Relationship Between Susceptivity to Triclosan Sensitization by Outer Membrane Permeabilization and Cell Surface Hydrophobicity Properties in Opportunistically Pathogenic *Serratia* Species

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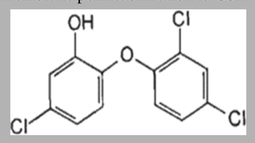
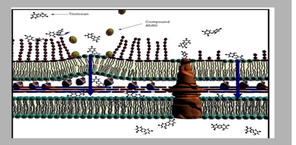
ABSTARCT

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 impermeability for triclosan differs dramatically among other opportunistically pathogenic *Serratia* species. Moreover, susceptivity to sensitization to triclosan by outer membrane premeabilization 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 premeabilization in selected species as we further characterize their cell surface properties in anticipation of their propensities to form *in vitro* biofilms. Three *Serratia* species (*marcescens*, *fonticola*, and *odorifera)* exhibiting disparate degrees of susceptivity to triclosan sensitization by outer membrane premeabilization were examined to determine their susceptibility levels to mechanistically-disparate hydrophobic molecules and their CSH properties. Intrinsic resistance to hydrophobic antibacterial agents was assessed using a standardized disk agar diffusion bioassay. CSH was determined using conventional crystal violet binding, hydrocarbon adherence, and 1-N-phenylnapthylamine uptake assays routinely employed in this laboratory. *S. marcescens* and *S. fonticola* were intrinsically resistant to all mechanistically-disparate hydrophobic antibacterial agents examined to include triclosan, while *S. odorifera* was susceptible. The CSH properties of all these differed only slightly, despite the disparate susceptivities of the two triclosan-resistant species to triclosan sensitization. These data suggest that phenotypic differences seen in three opportunistic *Serratia* species with regard to intrinsic resistance to hydrophobic antibacterial agents in general, and triclosan specifically are at least due in part to disparate abilities of their outer membranes to exclude hydrophobic substances. Moreover, susceptivity to triclosan sensitization by outer membrane premeabilization in the triclosan-resistant species *S. marcescens* and *S. fonticola* appears not to be influenced by differences in cell surface hydrophobicity properties.

INTRODUCTION

Triclosan (TCS) 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 *Pseudomonas aeruginosa* and *Serratia marcescens*. Previous work in our laboratory has shown that intrinsic resistance to TCS 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 susceptivity of sensitization to TCS by outer membrane permeabilization to CSH.





METHODS

Hydrocarbon adherence method

1. Prepare working cultures.
2. Inoculate starter cultures for 15-18hrs.
3. Inoculate 210 mL of MHB with starter cultures let grow to late exponential phase
4. Harvest cell suspensions and centrifuge (1200 x G) for 12 minutes
5. Aspirate supernatant and wash cells in 200 mL of cold PPMS buffer
6. Centrifuge washed cells as before
7. 1 ml of hexadecane and 4 mL of standardized cell suspension are added to 3 borosilicate tubes. A forth tube only gets 4 mL of cell suspension. Each mix is vortexed 4 times in 15 sec bursts.
8. Let sit for 15 mins.
9. Lower aqueous cell suspensions are removed from tubes and the turbidity is measured
10. Measure % Adherence

Crystal Violet Binding

1. Prepare working cultures
2. Streak Muller Hinton agar plates and Brain heart infusion plates .Incubate for 18 hrs. at 37 C
3. Gently flood plates with 8.0 mL of crystal violet solution for 2 min and decant
4. The binding of CV to hydrophobic colonies is observed by dark violet appearance . Hydrophilic colonies remain unstained and appear white.

