HEAT STABILITY, PERIODATE SENSITIVITY AND SOLUBILITY OF BACTERIAL ADHESINS AND NEMATODE RECEPTORS IN TWO *ANGUINA-RATHAYIBACTER* INTERACTIONS

S. Indarti[1] and I.T. Riley[2]

[1] *Entomology and Plant Pathology Department, Gadjah Mada University, Yogyakarta 55281, Indonesia*
[2] *Applied and Molecular Ecology, University of Adelaide, PMB1, Glen Osmond, South Australia 5064, Australia*

Summary. The heat stability and periodate sensitivity of the nematode receptors and bacterial adhesins and the solubility of the bacterial adhesins involved in the adhesion of *Rathayibacter toxicus* to *Anguina funesta* and *R. tritici* to *A. tritici* were examined. The data were consistent with *R. toxicus* having at least two adhesins for *A. funesta*, one heat labile and the other heat stable. The complementary receptors had a frequency of 50 and 15%, respectively, for the nematodes tested, and had the opposite heat stability. *R. tritici* appeared to have one heat labile (inactivated by 16 min at 100 °C) adhesin for *A. tritici*. The complementary, heat stable receptor occurred in 100% of the test population. Periodate treatment of either the nematode or the bacterium eliminated adhesion. There was no evidence that the bacterial adhesins were soluble.

A range of microorganisms can adhere to nematode cuticles either as the first step in killing or parasitising the nematode, as a source of nutrition, or in using the nematode as a vector. These include spores of fungal and bacterial endoparasites such as *Drechmeria* and *Pasteuria*, nematode trapping fungi such as *Arthrobotrys* (Stirling, 1991) and the fungal and bacterial plant pathogens such as *Dilophospora* and *Rathayibacter* (Bird and McKay, 1987; Riley and McKay, 1990; Riley and McKay, 1991). Given such organisms have the potential to provide the biological control of pest nematodes or, in the case of *Rathayibacter*, to increase the impact of its vector nematode, there has been some research on the chemistry of adhesion.

Treatment of nematodes with heat, periodate, protease enzymes or surfactants tends to reduce microbial adhesion (Bird *at al.*, 1989; Bird and Zuckerman, 1989; McClure and Spiegel, 1991; Jansson, 1993) while carbohydrates and carbolytic enzymes do not (McClure and Spiegel, 1991; Jansson, 1993). Similar results have been found with treatment of the associated microbes (Bird *at al.*, 1989; Bird and Zuckerman, 1989; McClure and Spiegel, 1991; Jansson, 1993; Jansson and Friman 1999). However, apart from a limited effect of treating *Rathayibacter* with wheat germ agglutinin (McClure and Spiegel, 1991), there have been no investigations of the chemistry of the bacterial component of *Rathayibacter-Anguina* interactions.

To provide further understanding of the bacterial adhesins involved in *Rathayibacter-Anguina* interactions, a study of the heat stability, periodate sensitivity of adhesins and associated receptors and the solubility of adhesins was undertaken for *Rathayibacter tritici* (Carlson *et* Vidaver, 1982) Zgurskaya *et al.*, 1993/*Anguina tritici* (Steinbuch, 1799) Chitw., 1935 and *Rathayibacter toxicus* (Riley *et* Ophel, 1982) Sasaki *et al.*, 1993/*Anguina funesta* Price *et al.*, 1979.

MATERIALS AND METHODS

Second-stage juveniles (J2) of *A. funesta* and *A. tritici* were obtained from seed galls from *Lolium rigidum* Gaudin (from Damboring, Western Australia) and *Triticum aestivum* L. (from Carnamah, Western Australia), respectively. To rehydrate the nematodes, intact galls were soaked overnight in separate wells in a 24 well plate containing phosphate buffer saline (PBS) 0.02M, pH 7.3 (0.5 ml) at 5 °C. In the case of the *Lolium* galls, the palea and lemma were removed before soaking. After soaking, the galls were dissected to release the J2 and the plant material removed. Dissecting instruments were cleaned between each gall.

