Bacteria Associated with Larvae and Adults of the Asian Longhorned Beetle (Coleoptera: Cerambycidae)[1]

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Abstract Bacteria representing several genera were isolated from integument and alimentary tracts of live Asian longhorned beetle, *Anaplophora glabripennis* (Motschulsky), larvae and adults. Insects examined were from infested tree branches collected from sites in New York and Illinois. *Staphylococcus sciuri* (Kloos) was the most common isolate associated with adults, from 13 of 19 examined, whereas members of the Enterobacteriaceae dominated the isolations from larvae. *Leclercia adecarboxylata* (Leclerc), a putative pathogen of Colorado potato beetle, *Leptinotarsa decemlineata* (Say), was found in 12 of 37 larvae examined. Several opportunistic human pathogens, including *S. xylosus* (Schleifer and Kloos), *S. intermedius* (Hajek), *S. hominis* (Kloos and Schleifer), *Pantoea agglomerans* (Ewing and Fife), *Serratia proteamaculans* (Paine and Stansfield) and *Klebsiella oxytoca* (Flugge) also were isolated from both larvae and adults. One isolate, found in 1 adult and several larvae, was identified as *Tsukamurella inchonensis* (Yassin) also an opportunistic human pathogen and possibly of Korean origin.. We have no evidence that any of the microorganisms isolated are pathogenic for the Asian longhorned beetle.

Key Words Asian longhorned beetle, *Anaplophora glabripennis*, bacteria

The Asian longhorned beetle, *Anoplophora glabripennis* (Motschulsky) a pest native to China and Korea, often has been found associated with wood- packing material arriving in ports of entry to the United States. The pest has many hardwood hosts, particularly maples (*Acer* spp.), and currently is established in isolated populations in at least 3 states - New York, NJ and Massachusetts (USDA-APHIS 2010). The beetle is reported to have several natural enemies in China (Smith et al. 2007), but none of these are native to the United States. Thus, in the United States the insect is under limited natural control and has the potential for becoming a very serious pest, particularly if it becomes established in sugar maple stands in the northeastern states and negatively impacts the maple sugar industry. At present, the only reliable control technology, with the possible exception of tree or soil injections with neonicotinoid systemic pesticides (Wang et al. 2005, Poland et al. 2006), is the cutting and incineration of infested trees (Haack et al. 2010). The availability of less socially disruptive and destructive control tactics is clearly desirable, including environmentally soft biopesticides that could be applied as either sprays or baits. The beetle's natural diseases

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have not been intensely studied in the United States, and there are relatively few reports on the microorganisms associated with the pest and the potential they may have for further development as biopesticides (Schloss et al. 2006). Geib et al. (2009) used an automated ribosomal interspacer analysis only to describe generically the Asian longhorned beetle microbial community. Some work has been done to establish the efficacy of various *Bacillus thuringiensis* (Berliner) products and toxins against the beetle but with limited success (D'Amico et al. 2004). Field experiments conducted with various fungal pathogens and products (Shimazu et al. 2002, Hajek et al., 2006, Dubois et al. 2008, Shanley et al. 2009) and entomopathogenic nematodes (Fallon et al. 2004) have shown promise but none are in operational use against the beetle in the United States. In a step toward a better understanding of the relationships between Asian longhorned beetle, its environment, and its microflora, and hopefully to uncover a microorganism amenable to development as a biopesticide, we isolated and identified to species bacteria associated with larval and adult stages of the insect collected from infestations in New York and Illinois. We report herein the results of this survey and discuss the significance of the isolations.

Materials and Methods

Beetle source. Live and putatively healthy Asian longhorned beetle larvae and adults, without regard to sex and age, were collected from infested norway maple (*Acer platanoides* Pax), silver maple (*Acer saccharinum* L.), sugar maple (*Acer saccharum* Marshall), and ash (*Fraxinus Americana* L.) logs held in the U.S. Forest Service Quarantine Facility, Ansonia, CT. Logs were from trees that had been felled in the Ravenswood neighborhood of Chicago, IL, and in Queens, NY, neighborhoods in compliance with a USDA-APHIS Asian longhorned beetle eradication program. Adult beetles were collected when they emerged from logs; whereas, beetle larvae were removed with sterile forceps from galleries in split logs. Seven adults and 2 larvae from the Queens logs and 12 adults and 35 larvae from the Chicago logs were examined.

