Relationship Between Cell Surface Hydrophobicity and Biofilm Adhesion in Opportunistic *Serratia* Species with Disparate Susceptivity to Triclosan Sensitization

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Abstract: We have recently reported that ten opportunistically pathogenic *Serratia* species markedly differ regarding susceptivity to sensitization to the hydrophobic biocide triclosan by outer membrane permeabilization. Representative organisms exhibiting slight (*Serratia marcescens*), complete (*Serratia fonticola*), transitorily complete (*Serratia liquefaciens*), and intermediate (*Serratia rubidaea*) susceptivity were selected for further analysis. The purpose of the present study was to determine if such phenotypic susceptivity is related to cell surface hydrophobicity properties and the proclivity to implement biofilm adhesion. Hydrocarbon adherence and hydrophobic fluorescent probe assays were employed to quantitate cell surface hydrophobicity properties, while an *in vitro* biofilm assay was used to assess adhesion of planktonic cells to a nonpolar substrate. While *S. rubidaea* was seen to be extremely hydrophobic, *S. marcescens* and *S. liquefaciens* were slightly to moderately hydrophobic, and *S. fonticola* was relatively hydrophilic. *S. rubidaea* adhered to the polystyrene substrate more readily than *S. fonticola* or *S. liquefaciens*, while *S. marcescens* adhesion was intermediate. These data do not support the notion that the degree of susceptivity to triclosan sensitization by outer membrane permeabilization is directly related to cell surface hydrophobicity. However, the initial adhesion stage of biofilm formation appears to be influenced at least in part by cell surface hydrophobicity properties.

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Introduction

Triclosan (5-choro-2-(2,4-dichlorophenoxy) phenol) is a hydrophobic diphenyl ether biocide (Figure 1). It possesses broad spectrum antibacterial activity that is utilized as an antiseptic or preservative in many medical, personal care, industrial, and household settings. Municipal water treatment processes are relatively ineffective at triclosan removal due to the stability of the biocide combined with its widespread use, thereby resulting in its environmental accumulation as a pollutant (Thompson et al. 2005; Bester 2005; Welsch and Gillock 2011; Dhillon et al. 2015; McNamara and Levy 2016). The mechanism of action of triclosan involves inhibition of enoyl-acyl carrier protein reductase, an essential cytoplasmic enzyme involved in bacterial fatty acid biosynthesis (McMurry et al. 1998). 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 passively partition through the phospholipid bilayer of the cytoplasmic membrane into the cytoplasm due to its hydrophobic nature.

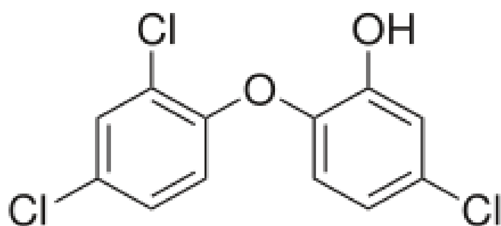

Figure 1. Molecular structure of triclosan.

As a broad-spectrum antibacterial agent (Dhillon et al. 2015), triclosan must passively traverse the outer membrane of gram-negative bacteria despite the fact most are typically impermeable to hydrophobic substances due to the asymmetric presence lipopolysaccharide in the outer leaflet (Nikaido 2003; Silhavy et al. 2010). However, the gram-negative nosocomial opportunists *Pseudomonas aeruginosa* and *Serratia marcescens* are markedly resistant (Jones et al. 2006; Schweizer 2001) and outer membrane impermeability has been shown to contribute significantly to the underlying resistance mechanism in both organisms (Boyina et al. 2023; Champlin et al. 2005; Ellison et al. 2007).

A plethora of published information exists regarding the basic biology and pathogenic properties of the *Serratia* type species *S. marcescens*. In contrast, a paucity of such research exists for other species in the genus which have also been shown to be etiologic agents in humans. Our laboratory has recently reported that 10 *Serratia* species capable of opportunistic pathogenicity differed dramatically regarding their relationship with triclosan by exhibiting phenotypes ranging from intrinsically resistant to extremely susceptible (Boyina et al. 2023). Moreover, resistant species exhibited disparate susceptivity levels to triclosan sensitization using compound 48/80, a cationic detergent that selectively permeabilizes the gram-negative outer membrane (Katsu et al. 1985). These data supported the overall notion that while outer membrane impermeability for hydrophobic substances was involved to different degrees in the resistance mechanisms of phenotypically disparate species, ancillary resistance mechanisms appeared to play supportive roles in some species and constitutive multi-drug efflux systems were suspected.