To confirm that the resultant nematode suspensions were not contaminated with *Rathayibacter* spp., which can be carried either inside or on the surface of galls, the multiwell plates were incubated at 25 °C for 1 h on an orbital shaker at 200 rpm. Immediately following this incubation, the nematodes in each well were examined for clumping, which occurs when adhering bacteria cause cross-linkage of the J2, and only J2 from apparently uncontaminated wells were used in the study (only a small proportion of *A. funesta* samples were found to be contaminated).

A. tritici J2 were combined, counted and diluted to 400 J2/ml in PBS and stored at 5 °C until used in adhesion experiments. As the proportion of the *A. funesta* population receptive to the adhesion of *R. toxicus* was found to be less than 30%, before combining the nematodes, subsamples (c. 100 J2) from individual wells were tested for adhesiveness. Only wells containing samples with 50% or more receptive J2 were combined and diluted in the same manner as the *A. tritici* J2.

Cultures of *R. toxicus* (CS14) and *R. tritici* (CS108) were grown in a glucose-yeast-casamino acid broth (Riley, 1987) at 25 °C for about five days depending on the

Table I. The effect of heat treatment on mean adhesion (measured by clumping reaction) of *Rathayibacter toxicus* to *Anguina funesta*.

Nematode treatment (minutes at 60 °C)		Bacterial treatment (minutes at 100 °C)									
		0	1	2	4	8	16	32	64	128	Autoclaved
0 (n = 8-12)	Clumping.(%)	50	45	45	45	45	40	15	15	10	10
	Strength	***	***	***	***	***	**	*	*	*	*
15 (n = 4)	Clumping.(%)	50				40	10	0	0	0	0
	Strength	***	-	-	-	***	*				
20 (n= 4)	Clumping.(%)	45	45	50	45	45	45				
	Strength	***	***	***	***	***	***	-	-	-	-
30 (n = 4)	Clumping.(%)	55	30	20	10	15	5	0	0		0
	Strength	***	**	**	*	*	*			-	

Clumping strength: *** strong; ** medium; * low; - Combination not tested

Table II. The effect of heat treatment on mean adhesion (measured by clumping reaction) of *R. tritici* to *A. tritici*.

Nematode treatment (minutes at 60 °C)		Bacterial treatment (minutes at 100 °C)							
		0	1	2	4	8	16	32	Autoclaved
0 (n=8-12)	Clumping.(%)	100	100	90	70	60	0	0	0
	Strength	***	***	***	***	***			
15 (n=4)	Clumping.(%)	100			40	40	5	0	0
	Strength	***	-	-	**	**	*		
20 (n=4)	Clumping.(%)	100	100	90	80	20			
	Strength	***	***	***	*	*	-	-	-
30 (n=4)	Clumping.(%)	100	75	25	10	5	0	5	0
	Strength	***	***	**	*	*		*	

Clumping strength: *** strong; ** medium; * low; - Combination not tested

growth rate. Cells were harvested by centrifugation (3,500 rpm for 15 min), washed twice in sterile PBS and diluted to an OD 1.0 at 600 nm for use in adhesion experiments.

Adhesion assays were conducted in multiwell plates with c. 200 J2 per well in 0.5 ml of PBS to which 20 ml of bacteria suspension was added. The plates were then incubated at 25 °C on an orbital shaker at 200 rpm for 1 h. Immediately following incubation, adhesion was estimated (to the nearest 5%) from clumping of J2 observed under a dissecting microscope (x40) as described by Riley and McKay (1990). For any given combination of nematode and bacteria, four to eight replicate wells were assessed.

