# RELATIONSHIP BETWEEN SUSCEPTIVITY TO TRICLOSAN SENSITIZATION BY OUTER MEMBRANE PERMEABILIZATION AND CELL SURFACE HYDROPHOBICITY PROPERTIES IN OPPORTUNISTICALLY PATHOGENIC *SERRATIA* SPECIES

By

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RELATIONSHIP BETWEEN SUSCEPTIVITY TO TRICLOSAN SENSITIZATION BY OUTER MEMBRANE PERMEABILIZATION AND CELL SURFACE HYDROPHOBICITY PROPERTIES IN OPPORTUNISTICALLY PATHOGENIC *SERRATIA* SPECIES

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TITLE OF STUDY: RELATIONSHIP BETWEEN SUSCEPTIVITY TO TRICLOSAN SENSITIZATION BY OUTER MEMBRANE PERMEABILIZATION AND CELL SURFACE HYDROPHOBICITY PROPERTIES IN OPPORTUNISTICALLY PATHOGENIC SERRATIA SPECIES

Major Field: BIOMEDICAL SCIENCES

Abstract: The nosocomial opportunists *Pseudomonas aeruginosa* and *Serratia marcescens* are atypically resistant to the hydrophobic biocide triclosan due largely to outer membrane impermeability properties for hydrophobic substances. However, we have recently shown that the degree of cell envelope exclusivity differs among other opportunistically pathogenic *Serratia* species. Moreover, susceptivity to sensitization to triclosan by outer membrane permeabilization also differs among other intrinsically resistant species. The purpose of the present study was to determine if cell surface hydrophobicity (CSH) properties underlie susceptivity to triclosan sensitization by outer membrane permeabilization in selected species. Three *Serratia* species (*marcescens, fonticola,* and *odorifera)* exhibiting disparate degrees of intrinsic susceptibility to hydrophobic antibacterial agents and susceptivity to triclosan sensitization by outer membrane permeabilization were examined to determine their ability to associate with hydrophobic substances. CSH was determined using conventional crystal violet binding, hydrocarbon adherence, and n-phenylnapthylamine uptake methods. *S. marcescens* and *S. fonticola* were intrinsically resistant to hydrophobic antibacterial agents including triclosan, while *S. odorifera* was susceptible. Their cell surface hydrophobicity properties differed only slightly regardless of disparate susceptivity to triclosan sensitization. These data suggest that phenotypic differences seen in three opportunistic *Serratia* species with regard to intrinsic resistance to triclosan are in at least part due to disparate outer membrane exclusion potential. Moreover, susceptivity to triclosan sensitization by outer membrane permeabilization appears not to be influenced by cell surface hydrophobicity properties.

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# CHAPTER I

## INTRODUCTION

Triclosan (5-choro-2-(2,4-dichlorophenoxy)phenol) is a hydrophobic diphenyl ether biocide (Figure 1) with broad spectrum antibacterial activity that is utilized as an antiseptic or preservative in many medical, personal care, industrial, and household settings (1). Municipal water treatment processes are relatively ineffective in triclosan removal due to the stability of the biocide combined with its widespread use, thereby resulting in its environmental accumulation as a pollutant (2,3).

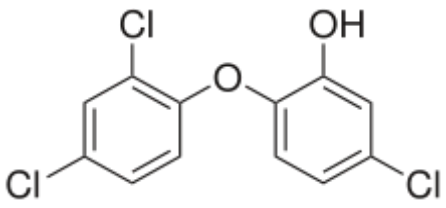


Figure 1. Molecular structure of triclosan.

The mechanism of action of triclosan involves inhibition of enoyl-acyl carrier protein reductase, an essential cytoplasmic enzyme involved in bacterial fatty acid biosynthesis (4) (Figure 2). In order to reach its mechanistic target in gram-negative bacteria, the biocide must transverse the outer membrane to enter the periplasmic space where it can

then, due to its hydrophobic nature, passively partition through the phospholipid bilayer of the cytoplasmic membrane into the cytoplasm.

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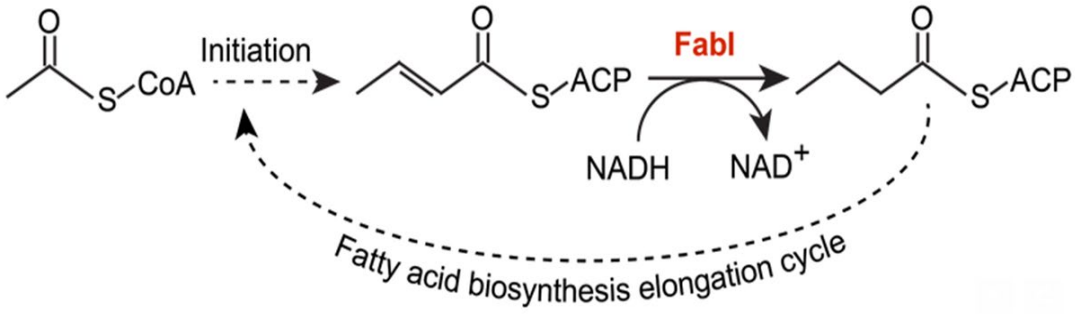


Figure 2. Cytoplasmic enoyl-acyl carrier protein reductase (FabI) catalyzed reaction of bacterial fatty acid biosynthesis (Logan Vinson, University of California, Davis)

As seen in figure 3, the gram-negative outer membrane consists of an inner leaflet composed of phospholipids and an outer leaflet primarily composed of lipopolysaccharides (LPSs). The LPS confers a highly polar nature to the cell surface due to its net negative charge of phosphate in the both the core and lipid A region, as well as the sugar residues in both the core and the O-side chain regions. This unique membrane surface is thereby able to prevent association with hydrophobic molecules in the extra cellular environment (5,6) Low molecular weight polar solutes such as nutrients are able to access the periplasm through a hydrophilic pathway by passively diffusing through water-filled outer membrane porins, while larger molecules are unable to pass thru the

porins regardless of their polarity (5,7). In contrast, diffusion of hydrophobic substances into the periplasm is dependent on the presence of a hydrophobic pathway whereby they associate with and partition through regions of exposed phospholipid bilayers (8,9). The hydrophobic pathway is absent in intact outer membranes of most gram-negative bacteria, thereby allowing them to protect the underlying protoplast from lipophilic compounds.

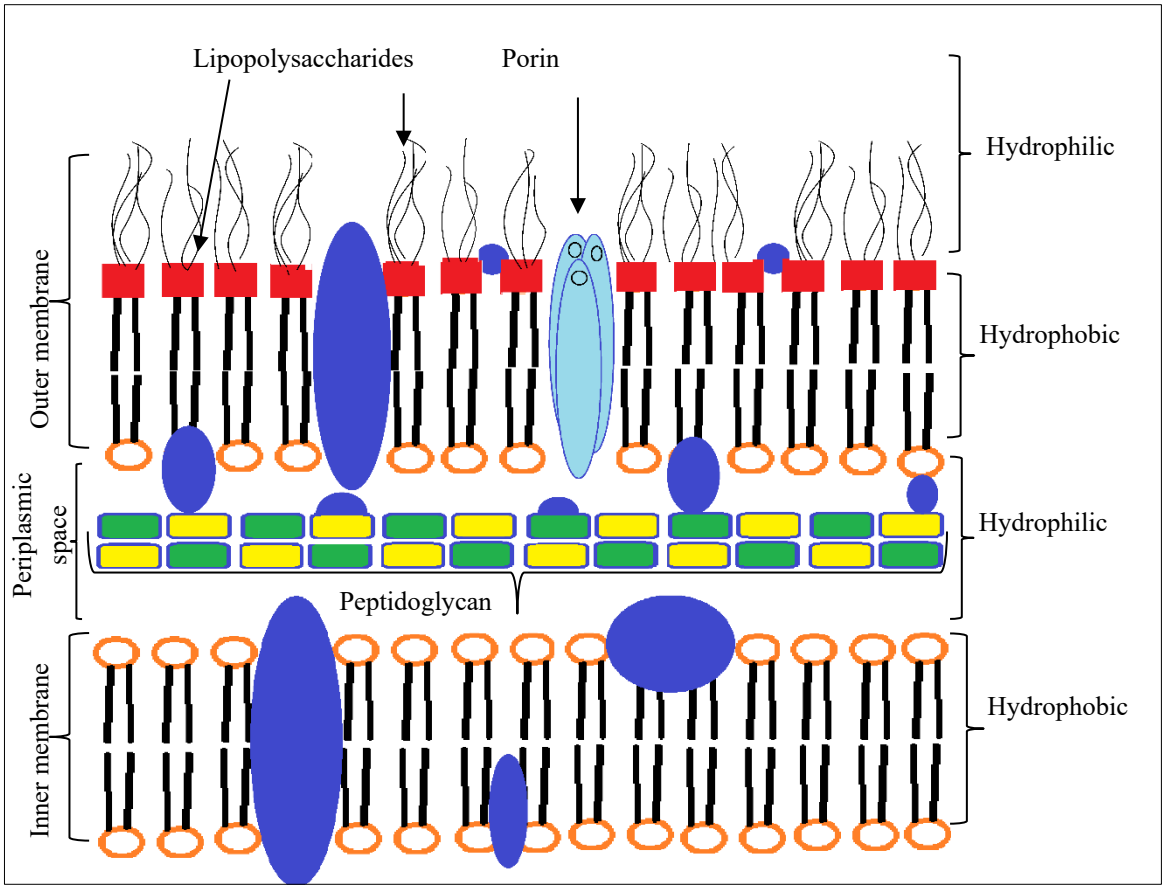


Figure 3. Schematic of the gram-negative bacterial cell envelope. (Abby Rigsbee, Oklahoma State University Center for Health Sciences).

Given its hydrophobic nature, the ability of triclosan to transverse the outer membranes of most gram-negative bacteria is atypical and not well understood (10). However, once it appears in the periplasm it transverse the cytoplasmic membrane phospholipid bilayer where it can then access enoyl-acyl carrier protein reductase as mentioned above in Figure 2.