The disparate synergistic relationships between triclosan and compound 48/80 seen among the refractory species are suggestive of potential outer cell envelope characteristics which could influence the expression of virulence factors such as the proclivity to form biofilms. In order to more fully investigate this possibility, species representing the different susceptivity phenotypes were selected for further analysis of their cell surface hydrophobicity (CSH) and biofilm formation properties in the present study. CHS 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 (Krasowska and Sigler 2014). Evidence for this relationship has been published for *S. marcescens* (Rosenberg et al. 1986), but similar research is lacking for other known biofilm-forming *Serratia* species

to include *Serratia liquefacians* (Remuzgo-Martinez et al. 2015), *Serratia plymuthica* (Van Houdt et al. 2005), and *Serratia proteamaculans* (Alavi and Hansen 2013).

Additional research is necessary to determine if the physiological consequences of gram-negative CSH properties influence the ability of pathogenic *Serratia* species to adhere to substrates in the initial adhesion step of biofilm formation. We have previously shown CSH to be particularly important in this regard for the pulmonary gram-negative opportunist *Burkholderia multivorans* (Ruskoski and Champlin 2017; Ruskoski et al. 2024). While production of extracellular polysaccharide (EPS) was seen to be necessary for the maturation of stable *in vitro* biofilms, its production appeared to be down regulated in order not to interfere with the initial attachment stage.

We hypothesized that CSH properties underlie (a) the disparate susceptivity to triclosan sensitization by perturbation of outer membrane impermeability for hydrophobic substances and (b) the initial adhesion stage of biofilm formation in opportunistically pathogenic *Serratia* species. The purpose of the present investigation was to quantify the CHS properties of four triclosan sensitization variant *Serratia* species and examine their respective proclivities for initiating biofilm formation on a hydrophobic polystyrene substrate.

Materials and Methods

Bacterial isolates, maintenance, and cultivation conditions: *S. marcescens* ATCC 13880, *Serratia fonticola* ATCC 29844, *S. liquefaciens* ATCC 27592, and *Serratia rubidaea* ATCC 27593 (Table 1) were obtained from the American Type Culture Collection (Manassas, VA) and are the type strains of their respective species. *E. coli* ATCC 25922 and *Pasteurella multocida* P-1581 are maintained in our laboratory and were included for comparative control purposes. All cultures were stored under cryoprotective conditions as described elsewhere (Darnell et al. 1987).

Working cultures were obtained by streak inoculating Mueller Hinton Agar (MHA; Becton, Dickinson and Co., Sparks, MD) plates with cells from cryopreserved stock cultures and incubating for 18 h at 37°C prior to storing at 4°C until needed. Starter cultures were prepared by inoculating 20 mL of Mueller Hinton Broth (MHB: Becton Dickinson and Co.) contained in 125-mL screw-capped glass Erlenmeyer flasks with working culture cells and incubating for 12-15 h at 37°C and 180 rpm (Excella E24 incubator shaker; New Brunswick Scientific, Edison, NJ) to provide stationary-phase inocula for experimental test cultures.

Disk agar diffusion bioassay: Susceptibility of *S. marcescens* ATCC 13880 and *E. coli* ATCC 25922 to triclosan and four hydrophobic antibiotics was assessed using a standardized disk agar diffusion bioassay routinely employed in our laboratory (Clayborn et al. 2011). Starter culture cells were used to inoculate MHB test cultures which were incubated at 37°C and 180 rpm (Excella E24 shaker incubator) until an OD_{620} of 0.10 (Spectronic 20D+ optical spectrophotometer; Thermo Fisher Scientific Inc., Waltham, MA) was obtained.

Adhesive ring formation: Overnight starter cultures of the test *Serratia* spp. were examined visually for rings of biomass adhering to glass growth flasks after incubation at 37°C for 12-15 h with rotary aeration at 180 rpm in an Excella E24 shaker incubator.

Hydrocarbon adherence: CHS properties were quantitated by assessing the degree to which test culture cells adhered to the hydrocarbon n-hexadecane using the method of Rosenberg et al. (1980) as modified for use in our laboratory (Thies and Champlin 1989; Ruskoski and Champlin 2017). Test culture inocula were prepared by inoculating 210 mL of MHB with starter culture cells to an OD_{620} of 0.05 (Spectronic 20D+ optical spectrophotometer). Aliquots of 100 mL were dispensed into each of two 250-mL screw-capped culture flasks which were incubated at 37°C and 180 rpm (Excella E24 shaker incubator) until late exponential-phase growth was obtained (OD_{620} of approx.