To test the heat stability of the bacterial adhesins, *R. toxicus* and *R. tritici* heated for various times from 0 to 128 min (see Tables I and II for the times used) in a boiling water bath (100 °C, 5 ml of suspension in a 10 ml polypropylene tube) or autoclaved for 20 min at 121 °C were assayed for adhesiveness. The assays were conducted with untreated or nematodes heated for 15, 20 or 30 min at 60 °C (30 min was required to heat kill the J2). To examine the effect of periodate on adhesion, bacteria and nematodes were incubated separately in freshly prepared 0.05M $NaIO_4$ at 20 °C for 1 h in the dark. Treated bacteria and nematodes were washed twice in PBS by centrifugation (as described above) before including in assays in factorial combinations with untreated suspensions. To test the solubility of the bacterial adhesions, filtered (0.45 mm) supernatants (3,500 rpm, 15 min) of bacterial suspensions (OD 1.0, 600 nm, PBS) incubated at 5 and 25 °C for 1 and 24 h replaced the bacterial suspension in modified adhesion assays (c. 200 J2 in 100 ml plus 400 ml supernatant).

RESULTS

Table I and II show the effects of heat treatments on the adhesion of *R. toxicus* and *R. tritici* to *A. funesta* and *A. tritici*, respectively. With untreated nematodes and bacteria, addition of *R. toxicus* caused strong clumping of about 50% of the J2 and *R. tritici* about 100%.

Heating of *R. toxicus* for up to 16 min had a minimal effect on clumping. However, heating for 32 min or above resulted in reduced clumping (10 to 15% instead of 50%). The clumps formed were clearly cross-linked and consistent with the reaction associated with adhesion, but were relatively weakly linked compared to untreated controls. Examination of these J2 with a compound microscope (x400) revealed much fewer adhering cells than in the controls. Heating of *A. funesta* had no apparent effect on adhesion of untreated bacteria. However, heating the J2 for 15 min or more in combination with heating *R. toxicus* for 32 min or more, largely eliminated clumping. A partial reduction in adhesion with heating of the J2 occurred even for *R. toxicus* that had been heated for 8 or 16 min but the response was not consistent.

The response to heat treatment of *R. tritici* was less complicated. Heating the *R. tritici* for 16 min or more largely eliminated adhesion irrespective of the treatment of the J2. Some decline in clumping was evident after 2 to 4 min heating of the bacterium. This reduction in adhesion was more pronounced for J2 heated for 30 min. However, the heat treatment of the J2 had no effect when untreated bacteria were added.

Periodate treatment (Table III) eliminated adhesion with treatment of either the nematode or bacterium in both species associations. A low level of clumping seen

Table III. The effect of sodium periodate on mean adhesion (measured by clumping reaction, n=8) of *R. toxicus* to *A. funesta* and *R. tritici* to *A. tritici*.

Periodate treatment		Clumping (%)	
Nematode	Bacteria	*R. toxicus*	*R. tritici*
-	-	50	100
+	-	0	0
-	+	5	0
+	+	0	0

+, incubated in 0.05M NaIO4 for 1 h at 20 °C in the dark; - untreated

Table IV. Deduced heat stability and frequency of the adhesin-receptor pairs in the interactions between *R. toxicus* and *A. funesta* and *R. tritici* and *A. tritici*.

Bacterium/nematode interaction	Bacterial adhesin	Nematode receptor	Frequency in nematode population (%)
R. toxicus/A. funesta	Labile	Stable	50
	Stable	Labile	10-15
R. tritici/A. tritici	Labile	Stable	100

with periodate-treated *R. toxicus* can be attributed to non-specific aggregation.

The filtered supernatants of bacterial suspensions gave no clumping response when added to the J2 of either *Anguina* species.

DISCUSSION

Since both nematode species remained receptive to adhesion by untreated bacteria, regardless of the period of heating of the nematode, this indicates that the nematode receptors involved are heat stable, at least within the thermal exposure tested. In contrast, adhesion of *Pasteuria penetrans* spores to *Meloidogyne javanica* was reduced by about 60% after the nematode had been exposed to 60 °C for 30 min (Bird *et al.*, 1989). In contrast, the bacterial adhesins appear to be heat labile, with the adhesin in *R. tritici* being more rapidly damaged than that in *R. toxicus*. Bird *et al.* (1989) also found that heat treatment (100 °C) of *P. penetrans* for 1 h reduced its adhesion about 50%.

