Biocontrol Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713409232

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Online publication date: 16 February 2010

To cite this Article Consolo, V. Fabiana, Mucci, Viviana, Salerno, Graciela L. and Berón, Corina M.(2010) 'Pathogenicity of bacterial isolates to *Cyclocephala signaticollis* (Coleoptera: Scarabaeidae)', Biocontrol Science and Technology, 20: 5, 475 — 482
To link to this Article: DOI: 10.1080/09583150903580537
URL: http://dx.doi.org/10.1080/09583150903580537

Biocontrol Science and Technology,
Vol. 20, No. 5, 2010, 475–482

Taylor & Francis
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SHORT COMMUNICATION

Pathogenicity of bacterial isolates to *Cyclocephala signaticollis* (Coleoptera: Scarabaeidae)

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(Received 30 September 2009; returned 12 November 2009; accepted 23 December 2009)

We describe the isolation and identification of pathogenic bacteria obtained from haemolymph of *Cyclocephala signaticollis* larvae. Two pathogenic bacteria, a *Bacillus thuringiensis* and an *Arthrobacter* sp. caused 90–100% mortality to *C. signaticollis* in 4 days after hemocoel injection, suggesting they might be useful as biological control agents.

Keywords: *Cyclocephala signaticollis*; scarab; bacteria

In many regions of the world, root-feeding white grubs cause significant damage to many agricultural crops (Alvarado 1980; Iannone 2006). In southeastern Buenos Aires Province, Argentina, *Cyclocephala signaticollis* Burmeister (Coleoptera: Scarabaeidae: Dynastinae) is the most damaging potato pest (Mondino, López, Alvarez Castillo, and Carmona 1997).

The prevailing method for white grub control involves the application of chemical insecticides as organochlorides, but the development of insect resistance, the emergence of secondary pests, the impact on non-target organisms, environmental pollution, and residues on the agriculture products and animals have increased interest in alternative strategies (Jackson 1999). Sustainable agriculture will increasingly rely on alternative interventions for pest management that are environmentally friendly and will minimize the use of chemical pesticides (Lacey, Frutos, Kaya, and Vails 2001). In particular, some bacterial species are pathogenic to specific white grub larvae and are used in biological control programs (Jackson 1992): for example, *Paenibacillus popilliae*, against *Popilia japonica* (Ogiwara et al. 1995), *Serratia entomophila* and *S. proteamaculans* against *Costelytra zealandica* (Trought, Jackson, and French 1982; Hurst, Glare, Jackson, and Ronson 2000), and *Xenorhabdus* spp. or *Photorhabdus* spp. that are vectored by entomopathogenic nematodes (Kaya and Gaugler 1993). We describe the isolation, identification and pathogenicity of bacterial isolates to *C. signaticollis* third instar larvae.

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ISSN 0958-3157 print/ISSN 1360-0478 online

DOI: 10.1080/09583150903580537
http://www.informaworld.com

Insect collection

Third instar larvae of *C. signaticollis* larvae were collected by digging under potato crops in commercial fields without pesticide treatment in the General Alvarado district (Buenos Aires) from March to October 2004–2005. Grubs were individually placed in plastic containers covered with soil and roots. The containers with the insects were kept at room temperature (about 20°C) for 40 days to allow the development of potential diseases. Therefore, all the bruised grubs, injured by digging, were removed from the test, and those showing bacterial infection symptoms, like changes in the colour and loss of body turgor, were selected. Grub cadavers were never detected in the field. Of 106 larvae subjected to quarantine, 60% showed bacterial infection symptoms, while the remaining were healthy (Figure 1). Surviving healthy larvae were used for bioassays.

Bacterial isolates and growth conditions

In order to obtain cultivable pathogenic bacteria from *C. signaticollis* larvae, mixtures of insect haemolymph were inoculated onto nutrient agar (Difco No. 003-01), a culture medium used for the development of a wide variety of microorganisms, without extreme requirements. Bacterial isolates were selected on the basis of conventional bacteriological techniques using two types of samples: (1) mixtures of haemolymph from healthy insects and (2) mixtures of haemolymph from insects which showed symptoms of bacterial infection. All larvae were surface sterilized by submerging in 0.1% sodium hypochlorite for 30 s, washing in sterile distilled water three times, before extraction of haemolymph from each insect.

