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Title: Cadmium resistance in *Staphylococcus sciuri*

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Staphylococcus sciuri is a member of the coagulase-negative staphylococci and its distribution is widespread in nature. Although primarily a pathogen of animals, this organism may colonize humans and its isolation from various clinical samples has been reported. In terms of heavy metal resistance, specifically cadmium resistance, most studies in the genus *Staphylococcus* have been confined to *S. aureus*; no investigations with *S. sciuri* have been reported. Three different cadmium resistance systems, designated CadA, CadB, CadD, have been reported in *S. aureus*. In this study, *S. sciuri* isolates obtained from a wild population of Gray Treefrogs were examined for cadmium resistance and it was shown that resistance levels among the isolates differ significantly. Using gene-specific primers based on *cadA* and *cadB/D*, amplicons were generated via the polymerase chain reaction which corresponded to the genes encoding CadA and CadB/D, respectively.

Cadmium Resistance in *Staphylococcus sciuri*

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PREFACE

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Introduction

Staphylococci are nonmotile, non spore forming, facultative anaerobic, Gram-positive cocci, ranging in size from 0.5 to 1.5 μm (25). Currently, the genus *Staphylococcus* is composed of 32 species and 15 subspecies (24). Although frequently isolated from the skin, mucous membranes, and gastrointestinal tracts of mammals and birds, staphylococci are ubiquitous in nature (22). The staphylococci are classified into two major groups: coagulase-positive and coagulase-negative, based on their ability to clot rabbit plasma. The most notorious member of the coagulase-positive staphylococci is *S. aureus*. This organism is a well recognized opportunistic pathogen and is a major cause of morbidity and mortality (28), as well as a major source of nosocomial infections (29). Clinical symptoms involving this organism range from simple skin infections, to more serious conditions such as pneumonia, osteomyelitis, and acute endocarditis (22). Food poisoning is often credited to an enterotoxin produced by *S. aureus*, as is toxic shock syndrome (TSS) (24). *Staphylococcus aureus* is also the most significant causative agent of bovine mastitis, which causes extensive economic losses in the dairy industry (33).

The coagulase-negative staphylococci (CoNS) as a group comprise a major component of the normal microflora of humans and animals. Although normally not pathogenic, members of this group can cause disease in immunocompromised hosts. Clinically, *S. epidermidis* has been well documented over the last two decades as a significant source of nosocomial infections (29).

Staphylococcus sciuri, a common CoNS in nature, is associated with a variety of animals (11,13,23,30). Several laboratories have reported regular isolation of this species from various foods, farm animals, rodents, marsupials, marine mammals, and birds (2,3,8,11,12,13,19,22,27,34,42,44,52,63,66). However, *S. sciuri* may also colonize humans and it has been implicated in a variety of infections, such as abscesses, boils, peritonitis, and endocarditis (1,18,31,37,65). Since many clinical laboratories do not identify CoNS to the species level, *S. sciuri* is rarely implicated as a human pathogen (67). It is considered one of the most primitive members of the genus *Staphylococcus* (8,9,23) and is distantly related to *S. aureus* along taxonomic lines (45,60). Current literature suggests its ubiquitous nature and broad range of environmental reservoirs (7,21,23,27,28,29,30) are due to its wide range of biochemical properties, such as its ability to use inorganic nitrogen as the sole source of nitrogen (7,9,23,27,28,30). Plasmids, common in *Staphylococcus*, are rarely found in *S. sciuri* (26). A 4.65-kb *S. sciuri* plasmid was identified which specified chloramphenicol resistance by encoding an inducible chloramphenicol acetyltransferase (46). Other plasmids carrying one or more resistance genes have also been identified (46,47,48).

The heavy metal cadmium is toxic to microbes and other types of life as well (5,35,38). Of all the microorganisms studied to date, *S. aureus* is the most effective in terms of cadmium resistance systems (59). Cadmium enters the cell through an Mn^{2+}-specific active transport system (35,62,68). Once inside the cell, cadmium ions bind to sulfhydryl groups on proteins essential in respiration, thereby inhibiting this process (64). The most prominent cadmium resistance systems in *S. aureus* involve the plasmid-

mediated CadA and CadB systems (39,51,53,54,56,57,59,61). CadA is usually encoded on a large plasmid such as pI258 by *cadA*. This system confers high level cadmium resistance by reducing the net uptake of Cd^{2+} through an increased energy-dependent efflux mechanism (39,55,56,57,62). Specifically, a 3.5-kb operon located on pI258 encodes two genes, designated *cadA* and *cadC* (39,49,50,62). *cadC* encodes a regulatory protein (16) essential for complete cadmium resistance, but its role in efflux remains unclear (6,70,71). Both *cadA* and *cadC* gene products are required for resistance to cadmium, and the *cadC* gene can be provided either in *trans* or *cis* (16,71). In contrast, the CadB system (encoded by *cadB*), confers low level cadmium resistance and has been confirmed on a large plasmid (pII147) and small multicopy plasmids as well (51). *Staphylococcus aureus* cells containing pII147 do not accumulate cadmium intracellularly, even though the Mn^{2+}-specific transport system is active (10). It has been suggested that CadB may protect the cell by binding cadmium in the membrane (40). The CadB determinant contains two genes, *cadB* and *cadX* (14,57). The later gene shows strong sequence similarity to *cadC* (16,71) and likewise, exerts positive regulatory effects (5,16). A recent study demonstrated a novel cadmium resistance gene similar to *cadB*, nominated *cadD*, was present on the large *S. aureus* plasmid pRW001. Its expression results in low-level resistance to cadmium (10). Chromosomal cadmium resistance determinants have also been reported in *S. aureus* distinct from the plasmid mediated systems (69). A CadB-like system has also been described on plasmid pLUG10 from *S. lugdunensis* (41).

A previous study demonstrated that Gray Treefrogs (*Hyla chrysoscelis*) contained a large proportion of *S. sciuri* as part of its normal flora (58). Since frogs, in general, are oftentimes considered as an indicator of a healthy environment, it was hypothesized that its microbial flora may also give insight into the health of the environment. Thus, the objective of this project was to investigate the level of cadmium resistance associated with 126 different *S. sciuri* isolates obtained from Gray Treefrogs (58). Our results indicate a significant variation of cadmium resistance among the isolates and that this resistance in some isolates is due in part to the well documented CadA and CadB resistance systems.

