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ORIGINAL PAPER

Diversity of entomopathogenic bacteria associated with the white grub, *Brahmina coriacea*

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Abstract A survey of potato fields located in the south-eastern region of Himachal Pradesh (India) was carried out in order to find out the natural pathogens infecting the white grub, *Brahmina coriacea*. About 88 % population of the infected grubs were found to exhibit symptoms of natural bacterial infection during the years 2007–2008. Hence, we attempted to isolate and characterize the most potent bacteria for the management of *B. coriacea* and tested their insecticidal activity. In this study, ten different bacterial isolates belonging to genera *Bacillus*, *Psychrobacter*, *Paracoccus*, *Paenibacillus*, *Mycobacterium*, *Staphylococcus* and *Novosphingobium* were isolated from *B. coriacea*. Bacterial species were identified based on morphology, biochemical tests and homologies of 16S rRNA gene sequences. Pathogenicity tests for all isolated bacteria at 1.0×10^8 cfu/ml of broth were performed on late first instar grubs. Among the bacteria tested, *Bacillus cereus* induced highest mortality level of 51.85 % within 7 days of treatment followed by *Psychrobacter pulmonis* (33.33 %), *Bacillus psychrodurans* (25.93 %), *Bacillus pumilus* (25.93 %), *Paenibacillus tylopili* (22.22 %) and *Novosphingobium capsulatum* (18.52 %). Mortality levels were further increased up to 100 % by *B. cereus* followed by 88.89 % by *P. pulmonis* after 30 days of treatment. Our results indicate that *B. cereus*, *P. pulmonis*, *B. psychrodurans*, *B. pumilus*, *P. tylopili* and *N. capsulatum* may be valuable biological control agents for white grubs, *B. coriacea*.

Keywords Entomopathogenic bacteria · 16S rRNA genes · Biochemical test · White grub · Potato

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Introduction

The geographical range of potato (*Solanum tuberosum* L.) is worldwide and is grown as a major source of food in most countries in temperate climates. Since potato is not a native crop of the sub-tropical region, it is therefore susceptible to many diseases and pest attacks prevalent in abundance in such agro-climates. Potato growing areas located in the north-western hills of India suffer serious damage by soil-dwelling pests belonging to the order Coleoptera including *Brahmina coriacea* (Hope) (Coleoptera: Scarabaeidae) (Sushil et al. 2008). Initially, first instar grubs of *B. coriacea* feed on the mother tuber and roots of developing plants. Second instar grubs tend to nibble potato tubers by making small superficial holes. However, the third instar is most active and causes severe damage by making large, shallow and circular holes in potato tubers, thus rendering them unfit for marketing. They remain concealed while feeding on the tubers, and plants continue

to grow normally without any reflection of injury on the aerial parts of the plants. Because of this concealed behavior, white grubs remain unnoticed in potatoes during crop development. Populations suddenly increases beyond the economic injury level in places having plentiful tubers. Up to 80 % yield losses in severely infested potato fields have been observed in the higher hills of Shimla in Himachal Pradesh (Chandel et al. 1993; Chandla et al. 2001).

A number of approaches have been employed for controlling the white grubs around the world. As a means of soil pest control, farmers use harmful chemicals without considering pest biology, the residual effect of pesticides in the soil environment or their effect on the health of the consumers. The use of the chemical insecticides is not advisable as the potato tubers are used as food (Ezekiel et al. 1999). Many cultural practices have been used for controlling the white grubs in Himachal Pradesh without an effective outcome (Misra and Chandel 2003). These factors have led to a focus on the development of alternative control measures instead of pesticides. Therefore, integrated pest management has placed great hopes on bio-control agents. The best possible alternatives can be microbial agents like bacteria, fungi, nematodes, viruses, protozoa, and botanical control agents. Microbial control is compatible with biological, toxicological, environmental, and social requirements. Only a few strains of bacteria like *Paenibacillus popilliae*, *Bacillus thuringiensis* and *Serratia entomophila* have been tested against white grubs of different scarab species (Suzuki et al. 1992; Hori et al. 1994; Alm et al. 1997; Hurst et al. 2000; Koppenhofer et al. 2000; Tan et al. 2006). *B. cereus* has been used successfully as a microbial control agent for the grubs of *Amphimallon solstitiale*, *Melolontha melolontha*, *Anomala dimidiata* and *Holotrichia seticollis* (Sezen et al. 2005; Selvakumar et al. 2007; Sushil et al. 2008). Other entomopathogenic bacteria isolated from Coleopteran insects include *B. circulans*, *B. sphaericus*, *P. polymyxa*, *Streptococcus* spp., *Micrococcus* spp., *Yersinia* spp. and *Enterobacter* spp. (Demir et al. 2002; Selvakumar et al. 2003; Sezen et al. 2005).

Given the variety of likely bacterial diseases associated with the white grubs in the south-eastern region of Himachal Pradesh, there is a likelihood of discovering several useful novel strains. Thus, this study was aimed at isolating and identifying potential bacterial pathogens for the management of the important white grub species of the region. Here, we report ten bacterial isolates associated with *B. coriacea*. Using biochemical tests and sequence analysis of the bacterial 16S rRNA gene, we identified these bacterial isolates by comparing them with those from GenBank. The insecticidal activities of these bacterial isolates were tested against late first instar grubs of *B. coriacea*. Six out of ten isolates exhibited significant insecticidal activity, suggesting their role as valuable potential bio-control agents.

