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Analysis of the Cell Surface of *Pseudomonas syringae* pv. *glycinea* with Monoclonal Antibodies

Vincent P. M. Wingate, Paul M. Norman, and Christopher J. Lamb

Plant Biology Laboratory, Salk Institute for Biological Studies, La Jolla, CA 92037 U.S.A.
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Murine hybridoma cell lines were generated against the cell surface of pathogenically defined races of *Pseudomonas syringae* pv. *glycinea*, including race 4 strain A29-2 containing the race 6 avirulence gene *avrA* (race 4 strain A29-2*). Generation of a high number of hybridoma cell lines (20 to 40%) secreting antibodies was critically dependent upon the immunization schedule. Radioimmunoassay and western blot analysis identified monoclonal antibodies (MAbs) to race 4-specific epitopes and race 4 strain-specific epitopes that were not present on race 1 strain R1, race 5 strain R5, and race 6 strain R6. Similarly, certain other MAbs identified epitopes found on race 1 strain R1, race 5 strain R5, and race 6 strain R6 that were not present on race 4 strains. In contrast, MAbs were not found that discriminated between the cell surfaces of race 1 strain R1, race 5 strain R5, and race 6 strain R6. With few exceptions, protease-sensitive epitopes were common to all *P. syringae* pathovars, whereas the majority of periodate-sensitive epitopes were only present in *P. s.* pv. *glycinea* races and were not found in other *P. syringae* pathovars. There was no correlation between MAbs recognizing race-specific bacterial cell surface epitopes and the pattern of infectivity of the bacterial races on the soybean cultivars, save that the surface of race 4, which is virulent on a wide range of soybean cultivars, was immunologically very distinct from the other races examined, which are virulent on more limited sets of cultivars. Likewise no MAbs were identified that gave a pattern of race reactivity exclusively correlated with expression of the *avrA* gene. These data indicate that the bacterial cell surface may not have a direct role in the expression of avirulence. However, differences in bacterial epitopes at the genus, species, pathovar, race, and strain levels were identified by this approach, indicating that MAb technology is a useful tool for dissecting the cell surface of genetically related bacteria.

Additional keyword: lipopolysaccharide.

Cell surfaces play key roles in cellular adhesion, recognition of self and nonself, and perception and transduction of extracellular signals (Frazier and Glaser 1979). In the case of phytopathogenic bacteria, roles for the cell surface in various aspects of pathogenesis include attachment to host plant cells and determination of host range and race-cultivar specificity (Keen 1982). Thus, dissection of the functional architecture of the cell surfaces of phytopathogenic bacteria is likely to be important for our understanding of molecular communication in plant-microbial pathogen interactions.

The outer membrane of the cell wall of gram-negative bacteria is a complex lipid bilayer containing glycoproteins, lipopolysaccharides (LPSs), and proteins. Phytopathogenic bacteria often have flagella and project threads of pili, and in some cases are covered by a loose extracellular exopolysaccharide slime. The LPS has been implicated in the initial attachment of *Agrobacterium* to the plant cell surface (Whatley *et al.* 1976). More recently, four genetic loci on the *Agrobacterium* chromosome have been identified that are involved in the attachment of *Agrobacterium* to the plant cell surface. These loci are involved in the synthesis of cellulose fibrils, cyclic glucan and acidic succinoglucan, and cell surface proteins and in the synthesis and excretion of β-1,2-glucan (Matthysee 1988). Some of these genetic loci are conserved in *Rhizobium*, suggesting that a number of bacterial cell surface components may be common in bacteria-plant interactions (Zambryski *et al.* 1989). In the *Rhizobium*-legume symbiotic interaction, some evidence suggests that bacterial polysaccharides and plant lectins may function in recognition (Long 1989). Thus, a mutant *R. leguminosarum* bv. *phaseoli* with an altered LPS is unable to form a normal infection thread (Carlson *et al.* 1987), and a pea lectin gene expressed in white clover roots caused the roots to be nodulated by *R. l.* bv. *viciae*, which normally is unable to form nodules on white clover (Diaz *et al.* 1989).

Similarly, cell surface antigens of phytopathogenic bacteria may play an important role in plant-bacteria recognition systems involved in determining host range and race-cultivar specificity conditioned by gene-for-gene interactions between specific bacterial avirulence (*avr*) genes and plant disease resistance genes (Keen 1982). However, there is relatively little information on the functional architecture of the cell surface of phytopathogenic bacteria in relation to recognition by the plant. A number of elicitors of the hypersensitive response or other aspects of the plant's defense mechanisms have been identified in preparations from bacterial cell walls or culture fluids; however, in general, these elicitors are not race specific, and their physiological roles in recognitional events, especially those governed by gene-for-gene interactions, have not been established (Keen 1982).

Current address of V. P. M. Wingate: Rhone-Poulenc Agrochimie, 14-20 Rue Pierre Baizet B.P. 9163, 69263 Lyon Cedex, France.
Address correspondence to C. J. Lamb: Plant Biology Laboratory, Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037 U.S.A.

