Russian Journal of Nematology, 1994, 2 (2),99 - 104

Coryneform bacteria from plant galls induced by nematodes of the subfamily Anguininae

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Accepted for publication 25 April 1994

Summary. New associations are reported of coryneform bacteria with the gall-forming nematodes, *Anguina agropyri*, *Heteroanguina graminophila*, *Mesoanguina picridis* and *Subanguina radicicola*. Bacteria isolated were identified as *Aureobacterium* sp., *Curtobacterium* sp. and *Rathayibacter* sp. in accordance with the properties of the strains and bacterial classification. Three strains obtained from stem galls of *Elytrigia repens* and leaf galls of *Calamagrostis neglecta* could not be assigned to known genera. The taxonomic position of these three strains has to be determined. Bacteria-nematode associations appear to be more wide-spread among anguinids then has been previously described.

Key words: bacteria, gall, nematodes, Anguininae.

Currently only three nematode species belonging to the genus *Anguina*, *A. tritici*, *A. agrostis* (syn. *A. funesta*) and *A. agropyri* (syn. *A. pacifica*), are known to carry bacteria into plant-hosts. These nematodes invade plants where they induce galls, which with the gall cavity and gall cells are colonised by coryneform bacteria (Bird, 1981; Cid Del Prado-Vera & Maggenti, 1984; Wen & Viglierchio, 1992). Three species of coryneform bacteria, *Clavibacter tritici*, *Cl. rathayi* and *Cl. toxicus* have been clearly shown to be associated with anguinids (Davis et al., 1984; Collins & Bradbury, 1986, 1991; Riley & Ophel, 1992).

In 1993, a new genus of bacteria, *Rathayibacter*, was proposed to accommodate the above mentioned species, as well as *Cl. iranicus*. Species of this genus form a phenetic cluster which is distinct from *Clavibacter* spp. at a level of 71% and exhibit 7-9% DNA reassociation with strains of *Clavibacter* spp. The properties of members of *Rathayibacter* include: coryneform morphology, peptidoglycan based on 2,4-diaminobutyric acid (DAB), predominant menaquinones of the MK-10 type, phosphatidylglycerol and diphosphatidylglycerol as basic polar lipids, 63 to 72 mol% G+C in their DNA, and several physiological characteristics (Zgurskaya et al., 1993).

In this paper we report new sets of gram-positive bacteria recovered from nematode galls induced in different plants which grow widely in various regions of the former USSR.

MATERIALS AND METHODS

We obtained the following nematode galls for investigation: leaf galls from *Centaurea leucophylla* Bied. and *Cousinia* sp. induced by *Mesoanguina picridis* (Kirjanova, 1944) Chizhov & Subbotin, 1985, collected in Northern Caucasia and Turkmenistan respectively, root galls from *Poa annua* L. induced by *Subanguina radicicola* (Greff, 1872) Paramonov, 1967, collected in Moscow region, leaf galls from *Achillea millefolium* L. induced by *Mesoanguina millefolii* (Low, 1874) Filipjev, 1936, collected in Moscow region, leaf galls from *Calamagrostis neglecta* P.B. induced by *Heteroanguina graminophila* (Goodey, 1933) Chizhov, 1980, collected in Moscow region, stem galls from *Elytrigia repens* L. induced by *Anguina agropyri* Kirjanova, 1955, collected in Moscow region and leaf galls from *Ferula* sp. induced by *Heteroanguina ferulae* (Ivanova, 1977) Chizhov &

Table 1. Bacteria isolated from nematode galls.