Materials and methods

Soil sampling and rearing of white grubs

Soil sampling for white grub incidence infesting potato crop was carried out in the potato farms in the south-eastern region of Himachal Pradesh, India, including Shimla (31°N, 77°E, 2,202 m amsl), Shillaroo (31°N, 77°E, 1,820 m amsl) and Kheradhar (30°N, 77°E, 1,048 m amsl) during June to September 2007 and 2008. Sampling was done from the root zone of randomly selected potato plants in 30-cm^2 areas up to 20-cm depth and minimum of ten samples from each quadrate of selected location were taken. The samples were placed immediately in containers with soil from the collection site and transported to the laboratory for further studies. Grubs were identified on the basis of (1) body length, (2) head capsule width, and (3) raster pattern (Misra and Chandel 2003). Grubs collected from different locations were reared and kept according to their developmental stage. First instar grubs were reared in trays with maize seedlings as feed (20 grubs/tray). Second and third instar grubs were kept individually in pots, containing a mixture of fine soil and well-rotted farm yard manure (FYM) (1:1) and fed with sliced potato. Collected grubs were checked periodically for any infection until the emergence of adults. Dead larvae were carefully separated and transferred into sterilized vials in order to assess the causes of mortality, whether due to bacteria or any other reasons.

Isolation and identification of bacteria

Grubs showing symptoms of bacterial infection and cadavers of dead grubs were surface-sterilized using 95 % ethanol and 1 % sodium hypochlorite for 2 min each, and then thoroughly washed with sterile distilled water. For the isolation of different pathogenic bacteria combination of methods were used (Fuxa et al. 1997; Travers et al. 1987). Initially non-spore-forming bacteria were isolated by sampling hemolymphs from each larva as the first step, and then each of the same larva was used for the isolation of endospore-forming bacilli. The hemolymph was collected into a microtube by puncturing the grub's body with a hypodermic needle, and then serially diluted in sterile distilled water and plated on Standard nutrient agar (Sigma Aldrich) media plates. For the isolation of endospore-forming bacilli, surface-sterilized specimens were homogenized in 0.1 % sterile tryptose (Sigma Aldrich) and serially diluted 1,000 times. The supernatant was heated at 80 °C for 10 min, plated on Standard nutrient agar media plates and incubated at 30 °C for 24 h. Bacterial colonies showing distinctive morphologies were isolated on fresh agar plates and subsequently maintained on nutrient agar

slants for further use. These served as pure stock cultures for subsequent Gram staining and biochemical characterization. Single colony was subcultured in a tube of nutrient broth and this subculture was used to inoculate test media. Purification was confirmed by Gram staining. Spore staining and acid fast staining were done using staining kits. The sterility of each test medium was carried out by incubating one uninoculated test medium along with the inoculated test medium. Further, biochemical characterization was carried out according to Bergeys' manual of systematic bacteriology (Kocur 1984; Wayne and Kubica 1986; Juni 2005; Yabuuchi and Kosako 2005; Logan and De Vos 2009; Priest 2009; Schleifer and Bell 2009).

16S rRNA sequence-based identification of bacteria

Genomic DNA was isolated from ten bacterial isolates by CTAB method with some modifications (Doyle and Doyle 1990). Partial fragments of 16S rDNA from genomic DNA samples of bacteria were amplified by PCR utilizing a pair of universal bacterial primers BCF1 5′-CGGGAGGCAG CAGTAGGGAAT-3′ and BCR2 5′-CTCCCCAGGCG GAGTGCTTAAT-3′ (Cano et al. 1994). PCR was performed using 1 µl of the genomic DNA, 0.5 µmol each primer, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 1–2 U *Taq* polymerase (Qiagen, Germany) in a 50-µl reaction. PCR was performed in a thermal cycler (Bio-Rad, USA) under conditions: initial denaturation at 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 54 °C for 40 s and 72 °C for 1 min; followed by a final extension of 7 min at 72 °C. PCR products were analyzed on 1.5 % agarose gel with ethidium bromide staining. The bands of desired size were cloned into pGEM®-T easy vector (Promega, USA) and sequenced. Sequencing was performed using Big Dye® Terminator cycle sequencing kit (v.3.1; Applied Biosystems, USA) on an automated DNA Sequencer (ABI Prism 3130*xl*,; Applied Biosystems, USA). Sequences were analyzed using BLASTN program (Zhang et al. 2000) at NCBI (www.ncbi.nlm.nih.gov). Phylogenetic and molecular evolutionary analyses were performed using MEGA 3.1 software (Kumar et al. 2004). The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987) using the distance matrix from the alignment, and distances were calculated by the Kimura 2-parameter (Kimura 1980). The reliability of the tree was measured by bootstrap analysis with 1,000 replicates (Felsenstein 1985).

