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Bacterial associates of *Anguina* species isolated from galls

Yong Chang WEN and David R. VIGLIERCHIO

Department of Nematology, University of California, Davis, CA 95616, USA

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Summary — Certain animal and plant diseases are well known to be a consequence of a particular nematode and bacterial species involved in an intimate relationship. For several such insect diseases, reports indicate that the nematode is unable to survive in the absence of the specific partner. That is not the case for the plant parasite group, *Anguina* in which each partner may survive independently. This report suggests that the relationship with *Anguina* is opportunistic in that if the appropriate bacteria share the nematode community, the characteristic disease appears. In the absence of that particular bacterial species other bacterial species in the nematode niche can substitute; however the relationship is benign. This indicates that although specificity in partners is required for a specific disease to emerge the nematode may develop intimate relationships with a range of non-threatening bacteria. Therein may lie part of the explanation of why the " sheep staggers " disease so serious to livestock in Australia is not so in the US Pacific Northwest which harbors the same nematode, *Anguina agrostis*.

Résumé — ***Bactéries associées à certaines espèces d'*Anguina *isolées de galles*** — Certaines maladies des animaux et des plantes sont bien connues pour être causées par des espèces particulières de nématodes et de bactéries étroitement associées. Dans le cas de plusieurs maladies d'insectes, les observations indiquent que le nématode ne peut survivre en l'absence de son partenaire spécifique. Ce n'est pas le cas des associations avec les nématodes phytoparasites du groupe *Anguina* dans lesquelles chacun des deux partenaires peut survivre indépendamment de l'autre. La présente étude suggère que la relation de la bactérie avec *Anguina* est opportuniste : si la bactérie appropriée est en communauté avec le nématode la maladie caractéristique apparaîtra, mais en l'absence de cette bactérie particulière, d'autres espèces bactériennes peuvent s'y substituer et une telle association demeurera bénigne. Ceci indique que si la spécificité des organismes associés est nécessaire pour que la maladie spécifique apparaisse, le nématode peut développer des relations étroites avec un éventail de bactéries non dangereuses. Ce phénomène peut fournir une explication partielle au fait que la maladie des moutons dite « sheeps staggers », très grave en Australie, ne l'est pas dans la région du nord-ouest pacifique des USA où le même nématode, *Anguina agrostis*, est présent.

Key-words : *Anguina*, bacteria, associations.

In multicomponent disease complexes, microorganismal species have been associated with nematodes for the expression of a particular disease (Poinar, 1983). Although many such associations give rise to interactive relations whereby the consequences of the prime pathogen may be modified by a secondary one, the resultant effect often tends to be reflected in the degree of disease severity (Dropkin, 1980). With some nematode-microorganismal complexes, however, the association is very specific and obligatory, e.g., *Steinernema carpocapsae-Xenorhabdus nematophilus*, an insect pathogenic complex (Kaya, 1985; Poinar, 1990; Akhurst & Boemare, 1990), or *Anguina agrostis-Corynebacterium rathayi*, a toxicity complex for live stock (Bird, 1981).

Anguina appears to be a genus with several representatives that exhibit a strong specificity with certain bacteria, e.g., *Anguina tritici-Corynebacterium tritici* and *Anguina agrostis-Corynebacterium rathayi* (Bird, 1981). *A. agrostis* has been long known to infect pasture grasses (*Agrostis tenuis, Lolium rigidum* and others) of the central and northern Pacific coast of the USA; however, reports of neurotoxic poisoning of live stock from the complex *A. agrostis* and *C. rathayi* in seed galls, common in Australia, have been infrequent.

A related nematode, *A. pacifica*, has recently been described from *Poa annua* stem galls from several golf courses in the San Francisco area and has also been reported to be associated with unidentified bacteria (Cid Del Prado V. & Maggenti, 1984). It was of interest therefore, to characterize to genus, bacterial isolates from *A. agrostis* seed galls (from a bulk supply provided by H. J. Jensen, Oregon State University) and *A. pacifica* (from the San Francisco Golf Club).

Materials and methods

A. agrostis seed galls were hand picked from a mass stock supply, placed between alcohol sterilized large rubber stoppers and, in a laminar flow hood rubbed gently to remove the gall plant tissue covering. The exposed nematode galls (five replicates, 50 galls/replicate) were wrapped in a sterile thin nylon net bag and immersed in 1 % sodium hypochlorite, submitted to aspirator vacuum for 5 min, then removed and washed six times with sterile distilled water. After the sixth wash, the galls of one replicate were distributed to the surface of nutrient agar (NA) medium in a Petri dish, those of a second replicate onto a yeast glucose carbonate (YGC) medium, the third on to peptone yeast extract (PYE) medium, the fourth, potato dextrose agar (PDA) medium, and the fifth, tryptose blood agar base (TBAB) medium, then incubated at 25-28 °C for 2-3 days. After

incubation, each gall community was evaluated in terms of kinds of organisms present. Samples from each community containing live bacteria and nematodes were resuspended in saline, then replated on the respective media in a dilution series to obtain individual colonies for subsequent stock culture and further evaluation.

