Published in final edited form as:

J Environ Sci Health A Tox Hazard Subst Environ Eng. 2011 ; 46(4): 436–440. doi:
10.1080/10934529.2011.549407.

Triclosan- resistant bacteria isolated from feedlot and residential soils

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Abstract

Triclosan is an antimicrobial agent that is currently incorporated into hundreds of consumer and medical products. It can be either a bacteriostatic or bactericidal agent, depending on its formulation. It has activity against Gram-positive and Gram-negative bacteria, as well as some viruses and protists. The purpose of this study was to determine whether triclosan-resistant bacteria could be isolated from the soil. Soils from cattle feedlots and residential lawns were collected and assayed for the presence of these organisms by plating samples on growth media containing triclosan. Organisms were subsequently identified by partial 16S rRNA sequencing analysis. All the organisms isolated in this study were Gram-negative rods, with members of genus *Pseudomonas* being particularly well represented. This result may not be surprising because Gram-negative organisms are generally more resistant to triclosan, and since *Pseudomonas* bacteria are known to have numerous efflux mechanisms for dealing with harmful substances.

Keywords

Triclosan-resistance; feedlots; soils

INTRODUCTION

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether) is an antimicrobial agent found widely in hundreds of commercially available products (Figure 1). It is included in items such as deodorants, antibacterial soaps, cosmetics, toothpaste, fabrics and many plastic items. It is marketed under several different names including Irgasan DP-300, Microban, Lexol-300, Cloxifenolum, and Biofresh.^[1] Triclosan was first introduced in 1965 and was shown to have a very high stability, including the ability to resist degradation in dilute acid and alkaline solutions.^[2] It has strong activity against Gram-positive bacteria, however its activity against yeasts and Gram-negative bacteria can be increased by changing its formulation. For instance it has been shown that triclosan can be combined with EDTA to increase its permeability against the outer membrane of Gram-negative bacteria.^[3] Triclosan also exhibits activity against certain infectious protists such as *Toxoplasma gondii* and *Plasmodium falciparum*.^[4] At lower concentrations using in vitro assays, the activity of

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triclosan is mostly bacteriostatic, while at higher concentrations it exhibits bactericidal capabilities.^[2] The activity of triclosan against staphylococci, some streptococci, certain mycobacteria, *Proteus* spp. and *Escherichia coli* is quite high, with minimum inhibitory concentrations (MIC) in the range of 0.01 to 0.1 mg/L. Some strains of methicillin-resistant *Staphylococcus aureus* (MRSA) show an elevated triclosan MIC, while others do not. Members of genus *Enterococcus* are not as susceptible to the effects of triclosan as members of *Staphylococcus*. The widely disseminated nosocomial pathogen *Pseudomonas aeruginosa* is highly resistant to triclosan.^[4]

When triclosan was first commercially introduced in the 1960's its exact mode of action was unknown. It was hypothesized that it acted in a non-specific manner by disrupting bacterial cell membranes.^[2] Today it is known that triclosan acts by inhibiting the NADH-dependant enoylacyl carrier protein reductase FabI, a critical component in the bacterial fatty acid synthesis pathway.^[5] The crystal structure of a complex created between FabI, NAD⁺, and triclosan from *Escherichia coli* showed that triclosan formed a stable ternary complex with amino acid residues in the active site of the enzyme.^[6] Other bacteria were later shown to produce enoyl reductases as well. These include *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* which produce FabK, *Bacillus subtilis* which produces FabL, and *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*, which both produce InhA. It has been shown that FabK and FabL enoyl reductases are resistant to the activities of triclosan, while InhA is not.^[7]