The 18 species (11,12) of the bacterial genus *Serratia* are gram-negative bacilli of soil origin which are facultatively anaerobic and members of the family Yersiniaceae (13,14). *Serratia marcescens* is the best-known opportunistic pathogen in the genus. It is typically resistant to a large number of disparate antibacterial agents to include antibiotics, as well the broad-spectrum hydrophobic biocide triclosan (13). It is notable for its ability to cause nosocomial infections related to implanted devices, as well as urinary tract and wound infections, septicemia, eye infections, and endocarditis. Other *Serratia* species that have been reported to cause infections in humans include *Serratia liquefacians, Serratia fonticola, Serratia rubidaea, Serratia proteamaculans, Serratia quinovorans, Serratia odorifera, Serratia ficaria, Serratia grimesii,* and *Serratia plymuthica* (13,14).

Cell surface hydrophobicity properties influence how bacteria interact with their environment and their ability to act as etiological agents of infection as they adhere to abiotic substances and biotic tissues (15). Furthermore, CSH affects outer cell envelope permeability for antibacterial solutes such as antibiotics and biocides (16,17,18). The cell surface hydrophobicity properties of *S. marcescens* have been previously documented in the literature (19, 20), while a paucity of research exists on this subject for other pathogenic *Serratia* species.

Biofilms are aggregate communities of bacterial cells enclosed in polysaccharide matrices that form through a process of adherence to a solid surface, production of micro colonies, excretion of extracellular polymers such as EPS that form an immobilizing matrix as the biofilm matures, and the eventual release of planktonic cells from microcolony breakdown (21). Biofilm formation is a cooperative process involved in gene transcription. This process occurs in 4 steps: attachment, sessile growth, maturation and detachment. These steps are regulated through quorum sensing, a mechanism in which bacterial cells secrete and respond to extracellular molecules that indicate physiological states of the interacting bacterial cells. Bacteria can sense when their population density is favorable for biofilm formation and release signal molecules that promote physiological processes in nearby bacterial cells that allow the formation of biofilms (22). The ability of pathogenic bacteria to form biofilms represents an important virulence factor in that they facilitate resistance to both antibiotics and host immune responses, as well as render them less susceptible to UV radiation, desiccation, and predation (21,23).Biofilm structure also retards diffusion of harmful compounds into the interior (24). Bacterial biofilms can be formed on abiotic surfaces, such as indwelling medical devices, as well as biological tissues of host organisms. As many as 80% of bacterial infections in humans involve biofilm production, thereby resulting in longer hospital stays, higher medical costs, and an increase in mortality from bacterial infections. (21). While *S. marcescens* (25)*, S. liquefacians* (26)*, S. plymuthica* (27), and *S. proteamaculans (*28) have been reported to form *in vitro* biofilms, there is a paucity of information in the literature regarding how CSH influences the proclivities of *Serratia*

species to form biofilms (29). We are not aware of published work regarding biofilm formation in other opportunistic Serratia species.

Additional research is necessary to better understand if physiological features of gram-negative cell surface properties such as hydrophobicity influence the ability of biofilm-forming species to adhere to substrates, the first step in biofilm formation (21). Work in our laboratory has shown CSH properties to be particularly important in this regard for the pulmonary opportunist *Bulkholderia multivorans* (30). Further research will also uncover how cell surface physiological properties impact the ability to maintain mature and stable biofilm integrity over time. Understanding the influence CSH properties in particular have on biofilms produced by opportunistic pathogenic *Serratia* species is essential to obtaining a better understanding of the cellular and molecular basis underlying their ability to infect human hosts.

We hypothesize in the present study that CSH properties underlie the ability of the outer cell surfaces of opportunistically pathogenic *Serratia* species to associate with hydrophobic antibacterial substances, thereby influencing their ability to inhibit the initiation of growth. Moreover, we further postulate that CSH properties are related to the susceptivity to sensitization to triclosan by chemical disruption of the outer membrane function. The goal of the present study was to test these hypotheses by first obtaining a better understanding of the relationship between *Serratia* species reported to be opportunistic pathogens in humans and hydrophobic antibacterial compounds having disparate mechanisms of action using a standardized disk agar diffusion bioassay developed and routinely employed in our laboratory (31). We next examined the CSH properties of three clinically-relevant *Serratia* species selected on the basis of their

disparate relationships with the hydrophobic biocide triclosan. The ability to associate with hydrophobic substances was quantitatively determined using crystal violet binding (32), hydrocarbon adherence (33), and 1-N-phenylnaphylanime (NPN) fluorescence probe partitioning (34) assays. These data were analyzed with the intention of the obtaining a better understanding relationship between selected *Serratia* species and hydrophobic antibacterial agents, as well as their proclivities of intrinsically resistant species to become sensitized to a synergistic combination of triclosan and a chemical outer membrane permeabilizer.

In summary, triclosan is a very stable hydrophobic compound effective against both gram-positive and gram-negative bacteria. It is atypically able to permeate the outer membrane of all gram-negative bacteria with the exception of certain organisms such as *Pseudomonas aeruginosa* and *S. marcescens*. Previous work in our laboratory has shown that intrinsic resistance to triclosan is due at least in part to outer membrane impermeability properties to hydrophobic compounds. The purpose of the present study was to examine the relationship between cell surface hydrophobicity properties and susceptivity to triclosan sensitization by outer membrane permeabilization of opportunistically pathogenic *Serratia* specie

# CHAPTER II

## REVIEW OF LITERATURE

The overall focus of work in our laboratory has been directed toward the mechanistic role of the gram-negative cell envelope in the cellular and molecular mechanisms underlying the intrinsic resistance of opportunistic pathogens to hydrophobic antibacterial agents such as novobiocin and the biocide triclosan. We have established that outer membrane exclusionary properties function in concert with multidrug efflux systems to render *P. aeruginosa* intrinsically resistant to triclosan (10,31,35,36,37). Ancillary work revealed the marked susceptibility to hydrophobic compounds seen in naturally-occurring *P. multocida* strains to be due to atypical permeability properties associated with the outer membrane (36). We more recently reported the biocidal nature of triclosan could be obviated by methylation (31), thereby revealing yet another potential resistance mechanism for *P. aeruginosa* and other organisms.

*Burkholderia mulitovorans* is the etiological agent of secondary pulmonary infections in cystic fibrosis and chronic granulomatous disease patients. It is phylogenetically closely related to *P. aeruginosa* and is also intrinsically resistant to hydrophobic molecules such as triclosan. We recently found that the expression of extracellular polysaccharide (EPS) and the hyper mucoid phenotype are not associated with either capsulation or the ability of cells to associate with hydrophobic substances (38). However, EPS is necessary for the maturation of stable *in vitro* biofilms, while interfering with the initial attachment stage (30).

Current research in our laboratory features efforts to better understand outer membrane permeability properties for hydrophobic antibacterial molecules in ten *Serratia* species which have been reported to be opportunistically pathogenic in humans (18). These include *S. marcescens* (13), *Serratia fonticola* (39), *Serratia liquefaciens* (40), S*erratia rubidaea* (41), *Serratia proteamaculans* (14), *Serratia quinovorans* (42), *Serratia odorifera* (43), *Serratia ficaria* (44), *Serratia grimesii* (45), and *Serratia plymuthica* (46). Preliminary research confirmed that while *S. marcescens* resembles *P. aeruginosa* in that it is intrinsically resistant to triclosan*, E. coli* is extremely susceptible in a manner consistent with most other gram-negative organisms. This despite the fact that both are phylogenetically closely-related members of the family Yersiniaceae and Enterobacteriaceae respectively (Figure 4).

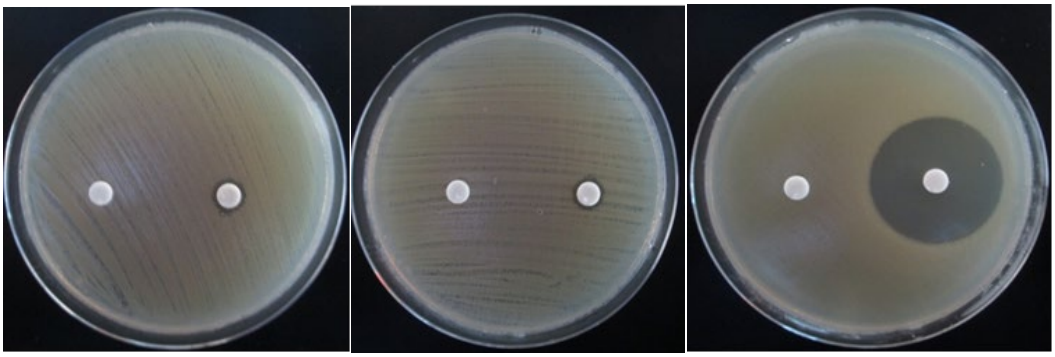

Figure 4. Representative disc agar diffusion bioassay. (L) *P. aeruginosa* PAO1, (C) *S. marcescens* ATCC 13880, (R) *E. coli* ATCC 25922. Discs: 1, control (95 % ethanol); 2, triclosan (Unpublished data).

