Influence of Cell Surface Hydrophobicity Properties on Susceptivity to Triclosan Sensitization by Outer Membrane Permeabilization in Opportunistically Pathogenic *Serratia* Species

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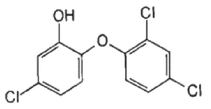
Abstract

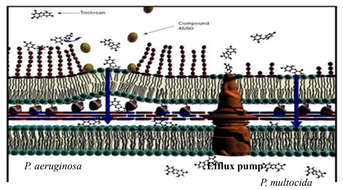
Unlike most hydrophobic molecules, the biocide triclosan is able to penetrate the gram-negative bacterial outer membrane. The nosocomial opportunists *Pseudomonas aeruginosa* and *Serratia marcescens* are atypically resistant to triclosan due largely to outer membrane impermeability properties for hydrophobic substances. However, we have recently shown that the degree of outer membrane involvement differs among disparate opportunistically pathogenic *Serratia* species. Cell surface hydrophobicity (CSH) properties underlie susceptivity to triclosan sensitization by outer membrane permeabilization in opportunistic *Serratia* species. : A model system consisting of opportunistic *Serratia* species (*marcescens, fonticola,* and *odorifera)* exhibiting disparate degrees of susceptivity to hydrophobic antibacterial agents and triclosan sensitization by outer membrane permeabilizer compound 48/80 were examined. Overall susceptibility to disparate hydrophobic antibacterial agents was determined using conventional disc agar diffusion and macrobroth dilution bioassays. Batch culture kinetics with triclosan and outer membrane permeabilizer compound 48/80 allowed analysis of cell envelope involvement in intrinsic triclosan resistance. CSH was determined using the hydrocarbon adherence method.. *S. marcescens* and *S. fonticola* were intrinsically resistant to hydrophobic antibacterial agents including triclosan, while *S. odorifera* was susceptible. CSH properties differed only slightly compared with control organisms, regardless of how susceptive they were to triclosan sensitization. These data suggest that the phenotypic differences seen in three opportunistic *Serratia* species with regard to intrinsic resistance to triclosan are at least partly due to disparate outer membrane exclusion potential. Moreover, susceptivity to triclosan sensitization by outer membrane permeabilization appears not to be influenced by CSH 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.



Triclosan



Materials and 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 (12000 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. Measurer % 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.

Results

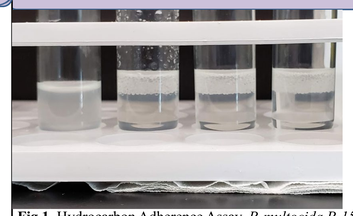


Fig 1. Hydrocarbon Adherence Assay. *P. multocida P-1581*

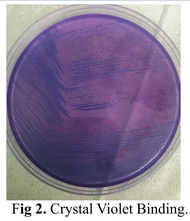


Fig 2. Crystal Violet Binding. *P. multocida P-1581*

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. *odorfera* 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).

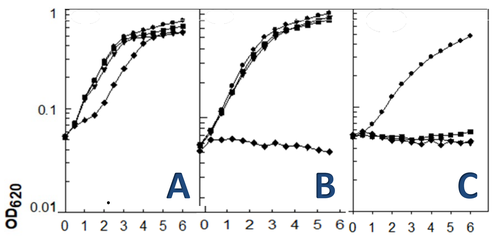


Fig 3. Batch cultural growth kinetics for representative *Serratia* species in the presence of triclosan and compound 48/80. (A) *S. marcescens ATCC 13880*, (B) *S. fonticola* ATCC 9844, (C) *S. odorifera* ATCC 33077. Symbols: (●) control MHB; (▼) compound 48/80 (2.5 µg/ml); (■) triclosan (2.0 µg/ml);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

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

Organism	Measurement of growth[1] 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.

Organism	Hydrocarbon Adherence Method	Crystal Violet Bioassay[1] MHA	BHIA
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. *odorfira* ATCC 33077	5.05 ± 1.32	-	-

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

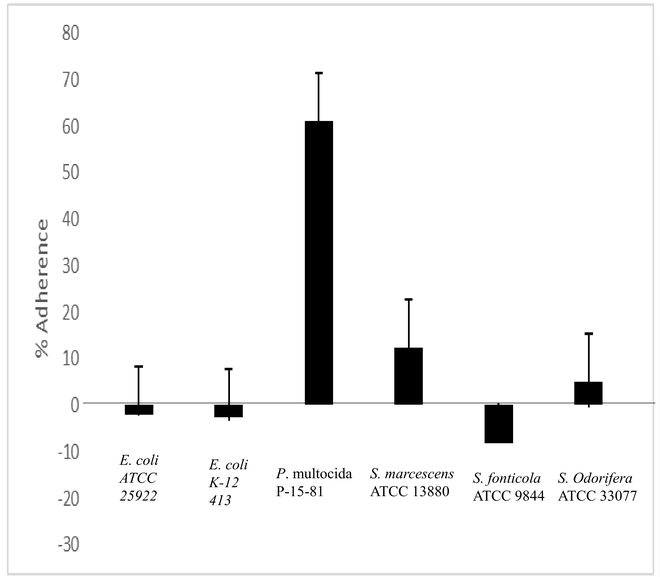


Fig 4. Hydrocarbon Adherence Assay Histogram.

Results Summary

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.

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

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