Bacterial resistance to triclosan has been shown to occur by several different mechanisms. The known mechanisms for triclosan resistance include: the expression of highly efficient efflux pumps that rid the cell of triclosan, the production of an enoyl reductase enzyme with low affinity for triclosan, or the expression of an enzyme that actively degrades triclosan.^[8] In several pathogenic bacteria, these mechanisms have been characterized. The triclosan efflux pump used by *Escherichia coli* is AcrAB-TolC, while in *Pseudomonas aeruginosa* the active efflux pumps include MexAB-OprM, MexCDOPrJ, and MexEF-OprN.^[9] In recent work by Chen et al,^[9] evidence indicated the increasingly important nosocomial agent *Acinetobacter baumannii* exhibits intrinsic active efflux as well as acquired resistance by *fabI* over-expression and mutation. Some bacterial strains have multiple enoyl-acyl carrier reductase enzymes that allow them to exhibit triclosan resistance. Zhu et al^[10] showed that *Pseudomonas aeruginosa* PAO1 expresses FabV, which is completely resistance to the activity of triclosan. This organism also carries a typical *fabI* gene, whose product is susceptible to triclosan. When the *fabI* gene was knocked out, the organism retained about 62% of its original triclosan resistance, leading these workers to hypothesize another mechanism was at work.^[10] Triclosan resistance by the production of enzymes that degrade triclosan has been reported for two organisms; *Pseudomonas putida* TriRY and *Alcaligenes xylosoxidans* subsp. *dentrificans* TR1. These two agents were also shown to have the ability to utilize triclosan as a sole carbon source.^[11]

The purpose of this study was to isolate triclosan-resistant microorganisms from non-clinical environmental sources, and to identify and characterize those organisms. Surface soil samples were collected from two types of locations; cattle feedlots and residential lawns.

We selected these two types of locations based upon expected bacterial loads. Feedlots were selected on the assumption that they would be heavily enriched with fecal bacteria that had been exposed to the antibiotics present in cattle feed, as well as the antibiotics the cattle received as part of their normal processing. Residential soils were selected based on the assumption that this type of soil would be most frequently encountered by people in communities. Feedlot samples were collected from ten different cattle feedlots in eight counties in central and southwestern Kansas. The feedlots ranged in size from small family operations of about 50 head of cattle, to large commercial facilities of up to 50,000.^[12] Residential soil samples were collected from six randomly selected locations in a Kansas community with a human population of approximately 20,000.

MATERIALS AND METHODS

Soil sampling and culturing

Soil sampling and culturing was conducted as described by Zwenger et al.^[12] Briefly, surface soil samples were collected during September 2006 from each site in sterile 50 mL screw cap polypropylene centrifuge tubes then transported back to the laboratory for culturing. Approximately four grams of soil were added to 10 mL of sterile phosphate buffered saline (PBS) in a sterile 15 mL screw cap polypropylene centrifuge tube. A slurry was produced by vigorously vortexing the soil and PBS mixture for 30 seconds. The slurry was allowed to settle for 10 minutes. Following settling, 100 μ L of the supernatant was plated, using a sterile glass L-rod, on Mueller-Hinton agar (Difco Laboratories, Detroit, Michigan) infused with triclosan at a concentration of 0.15% (w/v). Triplicates of all samples were plated. Plates were incubated under aerobic conditions at 37° C overnight. Resultant colonies were subcultured on Mueller-Hinton agar supplemented with 0.15% triclosan until pure cultures were obtained. Control organisms *Escherichia coli* ATCC 33625 and *Staphylococcus aureus* ATCC 25923 were used to verify triclosan activity in the growth media.

Bacterial Identification

Pure cultures of triclosan-resistant bacteria, grown on tryptic soy agar plates, were submitted to MIDI Labs (Newark, Delaware) for identification based on sequencing analysis of approximately the first 500 bases of the 16S rRNA gene.

Antibiotic resistance

In order to further distinguish between triclosan-resistant bacterial strains isolated from environmental samples, antibiotic resistance against two common broad spectrum antibiotics, ampicillin and cefoxitin, was determined. Antibiotic resistance against these two drugs was determined by the Kirby-Bauer disk diffusion assay, following Clinical and Laboratory Standards Institute guidelines.^[13]