0.40).

NPN uptake: Confirmation of CSH results was accomplished by assessing the degree to which test culture cells associated with the hydrophobic fluorescence probe 1-*N*-phenylnaphthylamine (NPN) using the method of Helander and Matilla-Sandholm (2000) as modified for use in our laboratory (Ellison and Champlin 2007; Ruskoski and Champlin 2017). Test culture inocula were prepared by inoculating 50 mL of MHB in 125-mL screw-capped culture flasks with starter culture cells to an OD_{620} of 0.05 (Spectronic 20D+ optical spectrophotometer). Flasks were incubated at 37°C and 180 rpm (Excella E24 shaker incubator) until mid-to-late exponential-phase growth was obtained (OD_{620} of approx. 0.30 to 0.40).

***In vitro* biofilm formation:** The proclivity of test culture cells to carry out the initial adhesion stage of *in vitro* biofilm formation was determined using a conventional μ-titer plate assay (O'Toole and Kolter 1998) as modified for use in our laboratory (Ruskoski et al. 2024). Test culture inocula were prepared by titrating 5.0-mL volumes of MHB contained in 18-mm diameter Kimax culture tube/sample holders with starter culture cells to an OD_{620} of 0.25 (Spectronic 20D+ optical spectrophotometer). Aliquots of 100 μL were dispensed into appropriate wells in hydrophobic (untreated) polystyrene 96-well microtiter plates (Cellstar; Greiner Bio-One Inc., Monroe, NC) which were placed in a closable plastic container with a beaker of deionized water and incubated at 37°C for 4 h.

Statistical analysis: All quantitative values represent the means of three-to-four independent determinations ± standard deviation. Differences between means were analyzed for statistical significance using SigmaPlot® version 15.0 (SigmaPlot Graphing Software, Palo Alto, CA) by determining *P* values using one-way ANOVA with Holm-Sidak comparisons. *P* values of less than 0.05 indicated statistical differences at the 5.0 % confidence level.

Results and Discussion

Previous work in our laboratory revealed outer membrane exclusionary properties of four different *S. marcescens* strains to be responsible to different degrees for their shared intrinsic resistance to the hydrophobic biocide triclosan (Boyina et al. 2023). Data in Figure 2 reveal the *S. marcescens* type strain to be resistant to five mechanistically disparate hydrophobic antibacterial agents in a manner like that seen for *E. coli* ATCC 25922 which differs only in being susceptible to triclosan. Such uniform resistance to antibacterial compounds having different mechanistic targets supports the notion that the *S. marcescens* outer membrane is generally impermeable to hydrophobic substances.

Boyina et al. (2023) reported that all but one of 10 *Serratia* species known to be capable of opportunistic pathogenicity are intrinsically

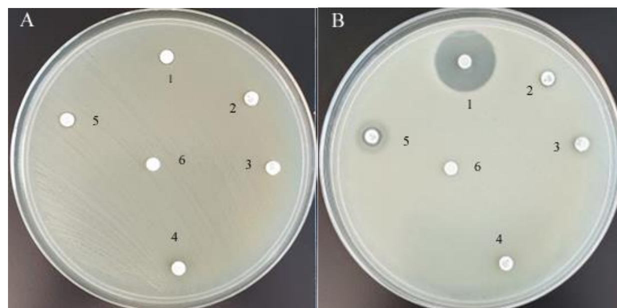

Figure 2. Disk agar diffusion bioassay for triclosan and representative hydrophobic antibiotics. Panel A, *S. marcescens* ATCC 13880; Panel B, *E. coli* ATCC 25922. Disk (potency): 1, triclosan (0.2 µg); 2, novobiocin (5.0 µg); 3, vancomycin (30 µg); 4, clindamycin (2.0 µg); 5, rifampin (5.0 µg), 6, ethanol control (95 %).

resistant to the hydrophobic biocide triclosan. However, they differed markedly regarding their susceptivity to triclosan sensitization by outer membrane permeabilization using the cationic detergent compound 48/80. These disparities formed the basis for the development of an intrinsic resistance model system consisting of species representing four phenotypically different groups to include (i) *S. marcescens* ATCC 13880, slight susceptivity; (ii) *S. fonticola* ATCC 29844, complete susceptivity; (iii) *S. liquefaciens* ATCC 27592, transitory complete susceptivity; and (iv) *S. rubidaea* ATCC 27593, moderate susceptivity (Table 1).