Pathogenicity test and analysis of data

Pathogenicity tests of bacterial isolates against late first instar grubs of *B. coriacea* were carried out as described by Jackson and Saville (2000) for soil-dwelling insect pests. Each bacterial isolate was grown on 300 ml of LB broth at 37 °C for 72 h, and subjected to constant shaking (150 rpm). The concentration of cfu in the broth culture was estimated by the spread plate technique after heat shocking of the culture broth, and was adjusted with sterile distilled water to obtain 1.0×10^8 cfu/ml of broth. A mixture of soil and FYM (1:1) autoclaved at 15 psi and 121 °C on three alternative days, was used as the substrate for testing isolates against late first instar grubs. A dose of 300 ml of broth culture (1.0×10^8 cfu/ml) was applied to 3 kg of the soil: FYM medium mixed thoroughly and placed 100 g in each plastic pot (15 cm). Final soil moisture content of 15–20 % was maintained by using sterile distilled water throughout the bioassay period by maintaining the initial gravimetric weight. Ten healthy late first instar grubs were released into each pot. Each treatment was replicated thrice. Maize seedlings were transplanted into the containers after the roots were dipped in the respective bacterial culture. Similarly controls were maintained on sterile soil and FYM mixture with maize seedlings. Disease incidences were determined at 7, 15, 21, and 30 days after the treatment. Grubs that appeared to be sluggish with characteristic symptoms of bacterial infection were categorized and monitored for mortality. Grub mortality was recorded for 4–6 weeks. The pathogenesis of all isolates was confirmed by re-isolation of the bacteria from the hemolymph and their bioassay through methodology detailed above. Re-isolation was done by taking three–four grubs as representative samples. Mortality was corrected using Abbott's formula (Abbott 1925). Corrected mortality data were statistically analyzed using one-way ANOVA using MSTAT-C software. The difference of two means between treatments exceeding critical difference value was taken as significant.

Results

Field survey for infection incidence of white grubs

A survey in potato fields in the south-eastern region of Himachal Pradesh involving Shimla, Shillaroo and Kheradhar was carried out in order to find natural pathogens infecting the white grub during 2007 and 2008. Grubs were found to exhibit a high occurrence of natural bacterial infection (Table 1). A total of 1,351 grubs were observed of which 148 (10.95 %) grubs were found to be infected with bacteria. Infected grubs were pale/light brown/yellow/milky white in color, with a shrunken body and sluggish movement (Fig. 1). Highest bacterial infection (11.99 %) was observed in the grubs collected from Shilaroo followed by Kheradhar (11.81 %), and least bacterial infection (6.96 %) was found in grubs collected from Shimla. The

Table 1 Natural bacterial infection found in white grub, *Brahmina coriacea* during years 2007 and 2008

Location	Years	Total number of grubs collected	Number of grubs Infected with bacteria	Percent bacterial infection
Shimla (31°N, 77°E 2,202 m amsl)	2007	158	11	6.96
	2008	187	17	9.09
Total	2007, 2008	345	28	8.12
Shillaroo (31°N, 77°E 1,820 m amsl)	2007	379	42	11.08
	2008	280	37	13.21
Total	2007, 2008	659	79	11.99
Kheradhar (30°N, 77°E 1,048 m amsl)	2007	180	19	10.56
	2008	167	22	13.17
Total	2007, 2008	347	41	11.81
Grand total (Shimla, Shillaroo and Kheradhar)	2007, 2008	1,351	148	10.95

Fig. 1 White grub, *Brahmina coriacea* showing natural bacterial infection

average body length of second and third instar grubs was 12.68 ± 0.09 × 5.04 ± 0.05 mm and 22.48 ± 0.23 × 9.14 ± 0.04 mm, respectively. Head capsule measured 2.35 ± 0.03 mm and 4.42 ± 0.01 mm. Grubs exhibit raster of two parallel rows of 12 pairs of long, stout, inwardly pointed setae on the last segment of the ventral side of the abdomen. They exhibit a Y-shaped anal slit and randomly placed long, straight and sharply pointed small setae with curved ends up to the anterior end of the palidia with random hairs on the dorsal anal lobe.

Isolation and Identification of bacterial isolates

A total of ten bacteria were isolated and characterized from white grubs, *B. coriacea*, first according to morphological and biochemical characteristics (Tables 2, 3). These results showed that some of the isolated strains were Gram-positive while others were Gram-negative. On the basis of Gram reaction and spore staining, five groups for identification were prepared, viz., Gram-positive cocci, Gram-positive and Gram-negative sporing bacilli, Gram-positive nonsporing bacilli and Gram-negative coccii. In the case of Gram-positive cocci, the catalase test was positive which differentiates Staphylococci from Streptococci. The alkaline phosphatase test was used to distinguish Staphylococci strains. *Paracoccus* was differentiated from *Staphylococcus* on the basis of production of β-galactosidase. One of the isolates was Gram-negative, non-motile, coccus-shaped cells that were catalase and oxidase-positive. It produced growth in 6.5 % NaCl with nonpigmented colonies. These characters were consistent with those of genus *Psychrobacter*. Gram-positive, spore-forming, thick rod-shaped isolates were identified as Bacilli. The colonies were large, raised irregularly and dull. The egg yolk test was used to differentiate strains of *Bacillus*. Gram-negative spore-forming rod-shaped *Bacillus* was identified as *Paenibacillus*. Gram-negative, non-spore-forming, motile rods were identified as *Novosphingobium*. Nonmotile, non-spore-forming, acid fast and Gram-positive rods were identified as *Mycobacterium massiliense*. Since the isolate was acid fast but nonpigmented and fast growing, the NaCl tolerance test was done which was found to be positive. The NaCl tolerance test is often used in the identification of rapidly growing *Mycobacteria*. Standard reactions as mentioned in Bergey's manual, like pigmentation, growth rate at 30 °C, nitrate reduction, pyrizinamidase, Tween 80 hydrolysis, arylsulfatase, and urease production were carried out for identification of *M. massiliense*.

