INFLUENCE OF METHYLATION ON THE ANTIBACTERIAL PROPERTIES OF TRICLOSAN IN *PASTEURELLA MULTOCIDA* AND *PSEUDOMONAS AERUGINOSA* VARIANT STRAINS

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2009

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 2011

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ACKNOWLEDGMENTS

Thank you to my advisor, Dr. Franklin R. Champlin, for allowing me to perform research in your laboratory and providing me with direction, knowledge, and advice.

Thank you to my committee members, Dr. Gerwald A. Kohler and Dr. Wendy L. Picking, for serving as sources of insight and helping to guide me in my graduate studies.

Thank you to Ciba Specialty Chemicals Corp. for providing triclosan (Irgasan DP 300).

This work was partially supported by Grant No. P2PRR016478 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH). Publication contents are solely the responsibility of the author and do not necessarily represent the official views of the NCRR or NIH.

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CHAPTER I

INTRODUCTION

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether) is a hydrophobic antimicrobial agent frequently incorporated into household and personal care products for its biocidal properties. While it likely exerts multiple mechanisms of action, the compound has been shown to specifically inhibit enoyl ACP-reductase (FabI) (McMurry *et al.*, 1998), an enzyme essential for fatty acid biosynthesis in bacteria. It exhibits an extremely broad antibacterial spectrum against disparate bacterial pathogens with the notable exception of *Pseudomonas aeruginosa* (Champlin *et al.*, 2005; Ellison *et al.*, 2007; Ellison and Champlin, 2007). This gram-negative organism is environmentally ubiquitous and a common opportunistic cause of nosocomial infections in chronically ill and immunocompromised patients. *P. aeruginosa* is intrinsically resistant to extremely high concentrations of triclosan due to multiple mechanisms (Hoang and Schweizer, 1999; Heath and Rock, 2000; Mima *et al.*, 2007) including exclusion by an outer membrane which is impermeable to nonpolar compounds (Champlin *et al.*, 2005). More recent work has revealed the transitory nature of *P. aeruginosa* sensitization to triclosan by the synergistic outer membrane permeabilizer compound 48/80 is likely due to a compensatory function of triclosan-recognizing efflux pumps rather than obviation of a hydrophobic diffusion pathway (Ellison *et al.*, 2007).

Previous work in this laboratory has shown the opportunistic pathogen *Pasteurella multocida* to be highly susceptible to triclosan despite its ultrastructurally typical gram-negative cell envelope (Champlin *et al.*, 2005). Subsequent research (Ellison and Champlin, 2007) determined such growth inhibition to be either bacteriostatic or bactericidal in a dose-dependent manner. Enhanced uptake of the hydrophobic probe 1-N-phenylnaphthylamine relative to the refractory control organisms *P. aeruginosa* and *Escherichia coli* suggested the presence of regions of surface-exposed phospholipid bilayer in the *P. multocida* outer membrane. It was concluded that the extreme susceptibility of *P. multocida* to triclosan (Champlin *et al.*, 2005) as well as other nonpolar antimicrobial agents (Hart and Champlin, 1988) is due to the general inability of the outer membrane to exclude hydrophobic molecules. Low triclosan concentrations which inhibited growth of untreated *P. multocida* cultures were only effective for *P. aeruginosa* when rendered susceptible using outer membrane-permeabilizing concentrations of polymyxin B-nonapeptide, compound 48/80, or ethylenediaminetetraacetate (Champlin *et al.*, 2005).

Because it is both a highly efficacious and safe antiseptic, triclosan is widely incorporated into hospital health care products. Protocols for surgical handwashing involving wash scrubs containing triclosan have been evaluated (Pereira *et al.*, 1997), and studies have found personnel handwashes formulated with triclosan to be mild yet effective, making such products ideal for general and high-risk areas within the healthcare setting (Zafar *et al.*, 1995; Jones *et al.*, 2000). A case study in which patient bathing solutions containing triclosan were used in a neonatal nursery to eradicate a methicillin-resistant *Staphylococcus aureus* outbreak was reported by Zafar *et al.* (1995). Triclosan has also been incorporated into products such as gloves (Mixon, 1999), sutures (Edmiston *et al.*, 2006; Fleck *et al.*, 2007), and surgical drapes (Microtek Medical, Inc, 2002) to reduce the risk of post-surgical infection.

It has become generally accepted that the frequent use of triclosan in health and personal care articles has resulted in its accumulation to demonstrable levels in human tissue fluids