Beetle dissection. Individual beetle adults and larvae were washed in 5.0 ml sterile physiological saline and the washings reserved. Adults and larvae were surface treated in 0.15% Zephiran Chloride® (Sanofi-Aventis U.S., Bridgewater, NJ) and washed exhaustively to remove traces of sterilant. Alimentary tracts (gut) of adult beetles were removed by carefully detaching the head of the beetle from its thorax and slowly teasing out the gut, ligating appropriately so as not to lose nor contaminate contents. Beetle larvae were pinned to a sterile silicone surface and dissected ventrolongitudinally. The larval gut was then carefully teased upward out of the body cavity, ligated, and removed. Each excised gut was slit along its length and its contents pooled in a sterile microfuge tube.

Isolation and identification of bacteria. A 10 μL sterile loop (Becton Dickinson, Sparks, MD containing gut contents from each insect was plated on Biolog Universal Growth Agar (Biolog Inc., Hayward, CA) enriched with 5% sheep blood, and plates were incubated aerobically at 37°C for 24 h. Beetle washings were similarly plated and incubated. No attempts were made to isolate either obligate anaerobes or microaerophilic microorganisms. Bacterial colonies clearly differing in morphology were picked and aqueous smears were prepared and Gram stained (Society of American Bacteriologists 1957). Bacterial cell suspensions were prepared according to Biolog (1999) protocol and pipetted into Biolog microplates™ containing 95 different carbon sources. Microplates were incubated at 37°C for 24 h, color reactions were read in a

Biolog MicroStation™ system, and species identified using MicroLog™ 4.0 bacterial data base software. The Biolog identification system is based solely on a microorganism's ability to use an array of carbon sources and in the process reduce a tetrazolium redox dye that turns the color of microplate wells purple. The software analyzes temporal and quantitative color changes in the wells to assign a species identification from a data base of over 800 g positive and gram negative aerobic bacteria.

Results and Discussion

The microbiological examination of Asian longhorned beetle larvae and adults revealed several species of aerobic and facultative anaerobic bacteria. Those found in insects from Chicago, IL, are listed in Table 1; those from Queens, NY, are listed in Table 2. The bacterium isolated most often from gut contents, i.e., from 11 adults from Chicago, IL and from 2 adults from Queens, NY, was *Staphylococcus sciuri* Kloos (Kloos et al. 1976). *Staphylococcus sciuri* is commonly found on the skin of squirrels and other rodents (Hauschild and Schwarz 2003) and is also associated with a variety of environmental sources (Kloos and Schleifer 1986). The species is very rarely isolated from humans and other primates but can be an opportunistic pathogen (Hedin and Widerström 1998). Because squirrels and other rodents share Asian longhorned beetle habitat, it was not unquestioable to have found this microorganism, but its high frequency of isolation was somewhat surprising. *Staphylococcus sciuri* also was isolated from the washings of 14 of the 19 adult beetles examined, indicating that this bacterium may become associated with the beetle through contact with contaminated bark; presumably the bark of the untreated logs from which they emerged in this study. *Staphyloccus sciuri* was not isolated from the gut contents of any of the larvae examined and only isolated from the washings from 1 larva. *Staphyloccus sciuri* has been isolated from other insects, e.g.,the planthopper, *Perigrinus maidis* (Ashmead), in which it is pathogenic by injection (Ammar et al. 1987) and the pea aphid, *Acyrthosiphon pisum* (Harris), in which it may interfere with gut cell metabolism (Grenier et al. 1994) Other members of the genus, e.g., *Staphylococcus xylosus* (Schleifer and Kloos), *Staphylococcus hominis* (Kloos and Schleifer), and *Staphylococcus intermedius* Hajek (Hajek 1976) were isolated less frequently from adult beetles, each only once from gut contents.. The latter 2 are associated with human skin and can be opportunistic pathogens in man. *Staphylococcus xylosus* is found primarily on the skin of lower primates and a variety of other mammals and has been implicated in a case of human acute pyelonephritis (Tselenis-Kotsowilis et al. 1982) as well as secondary infections following root canal surgery (Siqueria and Lima 2002). A single isolation of *Staphylococcus pasteuri* Chesneau (Chesneau et al. 1993) and 2 isolations of *Staphlococcus warneri* Kloos and Schleifer (Kloos and Schleifer 1975) were made from larval guts and 1 isolation of *Staphylococcus lentus* (Kloos) was made from larval washings; all can be opportunistic human pathogens. Singular isolations of *Staphylococcus carnosis* (Schleifer and Fischer), *Staphylococcus caseolyticus* (Schleifer), both associated with foods, and *Macrococcus equipercicus* Ballard (Kloos et al. 1998) from equine skin were made. Other gram positive cocci represented in the survey included *Leuconostoc mesenteroides* (Tsenkovskii) the causative agent of "ropey" milk, *Dienococcus radiodurans* (Brooks and Murray), whose most distinguishing characteristic is its ability to survive high doses of radiation, *Micrococcus luteus* (Schroeter) which can be a nosocomial pathogen (Peces et al. 1997), *Enterococcus casseliflavus* (Vaugham, Rigsby and Mundt), also a nosocomial pathogen (Reid et al. 2001) and *Globicatella sanguinis* Collins (Collins

