 10 μ M forward primer (5'-AGG AAA CAG CTA TGA CCA TGA TTA CGC CAA G-3'), 10 μ M reverse primer (5'-GCC CAA TAG GAA CCC ATG TAC CGT AAC ACT G-3'), 2 mM $MgCl_2$, and

Fig. 2. Schematic representation of the selection of lipopolysaccharide (LPS)-specific single-chain variable antibody fragments (scFvs) from a combinatorial antibody library using phage display. Helper phages, which contain the entire phage genome and a kanamycin-resistant gene, but lack an efficient packaging signal (M13-ori), are used to 'rescue' phagemids from a combinatorial antibody library. When both helper phage (kanamycin resistance) and phagemid (ampicillin resistance) are present within the same bacterium and the bacteria are cultured under selective pressure of both ampicillin and kanamycin, phage antibodies (PhAbs) are assembled that are phage particles that carry fusion proteins of scFv and minor coat protein (G3p) on their surface and contain the scFv-encoding phagemid vector. Consequently, within PhAbs, the genotype and the phenotype are linked! In order to select for antigen specificity, PhAbs rescued from a combinatorial antibody library are allowed to bind to immobilized LPS (panning). Washing removes the PhAbs that lack affinity for LPS. Bound PhAbs are eluted, and the selected PhAbs are enriched by sequential rounds of panning until the desired affinity is obtained.

50 mM Tris-HCl, pH 8. In a thermal cycler (Perkin Elmer Cetus, Norwalk, CT), 25 cycles (1 min at 94°C and 2 min at 72°C) were performed. From the PCR mix, 20 µl was added to 37.5 µl of H₂O and 6.5 µl of buffer H (Boehringer GmbH, Mannheim, Germany). After mixing, 10 U of *MvaI* (Boehringer GmbH) was added, and the mixture was incubated for 18 h at 37°C. The *MvaI* digestion patterns were analyzed on a 3% FMC Metaphor agarose gel (Episcience Technologies, Madison, WI).

Production and purification of scFvs. *E. coli* HB2151 was cultured according to Kerschbaumer et al. (12) and cells were pelleted. After incubation (5 min at 0°C) of the bacteria with 1/20 volume (referring to the original culture size) of 50 mM Tris-HCl buffer, pH 8, containing 30% sucrose and 1 mM EDTA, the scFvs were extracted (45 min at 0°C) from the periplasm with 1/20 volume (referring to the original culture size) of 5 mM MgSO₄. Secreted scFvs were purified from the periplasmic fraction using immobilized metal affinity chromatography (IMAC) according to Lindner et al. (15).

Routine protocol for the extraction of potato tubers. Potato extracts were made according to the legislation of the European Community (97/647/EG). Briefly, maceration buffer (4.26 g of Na₂HPO₄ and 2.72 g of KH₂PO₄ [50 mM PO₄] per liter of distilled water, pH 7.0) was added until 200 heel ends were fully submerged. The sample was incubated for 18 h at 6°C with shaking (75 rpm) and then passed through a 40- to 100-µm filter. Filtrate was collected after washing the filter with maceration buffer and centrifuged for 15 min at 10,000 × g at 6°C. The pellet was resuspended in 1 ml of 10 mM phosphate buffer (2.7 g of Na₂HPO₄·12H₂O per liter and 0.4 g of NaH₂PO₄·2H₂O per liter, pH 7.2). Glycerin was added to a final concentration of 15%, and samples were stored at -70°C until use.

ELISA. A phage ELISA was used to assess the specificity of the PhAbs after each round of panning according to standard methods (3,28), in which the plates were washed four times with PBST between each incubation step. Briefly, a 96-well microtiter plate was coated with LPS (2 µg/ml in 0.1 M NaCO₃) for 2 h at 37°C at 100 µl per well. After blocking with PBSTM-5% (PBST containing 5% skimmed milk powder) at 200 µl per well, the plates were incubated for 1 h with PhAbs in PBSTM-1% at 100 µl per well. After that, the plates were incubated with anti-M13 MAbs at 100 µl per well (MAb 12E4 diluted to 5 µg/ml in PBSTM-1%)

and finally with rat anti-mouse PABs conjugated to alkaline phosphatase (RaAM-AP; Jackson Immuno Research Laboratories, Inc., Westgrove, PA) diluted 1:2,000 in PBSTM-1%. The reaction with LPS was visualized by incubation with *p*-nitrophenyl phosphate substrate (*p*-NPP).