Materials and Methods

Bacterial Strains and Culture Conditions

One hundred and twenty six *Staphylococcus sciuri* isolates were obtained from a wild population of Gray Treefrogs (58). The isolates were routinely propagated in Tryptic Soy Broth (TSB) and Brain Heart Infusion (BHI) broth. For solid media, agar was added in the amount of 20 g/L. *Escherichia coli* and *S. aureus* were routinely maintained on Luria Bertani Agar (LBA) and Tryptic Soy Agar (TSA) plates, respectively. *Bacillus subtilis* was routinely maintained on TSA plates. Ampicillin in a final concentration of 100 μg/ml, and 5-Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside (X-Gal) in a final concentration of 20 μg/ml were added to LB medium when appropriate. Chloramphenicol in a final concentration of 15 μg/ml and cadmium in variable concentrations were added to media when needed.

Minimum Inhibitory Concentration Determination

The minimum inhibitory concentration (MIC) of cadmium was determined for all *S. sciuri* isolates. Briefly, each isolate was inoculated to a 0.05 McFarland standard into a series of TSB tubes containing variable amounts of $CdSO_4$. After incubation at 37°C with shaking at 250 rpm for 16 hours in New Brunswick Scientific shaker (Edison, NJ), results were recorded visually as growth, weak growth, or no growth, depending on the turbidity of the culture. Six resistant, 6 intermediate, and 6 sensitive isolates were selected randomly and used for further investigations.

Chromosomal DNA Isolation

Chromosomal DNA was isolated from *S. sciuri* strains using a modification of an established procedure (43). Briefly, 25 ml of BHI was inoculated with each isolate and incubated at 37°C with shaking at 250 rpm. Bacterial cells were precipitated by centrifugation at 4000 rpm and resuspended in 5 ml of Tris-Ethylenediamine (TE) buffer (10 mM tris, 1 mM EDTA; pH 8.0). Cell lysis was initiated by adding 100 µl of 10 mg/ml lysostaphin and subsequent incubation at 37°C for 20 minutes. The addition of 250 µl of 20% sodium dodecyl sulfate (SDS), followed by incubation at 60°C for 15 minutes completed cell lysis. RNA and proteins were degraded by adding 50 µl of RNase (final concentration 1 mg/ml) followed by incubation at 37°C for 15 minutes, and 75 µl of proteinase K (final concentration 1 mg/ml) and subsequent incubation at 60°C for 1 hour, respectively. Phenol extraction was performed by adding 6 ml of saturated phenol (pH 8). After mixing by inversion for at least 2-3 minutes, the solution was centrifuged at 4000 rpm for 5 minutes and the upper (aqueous) layer was removed and placed in a fresh conical tube. The phenol extraction was repeated as described above. Subsequently, an equal volume of chloroform/isoamyl alcohol (24:1) was added to the solution and the extraction performed as described above. DNA in the aqueous phase was precipitated upon the addition of ice-cold 95% ethanol. After mixing by inversion, a glass Pasteur pipette was used to remove the DNA, followed by drying in a Savant ISS 110 Integrated Speed Vac System (Holbrook, NY). Following resuspension in 500 µl of TE buffer, the DNA was stored at 4°C. The quantity and purity of chromosomal DNA were determined as indicated below.

Plasmid DNA Isolation

Plasmid DNA was isolated from *S. sciuri* using a QIAprep Spin Miniprep kit (Qiagen; Valencia, CA) with a slight modification. Briefly, the procedure was followed according the manufacturers' recommendations; however, 20 µl of 10 mg/ml lysostaphin was added along with the cellular resuspension buffer provided by the kit. After incubation at 37°C for 30 minutes, the remaining procedure was followed according to the manufacturers' instructions. The quantity and purity of plasmid DNA were determined as indicated below.

Quantification and Purity of DNA

The quantity and purity of DNA were determined using a Beckman DU530 spectrophotometer (Fullerton, CA). The DNA was diluted 50 fold by mixing 490 µl of TE buffer and 10 µl of the DNA to be analyzed in a Fisherbrand quartz cuvette (Fisher; St. Louis, MO). The absorbance at 260 nm and the absorbance ratio at 260 nm and 280 nm were performed to determine the quantity and purity, respectively. The quantity of DNA was determined using the following equation: (260 nm reading) (dilution factor) (50 µg/ml) = µg/ml DNA.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to visually detect plasmid DNA, chromosomal DNA, and PCR products according to standard conditions (43). Mini gels (12 wells) were prepared by dissolving 0.21 g of agarose in 30 ml of 1X TAE, which was prepared from a 50X stock consisting of 242 g tris, 57.1 ml acetic acid and 4 ml 0.5 M

EDTA/1L. One µl of 10 mg/ml solution of ethidium bromide was added to aid visualization of the DNA. Mid-sized gels (30 wells) were prepared by dissolving 0.84 g of agarose in 120 ml of 1X TAE containing 4 µl of ethidium bromide. The electrophoretic chambers employed were the MinicellEC370M and MaxicellEC360M (Fisher) and were powered by a BIO-RAD model 250/2.5 power supply (BIO-RAD; Hercules, CA). Agarose gels were visualized using a UV Intensity Transilluminator (Fisher) and documented with Panasonic CCD Ultra Lum camera and scion image software (UltaLum; Paramount, CA).

Purification of DNA from Agarose Gels

DNA was excised from agarose gels and purified using the QIAEX®II Gel Extraction Kit (QIAGEN) according to the manufacturers' recommendations.

Polymerase Chain Reaction

Chromosomal and plasmid DNA from *S. sciuri* isolates were used as template DNA in a polymerase chain reaction (PCR) to amplify the *cadA* and *cadB/D* genes using an MJ Research Minicycler (Watertown, Mass). Primers and amplification conditions used are listed in Table 1. Typical reaction mixtures consisted of 200 µM deoxynucleotide triphosphate (dNTP), 1.5 mM $MgCl_2$, 500 ng of plasmid or chromosomal DNA, 1 µl of Taq polymerase, 50 nM of *cadA* or *cadB/D* specific primers in buffer containing 50 mM Tris Cl, 50 mM KCL, and 0.01% Triton-X100 in a final volume of 100 µl. Agarose gel electrophoresis was used to analyze the amplified DNA as described above.

Table 1. PCR primers and reaction conditions.