RESULTS AND DISCUSSION

Cattle feedlot samples

In the feedlot samples a total of ten triclosan-resistant organisms were isolated and identified (Table 1). Neither of the control organisms showed any growth on the Mueller-Hinton agar supplemented with 0.15% triclosan. Four isolates were found in samples from Feedlot 1 (~50 head of cattle). Two of these were very similar strains of *Rahnella aquatilis*. The other two were *Pseudomonas straminea* and a *Stenotrophomonas* species. Three isolates were found in samples from Feedlot 2 (~12,500 head). Two were similar strains of *Providencia rettgeri* and one was *Enterobacter* species. Feedlot 4 (~1,000 head) had one isolate, a *Stenotrophomonas* species, and Feedlot 5 (~70,000 head) also had one isolate, *Pseudomonas straminea*. Feedlot 9 (~50 head) had one isolate, which was a *Shigella* species. In cases where two strains of the same organism were isolated from a single site, for instance the two *Rahnella aquatilis* samples from Feedlot 1, the Kirby-Bauer assay using cefoxitin and ampicillin was utilized to determine that they were indeed different strains, rather than duplicates of the same strain (Table 1).

Residential samples

From the residential soil samples, a total of six different triclosan-resistant bacterial strains were isolated. With the exception of the *Achromobacter xylosoxidans-denitrificans* from site 2, all isolates were members of genus *Pseudomonas* (Table 2). Site 1 contained *Pseudomonas asplenii/putida*, Sites 3 and 6 both contained *Pseudomonas straminea*, while Sites 4 and 5 contained *Pseudomonas aurantiaca* and *Pseudomonas aeruginosa*, respectively (Table 2).

Bacteria isolated

All the triclosan resistant isolates from both feedlot and residential sites were Gram-negative bacilli. This result is not surprising since triclosan exerts the greatest effect on Gram-positive bacteria. In addition, a high number of Gram-negative bacilli were expected in the feedlot samples due to the high fecal content of these soils. Due to the ability of the outer membrane to act as a barrier, many gram-negative bacteria possess intrinsic resistance to various antiseptics and disinfectants.^[3]

In the feedlot samples, it was somewhat surprising to isolate *Rahnella aquatilis*, as this organism's natural habitat is water. The original eleven samples of the organism were isolated from water samples in France.^[14] This bacterium is very rarely isolated in clinical specimens from humans, however when it is, it is usually from patients that are immunocompromised.^[15] There have been instances where the organism has been isolated from immunocompetent patients. In one such rare case, an individual was infected by treating himself with an intravenous vitamin solution prepared by a non-licensed pharmacist in a private drugstore in Korea.^[16] On the other hand, the isolation of triclosan-resistant *Pseudomonas straminea* from both feedlot and residential samples was not surprising. This organism was first isolated and described by Iizuka and Komagata from samples taken from Japanese rice paddies in 1963.^[17] Members of genus *Pseudomonas*, particularly

Pseudomonas aeruginosa, are well known for exhibiting intrinsic resistance to many different antimicrobial agents. In *Pseudomonas aeruginosa* the resistance to antimicrobial agents was originally attributed to the impermeability of its outer membrane. It is now known that the organism, as well as other members of genus *Pseudomonas*, constitutively expresses a number of efflux pumps that confer multiple drug resistance.^[18] There are no reports in the current literature of *Pseudomonas straminea* causing a human infection. In addition to *Pseudomonas straminea* found in both feedlot and residential soil samples, three other triclosan-resistant members of this genus were isolated only from the residential samples. These organisms were *Pseudomonas asplenii/putida*, *Pseudomonas aurantiaca*, and *Pseudomonas aeruginosa*. In a report from Meade et al,^[11] it was reported that *Pseudomonas putida* has the ability to inactivate triclosan in both liquid and solid substrates. In this same research, the investigators also found that *Alcaligenes xylosoxidans* subspecies *denitrificans* was able to inactivate triclosan in both liquid and solid substrates.^[11] This is particularly interesting since the genus currently known as *Achromobacter* was formerly named *Alcaligenes*. Therefore, it is not surprising that *Achromobacter xylosoxidans-denitrificans* should be present in the residential soil samples. Both *Pseudomonas putida* and *Achromobacter xylosoxidansdenitrificans* (again formerly *Alcaligenes xylosoxidans* subspecies *denitrificans*) were shown to be capable of growth on media which contained triclosan as the sole carbon source, indicating that the inactivation of triclosan is due to metabolic degradation of the agent.^[11] Human infections caused by *Achromobacter xylosoxidans* are relatively rare, although can be serious in immunocompromised individuals. Many strains that do cause human infection are multi-drug resistant, however can usually be treated with third generation cephalosporins, imipenem and trimethoprim-sulfamethoxazole, which are also used for severe *Pseudomonas* infections.^[19] Another common soil bacterium, *Pseudomonas aurantiaca*, was isolated from the residential soil samples. This organism is not considered a human pathogen, but is the subject of much research in the area of plant pathology. In work by Mandryk et al^[20], the strain *Pseudomonas aurantiaca* S-1 was shown to produce several antimicrobial compounds active against phytopathogens such as *Fusarium oxysporum* and *Pseudomonas syringae*. In field tests, a related strain, *Pseudomonas aurantiaca* SR1, was shown to colonize the roots and promote the growth of both wheat and maize, allowing less fertilizer to be used on these crops.^[21]