An indirect ELISA was used to assess the specificity of the recombinant scFvs according to standard methods (3,28), in which the plates were washed three times with PBST between each incubation step. Briefly, a 96-well microtiter plate was coated, either with LPS (2 µg/ml in 0.1 M NaCO₃) or with bacteria (OD₆₀₀ of 0.1 in 0.1 M NaCO₃), for 2 h at 37°C at 100 µl per well. After blocking with PBSTM-5% at 200 µl per well, the plates were incubated for 1 h with PABs PcA-9523 (a polyclonal antiserum that was produced against whole cells of *R. solanacearum* race 3 strain 1609 diluted 1:2,000 in PBSTM-1%; PAB-ELISA [ELISA in which detection of *R. solanacearum* is based on polyclonal antibodies]); or scFvs (c-myc-ELISA [ELISA in which binding of scFvs to *R. solanacearum* is detected through detection of the c-myc-tag {18} that is expressed on the C-terminus of the scFv {Fig. 1C}]) diluted in PBSTM-1% at 100 µl per well. After that, the plates were incubated at 100 µl per well with goat anti-rabbit PABs conjugated to alkaline phosphatase (diluted 1:2,000 in PBSTM-1%; PAB-ELISA;

Fig. 4. Characterization of four anti-lipopolysaccharide (LPS) clones by *MvaI* restriction fragment length polymorphism fingerprinting of the single-chain variable antibody fragments (scFv)-encoding DNA: lane 1, anti-LPS 3; lane 2, anti-LPS 8; lane 3, anti-LPS 12; and lane 4, anti-LPS 13.

A

B

Fig. 3. Recovery and specificity of lipopolysaccharide (LPS)-binding phage antibodies (PhAbs) in four sequential rounds of panning. PhAbs were applied to LPS-coated tubes and allowed to bind. Bound PhAbs were eluted after washing and used to infect *Escherichia coli*. After phage rescue with helper phage, the PhAbs (anti-LPS enriched) were used for a new round of panning. **A**, The number of applied and recovered PhAbs was counted, and the recovery was plotted for each subsequent panning round. **B**, A phage ELISA was done with equal amounts of the selected anti-LPS PhAbs and compared with comparable amounts of control PhAbs (derived from a panning against another antigen) to show that the binding was LPS specific.

Sigma Chemical Co., St. Louis) or anti-c-myc MABs (MAb 9E10 [18] diluted to 1 µg/ml in PBSTM-1%; c-myc-ELISA [9]) and finally with RaAM-AP diluted 1:2,000 in PBSTM-1%. The reaction with LPS was visualized by incubation with p-NPP.

The detection limits of the scFvs and polyclonal antiserum PcA-9523 for *R. solanacearum* were determined with an indirect double-antibody sandwich ELISA, in which the plates were washed three

Fig. 5. Reaction of purified monoclonal single-chain variable antibody fragments (scFv) (anti-LPS 3, anti-LPS 8, anti-LPS 12, and anti-LPS 13) with lipopolysaccharide (LPS) in enzyme-linked immunosorbent assay (ELISA). ELISA plates were coated with LPS derived from *Ralstonia solanacearum*. scFvs were applied and the binding to LPS was detected by incubations with anti-c-myc monoclonal antibodies, rat anti-mouse polyclonal antibodies conjugated with alkaline phosphatase, and para-nitrophenylphosphate.

times with PBST between each subsequent incubation step. Briefly, ELISA plates (intermediate binding capacity, cat. no. 655001; Greiner Labortechnik GmbH, Frickenhausen, Germany) were coated with PAb PcA-9523 (diluted 1:2,000 in 0.1 M NaCO₃) at 100 µl per well for 2 h at 37°C. After blocking with PBSTM-5% for 30 min at 200 µl per well, the plates were incubated with 10-fold serial dilutions of *R. solanacearum* bacteria (diluted in either PBS or potato tuber extract) at 100 µl per well for 2 h at 37°C. Subsequently, the plates were incubated with either PcA-9523-AP conjugate (diluted 1:2,000 in PBM) or with scFv anti-LPS 12 (diluted to 5 µg/ml