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Since the bacterial cell surface is such a complex array of antigens, we decided to use hybridoma technology to obtain monoclonal antibody (MAb) probes to bacterial cell surface epitopes. This approach has been used previously to examine the cell surface antigens of a number of bacterial phytopathogens (De Boer and Wieczorek 1984; Alvarez *et al.* 1985). The bacterial pathogen chosen in our study was *Pseudomonas syringae* pv. *glycinea* (Coerper) Young *et al.* because it had a number of distinct advantages. Thus, this pathovar of *P. syringae* may be divided into a number of distinct races, defined on the basis of the pattern of virulence or avirulence on a panel of soybean cultivars (Fett and Sequeira 1981), and for a number of these races there is more than one isolate or strain available. In addition, a number of genetic loci have been cloned that are required for pathogenicity (Lindgren *et al.* 1988) and the expression of avirulence (*avr* genes) in a hypersensitive response (Staskawicz *et al.* 1984, 1987). We were particularly interested in determining if the presence of the *P. s* pv. *glycinea avrA* gene in a different genetic background would alter the molecular architecture of the bacterial cell surface.

MATERIALS AND METHODS

Bacterial strains and media. The sources and characteristics of bacterial strains are described in Table 1. Bacteria were grown on King's B medium (King *et al.* 1954) unless otherwise specified. Isolates of *P. s.* pv. *glycinea* were confirmed with respect to their race identity by infectivity patterns on test soybean cultivars by Brian Staskawicz (University of California, Berkeley) (Table 2). Race 4 strain A29-2 containing the cosmid clone pPg6L3 of race 6 strain R6 DNA that carries the *avrA* gene (Staskawicz *et al.* 1984) was kindly provided by Brian Staskawicz and is referred to here as race 4 strain A29-2*.

MAb production. Bacterial isolates were grown on a liquid minimal medium (Holliday and Keen 1982). The bacteria were harvested during late log phase (cell density 5×10^8 to 10^9 per milliliter), centrifuged at $8,000 \times g$ for 10 min at 4° C, and washed twice in phosphate-buffered saline (PBS) prior to resuspension in PBS at a concentration of either 5×10^9 bacteria per milliliter for intravenous injection or 2×10^9 bacteria per milliliter for intraperitoneal injection. Balb/c mice (8 wk old, bred by the Salk Institute Animal Facility) were immunized with freshly prepared bacterial suspensions. Unless otherwise specified, the immunization protocol consisted of one intraperitoneal injection of 10^9 bacteria followed 28 days later by one intravenous injection of 10^9 bacteria. The mice were killed 3 days after the final injection, and the splenic lymphocytes were fused with S194/5.XXO.BU1 myeloma cells as described by Trowbridge (1978).

Radioimmunoassay of antibody binding. Radioimmunoassays (RIAs) were performed at 4° C. Freshly prepared bacteria grown on minimal medium were harvested, washed as described above, and resuspended at a concentration of 10^9 bacteria per milliliter in PBS containing 0.1% sodium azide and 1 mg/ml of bovine serum albumin (PBS-A-BSA). The bacterial suspension was incubated with appropriate hybridoma culture supernatants for 45 min in 96-well V-bottomed polystyrene plates (Dynatech Laboratories, Alexandria, VA) (50 μl of bacterial suspension and 50 μl of hybridoma culture supernatant per well). The bacteria were then incubated for 45 min with ^{125}I-labeled rabbit anti-mouse antibody diluted in PBS-A-BSA (50 μl containing 5×10^5 cpm per well). The ^{125}I-labeled antibody was prepared by the chloramine-T method (Greenwood *et al.* 1963) with carrier-free ^{125}I (Amersham, Arlington Heights, IL). The plates were washed three times, and the final bacterial pellet was then washed into transfer tubes for gamma counting. Control assays in which the first antibody was replaced with either Dulbecco's modified Eagle's medium or a MAb directed against a plant cellular membrane preparation routinely gave values below 1,000 cpm. All antibody supernatants were tested in triplicate, and the average counts per minute binding to the bacterial cell surface were determined. To obtain an index of the amount of antibody specifically bound to the bacteria, a signal-to-noise (S/N) ratio was determined for each antibody supernatant as follows:

$$\frac{\text{[binding of test antibody (cpm)]}}{\text{[binding of an unrelated control antibody (cpm)]}}$$

Table 1. Description and source of *Pseudomonas syringae* and *P. solanacearum*

Designation	Description	Source or reference
Pseudomonas syringae		
pv. *glycinea* R1	Race 1	Barton-Willis *et al.* 1984
pv. *glycinea* R4	Race 4	Barton-Willis *et al.* 1984
pv. *glycinea* A29-2	Race 4	Staskawicz *et al.* 1984
pv. *glycinea* A29-2*	Race 4, containing the cosmid clone pPg6L3 bearing the *avrA* gene	Staskawicz *et al.* 1984
pv. *glycinea* R5	Race 5	Barton-Willis *et al.* 1984
pv. *glycinea* R6	Race 6	Barton-Willis *et al.* 1984
pv. *lachrymans*	PHW214-6, isolated from cucumber	L. Sequeira[a]
pv. *tabaci*	Race 0, 11528, isolated from tobacco	L. Sequeira
P. solanacearum	Race 2, 333, isolated from banana	L. Sequeira

[a] University of Wisconsin, Madison.

Table 2. Race-cultivar interactions between strains of *Pseudomonas syringae* pv. *glycinea* and soybean

		Cultivar					
Race	Strain	Harasoy	Acme	Peking	Norchief	Flambeau	Centennial
1	R1	I[a]	C[b]	C	I	C	C
4	R4	C	C	C	C	C	C
4	A29-2	C	C	C	C	C	C
4	A29-2*	I	I	I	C	C	I
5	R5	C	I	C	I	I	I
6	R6	I	I	I	C	C	I

[a] I, incompatible interaction.
[b] C, compatible interaction.