(Allmyr *et al.*, 2006) and the environment (Líndström *et al.*, 2002; Bester, 2005; DeLorenzo *et al.*, 2008) to include hospital effluent (Gómez *et al.*, 2007). One additional fate of triclosan has been postulated to be its catalytic methylation potentiated by microorganisms in various environments (Bester, 2005; DeLorenzo *et al.*, 2008; Leiker *et al.*, 2009). As a result, methyl triclosan has concomitantly been reported to be increasingly present in disparate environmental systems such as surface waters (Líndström *et al.*, 2002; Bester, 2005; DeLorenzo *et al.*, 2008; Leiker *et al.*, 2009) and sewage (Líndström *et al.*, 2002; Bester, 2005; Farré, 2008), as well as biological systems (Leiker *et al.*, 2009; Balmer *et al.*, 2004). Líndström *et al.* (2002) described the presence of triclosan in lakes and a river in Switzerland and reported that triclosan and its methyl derivative were only found in the lakes studied which had anthropogenic sources, while both were absent in a remote mountain lake. This finding supports the notion that environmental triclosan accumulation is due to frequent incorporation into products for human use and that methyl triclosan results as consequence of biological modification. Bester (2005) reported the presence of triclosan in surface waters was the result of sewage treatment plant effluent, and also suggested that environmental methyl triclosan is a result of microorganism-catalyzed triclosan transformation. In an effort to determine the toxicity of triclosan in estuarine systems, DeLorenzo *et al.* (2008) examined adult shrimp in order to assess bioaccumulation. After a 14-day exposure to triclosan, investigators observed the accumulation of methyl triclosan, thereby suggesting formation of this triclosan metabolite in the seawater environment. Methyl triclosan was also found by Balmer *et al.* (2004) and Leiker *et al.* (2009) to be present in surface waters and to be accumulating in fish tissues. Both DeLorenzo *et al.* (2008) and Leiker *et al.* (2009) have suggested based on their work that the consequences of the accumulation of environmental metabolites of triclosan warrant further research.

The triclosan minimal inhibitory concentration for *P. aeruginosa* far exceeds its solubility in aqueous media (Champlin *et al.*, 2005). It was therefore necessary to determine the effect of

methylation on triclosan efficacy using susceptible organisms such as *P. multocida* for bioassay purposes. Wild-type *P. aeruginosa* K799, mutant *P. aeruginosa* Z61 (a genetic variant possessing a permeable outer membrane phenotype) (Nikaido and Vaara, 1985; Zimmermann, 1979), and outer membrane permeabilized wild-type *P. aeruginosa* PA01 were also examined in order to assess the possible involvement of outer membrane exclusion (Champlin *et al.*, 2005) in the mechanism underlying the relationship between methyl triclosan and the gram-negative cell surface.

CHAPTER II

METHODOLOGY

Bacterial Strains, Maintenance, and Cultivation

P. multocida ATCC 11039, *Serratia marcescens* ATCC 8100, *E. coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Burkholderia multivorans* ATCC BAA-247, *P. aeruginosa* Z61 (ATCC 35151), *Bacilus subtilus* ATCC 6633, and *Staphylococcus aureus* ATCC 25923 were purchased from the American Type Culture Collection (Manassas, VA). *P. multocida* ATCC 11039/iso is a capsule-negative variant derived from *P. multocida* ATCC 11039 (Champlin *et al*, 1999), *P. multocida* P-1581 was obtained from the USDA National Animal Disease Center (Ames, IA), *P. aeruginosa* PA1211 was provided by Dr. A.M. Zelazny of the National Institute of Allergy and Infectious Diseases (NIH, Bethesda, MD), *P. aeruginosa* K799 was provided by Dr. R.E.W. Hancock (University of British Columbia, Vancouver, Canada), *P. aeruginosa* PA01 is maintained in this laboratory as a reference organism, and *S. aureus* T-5706 was provided by Dr. Frank Austin (Mississippi State University College of Veterinary Medicine). All bacteria are maintained as cryopreserved stock cultures as described previously (Darnell *et al.*, 1987) and cultivated on Mueller Hinton Agar (MHA; Difco Laboratories, Detroit, MI) plates for 18 h at 37°C to provide working culture sources for inocula. Starter and test cultures were prepared using Mueller Hinton Broth (MHB; Difco Laboratories) or MHA and incubated at 37°C

under conditions described previously (Darnell *et al.*, 1987; Champlin *et al.*, 2005; Ellison *et al.*, 2007; Ellison and Champlin, 2007).

Chemicals

Ethanol (95% denatured alcohol; Fisherbrand, Pittsburgh, PA) was employed for control and triclosan solubilization purposes. Stock solutions of triclosan (Irgasan DP 300; Ciba Specialty Chemicals Corp., High Point, NC) and methyl triclosan (Triclosan-methyl; Sigma-Aldrich Chemical Co., St. Louis, MO) were prepared to desired concentrations in ethanol and stored tightly capped at 4ºC until needed.

Disk Agar Diffusion Bioassay

Triclosan is not soluble in aqueous media at concentrations greater than 32 µg/ml, a value far less than its minimal inhibitory concentration for *P. aeruginosa* (Champlin *et al.*, 2005; Ellison *et al.*, 2007). Therefore, the effect of triclosan and methyl triclosan on the ability to initiate growth was determined using a modification of a standardized disk agar diffusion bioassay (Hart and Champlin, 1988). Test cultures were obtained by inoculating culture tubes (18 x 150 mm, Kimax) each containing 5.0 ml of MHB with stationary-phase starter culture cells to an initial optical density at 620 nm (OD_{620}) of 0.025 (Spectronic 20 optical spectrophotometer; Thermo Electron Corp., Madison, WI) and incubating under standard conditions at 37°C with rotary aeration at 180 rpm (Excella E24 benchtop incubator shaker; New Brunswick Scientific Co., Inc, Edison, NJ) until an OD_{620} of 0.10 was reached. Standard (100 x 15 mm) or large (150 x 15 mm) MHA plates were seeded by aseptically transferring 150 or 250 µl, respectively, of appropriate test culture to the center of each plate. A cotton swab saturated with sterile MHB was then used to spread inoculate each plate in a uniform manner. Sterile paper disks (6.0-mm