Sequence analysis of the 16S rRNA of isolates indicated that they shared high homologies with other species. All these sequences were submitted to NCBI gene databank, and accession numbers of the isolates were obtained. The partial 547 bp of 16S rRNA sequence of *Bacillus psychrodurans* strain CPRI3 (GU565559) showed 100 % identity to the strain of *B. psychrodurans* TSC11. The partial 546 bp of 16S rRNA sequence of *Psychrobacter pulmonis* strain CPRI4 (GU565560) showed 99 % identity to the strain of *P. pulmonis* a141. The partial 520 bp of 16S rRNA sequence of *Paracoccus marcusii* strain CPRI6 (GU565562) showed 98 % identity to the strain of

Table 2 Results of colony characters, Gram staining reaction, spore staining and acid fast staining reaction of bacterial isolates

Strain no.	Isolate	Colony characters	Gram staining reaction	Spore staining	Acid fast staining
1.	*Bacillus cereus* strain CPRI14	Large, raised, dull colony with irregular margins	+ Thick bacilli arranged end to end in long chains	+	−
2.	*Bacillus psychrodurans* strain CPRI3	Medium sized with irregular margins	+	+	−
3.	*Bacillus pumilus* strain CPRI8	Small circular, white, mucoid with regular margins	+	+	−
4.	*Psychrobacter pulmonis* strain CPRI4	Medium sized, opaque and circular colonies	−	−	−
5.	*Paracoccus marcusii* strain CPRI6	Single, smooth, round bright orange colony	− Cocci	−	−
6.	*Paenibacillus tylopili* strain CPRI7	Round, whitish and glossy colonies	− Bacilli	+	−
7.	*Mycobacterium massiliense* strain CPRI10	Small, Smooth and white colonies	+ Bacilli	−	+
8.	*Staphylococcus epidermidis* strain CPRI9	Medium sized translucent white colony	+ Cocci arranged in clusters	−	−
9.	*Staphylococcus pasteuri* strain CPRI11	Medium sized translucent white colony	+ Cocci arranged in clusters	−	−
10.	*Novosphingobium capsulatum* strain CPRI12	Yellow, medium sized colonies with regular margins	−	−	−

P. marcusii. The partial 546 bp of 16S rRNA sequence of *Paenibacillus tylopili* strain CPRI7 (GU565563) showed 98 % identity to the strain of *P. tylopili* MK2. The partial 547 bp of 16S rRNA sequence of *Bacillus pumilus* strain CPRI18 (GU565570) showed 100 % identity to *B. pumilus* ZH20. The partial 16S rRNA sequence of 547 bp of *Staphylococcus epidermidis* strain CPRI9 (GU565564) showed 99 % identity to the strain of *S. epidermidis* FUA2087. The partial 16S rRNA sequence of 547 bp of *Staphylococcus pasteuri* strain CPRI11 (GU565566) showed 100 % identity to the strain of *S. pasteuri* Sp-12. The partial 16S rRNA sequence of 548 bp of *B. cereus* strain CPRI14 (GU565569) showed 100 % identity to *B. cereus* WQ9-2. The partial 16S rRNA sequence of 548 bp of *Mycobacterium massiliense* strain CPRI10 (GU565565) showed 99 % identity to *M. massiliense* INCQS 594. The partial 16S rRNA sequence of 521 bp of *Novosphingobium capsulatum* strain CPRI12 (GU565567) showed 99 % identity to *N. capsulatum* RFNB21. Based on genetic identities, the isolates were selected to construct the phylogenetic tree (Fig. 2).

Pathogenicity bioassay of bacteria

The results of the insecticidal activities of the ten bacterial isolates at 1.0×10^8 cfu/ml of broth are presented in Table 4 and Fig. 3. In the preliminary pathogenicity bioassay, six out of ten bacterial isolates were found capable of causing a high degree of mortality against late first instar grubs of *B. coriacea*. Mortality observed in control group was 10 % in 30 days. *B. cereus* strain CPRI14 was found to be the most pathogenic with the highest mortality rate of 51.85 % after 7 days of treatment. The mortalities recorded for *P. pulmonis* strain CPRI4, *B. psychrodurans* strain CPRI3, *P. tylopili* strain CPRI7, *B. pumilus* strain CPRI8, and *N. capsulatum* strain CPRI12 were 25.93, 22.22, 25.93, 18.52, and 33.33 %, respectively, after 7 days of treatment. Significantly low mortalities in the range 3.70–14.81 % were observed for *M. massiliense* CPRI10, *S. pasteuri* strain CPRI11, *S. epidermidis* strain CPRI9, and *P. marcusii* strain CPRI6. After 15 days of treatments, mortality of grubs was increased by all the isolates, viz., 77.78 % by *B. cereus* strain CPRI14, 55.56 % by *P. pulmonis* strain CPRI4, 44.44 % by *B. pumilus* strain CPRI8, 37.04 % by both, *B. psychrodurans* strain CPRI3 and *P. tylopili* strain CPRI7, and 33.33 % by *N. capsulatum* strain CPRI12. The rest of the isolates were able to cause 3.70–14.81 % mortality after 15 days of treatment. Mortality was further increased up to 96.30 % in the case of *B. cereus* strain CPRI14, 74.07 % by *P. pulmonis* strain CPRI4, 66.67 % by *B. pumilus* strain CPRI8, 62.96 % by *B. psychrodurans* strain CPRI3, and 59.26 % by each *P. tylopili* strain CPRI7 and *N. capsulatum* strain CPRI12. Mortality in the case of other isolates