Initial evidence for the possibility that these species might differ regarding their proclivities to bind to nonpolar surfaces was provided by the observation that they bound borosilicate glass growth flasks to different degrees during batch cultural growth (Figure 3). When viewed with the data presented in Figure 5 below, it can be seen that the more hydrophobic organism *S. rubidaea* formed a heavy biomass ring around the growth flask, while the less hydrophobic species *S. marcescens* and *S. liquefaciens* formed slight to moderate rings. The hydrophilic organism *S. fonticola* failed to form a biomass ring. Because glass is more hydrophobic than the aqueous medium, these data support the conclusion that ring formation is dependent on the CSH properties of the organisms.

These observations allowed for the hypothesis that outer CSH properties underlie the disparate susceptivity levels to triclosan sensitization by perturbation of outer membrane impermeability for hydrophobic substances represented by the four species under examination. This hypothesis was tested by quantitatively determining their cell surface hydrophobicity properties by assessing the degree to which they were able to adhere to the hydrocarbon n-hexadecane using the hydrocarbon adherence method of Rosenberg et al. (1980) as modified for use in our laboratory (Thies and Champlin 1989; Ruskoski and Champlin 2017). This is a more direct measure of cell surface hydrophobicity than other methods because it is a simple assessment of the amount of biomass that is able to partition into a nonpolar hydrocarbon phase within the restrictions of the assay protocol. There are fewer if any opportunities for secondary effects which could result in anomalous falsely positive results. Validation was provided by conducting a control experiment that employed two organisms for which this method had previously been employed (Figure 4).

As can be seen in Figure 5, *S. fonticola* is hydrophilic relative to the other organisms as it exhibited very little partitioning into the hydrocarbon. *S. marcescens* and *S. liquefaciens* are moderately hydrophobic while *S. rubiaea* can be seen to be extremely hydrophobic with over 80 % of its biomass partitioned into the hydrocarbon. These data do not support the notion that the degree of susceptivity to triclosan sensitization by outer membrane permeabilization (Boyina et al. 2023) is directly related to cell surface hydrophobicity properties due to the absence of correlation.

The relationship between CSH properties and triclosan sensitization by perturbation of outer membrane impermeability for hydrophobic substances was further tested by assessing the degree to which the selected *Serratia* species were able to associate with the hydrophobic fluorescence probe NPN using the method

Figure 3. Representative starter cultures exhibiting disparate degrees of biomass ring formation on flask sides.

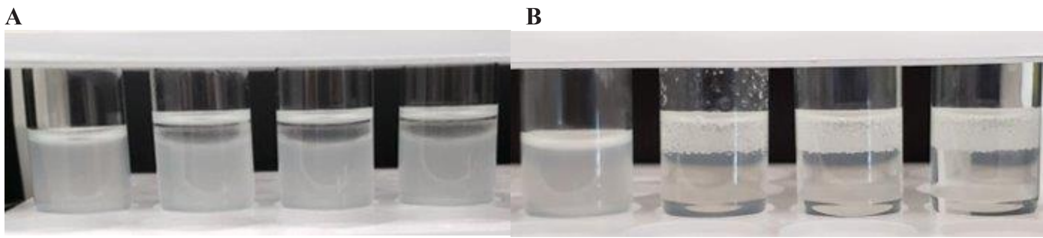

Figure 4. Control hydrocarbon adherence assays. Panel A, *E. coli* ATCC 25922 (hydrophilic; Rosenberg et al. 1980); Panel B, *P. multocida* P-1581 (hydrophobic, Darnell et al. 1987).

of Helander and Mattila-Sandholm (2000) as modified for use in our laboratory (Ellison and Champlin 2007; Ruskoski and Champlin 2017) (Figure 6). *S. marcescens*, *S. fonticola*, and *S. liquefaciens* exhibited similar CSH levels which were clearly less than that of *S. rubidaea*. These data support the conclusion reached with the hydrocarbon adherence method in that there is no direct correlation between association with the hydrophobic probe and susceptivity to triclosan sensitization by outer membrane permeabilization (Boyina et al. 2023).

Based on these findings, a hypothesis was formulated that posited outer cell surface hydrophobicity properties underlie the initial adhesion stage of biofilm formation in opportunistically pathogenic *Serratia* species. This hypothesis was tested by determining the degree to which the selected *Serratia* species were able carry out the initial adhesion stage of biofilm formation by assessing adherence to polystyrene at 4 h post-inoculation using a conventional μ-titer plate assay (O'Toole and Kolter 1998) as modified for use in our laboratory (Ruskoski et al. 2024) (Figure 7).