diameter; Becton, Dickinson and Co., Sparks Glencoe, MD) were impregnated with different concentrations of triclosan or methyl triclosan dissolved in ethanol (95%) or ethanol (95%) alone (control), air-dried overnight under flowing sterile air, and aseptically placed on seeded agar plates. Plates were placed at 4°C for one hour prior to incubation at 37°C for 18 h, after which zones of growth inhibition surrounding each disk were measured with the aid of electronic calipers.

Turbidimetric Growth Kinetics Bioassay

The effect of triclosan and methyl triclosan on total cell density growth kinetics was assessed turbidimetrically using a slight modification of the method described by Champlin *et al.*, 2005 and Ellison and Champlin, 2007. Stationary-phase cells from overnight starter cultures were employed to inoculate sterile MHB to an initial OD_{620} of 0.025 (Spectronic 20 optical spectrophotometer). Test cultures were then prepared by dispensing 5.0-ml aliquots into each culture tube (18.0-mm diameter, Kimax) and immediately incubating. Growth was assessed by monitoring OD_{620} at 30-minute intervals for 6.0 h. Biocide treatments were added post-inoculation at 1.5 h during early exponential phase by aseptically pipetting triclosan or methyl triclosan to final concentrations of 0.2 or 2.0 µg/ml. OD_{620} measurements for each test culture were plotted against time on semi-logarithmic graph paper in order to visualize the effects of treatment on culture growth.

P. aeruginosa Outer Membrane Permeabilization

The ability of compound 48/80 to synergistically inhibit cultural growth in the presence of either triclosan or methyl triclosan by outer membrane permeabilization (Champlin *et al.*, 2005; Vaara, 1992) was evaluated using turbidimetric assessments of batch cultural growth

kinetics. A previously described protocol of (Champlin *et al.*, 2005) with slight modifications as described above was employed to comprise a total cell density growth kinetics bioassay with the addition of compound 48/80 to the treatments at 1.5 h post-inoculation such that the final triclosan or methyl triclosan concentration was 2.0 µg/ml and the final compound 48/80 concentration was 5.0 µg/ml in indicated test cultures.

CHAPTER III

FINDINGS

Previous reports from this laboratory have revealed that *P. multocida* is markedly susceptible to the hydrophobic biocide triclosan (Champlin *et al.*, 2005; Ellison and Champlin, 2007), despite exhibiting typically gram-negative cell envelope ultrastructure. In contrast, *P. aeruginosa* possesses intrinsic resistance to extremely high concentrations of the compound (Champlin *et al.*, 2005; Hoang and Schweizer, 1999; Heath and Rock, 2000; Mima *et al.*, 2007). The present study was undertaken to determine the influence of methylation on the antibacterial properties of triclosan in both triclosan susceptible and resistant organisms in order to better understand the environmental ramifications of the stable triclosan biotransformation product methyl triclosan. It has been published in the *Journal of Hospital Infection* (Clayborn *et al.*, 2011).

In vitro Susceptibility to Triclosan and Methyl Triclosan

A disk agar diffusion bioassay (Figure 1) was employed to determine the relative abilities of triclosan and methyl triclosan to inhibit the initiation of test organism growth under *in vitro* conditions. All *P. multocida* strains examined were susceptible to triclosan at concentrations as low as 0.02 µg/disk regardless of host source or degree of capsulation (Figure 1, Table 1). Two genetically-unrelated wild-type *P. aeruginosa* strains were resistant to triclosan at all concentrations employed. *B. multivorans* and *S. marcescens* were least susceptible to triclosan

with growth only slightly inhibited by as much as 200 µg/disk, while *E. coli* and *S. typhimurium* exhibited intermediate levels of susceptibility. Methyl triclosan failed to inhibit growth of any of the organisms examined at concentrations ranging from 0.02 to 200 µg/disk (Figure 1, Table 2).

Gram-positive bacteria possess no outer membrane and are highly susceptible to hydrophobic triclosan (Microtek-Medical, Inc, 2002). To provide an assessment of a possible role for the outer membrane in the inability of methyl triclosan to inhibit growth in gram-negative bacteria, the disk agar diffusion bioassay was also employed to determine the susceptibility of three gram-positive organisms to either triclosan or methyl triclosan at concentrations of 0.02, 0.2, and 2.0 µg/disk of each. *B. subtilus* ATCC 6633, *S. aureus* T-5706, and *S. aureus* ATCC 25923 were seen to be susceptible to triclosan in a dose-dependent manner, while methyl triclosan caused no inhibition of growth (Table 3) in a manner consistent with that seen for gram-negative bacteria (Table 1, Table 2).