Although a heat labile adhesin is indicated in both bacterial species, the low frequency but persistent adhesion (even with autoclaved cells) seen in *R. toxicus* is consistent with this bacterium having at least two independent adhesins. These heat stable adhesins appear to attach to heat labile receptors as heat treatment of the nematode eliminates this adhesion. Table IV provides a possible interpretation of the results, with at least two receptor-adhesin combinations for *R. toxicus/A. funesta* and one for *R. tritici/A. tritici*. Multiple receptors or adhesins occurring within a population of nematode or strain bacterium, respectively, has previously been proposed by Riley and McKay (1990) based on reaction seen in factorial combinations of untreated nematode populations and bacterial strains.

Although periodate treatment of *A. funesta* and *A. tritici* was known to eliminate adhesion by *Rathayibacter* spp. (McClure and Spiegel, 1991), the periodate-sensitivity of bacterial adhesins had not been tested. This study confirms the earlier findings and demonstrates that the bacterial adhesions are also periodate-sensitive. Therefore, the bacterial adhesins have active sites with carbohydrate residues and are likely to be glycoproteins.

This work provided no evidence that the bacterial adhesins are soluble or, if soluble, that they can give cross-linking of the nematodes when separated from the bacterial capsule. The soluble antigens of intact *Rathayibacter* cells (Riley, 1987) may not be compounds involved in adhesion. It is probably reasonable to postulate that adhesins would be tightly associated with the capsule and not readily dissolved. However, adhesin may contain only one active site, so may not be able to cross-link nematodes. In which case, their solubility could be confirmed by demonstrating a reduction in adhesion of bacterial cells to nematodes pre-treated with a bacterial supernatant.

Although this work provides some additional information on receptor-adhesins in nematode-microbe interactions, a full understanding will only come with purification and characterisation of the compounds.

ACKNOWLEDGMENTS

The Australian Academy of Technological Sciences and Engineering through the Crawford Fund provided financial support for the senior author to undertake training at the University of Adelaide which included this research. The salary of I. T. Riley is in part funded by the Grains Research and Development Corporation.

LITERATURE CITED

Bird A.F. and McKay A.C., 1987. Adhesion of conidia of the fungus *Dilophospora alopecuri* to the cuticle of the nematode *Anguina agrostis*, the vector in annual ryegrass toxicity. *International Journal for Parasitology, 17*: 1239-1247

Bird A.F., Bonig I. and Bacic A., 1989. Factors affecting the adhesion of micro-organisms to the surfaces of plant-parasitic nematodes. *Parasitology, 98*: 155-64.

Bird A.F. and Zuckerman B.M., 1989. Studies on surface coat (glycocalyx) of the dauer larva of *Anguina agrostis*. *International Journal for Parasitology, 19*: 235-240.

Jansson H.B., 1993. Adhesion to nematodes of conidia from the nematophagus fungus *Drechmeria coniospora*. *Journal of General Microbiology, 139*: 1899-1906.

Jansson H.B. and Friman E., 1999. Infection-related surface proteins on conidia of the nematophagous fungus *Drechmeria coniospora*. *Mycological Research, 103*: 249-256.

McClure M.A. and Spiegel Y., 1991. Role of the nematode surface coat in adhesion of *Clavibacter* sp. to *Anguina funesta* and *Anguina tritici*. *Parasitology, 103*: 421-427.

Riley I.T., 1987. Serological relationships between strains of coryneform bacteria responsible for annual ryegrass toxicity and other plant-pathogenic corynebacteria. *International Journal of Systematic Bacteriology, 35*: 153-159.

Riley I.T. and McKay A.C., 1990. Specificity of the adhesion of some plant pathogenic micro-organisms to the cuticle of nematodes in the genus *Anguina* (Nematoda: Anguinidae). *Nematologica, 36*: 90-103.

Riley I.T. and McKay A.C., 1991. Susceptibility of *Anguina funesta* (Nematoda: Anguinidae) populations to adhesion by the toxigenic *Clavibacter* sp. responsible for annual ryegrass toxicity. *Nematologica, 37*: 439-446.

Stirling G.R., 1991. *Biological control of plant parasitic nematodes*. CAB International, Wallingford, U.K., pp. 282.

Accepted for publication on 7 July 2003.