Two of the isolates from feedlot soils were shown to be undetermined members of genus *Stenotrophomonas*. The isolation of these organisms from soil samples is not surprising since they are widely found in the environment, especially among soils and plants. Of the eight described members of the genus *Stenotrophomonas*, only *Stenotrophomonas maltophilia* is recognized as a human pathogen, which is interesting since it is usually the dominant member of the genus isolated from the soil.^[22] Since *Stenotrophomonas maltophilia* is common in the rhizosphere, multiple antibiotic resistance is a useful adaptation for competition in this environment. It has been shown that numerous clinical and environmental endophytic isolates possess a number of efflux pumps for antibiotic resistance.^[22] In work by Sanchez et al,^[23] it was demonstrated that triclosan selects

Stenotrophomonas maltophilia mutants that overproduce the multidrug efflux pump SmeDEF.

An organism we isolated twice from the same feedlot was *Providencia rettgeri*. Since these two organisms exhibited differences in antibiotic resistance, they are taken to be two separate strains of *Providencia rettgeri*. Members of genus *Providencia* are commonly associated with nosocomial infections, with *Providencia stuartii* being the most common. *Providencia rettgeri* is commonly associated with infections of the urinary tract, although not as commonly as *Providencia stuartii*. It has been shown that *Providencia rettgeri* is widely distributed in the environment, as it can be isolated from poultry, the feces of reptiles and amphibians, and from surface waters.^[24] In work done by Williams and Stickler,^[25] it was shown that a clinical isolate of *Providencia rettgeri* from a patient with a long-term urinary catheter exhibited increased resistance to triclosan when it was used in a model of a catheterized bladder.

Triclosan resistant strains of *Enterobacter cloacae* and a member of *Shigella* were also isolated from the feedlot soils. Like many other Gram-negative bacteria, members of both *Enterobacter* and *Shigella* genera have been shown to harbor numerous efflux mechanisms.^{[26][27][28]} It has been shown that *Enterobacter cloacae*, a known human pathogen, harbors the gene for AcrB, a member of the RND group of efflux pumps.^[26] Other Gram-negative rods that harbor the RND efflux systems, such as *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Campylobacter jejuni*, *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Stenotrophomonas maltophilia* have the ability to efflux triclosan molecules.^[27] However, it has not been definitively demonstrated that the RND efflux systems found in *Enterobacter cloacae* have this capability. The efflux components AcrA, AcrB and TolC have also been identified in *Shigella flexneri*, another human pathogen.^[26] It is not known whether these systems are present in all members of genus *Shigella*, but if so could account for the triclosan resistance seen in the *Shigella* sp. organism isolated from feedlot soils in this study.

