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LIPID CONTENT OF SERRATIA MARCESCENS, SENSITIVE AND RESISTANT TO POLYMYXIN B[a]

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The lipid composition of Serratia marcescens, sensitive and resistant to polymyxin B was analyzed. No differences were observed for the contents of total extractable lipid, the phospholipids and their components. However, there were significant differences in the fatty acid composition of the lipid fractions. The sensitive strains had a higher proportion of unsaturated fatty acids, particularly in the phospholipid fraction. Our results suggest that an increase of unsaturated fatty acid in sensitive strains may facilitate penetration of polymyxin B to the site of lethal action. On the other hand, an increased amount of saturated fatty acid in the resistant strains might promote more hydrophobic interactions with the hydrophobic site (6-methyloctanoic acid) of the polymyxin B and, hence, prevent the antibiotic from exerting its lethal action.

Serratia marcescens, once considered to be nonpathogenic, is being recognized with increasing frequency as causing various clinical diseases (Clayton and von Graevenitz, 1966; Wilfert et al., 1970; Maki et al., 1973). Most strains of S. marcescens that have been isolated from hospital patients are nonpigmented and have been found to be resistant to multiple drugs (Clayton and von Graevenitz, 1966; Thornton and Andriole, 1969; Thronton and Cramer, 1970; Maki et al., 1973). The fact that very few of these clinical isolates are pigmented indicates that the pigmented strains are probably more sensitive to antibiotic treatment (Clayton and von Graevenitz, 1966; Winshell and Neu, 1974). This suggests that the pigment, prodigiosin, may contribute to this increased susceptibility. Recent studies, however, do not indicate that a relationship exists, in general, between pigment formation and antibiotic sensitivity (Winshell and Neu, 1974; Button et al., 1974). Another factor which has been related to antibiotic susceptibility is the lipid content. It has been suggested that differences in the chemical composition of the cell envelope, especially total lipid, phospholipid and

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fatty acid content, may contribute to variations in antibiotic resistance (Hugo and Stretton, 1966; Dunnick and O'Leary, 1970; Norrington and James, 1970; Chang et al., 1972; Miller et al., 1973). There are, however, studies with gram-negative bacteria that have shown no significant changes in membrane lipid or fatty acid composition accompanying development of antibiotic resistance (Bishop and Bermingham, 1973). Furthermore, S. marcescens cells with different bacteriocin types contained similar amounts of total lipid and phospholipid (Winshell and Neu, 1974). Many of the clinical isolates of S. marcescens studied have been found to be uniformly resistant to the polymyxins, agents which interact with both the inner and outer membranes of the bacterial cell envelope. It has been suggested that the lipids and phospholipids of the readily extractable cell envelope may be important in the mechanism of resistance to polymyxins (Sud and Feingold, 1972; HsuChen and Feingold, 1973). Since the relationship between the lipid content of the cell envelope and antibiotic resistance has not been conclusively determined in S. marcescens, we have undertaken this study to clarify the relationship by comparing the lipid composition of several strains of S. marcescens which are either sensitive or resistant to polymyxin B.

METHODS AND MATERIALS

Three resistant strains, 08 (pigmented, nonclinical), #2736 (pigmented, clinical), and #6292 (nonpigmented, clinical) had minimum inhibitory concentrations of 1000 μg per ml or greater (Tsang et al., 1974). These were compared to two strains, Bizio (nonpigmented, nonclinical) and #13378 (pigmented, clinical) which were sensitive to 7.8 and 15.6 μg per ml, respectively. All cells were grown on an enriched medium (Tsang et al., 1974) with aeration and harvested during the late log phase. Lyophilized whole cells were extracted with chloroform:methanol (2:1, v/v) (Huston and Albro, 1964), washed according to Folch (Folch et al., 1957), dried and then weighed. The extracted lipids were reconstituted in chloroform:methanol (2:1, v/v) and then separated preparatively into neutral lipid and phospholipid fractions by thin-layer chromatography (TLC) on Silica Gel G using the solvent system hexane:diethyl ether:acetic acid (90:10:0.1, v/v/v). The phospholipids were further separated into their components by one-dimensional TLC using chloroform:methanol:water (65:25:4, v/v/v). The phospholipid content of the extracted lipids and the percent phospholipid component distribution were determined by the method of microphosphorus analysis (Bartlett, 1958). The total extractable lipids and the total phospholipids were hydrolyzed in 6 N HCl for 24 hours, and the fatty acid methyl esters were prepared for analysis of the fatty acid methyl esters was performed on a Varian Aerograph (model 1400) with a flame ionization detector and a column containing 15% diethylene glycol succinate polyester on Chromosorb W (60-80 mesh).

RESULTS AND DISCUSSION

Contrary to previous reports (Hugo and Stretton, 1966; Dunnick and O'Leary, 1970; Norrington and James, 1970), recent studies with strains of Serratia marcescens, sensitive and resistant to multiple drugs, do not show a direct relationship between lipid content and antibiotic resistance (Winshell and Neu, 1974). However, morphological changes have been observed in S. marcescens susceptible to polymyxin B (Tsang et al., 1974). The defects

produced were localized in the membranes and resulted in the collapse of these structures in sensitive bacteria. Our objective was to determine if there is a correlation between extractable membrane lipid composition and antibiotic resistance of S. marcescens, including several clinical isolates.