Name	Sequence 5'→3'	Amplification conditions[a]
cadA	F: ATGTCTGAACAAAAGGTTAAACTAATGGAA R: CTATTTATCCTTCACTCTCATCAGTCGTAA	94°C 1 min 52°C 1 min 72°C 2 min
cadB/D	F: TTTGCTAGAGCAAAGACTAGAAAAGAATAT R: AAATCCTAAAATTGTTTGAATAGTGTCAATTTC	94°C 1 min 52°C 1 min 72°C 30 sec

[a]Conditions listed represent one cycle. Thirty cycles were used. All PCR conditions included an initial 94°C for 5 min and a final 72°C for 5 min.

Ligation and Transformation

DNA fragments to be cloned were ligated into the appropriate plasmid using DNA ligase. Briefly, variable amounts of the DNA fragments were mixed with approximately 50 ng of restriction enzyme digested plasmid DNA, and 1 µl of DNA ligase (3 U/µl) in a buffer consisting of 66 mM Tris, 6.6 mM $MgCl_2$, and 10 mM DTT (pH 7.6). Reaction mixtures were incubated for 2 h at 22°C. Transformation of *E. coli* was accomplished according to standard conditions (43).

DNA Sequencing

The nucleotide sequence of cloned inserts was determined at the DNA Sequencing Facility located at Kansas State University, Manhattan, KS. The DNA sequence was analyzed by the basic local alignment search tool (BLAST) (4).

Ethidium Bromide (EtBr) Curing

Staphylococcus sciuri isolates were inoculated into series of TSB tubes containing variable amounts of EtBr. After incubation at 37°C with shaking for 16 hours, cells were streak plated from the tube with growth containing the highest concentration of EtBr. A single colony obtained from this plate was referred to as cure 1. An inoculum obtained from cure 1 was used to inoculate a series of TSB-EtBr tubes containing higher concentrations of EtBr. The tube with the highest concentration of EtBr showing growth was used for streak plating in order to obtain single colonies. Cells from this plate were labeled cure 2. The same procedure was repeated to obtain cure 3 and cure 4.

Preparation of *Bacillus subtilis* Competent Cells

A fresh culture of *B. subtilis* Marburg growing on TSA was used to inoculate 30 ml of SPI medium (90 ml of sterile ddH_2O, 10 ml of 10X subtilis salts which consisted of 20 g of $(NH_4)_2SO_4$, 140 g of K_2HPO_4, 60 g of KH_2PO_4 and 10 g of $Na_3C_6H_5O_7 \cdot 2H_2O$ per liter, 200 µl of 1 M $MgCl_2$, 1.0 ml of 10% yeast extract, 1.0 ml of 2% casein hydrolysate, 3.0 ml of 20% glucose, and 1.0 ml of 10mg/ml tryptophan). After incubation with shaking at room temperature overnight, 70 ml of SPI medium was added to the culture and growth continued at 37°C with shaking at 250 rpm for approximately 1 hour. This time was referred to as T_0. Growth was allowed to continue for 90 minutes past T_0 (T_{90}). At this point, 10 ml of cells were transferred to 90 ml of SPII (90 ml of SpI containing 50 µl of 0.1 M $CaCl_2$) in a fresh flask, followed by incubation for 90 minutes at 37°C with shaking at 250 rpm. Cells were harvested by centrifugation at 5,000 rpm for 5 minutes in a J2-HS Centrifuge (Beckman; Palo Alto, California). Cells pellets were resuspended in 10 ml of the spent supernatant followed by the addition of 2.5 ml of 100% sterile glycerol. The competent cells were divided into 250 µl aliquots and stored at -70°C until needed.

Transformation of Competent *Bacillus subtilis*

Bacillus subtilis competent cells (100-250 µl) were thawed and transferred to a sterile 50 ml conical tube containing 100-400 ng of plasmid DNA. The total volume was brought up to 1 ml by the addition of TSB. This solution was incubated at 37°C with shaking at 250 rpm for 1 hour prior to spread plating of 100 µl on agar media containing the appropriate selective agent.

Preparation of *Staphylococcus aureus* RN4220 Competent Cells

10 ml of TSB was inoculated with a single colony of *S. aureus* RN4220 and grown overnight at 37°C with shaking at 250 rpm. Two hundred ml of fresh TSB was inoculated with 4 ml of the overnight culture and grown to an OD_{600} of 0.4 at 37°C with shaking at 250 rpm. Cells were harvested by centrifugation at 5500 rpm for 20 minutes and pellets resuspended in 10 ml of ice-cold 0.5 M sucrose. After resuspension, 190 ml of sterile ice-cold 0.5 M sucrose was added followed by centrifugation at 5500 rpm for 20 minutes. Precipitated cells were resuspended in 10 ml ice-cold 0.5 M sucrose, followed by the addition of 90 ml of sterile ice-cold 0.5 M sucrose. After incubation on ice for 30 minutes, cells were harvested by centrifugation at 5500 rpm for 20 minutes and resuspended in 10 ml ice-cold 0.5 M sucrose. After resuspension, an additional 10 ml of sterile ice-cold 0.5 M sucrose was added. Cells were harvested by centrifugation at 5500 rpm for 20 minutes. Pellets were resuspended in 400 µl of ice-cold 0.5 M sucrose and stored at -70°C until needed.

Transformation of Competent *Staphylococcus aureus* RN4220

100 µl of competent *S. aureus* RN4220 was mixed with variable quantities of plasmid DNA. The cell/DNA mixture was placed into a disposable cuvette (2.00 mm gap) (Molecular Technologies; St. Louis, Missouri) contained in an Eppendorf Electroporator 2510 (San Louis Obispo, California) and pulsed according to previously documented conditions (32). Nine hundred and twenty µl of SMMP broth (equal volumes of 2X SMM, which consisted of 34.2 g of sucrose, 0.46 g of maleic acid, and 0.81 g of $MgCl_2$ in a final volume of 100 ml (pH 6.5), and 4X PAB, which consisted of

17.5 g of antibiotic medium # 3 (Difco; Sparks, MD) in a final volume of 250 ml) was added immediately to the cuvette. The contents were transferred to a sterile 15 ml conical tube and after incubation for 1 hour at 37°C, 100 µl aliquots were plated directly onto selective media.