The outer membrane exclusionary properties of four disparate *S. marcescens* strains were shown to be responsible to different degrees for their intrinsic resistance to triclosan (Figure 5) (18). The examination of nine other opportunistically pathogenic *Serratia* species revealed marked disparities with regard to intrinsic resistance to hydrophobic molecules in general, and susceptivity to triclosan sensitization by outer membrane permeabilization in resistant species. These disparities allowed the development of a model system consisting of species representing three different phenotypic groups: (i) *S. marcescens* ATCC 13880 represented species intrinsically resistant to triclosan and slightly susceptive to triclosan sensitization, (ii) *S. fonticola* ATCC 29844 represented species intrinsically resistant to triclosan and markedly susceptive to triclosan sensitization, and (iii) *S. odorifera* ATCC 33077 representing the only species intrinsically susceptible to hydrophobic molecules in general (Figure 5). *S. marcescens* ATCC 13880 and *S. fonticola* ATCC 29844 can be further differentiated in that while

both are intrinsically resistant to triclosan, *S. marcescens* is able to overcome triclosan sensitization significantly and resume a normal growth rate, while *S. fonticola* is completely inhibited by triclosan sensitization in a bactericidal manner. *S. marcescens* has been reported to be able to form *in vitro* biofilms (25), while this behavior as not been studied for either *S. fonticola* or *S. oderifera.*

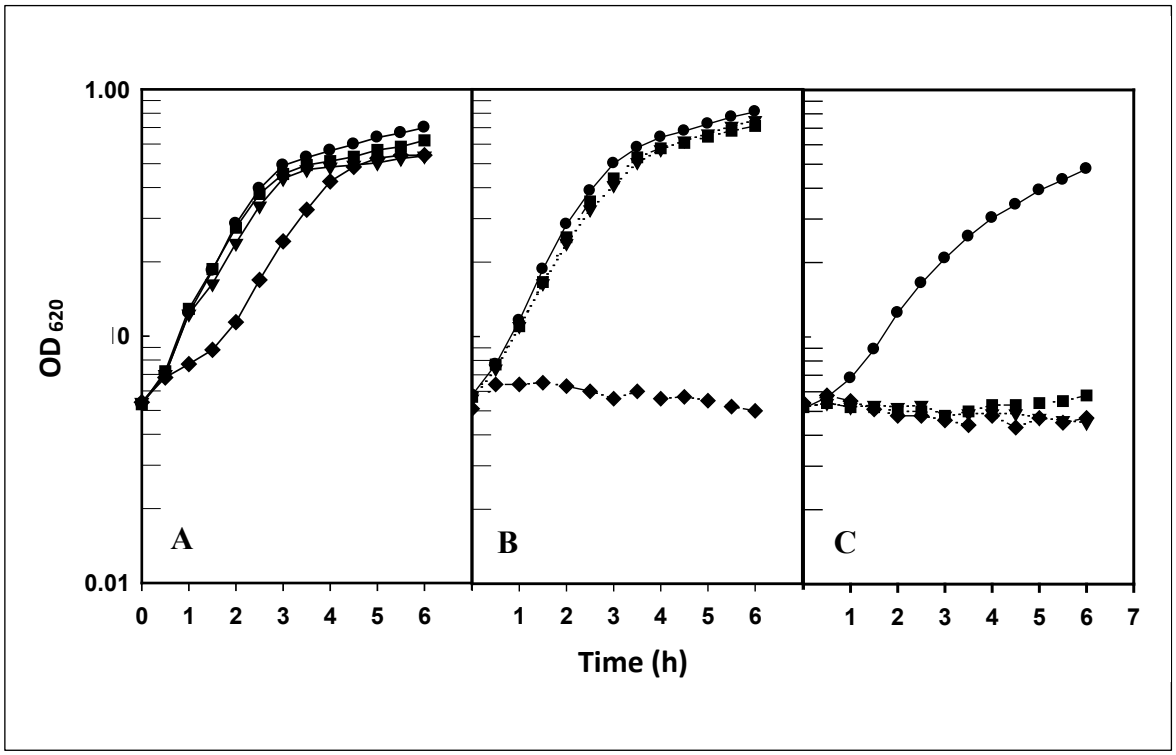


Figure 5. Batch cultural growth kinetics for model system *Serratia* spp. in the presence of compound 48/80 and triclosan. Panel A, *S. marcescens ATCC 13880*; Panel B, *S. fonticola* ATCC 29844; Panel C, *S. odorifera* ATCC 33077. Symbols: ●, control MHB; ▼, compound 48/80 (2.5 µg/mL); ■, triclosan (2.0 µg/mL); ◆, compound 48/80 (2.5 µg/mL) plus triclosan (2.0 µg/mL) (Boyina *et al.*, 2020).

# CHAPTER III

# METHODOLOGY

## Bacterial Isolates and Maintenance

Type strains of all ten *Serratia* species which have been shown capable of causing opportunistic infections in humans were obtained from the American Type Culture Collection (Manassas, VA). *S. marcescens* ATCC 13880*, S. fonticola* ATCC 29844, and *S. odorifera* ATCC 33077 were selected for inclusion in a model system for further examination in the present study on the basis of their respective relative susceptivities to triclosan sensitization by outer membrane permeabilization as established previously (18). *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, and *P. multocida* ATCC 11039 were purchased from the American Type Culture Collection and employed for comparative control purposes. Other comparative controls organisms included *P.* aeruginosa PAO1(this laboratory), *E. coli* K12 413 (Dr. Gerwald Köhler, Oklahoma State University Center for Health Sciences), and *P. multocida* P-1581. (National Animal Disease Center, Ames, IA). Stock cultures were maintained using cryopreservation in the presence of 15 % glycerol at -80°C as previously detailed by Darnell *et al.* (1987). Organisms comprising this experimental model system are described in Table 1.

Table 1. Control organisms and experimental *Serratia* spp. compromising experimental model system.

| Organism | Source | Pertinent phenotype | Reference |
|---|---|---|---|
| **Reference organisms** [a] | | | |
| *Pseudomonas aeruginosa* ATCC 27853 | Human | Susceptive to triclosan sensitization [b] | Boyina *et al,* 2020 |
| *Pseudomonas aeruginosa* PAO1 | Human | Susceptive to triclosan sensitization [b] | Champlin *et al.,* 2005; Ellison *et al.,* 2007 |
| *Escherichia coli* ATCC 25922 | Human | Intrinsically susceptible to triclosan | Boyina *et al.,* 2020 |
| *Escherichia coli* K12 413 | Human | Intrinsically susceptible to triclosan | Present study |
| *Pasteurella multocida* ATCC 11039 | Avian | Intrinsically susceptible to hydrophobic molecules in general | Hart and Champlin, 1988; Ellison and Champlin, 2007 |
| *Pasteurella multocida* P-1581 | Avian | Intrinsically susceptible to hydrophobic molecules in general | Hart and Champlin, 1988 |
| **Experimental *Serratia* spp.** [c] | | | |
| *Serratia marcescens* ATCC 13880 | Environment | Slightly susceptive to triclosan sensitization [b] | Boyina *et al.,* 2020 |
| *Serratia fonticola* ATCC 29844 | Environment | Susceptive to triclosan sensitization [b] | Boyina *et al,* 2020 |
| *Serratia odorifera* ATCC 33700 | Human | Intrinsically susceptible to triclosan and hydrophobic molecules in general | Boyina *et al.,* 2020; present study |

[a] Included for comparative and/or control purposes for pertinent experiments as needed.
[b] Outer membrane permeabilized for hydrophobic substances using compound 48/80.
[c] Representative *Serratia* spp. chosen for the present study on the basis of most extreme nonsusceptivity or susceptivity to triclosan sensitization by compound 48/80 permeabilization or intrinsic susceptibility to triclosan.

## Culture Conditions

Working cultures of each organism were prepared by inoculating cells from cryopreserved stock cultures onto Difco Mueller Hinton Agar (MHA; Becton, Dickson and Co., Sparks, MD) plates, incubating at 37°C for 18 ± 1 h, and storing at 4°C until needed. Overnight starter cultures were prepared by inoculating 20 mL of sterile Difco Mueller Hinton Broth (MHB; Becton, Dickson and Co.) in 125-mL culture flasks with cells from working cultures. These were incubated for 15 ± 1 h at 37°C with rotary aeration at 180 rpm in an Excella E24® environmental shaker incubator (New Brunswick Scientific Co., Edison, NJ) to obtain stationary-phase inocula. Experimental cultures were prepared by inoculating MHB with stationary-phase starter culture cells to an initial optical density ($OD_{620}$) of either 0.025 or 0.05 with the aid of a Spectronic 20D+ optical spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) depending on the bioassay (see below). Cultures were transferred to sterile culture/spectrophotometer tubes (18 x 150-mm) in 5.0-mL aliquots and incubated at 37° C and 180 rpm until desired optical densities were obtained.

**Disc Agar Diffusion Bioassay**

Susceptibilities to mechanistically disparate hydrophobic antibacterial agents of all *Serratia* species reported to be pathogenic in humans (18) were examined using a conventional disk agar diffusion bioassay as standardized in our laboratory (31).Test cultures were prepared by inoculating 5.0 mL of MHB with starter culture cells to an initial $OD_{620}$ of 0.025 in sterile 18 x 150-mm culture tubes and incubating at 37°C and 180 rpm (Excella E24® environmental shaker incubator) until exponential phase was obtained ($OD_{620}$ of 0.10). Solvent control disks were aseptically prepared by impregnating sterile Blank Paper Discs (6.0-mm diameter; Becton, Dickinson and Co.) with 10 µL of ethanol (95%). Triclosan test disks (potency of 0.2 µg/disk) were prepared by impregnating sterile disks with 10 µL of a triclosan stock solution (20.0 µg/mL). All disks were allowed to dry under flowing sterile air. Commercially-prepared Sensi-Discs® containing mechanistically-disparate hydrophobic antibiotics were obtained from Becton, Dickinson and Co. MHA plates (15 x 150 mm diam.) were each uniformly seeded with 250 µL each of an indicated exponential-phase test culture suspension ($OD_{620}$ of 0.10) and spread inoculated with a consistent pattern using a sterile cotton swab saturated with sterile MHB. Ethanol control and triclosan test disks were aseptically applied with alcohol flame-sterilized forceps. Commercially-prepared disks were applied with the aid of a BBL Sensi-Discs® 12-place dispenser (Becton, Dickinson and Co.). Plates were stored at 4°C for 1.0 h to allow the hydrophobic antibacterial agents to diffuse in the absence of growth prior to incubation at 37°C for 18 ± 1 h. Zones of

growth inhibition were measured using Traceable ® digital calipers (Fisher Scientific, Hampton, NH).