Table 3 Results of biochemical tests for the identification of different isolates

Biochemical test	*B. cereus* strain CPRI14	*B. psychrodurans* strain CPRI3	*B. pumilus* strain CPRI8	*P. tylopili* strain CPRI7	*P. marcusii* strain CPRI6	*S. epidermidis* strain CPRI9	*S. pasteuri* strain CPRI11	*P. pulmonis* strain CPRI4	*N. capsulatum* strain CPRI12	*M. massiliense* strain CPRI10
Fermentation of										
Glucose	+	−	+	+	ND	ND	ND	+	+	ND
Lactose	ND	ND	ND	ND	+	+	+	ND	ND	ND
Maltose	ND	ND	ND	ND	+	+	+	+	+	ND
Trehalose	ND	ND	ND	ND	+	−	+	ND	+	ND
Arabinose	+	+	+	+	+	−	−	+	ND	ND
Sucrose	+	+	+	+	+	+	+	ND	ND	ND
Raffinose	ND	ND	ND	ND	−	−	−	ND	ND	ND
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	−	+	−	+	+	−	−	+	+	+
MR	−	+	−	+	+	−	−	ND	−	ND
VP	+	−	+	−	−	+	+	ND	+	ND
Hydrolysis of										
Starch	+	+	−	+	ND	ND	ND	ND	ND	ND
Tween 80	ND	ND	ND	ND	ND	ND	ND	ND	ND	−
Citrate, Simmon's	+	−	+	−	ND	ND	ND	−	ND	ND
Nitrate reduction	+	+	−	−	ND	ND	ND	+	+	−
Arginine utilization	−	+	+	−	−	+	+	ND	−	ND
Alkaline phosphatase	ND	ND	ND	ND	+	+	−	−	ND	ND
Urease	ND	ND	ND	ND	−	+	+	−	−	ND
Pigmentation	−	−	−	−	−	−	−	−	−	−
Egg yolk test	+	−	−	+	ND	ND	ND	ND	ND	ND
Aryl sulphatase	ND	ND	ND	ND	ND	ND	ND	ND	ND	+
Pyrizinamidase										
Growth at 30 °C	+	+	+	+	+	+	+	+	+	+
Indol production	ND	ND	ND	ND	ND	ND	ND	ND	ND	−
Mannitol	−	−	+	+	+	−	+	ND	ND	ND

Table 3 continued

Biochemical test	*B. cereus* strain CPRI14	*B. psychrodurans* strain CPRI3	*B. pumilus* strain CPRI8	*P. tylopili* strain CPRI7	*P. marcusii* strain CPRI6	*S. epidermidis* strain CPRI9	*S. pasteuri* strain CPRI11	*P. pulmonis* strain CPRI4	*N. capsulatum* strain CPRI12	*M. massiliense* strain CPRI10
Malate	ND	ND	ND	ND	ND	ND	ND	–	+	ND
Malonate	–	–	–		ND	ND	ND	ND	ND	ND
Gluconate	ND	ND	ND	ND	ND	ND	ND	ND	+	ND
Indole production	ND	ND	ND	ND	ND	ND	ND	ND	ND	–
Motility	+	–	–	+	–	–	–	–	–	–
β-Galactosidase	ND	ND	ND	ND	+	–	–	ND	+	+
5 % NaCl tolerance	ND	ND	ND	ND	ND	ND	ND	ND	ND	+
6.5 % NaCl tolerance	ND	ND	ND	ND	ND	ND	ND	+	ND	ND

+ Positive, − negative, *ND* not done for the isolate

was not increased much and reached up to 14.81–25.93 % only. After 30 days of treatment, *B. cereus* strain CPRI14 was able to cause 100 % mortality in first instar grubs followed by 88.89 % by *P. pulmonis* strain CPRI4, 81.48 % by each *B. pumilus* strain CPRI8 and *B. psychrodurans* strain CPRI3, 70.37 % by *N. capsulatum* strain CPRI12, and 66.67 % by *P. tylopili* strain CPRI7. The rest of the species caused mortalities in the range 7.04–29.63 after 30 days. When re-isolation was carried out, it was possible to get inoculated bacteria, and the inoculated species were found to be predominating (above 98 % in case of all the isolates) as compared to other bacterial species.

Discussion

Recently, there has been an increasing interest in finding more pathogenic and safer bacterial isolates against insect pests. Although there have been several studies for controlling *B. coriacea* grubs, studies on its microbiota have so far been neglected. Studies on insect microbiota can be the best way to utilize the pathogens of harmful insects for their own biological control. To date, no study has been carried out on the determination of bacterial isolates and the investigation of biological control agents of *B. coriacea*. For the present study, we employed a range of growth media (data not shown) to examine different samples obtained from diseased grubs, and recovered a total of ten strains of bacteria. Although the majority of biochemical tests were similar to those available in the literature, the increasing availability of gene sequences has contributed greatly to the characterization of bacterial species. For bacterial identification, conventional results are sufficient most of the time for identifying a new bacterial isolate, but occasionally it is very difficult to identify some bacteria based only on conventional tests (Bahar and Demirbag 2007). Hence, we supported identifications of bacterial isolates using 16S rRNA gene sequence analysis, and determined their pathogenicity. Since the 1980s, sequencing of the 16S rRNA gene has been used as an important tool for phylogenetic analysis and classification of bacteria (Lane et al. 1985). The 16S rRNA gene contains regions well conserved in all organisms that are ideal for primer design, PCR, or sequencing and sequence alignment. It also contains species-specific variable regions that allow species identification. Therefore, sequence analysis of the 16S rRNA gene has become a powerful technology for identification of bacterial isolates.