Conjugation between *Staphylococcus sciuri* and *Staphylococcus aureus* RN4220

Each *S. sciuri* strain (donor), and *S. aureus* RN4220 (pLI50) (recipient) were propagated for approximately 12 hours in TSB at 37°C without shaking. Two hundred µl of each *S. sciuri* isolate was mixed with 200 µl of *S. aureus* RN4220 (pLI50) in a sterile 15 ml conical tube. Following incubation for 6 hours at 37°C without shaking, 50 µl of the conjugation mixture was spread plated on TSA containing 15 µg/ml of chloramphenicol and 100 µg/ml of cadmium. Plates were incubated overnight at 37°C. Prolonged incubation at 37°C was also carried out in order to identify slower growing organisms. The initial conjugation mixture was also allowed to incubate an additional 10 hours followed by spread plating as described.

Results

Minimum Inhibitory Concentration (MIC) Determination

The MIC of cadmium for all 126 *S. sciuri* isolates was determined (Table 2). All isolates were categorized based on their MIC results. Resistant organisms had an MIC of 200 – 300 μg/ml, intermediate organisms were categorized with MIC of 10 – 150 μg/ml, and the MIC for sensitive organisms was < 10 μg/ml. Based on these criteria, 21 isolates were classified as resistant, 90 as intermediate, and 15 as sensitive. Six resistant, 6 intermediate, and 6 sensitive isolates were picked randomly (Table 3) and used for further investigations.

Plasmid DNA

To determine if plasmids were present in the 6 resistant, 6 intermediate, and 6 sensitive organisms, plasmid DNA was isolated from each isolate. Agarose gel electrophoresis was used to visually detect the plasmid DNA if present. Plasmid DNA was successfully isolated from 3 resistant isolates (F7C13, F9C10, and F9C11), 2 intermediate (F1C20 and F2C8), and all sensitive isolates (data not shown).

Ethidium Bromide (EtBr) Curing

In an attempt to cure plasmid DNA from the cell, *S. sciuri* isolates were inoculated into a series of TSB tubes containing variable amounts of EtBr. All cure 4 isolates obtained were tested for cadmium resistance. No difference in cadmium resistance was observed among the *S. sciuri* isolates before the curing experiment and

Table 2. MIC of cadmium for *S. sciuri* strains[a].

Strain	0 µg/ml	10 µg/ml	50 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml	300 µg/ml
F1C1	+	-	-	-			
F1C3	+	-	-	-			
F1C6	+	+	+	w	w	w	w
F1C7	+	w	-	-			
F1C8	+	+	w	-			
F1C9	+	-	-	-			
F1C10	+	+	-	-			
F1C11	+	+	+	+	w	w	-
F1C12	+	+	+	-			
F1C13	+	+	+	w	-	-	-
F1C14	+	+	-	-			
F1C15	+	+	+	-			
F1C16	+	+	+	-			
F1C17	+	+	+	-			
F1C18	+	+	+	-			
F1C19	+	w	-	-			
F1C20	+	+	+	+	w	-	-
F2C1	+	+	-	-			
F2C2	+	-	-	-			
F2C3	+	+	-	-			
F2C4	+	-	-	-			
F2C5	+	-	-	-			
F2C6	+	+	+	+	w	w	-
F2C7	+	+	+	w	w	w	w
F2C8	+	+	+	+	w	-	-
F2C9	+	+	+	-			
F2C10	+	+	+	+	+	-	-
F2C11	+	+	-	-			
F2C12	+	+	+	+	+	w	-
F2C14	+	+	+	-			
F2C13	+	+	-	-			
F2C17	+	-	-	-			
F2C18	+	+	+	+	+	w	-
F2C19	+	+	w	-			
F2C20	+	-	-	-			
F3C1	+	+	w	-			
F3C2	+	+	-	-			

F3C3	+	+	-	-			
F3C4A	+	-	-	-			
F3C4B	+	-	-	-			
F3C5	+	-	-	-			
F3C6	+	-	-	-			
F3C7A	+	-	-	-			
F3C7B	+	+	-	-			
F3C8	+	+	-	-			
F3C9	+	+	-	-			
F3C10	+	+	-	-			
F3C11	+	+	-	-			
F3C12	+	w	-	-			
F3C13	+	-	-	-			
F3C14	+	+	-	-			
F3C15	+	+	-	-			
F3C16	+	+	-	-			
F3C17	+	+	+	+	w	-	-
F3C18	+	+	+	-			
F3C19	+	+	+	w	-	-	-
F3C20	+	+	+	w	-	-	-
F3C21A	+	+	-	-			
F3C21B	+	+	w	-			
F5C1	+	+	-	-			
F5C2	+	+	+	+	-	-	-
F5C3	+	+	+	-			
F5C4	+	+	+	-			
F5C5	+	+	+	+	w	w	-
F5C6	+	+	+	+	w	w	w
F5C7	+	+	+	+	w	w	w
F5C8	+	+	+	+	w	w	-
F5C9	+	+	+	w	-	-	-
F5C10	+	+	+	+	w	-	-
F5C11	+	+	+	-			
F5C12	+	+	-	-			
F5C13	+	+	-	-			
F5C14	+	+	+	+	w	w	-
F5C15	+	+	+	+	w	w	w
F5C16	+	w	-	-			
F5C18	+	+	+	w	-	-	-

F5C19	+	+	-	-			
F5C20	+	+	+	+	+	+	+
F6C5	+	+	+	-			
F6C6	+	+	-	-			
F6C7	+	+	+	w	-	-	-
F6C9	+	+	w	-			
F6C10	+	+	+	w	-	-	-
F6C11	+	+	+	w	-	-	-
F6C12	+	+	w	-			
F6C13	+	+	-	-			
F6C14	+	+	-	-			
F6C15	+	+	+	w	-	-	-
F6C16	+	+	-	-			
F6C17	+	+	+	+	w	w	-
F6C18	+	-	-	-			
F6C19B	+	+	-	-			
F7C10	+	w	-	-			
F7C11	+	+	+	+	+	+	+
F7C13	+	+	+	+	+	+	w
F8C1	+	+	-	-			
F8C3	+	+	-	-			
F8C4	+	+	-	-			
F8C5	+	+	-	-			
F8C8	+	+	-	-			
F8C9	+	+	+	-			
F8C17	+	+	w	-			
F8C19	+	w	-	-			
F8C20	+	+	+	+	-	-	-
F9C3	+	+	+	+	-	-	-
F9C8	+	+	w	-			
F9C9	+	+	+	+	-	-	-
F9C10	+	+	+	+	+	+	w
F9C11	+	+	+	+	+	+	w
F9C12	+	+	+	+	-	-	-
F9C13	+	+	+	+	w	-	-
F9C15	+	+	+	w	-	-	-
F9C16	+	+	+	+	+	+	+
F9C17	+	+	+	-			
F9C19	+	+	+	-			