Table 3. Optimization of immunization protocol for the production of hybridoma cell lines secreting antibodies recognizing bacterial cell surface antigens

Immunization schedule (Days)											
1	4	8	15	22	25	28	29	30	31	34	S/N >3[a] (%)
10^9 [b]		10^9	10^9		S						0–1
10^9		10^9	10^9	5×10^9	S						0–1
10^8		10^8	10^8	10^8	S						0–1
10^9		10^9				5×10^8	5×10^8	5×10^8	5×10^8	S	0–1
10^8	S[c]										0
10^9	S										0
10^9						10^9			S		1–10
10^8						10^9			S		0–1
10^8						10^8			S		0–1
10^9						10^9 [d]			S		20–40

[a] S/N, signal-to-noise ratio.
[b] Injections were intraperitoneal, except where designated. The number of bacteria in each injection is given in each case.
[c] S denotes splenectomy.
[d] Intravenous injection.

Table 4. Immunization schedule for production of specific MAbs

MAb	Bacterial race inoculated	
	Day 1	Day 28
63.4B1	4 (strain R4)	4 (strain R4)
63.3C3	4 (strain R4)	4 (strain R4)
75.1C2	4 (strain A29-2*)	4 (strain A29-2*)
75.3A6	4 (strain A29-2*)	4 (strain A29-2*)
58.3A2	5 (strain R5)	5 (strain R5)
25.3C2	6 (strain R6)	6 (strain R6)
29.2A3	6 (strain R6)	6 (strain R6)
34.2A2	6 (strain R6)	6 (strain R6)
40.2B2	6 (strain R6)	6 (strain R6)
40.2C2	6 (strain R6)	6 (strain R6)
42.3B3	6 (strain R6)	6 (strain R6)
42.3C6	6 (strain R6)	6 (strain R6)
50.1D4	6 (strain R6)	6 (strain R6)
51.1A5	6 (strain R6)	6 (strain R6)
51.2B3	6 (strain R6)	6 (strain R6)
52.1B5	6 (strain R6)	6 (strain R6)
52.1D2	6 (strain R6)	6 (strain R6)
52.3C3	6 (strain R6)	6 (strain R6)
71.4C2	6 (strain R6)	6 (strain R6)
77.2B5★[a]	4 (strain A29-2*)	6 (strain R6)
77.4C5	4 (strain A29-2*)	6 (strain R6)
78.2D2★[a]	4 (strain A29-2*)	6 (strain R6)

[a] In each case splenectomy was performed on day 31; ★ denotes nonclonal antibody supernatant.

An S/N >3 for an antibody supernatant was taken to indicate production of antibody at a useful level by the corresponding hybridoma cell line. Maintenance of an S/N >3 was also found to be a useful indicator of the stability of a hybridoma cell line for the production of antibody.

Western blots of bacterial lysates. Bacteria were harvested during late log phase, centrifuged at 8,000 × *g* for 10 min at 4° C, and washed twice with PBS-A, and the final bacterial pellet was solubilized at a concentration of 10^{10} bacteria per milliliter in sample buffer (Hitchcock and Brown 1983). The bacterial lysate was incubated at 100° C for 5 min and then stored at −70° C until required. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the bacterial lysates was performed by the method of Laemmli (1970). Electrophoretic transfer of the bacterial extract to a nitrocellulose membrane was performed at room temperature at 60 V for 3 hr in a transfer buffer composed of 80 mM Tris, pH 7.5, 13 mM glycine, and 20% methanol. Immunoanalysis of the western blots was performed by the protocol of Brewin *et al.* (1985) using horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories, Richmond, CA). Where appropriate, bacterial lysates in sample buffer were digested with proteinase K (1 mg/ml) at 60° C for 1 hr (Hitchcock and Brown 1983), or western blots were incubated with 20 mM periodic acid (Woodward *et al.* 1985).

RESULTS

Optimization of the immunization schedule. The frequency of immunization and the amount of immunogen were found to be critical factors for the generation of useful numbers of hybridoma cell lines secreting antibodies. An immunization protocol of one intraperitoneal injection of 10^9 bacteria on day 1 and one intravenous injection on day 28, followed by splenectomy and cellular fusion 3 days later, was the most successful. With this protocol, 20 to 40% of the cryopreserved cell lines produced an antibody-containing supernatant giving an S/N >3 in RIA. In contrast, other protocols with more frequent immunizations resulted in less than 1% of the hybridoma cell lines secreting antibodies directed against bacterial cell surface antigens (Table 3). Moreover, antiserum titer was not a reliable guide to the subsequent production of MAbs. Higher antiserum titers in immunized mice were obtained with the more frequent bacterial injections, which gave very low numbers of secreting hybridoma cell lines.

MAb production and initial characterization. Initially, individual mice were immunized with one of the *P. s.* pv. *glycinea* strains: A29-2* (race 4), R4 (race 4), R5 (race 5), or R6 (race 6), which were administered singly. Other secreting hybridoma cell lines were obtained from mice

immunized with race 4 strain A29-2* on day 1, followed by race 6 strain R6 on day 28, prior to splenectomy and cell fusion on day 31. The immunization protocols used in the generation of specific MAbs and nonclonal cell lines described in the text are presented in Table 4. Antibody supernatants that gave an S/N >3 were further investigated by RIA of binding to race 1 strain R1; race 4 strains A29-2, A29-2*, and R4; race 5 strain R5; and race 6 strain R6. The most striking observation from this analysis was that the cell surfaces of the race 4 strains were epitopically distinct from race 1 strain R1, race 5 strain R5, and race 6 strain R6.