Bacteria	Strain	Nematode	Plant-host	Gall locality	Region
Genus sp.	4G1	*Anguina agropyri*	*Elytrigia repens*	stems	Moscow region
Genus sp.	5G3	*Heteroanguina graminophila*	*Calamagrostis neglecta*	leaves	Moscow region
Genus sp.	5G1	*H. graminophila*	*C. neglecta*	leaves	Moscow region
Aureobacterium sp.	3G1a	*Subanguina radicicola*	*Poa annua*	roots	Moscow region
Curtobacterium sp.1.	3G1b	*S. radicicola*	*P. annua*	roots	Moscow region
Curtobacterium sp.2.	1G	***Mesoanguina picridis***	*Centaurea leucophylla*	leaves	Northern Caucasia
Curtobacterium sp.2.	2G	***M. picridis***	***C. leucophylla***	leaves	Northern Caucasia
Curtobacterium sp.2.	4G	*M. picridis*	*C. leucophylla*	leaves	Northern Caucasia
Rathayibacter sp.	KUZ1	*M. picridis*	*Cousinia* sp.	leaves	Turkmenistan
Rathayibacter sp.	KUZ2	*M. picridis*	*Cousinia* sp.	leaves	Turkmenistan

Subbotin, 1985, collected in Uzbekistan.

For transmission electron microscopy galls were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH=7.2) and then post-fixed in 1% osmium tetroxide, dehydrated in an ethanol series and embedded in Epon resin. Utrathin gall sections were cut with an LKB ultramicrotome IV, stained with uranyl acetate and lead citrate. Sections were examined in a Tesla BS-500 transmission electron microscope.

Surfaces of dry or fresh galls were sterilised with 30% H_2O_2 (for two minutes). Subsequently, the galls were cut into pieces, and added to 3 ml of sterile physiological saline and milled. Additionally, for inhibition of gram-negative bacteria, which are widespread on plant surfaces, the gall-mill suspension had KOH added to give a final concentration of 0.1 M and then incubated for 15 minutes. One drop of this alkaline suspension was plated onto nutrient agar (2 g peptone, 1 g glucose, 1 g yeast extract, 1 g casein hydrolysate, 10 ml glycerol, 100 ml wort, 5 g chalk and 1 litre of distilled water [pH=7.4]) and incubated for two weeks at room temperature (18-24° C). Suspensions without pre-treatment were plated as controls.

All bacteria strains isolated were maintained on modified Prauser's medium 79 (10 g glucose, 5 g peptone, 2 g yeast extract, 2 g casamino acids, 6 g NaCl, 15 g agar and 1 litre of distilled water [pH= 7.4]).

Morphological characteristics were studied by phase-contrast microscopy in 3- and 7-day-old cultures grown on Prauser's medium 79. Physiological properties were examined at 24° C as previously described (Zgurskaya et al., 1993).

Peptidoglycan preparations were extracted from intact cell walls by the method of Schleifer and Kandler (1972). Quantitative determination of amino acids and amino sugars composition of peptidoglycans were performed using an LC 600 amino acid analyzer (Biotronic, Munich, Germany). The presence of 2,4-DAB was controlled by the TLS method (Bousfeld et al., 1985). Sugar composition of the cell wall was determined by the method described by Bousfield et al. (1985). Isoprenoid quinones (menaquinones) were extracted and purified by the method described by Collins (1985) and their composition was assayed by thin layer chromatography (TLC), and with a MAT 8430 mass spectrometer (Finnigan, Bremen, Germany).

RESULTS AND DISCUSSION

Electron microscopic study of nematode galls revealed the large accumulations of rod-like bacteria in the cavities, intercellular space and destroyed cells of most galls induced by *Anguina agropyri*, *Heteroanguina graminophila*, *Mesoanguina picridis* (Fig. 1).

Among the bacterial colonies which grown on most of the plates, two types of colonies predominated:

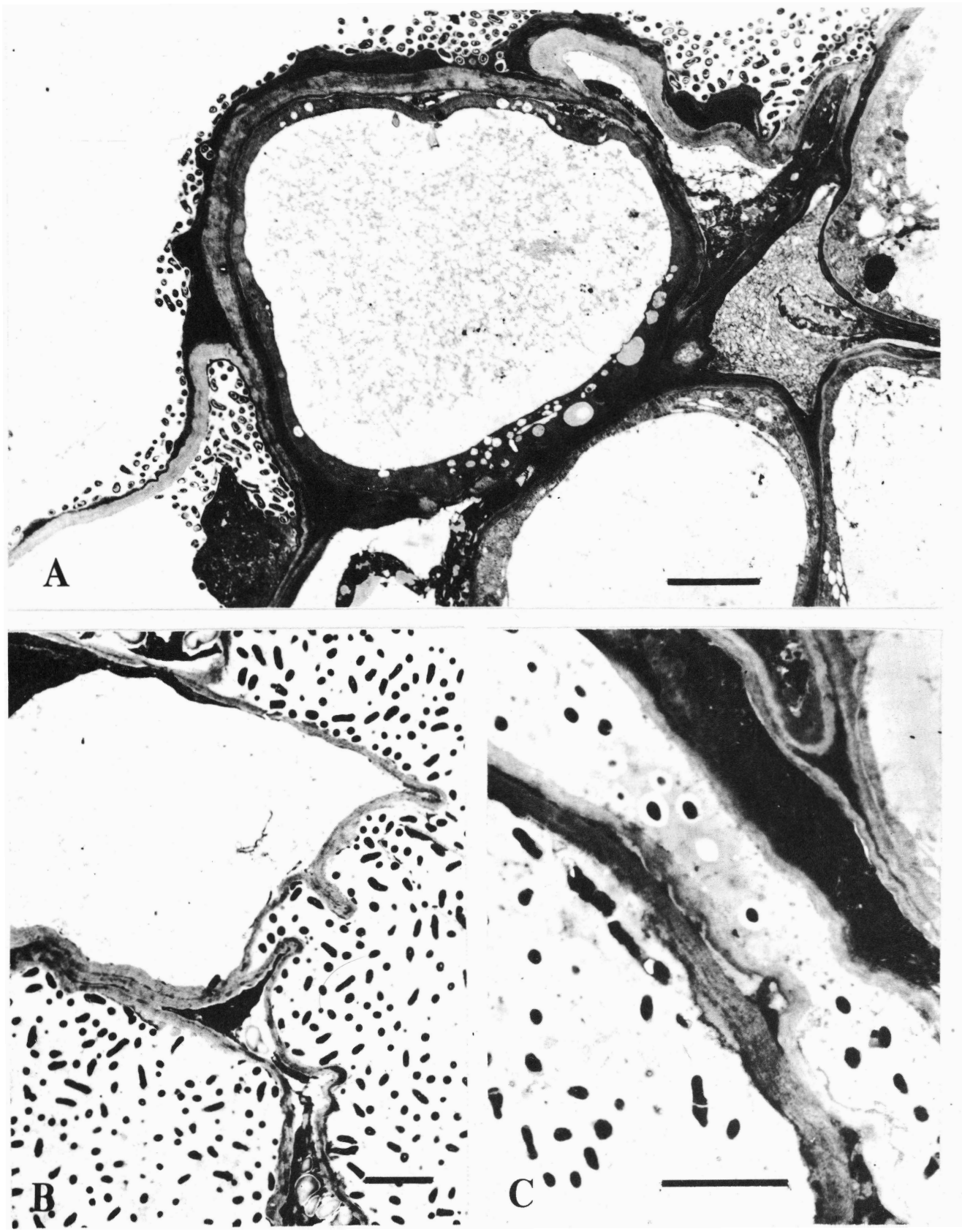

Fig. 1. Coryneform bacteria inside of galls induced by nematodes. A: *Mesoanguina picridis*; B: *Anguina agropyri*, C: *Heteroanguina graminophila*. Scale bars - 3 μm.

Table 2. Characteristics of bacterial strains containing DAB and/or ornithine in their cell walls.