F9C20	+	+	+	-			
Dry 16	+	+	+	-			
Dry 17	+	+	+	-			
Dry 19A	+	+	+	-			
Dry 19B	+	w	w	-			
Fresh 11	+	+	-	-			
Fresh 13	+	+	+	+	+	+	w
Fresh 16	+	+	+	w	-	-	-
Fresh 17	+	+	+	+	+	+	w
Fresh 18	+	+	+	w	-	-	-
Fresh 19	+	+	+	-			

[a] Minimum Inhibitory Concentration (MIC) results. +, growth; w, weak growth; -, no growth.

Table 3. *Staphylococcus sciuri* isolates (6 cadmium resistance, 6 intermediate, and 6 sensitive) used for further study.

Isolate	MIC[a]
F5C20	R
F7C11	R
F7C13	R
F9C10	R
F9C11	R
F9C16	R
F1C20	I
F2C8	I
F9C3	I
F9C9	I
F9C12	I
F9C17	I
F1C1	S
F1C3	S
F1C9	S
F2C2	S
F2C4	S
F2C5	S

[a]MIC: R, resistant (200 – 300 μg/ml); I, intermediate (10 – 150 μg/ml); S, sensitive (< 10 μg/ml).

those after the curing experiment (Table 4). Also, plasmid DNA isolated from each cure 4 isolate revealed a plasmid profile similar to the profile obtained before the curing experiment (data not shown).

Transfer of Cadmium Resistance

A. Transformation of *Bacillus subtilis*

In an attempt to transfer the genes of cadmium resistance from *S. sciuri* to *B. subtilis*, transformation of *B. subtilis* was performed using plasmid DNA from the *S. sciuri* isolates. Cadmium resistance could not be transferred to *B. subtilis* (data not shown). Control experiments verified the *B. subtilis* cells were competent.

B. Electroporation of *Staphylococcus aureus* RN4220

Plasmid DNA from the *S. sciuri* isolates was used in an attempt to transfer the genes of cadmium resistance from *S. sciuri* to *S. aureus* RN4220 by electroporation. Cadmium resistance genes could not be transferred to *S. aureus* RN4220 (data not shown). Control experiments verified the *S. aureus* RN4220 cells were competent.

C. Conjugation between *Staphylococcus sciuri* and *Staphylococcus aureus* RN4220

Conjugation was used in an attempt to transfer cadmium resistance genes from each *Staphylococcus sciuri* isolate to *S. aureus* RN4220. Cadmium resistance could not be transferred (data not shown). Control experiments verified the *S. aureus* RN4220 was a suitable recipient.

Table 4. MIC for cadmium after EtBr curing for the 6 resistant, 6 intermediate, and 6 sensitive *S. sciuri* isolates.

Strain	MIC[a]	Curing[b]	EtBr[c] (μg/ml)	Plasmid DNA[d]	$CdSO_4$ Concentration after EtBr Curing Experiment		
					0 μg/ml	200 μg/ml	300 μg/ml
F5C20	R	1	10		+	+	+
		2	20		+	+	+
		3	40		+	+	+
		4	70	-	+	+	+
F7C11	R	1	6.7		+	+	+
		2	13.3		+	+	+
		3	30		+	+	+
		4	50	-	+	+	+
F7C13	R	1	3.3		+	w	w
		2	10		+	+	-
		3	30		+	+	-
		4	60	+	+	+	w
F9C10	R	1	10		+	w	-
		2	20		+	w	-
		3	40		+	w	w
		4	70	+	+	w	-
F9C11	R	1	10		+	+	w
		2	20		+	+	-
		3	50		+	+	+
		4	60	+	+	+	-
F9C16	R	1	10		+	+	+
		2	20		+	+	+
		3	50		+	+	+
		4	80	-	+	+	+
					0 μg/ml	100 μg/ml	150 μg/ml
F1C20	I	1	10		+	+	+
		2	16.7		+	+	+
		3	50		+	+	+
		4	80	+	+	+	+
F2C8	I	1	6.7		+	+	+
		2	13.3		+	+	+
		3	30		+	+	+

		4	40	+	+	+	+
					0 µg/ml	50 µg/ml	100 µg/ml
F9C3	I	1	10		+	+	+
		2	20		+	+	+
		3	50		+	+	+
		4	80	-	+	+	+
F9C9	I	1	6.7		+	+	w
		2	16.7		+	+	+
		3	50		+	+	+
		4	80	-	+	+	+
F9C12	I	1	6.7		+	+	+
		2	16.7		+	+	w
		3	40		+	+	+
		4	60	-	+	+	w
					0 µg/ml	10 µg/ml	50 µg/ml
F9C17	I	1	10		+	+	+
		2	20		+	+	+
		3	50		+	+	+
		4	80	-	+	+	+
					0 µg/ml	10 µg/ml	
F1C1	S	1	10		+	-	
		2	20		+	-	
		3	50		+	-	
		4	80	+	+	-	
F1C3	S	1	10		+	-	
		2	20		+	-	
		3	50		+	-	
		4	80	+	+	-	
F1C9	S	1	10		+	-	
		2	20		+	-	
		3	50		+	-	
		4	80	+	+	-	
F2C2	S	1	10		+	w	
		2	20		+	-	
		3	50		+	-	
		4	80	+	+	-	

F2C4	S	1	10		+	w	
		2	20		+	-	
		3	50		+	w	
		4	80	+	+	-	
F2C5	S	1	6.7		+	-	
		2	16.7		+	-	
		3	40		+	-	
		4	70	+	+	-	

[a]MIC: R, resistant (200 – 300 μg/ml); I, intermediate (10 – 150 μg/ml); S, sensitive (< 10 μg/ml). +, growth; w, weak growth; -, no growth.

[b]The curing procedure was done for 4 rounds.

[c]The highest concentration of EtBr for each curing experiment.

[d] Plasmid DNA isolation for cure 4. No plasmid DNA isolation was done for cures 1, 2, and 3.