Effect of Triclosan and Methyl Triclosan on Growth Kinetics

To eliminate the possibility that variation in compound diffusibility in the disk agar diffusion bioassay was responsible for the disparate effects observed for triclosan and methyl triclosan, turbidimetric measurements of total cell densities of the treated *P. multocida* and *P. aeruginosa* type strains were employed. As can be seen in Figure 2, *P. multocida* ATCC 11039 was susceptible to triclosan in a concentration-dependent manner, while *P. aeruginosa* PA01 was resistant to triclosan at the same concentrations (0.2 and 2.0 µg/ml). Neither organism was susceptible to methyl triclosan at either 0.2 or 2.0 µg/ml. The apparent inability of methylated triclosan to inhibit bacterial growth (Table 2) was confirmed by these data.

Outer Membrane Permeabilization of *P. aeruginosa*

The role of outer membrane impermeability in triclosan resistance has been established previously by chemical permeabilization of intrinsically resistant *P. aeruginosa* cells (Champlin

et al., 2005; Ellison *et al.*, 2007). This conclusion is supported by the inherent susceptibility seen in intact *P. multocida* cells which possess an outer cell envelope intrinsically permeable to nonpolar molecules (Ellison and Champlin, 2007) Another disk agar diffusion bioassay protocol in which a wild-type *P. aeruginosa* parental strain and a mutant exhibiting a permeable outer membrane phenotype was performed to investigate the potential dependency of methyl triclosan resistance on outer membrane permeability. Data presented in Table 4 reveal that wild-type *P. aeruginosa* K799 was resistant to triclosan concentrations ranging over two orders of magnitude, while outer membrane permeability mutant *P. aeruginosa* Z61 was susceptible to triclosan in a concentration-dependent manner. However, both wild-type and mutant strains were resistant to methyl triclosan at all concentrations employed.

Similarly, using outer membrane permeabilizer compound 48/80 to chemically disrupt the *P. aeruginosa* PA01 outer cell envelope with regard to its impermeability properties for nonpolar antimicrobial agents failed to confer susceptibility to methyl triclosan at concentrations as high as 2.0 µg/ml (Figure 3). This is in stark contrast with the demonstration that compound 48/80 sensitized *P. aeruginosa* to the same concentration of triclosan in an initially bactericidal manner.

Table 1. Susceptibility of gram-negative test organisms to triclosan.

Organism	Inhibition zone diameter[a] (mm) ± SD					
	Control[b]	0.02 μg	0.2 μg	2.0 μg	20 μg	200 μg
P. multocida ATCC 11039	0.0 ± 0.0	7.3 ± 1.1	20.2 ± 1.7	35.7 ± 3.2	46.0 ± 1.3	48.6 ± 1.2
P. multocida ATCC 11039/iso	0.0 ± 0.0	4.6 ± 0.7	12.3 ± 1.0	22.9 ± 1.7	32.5 ± 1.7	36.1 ± 1.9
P. multocida P-1581	0.0 ± 0.0	4.7 ± 1.0	18.9 ± 1.4	35.2 ± 1.7	46.5 ± 1.2	47.7 ± 1.3
P. aeruginosa PA01	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P. aeruginosa PA1211	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
S. marcescens ATCC 8100	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 0.2
E. coli ATCC 25922	0.0 ± 0.0	0.0 ± 0.0	4.0 ± 0.6	16.3 ± 0.6	25.3 ± 0.2	26.9 ± 0.4
S. typhimurium ATCC 14028	0.0 ± 0.0	0.0 ± 0.0	2.3 ± 0.1	13.4 ± 0.3	21.2 ± 0.2	23.4 ± 0.4
B. multivorans ATCC BAA-247	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.2	2.1 ± 0.2

[a]Diameter of growth inhibition zone minus disk diameter (6.0 mm); each value represents the mean of three to five independent determinations ± standard deviation.

[b]Ethanol (95%) was used to facilitate triclosan solubilization, therefore control disks were prepared by impregnating with solvent alone and allowing to air dry prior to plate application.

Table 2. Susceptibility of gram-negative test organisms to methyl triclosan.

Organism	Inhibition zone diameter[a] (mm) ± SD					
	Control[b]	0.02 μg	0.2 μg	2.0 μg	20 μg	200 μg
P. multocida ATCC 11039	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P. multocida ATCC 11039/iso	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P. multocida P-1581	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P. aeruginosa PA01	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P. aeruginosa PA1211	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
S. marcescens ATCC 8100	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
E. coli ATCC 25922	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
S. typhimurium ATCC 14028	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
B. multivorans ATCC BAA-247	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

[a]See Table 1.

[b]See Table 1.