The bacterial genera analyzed in this study encompassed a range of entomopathogenic, phytopathogenic, and soil saprophytic organisms. Insect-associated bacterial flora have been reported earlier on several insects, but bacterial isolates identified in the present study are all new records from grubs of *B. coriacea*. However, a few of them have already been

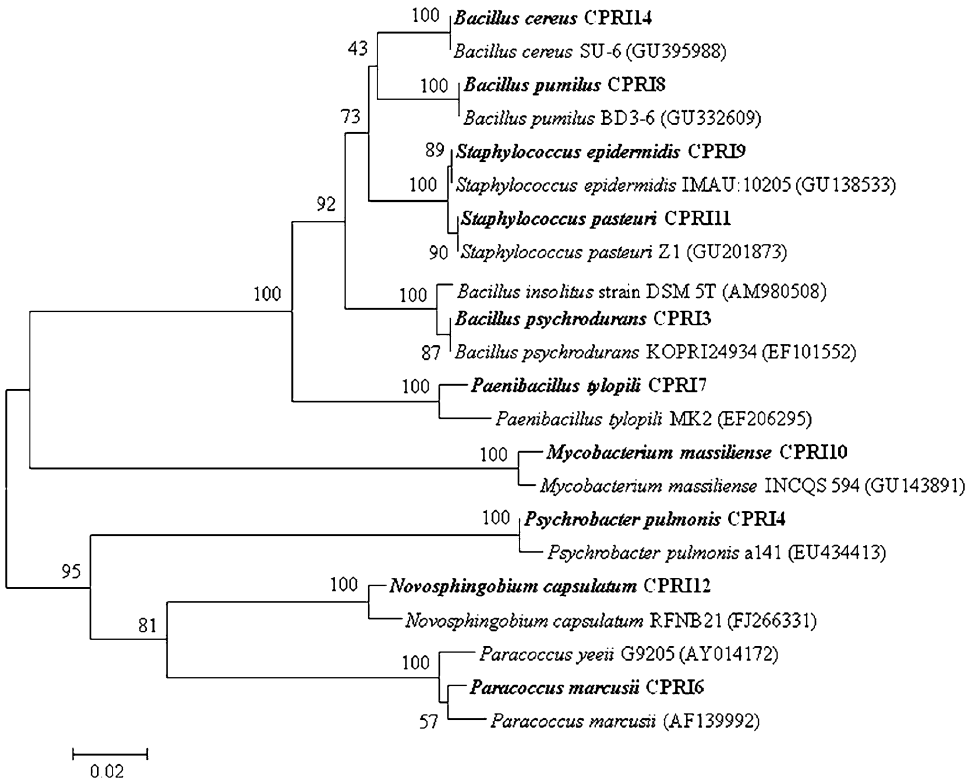

Fig. 2 Phylogenetic tree of bacterial isolates obtained from white grubs. Isolate designations are shown in *bold*. The optimal tree with the sum of branch length = 0.74379318 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches

Table 4 Preliminary pathogenicity test of locally isolated bacteria against late first instar white grubs

Bacterial treatment at 1.0×10^8 cfu/ml of broth	Mortality (%) ± SE			
	7 DAT	15 DAT	21 DAT	30 DAT
B. cereus strain CPRI14	51.85 ± 3.70 a	77.78 ± 6.41 a	96.30 ± 3.70 a	100.0 ± 0.00 a
B. pumilus strain CPRI8	25.93 ± 3.70 bc	44.44 ± 6.41 bc	66.67 ± 6.41 b	81.48 ± 3.70 bc
P. pulmonis strain CPRI4	33.33 ± 6.41 b	55.56 ± 3.70 b	74.07 ± 3.70 b	88.89 ± 0.00 ab
B. psychrodurans strain CPRI3	25.93 ± 3.70 bc	37.04 ± 3.70 c	62.96 ± 9.80 b	81.48 ± 7.40 bc
P. tylopili strain CPRI7	22.22 ± 6.41 bcd	37.04 ± 9.70 c	59.26 ± 9.80 b	66.67 ± 6.41 c
N. capsulatum CPRI12	18.52 ± 3.70 cde	33.33 ± 6.41 c	59.26 ± 9.80 b	70.37 ± 9.80 c
M. massiliense strain CPRI10	11.11 ± 6.41 def	14.81 ± 3.70 d	22.22 ± 0.00 c	29.63 ± 3.70 d
S. pasteuri strain CPRI11	7.41 ± 3.70 ef	11.11 ± 6.41 d	25.93 ± 7.40 c	29.63 ± 3.70 d
S. epidermidis strain CPRI9	3.70 ± 3.70 f	7.41 ± 3.70 d	14.81 ± 3.70 c	22.22 ± 6.41 d
P. marcusii strain CPRI6	3.70 ± 3.70 f	3.70 ± 3.70 d	14.81 ± 3.70 c	18.52 ± 3.70 d
CD (5 %)	12.22	17.24	19.82	16.45

Each value is mean of three replications; different lowercase letters in columns denote statistical differences between treatments