A. pacifica stem leaf-sheath galls were hand picked, the sheath excised just below and above the gall, and the explant wrapped in the nylon cloth bag as previously described. The bag was then immersed in 0.05 % sodium hypochlorite solution and submitted to vacuum for 5 min and then washed with sterile distilled water six times as was done with seed galls. The axenized galls were then distributed on nutrient agar or yeast glucose carbonate medium and incubated at 25 °C. After 24 h of incubation, galls showing no medium contamination were opened axenically and placed on fresh media plates to release internal contents for culture. As previously described, samples of each gall community were suspended in solution, replated on the respective media in a dilution series to obtain individual colonies for subsequent stock culture and further evaluation.

The identification to genus incorporated tests of colony characteristics, morphology, and physiology; classification was determined according to Bergey's Manual of Systematic Bacteriology (Krieg, 1984; Sneath, 1986), The Prokaryotes Handbook (Starr *et al.*, 1981), Manual of Methods for General Bacteriology (Gerhardt, 1981) and the API 20 E System. Morphological characteristics, the presence of endospore formation and cell motility were determined by examination of whole cell preparations with the aid of an optical microscope. Colony fluorescence was verified by examination of plates cultured for 24, 48 and 72 h by use of UV illumination (254 nm). Bacterial growth in liquid YGC medium was determined by sample examination with the aid of the optical microscope.

TEM micrographs (run on electron microscope Model JEOL 100 S) were taken of whole cell preparations obtained from isolates cultured 24 h at 28 °C in YGC broth then negatively stained with 2 % phosphotungstic acid (PTA) for 30 s.

Results

The incidence of kinds of organisms associated with *A. agrostis* seed galls (Table 1) indicate that bacteria and nematodes together occur most frequently followed by nematodes alone at an intermediate frequency and bacteria or fungi or no organisms at all at a very low frequency. It is of substantial relevance to note that in five media, one quarter to one third of all seed galls were axenic, exhibiting only nematodes but no bacteria or fungi. Turf grass galls cultured on nutrient agar or yeast glucose carbonate all contained bacteria wherein a substantial number of those galls also contained live nematodes.

Table 1. Incidence of organisms associated with *A. agrostis* seed galls in different media. B + N = Bacteria and nematodes, N = Nematodes only, B = Bacteria only, F = Fungi only, F + B or F + N, O = no B, N or F.

Medium	*Incidence of organisms associated with galls (Percent)*				
	B + N	*N*	*B*	*F*	*O*
Nutrient Agar	55	35	5	5	0
Yeast Glucose Carbonate	60	25	10	5	0
Peptone Yeast Extract	65	25	0	5	5
Potato Dextrose Agar	52	33	0	14	0
Tryptose Blood Agar Base	33	43	10	10	4

The characteristics exhibited by colonies of isolates on different media suggested that San Francisco Golf Club turf galls provided ten different isolates and that the Oregon Bent grass seed galls provided seven different isolates; two of these seven isolates which on preliminary trials seemed different, manifested identical colony characteristics on four media.

Morphological characteristics (shape and size) and growth characteristics indicated that isolates SF 2 and SF 3 were similar but not identical; whereas, Oregon isolates OR 2 and OR 3 appeared indistinguishable.

In summary, of the seventeen bacterial isolates, there were fourteen Gram-negative rod shaped bacteria belonging to four genera, two Gram-positive rod shaped bacteria, and one Gram-positive coccus.

Genera of Gram-negative aerobic rod shaped bacteria

Pseudomonas Migula, 1894

Nine isolates designated SF 5-SF 9 and OR 2-OR 5 have common characteristics : motile (polar flagella); both methyl red (M.R.) and Voges-Proskauer (V.P.) negative; oxidase positive, oxidation positive and fermentation negative; reduction of nitrate to ammonia positive; and growth at 40 °C positive for most of the isolates.

Xanthomonas Dowson, 1939

Two isolates, OR 6 and OR 7 are motile with one polar flagellum. Most other characteristics are similar to *Pseudomonas* except *Xanthomonas* are oxidase negative and cannot grow at 40 °C. There is also one species of *Pseudomonas* that is oxidase negative. *Pseudomonas maltophilia* (Krieg, 1984) OR 6, was identified to be *Pseudomonas maltophilia* by API 20 E multi-test system. Taxonomic uncertainty exists for this organism as to

Table 2. Colonial characteristics of bacterial isolates and different media.