Strains	*Rathayibacter* sp.		*Curtobacterium* spp.				*Aureobacterium* sp.	*Genus* sp.		
	KUZ1	KUZ2	1G	4G	2G	3G1b	3G1a	4G1	5G3	5G1
Color of colony*	CY	CY	YO	YO	YO	YO	YO	ORB	ORB	ORB
Motility	—	—	+	+	+	+	+	—	—	—
Peptidoglycan amino acids (mol.ratio):										
Alanine	0.73	0.92	0.92	1.0	0.88	0.91	0.94	2.0	1.98	1.82
Glutamic acid (Glu)	1.32	1.09	1.0	1.4	1.31	1.15	0.47	0.31	0.51	0.57
Hug**	—	—	—	—	—	—	0.57	0.82	0.74	0.58
Glu+Hug	—	—	—	—	—	—	1.04	1.10	1.24	1.15
Glycine	0.88	1.21	1.2	1.4	1.19	1.16	2.0	4.26	2.21	1.93
Homoserine	—	—	0.5	0.6	0.36	0.55	0.48	—	—	—
Diaminobutinic acid	2.0	2.0	—	—	—	—	—	1.13	1.0	1.0
Ornithine	—	—	1.0	1.0	1.0	1.0	1.31	1.0	1.0	0.72
Peptidoglycan type***	B2γ	B2γ	B2β	B2β	B2β	B2β	ND	ND	ND	ND
Sugars of cell wall:										
Glucose	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	—	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+
Xylose	+	+	—	—	—	—	+	—	—	—
Fucose	—	—	—	—	—	—	—	+	+	+
Rhamnose	+	+	+	+	+	+	+	+	+	+
Major menaquinone	MK-10	MK-10	MK-9	MK-9	MK-9	MK-9	MK-11	MK-10	MK-10	MK-10

* - CY - cream-yellow; YO - yellow-orange, ORB - orange to red-brown
** - 3-hydroxyglutamic acid
*** - (ND) No data

Pseudomonas-like (pellucid, non or weakly pigmented) and others which were similar to colonies of gram-positive coryneform bacteria (yellow-orange-rose pigmented, glistening, soft, pasty). Coryneform bacteria grew only (about 20-40 per plate), if the gall suspension had been pre-treated with alkali. Most of the bacteria from *Elytrigia repens, Poa annua, Calamagrostis neglecta, Centaurea leucophylla*, were orange or orange-yellow colored. Yellow colonies dominated in the incubation plates seeded with gall-mill from *Cousinia* sp. Rose colonies were present in all plates.

Fifty strains were isolated and purified for further study. Ten strains, which represented the predominant groups of isolates, were studied in detail (Table 1).

The principal cultural, morphological, chemotaxonomic, and physiological characteristics of the strains studied are shown in Tables 2 and 3.

Two strains, KUZ1 and KUZ2, from *Cousinia* sp. were similar in their basic chemotaxonomic features (Table 2) and these bacteria were identified as belonging to the genus *Rathayibacter*. They had alanine, glycine, glutamic acid, and DAB in cell-wall peptidoglycan (molar ratio ca. 1:1:1:2) and predominant unsaturated menaquinones with ten isoprene units, MK-10. The above-mentioned strains differed from bacteria of all known species of

Table 3. Characteristics differentiating bacterial strains KUZ1 and KUZ2 (*Rathayibacter* sp.) from other species in the genus *Rathayibacter*.

Characteristics	*R. iranicus*	*R. rathayi*	*R. tritici*	*R.toxicus*	*Rathayibacter* sp.
Color of colony:					
yellow	+	+	+	+	—
cream-yellow	—	—	—	—	+
Rod-coccus cycle	—	—	—	—	+
Utilization of carbohydrates:					
Arabinose	—	+	+	—	+
Lactose	—	+	+	—	+
Mannitol	—	+	+	—	+
Melibiose	—	—	—	+	
L-Rhamnose	—	—	—	—	+
Lyxose	—	—	—	—	+
Growth at 37° C	—	—	—	—	+
Methyl-red test	—	—	—	—	+
Foges-Proskauer	—	—	—	—	+
Growth at 6% NaCl	—	—	—	—	+
Coagulation of casein	—	—	—	—	+

Rathayibacter by having cream-yellow pigmented colonies, a distinct rod-coccus cycle, large cell size in young cultures (0.7-0.9 μm x 0.7-3.5 μm) and an ability to utilize a wide range of carbon sources as well as fermentation activities (Table 3). These two strains may in future be described as new species of the genus *Rathayibacter*.