Each haemolymph mixture was serially diluted, plated on nutrient broth or nutrient agar and incubated at 28°C. Bacterial cultures were microscopically identified and cultured. Biochemical tests were performed, according to Bergey's Manual (Mac Faddin 1986; Holt, Krieg, Sneath, Staley, and Williams 1994) and shown in Table 1. We identified the same bacterial species in both groups of insects (seven bacterial isolates in total). In addition, two different bacterial strains were isolated only from larvae showing bacterial infection symptoms.

16 rRNA sequence analysis

Molecular characterization was used to confirm the bacterial identifications. Small-subunit rRNA genes (16S rDNA) were amplified by PCR using universal bacteria

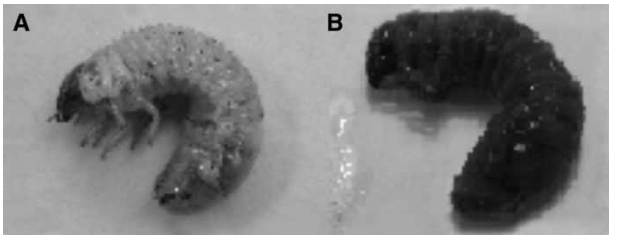

Figure 1. *C. signaticollis* third-instar larvae. (A) Healthy and (B) unhealthy larvae.

Table 1. Biochemical characterization of the bacterial strains isolated from *C. signaticollis*.

Strain	Source[1]	Gram stain	Catalase test	Thioglycolate broth	OF[2]	Motility (30°C)	Carbohydrate use (24 h)[3]					
							Glc	Lac	Cel	Man	Sac	Mn
B.1	HL/IL	+	+	−	−	−	−	−	−	−	−	−
B.2	HL/IL	−	+	−	F/O	−	−	−	−	−	−	−
B.3	HL/IL	−	+	−	F	−	−	−	−	−	−	−
B.4	IL	+	+	−	−	+	+	−	+	−	−	+
B.6	HL/IL	+	+	−	−	+	−	−	−	−	−	
7.3	IL	+	+	−	O	−	−	−	+	−	−	−
7.4	HL/IL	−	+	−	−	−	−	−	−	−	−	−
S.1	HL/IL	+	+	−	−	−	−	−	−	−	−	−
S.2	HL/IL	+	+	−	−	−	−	−	−	−	−	−

[1]HL healthy larvae and IL larvae showing bacterial infection symptoms.
[2]OF, oxide-fermentation test: F, fermentative; O, oxidative.
[3]Carbohydrate: *Glc*, glucose; *Lac*, lactose; *Cel*, celobiose; *Man*, manitol; *Sac*, sucrose; *Mn*, manose.

specific primers, 43f and 1387r (Marchesi et al. 1998; Mignard and Flandrois 2006), using genomic DNA of bacterial strains isolated as previously described (Cai and Wolk 1990). The complete 16S rDNA-fragment (≈1.3 kb DNA) was cloned by standard protocols (Sambrook and Russell 2001) and duplicates of bacterial isolate, representing each of the bacterial species were sequenced (Macrogen, Korea). DNA sequence data sets were analyzed by BLAST N and multiple-sequence alignment (Altschul, Gish, Miller, Myers, and Lipman 1990).

The nine different bacterial isolates were identified as *Microbacterium* sp., *Ochrobactrum* sp., *Chryseobacterium* sp., *Bacillus* sp. (three isolates), *Arthrobacter* sp., *Pseudomonas* sp. and *Streptomyces* sp. Details of the analyses are shown in Table 2.

Good agreement between biochemical test and molecular analysis was mostly observed. However, the molecular approach was very useful for the final confirmation of *Chryseobacterium* sp. and *Streptomyces* sp.

According to the molecular characterization using 16S rDNA, isolate B.4 was identified as *B. thuringiensis* or *B. cereus*. *B. thuringiensis* can not be distinguished from *B. cereus* on the basis of genetic evidence, because the only established difference between them is the presence of genes coding for the insecticidal toxins, usually present in plasmids in *B. thuringiensis*. If these plasmids are lost, both species can no longer be distinguished (Helgason et al. 2000). Microscopic inspection of B.4 isolate shown typical parasporal crystalline inclusion described for *B. thuringiensis* strains (de Maagd, Bravo, Berry, Crickmore, and Schnepf 2003; data not shown). Thus, we identified B.4 as a *B. thuringiensis* strain.