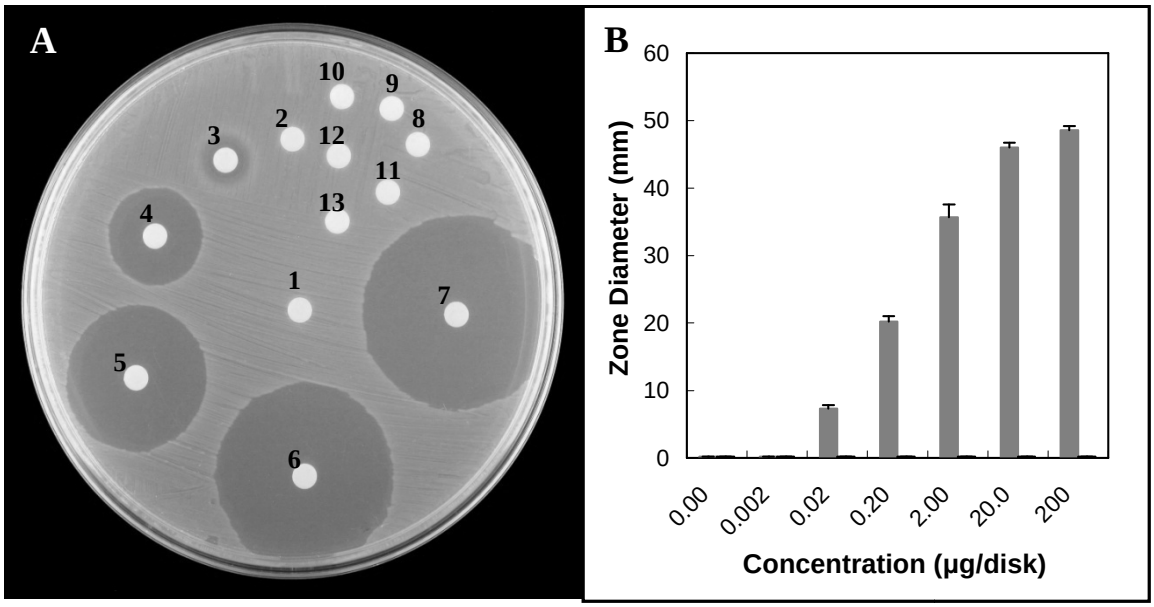

Figure 1. Representative disk agar diffusion bioassay for *P. multocida* ATCC 11039. (A) Disks: 1, control (95% Ethanol); 2, 0.002 µg TCS; 3, 0.02 µg TCS; 4, 0.2 µg TCS; 5, 2.0 µg TCS; 6, 20 µg TCS; 7, 200 µg TCS; 8, 0.002 µg MeTCS; 9,0.02 µg MeTCS; 10, 0.2 µg MeTCS; 11, 2.0 µg MeTCS; 12, 20 µg MeTCS; 13, 200 µg MeTCS. (B) Susceptibility to TCS (■) and MeTCS (■). Diameter of growth inhibition zone minus disk diameter (6.0 mm); each value represents the mean of three to five independent determinations plus standard error.

Table 3. Susceptibility of gram-positive test organisms to triclosan or methyl triclosan.

Organism	Inhibition zone diameter[a] (mm) ± SD						
	Control[b]	TCS (μg)			MeTCS (μg)		
		0.02	0.2	2.0	0.02	0.2	2.0
B. subtilus ATCC 6633	0.0 ± 0.0	0.0 ± 0.0	3.0 ± 0.13	13.2 ± 0.21	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
S. aureus T-5701	0.0 ± 0.0	2.9 ± 0.16	21.1 ± 0.41	36.3 ± 0.91	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
S. aureus ATCC 25923	0.0 ± 0.0	6.4 ± 0.26	22.4 ± 0.14	38.6 ± 0.17	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

[a]See Table 1; each value represents the mean of three independent determinations ± standard deviation.

[b]See Table 1.