**Crystal Violet Binding Hydrophobicity Assay**

Qualitative determinations of cell surface hydrophobicity properties of model system organisms were assessed based on the degrees to which microscopic colonies bound crystal violet using the method described by Bhaduri *et al.* (1987). MHA and Difco Brain Heart Infusion Agar (BHIA; Becton, Dickinson and Co.) plates were streak inoculated for isolation with bacteria from working cultures and incubated at 37°C for 18 ± 1 h. Plates were flooded with 8.0 mL of an aqueous solution of crystal violet (85.0 µg/mL; Fischer Chemical Co., Fair Lawn, NJ), which was carefully decanted using Pasteur pipet aspiration after 2.0 min. Hydrophobic colonial growth appeared dark violet due to crystal violet uptake, while hydrophilic colonies remained colorless as visualized immediately with the aid of a Darkfield Quebec® Colony Counter. Degree of crystal violet binding by isolated colonies graded usually as: -, none (hydrophilic); +, slight (intermediate); ++, or heavy (hydrophobic).

**Hydrocarbon Adherence Hydrophobicity Assay**

Cell surface hydrophobicity properties of model system organisms were quantitatively accessed on the basis of the degrees to which cells partitioned into n-hexadecane using the hydrocarbon adherence method developed by Rosenburg *et al.* (1980) as modified for use in our laboratory by Darnell *et al*., (1987) and Ruskoski and Champlin (2017). Stationary-phase starter cultures were used to inoculate 210 mL of MHB to an initial $OD_{620}$ of 0.05. Aliquots of 100 mL were dispensed into each of two 250-mL flasks and incubated at 37°C at 180 rpm (Excella E24® environmental incubator shaker) until late-exponential phase ($OD_{620}$ of approx. 0.30-0.40). Cell suspensions were harvested by combining both cultures into one 250-mL centrifuge bottle and centrifuging at 12,000 x g for 15 min at 4°C (Sorvall Legend XTR Centrifuge; Thermo Fisher Scientific, Inc., Waltham, MA). Supernates were carefully decanted and pellets were washed once in 200 mL of chilled (4°C) potassium phosphate magnesium sulfate buffer (PPMS; 6.97 g of $K_2HPO_4$, 2.99 g of $KH_2PO_4$, and 0.2 g of $MgSO_4 \bullet 7H_2O$ per liter of deionized water at pH 7.2). Washed cell pellets were suspended in ambient temperature PPMS to an $OD_{620}$ of 0.50 as determined in 13 x 100-mm borosilicate sample tubes using a Spectronic 20+ optical spectrophotometer. Standardized cell suspensions of 4.0 mL were dispensed into each of four round bottom, borosilicate culture tubes (20 x 150 mm). A 1.0-mL volume of n-hexadecane (Sigma-Aldrich, St. Louis, MO) was transferred to each of three tubes leaving the forth as an untreated control. Aqueous bacterial suspensions and oil phases were mixed using vortex agitation for four 15-sec bursts for a total of 1.0 min before allowing 15 min for phase separation at ambient temperature. Lower aqueous-phase cell suspensions were transferred to 13 x 100-mm borosilicate disposable sampler holders

using 9-inch Pasteur pipets and $OD_{620}$ values of control and experimental cell suspensions were determined spectrophotometrically. Cell surface hydrophobicity properties were established on the basis of the portion of the cells that had partitioned into the hydrocarbon phase in relation to untreated control cells and reported as percent adherence to n-hexadecane using the follo`wing formula:

$$\% \text{ Adherence} = \left(1 - \frac{\text{OD}_{620} \text{ treated cell suspension}}{\text{OD}_{620} \text{ untreated cell suspension}}\right) \text{x } 100$$

**1-*N*-phenylnapthylamine Uptake Hydrophobicity Assay**

The potential degrees to which hydrophobic regions of model system organism cell surfaces were able to associate with the hydrophobic fluorescent probe 1-N-phenylnapthylamine (NPN; Sigma–Aldrich Chemical Co., St. Louis, MO) were examined using our modification (36,38) of the method of Helander and Mattila-Sandholim (2000). Experimental cultures were prepared by inoculating test cultures consisting of 125-mL screw-capped culture flasks each containing 50 mL of MHB with stationary-phase starter culture cells to an initial $OD_{620}$ of 0.025. Cultures were incubated at 37°C with rotary aeriation at 180 rpm using an Excella E24® in environmental shaker incubator until late-exponential phase ($OD_{620}$ of aprrox. 0.30-0.40). Cells were harvested by centrifugation at 12,000 x g (Sorvall Legend XTR Centrifuge) for 15 min at 4°C. Supernates were carefully aspirated and cells were resuspended in 5.0 mM of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Sigma–Aldrich Chemical Co.) buffer and adjusted to pH 7.2 using 1.0 N sodium hydroxide to an $OD_{620}$ of 0.05. An NPN (Certified ACS; Fisher Scientific, Fair Lawn, NJ) stock solution was prepared in acetone (Fisher Chemical Co.) to a final concentration of 0.5 mm. The NPN stock solution was diluted in HEPES buffer to a final concentration of 40 µM. Polystyrene microtiter plates (Coster 96-well black sided, clear bottom; Corning Inc., Lowell, MA) were loaded with the following samples (Figure 6): MHB control (200 µL of MHB); HEPES control (200 µL of HEPES buffer), NPN control (150 µL of HEPES and 50 µL of NPN), test culture cell suspensions control (100 µL of HEPES and 100 µL of indicated standardized cell suspension). Test wells contained 50 µL of HEPES, 50 µL of NPN (40µM), and 100 µL of the indicated standardized cell suspension).

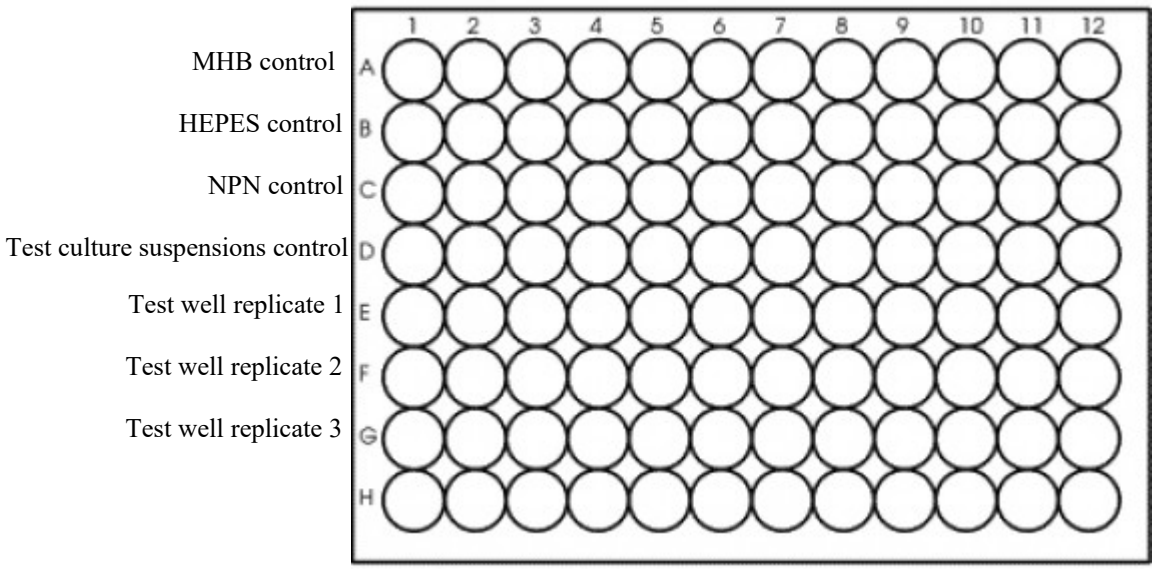


Figure 6. NPN uptake assay plate. Columns: 1, *P. aeruginosa* ATCC 27853; 3, *E. coli* ATCC 25922; 5, *P. multocida* P-1581; 7, *S. marcescens* ATCC 13880; 9, *S. fonticola* ATCC 29844; 11, *S. odorifera* ATCC 33077. Rows: A, MHB control ; B, HEPES control; C, NPN control; D, test culture cell suspension control; E, test well replicate 1 containing 50 µL of HEPES, 50 µL of NPN (40µM), and 100 µL of the indicated standardized cell suspension); F, test well replicate 2; G, test well replicate 3.

Relative fluorescence intensity was immediately measured using a Synergy 2 Multi-Detection Microplate Reader (BioTek Instruments Inc., Winooski, VT) using an excitation wavelength of 340 nm and an emission wavelength of 420 nm. Relative fluorescence was calculated using the following equation:

$$\text{Relative fluorescence} = \frac{\text{test culture with NPN} - \text{test culture control}}{\text{NPN control} - \text{HEPES control}}$$

# CHAPTER IV

# FINDINGS

## Intrinsic susceptibility of *Serratia* species to hydrophobic antibacterial agents

A standardized disk agar diffusion bioassay was employed to determine the relative susceptibilities of opportunistically pathogenic species of *Serratia* to a panel of hydrophobic antibacterial agents in order to provide presumptive evidence for the involvement of the gram-negative outer membrane in intrinsic resistance to non-polar substances including triclosan. Zones of growth inhibition having diameters < 5.0 mm were considered relativity resistant, while those > 5.0 mm were considered indicative of relative susceptibility. Mechanistically disparate hydrophobic antibiotics and one biocide (triclosan) were employed in order to determine if overall resistance was a product of specific resistance mechanisms to the compounds themselves or if intrinsic resistance was due to the general impermeability properties of the gram-negative outer membrane.*P. aeruginosa* is highly resistant to hydrophobic compounds due to outer membrane exclusivity (10,35,36,37,31). Strains ATCC 27853 and PAO1 were included

in the present study to serve as positive refractory controls. *E. coli* is a member of the family Enterobacteriaceae and phylogenetically-related to *S.marcescens* and therefore resistant to hydrophobic compounds in general (5,6,8). Unlike *S. marcescens, E. coli is* susceptible to triclosan (Figure 7), so strains ATCC 25922 and K12 413 were examined as positive comparative controls for triclosan susceptibility. *P. multocida* is a gram-negative bacterium with an outer membrane that is atypically permeable to all hydrophobic molecules (36). Strains ATCC 11039 and P-1581 was employed as overall negative comparative controls.