Table 1. Frequency of occurrence* of aerobic bacterial species isolated from integumental washings and alimentary tracts of 35 Asian longhorned beetle larvae and 12 adults sampled from logs cut from infested trees in Chicago IL.

	Larvae		Adults	
Microorganism	Washings	Gut	Washings	Gut
Bacillaceae *Bacillus mycoides*	–0-	–0-	1/12	–0-
Psuedomonadaceae				
Psuedomonas putida	1/35	–0-	–0-	–0-
Enterobacteriaceae				
Enterobacter nimipressuralis	–0-	1/35	–0-	–0-
Pantoea agglomerans	2/35	1/35	–0-	1/12
Serratia proteamaculans	–0-	–0-	–0-	1/12
Serratia rubidae	–0-	1/35	–0-	–0-
Serratia ficaria	1/35	–0-	–0-	–0-
Klebsiella oxytoca	5/35	5/35	–0-	–0-
Klebsiella terrigena	2/35	1/35	–0-	–0-
Klebsiella pnuemoniae	–0-	2/35	–0-	–0-
Klebsiella spp.	3/35	2/35	–0-	–0-
Leclercia adecarboxylata	8/35	7/35	–0-	–0-
Rahnella aquatilis	2/35	1/35	–0-	–0-
Leuconostocaceae				
Leuconostoc mesenteroides	–0-	–0-	–0-	1/12
Aerococcaceae				
Globicatella sanguinis	1/35	–0-	–0-	–0-
Enterococcaceae				
Enterococcus casseliflavus	1/35	2/35	–0-	–0-
Micrococcaceae				
Micrococcus luteus	–0-	–0-	–0-	1/12
Dienococcaceae fam. Nov.				
Dienococcus radiodurans	–0-	–0-	–0-	1/12

Table 1. Continued

Microorganism	Larvae		Adults	
	Washings	Gut	Washings	Gut
Staphylococcaceae				
Staphylococcus sciuri	1/35	–0-	8/12	11/12
Staphylococcus lentus	1/35	–0-	–0-	–0-
Staphylococcus xylosus	–0-	–0-	–0-	1/12
Staphylococcus intermedius	–0-	–0-	–0-	1/12
Staphylococcus caseolyticus	–0-	–0-	1/12	–0-
Staphylococcus carnosus	–0-	–0-	1 /12	–0-
Staphylococcaceae				
Staphylococcus pasteuri	–0-	1/35	–0-	–0-
Staphylococcus warneri	–0-	2/35	–0-	–0-
Macrococcus equipercicus	–0-	–0-	–0-	1/12
Staphylococcus spp.	2/35	2/35	–0-	–0-
Microbacteriaceae				
Microbacterium spp. (CDC A-4)	–0-	1/35	–0-	1/12
Curtobacterum flaccumfaciens	3/35	1/35	–0-	–0-
Curtobacterium citreum	–0-	1/35	–0-	–0-
Tsukamurellaceae				
Tsukamurella inchonensis	4/35	3/35	–0-	1/12

* No. of isolates/ No. of larvae or adults

et a. 1992) which has been shown to cause meningitis in humans (Seegmüller et al. 2007). Isolations reported as *Staphylococcus* spp. in Table 1 could not be identified with certainty but based upon similarity probabilities in the data base analysis most likely represented those species described above.