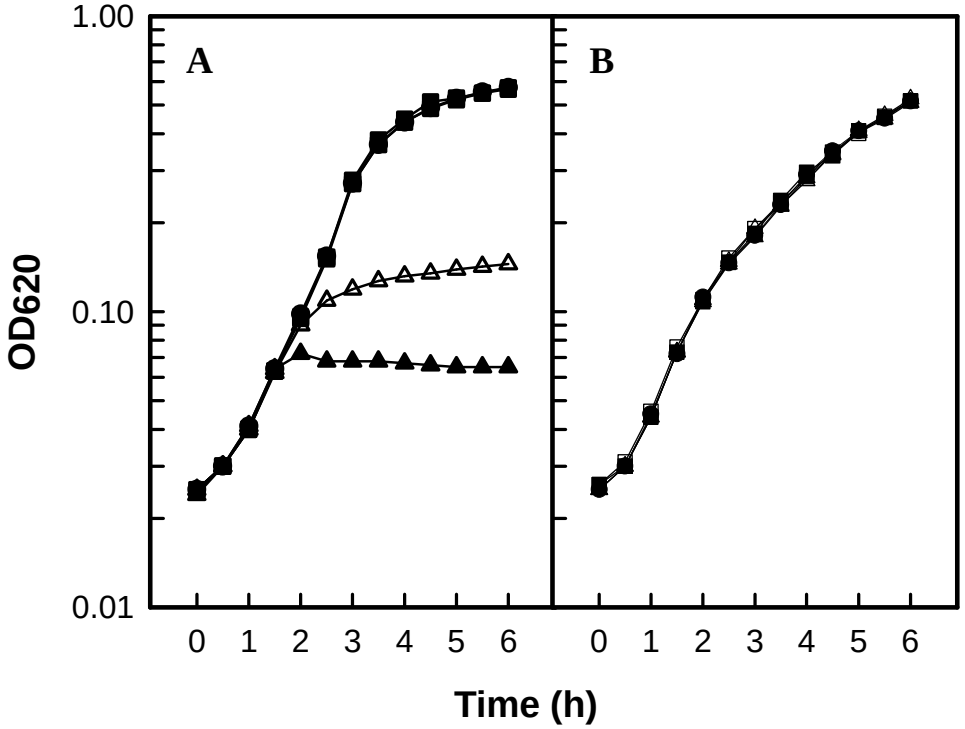

Figure 2. Total cell density growth kinetics of *P. multocida* ATCC 11039 (A) and *P. aeruginosa* PA01 (B) in the presence of either TCS or MeTCS. Each value represents the mean of three to four independent determinations. Symbols: ●, ethanol (0.4%, control); △, TCS (0.2 µg/ml); ▲, TCS (2.0 µg/ml); □, MeTCS (0.2 µg/ml); ■, MeTCS (2.0 µg/ml).

Table 4. Susceptibility of *P. aeruginosa* permeability variants to triclosan or methyl triclosan.

Strain	Inhibition zone diameter[a] (mm) ± SD						
	Control[b]	TCS (μg)			MeTCS (μg)		
		2.0	20	200	2.0	20	200
P. aeruginosa K799 (wild-type)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P. aeruginosa Z61 (permeable mutant)	0.0 ± 0.0	0.0 ± 0.0	2.3 ± 0.3	5.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

[a]See Table 1; each value represents the mean of three independent determinations ± standard deviation.

[b]See Table 1.

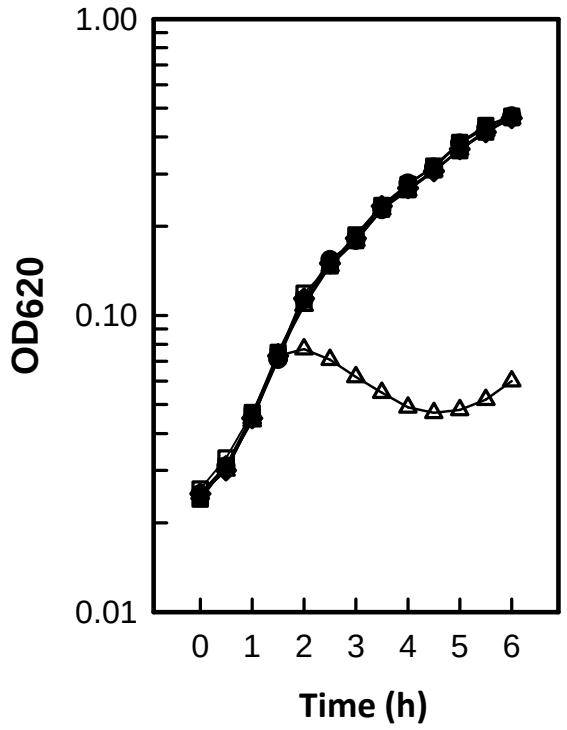

Figure 3. Total cell density growth kinetics of outer membrane-permeabilized *P. aeruginosa* PA01 in the presence of either TCS or MeTCS. Each value represents the mean of three independent determinations. Symbols: ●, ethanol (0.4%, control); ◆, compound 48/80 (5.0 µg/ml); ▲, TCS (2.0 µg/ml); ■, MeTCS (2.0 µg/ml); △, compound 48/80 (5.0 µg/ml) plus TCS (2.0 µg/ml); □, compound 48/80 (5.0 µg/ml) plus MeTCS (2.0 µg/ml).

CHAPTER IV

CONCLUSIONS

These data support the notion that methylation of triclosan renders the compound unable to inhibit growth of disparate gram-negative and gram-positive bacterial pathogens exhibiting variant cell envelope permeability phenotypes in a manner independent of an intact outer membrane. This finding is underscored by the fact *P. multocida* cell surface variant strains were seen to be resistant to methyl triclosan despite possessing an outer cell envelope that is remarkably permeable to nonpolar antimicrobial agents (Hart and Champlin, 1988). That gram-positive organisms exhibit no outer membrane, yet remain resistant to methyl triclosan validates this finding.

Meade *et al*.(2001) isolated two bacterial soil isolates which are able to employ triclosan as their sole carbon source in chemically-defined growth media, thereby explaining their ability to inactivate the biocide in both batch culture and impregnated plastic. Biotransformation of triclosan by methylation in the environment represents a means by which the efficacy of the biocide would be decreased. Obviation of the bactericidal properties of triclosan in such a manner might also be expected to mitigate potential selection of triclosan resistance due to alteration of the FabI enzyme (Heath *et al*., 1999) or the acquisition of adaptive cross-resistance to other antimicrobial compounds (Braoudaki and Hilton, 2004) as possible consequences of the presence of the compound in the human wastestream. Moreover, it can also be concluded that biocide modification represents a potential mechanism contributing to the high levels of intrinsic triclosan resistance seen in *P. aeruginosa*.