TABLE 1. Comparison of single-chain variable antibody fragments (scFv) anti-lipopolysaccharide (LPS) 12 and polyclonal antiserum PcA-9523 for their detection limits for *Ralstonia solanacearum* race 3 in enzyme-linked immunosorbent assay (ELISA)

<i>R. solanacearum</i> concentration (CFU/ml)	ELISA substrates ^a			
	p-NPP (OD ₄₀₅ value)		FDP (relative fluorescence)	
	anti-LPS 12 ^b	PcA-9523 ^c	anti-LPS 12 ^d	PcA-9532 ^d
4 × 10 ⁵	2.8 ^e	2.9	3.3	2.9
4 × 10 ⁴	0.86	0.9	0.7	2.5
4 × 10 ³	0.21	0.42	0.14	0.83
4 × 10 ²	0.15	0.35	0.10	0.69
0	0.16	0.20	0.09	0.68

^a Cells of strain 1609 were suspended in phosphate-buffered saline and tested at different concentrations. The reactions were visualized using the chromatogenic substrate *p*-nitrophenyl phosphate substrate (p-NPP) or the fluorogenic substrate fluorescein diphosphate (FDP).

^b Incubation time with substrate was 120 min.

^c Incubation time with substrate was 40 min.

^d Incubation time with substrate was 80 min.

^e The data were obtained from a representative experiment and the standard deviations (*n* = 2) were smaller than 0.1 for all presented data. ELISA signals that exceeded at least threefold the background value were regarded as a positive response.

Fig. 6. **A**, Immunofluorescence staining of whole *Ralstonia solanacearum* bacteria showing that a surface epitope is recognized by single-chain variable antibody fragment (scFv) anti-lipopolysaccharide (LPS) 12 (12.5 µg/ml) and **B**, a Western blot showing that the epitope that is recognized is located on the LPS of *R. solanacearum*. Purified LPS, derived from *R. solanacearum* (tracks 1 and 3) and *R. picketti* (tracks 2 and 4) was blotted onto nitrocellulose and stained with silver (Bio-Rad Laboratories) or with scFv anti-LPS 12 (tracks 3 and 4), respectively.

in PBM-2%) at 100 µl per well for 1 h at 37°C. This was followed by incubations with anti-myc MAb 9E10 (diluted to 1 µg/ml in PBM-2%) and RaAM-AP (diluted 1:2,000 in PBM-2%) at 100 µl per well for 1 h. The reactions were visualized by incubation with either p-NPP or with fluorescein diphosphate (FDP) substrate. Optical densities at 405 nm were measured with an ELISA reader and fluorescence was measured at 510 nm with a fluorometer (ABI PRISM 7700 sequence detection system, Perkin-Elmer Applied Biosystems, Foster City, CA).

Western blotting. Purified LPS (0.5 µg per lane) was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes as described by van der Wolf et al. (29). Reactive groups on the membrane were blocked with PBMT-5%. The reactivity of the scFv towards LPS was shown by incubations of the blot with anti-LPS scFv, anti-c-

myc MAb 9E10 (diluted to 10 µg/ml in PBM-2%), RaAM-AP (diluted 1:2,000 in PBM-2%), and 5-bromo-4-chloro-3-indoylphosphate/nitroblue tetrazolium chloride substrate.

IF. IF was performed according to van der Wolf et al. (29). For IF, the bacteria were coated on microscope slides and incubated with anti-LPS scFv (diluted to 12.5 to 125 µg/ml in PBS) and anti-c-myc MAb 9E10 (diluted to 30 µg/ml in PBS), which was conjugated with fluorescein isothiocyanate.

RESULTS

Selection of LPS-binding clones from a combinatorial antibody library. From the first panning round in which 2.5×10^{13} PhAbs (expressing 1.4×10^{10} different scFvs) were applied to the LPS-coated immunosorbent tube, approximately 1.5×10^6 PhAbs were recovered (Fig. 3A). In the subsequent rounds of selection, enrichment factors of 100- to 1,000-fold were obtained as the stringency during the selection was gradually increased. The increased efficiencies of recovery during panning were paralleled by increasing signals in phage ELISA (Fig. 3B). This result indicates that phages binding to LPS were enriched during the selection procedure.