Bacteria representing the family Enterobacteriaceae dominated the isolations from larvae, from both integument washings and guts. These Gram negative facultative anaerobes can grow in either the presence or absence of oxygen and thus find the larval gut with its low oxygen tension acceptable for growth and reproduction. The enteric organism isolated most frequently was *Leclercia adecarboxylata* (Leclerc). This bacterium, which is widespread in nature, has been recognized as a secondary pathogen in humans (Hess et al. 2008) and shown to be present in the pink sugarcane mealybug, *Saccharicoccus sacchari* (Cockerell), by culture-independent molecular methods (Franke-Whittle et al. 2004). Only recently has it been cultured from an insect, the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), for which it was shown to be pathogenic per os (Muratoglu et al. 2009). These authors did not

Table 2. Frequency of occurrence* of aerobic bacterial species isolated from integumental washings and alimentary tracts of 2 Asian longhorned beetle larvae and 7 adults sampled from logs cut from infested trees in Queens NY.

	Larvae		Adults	
Microorganism	Washings	Gut	Washings	Gut
Enterobacteriaceae				
Klebsiella oxytoca	–0-	–0-	–0-	1/7
Klebsiella ornithinolytica/ planticola	–0-	–0-	1/7	1/7
Klebsiella terrigena	–0-	–0-	–0-	1/7
Klebsiella pnuemoniae	–0-	1/2	–0-	–0-
Klebsiella spp.	–0-	–0-	2/7	1/7
Staphylococcaceae				
Staphylococcus sciuri	–0-	–0-	1/7	3/7
Staphylococcus hominis	–0-	–0-	–0-	1/7
Microbacteriaceae				
Curtobacterium flaccumfaciens	1/2	–0-	1/7	–0-
Tsukamurellaceae				
Tsukamurella inchonensis	1/2	–0-	–0-	–0-

* No. isolates/ No. larvae or adults

specifically indicate the source of the microorganism (living or dead insects) but suggested the bacterium had potential for development as a microbial control agent against *L. decemlineata.* They have not reported on any further effort in that regard. In our study all *L. adecarboxylata* isolates were from living, putatively healthy larvae representing a range of instars and a few of them approaching pupation. We did not test for pathogenicity but in light of the Turkish study referenced above it may be instructive to challenge *A. glabripennis* with strains of *L .adecarboxylata.*

Members of the genus *Klebsiella* (Trevisan) are often isolated from insects and often implicated in nosocomial disease (Fotedar et al. 1991, Bouamama et al. 2010). In this study we isolated several species. *Klebsiella oxytoca* (Flügge), *K. pnuemoniae* (Schroeter), *Klebsiella terrigena* (Izard) and *Klebsiella* spp. were isolated from both Chicago and Queens insects; whereas, *K. ornithinolytica* Sakazaki (Sakazaki et al. 1989) was isolated from 1 adult beetle from queens. *Klebsiella oxytoca* and *K. pnuemoniae* are intestinal microorganisms that often are associated with various secondary pathological processes in humans. The other members of the genera are associated mainly with botanical, aquatic and soil environments (Izard et al. 1981)

Members of the genus *Serratia* (Bizio), *Serratia proteamaculans* (Paine and Stansfield), *Serratia rubidae* (Stapp) and *Serratia ficaria* (Grimont), were found in 3 different

Chicago insects. *Serratia proteamaculans* is a true insect pathogen (Glare et al. 1993) and has been implicated in human disease (Bollet et al. 1993). *Serratia rubideae* has not been reported as being associated with insects but can be a secondary invader in humans (Ursua et al. 1996). *Serratia ficaria* has been isolated from the fig wasp, *Blastophaga psenes* L. (Grimont et al. 1979), and has been implicated in a human infection (Gill et al. 1981). At the time of their isolation, none of these species appeared to be causing any disease in the insects examined.