More than 500 antibody supernatants were analyzed by RIA. A number of representative hybridoma cell lines, whose antibody supernatants showed differential binding to individual *P. s.* pv. *glycinea* races, were cloned by limiting dilution. Twenty-one hybridoma cell lines successfully passed through cloning by limiting dilution. The clonal cell lines showed considerable variation in the stability of antibody production.

The patterns of binding of these MAbs to the cell surfaces of *P. s.* pv. *glycinea* race 1 strain R1, race 4 strains R4, A29-2, and A29-2*, race 5 strain R5, and race 6 strain R6 are shown in Table 5. In the RIA, the MAbs showed both quantitative and qualitative differences in the amount of antibody bound to the cell surface of the various races. The reactivity of the MAbs could be grouped into five categories:

(I) Four MAbs recognized all of the races. MAb 25.3C2 bound to a similar extent to all races. In contrast, MAb 77.4C5 showed markedly less binding to race 5 strain R5 and race 6 strain R6 compared to race 1 strain R1 and race 4 strains R4, A29-2, and A29-2*. MAb 77.4C5 was the only MAb that showed a quantitative difference in binding between race 1 strain R1 and race 5 strain R5 and race 6 strain R6.

(II) Seven MAbs recognized only race 1 strain R1, race 5 strain R5, and race 6 strain R6, but did not recognize race 4 strains R4, A29-2, and A29-2*. Thus the race 4 strains, which are virulent on all soybean cultivars, do not have a number of epitopes that are common to the less virulent race 1 strain R1, race 5 strain R5, and race 6 strain R6.

(III) Two MAbs specifically bound to race 4 strains R4 and A29-2, but did not bind to race 1 strain R1, race 5 strain R5, and race 6 strain R6. MAb 75.3A6 showed quantitatively similar binding to each of the race 4 strains, whereas MAb 75.1C2 showed less binding to strain R4 compared to strains A29-2 and A29-2*.

(IV) Two MAbs bound to epitopes unique to race 4 strain R4. Thus MAb 63.4B1 did not bind to other races, or to race 4 strain A29-2, and hence can distinguish between different strains of the same physiological race.

(V) Six MAbs bound to race 1 strain R1, race 4 strain R4, race 5 strain R5, and race 6 strain R6, but did not bind to race 4 strains A29-2 and A29-2*. MAb 52.3A3 gave quantitatively similar binding to *P. s.* pv. *glycinea* race 1 strain R1, race 4 strain R4, race 5 strain R5, and race 6 strain R6, whereas MAb 40.2B2 showed less binding to race 4 strain R4 compared to race 1 strain R1, race 5 strain R5, and race 6 strain R6. In contrast, race 4 strains A29-2 and A29-2* were identical to each other in their reactivity to these MAbs.

Two hybridoma cell lines (77.2B5 and 78.2D2), which

Table 5. Comparative radioimmunoassay of MAb binding to *Pseudomonas syringae* pv. *glycinea* races

		Antibody binding to race (cpm)[a]					
Group	**MAb**	**1 (R1)**	**4 (R4)**	**4 (A29-2)**	**4 (A29-2*)**	**5 (R5)**	**6 (R6)**
I	25.3C2	+++	+++	+++	+++	+++	+++
	51.1A5	+	+	+	+	+	+
	71.4C2	++	++	++	++	++	++
	77.4C5	++	++	++	++	+	+
II	29.2A3	+++	±	−	−	+++	+++
	34.2A2	+	−	−	−	+	+
	40.2C2	+	−	−	−	+	+
	42.3C6	+++	±	−	−	+++	+++
	50.1D4	+++	±	−	−	+++	+++
	51.2B3	+++	±	−	−	+++	+++
	52.3C3	+++	±	−	−	+++	+++
III	75.1C2	−	+	+++	+++	−	−
	75.3A6	−	+++	+++	+++	−	−
IV	63.4B1	−	+++	−	−	−	−
	63.3C3	−	+++	−	−	−	−
V	40.2B2	+++	+	±	±	+++	+++
	42.3B3	+++	+++	−	−	+++	+++
	52.1B5	++	++	±	±	++	++
	52.1D2	+++	+++	−	−	+++	+++
	52.3A3	+++	+++	−	−	+++	+++
	58.3A2	+++	+++	−	−	+++	+++

[a] The amount of antibody bound is recorded on a scale of − (<1,000 cpm, no binding), ± (2,000 to 3,000 cpm), + (3,000 to 5,000 cpm), ++ (5,000 to 8,000 cpm), and +++ (>8,000 cpm). (Strain designations are given within parentheses.)

did not survive cloning by limiting dilution, showed very little binding to race 4 strain A29-2, but significant binding to race 4 strain A29-2* and race 6 strain R6. Analysis of serial dilutions of the nonclonal antibody supernatants 77.2B5 and 78.2D2 showed similar titers for antibody binding to race 4 strain A29-2* and race 6 strain R6. In contrast, MAb 25.3C2 showed the same amount of antibody binding to all three strains (Fig. 1). However, the nonclonal antibody supernatants 77.2B5 and 78.2D2 showed a similar level of antibody binding to race 1 strain R1, race 4 strain R4, and race 5 strain R5 as that observed with race 6 strain R6 or race 4 strain A29-2* (data not shown). Hence, although introduction into race 4 strain A29-2 of a cosmid clone carrying the *avrA* gene from race 6 strain R6 concomitantly causes a major increase in binding of antibodies from cell lines 77.2B5 and 78.2D2, there is no simple, direct relationship between the presence and functional expression of the *avrA* gene and the presence of these epitopes on the cell surfaces of different *P. s.* pv. *glycinea* races.