A significant concern raised by the overuse of triclosan in commercial products is that bacteria which become resistant to triclosan will also become resistant to antibiotics. There is evidence that indicates this concern is valid. In work done by Braoudaki and Hilton^[29], it was shown that *Escherichia coli* O157 strains that were repeatedly passaged in media containing increasing concentrations triclosan were able to develop resistance to high levels of the antibiotics amoxicillin-clavulanic acid, amoxicillin, chloramphenicol, erythromycin, imipenem, tetracycline, and trimethoprim. These investigators also showed that *Salmonella enterica* serovar Typhimurium grown in increasing concentrations of erythromycin will develop resistance to triclosan. It is especially interesting to note that the strains of *Escherichia coli* O157 and *Salmonella enterica* that developed adaptive resistance maintained that resistance even after more than 30 passages in media that did not contain biocide or antibiotics.^[29] Conversely in other work, it was shown that triclosan-tolerant strains of certain bacteria did not have increased resistance to the antibiotics tested.^[30] In these experiments, triclosan-tolerant strains of *Escherichia coli*, *Staphylococcus aureus*, and *Acinetobacter johnsonii* were screened against a large panel of antibiotics, representing

several groups, including β -lactams, aminoglycosides, macrolides, fluoroquinolones, tetracyclines and others. Most of the triclosan-tolerant mutants showed essentially no increase in minimum inhibitory concentrations of antibiotics. Interestingly, the triclosan-tolerant *Escherichia coli* mutant showed an increased susceptibility to the aminoglycoside antibiotics, including gentamicin, amikacin, framycetin, streptomycin, and kanamycin. It is hypothesized that in these cells the increased tolerance to triclosan caused a loss of plasmid DNA, and concomitant loss of the ability to produce aminoglycoside-modifying enzymes.^[30]

It is clear from this study that microorganisms resistant to triclosan can be isolated from common environmental sites. Many of these organisms seem to possess an intrinsic resistance to triclosan, although we did not determine the mechanisms used by the organisms we isolated. One concern raised by the isolation of these organisms is that they may have the capability to confer triclosan-resistance, via horizontal gene transfer, to organisms that are currently sensitive to triclosan.

Acknowledgments

The authors thank Sam Zwenger for collecting feedlot soil samples and Shingo Ishihara for expert laboratory assistance. This project was supported by the Kansas Idea Network for Biomedical Research Excellence Grant NIH P20RR016475 from the K-INBRE program of National Center for Research Success.

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Figure 1. Structural formula of the antimicrobial agent triclosan, (2,4,4'-trichloro-2'-hydroxydiphenyl ether).

Table 1

Results from soil samples collected at cattle feedlots. Strain identification was determined by partial 16S rRNA gene sequencing by MIDI Labs. Kirby-Bauer values for cefoxitin 30 µg (FOX30) and ampicillin 10 µg (AM10) are given as zone diameters in millimeters. The disk diameters were six millimeters, therefore values of 6 in the table indicate there was no zone of inhibition around the disk.

Isolate	Strain Identification	FOX30	AM10
FL1-1	<i>Rahnella aquatilis</i>	18	14
FL1-2	<i>Rahnella aquatilis</i>	14	8
FL1-3	<i>Pseudomonas straminea</i>	6	6
FL1-5	<i>Stenotrophomonas sp.</i>	10	6
FL2-1	<i>Providencia rettgeri</i>	16	12
FL2-2	<i>Enterobacter cloacae</i>	20	16
FL2-3	<i>Providencia rettgeri</i>	18	16
FL4-2	<i>Stenotrophomonas sp.</i>	6	10
FL5-1	<i>Pseudomonas straminea</i>	6	8
FL9-1	<i>Shigella sp.</i>	6	10

Table 2

Results from soil samples collected at residential sites. Strain identification was determined by partial 16S rRNA gene sequencing by MIDI Labs. Kirby-Bauer values for cefoxitin 30 µg (FOX30) and ampicillin 10 µg (AM10) are given as zone diameters in 368 millimeters. The disk diameters were six millimeters, therefore values of 6 in the table indicate there was no zone of inhibition around the disk

Isolate	Strain Identification	FOX30	AM10
NTWH	<i>Pseudomonas asplenii/putida</i>	20	14
WTWH	<i>Achromobacter xylosoxidans-denitrificans</i>	6	9
FPT9	<i>Pseudomonas straminea</i>	6	6
BPP	<i>Pseudomonas aurantiaca</i>	12	6
W27th	<i>Pseudomonas aeruginosa</i>	6	6
FHSULib	<i>Pseudomonas straminea</i>	6	6