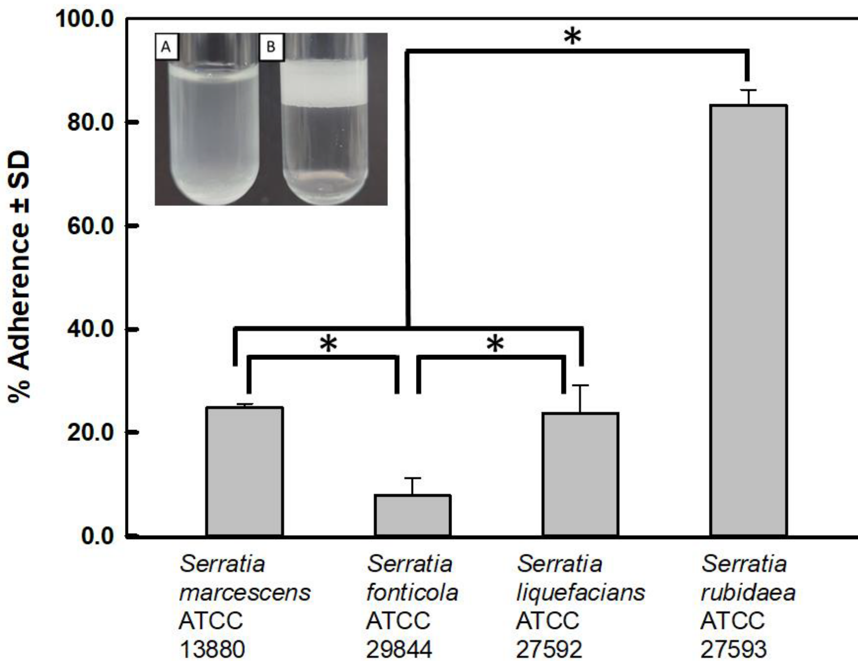

Figure 5. Cell surface hydrophobicity properties determined based on the degree to which cells associated with n-hexadecane as measured turbidimetrically using the method of Rosenberg et al. (1980) as modified by Ruskoski and Champlin (2017). Inset: Representative untreated control (A) and test (B) samples of *S. marcescens* ATCC 13880.

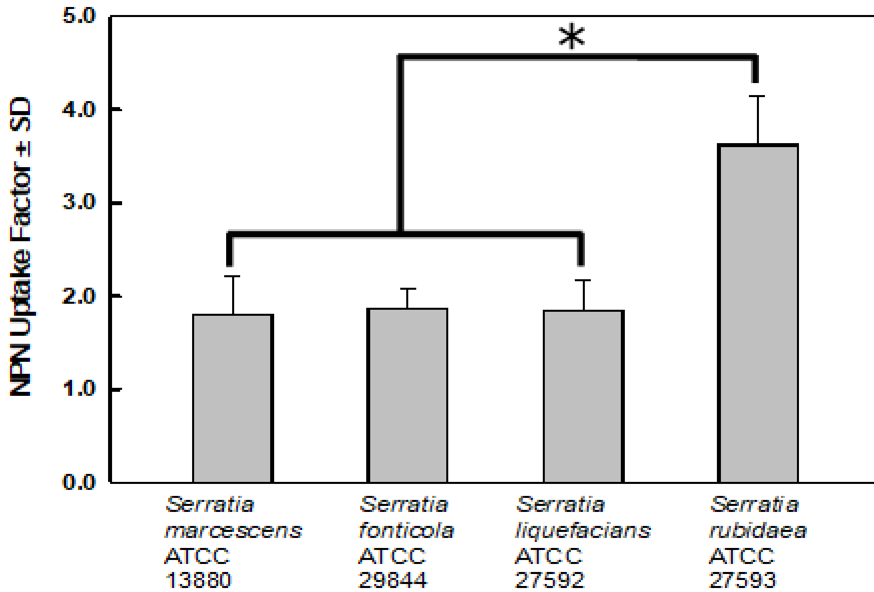

Figure 6. Cell surface hydrophobicity properties determined based on the degree to which NPN partitioned into outer membranes as measured fluorometrically using the method of Helander and Mattila-Sandholm (2000) as modified by Ruskoski and Champlin (2017).