The value of the work summarized herein lies in the acquisition of a better understanding of biocide modification, namely microorganism-catalyzed methylation, and the ability to inactivate the growth-inhibitory properties of a compound known to exhibit broad spectrum antibacterial activity. Implications of the study also include the notion that triclosan resistance in *P. aeruginosa* may in part be due to its ability to catalyze such modifications. One important future extension of this work should involve an evaluation of the effects of triclosan on the morphology and ultrastructure of these test organisms. A comparative analysis with methyl triclosan should be performed to determine if, despite its inability to inhibit growth, it confers morphological or ultrastructural changes to otherwise recalcitrant cells by virtue of its hydrophobic nature. The demonstration that either or both compounds exert such effects would support the hypothesis that triclosan likely acts upon multiple mechanistic targets to include the cytoplasmic membrane and that this property is altered or obviated by methylation of the compound.

REFERENCES

1. Allmyr M., Adolfsson-Erici M., McLachlan M.S., Sandborgh-Englund G. (2006). "Triclosan in plasma and milk from Swedish nursing mothers and their exposure via personal care products." Sci Tot Environ 372: 87-93.

2. Balmer M.E., Poiger T., Droz C., *et al*. (2004). "Occurrence of methyl triclosan, a transformation product of the bactericide triclosan, in fish from various lakes in Switzerland." Environ Sci Technol 38: 390-395.

3. Bester K. (2005). "Fate of triclosan and triclosan-methyl in sewage treatment plants and surface waters." Arch Environ Contam Toxicol 49: 9-17.

4. Braoudaki M., Hilton A.C. (2004). "Adaptive resistance to biocides in *Salmonella enterica* and *Escherichia coli* O157 and cross-resistance to antimicrobial agents." J Clin Microbiol 42:73-78.

5. Champlin F.R., Ellison M.L., Bullard J.W., Conrad R.S. (2005). "Effect of outer membrane permeabilisation on intrinsic resistance to low triclosan levels in *Pseudomonas aeruginosa*." Int J Antimicrob Agents 26: 159-164.

6. Champlin F.R., Patterson C.E., Austin F.W., Ryals P.E. (1999). "Derivation of extracellular polysaccharide-deficient variants from a serotype A strain of *Pasteurella multocida*." Curr Microbiol 38: 268-272.

7. Clayborn A.B., Toofan S.N., Champlin F.R. (2011). "Influence of methylation on the antibacterial properties of triclosan in *Pasteurella multocida* and *Pseudomonas aeruginosa* variant strains." J Hosp Infect 77: 129-133.

8. Darnell K.R., Hart M.E., Champlin F.R. (1987). "Variability of cell surface hydrophobicity among *Pasteurella multocida* somatic serotype and *Actinobacillus lignieresii* strains." J Clin Microbiol 25: 67-71.

9. DeLorenzo M.E., Keller J.M., Arthur C.D., *et al*. (2008). "Toxicity of the antimicrobial compound triclosan and formation of the metabolite methyl-triclosan in estuarine systems." Environ Toxicol 23: 224-232.

10. Edmiston C.E., Seabrook G.R., Goheen M.P., *et al.* (2006). "Bacterial adherence to surgical sutures: can antibacterial-coated sutures reduce the risk of microbial contamination?" J Am Coll Surg 203: 481-489.

11. Ellison M.L., Champlin F.R. (2007). “Outer membrane permeability for nonpolar antimicrobial agents underlies extreme susceptibility of *Pasteurella multocida* to the hydrophobic biocide triclosan.” Vet Microbiol 124: 310-318.

12. Ellison M.L., Roberts A.L., Champlin F.R. (2007). “Susceptibility of compound 48/80-sensitized *Pseudomonas aeruginosa* to the hydrophobic biocide triclosan.” FEMS Microbiol Lett 269: 295-300.

13. Farré M., Asperger D., Kantiani L., González S., Petrovic M., Barceló D. (2008). “Assessment of the acute toxicity of triclosan and methyl triclosan in wastewater based on the bioluminescence inhibition of Vibrio fischeri.” Anal Bioanal Chem 390: 1999-2007.

14. Fleck T., Moidl R., Blacky A., *et al*.(2007) “Triclosan-coated sutures for the reduction of sternal wound infections: economic considerations.” Ann Thorac Surg 84: 232-236.

15. Gómez M.J., Agüera A., Mezcua M., Hurtado J., Mochilí F., Fernández-Alba A.R. (2007). “Simultaneous analysis of neutral and acidic pharmaceuticals as well as related compounds by gas chromatography-tandem mass spectrometry in wastewater.” Talanta 73: 314-320.

16. Hart M.E., Champlin F.R. (1988). “Susceptibility to hydrophobic molecules and phospholipid composition in *Pasteurella multocida* and *Actinobacillus lignieresii*.” Antimicrob Agents Chemother 32: 1354-1359.

17. Heath R.J., Rock C.O. (2000). “A triclosan-resistant bacterial enzyme.” Nature 406: 145-146.

18. Heath R.J., Rubin J.R., Holland D.R., Zhang E., Snow M.E., Rock C.O. (1999). “Mechanism of triclosan inhibition of bacterial fatty acid synthesis.” J Biol Chem 274: 11110-11114.

19. Hoang T.T., Schweizer H.P. (1999). “Characterization of *Pseudomonas aeruginosa* enoyl-acyl carrier protein reductase (FabI): a target for the antimicrobial triclosan and its role in acylated homoserine lactone synthesis.” J Bacteriol 181: 5489-5497.