The total extractable lipid (TEL) and phospholipid composition of the TEL are reported in Table 1. With the exception of one sensitive strain (Bizio), no major differences were observed between the contents of TEL (7-8%) and phospholipid (75-80%) of the sensitive and resistant strains. Likewise, the phospholipid component distribution, shown in Table 2, indicated no significant differences between strains of different antibiotic sensitivities. These results are consistent with those of several previous reports (Bishop and Bermingham, 1973; Winshell and Neu, 1974). In relation to pigmentation, Kates (Kates et al., 1964) observed that if pigmented or nonpigmented cells of the same strain of S. marcescens are of the same physiological age, their lipid content would be similar. Our results are not only consistent with these observations of Kates et al., but also with the lipid analyses reported by Button et al. (Button et al., 1974) on the lipid composition of a pigmented strain and its nonpigmented variants.

The fatty acid composition of the membrane lipid has been previously implicated in the resistance to antibiotics (HsuChen and Feingold, 1973). It was suggested that hydrophobic interactions could occur between the fatty acids of the membrane lipids and hydrophobic groups, such as 6-methyloctanoic acid of polymyxin B. Therefore, we examined the fatty acid distribution of the total extractable lipid and the phospholipids. The results are reported in Table 3. The major fatty acid of the resistant strains is C_{16} (saturated) while the predominant component for the sensitive strains is C18:1 (unsaturated). More significantly, the ratio of saturated to unsaturated fatty acid, particularly for the phospholipid fraction, shows a distinct difference between sensitive and resistant strains.

Our results may suggest that an increase in the proportion of unsaturated fatty acid in the phospholipid may be associated with an increase in the sensitivity to polymyxin B. This could be due to decreased hydrophobic interactions between membrane fatty acids and the 6-methyloctanoic acid of the polymyxin B, enabling the antibiotic to penetrate with greater ease. A similar effect of the degree of unsaturation was reported by Rosen and Hackette (Rosen and Hackette, 1972). It was observed that the permeability of the outer membrane of Escherichia coli was increased by the substitution of unsaturated fatty acids for saturated fatty acids. This permitted the penetration of lysozyme and other agents and facilitated the release of periplasmic proteins following osmotic shock. It has also been observed in other membranes (McElhaney et al., 1973) that a change in the permeability of the outer membrane occurs when the packing of the hydrophobic molecules is disrupted by the introduction of unsaturated fatty acids. This increased permeability could also lead to an increased penetration of polymyxin B, making it easier for the antibiotic to reach the inner membrane and exert its lethal effect. Conversely, the increased proportion of saturated fatty acid in the resistant strains could be promoting hydrophobic interactions between outer membrane lipid fatty acids and the hydrophobic site (6-methyloctanoic acid) of polymyxin B. This would prevent the antibiotic from penetrating to the inner membrane where it has a lethal action.

TABLE I. Lipid contents of pigmented and nonpigmented strains of *Serratia marcescens* sensitive and resistant to polymyxin B

Strains	Pigmentation	Minimum Inhibitory Concentration of Polymyxin B μg/ml	Total Extractable Lipid[a] %	Composition of TEL	
				Neutral Lipid[b] %	Phospholipid[c] %
08	+	1,000	8.5[d]	18.4[e]	81.6[e]
2736	+	1,000	8.4	25.4	74.6
6292	-	1,000	7.3	26.3	73.7
Bizio	-	7.8	11.3	19.7	80.3
13378	+	15.6	8.0	17.1	82.9

[a]Expressed as percent of dry cells

[b]Calculated from the difference between total extractable lipid and phospholipid

[c]Expressed as percent of total extractable lipid

[d]Average of at least five runs

[e]Average of at least three runs

TABLE 2. Phospholipid component distribution of Serratia marcescens sensitive and resistant to polymyxin B

Strains	Phospholipid Distribution PE[a] %	PG %	CL %
Resistant			
08	66.4[b]	15.8	17.8
2736	67.8	18.3	13.9
6292	72.6	14.7	12.7
Sensitive			
Bizio	63.2	16.4	20.5
13378	66.9	21.4	11.7

[a] PE = Phosphatidylethanolamine
PG = Phosphatidylglycerol
CL = Cardinolipin (Polyglycerol phosphatide)

[b] Average of two runs

In order to better understand the antibiotic mechanisms of polymyxin B, a study of other lipid-containing components in the outer membrane, such as lipopolysaccharides (LPS), could also be informative. An interaction between LPS and polymyxin B has been reported (Lopes and Inniss, 1969) in which treatment of isolated LPS by polymyxin B caused a dissociation of the LPS molecules. In addition, polymyxins are known to have sites of activity at both the outer membrane and the cytoplasmic membrane (Sud and Feingold, 1972). Thus, it may also be necessary to isolate these two membrane fractions and analyze the lipid composition of each membrane fraction.

TABLE 3. Fatty acid composition of the total extractable lipid and phospholipid of Serratia marcescens sensitive and resistant to polymyxin B

Strains	Fatty Acid %[a]									
	Total Extractable Lipid					Total Phospholipid				
	C_{14}	C_{16}	$C_{16:1}$	$C_{18:1}$	sat. fatty acid / unsat. fatty acid	C_{14}	C_{16}	$C_{16:1}$	$C_{18:1}$	sat. fatty acid / unsat. fatty acid
Resistant										
08	3.8	40.8	25.3	29.9	0.81	3.4	51.4	28.1	17.2	1.21
2736	2.7	39.7	22.4	35.3	0.73	4.2	56.8	7.7	31.2	1.55
6292	5.7	44.2	14.1	36.0	0.99	4.6	41.5	14.6	39.3	0.86
Sensitive										
Bizio	3.1	35.8	18.3	42.8	0.64	2.3	36.3	17.4	44.0	0.63
13378	2.4	37.0	12.6	48.0	0.65	2.1	35.3	11.5	51.1	0.60

[a]Average of two runs

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