Figure 7 contains two representative disk agar diffusion bioassay plates for *marcescens* 13880 (type strain) and *E. coli* ATCC 25922. Both organisms can be seen to be resistant to the four mechanistically-disparate hydrophobic antibiotics novobiocin, vancomycin, clindamycin and rifampin. These data confirm that all these bacteria possess outer membranes which are typically impermeable to hydrophobic substances in general. *E. coli* is a representative of most gram-negative bacteria by virtue of having an outer cell envelope which is freely permeable to the hydrophobic biocide triclosan. In contrast, *S. marcescens* is resistant by virtue of its refractory outer membrane (18). As can be seen for the reference organisms in Table 2, *P. aeruginosa* strains ATCC 27853 and PAO1 were intrinsically resistant to all hydrophobic antibiotics employed as well as the hydrophobic biocide triclosan with the exception that the latter strain which was susceptible to chloramphenicol. *E. coli* ATCC 25922 and K12 413 exhibited a similar profile with the exception of being susceptible to triclosan. *P. multocida* ATCC 11039 and P-1581 were susceptible to all hydrophobic antibacterial agents examined with the exception of vancomycin.

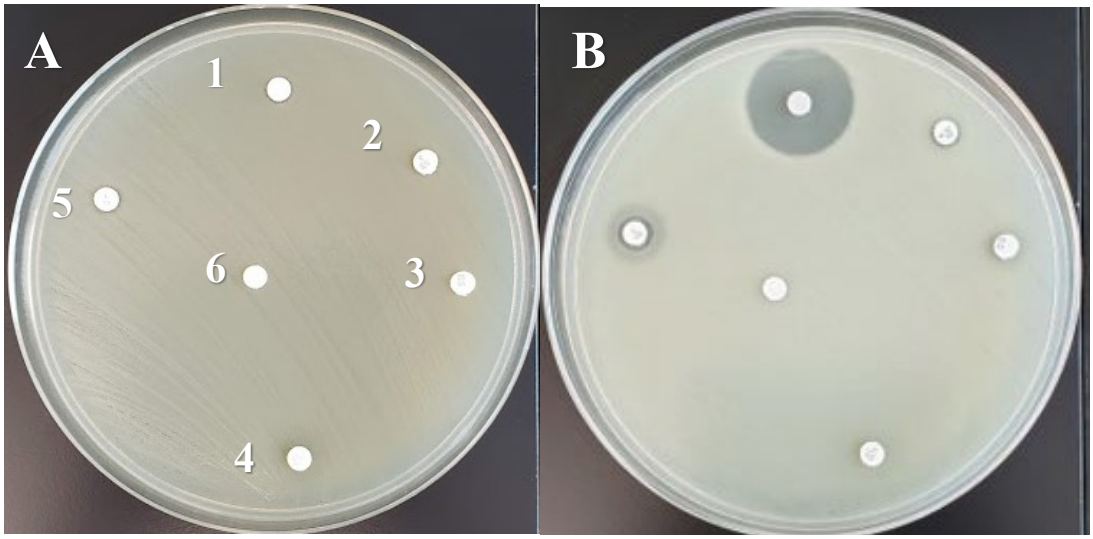


Figure 7. Representative disk agar diffusion bioassay plates. Panel A, *S. marcescens*; Panel B, *E. coli* ATCC 25922. Disk (potency): 1, triclosan (0.2 µg); 2, novobiocin (5.0 µg); 3, vancomycin (30 µg); 4, clindamycin (2.0 µg); 5, rifampin (5.0 µg); 6, Ethanol control (95%).

Table 2. Disk agar diffusion bioassay of mechanistically-disparate hydrophobic antibacterial agents for reference bacteria.

| Organism | Inhibition zone (mm ± SD)[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | ETOH[b] | TSC | CLI | CHL | NOV | VAN | RIF |
| *Pseudomonas aeruginosa* ATCC 27853 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 2.5 ±1.9 |
| *Pseudomonas aeruginosa* PAO1 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 15.5 ±3.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 2.6 ± 0.8 |
| *Escherichia coli* ATCC 25922 | 0.0 ± 0.0 | 28.7 ± 0.4 | 0.0 ± 0.0 | 18.5 ± 2.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 4.6 ± 0.5 |
| *Escherichia coli* K12 413 | 0.0 ± 0.0 | 31.5 ± 0.5 | 0.0 ± 0.0 | ND | 0.0 ± 0.0 | 4.3 ± 2.0 | 6.0 ± 1.0 |
| *Pasteurella multocida* ATCC 11039 | 0.0 ± 0.0 | 55.4 ± 2.2 | 1.7 ± 1.7 | 29.2 ±1.6 | 13.7 ± 2.7 | 0.0 ± 0.0 | 17.6 ±3.6 |
| *Pasteurella multocida* P-1581 | 0.0 ± 0.0 | 45.7 ± 1.8 | 0.0 ± 0.0 | ND | 23.5 ± 2.5 | 0.0 ± 0.0 | 18.9 ± 0.5 |

[a]The diameter of the zones of inhibition values were obtained after subtracting 6.0 mm, the diameter of the discs from the observed diameter. Each value is the mean of no fewer than 3 independent determinations.

[b]ETOH discs were prepared as control discs by aseptically loading sterile blank discs with 20µl of 95% ethanol then allowing to air day before application.

Abbreviations (potency): TCS, Triclosan (0.2 µg); CLI, Clindamycin (2 µg); CHL, Chloramphenicol (30µg); NOV, Novobiocin (5 µg); Van, Vancomycin (30 µg); RIF, Rifampin (5 µg).

[c] Boyina *et al*., unpublished data.

The opportunistically pathogenic *Serratia* species examined comprised three groups with regard to their relationships with hydrophobic antibacterial agents (Table 3).The first group (Table 3, Group 1) included *S. marcescens* ATCC 13880, dbll and, COA-1, *S. fonticola* ATCC 29844, *S. liquefaciens* ATCC 27592 and 35551, *S. ficaria* ATCC 33105, *S. grimessi* ATCC 14460, all of which were intrinsically resistant to all hydrophobic molecules tested including triclosan, despite the disparate mechanisms of action of the molecules. Such overall resistance to hydrophobic antibacterial compounds is consistent with the presence of refractory outer membranes having impermeability properties similar to those of *P. aeruginosa* ATCC 27853 and PAO1 (Table 2). Their antibiograms profiles differ from those of the two *E. coli.*

*S. plymuthica* ATCC 183 and 385, *S. rubidaea* ATCC 27593 and 33670, *S. quinovorans* ATCC 33763, and *S. entomophilia* ATCC 43750 comprised the second group (Table 3, Group 2). All were relatively resistant to some hydrophobic antibacterial agents, while being susceptible to others to include triclosan. These organisms lack the overall impermeability properties seen for the outer membranes of most gram-negative bacteria for hydrophobic substances. Intrinsic resistance for some of the compounds examined suggests the presence of specific resistant mechanisms for these compounds

The third group (Table 3, Group 3) included *S. odorifera* ATCC 33077, *S. proteamaculans* ATCC 19323, and *S. entomophilia* ATCC A1M02 which were susceptible to all hydrophobic antibacterial agents examined to include triclosan. These results are indicative of outer membranes which are atypically permeable for hydrophobic substances in organisms that lack resistant mechanisms for specific antibacterial compounds.

Table 3. Disk agar diffusion bioassay of mechanistically-disparate hydrophobic antibacterial agents for experimental *Serratia* spp.

| Organism | Inhibition zone (mm ± SD)[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | ETOH[b] | TSC | CLI | CHL | NOV | VAN | RIF |
| Group 1 | | | | | | | |
| *Serratia marcescens* ATCC 13880[c] | 0.0 ± 0.0 | 2.3 ± 0.2 | 0.0 ± 0.0 | 17.1 ± 0.8 | 0.2 ± 0.2 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| *Serratia marcescens* db11[c] | 0.0 ± 0.0 | 0.4 ± 0.6 | 0.00 ± 0.0 | 20.4 ± 1.1 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.4 ± 0.2 |
| *Serratia marcescens COA-1* | 0.0 ± 0.0 | 1.0 ± 1.7 | 0.0 ± 0.0 | 26.9 ± 1.9 | 4.0 ± 0.2 | 0.0 ± 0.0 | 5.9 ± 1.0 |
| S*erratia fonticola* ATCC 29844 | 0.0 ± 0.0 | 1.8 ± 0.4 | 0.0 ± 0.0 | 24.1 ± 1.5 | 0.0 ± 0.0 | 0.0 ± 0.0 | 1.8 ± 0.8 |
| S*erratia liquifacians* ATCC 27592 | 0.0 ± 0.0 | 2.7 ± 0.3 | 2.4 ± 1.2 | 21.5 ± 1.4 | 0.00 ± 0.0 | 0.00 ± 0.0 | 2.2 ± 0.3 |
| S*erratia liquifacians* ATCC 35551 | 0.0 ± 0.0 | 3.2 ± 0.2 | 4.8 ± 0.7 | 27.7 ± 1.8 | 0.0 ± 0.0 | 0.0 ± 0.0 | 2.4 ± 0.5 |
| S*erratia ficaria* ATCC 33105 | 0.0 ± 0.0 | 1.9 ± 0.1 | 16.3 ± 0.6 | 22.1 ± 1.2 | 0.0 ± 0.0 | 0.0 ± 0.0 | 1.5 ± 1.3 |
| S*erratia grimesii* ATCC 14460 | 0.0 ± 0.0 | 2.7 ± 0.6 | 3.5 ± 0.2 | 26.2 ± 4.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 2.4 ± 1.8 |

Table 3. Continued.