Three additional members of the Enterobacteriaceae, *Enterobacter nimipressuralis* (Carter), *Pantoea agglomerans* (Ewing and Fife), and *Rahnella aquatilis* (Izard) were found in Chicago insects. The former is a plant pathogen (Carter 1945) and the latter 2 have been implicated in human disease (Cruz et al. 2007, Maraki et al. 1994, Goubau et al. 1988). *Rahnella aquatilis* has been isolated from other longicorn beetles (Park et al. 2007), and Vasanthakumar et al. (2006) suggested that this bacterium may play a nitrogen-fixation role for developing larvae of the Southern pine beetle, *Dendroctonas frontalis* Zimmermann.

One member of the genus *Microbacterium* was encountered in our study. *Microbacterium* spp. (CDC 4-A) (Hollis and Weaver) was isolated from both a larva and an adult from Chicago logs. This microorganism has been isolated from hospital environments and implicated as a secondary pathogen (Esteban et al. 1998). Two other members of the Microbacteriacea also were isolated; *Curtobacterium flaccumfaciens* (Hedges) was found associated with insects from both Queens and Chicago, whereas only 1 isolation of *Curtobacterium citreum* (Komagata and Iizuka) was made from the gut of a Chicago larva. Most members of the genus *Curtobacterium* have been isolated from plants but only strains of *C. flaccumfaciens* are reported to be phytopathogenic (Komagata and Suzuki 1986).

Single isolations from the families Bacillaceae (*Bacillus mycoides* Flügge) and Psuedomonadaceae (*Psuedomonas putida* Trevisan) were made from Chicago insect washings. Both are widely distributed in nature and are not considered entomopathogens.

Of the bacteria isolated from the insects in this study one of the most noteworthy was *Tsukamurella inchonensis*. This bacterium, to our knowledge, has not been isolated previously from any source in the United States. It has been isolated from the red mite, *Dermanyssus gallinae* De Geer, collected on poultry farms in France (Moro et al. 2009) and from the surfaces of the German cockroach, *Blattella germanica* L., infesting households in Botswana (Mpuchane et al. 2006). It is a relatively recently described species (Yassin et al. 1995) originally isolated from the blood of a patient in a hospital in Inchon, South Korea, and subsequently described as an opportunistic pathogen in a variety of clinical cases (Kattar et al. 2001). This microorganism is closely related to *Corynebacterium paurometabolum* first isolated from mycetomas and ovaries of bed bugs, *Cimex lectularius* L., by Steinhaus (1941) and later described as *Tsukamurella paurometabolum* by Collins et al. (1988). Corynebacteria are known symbionts of beetles (Douglas 2009) but assignation of any symbiotic role for *T. inchonensis* was outside the scope of this study.

Results of this study should be viewed as a preliminary probing for microorganisms from a limited number of insects and one in which there was little or no opportunity to select individual infested trees from which to sample insects. Thus, any rigorous comparisons of bacterial isolations relative to tree species or collection site were not appropriate. However, there are some findings on which to speculate and on which future studies might be based. The incidence of *L.adecarboxylata* in Asian longhorned beetle populations should be confirmed along with any role it may play, either symbiotic

or pathogenic, in beetle dynamics. In their culture independent study, Franke-Whittle et al. (2004) concluded that *L. adecarboxylata* along with *E. nimipressuralis*, one of the other enterics found in this study, shared a close relationship with pink sugarcane mealybug endosymbionts. And, as mentioned above, Muratoglu et al. (2009) found *L. adecarboxylata* pathogenic for Colorado potato beetle; whether it is pathogenic for Asian longhorned beetle should be determined. Further, a systematic search for *T. inchonensis* across several Asian longhorned beetle populations may shed some light on any role this microorganism may play in beetle dynamics. Finally, though many of the bacteria encountered in this study are either primary or secondary entomopathogens, none appeared to be causing any pathologies in either beetle adults or larvae at the time they were sacrificed for examination. Thus, researchers should be alert for diseased larvae and adults, either in nature or in laboratory colonies, that can be examined for microorganisms that may have potential for future development as microbial control agents.

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