Characterization of monoclonal LPS-binding scFv. Four bacterial clones, which produced scFvs with high activity in the c-myc-ELISA (designated anti-LPS 3, anti-LPS 8, anti-LPS 12, and anti-LPS 13), were further characterized. The variability within the scFv genes was established using RFLP of PCR-amplified DNA. Four different *MvaI* restriction patterns were observed (Fig. 4). Although the patterns of anti-LPS 12 and anti-LPS 13 looked quite similar, nucleotide DNA sequencing of plasmid DNA revealed that these scFvs were different (data not shown). The four different scFvs were purified by IMAC. Anti-LPS 3 could only be purified in low amounts; the maximum yield was 50 µg/liter, and SDS-PAGE indicated that the bulk of this was degraded, as it did not have the expected molecular mass of 27 kDa (data not shown). This was in contrast to the scFvs anti-LPS 8, 12, and 13, which yielded up to 1.5, 3, and 2.5 mg of intact scFvs per liter, respectively.

TABLE 2. Values obtained in an enzyme-linked immunosorbent assay (ELISA) with single-chain variable antibody fragments (scFv) anti-lipopolysaccharide (LPS) 12 against *Ralstonia solanacearum* race 3

<i>R. solanacearum</i> concentration (CFU/ml)	ELISA substrates (units) ^a			
	p-NPP (OD ₄₀₅) ^b		FDP (relative fluorescence) ^b	
	Pure culture	Tuber extract	Pure culture	Tuber extract
5×10^5	>3 ^c	>3	3.1	2
5×10^4	1.4	1.1	1.9	1.6
5×10^3	0.44	0.44	0.51	0.31
5×10^2	0.33	0.36	0.19	0.18
0	0.28	0.31	0.11	0.1

^a Different concentrations of *R. solanacearum* bacteria strain 1609 were suspended in phosphate-buffered saline (pure culture) or in a potato tuber extract, and the detection limits were assayed with a double-antibody sandwich ELISA. The reactions were visualized using the chromatogenic substrate *p*-nitrophenyl phosphate substrate (p-NPP) or the fluorogenic substrate fluorescein diphosphate (FDP).

^b Incubation time with substrate was 120 min.

^c The data describe a typical experiment and the standard deviations ($n = 2$) were smaller than 0.1. ELISA signals that exceeded at least threefold the background value were regarded as a positive response.

TABLE 3. Comparison of the reactivity of monoclonal single-chain variable antibody fragments (scFv) (anti-lipopolysaccharide [LPS] 12) and polyclonal anti-serum (PcA-9523) with *Ralstonia solanacearum* strains in enzyme-linked immunosorbent assay (ELISA) and immunofluorescence cell staining (IF)

Strain ^a	Country where isolated	IPO number ^b	Original number	Race	Biovar	ELISA ^c		IF ^d	
						scFv	PcA	scFv	PcA
<i>R. solanacearum</i>	Brazil	1657	PD1414	1	1	—	+	—	nd
<i>R. solanacearum</i>	Costa Rica	1653	PD507	1	3	+	+	+	+
<i>R. solanacearum</i>	Panama	1655	PD1255	1	3	—	+	—	nd
<i>R. solanacearum</i>	France	1656	PD1258	1	3	—	+	—	nd
<i>R. solanacearum</i>	France	1669	PD1256	1	3	—	+	—	nd
<i>R. solanacearum</i>	Indonesia	1670	PD278	1	3	—	+	+	nd
<i>R. solanacearum</i>	Hawaii	1654	PD508	1	4	—	+	—	nd
<i>R. solanacearum</i>	Japan	1658	PD1419	1	4	+	+	+	+
<i>R. solanacearum</i>	Panama	1665	PD1445	2	1	—	+	—	nd
<i>R. solanacearum</i>	Costa Rica	1666	PD1446	2	1	—	+	—	nd
<i>R. solanacearum</i>	Egypt	933	PD445	3	2	+	+	+	+
<i>R. solanacearum</i>	Netherlands	1608	PD2762	3	2	+	+	+	+
<i>R. solanacearum</i>	Netherlands	1609	PD2763	3	2	+	+	+	+
<i>R. solanacearum</i>	Netherlands	1610	PD2764	3	2	+	+	+	+
<i>R. solanacearum</i>	Egypt	1661	PD426	3	2	+	+	+	+
<i>R. solanacearum</i>	India	1662	PD1254	3	2	+	+	+	+
<i>R. solanacearum</i>	France	1663	PD1260	3	2	+	+	+	+
<i>R. solanacearum</i>	Chile	1664	PD1408	3	2	+	+	+	+
<i>R. solanacearum</i>	Netherlands	1676	PD2822	3	2	+	+	+	+
<i>R. solanacearum</i>	Netherlands	1677	PD2828	3	2	+	+	+	+
<i>R. solanacearum</i>	Netherlands	1678	PD2831	3	2	+	+	+	+
<i>R. solanacearum</i>	United Kingdom	1681	PD2474	2	2	+	+	+	+
<i>R. solanacearum</i>	United Kingdom	1684	PD2477	2	2	+	+	+	+
<i>R. solanacearum</i> ?	Unknown	1652	NIZO96	—	—	—	+	nd	nd
<i>R. solanacearum</i>	France	1711	1310	+	+	+	+	+	+
<i>R. solanacearum</i>	France	1712	1361	+	+	+	+	+	+

