Immuno-capture of *Ralstonia solanacearum* by an EPS-specific monoclonal antibody enhances sensitivity of PCR

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Abstract

Detection of *Ralstonia solanacearum* (*Rs*) in field soil and irrigation water is complicated by significant heterogeneity within the species complex and insufficient bacterial populations for most detection assays. DNA-based methods, though sensitive and specific, are not very practical since they only accommodate a small sample volume, usually 1-5 microliters, which may not contain enough bacterial DNA for detection. Therefore, an anti-*Rs* antibody was developed with the aim of concentrating low populations of bacteria from large volumes of irrigation or drainage water. The antibody was developed using traditional monoclonal hybridoma technology with a pool of 10 *Rs* strains as the antigen. The strong reacting antibody 3.H7 (IgG$_3$ with kappa light chain) is specific for all *Rs* EPS (109 strains from diverse hosts and geographical origins, representing 3 phylotypes) with only one exception (one Race 2 strain from banana). Detection of low *Rs* populations was not possible when aliquots from large water samples were directly added to PCR (using *fliC* or 759/760 primers) reactions; however, aliquots of 3.H7-immuno-captured cells were successfully amplified by PCR from these same water samples.

Introduction

Rs is a heterogeneous species with a wide host range. Detecting this pathogen at low population levels is critical for disease management strategies. Antibody-based assays (ELISA, dye-based dipsticks, etc.) are robust and technically simple methods for detecting plant pathogens, but they often lack the specificity of DNA detection methods. PCR, while highly specific, can only accommodate small sample volumes (usually 1-5 ul). In addition, PCR is often inhibited by soil and plant extracts. These drawbacks limit the utility of PCR for detecting *Rs* in large sample sets (from farms, exported and imported agricultural products). Immunocapture-PCR methods use the robustness of antibodies to concentrate target organisms while simultaneously removing PCR inhibitors effectively lowering detection limits. Therefore, a monoclonal antibody (MAB) was developed for enhancing the sensitivity of PCR detecting *Rs* in large environmental samples.

Methods

MAB was generated as previously described with some modifications[1]. Ten formalin-fixed (15 minutes in PBS with 0.5% formalin) *Rs* Race 3 biovar 2 (R3B2) strains (Table 1, bold) were washed in PBS and adjusted to 0.1 OD$_{600}$, pooled equally and injected (0.2 ml) into the peritoneum of 2 Balb/c mice 3 times a week for 3 weeks. After a two-week rest period, the mice were injected once more. Following a 5-day resting period, the mice were sacrificed by cervical dislocation, their spleens removed and splenocytes fused with P3 cells using traditional hybridoma methodology (5:1 splenocyte/ P3 ratio, 1500-4000 MW polyethylene glycol) as previously described. Clones were screened by ELISA using 96-well plates (Costar Cat# 2595) coated with *Ralstonia* strains adjusted to 0.1 OD$_{600}$ in carbonate bicarbonate (CBC) buffer (15 mM Na$_2$CO$_3$, 35 mM NaHCO$_3$, pH 9.6), plated (100 ul/ well) on and dried overnight in a 37° C incubator. Isotyping was performed using Isotype strips (Envirologix cat# RS200 Kit 25). Immunocapture-PCR was performed by soaking hole-punched nitrocellulose discs in purified (protein G chromatography) 3.H7 antibody overnight. The discs were blocked for 30 minutes in 5% blotto before incubating overnight in 1-liter bacterial suspensions of the indicated concentration. PCR (primers as indicated, 5′ @ 95°C, 35 cycles of 15″ @ 95°C, 30″ @ 55°C, 30″ @ 72°C, with a final 7′ 72°C extension) was performed using 5 ul template aliquots directly from the bacterial suspensions (before disc incubation) or from disc extractions (discs rinsed twice in PBS, placed in 50 ul sterile water and boiled for 5 minutes). Dot blots were performed by spotting 5 ul of the indicated strains at the indicated concentration. Removal of EPS was performed by pelleting (by centrifugation) and resuspending cells 3x in H$_2$O. A rabbit polyclonal antibody was generated at the University of Georgia. Magnetic beads were generated as outlined in Figure 3[2].