Polymerase Chain Reaction of *cadA* and *cadB/D*

Chromosomal DNA from *S. sciuri* was used as template DNA in a PCR in an attempt to amplify the *cadA* gene. Amplicons of approximately 2.5 kb were observed in the F5C20 resistant strain and in the F1C20 intermediate strain (Figure 1). Also, the *cadB/D* gene amplicon of approximately 540 bp was observed in the F1C20 intermediate strain and in both F2C4 and F2C5 sensitive strains (Figure 2). When plasmid DNA from *S. sciuri* was used as template DNA in a PCR using *cadA* primers, no amplicons were observed (data not shown). However, when *cadB/D* primers were used, an amplicon of approximately 540 bp was observed in the F1C20 intermediate strain (Figure 3). Agarose gel electrophoresis was used to visually detect DNA amplicons in each experiment.

Cloning and DNA Sequencing of *cadA* and *cadB/D* Genes

Nucleotide sequencing of the amplicon generated via PCR using gene-specific primers based on the CadA system followed by BLAST analysis demonstrated the amplified gene in the *S. sciuri* F5C20 resistant strain was homologous to *cadA*. The partial nucleotide sequence is shown in Figure 4. Similarly, nucleotide sequencing of the 5' region of the amplicon obtained using the *cadB/D* primers and subsequent BLAST analysis in the *S. sciuri* F1C20 intermediate strain revealed it was homologous to *cadB/D* from *S. aureus* (Figure 5).

Figure 1. PCR of *S. sciuri* isolates using chromosomal DNA and *cadA* gene specific primers. Lane 1, molecular weight markers; lanes 2-3, 2.5 kb amplicon from strain F5C20 and F1C20, respectively.

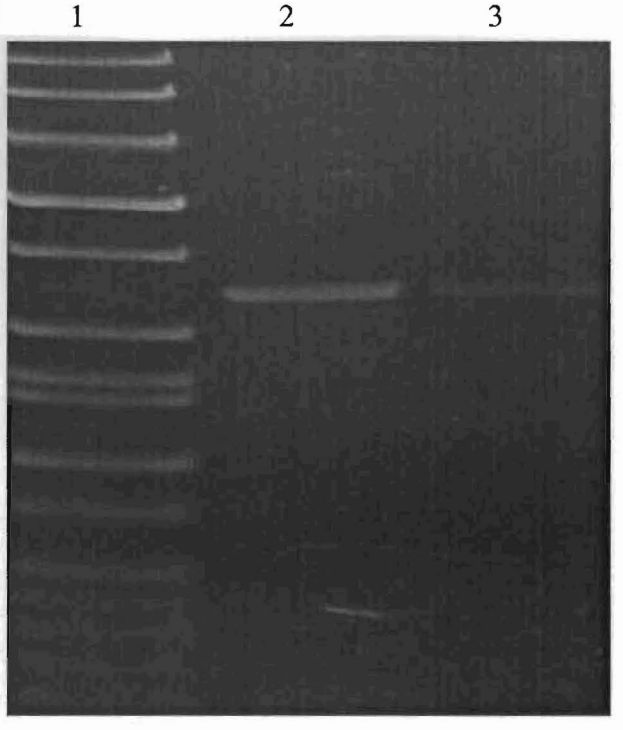
1
2
3

Figure 2. PCR of *S. sciuri* isolates using chromosomal DNA and *cadB/D* gene specific primers. Lane 1, molecular weight markers; lanes 2-4, 540 bp amplicon from strain F1C20, F2C4, and F2C5, respectively.

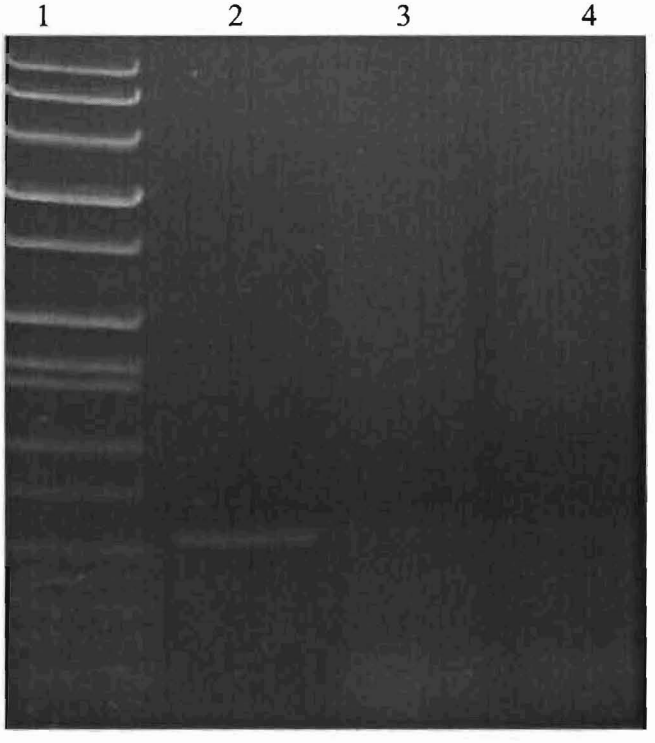
1
2
3
4

Figure 3. PCR of *S. sciuri* isolates using plasmid DNA and *cadB/D* gene specific primers. Lane 1, molecular weight markers; lane 2, 540 bp amplicon from strain F1C20.

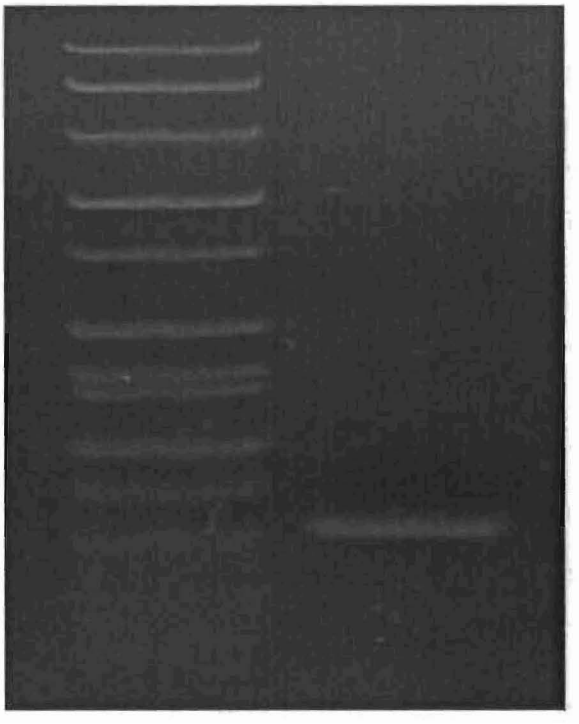
1
2

Figure 4. DNA sequence corresponding to *cadA*.

