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SHORT COMMUNICATION

Prevalence of cadmium resistance in *Staphylococcus sciuri* isolated from the Gray Treefrog, *Hyla chrysoscelis* (Anura: Hylidae)

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Amphibians are good bioindicators of environmental pollution. Recent declines in amphibian populations likely were produced by a combination of factors; nonetheless, habitat degradation resulting from environmental metal and chemical contamination is well established as one causative agent (Clark *et al.* 1998, Goleman *et al.* 2002, Levy *et al.* 2004, Sharma and Patino 2008). These observations are of grave concern, but it is even more distressing that some pollutants also can be transferred to predatory organisms (Burger and Snodgrass 2001), thereby amplifying their effects. The effects of contaminants on the resident microbial communities often are forgotten.

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Cadmium is toxic to most life forms (Lane *et al.* 2005). Of all the microorganisms studied to date, the cadmium-resistance systems of *Staphylococcus aureus* have been investigated most thoroughly (Smith and Novick 1972, Nies 1992, Nies and Silver 1995). The plasmid-mediated CadA and CadB systems are the most prominent cadmium-resistance mechanisms in *S. aureus* (Perry and Silver 1982, Silver and Misra 1988, Tisa and Rosen 1990). CadD, which shares significant homology to CadB, specifies low-level resistance to cadmium (Crupper *et al.* 1999). Furthermore, chromosomal cadmium-resistance determinants also are known in staphylococci distinct from plasmid-mediated systems (Witte *et al.* 1986).

Gray Treefrogs (*Hyla chrysoscelis*) contain large numbers of *Staphylococcu sciuri* as part of their normal flora (Slaughter *et al.* 2001). Given the well-documented cadmium-resistance systems

present in *S. aureus*, we hypothesized that these systems also might be present *S. sciuri*. A limited investigation revealed that a small subset of isolates of *S. sciuri* from *H. chrysoscelis* was resistant to elevated concentrations of cadmium. Because resistance to this heavy metal had not been documented in *S. sciuri*, we investigated the prevalence of cadmium resistance in 126 different isolates of *S. sciuri* from *H. chrysoscelis.* Additional experiments were designed to determine if CadA, CadB, or CadD caused the resistance.

Staphylococcus sciuri is a common coagulase-negative staphylococcus (CoNS) that is isolated regularly from various animal sources (Devriese *et al.* 1985, Adegoke 1986, Shimizu *et al.* 1992). This organism also colonizes humans and has been implicated in a variety of infections (Adegoke 1986, Kolawole and Shittu 1997, Hedin and Widerstrom 1998, Marsou *et al.* 1999). *Staphylococcus sciuri* is considered to be one of the basal members of the genus *Staphylococcu*s (Kloos *et al.* 1997, Couto *et al.* 2000) and to be a distant relative of *S. aureus* (Schleifer and Kroppenstedt 1990, Takahashi *et al.* 1999). Plasmids, common in *Staphylococcus* as a group, rarely are found in *S. sciuri* (Kloos *et al.* 1981), but their isolation has been reported (Schwarz *et al.* 1990, Schwarz and Noble 1994).

The minimum inhibitory concentration (MIC) of cadmium was determined for each isolate of *Staphylococcus sciuri*. Each organism was inoculated to a 0.05–McFarland standard into a series of tryptic soy-broth (TSB) tubes containing variable amounts (0–300 µg/ml) of $CdSO_4$. After incubation at 37°C with agitation at 250 rpm for 16 hr, results were recorded visually as "growth," "weak growth," or "no growth," depending on the turbidity of the culture. Resistant organisms were defined as having a MIC of 200–300 µg/ml; a MIC of 10–200 µg/ml indicated intermediate resistance and a MIC less than 10 µg/ml represented sensitive organisms. Based on the criteria set, 21 isolates were classified as resistant, 90 as intermediate, and 15 as sensitive. Six resistant, six intermediate, and six sensitive test isolates were picked randomly (Table 1) and used for further investigations.

Because the well-documented cadmium resistance systems in *Staphylococcus* are plasmid-mediated, we undertook experiments to determine if plasmids were present in the randomly chosen test strains. Plasmid DNA was successfully isolated from three resistant isolates, two intermediate, and all six sensitive isolates (Table 1). We did not delineate the sizes of plasmids and repeated attempts to isolate plasmid DNA from apparent negative strains were unsuccessful. Often it is difficult to obtain detectable amounts of large, low-copy number plasmids; therefore, we cannot rule out the possibility that these types of plasmids existed in the cell.

To examine the putative genetic location of the cadmium-resistance determinant, we attempted to cure the plasmids from each test isolate and measure of their cadmium resistance. Test isolates of *Staphylococcus sciuri* were inoculated into a series of TSB tubes containing variable amounts (0–80 µg/ml) of ethidium bromide, a useful agent for removing plasmids from a cell. This procedure was performed on each test isolate, including those lacking plasmid DNA. After four rounds of curing, each isolate was tested for cadmium resistance. No difference in cadmium resistance was observed in the pre- and post-ethidium–bromide isolates of *S. sciuri* (Table 1). Also, the plasmid profile of DNA from the post-ethidium–bromide isolates resembled that obtained before exposure of the test isolate to the curing agent (data not shown). In a similar experiment in which novobiocin was used as the curing reagent, only about 1% of the resulting *S. lugdunensis* clones lost their plasmids and were found to be sensitive to cadmium (Poitevin-Later *et al.* 1992).