Antibody binding to western blots of bacterial lysates. The RIA was an appropriate method to screen for MAb-recognizing bacterial cell surface epitopes and to identify major epitopic differences in the cell surface architecture of the various *P. s.* pv. *glycinea* races. The patterns of antibody binding were then analyzed in western blots of whole bacterial lysates to obtain information on the molecular weight, size distribution, heterogeneity, and biochemical

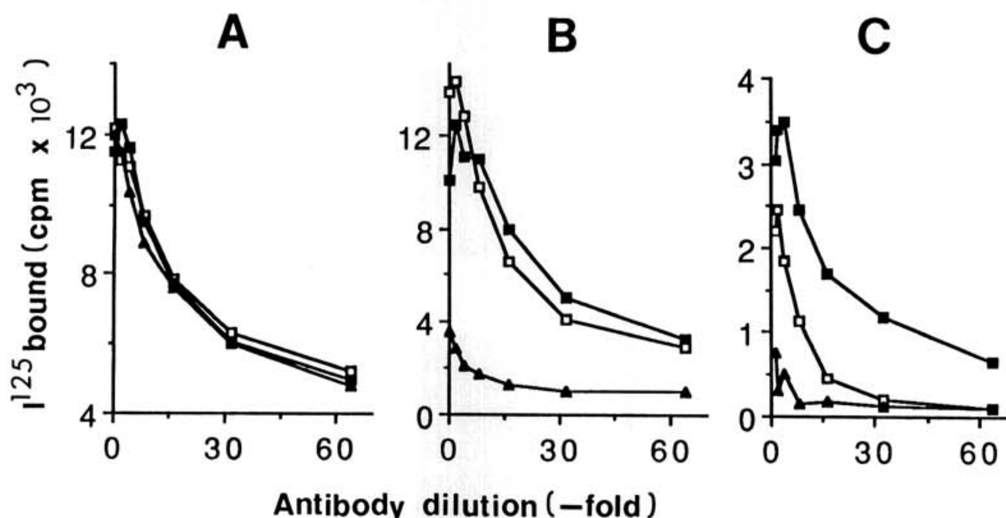

Fig. 1. Titration of MAb 25.3C2 (**A**) and nonclonal antibody supernatants 78.2D2 (**B**) and 77.2B5 (**C**) with cell surface epitopes of *Pseudomonas syringae* pv. *glycinea* race 4 strain A29-2 (▲), race 6 strain R6 (■), and race 4 strain A29-2* containing race 6 cosmid clone pPg6L3 carrying the *avrA* gene (□).

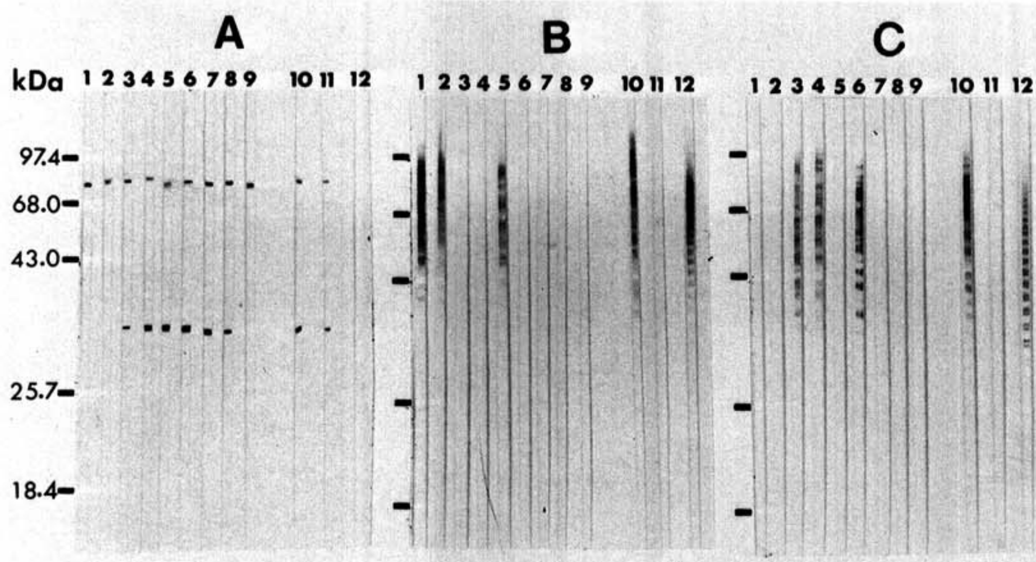

Fig. 2. Immunoanalysis of western blots of whole bacterial cell lysates with MAbs 77.4C5 (**A**), 29.2A3 (**B**), and 75.3A6 (**C**). Lanes 1–6 contain *Pseudomonas syringae* pv. *glycinea* race 6 strain R6, race 5 strain R5, race 4 strain A29-2, race 4 strain A29-2*, race 1 strain R1, and race 4 strain R4, respectively; lane 7 contains *P. s.* pv. *lachrymans*; lane 8, *P. s.* pv. *tabaci*; and lane 9, *P. solanacearum*. Lanes 10, 11, and 12 in panels **A** and **C** contain *P. s.* pv. *glycinea* race 4 strain A29-2*, and in panel **B** they contain *P. s.* pv. *glycinea* race 6 strain R6. In lane 11 the western blots were periodate treated, and in lane 12 the bacterial lysate was digested with proteinase K prior to electrophoresis.

properties of the cellular components carrying the corresponding epitopes.