1ATA TGT CTG AAC AAA AGG TTA AAC TAA TGG AAG AAG AAA TGA ACG
46TCT ATC GGG TCC AAG GAT TTT CAT GTG CAA ATT GTG CAG GAA AGT
91TTG AGA AAA ATG TTA AAA AGA TTC CAG GCG TTC AGG ACG CAA AAG
136TAA ATT TTG GAG CTT CAA AAA TTG ATG TCT TCG GCA GTG CAA CTG
181TTG AAG AAC TAG AAA AGG CTG GTG CTT TTG AAA ATC TTA AAG TGG
226CAC CAG AGA AAC CTA AAA GAC GGG TAG AAC CTG TGG TAA TTA
268AAG ATA AAA ACG TTT ACC GTG TGG AAG GAT TTT CCT GCG CAA ATT
313GTG CGG GGA AGT TTG AAA AAA ATG TAA AAC AAA TAG CTG GAG
355TTG AGG ATG CAA AAG TAA ACT TTG GCG CTT CTA AAA TTG ATG TAT
400ATG GAA ATG CAT CGG TTG AAG AAC TTG AAA AAG CAG GTG CTT TTG
445AGA ATC TAA AAG TAT CTC CTG AAA AAC TAG CGA ATC AAA CGA TAC
490AAA GGG TTA AAG ATG ACA CTA AGG CTC ATA AAG AAG AGA AAA
532CAC CAT TTT ATA AAA AAC ATA GTA CAT TGC TGT TTG CCA CAC TAC
577TAA TTG CTT TTG GTT ACC TTT CTC ACT TTG TAA ATG GAG AAG ATA
622ACC TCG TAA CTT CCA TGT TAT TTG TAG GTT CTA TTG TAA TTG GCG
667GAT ATT CAT TAT TTA AAG TCN GTT TTC AAA ATT TGA TAC GCT TTG
712ATT TCG ACA TGA AAA CCC TGA TGA CCC GTT GCC CGT TAT TGG AGC
757TGC CAT TAT TGG TGA ATG GGC CAA AGC ATC TAT TGG TGG TAT TCT
802TTT TGC AAT CAG TGG AAG CAC TTG AAC

Figure 5. DNA sequence corresponding to *cadB/D*.

1ATA AAT CCT AAA ATT GTT TGA ATA GTG TCA TTT TCA ATA ATA ATA
46AAT AAA CCT AAA GCT ATA TAA ATA ACA GCC ATA ATC CAA CGA CTA
91AAT TTC TCA ACA ATT TCT CCA ACT CCT GGA ATA TTA GCT AAT TTT
136TGT GCA GTA AAT ACC AAG AAG AAA ATT AAA ATT AAA AAG ACA
178AAC AAA GTA ATT AAT AAA TTA GTA ACA CTT AAT GTC ACA AAA TAT
223GGA ACA AAT AAA CCA ATA TTA TCA GCA CCA CAA CTT GCT ATC GTA
268ACA ATT GCA ACC GTA CCA ACT AAT TTA GAC AAT CCT TTT TCA TTC
313AAT TCT TTT TTA GCT CTC TTT TCT CCT TCA CAA TCA TCA TAA ATA
358GCC ACT TTA ATT CCA AGA TAA ATT GGT ATT AAA CCC AAT AAA CCT
403AAT ATC CAC TTC TCT GGA ACA TAA TTT AAT ACA AAA GCT AAA AAC
448AAA CTA ACT AAT ATT AAA ATA ATA GAC CCT AAA TAT TGA CCA ACA
493TAA ATA TCT CTA TAT TCT TTT CTA GTC TTT GCT CTA GCA AAA TCT
538GAA TTC GTC GAC AAG CTT CTC GAG CCT AGG CTA GCT CTA GAC CAC
583ACG TGT GGG GGC CCG AGC TCG CGG CCG CAC AAT TCA CTG GCC GTC
628GTT TTA CAA CGT CGT GAC TGG GAA AAC CCT GGC GTT CCC AAC TTA
673ATC GCC TTG CAG CAC ATC CCC CTT TCG CCA GCT GGC GGT AAT AGC
718GAA GAA GCC CGC ACC GAT CGC CCT TCC CAA CAG TTG CGC AGC CTG
763AAT GGG GGA ATG GAA ATT GTA AGC GTT AAT ATT TTG GTT AAA ATC
808GCG TTA AAT TTT TGT TAA ATC AGC TCA TTT TTT

Discussion

The most distinct mechanisms of cadmium resistance in *S. aureus* involve the plasmid-mediated *cadA* and *cadB* genes (39,51,53,54,56,57,59,61). The *cadA* determinant, and its associated regulatory gene *cadC*, confers high level cadmium resistance through an increased energy-dependent efflux system, which prevents the internal accumulation of cadmium ions (39,55,56,57,62). In contrast, *cadB*, and its associated regulatory gene *cadX*, confers a lower level of cadmium resistance by a mechanism potentially involving cadmium binding in the membrane (40). The CadA system has been localized on several different plasmids, including pXU5, pI258, pI524, and pSK57 (36,41). CadB is encoded on the plasmids pIP983 (15) and pOX6 (53). Plasmid pII147 is notable in that it carries both *cadA* and *cadB* genes (17). A less documented cadmium resistance system is designated CadD. It is similar to CadB, confers low-level resistance to cadmium, and is encoded on the large *S. aureus* plasmid pRW001 (10).

Cadmium resistance systems are not confined to *S. aureus* and have been found in a variety of different hosts. For example, homologous gene sequences to *S. aureus cadB* are present on plasmids isolated from *S. lugdunensis* (41). An additional study demonstrated cadmium resistance in *Listeria monocytogenes* is mediated by two genes similar to the *cadAC* determinants of *S. aureus* (35). To date, investigations dealing with *S. sciuri* are minimal, and no reports concerning cadmium resistance are available. Thus, data contained herein are potentially the first to examine cadmium resistance in *S. sciuri*.