Table 1. MAB 3.H7 Reactivity List

Avg OD	Other ID	Strain	Geographic Origin	Host
0.3535*		Bacillus megaterium	Hawaii	Soil
0.3565*		Enterbacter asburiae	Hawaii	Ginger
0.475*	cc26	Erwinia carotovora	Hawaii	Aglaonema
0.196		Escheria coli	Hawaii	Soil
0.2005	EMS-1	P. corrugata	Idaho	Tomato
0.171		X. campestris	Hawaii	Cabbage
0.189	164	Pseudomonas syringae	South Africa	Rice
1.235	PS-11	R3B2	Guatemala	Geranium
1.951	**PS-12**	**R3B2**	**Guatamala**	**Geranium**
0.7825	PS-13	R3B2	Guatemala	Geranium
0.9925	PS-14	R3B2	Guatemala	Geranium
1.437	PS-16	R3B2	Guatemala	Geranium
0.729	PS-17	R3B2	Guatemala	Geranium
1.593	PS-20	R3B2	Guatemala	Geranium
0.903	PS-22	R3B2	Guatemala	Geranium
1.633	PS-31	R3B2	Guatemala	Geranium
2.964	19	R3B2	Columbia	Potato
2.7345	**UW19**	**R3B2**	**Colombia**	**Potato**
3.0745	**UW23**	**R3B2**	**Israel**	**Potato**
3.57	UW24	R3B2	Israel	Potato
2.9625	UW37	R3B2	Colombia	Potato
3.039	**UW72**	**R3B2**	**Greece**	**Potato**
3.139	UW73	R3B2	Ceylon	Potato
2.7305	UW80	R3B2	Columbia	S. phureja
2.235	**UW81**	**R3B2**	**Colombia**	**Potato hybrid**
2.174	UW120	R3B2	Costa Rica	Potato
1.845	UW145	R3B2	Australia	Potato
1.1345	UW150	R3B2	Australia	Potato
2.4545	UW220a	R3B2	India	Potato
2.451	UW221	R3B2	Kenya	Solanum Nigrum
3.383	**UW224**	**R3B2**	**Australia**	**Potato**
2.399	UW257	R3B2	Costa Rica	Potato
2.5315	UW260	R3B2	Peru	Potato
4.22	UW272	R3B2	Costa Rica	Potato
2.736	UW276	R3B2	Mexico	Potato
2.6075	UW344	R3B2	Brazil	Potato
2.463	UW347	R3B2	Brazil	Potato
2.904	**UW348**	**R3B2**	**Brazil**	**Potato**
3.266	UW349	R3B2	Brazil	Potato
2.915	**UW365**	**R3B2**	**China**	**Potato**
1.4665	UW408	R3B2	Australia	Potato
0.79	UW437	R3B2	Australia	Tomato
2.3505	UW441	R3B2	Australia	Potato
2.707	UW443	R3B2	Australia	Tomato
2.318	UW448	R3B2	Burundi	Potato
3.594	UW449	R3B2	Peru	Potato
1.858	UW453	R3B2	Nigeria	Potato
4.028	UW464	R3B2	Brazil	Potato
2.112	UW473	R3B2	Peru	Potato
4.022	UW487	R3B2	Peru	Potato
2.0505	UW489	R3B2	Chile	Potato
2.361	UW490	R3B2	Chile	Potato
0.565	UW491	R3B2	Colombia	Potato
3.281	UW492	R3B2	Peru	Potato
2.4045	UW523	R3B2	France	Tomato
1.9395	UW550	R3B2	Netherlands	Potato
2.923	**UW551**	**R3B2**	**Kenya**	**Geranium**
0.255	EPS-	R3B2	Kenya	Geranium
1.116	LPS-	R3B2	Kenya	Geranium
0.556	**UW552**	**R3B2**	**Guatamala**	**Geranium**
2.829	UW556	R3B2	Guatemala	Potato
1.378	UW560	R3B2	Guatemala	Potato
3.728	UW573	R3B2	Guatemala	Potato
0.7085	UW596	R3B2	Guatemala	Potato
0.7685	UW598	R3B2	Guatemala	Potato
2.593	UW600	R3B2	Guatemala	Potato
2.84	UW604	R3B2	Guatemala	Tomato
1.917	UW610	R3B2	Guatemala	Tomato
2.584	UW612	R3B2	Guatemala	Soil / Tomato field
3.2325	GMI1000	Race 1	Guyana	Tomato
3.0545	30	Race 1	Peru	Tomato
0.572	130	Race 1	Peru	Tomato
3.918	UW27	Race 1	Florida	Tobacco
2.658	UW97	Race 1	Hawaii	Ginger
3.569	UW329	Race 1	Taiwan	Tobacco
1.747	UW361	Race 1	China	Mulberry
3.307	UW369	Race 1	China	Peanut
3.872	UW386	Race 1	Nigeria	Soil
3.682	UW451	Race 1	Peru	Potato
3.173	UW457	Race 1	Peru	Soil
3.064	UW458	Race 1	Peru	Potato
2.707	UW459	Race 1	Peru	Potato
3.808	UW461	Race 1	Brazil	Potato
3.731	UW462	Race 1	Brazil	Potato
2.335	UW465	Race 1	Brazil	Potato
2.823	UW466	Race 1	Brazil	Potato
3.522	UW467	Race 1	Brazil	Potato
3.719	UW470	Race 1	Brazil	Potato
4.011	UW471	Race 1	Peru	Potato
2.057	UW472	Race 1	Nigeria	Potato
3.172	UW475	Race 1	Peru	Eggplant
3.86	UW477	Race 1	Peru	Potato
2.069	UW484	Race 1	Peru	Potato
3.759	UW485	Race 1	Peru	Potato
2.387	UW488	Race 1	Brazil	S. americanum
3.655	UW497	Race 1	Peru	Tomato
3.891	UW576	Race 1	Florida	Tomato
1.419	UW581	Race 1	Florida	Hydrangea
2.198	UW585	Race 1	Florida	Pepper
2.647	167	Race 2	Costa Rica	Banana
0.1985	91/04	Race 2	Jamaica	Banana
1.508	UW9	Race 2	Costa Rica	Heliconia
3.226	KV2.2	Race 2	Oahu	Heliconia
3.0755	DP-6	Race 4	Hawaii	Ginger
3.3975	GW-1	Race 4	Hawaii	Ginger
3.345	PO-7	Race 4	Hawaii	Ginger
2.9	BD-1	BD	Indonesia	Banana
3.5485	BD-1 Mu	BD	Indonesia	Banana
1.888	BD-2	BD	Indonesia	Banana
3.761	UW84	N/A (Ralstonia)	Canada	Tomato
2.664	UW355	N/A (Ralstonia)	China	Sweet Potato
3.71	UW517	N/A (Ralstonia)	Brazil	Eucalyptus