Isolates	*Nutrient agar*	*Yeast glucose carbonate*	*Potato dextrose agar*	*Peptone yeast extract*
OR 1	Growth very fast, pale yellow to light brownish yellow, flat, thick, shiny	Growth very fast, light greenish yellow to light brownish yellow, convex, concentric, shiny	Growth fast, mustard yellow, flat, thick, shiny	Growth fast, pale milky yellow, flat, shiny
OR 2	Growth very fast, pale yellowish white, flat, shiny, transparent	Growth very fast, creamy pinkish white, concentric, shiny	Growth fast, pale yellowish white, flat, shiny	Growth fast, light yellowish white, flat and thin, shiny, transparent
OR 3	Growth very fast, pale yellowish white, flat, shiny, transparent	Growth very fast, creamy pinkish white, concentric, shiny	Growth fast, pale yellowish white, flat, shiny	Growth fast, light yellowish white, flat and thin, shiny, transparent
OR 4	Growth fast, light milky yellowish white, very thin, shiny, transparent	Growth very fast, light brownish yellow, flat, glistens	Growth fast, milky yellowish white, flat, shiny	Growth fast, colorless, flat and very thin, shiny
OR 5	Growth very fast, pale creamy yellowish white, convex, glistens, transparent	Growth very fast, pale pinkish white, convex, glistens	Growth very fast, pale creamy yellowish white, thick, glistens	Growth very fast, pale creamy yellowish white, thin, glistens, transparent
OR 6	Growth fast, creamy white, flat and very thin, shiny, transparent	Growth slow, pale greenish yellow, flat and thin, shiny, opaque	Growth slow, colorless, flat and very thin, shiny, transparent	Growth fast, pale creamy white to colorless, flat, very thin, transparent
OR 7	Growth fast, creamy white, flat and very thin, shiny, transparent	Growth fast, pale greenish yellow, flat, shiny, opaque	Growth slow, colorless, flat and thin, shiny, transparent	Growth fast, colorless, flat and thin, shiny, transparent

whether it should be designated as *Pseudomonas maltophilia* or *Xanthomonas maltophilia* (Krieg, 1984).

Flavobacterium Bergey *et al.*, 1923

Two isolates, SF 10 and OR 1 whose characteristics are : yellow pigmented colonies; motile with peritrichous flagella or non motile; oxidase negative; and fermentation and oxidation of glucose negative.

Erwinia Winslow *et al.*, 1920

One isolate F 4 with these characteristics : motile (peritrichous flagella); both M.R. and V.P. positive; oxidase negative; glucose fermentation positive; reduces nitrate to nitrite, ammonia, and nitrogen gas; mannitol fermentation positive; and growth on 7.5 % NaCl medium.

Through the API 20 E multi-test system a profile number of 1205111 was obtained, identifying this isolate as *Enterobacter agglomerans*. This organism is also called *Erwinia herbicola* Dye, 1964 (Starr *et al.*, 1981).

GENUS OF GRAM-POSITIVE AEROBIC ROD SHAPED BACTERIA

Corynebacterium Lehman & Neuman, 1896

This genus was isolated only from the San Francisco golf grass sample as isolates SF 2 and SF 3 whose characteristics are : curved-shaped rod; non-motile; catalase positive; oxidase negative; both M.R. and V.P. negative; oxidation and fermentation positive; nitrate reduction negative; gelatin hydrolysis negative; and acid-fast staining negative.

GENUS OF GRAM-POSITIVE COCCI

Staphylococcus Rosenbach, 1884

Isolate SF 1 whose characteristics are : round shaped; non-motile; catalase positive; both M.R. and V.P. negative; glucose fermentation positive; mannitol fermentation negative; oxidase negative; nitrate reduction to nitrite positive (no ammonia); gelatin hydrolysis negative; and growth on 7.5 % NaCl medium.

Discussion

From the *Anguina agrostis* seed galls of *Agrostis tenuis* (bent grass) obtained from Oregon at least six different bacterial associates were isolated, none of which were *Corynebacterium rathayi*, noted for producing a neurotoxin. Not only was the genus *Corynebacterium* absent but a significant number of galls appeared free of any

microorganisms. That is not to imply that *C. rathayi* never occurs; it suggests, however, that the frequency of incidences is low in the Pacific coastal area and therefore may explain the infrequency noted for live stock toxication.

The stem galls of *A. pacifica* have a wide range of bacterial associates, two of which were *Corynebacterium* species. Whether these produced neurotoxins remains unknown. The wide range of bacteria that Pacific coast *A. agrostis* or *A. pacifica* are able to introduce into plant tissue suggest a lack of specificity but rather a propensity for opportunistic events. It would appear likely that in areas with different bacterial genera, these nematodes would be able to introduce other bacteria into their respective galls.

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