Approximately 250 antibody supernatants and 21 MAbs were used in the western blot analysis, with particular emphasis on antibody supernatants that had been shown by RIA either to bind to epitopes on race 4 strain A29-2* or to differentiate between races of *P. s.* pv. *glycinea*. Approximately 10% of the antibody supernatants that were positive in the RIA did not give a signal on western blots, including the nonclonal supernatants 77.2B5 and 78.2D2, as well as MAbs 34.2A2, 40.2C2, 51.2B3, and 52.3C1. Consequently, the molecular basis underlying the differential binding of 77.2B5 and 78.2D2 to race 4 strains A29-2 and A29-2* could not be studied by this technique.

Overall, the immunoanalysis of western blots closely correlated with the observations made by RIA. Representative western blots are shown in Figure 2. Western blots defined five classes of MAbs that showed differences in their reactivity toward the *P. s.* pv. *glycinea* races, other *P. syringae* pathovars, and other *Pseudomonas* species investigated (Table 6). These five are as follows:

(I) A number of MAbs (for example MAbs 25.3C2 and 71.4C2) recognized epitopes common to all *P. syringae* pathovars. In some cases (for example MAbs 51.1A5 and 77.4C5), antibodies recognized epitopes common to all the *Pseudomonas* species tested. Interestingly, MAb 77.4C5 (Fig. 2A) recognized the same epitope on two different-sized species, subunit molecular sizes of 36 and 80 kDa, and while reactivity with the 80-kDa species was found with all *P. syringae* pathovars and with *P. solanacearum* (Smith) Smith, reactivity with the 36-kDa species was detected only in *P. syringae* pathovars and not in *P. solanacearum*.

(II) A set of MAbs (for example MAb 29.2A3) recognized epitopes common to *P. s.* pv. *glycinea* race 1 strain R1, race 5 strain R5, and race 6 strain R6, but absent from other races and other *P. syringae* pathovars (Fig. 2B).

(III) Another set of MAbs (for example MAb 75.3A6) recognized epitopes present in all strains of *P. s.* pv. *glycinea* race 4, but absent from other races and other *P. syringae* pathovars (Fig. 2C).

(IV) A number of MAbs (for example MAb 63.4B1) recognized epitopes unique to *P. s.* pv. *glycinea* race 4 strain R4.

(V) A further set of MAbs (for example MAb 58.3A2) recognized epitopes present only in race 1 strain R1, race 4 strain R4, race 5 strain R5, and race 6 strain R6, but not found in other *P. s.* pv. *glycinea* races and other *P. syringae* pathovars.

Following protease digestion of the bacterial extracts prior to electrophoresis, only polypeptide fragments smaller than 10 kDa were detected by staining with Coomassie Brilliant Blue R 250 (data not shown). With few exceptions, where immunoreactivity was destroyed by protease digestion of the antigens, the corresponding epitopes were found to be common to all *P. syringae* pathovars (Table 6). In contrast, periodate-sensitive epitopes were generally specific for races of *P. s.* pv. *glycinea*, for example reactivity patterns of MAbs 29.2A3, 42.3C6, and 75.3A6. Only MAbs 25.3C2 and 51.1A5 recognized periodate-sensitive epitopes present on all the *P. s.* pv. *glycinea* races and other *P. syringae* pathovars tested. In a number of cases, periodate-

Table 6. Immunoanalysis of western blots of *Pseudomonas syringae* pv. *glycinea* races, *P. s.* pv. *lachrymans*, *P. s.* pv. *tabaci*, and *P. solanacearum*

Group	MAb	Mol. size (kDa)	Protease sensitivity	Periodate sensitivity	Designation[a]								
					1 (R1)	4 (R4)	4 (A29-2)	4 (A29-2*)	5 (R5)	6 (R6)	Pl	Pt	Ps
I	25.3C2	41–97	+	+	+	+	+	+	+	+			
		36–64									+	+	
I	51.1A5	>200		+	+	+	+	+	+	+	+	+	+
		<14.5		+	+	+	+	+	+	+	+		
I	71.4C2	32	+		+	+	+	+	+	+	+	+	
I	77.4C5	80	+	+	+	+	+	+	+	+	+	+	+
		36		+	+	+	+	+			+	+	
II	29.2A3	39–100		+	+				+	+			
II	42.3C6	39–110		+	+				+	+			
II	50.1D4	39–110		+	+				+	+			
III	75.1C2	37–97		+		W+[b]	+	+					
III	75.3A6	37–97		+		+	+	+					
IV	63.4B1	38–68		+		+							
IV	63.3C3	43–68		+		+							
V	40.2B2	45–97		+	+				+	+			
V	42.3B3	36–>100		+	+				+	+			
		45–97			W+								
V	52.1B5	80	+		W+	W+			W+	W+			
V	52.1D2	39–100		+	+	+			+	+			
V	52.3A3	39–>100		+	+	+			+	+			
V	58.3A2	26–>200		+	+	+			+	+			

[a] *P. s.* pv. *glycinea* race and strain designations are as follows: 1 (R1), race 1 strain R1; 4 (R4), race 4 strain R4; 4 (A29-2), race 4 strain A29-2; 4 (A29-2*), race 4 strain A29-2*; 5 (R5), race 5 strain R5; and 6 (R6), race 6 strain R6. Pl, *P. s.* pv. *lachrymans*. Pt, *P. s.* pv. *tabaci*. Ps, *P. solanacearum*.