Bold = strains used for injecting mice
* = strains with a high background without antibody exposure
N/A = not available (uncharacterized *Ralstonia* strains)

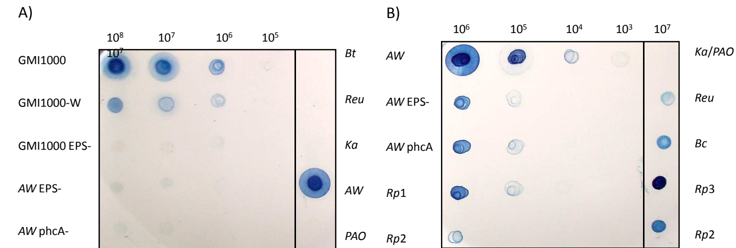

Figure 1. MAB 3.H7 specifically detects *Rs*, but not EPS- *Rs*, cells. MAB 3.H7 (A) or rabbit polyclonal sera (B) was used to probe a membrane spotted with the indicated strains and concentrations. *Bt* = *Burkholderia thailandensis*; *Reu* = *Ralstonia eutropha* CH34; *Rp* = *Ralstonia pickettii* (1-3 are different strains); *Bc* = *Burkholderia cepacia* 2735; *PAO* = *Pseudomonas aeruginosa*; *Ka* = *Klebsiella aerogenes*; -W = washed to remove non cell bound EPS.