^a Strains were obtained from J. D. Janse (Plant Protection Service, Wageningen, the Netherlands) and A. C. Le Roux (INRA, Rennes, France).

^b IPO = Institute of Plant Protection, Wageningen, the Netherlands.

^c ELISA signals: — = reaction below background; and + = strong reaction (at least threefold above background).

^d IF signals: — = no fluorescence; + = strong fluorescence; and nd = not done.

Purified scFvs anti-LPS 8, 12, and 13 gave high signals when tested in c-myc-ELISA with purified LPS (Fig. 5) and with *R. solanacearum* cells (data not shown), whereas a weak reaction was found with scFv anti-LPS 3. Similar results were obtained with IF. The scFvs anti-LPS 8, 12, and 13, but not scFv 3, gave bright fluorescent staining of *R. solanacearum* at 12.5 µg/ml (Fig. 6A) and brilliant fluorescence at 125 µg/ml (data not shown). It was confirmed that the scFvs indeed reacted with LPS of *R. solanacearum* by Western blotting, whereas no reaction with LPS from *R. picketti* was found (Fig. 6B).

The detection limit for *R. solanacearum* of scFv anti-LPS 12, the most reactive of the isolated scFvs, was comparable to polyclonal antiserum PcA-9523. In a double-antibody sandwich ELISA, both could detect as few as 4×10^4 cells per ml when in pure culture (Table 1). No difference in sensitivity was observed between the chromatogenic substrate p-NPP and the fluorogenic substrate FDP. The signals obtained were higher for PcA-9523 than for scFv anti-LPS 12, but the background value of scFv anti-LPS 12 was lower, especially when FDP substrate was used instead of p-NPP.

To evaluate scFv anti-LPS 12 for use in routine tuber testing, *R. solanacearum* cells were diluted into a potato tuber extract and compared with serial dilutions of a pure culture. The lowest con-

centration of *R. solanacearum* cells that could be detected in the sandwich ELISA with scFv anti-LPS 12 was 5×10^3 cells per ml in the potato tuber extract (Table 2). Even though high numbers of saprophytic bacteria were present in the tuber extract, the signals were only slightly reduced and the sensitivity was comparable to the pure culture.

Testing of the scFvs against 26 *R. solanacearum* strains, both in ELISA and IF (Table 3), revealed that they reacted with all strains of race 3, but only with some of the race 1 strains. No difference was observed between the specificities of the anti-LPS 8, anti-LPS 12, and anti-LPS 13 scFvs, and, therefore, only the results with scFv anti-LPS 12 are shown.

The cross-reactivity of the recombinant antibodies was compared with PcA-9523 using a panel of plant-pathogenic and saprophytic bacteria (Table 4) that had been shown to cross-react with polyclonal antisera in ELISA, IF, or IFC. In ELISA and IF tests, scFvs cross-reacted with the BBD, *R. syzygii*, *Serratia fonticola*, two strains belonging to the *Enterobacteriaceae*, and an unidentified bacterial strain 1713, (Table 4). As was shown in Table 4, PcA-9523 cross-reacted with several bacterial strains that were not recognized by the scFvs. Thus, the specificity of the scFvs was higher than that of PcA-9523, the polyclonal antiserum that is used routinely for the detection of *R. solanacearum* in the Netherlands.