The strains 1G, 2G, 3G1b, 4G from leaf galls of *Centaurea leucophylla* were identified as *Curtobacterium* sp. (Komagata & Suzuki, 1986a) on the basis of the following general characteristics (Table 2): yellow-orange coloured colonies, motile cells (0.5-0.6 μm dia. and 1.5-3.5 μm in length), bending type of cell division, amino acid composition of cell wall peptidoglycan similar to the B2β type ([L-hsr]-Glu-Gly-D-Orn) (Schleifer & Kandler, 1972) and unsaturated menaquinones with nine isoprene units (MK-9). These strains were similar to the species *Curtobacterium flaccumfaciens* in the colour of their colonies (Yokota et al., 1993).

The basic properties of strain 3G1a (Table 2) were similar to those belonging to the genus *Aureobacterium* (Komagata & Suzuki, 1986b; Yokota et al., 1993). In young cultures, irregular rods (0.6 to 0.8 x 2.5 to 3.5 μm) occurred; some cells were arranged at an angle, forming V-shape motile cells. In older cultures (5 to 7 days) the rods were shorter; a marked rod-coccus cycle was not observed. The cell wall peptidoglycan was of B2β' type (Schleifer & Kandler, 1972) containing ornithine, homoserine, glutamic acid and 3-hydroxyglutamic acid, glycine and alanine (molar ratio, ca. 1:0.5:1:2:1). The whole cell sugars were glucose, galactose, mannose, xylose, and rhamnose. Unsaturated menaquinones of the MK-11 type were present. It had yellow-orange colored colonies similar to the single orange coloured species of the genus *Aureobacterium*, *A. testaceum* (Yokota et al., 1993).

Colonial morphological and chemotaxonomic features of strains 4G1, 5G1, 5G3 isolated from stem galls of *Elytrigia repens* and leaf galls of *Calamagrostis neglecta* revealed their taxonomic uniqueness. These three strains had orange (to red-brown in old cultures) coloured colonies containing DAB, ornithine, glutamic and 3-hydroxyglutamic acid with glycine and alanine in their peptidoglycans (molar ratio, ca. 1:1:1:4:2 or 1:1:1:2:2). This composition of bacterial mureins has not been previously reported. Their cell walls contained glucose, galactose, mannose, fucose, and rhamnose. Menaquinones were of the MK-10 type.

The unique composition of peptydoglycan and the other significant differences of these strains from known genera indicate that these three strains may

represent a new species of a new genus of coryneform bacteria.

The characteristics of bacterial strains isolated during our study allowed us to identify the bacteria as belonging to the genera *Aureobacterium*, *Curtobacterium*, *Rathayibacter*. Further investigations are required to confirm if some of the bacterial strains represent new species and a new genus.

Nevertheless, our study of new sets of bacterial strains isolated from plant-nematode-bacteria complexes have revealed the existence of a greater taxonomic diversity of bacteria from such sources than has been previously described.

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Евтушенко Л.И., Дорофеева Л.В., Добровольская Т.Г., Субботин С.А. Коринеформные бактерии из галлов, индуцируемых нематодами подсемейства Anguininae.

Резюме. Обнаружены новые ассоциации коринеформных бактерий с галлообразующими нематодами *Anguina agropyri*, *Heteroanguina graminophila*, *Mesoanguina picridis* и *Subanguina radicicola*. Выделенные из галлов штаммы бактерий были определены как *Aureobacterium* sp., *Curtobacterium* sp. и *Rathayibacter* sp. Таксономическое положение трех штаммов бактерий из стеблевых галлов *Elytrigia repens* и листовых галлов *Calamagrostis neglecta* осталось неопределенным и требует дальнейшего изучения. Ассоциации бактерий с ангвининами, вероятно, распространены значительно шире, чем это предполагалось ранее.