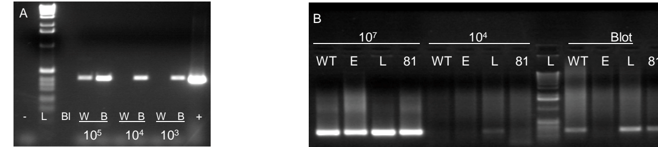

Figure 2. MAB 3.H7 captures *Rs*, but not EPS-, cells enhancing sensitivity of PCR. One liter volumes with the indicated concentration of *Rs* GW-1 (cells/ml) were incubated with a 3.H7 soaked membrane and then subjected to PCR using *flic* primers (A). WT (WT), EPS- (E) and LPS- (L) mutants of UW551 and UW81 (81) were adjusted to 10^7 cells/ ml, then 1 ml was added to 1 liter of water. A 3.H7 soaked membrane was used to capture cells followed by PCR using 759/ 760 primers (B). (-) = water negative control, Bl= blank lane, W= 1 liter water sample, B= blot captured cell sample, (+) = 10^8 cells/ ml positive control.

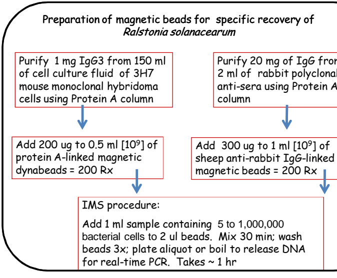

Figure 3. Schematic of magnetic bead preparation.

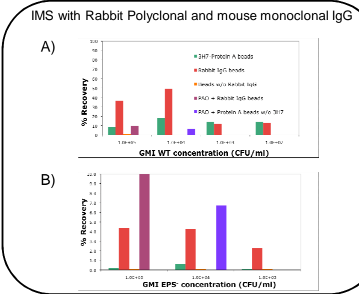

Figure 4. MAB 3.H7 vs. rabbit polyclonal antibody magnetic bead capture of *Rs*. The percent recovery of the indicated concentration of cells were calculated based on plate counts. Beads with (green and red) and without (orange and blue) antibody were assessed for non-specific binding. *PAO = Pseudomonas aeruginosa.*

Results

The monoclonal antibody 3.H7 reacts with all *Ralstonia* strains tested thus far except a Race 2 strain from banana, an uncharacterized clove strain and the UW551 EPS- mutant (Table 1). By dot blot analysis, 3.H7 can detect 10^6 *Rs* but not EPS- mutants (Figure 1). In addition, the detection of washed (removes most EPS) cells is reduced. However, 3.H7 does not react with other genera, whereas a rabbit polyclonal antiserum does (Table 1 and Figure 1). The 3.H7 MAB effectively concentrated *Rs* cells in large volumes of water, generating a positive PCR signal for WT *Rs* but not EPS- cells (Figure 2). 3.H7-conjugated magnetic beads captured as few as 10 *Rs* cells from a 100 cells/ml sample (Figure 4). However, the detection of washed cells or EPS- mutants was significantly lower. The rabbit polyclonal magnetic beads was able to effectively detect EPS- cells but also bound non-*Rs* when these cells were at very high concentrations.

Conclusions

Rs can be difficult to detect in large environmental sample sets that may be too low in concentration or contain inhibitors that reduce the effectiveness of PCR. Therefore, we developed a MAB to capture *Rs* cells, remove them from inhibitors and concentrate into a small volume able to be detected by PCR. 3.H7 is limited due to the lack of binding to washed or EPS- mutated cells. However, a magnetic bead capture assay was able to capture 10% from 100 cells/ ml. Future work will optimize a capture/ real-time PCR detection assay for *Rs* in environmental samples.

References

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2) Walcott et al., "Detection of *Acidovorax avenae* subsp. *citrulli* in watermelon seed using immunomagnetic separation and the polymerase chain reaction," Plant Disease, 84(4):470-474 (Apr. 2000).

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