TABLE 4. Comparison of the reactivity of monoclonal single-chain variable antibody fragments (scFv) (anti-lipopolysaccharide [LPS] 12) and polyclonal antiserum (PcA-9523) with various bacterial strains in enzyme-linked immunosorbent assay (ELISA) and immunofluorescence cell staining (IF)

Family ^a , genus or species	Strain	Origin	IPO number ^b	Original number	Immunoassay			
					ELISA ^c		IF ^d	
					scFv	PcA	scFv	PcA
<i>Ralstonia solanacearum</i>	Netherlands		1609	PD2763	+	+	+	+
Banana blood disease	Sulawesi		S303	PD2100	+	+	+	+
<i>Ralstonia picketti</i>	United Kingdom		1720	Pr1150	–	+	–	+
<i>Ralstonia syzygii</i>	Indonesia		S302	PD2093	+	+	+	+
<i>Pseudomonas marginalis</i> pv. <i>marginalis</i>	France		1714	CFBP1538	–	+	–	–
Unknown	France		1713	1531	+	+	–	–
<i>Ochrobactrum antropi</i>	Spain		1682	1521101	–	–	–	–
<i>Rathayibacter tritici</i>	Spain		1690	15803	–	+	–	+
<i>Bacillus macerans</i>	Spain		1691	15803	–	–	–	–
<i>Aureobacterium liquefaciens</i>	Spain		1692	15807	–	+	–	–
<i>Aureobacterium liquefaciens</i>	Spain		1693	158010	–	+	–	+
<i>Serratia fonticola</i>	Spain		1694	1534.c	+	+	+	+
<i>Ralstonia</i>	Netherlands		1723	PD2778	–	–	–	+
Unknown	France		1715	R3	–	–	–	+
<i>Ochrobactrum antropi</i>	Unknown		S306	PD2808	–	–	–	+
<i>Enterobacteriaceae</i>	Netherlands		S339	PK15a	+	+	+	+
<i>Rhodococcus</i>	Netherlands		S340	PK10a	–	–	–	–
<i>Xanthomonas maltophilia</i>	Netherlands		S341	PK06b/b	–	–	–	–
<i>Enterobacteriaceae</i>	Netherlands		S342	IV137	+	+	+	+
<i>Erwinia herbicola</i>	Netherlands		S343	PK18a	–	–	–	–
<i>Enterobacteriaceae</i>	Netherlands		S345	PK04a	–	–	–	+
<i>Bacillus</i>	Netherlands		S346	PK2/455	–	–	–	+
<i>Rhodococcus</i>	Netherlands		S347	PK2/76c	–	–	–	–
Unknown	Sweden		S348	PK2/76b	–	–	–	+
<i>Bacillus polymyxa</i>	France		1721	CFBP1954	–	–	–	–
<i>Ochrobactrum antropi</i>	United Kingdom		1722	T-3b-7	–	+	–	–
<i>Sphingobacterium multivorum</i>	Unknown		1717	R1c-3a	–	–	–	–
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>	France		1710	233	–	–	–	+
<i>Burkholderia andropogonis</i>	United Kingdom		1704	NCPBP1127	–	+	–	–
<i>Burkholderia caryophylli</i>	United Kingdom		1705	NCPBP2151	–	+	–	+
<i>Burkholderia caryophylli</i>	United Kingdom		1701	NCPBP353	–	–	–	+
<i>Burkholderia cepacia</i>	United Kingdom		1702	NCPBP945	–	–	–	–
<i>Burkholderia cepacia</i>	United Kingdom		1703	NCPBP946	–	+	–	–
<i>Burkholderia cepacia</i>	Unknown		1718	R5d-1	–	–	–	–
<i>Burkholderia glumae</i>	United Kingdom		1708	NCPBP3708	–	+	–	+
<i>Burkholderia plantarii</i>	United Kingdom		1707	NCPBP3590	–	–	–	+

^a Fatty acid analysis was used for strain identification, and the best matches with the database of the Microbial Identification System are given. Sometimes characterization was only up to the family level as no closer match (at least 50% identity) was found. Strains were obtained from J. D. Janse (Plant Protection Service, Wageningen, the Netherlands), M. M. Lopez (IVIA, Valencia, Spain), A. C. Le Roux (INRA, Rennes, France), and J. G. Elphinstone (Central Science Laboratory, York, United Kingdom).