| Organism | Inhibition zone (mm ± SD)[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | ETOH[b] | TSC | CLI | CHL | NOV | VAN | RIF |
| Group 2 | | | | | | | |
| S*erratia plymuthica* ATCC 183 | 0.0 ± 0.0 | 16.8 ± 2.4 | 17.0 ± 1.5 | 39.7 ±6.3 | 10.9 ± 1.2 | 0.00 ± 0.0 | 10.8 ± 1.0 |
| S*erratia plymuthica 385* | 0.0 ± 0.0 | 17.1 ± 0.7 | 20.6 ± 0.6 | 29.6 ± 0.4 | 30.8 ± 1.4 | 17.5 ± 0.4 | 31.9 ± 1.0 |
| S*erratia rubidaea* ATCC 27593 | 0.0 ± 0.0 | 10.2 ± 1.8 | 0.0 ± 0.00 | 24.8 ± 1.0 | 6.8 ± 0.3 | 0.00 ± 0.0 | 2.8 ± 0.1 |
| S*erratia rubidaea* ATCC 33670 | 0.0 ± 0.0 | 18.1 ± 3.5 | 16.1 ± 4.3 | 23.1 ± 0.4 | 18.9 ± 8.1 | 13.7 ± 3.9 | 22.5 ± 6.2 |
| S*erratia quinovorans* ATCC 33763 | 0.00 ± 0.0 | 10.0 ± 2.8 | 4.9 ± 1.3 | 34.1 ± 2.1 | 0.1 ± 0.1 | 0.0 ± 0.00 | 9.6 ± 0.4 |
| Serratia entomophilia ATCC 43705 | 0.0 ± 0.0 | 1.2 ± 0.7 | 6.7 ± 0.4 | 17.1 ± 0.8 | 0.2 ± 0.4 | 0.00 ± 0.0 | 0.00 ± 0.0 |
| Group 3 | | | | | | | |
| S*erratia odorifera* ATCC 33077 | 0.0 ± 0.0 | 28.3 ± 1.5 | 4.1 ± 7.1 | 18.1 ± 2.8 | 4.12 ± 7.2 | 10.5 ± 1.5 | 11.7 ± 4.5 |
| *Serratia proteamaculans* ATCC 19323 | 0.0 ± 0.0 | 14.0 ± 1.51 | 19.8 ± 0.5 | 28.4 ± 1.3 | 26.8 ± 1.40 | 16.6 ± 1.3 | 30.8 ± 2.1 |
| S*erratia entomophilia* A1M02 | 0.0 ± 0.0 | 16.6 ± 0.9 | 23.2 ± 1.8 | 31.1 ± 2.5 | 30.1 ± 0.7 | 19.6 ± 1.2 | 33.4 ± 2.9 |

[a,b] See Table 6.

**Crystal violet binding hydrophobicity assay**

Cell surface hydrophobicity properties were determined on the basis of the degree to which macroscopic colonies of *Serrata* species were able to bind to the hydrophobic cationic stain crystal violet. Hydrophobic *P. multocida* P-1581 colonies in Figure 8B can be seen to bind to the crystal violet and appear dark violet, while hydrophilic *E. coli* ATCC 25922 colonies remain unstained and appeared white (Figure8 A). Data contained in Table 4 reveal colonies of hydrophilic control organisms *P. aeruginosa* ATCC 27853 (16) and *E. coli* ATCC 25922 (33) to have bound little or no crystal violet. In contrast, hydrophobic *P. multocida* P-1581 (16) colonies bound crystal violet and appeared dark violet. *Serratia* species selected on the basis of their disparate relationships with triclosan included *S. marcescens* ATCC 13880 appeared hydrophilic and slightly hydrophilic when cultured on MHA and BHIA respectively. *S. fonticola* ATCC 29844, and *S. odorifera* ATCC 33077 both remained unstained after growth on both media and were judged to be hydrophilic.

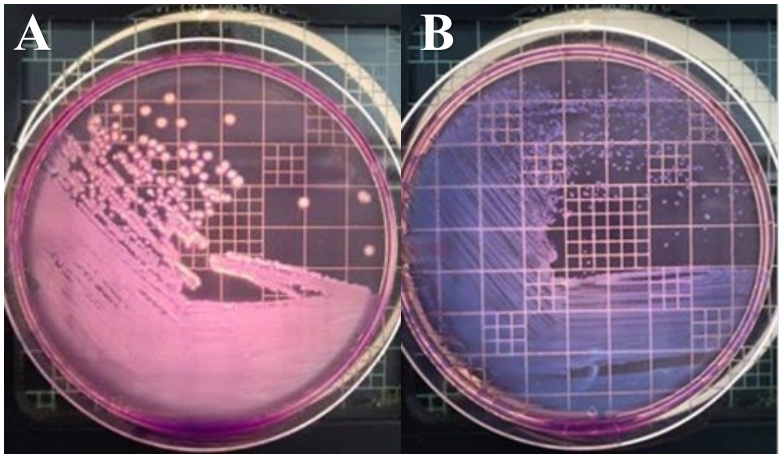


Figure 8. Representative crystal violent binding assay plates. Panel A, *E. coli* ATCC 25922 (hydrophilic); Panel B, *P. multocida* P-1581 (hydrophobic).

Table 4. Crystal violet binding bioassay of reference bacteria and experimental Serratia spp.

| Organism | CV binding [a] | |
|---|---|---|
| | MHA | BHIA |
| **Reference Organisms** | | |
| *Pseudomonas aeruginosa* ATCC 27853 | + | - |
| *Escherichia coli* ATCC 25922 | - | - |
| *Pasteurella multocida* P-1581 | ++ | ++ |
| **Experimental *Serratia* spp.** | | |
| *Serratia marcescens* ATCC 13880 | - | + |
| *Serratia fonticola* ATCC 29844 | - | - |
| *Serratia odorifera* ATCC 33077 | - | - |

[a]Degree of crystal violet binding by isolated colonies graded usually as: -, none (hydrophilic); +, slight (intermediate); ++, heavy (hydrophobic). Abbreviations: MHA, Mueller Hinton agar; BHIA, Brain Heart Infusion Agar.

**Hydrocarbon adherence assay**

Cell surface hydrophobicity properties of experimental *Serratia* species chosen on the basis of their disparate relationships with triclosan were determined on the basis of the degree to which they partition into n-hexadecane using the hydrocarbon adherence method developed by Rosenburg *et al*. (1980) as modified for use in our laboratory by Darnell *et al*.(1987) and Ruskoski and Champlin (2017). Figure 9 provides representative results for hydrophilic control organism *E. coli* ATCC 25922 (33) and hydrophobic control organism *P. multocida* P-1581 (16).

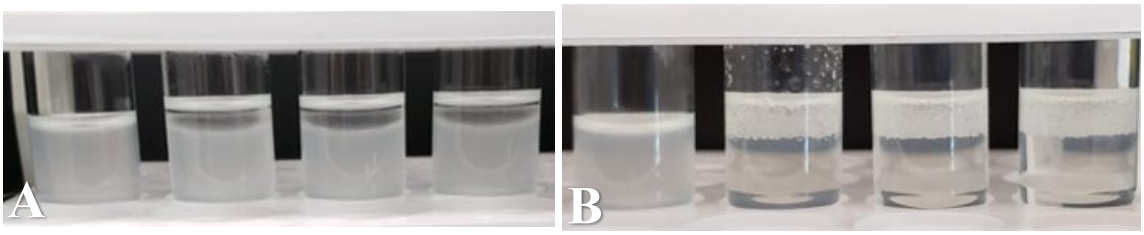


Figure 9. Representative hydrocarbon adherence assays. Panel A, *E. coli* ATCC 25922 (hydrophilic); Panel B, *P. multocida* P-1581 (hydrophobic).

The reference organisms *P. aeruginosa* ATCC 27853 (16) and *E. coli* ATCC 25922 (33) were used as hydrophilic controls and can be seen to be unable to partition into n-hexadecane in Figure 10, while *P. multocida* P-1581 served as a hydrophobic control (36). *S. marcescens* ATCC 13880, *S. fonticola* ATCC 29844, and *S. odorifera* ATCC 33077 cells partitioned into the n-hexadecane phase significantly less than did the hydrophobic control *P. multocida* P1581 and to a degree similar to that of the hydrophilic controls *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 .

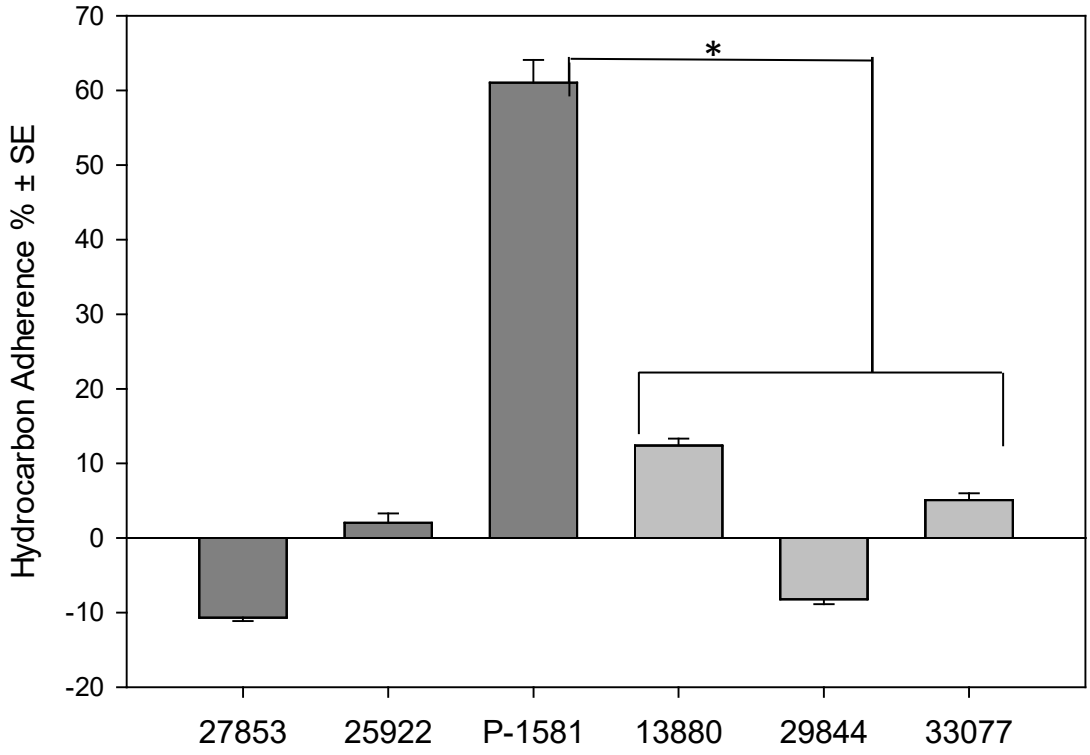


Figure 10. Hydrocarbon adherence bioassay. Cell surface hydrophobicity properties of reference and model system organisms were quantitatively accessed on the basis of the degrees to which cells partitioned into the n-hexadecane. Reference organisms (dark): *P. aeruginosa* ATCC 27853; *E .coli* ATCC 25922; *P. multocida* P-1581. Experimental organisms(light): *S. marcescens* ATCC 13880; *S. fonticola* ATCC 29844; *S. odorifera* ATCC 33077. Each value represents a mean of three to nine independent determinations ± SE. *, denotes $P< 0.001$ as determined using a one-way ANOVA and Tukey's post-hoc pairwise comparison.