20. Jones R.D., Jampani H.B., Newman J.L., Lee A.S. (2000). “Triclosan: a review of effectiveness and safety in health care settings.” Am J Infect Control 28: 184-196.

21. Leiker T.J., Abney S.R., Goodbred S.L., Rosen M.R. (2009). “Identification of methyl triclosan and halogenated analogues in male common carp (*Cyprinus carpio*) from Las Vegas Bay and semipermeable membrane devices from Las Vegas Wash, Nevada.” Sci Tot Environ 407: 2102-2114.

22. Líndström A, Buerge IJ, Poiger T, Bergqvist P-A, Müller MD, Buser H-R. (2002). "Occurrence and environmental behavior of the bactericide triclosan and its methyl derivative in surface waters and in wastewater." Environ Sci Technol 36: 2322-2329.

23. Meade M.J., Waddell R.L., Callahan T.M. (2001). "Soil bacteria *Pseudomonas putida* and *Alcaligenes xylosoxidans* subsp. *dentrificans* inactivate triclosan in liquid and solid substrates." FEMS Microbiol Lett 204: 45-48.

24. McMurry L.M., Oethinger M., Levy S.B. (1998). "Triclosan targets lipid synthesis." Nature 394: 531-532.

25. Microtek Medical, Inc. Columbus, MS. (2002). "Isodrape surgical film drapes featuring Microban." Manufacturer's brochure. Literature No.: LIT013183-2500.

26. Mima T., Joshi S., Gomez-Escalada M., Schweizer H.P. (2007). "Identification and characterization of TriABC-OpmH, a triclosan efflux pump of *Pseudomonas aeruginosa* requiring two membrane fusion proteins." J Bacteriol 189: 7600-7609.

27. Mixon G.C., Inventor. (May 25, 1999)"Antimicrobial gloves and a method of manufacture thereof." United States patent US 5906823.

28. Nikaido H., Vaara M. (1985). "Molecular basis of bacterial outer membrane permeability." Microbiol Rev 49: 1-32.

29. Pereira L.J., Lee G.M., Wade K.J. (1997). "An evaluation of five protocols for surgical handwashing in relation to skin condition and microbial counts." J Hosp Infect 36: 49-65.

30. Vaara M. (1992). "Agents that increase the permeability of the outer membrane." Microbiol Rev 56: 395-411.

31. Zafar A.B., Butler R.C., Reese D.J., Gaydos L.A., Mennonna P.A. (1995). "Use of 0.3% triclosan (Bacti-Stat) to eradicate an outbreak of methicillin-resistant *Staphylococcus aureus* in a neonatal nursery." Am J Infect Control 23: 200-208.

32. Zimmermann W. (1979). "Penetration through the gram-negative cell wall: a co-determinant of the efficacy of beta-lactam antibiotics." Int J Clin Pharmacol Biopharm 17: 131-134.

VITA

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Thesis: INFLUENCE OF METHYLATION ON THE ANTIBACTERIAL PROPERTIES OF TRICLOSAN IN *PASTEURELLA MULTOCIDA* AND *PSEUDOMONAS AERUGINOSA* VARIANT STRAINS

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Major Field: Biomedical Sciences

Scope and Method of Study:

The opportunistic bacterium *Pasteurella multocida* is extremely susceptible to the hydrophobic biocide triclosan by virtue of its markedly permeable outer membrane, while the opportunistic pathogen *Pseudomonas aeruginosa* is intrinsically resistant to levels far exceeding the triclosan aqueous solubility limit. Widespread incorporation of triclosan in health and personal care products has resulted in its concomitant accumulation with metabolites such as methyl triclosan in environmental and biological systems. The present study was undertaken to investigate the possibility that methylation of triclosan may mitigate its efficacy and represent a potential resistance mechanism. Comparative standardized disk agar diffusion and batch cultural turbidimetric bioassays were employed to assess the relationship between triclosan susceptible or resistant bacteria and methyl triclosan. A wild-type *P. aeruginosa* parental strain and a mutant exhibiting a permeable outer cell envelope phenotype were examined in concert with a refractory wild-type strain sensitized to triclosan susceptibility using outer membrane permeabilizer compound 48/80. Gram-positive organisms were also evaluated to further assess the possibility of a role for the gram-negative outer membrane in the inability of methyl triclosan to affect bacterial growth.

Findings and Conclusions:

All organisms examined were resistant to methyl triclosan, while all organisms excluding *P. aeruginosa* were susceptible to triclosan over a wide concentration range. The permeable outer membrane phenotype in both mutant and chemically-sensitized wild-type strains rendered *P. aeruginosa* susceptible to triclosan, but not methyl triclosan. These data support the notion that methylation of triclosan renders the compound unable to inhibit the growth of disparate bacterial pathogens in a manner independent of an intact outer membrane. This is substantiated by the fact that gram-positive bacteria do not possess an outer membrane and exhibit no inhibition of growth in the presence of methyl triclosan. It can also be postulated that biocide modification may contribute to the intrinsic resistance of *P. aeruginosa* to triclosan.

ADVISER'S APPROVAL: _____

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