Because we could not cure plasmids from cells harboring them, we investigated the transfer of cadmium resistance from the test strains to recipient strains. We designed experiments using electroporation (Kraemer and Iandolo 1990) and conjugation (Khan *et al.*

Table 1. Characterization of test isolates of *Staphylococcus sciuri*. Visual detection of plasmid DNA is indicated by "+," whereas "–" indicates that plasmid DNA was not detected by agarose gel electrophoresis or by absorbance at 260 nm. Presence/absence of a 2.5 kb amplicon of *cadA* or 0.54 kb amplicon of *cadB* is indicated by "+" or "–." The MIC of cadmium was determined before and after four rounds of curing with ethidium bromide; for comparison, *S. aureus* RN4220 containing either *cadA* from pI258 or *cadB* from pII147 grew in 300 µg/ml $CdSO_4$.

Strain number	MIC cadmium (µg/ml)		Plasmid DNA	PCR amplification	
	Pre-EtBr	Post-EtBr		*cadA*	*cadB*
F5C20	300	300	–	+	–
F7C11	300	300	–	–	–
F7C13	300	300	+	–	–
F9C10	300	300	+	–	–
F9C11	300	300	+	–	–
F9C16	300	300	–	–	–
F1C20	150	150	+	+	+
F2C8	150	150	+	–	–
F9C3	100	100	–	–	–
F9C9	100	100	–	–	–
F9C12	100	100	–	–	–
F9C17	50	50	–	–	–
F1C1	≤10	≤10	+	–	–
F1C3	≤10	≤10	+	–	–
F1C9	≤10	≤10	+	–	–
F2C2	≤10	≤10	+	–	–
F2C4	≤10	≤10	+	–	+
F2C5	≤10	≤10	+	–	+

2000) to transfer genes encoding cadmium resistance from *Staphylococcus sciuri* to *S. aureus* RN4220. Neither technique resulted in the transfer of a cadmium resistant phenotype, whereas control reactions were successful (data not shown). These data imply either that the cadmium-resistance genes are located on chromosomes or that they might be encoded on a plasmid with a replication system incompatible with *S. aureus*. Although we could not transfer cadmium resistance using these methodologies, they have been used successfully in other studies. For example, plasmids transferred from *S. lugdunensis* into competent *S. aureus* RN4220 by electroporation, conferred a cadmium resistance phenotype (Chaouni *et al.* 1996). Con-

Table 2. PCR primers and reaction conditions for one of thirty cycles.

Name	Sequence 5′ 3′	Reaction conditions
cadA	F: ATGTCTGAACAAAAGGTTAAACTAATGGAA	94°C/1 min
	R: CTATTTATCCTTCACTCTCATCAGTCGTAA	52°C/1 min
		72°C/2 min
cadB/D	F: TTTGCTAGAGCAAAGACTAGAAAAGAATAT	94°C/1 min
	R: AAATCCTAAAATTGTTTGAATAGTGTCAATTTC	52°C/1 min
		72°C/30 sec

jugation also was a successful method to transfer *ermA* and *ermC,* the two most common resistance determinants of erythromycin resistance to recipient *S. aureus* strains (Khan *et al.* 2000). Although these reports document successful exchange of genetic information into *S. aureus* from a foreign host, our experiments to transfer cadmium resistance were unsuccessful.

Based on published gene sequences for *cadA* (Wang and Novick 1987) and *cadB* (Chaouni *et al.* 1996), we designed DNA primers to amplify the respective genes in test strains of *Staphylococcus sciuri* (Table 2). Because CadD shares significant homology with CadB (Crupper *et al.* 1999), we designed the primers such that amplification with this primer pair also would detect *cadD.* By using chromosomal DNA from test isolates of *S. sciuri* in combination with *cadA* primers, we observed amplicons of approximately 2.5 kb in each of two strains (Table 1). Use of *cadB* primers produced an amplicon of approximately 540 bp in each of three strains. No amplicons were observed when we used plasmid DNA from *S. sciuri* as template DNA in a PCR with *cadA* and *cadB* primers. We included positive controls from *S. aureus* for *cadA* and *cadB* in all PCRs. We demonstrated that the amplicons were homologous to the respective gene by sequencing nucleotides of the PCR products and performing a BLAST analysis (Altschul *et al.* 1990) (data not shown).

The data presented herein demonstrate that cadmium resistance is widespread among isolates of *Staphylococcus sciuri* obtained from the anuran *Hyla chrysoscelis*. We neither demonstrated the exact genetic location of the resistance conclusively nor explained why amplicons were not generated in all resistant strains. Although a distinct mode of cadmium resistance may account for the resistance, nucleotide sequence heterogeneity may have prevented primer binding. Also, the considerable variability noted in cadmium resistance among the entire collection of strains may reflect differential regulation of the cadmium-resistance genes. Studies like this one are important to demonstrate the profound effect heavy metals can have on the resident microbial populations of plants and animals experiencing declines because low-level concentrations of cadmium are ubiquitous in both aquatic and terrestrial environments. Furthermore, data describing the microbial communities associated with populations of plants and animals may aid in the development of plans to help restore declining populations.

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