^b IPO = Institute of Plant Protection, Wageningen, the Netherlands.

^c ELISA signals: – = reaction below background; and + = strong reaction (at least threefold above background).

^d IF signals: – = no fluorescence; + = strong fluorescence; and nd = not done.

DISCUSSION

Four different scFvs were isolated from a combinatorial antibody library and evaluated for their use in a diagnostic assay for the detection of *R. solanacearum* race 3. Three of these, anti-LPS 8, anti-LPS 12, and anti-LPS 13, were promising candidates for routine application based on their ease of production in *E. coli* and their specificity in IF and ELISA. The affinity of anti-LPS 12 was sufficient for sensitive detection of *R. solanacearum*; threshold levels between 5×10^3 and 4×10^4 bacteria per ml were reached in ELISA and bright fluorescent staining was achieved in IF.

The selected antibodies showed a higher degree of specificity than did PcA-9523, the polyclonal antiserum that is currently used in routine testing. Apparently, the LPS epitopes recognized by the scFvs do not occur as frequently in the bacteria tested (Table 4) as do other epitopes recognized by PcA-9523. It is interesting that all four different scFvs recognize the same bacterial strains and, therefore, probably react with the same epitope. However, this was not validated, as no epitope mapping was carried out. The cross-reactions (Table 4) observed with BBD and *R. syzygii* will not compromise a diagnostic test for potato brown rot, because these species are not present in temperate areas where this test will be used. In contrast, the cross-reactions observed with the four other saprophytic bacteria from potato and soil can possibly cause false-positive results, because they have been isolated in temperate areas. However, as the number of cross-reacting species is reduced relative to the PABs in PcA-9523, it is anticipated that the use of these antibodies in seed testing will result in a decreased number of false positives.

The sensitivity observed in ELISA, comparable to high quality PABs, is promising. In IF, relatively high quantities of scFv were required to obtain the same signal as the PABs. This is probably because the scFvs have one antigen-binding site and bind monovalently to the antigen. In contrast, full-size antibodies have two antigen-binding sites that can bind with two epitopes simultaneously. According to Neri et al. (19), this avidity effect can increase binding up to 100-fold and results in improved performance in immunoassays. Single-chain antibodies can be engineered in a format allowing bivalent binding (21). Bivalent molecules can also be established through a genetic fusion with *E. coli* alkaline phosphatase, a dimeric enzyme (13,14). Thus, if one of these avidity effects is added, it may further increase the sensitivity of our immunoassay. Alkaline phosphatase may be the most convenient choice for detection purposes. The production of scFv enzyme fusion proteins may result in improved reproducibility for the detection of antigens in various immunoassays, since the variation due to repeated antibody enzyme conjugation is avoided.

Engineering the format of scFvs might also be advantageous for the development of routine assays for the detection of *R. solanacearum*. Single-chain antibodies are small molecules that are often inactivated upon coating to ELISA plates (12). Fusion to large proteins like glutathione-S-transferase, amphipathic helices, or to other immunoglobulin domains may enhance the coating efficiency while retaining the full binding activity (12).

The monoclonal recombinant scFvs described here are applicable in routine assays for *R. solanacearum* race 3, even though some cross-reactions with other species were observed. The selection of other anti-LPS scFvs that do not cross-react with these strains remains an option. The difficulty lies in the nature of the LPS of the cross-reacting bacteria. Although LPS are highly variable molecules, the possibility cannot be excluded that LPS epitopes of cross-reacting bacteria are at least partially identical to the target bacteria. For the isolation of even more specific antibodies, consideration may be given to outer membrane proteins or extracellular polysaccharides as antigens. We used a straightforward selection protocol that resulted in the most specific antibodies found thus far for *R. solanacearum* race 3. However, phage display allows easy adaptation of the selection protocols such as the inclusion of

cycles of negative (against unwanted bacteria) and positive (for different races) selection. In addition, the current antibody libraries cover the complete immune repertoire, in contrast to the hybridoma technology in which only a part of the immune repertoire is tested. Antibody phage display in combination with protein engineering is highly promising for the selection of tailor-made MAbs and can be combined with the design of improved immunoassays for phytopathogenic bacteria.

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