[b] W+, weakly positive.

sensitive epitopes were found on a series of differently sized molecules, for example MAbs 29.2A3 and 75.3A6 recognized epitopes on molecules with subunit molecular sizes ranging from 39 to 100 kDa and 37 to 97 kDa, respectively. MAb 25.3C2 was unique in that neither protease treatment nor periodate treatment completely abolished the binding of the antibody to the bacterial molecules. Since the cell line underwent two rounds of cloning by limiting dilution, it is very likely to be monoclonal.

DISCUSSION

We have examined the cell surface of *P. s.* pv. *glycinea* and the relationship of surface architecture to host range and race-cultivar specificity using MAb probes. Our study revealed considerable differences in cell surface architecture between races and even between different strains of the same race. Although we identified MAbs that recognize differences in bacterial cell surface epitopes at the species, pathovar, race, and strain levels, no direct correlation between cell surface features and race-cultivar specificity was observed. In part, this may reflect the presence of immunodominant molecules unrelated to race-cultivar specificity.

There are numerous protocols for immunization with cellular antigens including whole bacteria (Alvarez *et al.* 1985; De Boer and Wieczorek 1984). In our study, it was empirically determined that one intraperitoneal injection followed 28 days later by one intravenous injection of *P. s.* pv. *glycinea* resulted in 20 to 40% of hybridoma cell lines secreting antibodies, whereas other protocols were not effective. Presumably this immunization schedule caused the splenic B cells to be in an optimal developmental stage for the continued production of antibodies following fusion with the myeloma cell line.

RIA with whole bacteria should identify antibodies directed against epitopes on the cell surface, since it is unlikely that antibodies were able to pass through the bacterial outer membrane, and any putative active transport mechanism was inhibited by azide in the RIA buffers. Moreover, the iodinated second antibody, either in the presence or absence of a negative control antibody, did not give a signal, suggesting that antibody was unable to diffuse into the bacteria. Finally, bacterial lysis leading to the release of intracellular antigens did not occur in the RIA procedure.

Overall, essentially the same patterns of reactivity toward different *P. s.* pv. *glycinea* races were observed by RIA and western blot analysis. This analysis allowed the identification of differences in cell surface epitopes at the genus, species, pathovar, race, and strain levels. While MAbs directed against protease-sensitive epitopes were generally common to all *P. syringae* pathovars, periodate-sensitive epitopes showed varying degrees of specificity between the different *P. s.* pv. *glycinea* races. These data indicate that the immunodominant periodate-sensitive epitopes of race 4 strain A29-2 differ from those of race 1 strain R1, race 5 strain R5, and race 6 strain R6. Race 4 strain R4 has some epitopes in common with the A29-2 strain, but also has some unique epitopes. Since the immunochemical analysis showed that each isolate of race 4 does not display identical cell surface epitopes, no obvious direct correlation between differences in cell surface topology and race-cultivar specific patterns could be identified.

Although the RIA was able to reveal cell surface differences between the various *P. s.* pv. *glycinea* races, it was unable to identify the molecules to which the antibodies bound. Thus, RIA was more useful as an initial screen to detect both MAbs and antibody supernatants directed against bacterial cell surface epitopes. In contrast, the western blots revealed both the molecular size and the chemical nature of the epitope. Protease digestion caused a slightly altered electrophoretic banding pattern of the periodate-sensitive molecules compared to the nondigested bacterial lysates. This was probably due to the lack of proteins in the protease-digested sample. The protease digestion gave greater resolution of these molecules, leading to antibody binding to a number of regularly spaced bands, in a pattern highly characteristic of LPS molecules with different lengths of *O*-linked glycosyl side chains (Bradbury *et al.* 1984; Hendrick and Sequeira 1984; Hitchcock and Brown 1983).

In our study, the majority of such putative LPS epitopes identified by MAbs were found only in *P. s.* pv. *glycinea* and not in other pathovars or other *Pseudomonas* species, suggesting that the *P. s.* pv. *glycinea* races have LPS with distinctive structural features. MAb 25.3C2 was an exception in that it recognizes a putative LPS epitope also found in *P. s.* pv. *lachrymans* (Smith and Bryan) Young *et al.* and *P. s.* pv. *tabaci* (Wolf and Foster) Young *et al.* This epitope may be present on the core carbohydrate of the LPS molecule rather than the *O*-antigen side chain. Studies of gram-negative bacteria have shown that there is less variation in the core carbohydrate than in the *O*-side chain (Jann and Jann 1977). While we found that MAbs distinguished the LPS of two isolates of *P. s.* pv. *glycinea* race 4 (virulent on all soybean cultivars), none were isolated that discriminated between the LPS of race 1 strain R1, race 5 strain R5, and race 6 strain R6 which have more restricted host cultivar ranges. Moreover, no MAbs or nonclonal antibody supernatants to LPS epitopes were found that distinguished between race 4 strains A29-2 and A29-2*. These data strongly suggest that racial differences in LPS epitopes are not related to expression of avirulence. However, we cannot exclude the possibility that race-specific LPS epitopes are not immunogenic to the murine immune system. Alternatively, putative race-specific LPS epitopes may only be expressed *in planta*, since differences in exopolysaccharide and LPS of bacteria grown in axenic culture and *in planta* have been identified (Brewin *et al.* 1986; Osman *et al.* 1986).