Significant variability in terms of cadmium resistance among the 126 *S. sciuri* isolates examined was observed (Table 2). The wide range of cadmium resistance may be due to differences in cadmium resistance mechanisms possessed by individual *S. sciuri* strains, or it may reflect differential expression of cadmium resistance genes. No attempt was made to address the variability of cadmium resistance among the isolates. Variable cadmium resistance among a group of isolates has been observed in other studies. For example, an investigation in which 35 *S. lugdunensis* strains were examined for cadmium resistance, 20 strains harbouring either a 2.9-, 3.2-, 3.4-, 3.5- or 3.7-kb plasmid encoding a *cadB* homologue were more resistant to cadmium than 11 plasmid-negative strains or four strains containing a 4.6-kb plasmid (41).

Since most cadmium resistance examined to date is plasmid-mediated, experiments were initiated to determine if plasmids were present in the 6 resistant, 6 intermediate, and 6 sensitive test strains. Plasmids were isolated and visualized in 3 resistant, 2 intermediate, and all 6 sensitive test strains. No attempt was made to delineate their size. Since not all resistant isolates contained a plasmid, these results suggested cadmium resistance in some strains may be encoded in the chromosome. Indeed, chromosomal cadmium resistance has been documented in *S. aureus* (69). To examine this possibility, an attempt was made to cure the plasmids from each cell that harbored them, followed by a measurement of cadmium resistance. After four rounds of the curing by growing each strain in increasing amounts of EtBr, isolates were tested for their cadmium resistance after each round of curing. Also, cells from the fourth round of curing (cure 4) were examined to determine if a plasmid was still present. Although the

cells adapted well to the increasing EtBr concentration, the MIC of cadmium resistance and plasmid DNA profile for cure 4 showed no difference as compared to cells examined before the curing experiment (Table 4). These results suggested that cadmium resistance determinants are encoded on the chromosome. It is possible, however, that EtBr may not have been the most appropriate way to cure the plasmids from the cells. In a previous study using novobiocin as the curing reagent, only approximately 1% of the resulting *S. lugdunensis* clones lost their plasmids and were found to be sensitive to cadmium (41). Also, to verify whether the cadmium resistance in *L. monocytogenes* strain Lm74 was plasmid borne, it was cured of its plasmid by high temperature treatment. A resulting 32-fold decrease in the MIC of cadmium was observed (35). Although we did not examine these additional methods of curing, EtBr has been well documented as a valid curing agent. In fact, some plasmids are stable in a cell regardless of the curing method employed. While the molecular nature of the plasmids in *S. sciuri* was not examined, the fact that they are not easily removed from the cell suggests they encode an essential cellular function.

The inability to cure plasmids from the cells prompted investigation into the transfer of plasmids from the test strains to recipient strains via transformation, electroporation, or conjugation. Electroporation of *S. aureus* RN4220 was performed using isolated plasmids from the test strains. No cadmium resistance could be transferred (data not shown). These results suggested the plasmids harbored by the test strains could be conjugative plasmids. Thus, each test strain was mated with *S. aureus* RN4220 (pLI50). No transconjugants resistant to cadmium were obtained (data not shown).

Since many staphylococcal plasmids will also replicate in *B. subtilis*, plasmid preparations from the test strains were used to transform *B. subtilis*. As observed with electroporation and conjugation of *S. aureus*, no *B. subtilis* transformants resistant to cadmium were obtained (data not shown). Taken together, these results suggest cadmium resistance may be chromosomal-borne. Alternatively, the resistance genes may be encoded on a plasmid, but contain a replication system incompatible with *S. aureus* and *B. subtilis*. A previous study demonstrated *B. subtilis* cells could be transformed with recombinant plasmid DNA from *L. monocytogenes* successfully (35). Also, recombinant plasmid DNA from *S. aureus* was electroporated into *S. aureus* RN4220 followed by identification of clones that conferred cadmium resistance (32). In another study, plasmids from *S. lugdunensis* were transferred into competent *S. aureus* RN4220 by electroporation (5). Conjugation between *S. aureus* isolates has also been performed successfully to transfer the *ermA* and *ermC* genes, the two most common resistance determinants of erythromycin resistance (20). While all these reports demonstrate successful exchange of genetic information into *S. aureus* or *B. subtilis* from a foreign host, experiments designed to transfer cadmium resistance in this study were unsuccessful.

Based on published gene sequences for *cadA* and *cadB/D*, DNA primers were designed for use in a PCR in an attempt to amplify the respective genes in the *S. sciuri* test strains. For example, by using chromosomal DNA from *S. sciuri* test strains as template DNA, amplicons of a size similar to that of *cadA* gene were observed in the resistant strain F5C20 and in the intermediate strain F1C20 (Figure 1). Also, amplicons

of a size similar to that of *cadB* gene were observed in the intermediate strain F1C20 and in both F2C4 and F2C5 sensitive strains (Figure 2). These results suggest that these genes are chromosomal-borne. Oftentimes, large plasmids contaminate chromosomal DNA preparations and this extraneous DNA may be responsible for the amplification. Also, using plasmid DNA from *S. sciuri* test strains as template DNA, an amplicon of a size similar to that of *cadB* gene was observed in the intermediate strain F1C20 (Figure 3). This suggested *cadB* is plasmid-mediated. Although *cadB* was amplified from the sensitive strains F2C4 and F2C5, these strains do not express cadmium resistance. No attempt was made to determine why a cadmium resistant phenotype was not observed. DNA sequencing and BLAST analysis confirmed the amplicons were indeed *cadA* (Figure 4) and *cadB/D* (Figure 5). Amplicons were not generated in all strains, even if cadmium resistance was detected. This suggested the *cadA* and/or *cadB/D* specific primers did not bind due to DNA sequence heterogeneity. Alternatively, a completely different cadmium resistance system may be present in these isolates.

In summary, this study shows that cadmium resistance among *S. sciuri* strains differs. Also, the well defined *cadA* and *cadB* genes were found to be present in some of the *S. sciuri* test strains. Future studies could investigate the nature of the cadmium resistance in the isolates in which *cadA* or *cadB* could not be amplified. Also, investigation of the resistance systems to other heavy metals, such as zinc, cobalt, or nickel, in *S. sciuri*, followed by a comparison to the cadmium resistance systems would be of interest.

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