**N-phenylnapthylamine partitioning assay**

Our modification (36,38) of the NPN uptake assay described by Helander and Mattila-Sandholim (2000) was employed to further confirm the cell surface hydrophobicity properties of the *Serratia* species chosen for additional experimentation. Relative fluorescence intensity was measured to assess the degree to which hydrophobic probe NPN was able to associate with and partition into hydrophobic portions of the outer cell surfaces.

*P. aeruginosa* ATCC 27853 (16) and *E. coli* ATCC 25922 (33) have outer cell surfaces which exhibited hydrophilic properties and were therefore used as negative controls. *P. multocida* P-1581 was employed as a positive control due its outer membrane accessibility to hydrophobic molecules (36). As can be seen in Figure 11, the relative fluorescence values of control bacteria reflected these properties when treated with NPN. The experimental *Serratia* species, *S. marcescens* ATCC 13880, *S. fonticola* ATCC 29844, and *S. odorifera* ATCC 33077 all exhibited relative fluorescence values comparable to those seen with the negative controls and were therefore determined to have a hydrophilic surface properties on the basis of this assay.

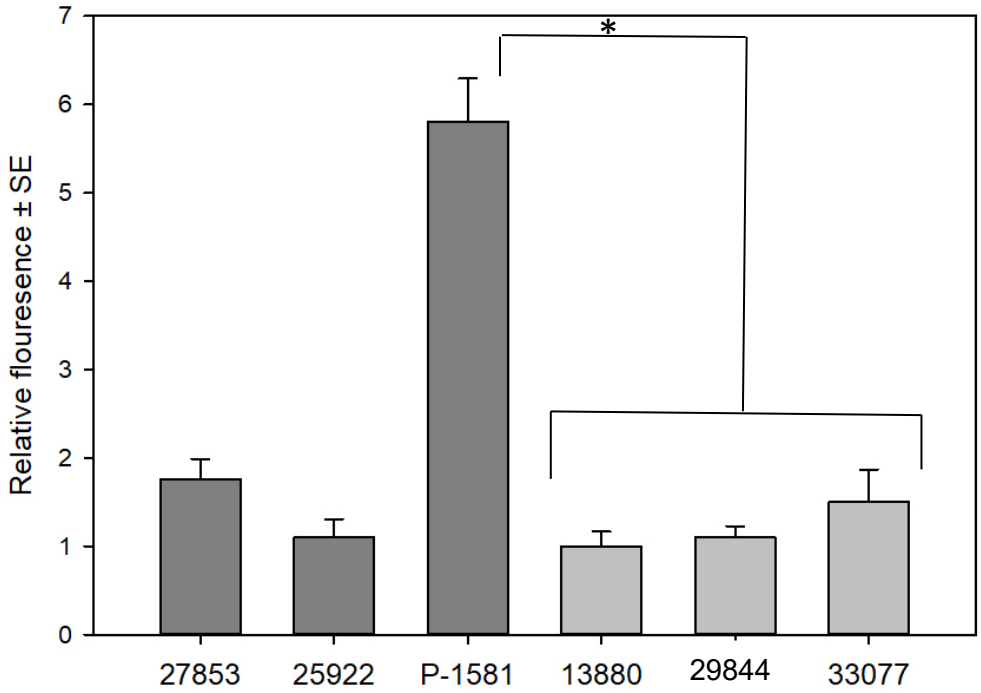


Figure 11. NPN uptake assay. Uptake by reference and model system organisms were quantitatively determined on the basis of the degree to which NPN partitioned into the outer cell envelope as assessed by relative fluorescence values after subtracting background levels. Reference organisms (dark): *P. aeruginosa* ATCC 27853; *E. coli* ATCC 25922; *P. multocida* P-1581. Experimental *Serratia* spp. (light): *S. marcescens* ATCC 13880; S. *fonticola ATCC 29844*; *S. odorifera* ATCC 33077. Each value represents the mean of three-to-nine independent determinations ± SD. *, denotes P< 0.001 as determined using a one-way ANOVA and Tukey's post-hoc pairwise comparison.

# CHAPTER V

# CONCLUSIONS

The disk agar diffusion bioassay data revealed that the *Serratia* species known to be able to infect humans exhibited disparate properties with regard to their ability to initiate growth in presence of mechanistically-disparate hydrophobic antibacterial agents. Because *Serratia* species *marcescens* ATCC 13880, *fonticola* ATCC 29844, liquefaciens ATCC 27592 and ATCC 35551, *ficaria* ATCC 33105, and *grimesii* ATCC 14460 were resistant to all hydrophobic antibacterial agents examined in a manner identical to that seen for the refractory control strains of *P. aeruginosa* we hypothesized that their respective outer membranes were impermeable to hydrophobic substances in general. However, these properties are not conserved among all pathogenic *Serratia* species. In order to examine the potential role of the gram-negative cell envelope in the intrinsic triclosan resistance of refractory species, their outer membrane were chemically modified using the outer membrane permeabilizer compound 48/80 in an effort to sensitize them to the hydrophobic biocide triclosan. A model system comprised of three

species representing the most dramatic examples of the disparate relationships withtriclosan was selected for further examination. *S. marcescens* ATCC 13880 and *S. fonticola* ATCC 29844 were both intrinsically resistant to hydrophobic compounds including triclosan. However, the former organism was only slightly susceptive to triclosan sensitization and was able overcome it and resume a normal growth rate, while the latter organism was extremely susceptive to sensitization. In contrast, *S. odorifera* ATCC 33077 represented the group of species that was susceptible to all hydrophobic molecules tested, including triclosan. Their cell surface hydrophobicity properties were then examined in order to obtain a better understanding of cell envelope physiology underlying the disparities seen amongst opportunistic pathogenic *Serratia* species and susceptivity or resistance to triclosan sensitization.

The crystal violet binding assay revealed that all three *Serratia* species possessed hydrophilic cell surfaces. Interestingly, *S. marcescens* ATCC 13880 was less hydrophilic than the others when cultivated on BHIA. The results were confirmed in a more quantitative manner by the hydrocarbon adherence and NPN assays. These data confirmed the hydrophilic nature of all three of the disparate *Serratia* species comprising the model system. Moreover, cell surface hydrophobicity properties are not directly related to susceptive to triclosan sensitization by chemical disruption of the outer membrane function or to being intrinsically resistant.

In summary, these data suggest that phenotypic differences seen in three opportunistic *Serratia* species with regard to intrinsic resistance to triclosan are at least partly due to disparate outer membranes exclusion potential. Moreover, susceptivity to triclosan

sensitization by outer membrane permeabilization appears not to be influenced by differences seen in cell surface hydrophobicity properties.

Future research will examine the influence of surface hydrophobicity of substrate surfaces on the propensities of model system organisms to form *in vitro* biofilms. Better understanding of how cell surface physiology properties of opportunistically pathogenic *Serratia* species relate to the proclivity to form and maintain biofilms will allow for a better understanding of the cellular and of the mechanisms underlying the pathogenicity of these organisms in human hosts.

# REFERENCES


1. Carey DE and Mcnamera PJ. 2015. The impact of triclosan on the spread of antibiotic resistance in the environment. Front Microbiol. 5:780. https://doi.10.3389/fmicb.2014.00780.

2. Bester K. 2005. Fate of triclosan and triclosan-methyl in sewage treatment plants and surface waters. Arch Envir Cotam Toxicol. 49:1-7. https://doi.10.1007/s00244-004-0155-4.

3. Singer H, Muller S, Tixier C, Pillonel L. 2002. Triclosan: occurrence and fate of a widely used biocide in aquatic environment: Field measurements in wastewater treatment plants, surface waters, and lake sediments. Environ Sci Technol. 36:4488-5004. https://doi. 10.1021/es025750i.

4. Mcmurry A, Oethinger M, Levy SB. 1998. Triclosan targets lipid synthesis. Nature. 394: 531-532. https://doi.10.1038/28970.

5. Nikaido H and Vaara M. 1976. Molecular basis of bacterial outer membrane permeability. Microbiol Rev. 49:1-32. https://doi. 10.1128/mmbr.67.4.593-656.2003.

6. Silhavy TJ, Kahne D, Walker S. 2010. The bacterial cell envelope. Cold Spring Harb Prespect Biol. 2:a000414. https://doi:org/10.1038/28970.

7. Delcour AH. 2009. Outer membrane permeability and antibiotic resistance. Biochim Biophys Acta. 1794(5): 808-816. https://doi.10.1016/j.bbapap.2008.11.005

8. Nikaido H. 1985. Outer membrane of *Salmonella typhimurium*. Transmembrane diffusion of some hydrophobic substance. Biochim Biophysics Acta. 433:118-132. https://doi. 10.1016/0005-2736(76)90182-6.

**9.** Hart ME and Champlin FR.1998. Susceptibility to hydrophobic molecules and phospholipid composition in *Pasteurella multocida* and *Actinobacillus lignieresii*. Antimicrob Agents Chemother. 32 (9):1354-9. https://doi. 10.1128/AAC.32.9.1354.

**10.** Champlin FR, Ellison ML, Bullard JW, Conrad RS. 2005. Effect of outer membrane permeabilisation on intrinsic resistance to low triclosan levels in *Pseudomonas aeruginosa.* Int J Antibacterial Agents. 26:59-164. https://doi.10.1016/j.ijantimicag.2005.04.020.