NPN Assay

1. Prepare working cultures.
2. Inoculate starter cultures for 15-18hrs.
3. Inoculate 50 mL of MHB with starter cultures and let grow until late-exponential phase.
4. Harvest cell suspensions and centrifuge (1200 x G) for 12 minutes.
5. Aspirate supernatant and suspend cells into HEPES buffer.
6. Using a black microtiter plate place control, NPN control, HEPES control and experimental cultures in appropriate wells.
7. Measure fluorescence intensity using BioTek microtiter plate reader.
8. Calculate relative fluorescence.

RESULTS

Fig 1. Hydrocarbon Adherence Assay. *P. multocida P-1581*

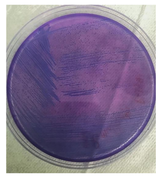


Fig 2. Crystal Violet Binding. *P. multocida P-1581*

Table 1. Susceptibility of Test Organisms to Triclosan.

Organism	Disc Agar Diffusion Inhibition Zone (mm ±SD[1])	Micro Broth Dilution MIC (µg/mL)	Macro Broth Dilution MBC (µg/mL)
Control			
E. coli ATCC 25922	0	<0.25	<0.25
E. coli K-12 413	ND	ND	ND
P. multocida P-1581	ND	ND	ND
Experimental			
S. marcescens ATCC 13880	2.30 ± 0.21	64	>64
S. *fonticola* ATCC 9844	1.81 ± 0.38	16	32
S. *odorifera* ATCC 33077	28.27 ± 1.46	4.0	8.0

[1]Diameter of the zones of growth inhibition after subtracting disc diameter (6.0 mm); each represented the mean of a minimum of three independent determinations ± SD.
Abbreviations (potency): TCS, triclosan (0.2 µg).

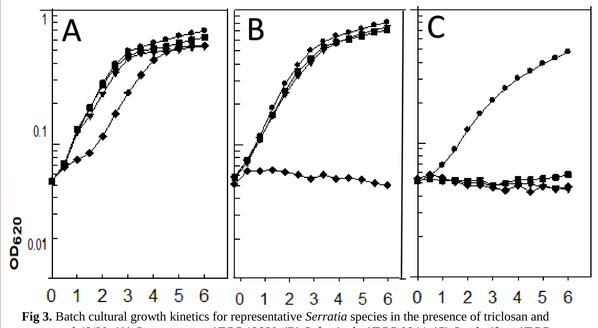


Fig 3. Batch cultural growth kinetics for representative *Serratia* species in the presence of triclosan and compound 48/80. (A) *S. marcescens ATCC 13880*, (B) *S. fonticola* ATCC 9844, (C) *S. odorifera* ATCC 33077. Symbols: (●) control MHB; (▼) compound 48/80 (2.5 µg/ml); (■) triclosan (2.0 µg/ml); (◆) triclosan plus compound 48/80.

Table 2. Effect of Compound 48/80 Outer Membrane Permeabilizer On Intrinsic Resistance To the Hydrophobic Biocide Triclosan.

	Measurement of growth[1]			
Organism	ETOH	Cpd 48/80	TCS	Cpd 48/80
Control				
E. coli ATCC 25922	+++	+++	0	0
E. coli K-12 413	ND	ND	ND	ND
P. multocida P-1581	ND	ND	ND	ND
Experimental				
S. marcescens ATCC 13880	+++	+++	+++	++
S. *fonticola* ATCC 9844	+++	+++	+++	0
S. *odorifera* ATCC 33077	+++	0	0	0

[1]Overall batch cultural growth obtained in the presence of compound 48/80, triclosan, and compound 48/80 plus triclosan as judged from turbidimetric growth curves (see Figures 1 and 2 for examples) and graded as 0, none; +, slight; ++, moderate; +++, control).
Abbreviations: ETOH, ethanol control; Cpd 48/80, Compound 48/80; TCS, triclosan.

Table 3. Cell Hydrophobicity Bioassays.