On the other hand, according to the molecular characterization using 16S rDNA, isolate 7.3 was 99% identical to *Arthrobacter woluwensis*.

Table 2. Phylogenetic affiliation of isolates obtained from the culture-based techniques based on 16S ribosomal RNA (rRNA) gene sequence.[1]

	Blast analysis			
Bacterial isolate	Nearest phylogenetic neighbor	Accession number	% Similarity	Expect value
B.1	*Microbacterium* sp.	AJ698726	97	2e-94
B.2	*Ochrobactrum* sp.	AJ550273	96	0.0
B.3	*Chryseobacterium* sp.	EU109723	97	0.0
B.4	*Bacillus thuringiensis*	AM747225	100	0.0
	Bacillus cereus	AY138271	100	0.0
B.6	*Bacillus* sp.	AJ439078	93	0.0
7.3	*Arthrobacter* sp.	AB244483	99	0.0
7.4	*Pseudomonas* sp.	EU266580	97	0.0
S.1	*Streptomyces* sp.	FJ486478	99	0.0
S.2	*Bacillus* sp.	EU184084	96	0.0

[1]The sequence analysis was done at the Ribosomal Database Project and the National Center for Biotechnology Information (www.rdp.cme.mse.edu, www. ncbi.nlm.nih.gov/BLAST, respectively) and is based on the 16S rRNA gene sequence.

Pathogenicity of bacteria isolates against *C. signaticollis*

Infection of *C. signaticollis* larvae was determined by a standard bioassay (Grkovic, Glare, Jackson, and Corbett 1995; Jackson and Saville 2000). Each bacterial isolate was assayed against healthy larvae. Cultures of bacterial isolates were grown on nutrient broth with continuous shaking at 28°C. After 72 h, cell density was determined by OD_{600} and direct counting using a Neubauer chamber. Bacterial cells were harvested by centrifugation at $7000 \times g$ for 10 min at 4°C. Pellets were resuspended in sterile saline solution (0.9% sodium chloride). All cultures were adjusted to the same OD_{600} and six serial dilutions were done for each bioassay. During the first screening, 10 individuals were inoculated with each bacterial isolate at 1×10^6 cell/mL. Inoculations were done by oral or intracoelomic injection of grub larvae according to Jackson and Saville (2000) with 2 μL of bacterial suspension. Experimental 50% lethal concentrations (LC_{50}) of the pathogenic bacterial isolates were determined by Probit analysis (Finney 1964), after correction for control mortality by Abbott's (1925) formula in six doses ranging from 10 to 10^6 bacteria/mL. All bioassays were performed three times in duplicates for each concentration. Larval mortality was monitored daily during 40 days. Control bioassays were done by oral inoculation or intracoelomic injection of larvae using sterile saline solution.

Seven out of nine bacterial isolates were non-pathogenic. Particularly, *Ochrobactrum* spp., *Chryseobacterium* spp. and *Streptomyces* spp. are widely distributed in the environment, mainly in soil. On the other hand, some species from *Pseudomonas* genus and *Microbacterium* sp. are commonly found in some insects (Holt et al. 1994; Demaio, Pumpuni, Kent, and Beier 1996; Liehl, Blight, Vodovar, Boccard, and Lemaitre 2006).

We found the same seven bacterial isolates in either healthy or unhealthy larvae. Currently, the path of entry of these bacteria into the haemolymph of healthy larvae is not completely understood. However, it is possible that mechanical injury during grub motility in the soil might facilitate bacterial colonization of haemolymph without causing any observable effect on the grubs' health. Insects are continuously exposed to potentially pathogenic microorganisms, but only a few encounters result in infection. Insects possess a complex and efficient system of defence against pathogens (Gillespie, Kanost, and Trenczek 1997). In contrast, some pathogenic bacteria like *S. entomophila*, *S. proteamaculans* and *B. thuringiensis* eventually invade the hemocoel, after gut disruption (Grkovic et al. 1995; de Maagd et al. 2003).