CD critical difference, *SE* standard error, *DAT* days after treatment

isolated from other insect and white grub species. Five bacterial isolates, viz. *P. pulmonis* strain CPRI4, *B. psychrodurans* strain CPRI3, *P. tylopili* strain CPRI7, *N. capsulatum* strain CPRI12, *M. massiliense* strain CPRI10, and *P. marcusii* strain CPRI6 had never been reported from any insect species. Earlier, *B. cereus* has been a well-known insect pathogen and had been isolated from grubs of *A. solstitiale*, *M. melolontha* and *A. dimidiata* (Sezen et al. 2005; Selvakumar et al. 2007; Sushil et al. 2008). *P. pulmonis* is a non-spore-forming, non-motile bacterium and can grow in 6.5 % Nacl. *P. pulmonis* had been isolated from *Dermanyssus gallinae* (Mesostigmata: Dermanyssidae) (Valiente et al. 2009). *B. pumilus* is known to exhibit antifungal activity and plant growth regulator activity, and its spores are extremely resistant to UV (Bottone and Peluso 2003; Joo et al. 2004; Link et al. 2004). This bacterium is non-toxic to human and animals (USEPA 2004); however,

Fig. 3 Mortalities of white grubs caused by **a** *Bacillus cereus* strain CPRI14, **b** *Novosphingobium capsulatum* strain CPRI12, **c** *Psychrobacter pulmonis* strain CPRI4, **d** *Bacillus psychrodurans* strain CPRI3, **e** *Bacillus pumilus* strain CPRI8, **f** *Paenibacillus tylopili* strain CPRI7, and **g** healthy grub

Bentur et al. (2007) has reported central nervous system infection in a child due to *B. pumilus*. *B. pumilus* had been reported from several Coleopteran species such as *Scolytus multistriatus* (French et al. 1984), *Limonius canus* (Lacey et al. 2007), *Dendroctonus micans* and *Rhizophagus grandis* (Yaman et al. 2010), but no report of its identification from white grub has been encountered. *B. psychrodurans* is spore forming, non motile bacteria and can grow at 30 °C to 35 °C. This bacterium was earlier identified from *D. gallinae* (Abd El-Rahman et al. 2002; Groudieva et al. 2003; Heuchert et al. 2004). *N. capsulatum* is a non-spore-formimg, aerobic, non-motile and chemo-organotrophic bacterium which inhabits soil, roots of rose tree, clinical specimens, stocked distilled water, coastal plain sediments, and fluidized bed reactors (Takeuchi et al. 2001). *P. tylopili* is a spore-forming, chitinolytic bacterium and has been isolated from the mycorhizosphere of *Tylopilus felleus* (Boletales: Boletaceae) (Kuisiene et al. 2008). *P. tylopili* is the species which has never been isolated from any arthropod. Four species isolated in the present studies: *S. epidermidis*, *S. pasteuri*, *P. marcusii*, and *M. massiliense* are well-known human pathogens. *S. pasteuri* has earlier been isolated from grubs of *Costelytra zealandica* (Ray et al. 2007). *S. epidermidis* has previously been recorded from many insects (Davidson et al. 2000; Zouache et al. 2009). *P. marcusii* has been shown to produce carotenoids and is under intellectual property protection (Harke et al. 1998). *M. massiliense* was validated as a species separate from the *M. chelonae*—*M. abscessus* group in 2006 (Euzeby 2007). It has been isolated from several pulmonary specimens (Adekambi et al. 2004; Simmon et al. 2007; Kim et al. 2007).

In the preliminary pathogenicity bioassays, six out of ten bacterial isolates, viz. *B. cereus* strain CPRI14, *B. psychrodurans* strain CPRI3, *B. pumilus* strain CPRI8, *P. tylopili* strain CPRI7, *P. pulmonis* strain CPRI4, and *N. capsulatum* strain CPRI12 were found to be highly pathogenic against late first instar grubs of *B. coriacea.* Other bacterial isolates were also found capable of causing varying degree of mortality. *B. cereus* strain CPRI14 caused 100 % mortality in first instar grubs after 30 days of treatment. *B. cereus* had already been reported to cause pathogenicity in other insects and a few white grub species. The mortalities of the grubs of *A. solstitiale* has been found to be 90 % with *B. cereus* isolated from *A. solstitiale* and 75 % with *B. cereus* from *M. melolontha* within 10 days (Sezen et al. 2005). *B. cereus* strain WGPSB-2 had been reported to cause 92 and 67 % mortality in grubs of *A. dimidiata* and *H. seticollis*, respectively (Selvakumar et al. 2007; Sushil et al. 2008). Though some strains of *B. cereus* have been known as opportunistic human pathogens with their ability to produce enteric toxins, some strains of *B. cereus* have many agronomically useful traits, such as antibiotic production for plant disease suppression (Handelsman et al. 1996). The insecticidal property of *B. cereus* has been attributed to the production of the lipase toxin phospholipase C (Lysenko 1972a, b) and the paralytic toxin sphingomyelinase C (Nishiwaki et al. 2004).