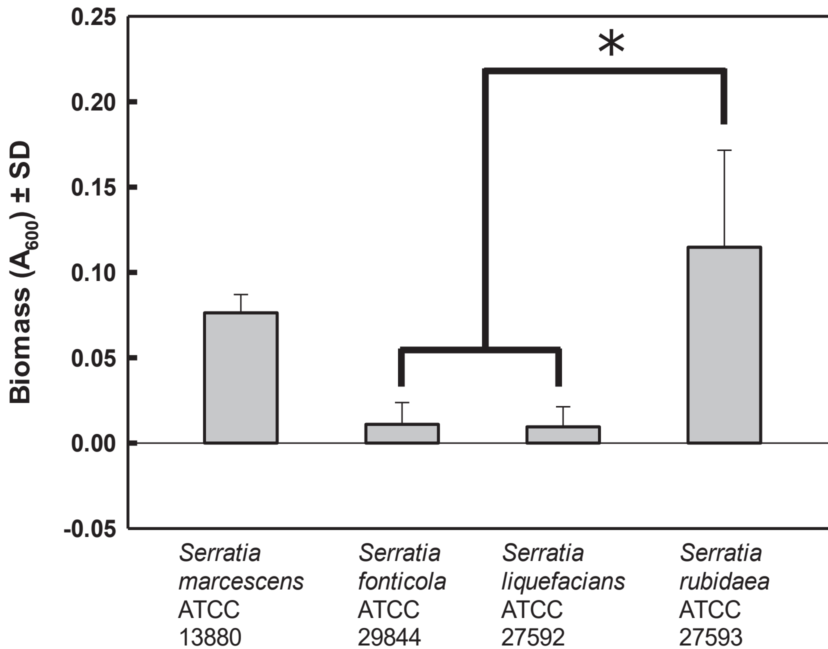

Figure 7. Initial biofilm adhesion (4.0 h) based on the amount of biomass adherence to hydrophobic (untreated) polystyrene microtiter plates as measured spectrophotometrically using the method of O'Toole and Kolter (1998) as modified by Ruskoski and Champlin (2024).

Table 1: Summarized results for *Serratia* model system species employed for this study.

Organisms[a]	Source	Phenotypic susceptivity to triclosan sensitization	Cell surface hydrophobicity	Biofilm adhesion
S. marcescens ATCC 13880	Environment	Slight susceptivity	Hydrophobic	Moderate
S. fonticola ATCC 29844	Environment	Complete susceptivity	Hydrophilic	Slight
S. liquefaciens ATCC 27592	Milk	Transitory complete susceptivity	Hydrophobic	Slight
S. rubidaea ATCC 27593	Unknown	Moderate susceptivity	Extremely hydrophobic	Heavy

[a]Representative *Serratia* species selected for the present study on the basis of the degree to which they exhibited susceptivity to triclosan sensitization with outer membrane permeabilizer compound 48/80 (Boyina et al. 2023).

S. rubidaea, the most hydrophobic organism in the model system (Figures 5 and 6), adhered most efficiently to the hydrophobic polystyrene substrate in the initial adhesion stage of biofilm formation. *S. marcescens* exhibited intermediate CSH properties and was the second most adherent. Both organisms formed heavy biomass rings in growth flasks (Figure 3). *S. liquefaciens* also exhibited intermediate CSH properties but was only slightly adherent to both glass growth flasks and the polystyrene biofilm substrate. *S. fonticola* was the most hydrophilic bacterium, produced no biomass rings, and was only slightly adherent to the polystyrene biofilm substrate. *Serratia odorifera*, an organism not included in the present model system, was determined to be the most hydrophilic bacterium tested, unable to form biomass rings on glass growth flasks, and nonadherent in the biofilm attachment assay (data not shown). These data suggest that factors exposed on the surfaces of these bacteria which influence CSH properties are involved in the cellular and molecular mechanisms underlying the first stage of biofilm formation on hydrophobic substrates (Table 1). However, they do not directly correlate with cell envelope factors which determine the susceptivity of the individual *Serratia* species to triclosan sensitization by chemical permeabilization of their outer membranes.

Conclusion

The degree of susceptivity to triclosan sensitization by outer membrane permeabilization in opportunistically pathogenic *Serratia* species as previously reported by our laboratory (Boyina et al. 2023) is not directly related to CSH properties. However, phenotypic differences in the initial adhesion stage of *in vitro* biofilm formation appear to be influenced to some degree by the propensity with which the bacterial cell surface is able to associate with hydrophobic substances.

Acknowledgements

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