Two bacterial isolates, *B. thuringiensis* strain and *Arthrobacter* sp. were pathogenic when inoculated separately by intracoelomic injection. Isolate B.4 was the most virulent isolate killing 100% of the larvae in 4 days, while *Arthrobacter* sp. isolate 7.3 produced a mortality of 90% in the same time frame. The average LC_{50} values were 3.3×10^5 and 7.5×10^5 cells/mL, respectively; these doses represent inoculations of 660 and 1500 bacterial cells, respectively (Table 3). No mortality was observed in control larvae. None of the nine bacterial isolates was pathogenic to *C. signaticollis* third-instar larvae when applied directly into the oral cavity. This was especially surprising for *B. thuringiensis* since insect feeding is commonly the first event during *B. thuringiensis* pathogenesis. The reason of this lack of pathogenicity is currently not understood and the current evidence does not allow us to discriminate whether these bacterial isolates are truly non-pathogenic to *C. signaticollis* via oral

Table 3. Probit analysis of three bioassays of bacterial strains B.4 and 7.3 against *C. signaticollis* third instar larvae.

Bacterial strain	Replicate number	Total number of larvae[1]	LC_{50} (cells/mL)	Cells applied (in 2 μL)	Slope ± SE	χ^2
B.4	1	70	9.8×10^4	196	1.23 ± 0.50	0.47
	2	70	4.47×10^5	894	0.18 ± 0.12	0.17
	3	70	4.47×10^5	894	0.18 ± 0.12	0.17
7.3	1	70	2.0×10^5	400	1.54 ± 0.73	1.16
	2	70	1.7×10^4	34	0.32 ± 0.14	0.48
	3	70	9.4×10^3	19	0.23 ± 0.12	1.02

[1]Total number of third instar larvae of *C. signaticollis* tested: 10 larvae per dosage, per six dosages from 10 to 10^6 cells mL^{-1} of bacterial strains B.4 and 7.3 plus 10 control larvae.

administration or if an effective dose was not achieved during the assays due to regurgitation or other reasons.

Several bacterial pathogens have been isolated from soil-dwelling pests and were discussed by Jackson and Saville (2000). Among them, some sub-species of *B. thuringiensis* were described as pathogens against some soil scarabaeid. However, the most common *B. thuringiensis* toxins show little effect against pests that have evolved in microbial-rich soil. On the other hand, *Bacillus* spp. were routinely recovered in the haemolymph of some larvae and adult insects like queen of *Solenopsis invicta* (Tufts and Bextine 2009).

In contrast, *Arthrobacter* sp. is widely distributed in the environment and sometimes represents the most numerous bacterial group in aerobic plate counts of specimens from the soil (Holt et al. 1994; Funke et al. 1996). More recently, *Arthrobacter* sp. was found as one of the most predominant bacterial components in internal tissues of some stages of *Acantholyda erytrocephala* (Zahner, Lucarotti, and McIntosh 2008) and in the diptera *Wohlfahrtia magnifica* (Tóth, Hell, Kovács, Borsodi, and Márialigeti 2006). On the other hand, *Arthrobacter woluwensis* was isolated from the digestive organ of the antlion *Myrmeleon bore* (Nishiwaki, Ito, Shimomura, Nakashima, and Matsuda 2007).

The scarcely of reports on microbial strains capable of causing disease to white grubs makes the finding reported herein a first step towards the development of biological control programs against *C. signaticollis*. Most formulations for biological control programs used enterobacteria, which enter the insect orally and cause intestinal infections. A different strategy of formulation should be used with biological control agents that have to reach the insects' haemolymph. To this regard future efforts should be directed to investigate both the port of entry of these pathogenic bacteria into *C. signaticollis*, whether it is unassisted or vectored by some other soil-dwelling organism(s).

Acknowledgements

We give special thanks to Dr Leonardo Curatti and Professor Ana Tassi for critical revision of the manuscript. This investigation was supported by Fundación para Investigaciones Biológicas Aplicadas and by the Universidad Nacional de Mar del Plata (grant 15E/329 EXA 382/07). V.F.C, G.L.S. and C.M.B. are career investigators at the Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Argentina.

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