**11.** Hejazi A, Falkiner FR. 1997. *Serratia marcescens*. J Med Microbiol. 46: 903-912. https://doi.10.1099/00222615-46-11-903.

**12.** Kampfer P, Glaeser SP. 2016. *Serratia aquatilis* sp. nov. isolated from drinking water systems. Int. J. Syst. Evol. Microbiol. 56: 407-413. https://doi:10.1099/ijsem.0.000731.

**13.** Mahlen SD, 2011. *Serratia* infections from military experiments to current practice. Clin Microbiol Rev 24:755-791. https://doi.10.1128/CMR.00017-11.

**14.** Stock I, Grueger T, Wiedemann B. 2003. Natural antibiotic susceptibility of strains of *Serratia marcescens* and the *S. liquefaciens* complex *S. liquefaciens* sensu stricto, *S. proteamaculans* and *S. grimesii.* Int J Antibacterial Agents. 22:35-47. https://doi.10.1016/S0924-8579.

**15.** Krasowska A, Sigler K. 2014. How microorganisms use hydrophobicity and what does this mean for human needs? Front Cell Infect Microbiol. 4: 112. https://doi:10.3389/fcimb.2014.00112.

**16.** Darnell FR Hart ME, Champlin FR. 1987. Variability of cell surface hydrophobicity among *Pasteurella multocida* and *Actinobacillus lignieresii*.. J Clin Microbiol. 25:67-71. https://doi.10.1128/JCM.25.1.67-71.1987.

**17.** Denyr SP, Millard JY. 2002. Cellular impermeability and uptake of biocides and antibiotics in Gram-negative bacteria. J. Appl. Microbiol. 92: 35S–45S. https://doi.org/10.1046/j.1365-2672.92.5s1.19.x.

**18.** Boyina K, King B, Rigsbee A, Yang J, Sprinkles W, Patel V, McDonald A, Katz Amburn A, Champlin FR. Influence of outer membrane permeabilization on intrinsic resistance to the hydrophobic biocide triclosan in opportunistic *Serratia* species. In preparation.

**19.** Rosendberg M, Blumberger Y, Judes H, Bar-Ness R, Rubinstein E, Mazor Y. 1986. Cell Surface Hydrophobicity of Pigmented and Nonpigmented Clinical *Serratia marcescens* Strains. Infect Immun. 51: 932-935. https://doi:10.1128/IAI.51.3.932-935.1986.

**20.** Bar-Ness , Avarahamy N, Matsuyama T, Rosenberg M.1988. Increased Cell Surface Hydrophobicity of a *Serratia marcescens* NS 38 Mutant Lacking Wetting Activity. J Bacteriol. 170: 4361-4364. https://doi:10.1128/jb.170.9.4361-4364.1988.

**21.** Donlan RM. 2002. Biofilms microbial life on surfaces. Emerg Infect Diseases. 8**:**881-890. https://doi.10.3201/eid0809.020063..

**22.** Crouzet, M, Le Senechal, C, Brözel, VS, Costagliolo P, Barthe C, Bonneu M, Garby B, Vilian S. 2014. Exploring early steps in biofilm formation: set-up of an experimental system for molecular studies. BMC Microbiol. 14:253. https://10.1186/s12866-014-0253-z.

.

**23.** Magana M, Sereti C, Ioannidis A, Mitchell CA, Ball AR, Magiorkinis E, Chatzipanagiotou S, Hamblin MR, Hadjifrangiskou M, Tegos GP. 2018 Options and limitations in clinical investigation of bacterial biofilms. Clin Microbiol. Rev.31:e00084-16. https://doi.10.1128/CMR.00084-16.

**24.** Stewart PS. 2003. Diffusion in biofilms. J Bacteriol.185(5):1485–1491 .https://doi:10.1128/JB.185.5.1485–1491.

**25.** Rice SA, Koh KS, Queck SY, Labbate M, Lam KW, Kjelleberg S. 2005. Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. J Bacteriol. 187: 3477-3485.https://doi:10.1128/JB.187.10.3477-3485.2005.

**26.** Labbate M, Queck SY, Koh KS, Rice SA, Givskov M, Kjelleberg S. 2004. Quorum sensing-controlled biofilm development in *Serratia liquefaciens* MG1. J Bacteriol. 186**:**692-698. https://doi:10.1128/jb.186.3.692-698.2004.

**27.** Houdt RV, Moons P, Jansen A, Vanoirbeek, Michiels C. 2005. Genotypic and phenotypic characterization of a biofilm-forming *Serratia plymuthica* isolate from a raw vegetable processing line. FEMS Microbiol Lett. 15:265-272. https://doi.10.1016/j.femsle.2005.04.016.

**28.** Daneshvar HE, Alavi & Hansen LT. 2013. Kinetics of biofilm formation and desiccation survival of *Listeria monocytogenes* in single and dual species biofilms with *Pseudomonas fluorescens*, *Serratia proteamaculans* or *Shewanella baltica* on food-grade stainless steel surfaces. Biofouling 29**:** 1253-1268. https://doi.org/10.1080/08927014.2013.835805.

**29.** Koh KS, Lam KW, Alhede M, Queck SY, Labbate M, Kjelleberg S, Rice SA. 2007. Phenotypic Diversification and Adaptation of *Serratia marcescens* MG1 Biofilm-Derived Morphotypes. J Bacteriol. 189: 119-130. https://doi:10.1128/JB.00930-06.

**30.** Ruskoski SA, Champlin FR. 2020. Genetic assessment of extracellular polysaccharide and its effect on biofilm formation in *Burkholderia multivorons***.** J Med Microbiol. In preparation.

**31.** Clayborn AB, Toofan SN, Champlin FR. 2011. Influence of methylation on the antibacterial properties of triclosan in *Pasturella multocida* and *Pseudomonas aeruginosa* variant strains. J Hosp Infect. **77:**129-133. https://doi.10.1016/j.jhin.2010.09.021.

**32.** Buaduri S, Conway LK, Lachica V. 1987. Assay of crystal violet binding for rapid identification of virulent Plasmid-Bearing Clones of *Yersina enterocolitica.* J Clin Microbiol 25:1039-1042. https://doi.10.1128/JCM.25.6.1039-1042.1987

**33.** Rosenburg M, Gutnick D, Rosenberg E. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol Let. 9: 29-33. https://doi.10.1111/j.1574-6968.1980.tb05599.x.

**34.** Hellander IM, Metilla-Sandholm T. 2000. Fluorometric assessment of gram-negative bacterial permebilization. J appl Microbiol. 88:213-219. https://doi.org/10.1046/j.1365-2672.2000.00971.x

**35.** Ellison ML, Roberts AL, Champlin FR. 2007. Susceptibility of compound 48/80-sensitized *Pseudomonas aeruginosa* to the hydrophobic biocide triclosan. FEMS Microbial Lett. 269:295-300. https://doi.org/10.1111/j.1574-6968.2007.00640.x.

**36.** Ellison ML, Champlin FR. 2007. Outer membrane permeability for nonpolar antimicrobial agents underlies extreme susceptibility of *Pasturella multocida* to the hydrophobic biocide triclosan. Vet Microbiol. 124:310-318. https://doi.10.1016/j.vetmic.2007.04.038.

**37.** Bullard JW, Champlin FR, Burkus J, Millar SY, Conrad RS. Triclosan-induced modification of unsaturated fatty acid metabolism and growth in Pseudomonas aeruginosa PAO1. Curr Microbiol. 2011(62):697-702. https://doi.10.1007/s00284-010-9765-4.

**38.** Ruskoski SA, Champlin FR. 2017. Cell surface physiology and outer cell envelope impermeability for hydrophobic substances in *Burkholderia multivorons***.** J Med Microbiol. 66:965–971. https://doi.10.1099/jmm.0.000532.

**39.** Aljorayid A, Viau R, Castellino L, Jump RLP. Serratia fonticola, pathogen or bystander? A case series and review of the literature. IDCases. 2016(5):6-8.https://doi. 10.1016/j.idcr.2016.05.003.

**40.** Grohskopf LA, Roth VR, Feikin DR, Arduino MJ, Carson LA, Tokars JI, et al. Serratia liquefaciens bloodstream infections from contamination of epoetin alfa at a hemodialysis center. The New Eng J Med. 2001;344(20):1491-1497. https://doi.10.1056/NEJM200105173442001.

**41.** Ursua PR, Unzaga MJ, Melero P, Iturburu I, Ezpeleta C, Cisterna R. 1996. Serratia rubidaea as an invasive pathogen. J Clin Microbiol.34(1):216-217. https://doi. 10.1128/JCM.34.1.216-217.1996.

**42.** Bollet C, Grimont P, Gainnier M, Geissler A, Sainty J-M, De Micco P. 1993. Fatal pneumonia due to Serratia proteamaculans subsp. quinovora. J Clin Microbiol.31(2):444-445. https://doi.10.1128/JCM.31.2.444-445.1993

**43.** Chmel H. *Serratia odorifera* biogroup 1 causing an invasive human infection. J Clin Microbiol. 26(6):1244-1245. https//:doi.10.1128/JCM.26.6.1244-1245.1988.

**44.** Anahory T, Darbas H, Ongaro O, Jean-Pierre H, Mion P. Serratia ficaria: a misidentified or unidentified rare cause of human infections in fig tree culture zones. J Clin Microbiol. 36(11):3266-3272. https://doi.10.1128/JCM.36.11.3266-3272.1998.

**45.** Balakrishna AM, Mahto SK, Goel M, Goel A, Mitrolia B. 2018, Liver abscess caused by Serratia grimesii in an immunocompetent patient. Int J Res Med Sci. 6(5):1831-1832. https://doi.org/10.18203/2320-6012.ijrms20181789.

**46.** Carrero P, Garrote JA, Pacheco S, Garcia AI, Gil R, Carbajosa SG. Report of six cases of human infection by Serratia plymuthica. J Clin Microbiol. 33(2):275-276. https://doi.10.1128/JCM.33.2.275-276.1995.

VITA

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