Control			
E. coli ATCC 25922	-2.02 ± 2.20	-	-
E. coli K-12 413	-2.70 ± 2.28	+/-	+/-
P. multocida P-1581	61.05 ± 8.61	++	++
Experimental			
S. marcescens ATCC 13880	12.36 ± 1.36	-	-
S. *fonticola* ATCC 9844	-8.20 ± 0.88	-	-
S. *odorifera* ATCC 33077	5.05 ± 1.32	-	-

[1]Symbols: -, hydrophilic; +/-, intermediate; ++, hydrophobic

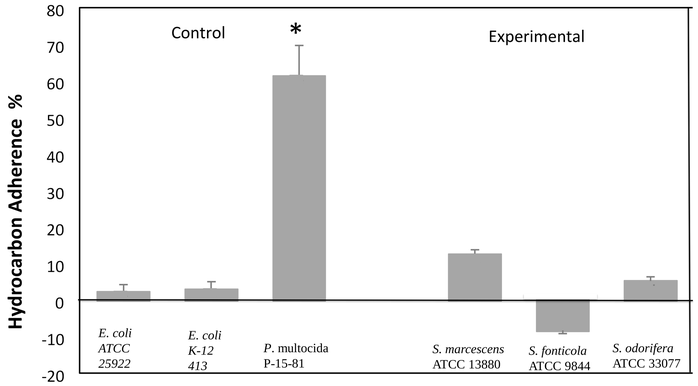


Fig 4. Hydrocarbon Adherence Assay Histogram.

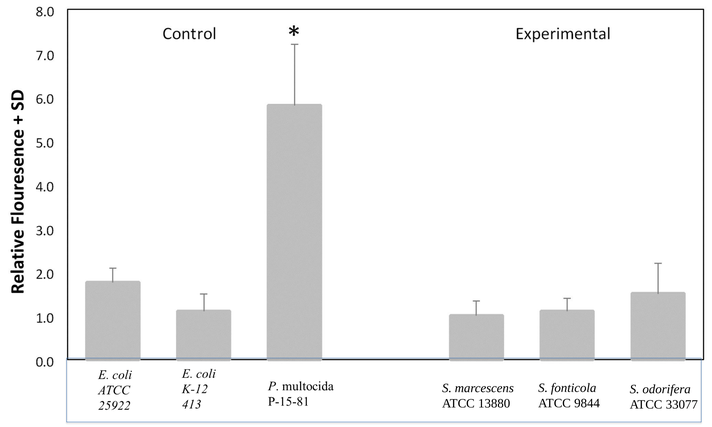


Fig 5. NPN histogram

CONCLUSION

A. Disparate *Serratia* species differed with regard to their intrinsic resistance levels to triclosan.

B. Disparate *Serratia* species differed with regard to the degree to which they were susceptive to triclosan by outer membrane permeabilization.

C. Hydrocarbon adherence assay results reveled that all *Serratia* species were relatively hydrophobic when compared to the hydrophobic control organism *P. multocida* P-1581.

D. Crystal violet binding results confirmed the hydrocarbon adherence data in that none of the *Serratia* species were able to absorb the hydrophobic stain.

E. NPN results further confirmed that all *Serratia* species examined exhibited no statistical differences in their cell surface hydrophobicity.

CONCLUSION

A. Intrinsic resistance to TCS is not phenotypically conserved amongst all *Serratia* species examined. Sensitization to TCS by all *Serratia species* by outer membrane permeabilization suggests that the outer membrane exclusionary properties are at least partially responsible for TCS resistance.

B. These data suggest the proclivity for sensitization to TCS with the outer membrane permeabilizer compound 48/80 appears not to be influenced by large differences in CSH.

REFERENCES

Champlin *et al. Antimicrobial Agents and Chemotherapy*. 2005.

Ellison and Champlin. *Veterinary Microbiology*. 2007.

Ruskoski and Champlin. *Journal of Medical Microbiology* 2017.

Clayborn *et al. Journal of Hospital Infection.* 2011.

Boyina *et al. PLOS ONE*. 2019. In preparation.