Similar conclusions were drawn from a chemical analysis of LPS by Barton-Willis *et al.* (1984). Thus LPS molecules isolated from a number of *P. s.* pv. *glycinea* races showed a high degree of size heterogeneity, and chemical analysis of the *O*-side chains showed that the ratio of the two major components, rhamnose and *N*-acetylglucosamine, was different for race 4 strain R4 compared to race 1 strain R1, race 5 strain R5, and race 6 strain R6. Further analysis revealed that the LPS *O*-side chain repeating units of race 1 strain R1, race 5 strain R5, and race 6 strain R6 are similar if not identical, and LPS isolated from race 4 strain

A29-2 contained the same residues in the same ratios (Barton-Willis *et al.* 1987).

The functions of the protease-sensitive species identified by MAb or antibody supernatant reactivity are unknown. In general, the epitopes recognized were common to all *P. syringae* pathovars suggesting basic functions for these polypeptides, such as porins involved in the transport of nutrient molecules across the outer membrane. There was no direct correlation with the racial expression of avirulence since the immunodetected polypeptides were expressed in all *P. s.* pv. *glycinea* races.

We used the bank of MAbs and nonclonal antibody supernatants to search for surface epitopes specifically associated with expression of the *avrA* gene by comparison of the binding to race 4 strain A29-2 with the binding to this race containing the cosmid clone pPg6L3 of race 6 strain R6 DNA, which carries the *avrA* gene (race 4 strain A29-2*). Two nonclonal hybridoma cell line supernatants (77.2B5 and 78.2D2) contained antibodies that showed weak binding to race 4 strain A29-2 but strong binding to both race 6 strain R6 and race 4 strain A29-2* carrying the *avrA* gene. However, these cell line supernatants also bound to other races that do not contain *avrA* and do not show the pattern of race-cultivar specificity characteristic of this avirulence gene. Hence no simple, direct relationship could be discerned between the presence and functional expression of the *avrA* gene and the presence of epitopes reactive with antibodies in the supernatants of the 77.2B5 and 78.2D2 cell lines. Since the cell lines were not clonal, we cannot formally exclude the presence of additional antibody specificities that react with race 1 strain R1, race 4 strain R4, and race 5 strain R5 and hence mask the binding specificities of an antibody directed toward an epitope dependent on expression of the *avrA* gene. In addition, since antibody screening detects the distribution of specific structural epitopes rather than entire macromolecules, the macromolecular structural environment(s) in which an epitope resides may be highly dependent on the genetic background. Hence, reactivity of antibodies from cell lines 77.2B5 and 78.2D2 might then reflect the presence of this epitope in the macromolecular structure directly involved in *avrA*-mediated race-cultivar specificity in race 6 strain R6 and race 4 strain A29-2*, whereas in race 1 strain R1 and race 5 strain R5, this epitope is present in different macromolecular structural contexts unrelated to *avrA* function. However, the strong reactivity of these antibodies with race 4 strain R4 argues against this possibility.

Cosmid clone pPg6L3 contains 27.2 kilobase pairs of race 6 strain R6 DNA and hence, in addition to the *avrA* gene, carries other genes that may perturb cell surface architecture but are unrelated to *avrA* gene function in race-cultivar specificity. All other antibody supernatants and MAbs did not discriminate between the cell surfaces of race 4 strains A29-2 and A29-2*. Moreover, since our data indicate that there are considerable differences in the surface architecture of race 4 (strains R4 and A29-2) compared to race 6 strain R6, it is clear that the phenotype conferred by *avrA* is not critically dependent on the structural architecture of the cell surface. The deduced amino acid sequence encoded by the *avrA* gene predicts a polypeptide of 100 kDa that is predominantly hydrophilic with no hydrophobic domains characteristic of membrane proteins and no charged signal peptide, suggesting that the gene product is not translocated to the cell surface (Napoli and Staskawicz 1987; Staskawicz *et al.* 1987). Likewise, our results suggest that the introduction of the cosmid clone pPg6L3 into race 4 strain A29-2 does not cause a major alteration in the antigenicity of the cell surface, although other features of the surface architecture not recognized by the antibodies may be altered.

This raises the question of how expression of the *avrA* gene affects race-cultivar specificity if it does not directly or indirectly influence major cell surface antigens. One possibility is that a weakly or nonantigenic surface component is altered. Alternatively, the *avrA* gene product might result in the production of a small diffusible molecule that is detected by the plant at a distance from the bacterium. Recent data indicate that the *avrD* gene from *P. s.* pv. *tomato* (Okabe) Young *et al.* is involved in the production of a diffusible race-specific elicitor (Keen *et al.* 1990). A similar species generated by the action of the *avrA* gene would not have been detected by the present immunoscreening approach.

In conclusion, while we have been able to generate MAbs that identify differences in bacterial cell surface epitopes at the genus, species, pathovar, race, and strain levels, no direct correlation was found between the presence of the avirulence gene *avrA* and the race-specific expression of a cell surface epitope. These results suggest that the molecular basis of host-pathogen specificity either does not involve the bacterial cell surface or involves only a minor antigen. However, we cannot exclude changes in cell surface components that are weakly or nonantigenic independent of gross cell wall architecture. In a similar study, MAbs raised against extracellular antigens of the soybean fungal pathogen *Phytophthora megasperma* f. sp. *glycinea* Kuan & Erwin were unable to identify epitopes that were either the direct or indirect products of fungal avirulence genes. The MAbs recognized carbohydrate-specific epitopes, and immunoanalysis of western blots showed very little difference between different fungal races in the pattern of molecules recognized by the MAbs (Wycoff *et al.* 1987).

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