A synergistic action between the antibiotic zwittermicin produced by *B. cereus* and the crystal toxins of *B. thuringeinesis* var. *kurstaki* has been reported in controlling the gypsy moth (Broderick et al. 2000). Recently, Abi Khattar et al. (2009) reported that *dlt* operon of *B. cereus* is required for resistance to cationic antimicrobial peptides and virulence in insects. Pathogenicity of *B. cereus* against other scarab grub species has also been reported previously. A high degree of mortality due to *P. pulmonis* strain CPRI4 (88.89 %) was recorded for the first time in the present study. There are no previous reports of such studies conducted on this bacterium or its association with any insect. Human infection by *Psychrobacter* species is rare; however, a few case reports of infection by other *Psychrobacter* species have been reported (Bowman et al. 1996; Guttigoli and Zaman 2000). Other isolates in the present study, *B. pumilus* strain CPRI8 and *B. psychrodurans* strain CPRI3, caused 81.48 % mortality in the larvae of *B. coriacea* after 30 days of treatment. *B. pumilus* is a known insect pathogen for possible use in controling insect pests (Thiery and Frachon 1997). An insecticidal activity of *B. pumilus* has been earlier reported by Erturk et al. (2008) and was found to be highly toxic to the larvae of *L. decemlineata* causing 95.7 % mortality. *B. psychrodurans* is recently discovered, psychrotrophic bacterium which is able to grow at lower temperature −2 °C to 0 °C (Abd El-Rahman et al. 2002). Thus, the use of *B. psychrodurans* may be a plus point at the temperate environments of potato growing areas. Pathogenicity test of *B. psychrodurans* against any insect had not been done earlier. No report of human infection by *B. psychrodurans* and *P. tylopili* has yet been encountered. *P. tylopili* is a chitinolytic bacterium (Kuisiene et al. 2008) and no insect pathogenicity studies have been previously carried out. *N. capsulatum* has also caused significant mortality of first instar grubs, but again there is no previous report of its pathogenecity for any insect. *N. capsulatum* is also a psychrophilic bacterium and no reports of human pathogenecity have been encountered till date. Significantly low mortalities (18.52–29.63 %) were caused by *M. massiliense* strain CPRI10, *P. marcusii* strain CPRI6, *S. pasteuri* strain CPRI11, and *S. epidermidis* strain CPRI9. *M. massiliense* and *P. marcusii* are well-known human pathogens (Chiba et al. 2000; Adekambi et al. 2004; Simmon et al. 2007; Leao et al. 2009), but have never been found associated with any insect, and their pathogenecity studies against any insect had not previously been carried out. However, the remaining two species, *S. pasteuri* and *S. epidermidis*, are also known for infections in human and other animals (Roth and James 1988; Kawamura et al. 1998; Olayide and Bamidele 2008; Nelson et al. 2009), but they have also been found associated with insects, and the pathogenicity of *S. epidermidis* has been reported in *Drosophila melanogaster* (Diptera: Drosophilidae) (Needham et al. 2004).

The long time taken by the grubs to die is attributed to the fact that grubs feeding in the soil consume spores which germinate in the larval gut and penetrate into the hemocoel. A period of vegetative growth is followed by asynchronous sporulation and death of the larvae. The time taken from ingestion to establishment of bacteria is a few days to several weeks during which grubs generally change in color, stop feeding, show sluggish movement, and die due to bacterial septicemia (Sushil et al. 2008; Mashtoly et al. 2009). As the feeding is stopped, because of the cascade initiated by the bacteria, even if the grub is not dead, no damage is expected from a dying grub, after dying and decomposition of the grub's body, billions of spores are released into the soil which can initiate further cascades in other grubs if ingested.

In the present study, most of the potential bacteria are spore-forming; however, *P. pulmonis* is a non-spore-forming bacterium with high pathogenesis. Spore-forming bacteria are generally known to be more effective, whereas non-spore-forming bacteria are considered less pathogenic when they occur in the digestive tract of an insect, but may be very pathogenic if they are able to enter the insect's hemocoel. Thus, diseases caused by non-spore-forming bacteria generally rely on a conditional factor to gain entrance into the hemocoel. Once in the hemocoel, many non-spore-forming bacteria multiply rapidly and may cause death of the insect from bacterial septicemia. According to Coppel and Martins (1977), non-spore-forming bacterial pathogens include all of the potential pathogens for the insects. Potential pathogens do not normally multiply in the gut, but they can establish themselves in the haemocoel if they have enough time to pass through the wall and enter susceptible cells. Several non-spore-forming bacteria, *Serratia entomophila*, *Serratia marcescens* and *Melissococcus pluton* are the well-known insect pathogens (Jackson et al. 1992; Yilmaz et al. 2006; Muratoglu et al. 2009; Sevim et al. 2010). Hence non-spore-forming bacteria like *P. pulmonis* and *N. capsulatum*, in addition to spore-formers, can prove beneficial in the control of *B. coriacea* grubs. Further testing of all the potential bacteria against second and third instar grubs, eggs, and beetles can provide more information on their pathogencity.

Conclusion

This is the first detailed investigation of isolation and identification of ten bacterial species belonging to seven genera associated with *B. coriacea* white grubs based on homologies of 16S rRNA gene sequences. All bacterial species encountered in the present study are new records

from white grubs except *B. cereus* and *S. pasteuri*, and some of the species viz., *B. psychrodurans*, *P. tylopili*, *P. pulmonis*, *M. massiliense*, and *P. marcusii*, have not previously been reported from any insect. In preliminary pathogenecity tests, some of them appear to be promising for use as bicontrol agents. The present studies have provided us a framework for further investigation and for generating hypothesis to conduct pathogenicity tests in different developmental stages of white grubs. Further studies are needed for strain optimization and development of better substrates for mass production and practical uses. Application of isolates on different stages of grubs including second and third instar grubs, eggs, and beetles are also needed to understand their mortality effects. Side effects and safety tests with beneficial insects, mammals, and human cells need to be conducted.

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