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Lipid Content of Antibiotic-Resistant and -Sensitive Strains of *Serratia marcescens*

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The lipid content of antibiotic-resistant, nonpigmented strain (Bizio) and antibiotic-sensitive, pigmented strain (08) of *Serratia marcescens* was studied. The resistant strain contains at least three times more total extractable lipid and phospholipid than the sensitive strain. Lysophosphatidylethanolamine, phosphatidylserine, lecithin, phosphatidylglycerol, phosphatidylethanolamine, and polyglycerolphosphatide were identified in the phospholipid fractions of both strains.

The cell wall is the first component of most bacteria to interact with the environment. The structure of the cell walls, in general, contains a rigid network of murein which consists of polypeptide and polysaccharide. For gram-negative bacteria, in addition to murein, the cell walls contain up to 25% of their weight in lipoprotein and lipopolysaccharide and a smooth, soft, lipid-rich outer covering.

The lipids in this outer layer are varied in bacteria, but, in general, the gram-negative bacteria contain three times more lipid than the gram-positive bacteria (21). It has also been found, in the case of certain antibiotic-resistant bacteria such as *Escherichia coli* (27), *Staphylococcus aureus* (15), *Rhizobium meliloti* (20), and *Streptococcus pyogenes* (13, 21), that the increase in the proportion of the extractable lipids (i.e., the outer layer lipids) closely paralleled an increase in resistance. It has been suggested that the high content of phospholipids in the extractable lipid of the cell envelope may create a permeability barrier for the passage of certain antibiotics to the cytoplasmic membrane (15, 20).

Serratia marcescens is one of the members of the gram-negative bacteria. Certain strains of *S. marcescens* produce a characteristic red pigment. Some mutant strains produce a rose pigment, whereas others are nonpigmented, such as the Bizio strain. In some cases, however, the bacteria of the same strain may exhibit different colors, depending on the age of the bacteria (17). *S. marcescens*, formerly considered nonpathogenic, has recently been recognized with increasing frequency as the cause of certain clinical diseases (7, 25). Most of the strains indicted as infectious were nonpigmented, including the Bizio strains (8, 22). Characteristically, these nonpigmented strains have been resistant to most antimicrobial agents (7, 22, 23).

The resistance of *S. marcescens* to antimicrobial drugs such as quaternary ammonium compound has been studied (5, 6). Simple staining by Sudan Black B and removal of resistance by the action of lipase supported the belief that the acquired resistance is dependent upon the increased lipid content of the resistant cell. Unfortunately, the method used was less than quantitative and precise. In this communication, we reported the results of lipid analysis from two strains of *S. marcescens*: the antibiotic-resistant, nonpigmented Bizio and the antibiotic-sensitive, pigmented 08 strain.

MATERIALS AND METHODS

Bacteria. Both the *S. marcescens* 08 (pigmented) and the Bizio (nonpigmented) strains, grown on either an inorganic or an enriched medium (1, 24), were supplied by General Biochemicals, Chagrin Falls, Ohio. The cells were harvested at the late log phase. Whole cells were isolated by washing the cell paste twice with distilled water, centrifugation at 10,000 rev/min for 10 min, and lyophilization. The dry whole cells were stored in a vacuum desiccator. Cell walls were isolated from lyophilized whole cells by sonic treatment and centrifugation by the method of Williams (26), as modified by Tsang (24).

Extraction of free lipids from whole cell and isolated cell wall. Lyophilized whole cells and cell walls (2 to 5 g) were extracted by the method of Huston (16) and washed by the method of Folch (12). The dry weights of the lipids were measured and recorded. The known quantity of lipids was then

redissolved in a measured quantity of the solvent chloroform-methanol (2:1, v/v) and stored at 5 C.

Separation of lipids. The lipids were separated on 20- by 20-cm thin-layer plates which had been spread with Silica Gel G (Brinkmann Instruments, Inc., Westbury, N.Y.) to a 0.25 mm thickness. The plates were heat activated for 1 hr at 110 C and stored in a cabinet where the humidity was controlled until ready for use. All procedures were carried out at room temperature. The lipids were first separated into phospholipid, free fatty acid, and triglyceride fractions by using the solvent system I: hexane-ether-acetic acid (90:10:1, v/v/v). The phospholipids were scraped off from the thin-layer plates and eluted from the Silica Gel G by using chloroform-methanol (2:1, v/v) solvent. In some cases, the neutral lipids were removed by acetone extraction directly, and the completeness of removal was determined by thin-layer chromatography on solvent system I. The phospholipids were dried, weighed, and redissolved in a known quantity of the chloroform-methanol (2:1, v/v) mixture. The known concentration of the phospholipid solution was examined by one- and two-dimensional thin-layer chromatography by using the solvent system II: chloroform-methanol-water (65:25:4, v/v/v) for the first dimension, and solvent system III: chloroform-methanol-water (80:20:2, v/v/v) for the second dimension. The first dimension required 70 min and the second dimension took about 50 min. The phospholipid standards consisted of cardiolipin, lysolecithin, lecithin, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin (Supelco, Inc., Bellefonte, Pa.). Molybdenum blue stain was used to detect the phospholipid. Ninhydrin spray (Brinkman Instruments, Inc., Westbury, N.Y.) was used to detect the phospholipid with the free amino group.

Micromethod for phosphorus determination. The method of Bartlett (3) was used with a little modification. A sample (1–3 ml) of phospholipid containing 25 to 75 μg was transferred to an acid-washed tube and evaporated to dryness on a steam bath. Then, 0.5 ml of 70% perchloric acid was added. The sample was digested at 200 to 250 C for 2 hr. After the tube was cooled to room temperature, 1.0 ml of water, 3.0 ml of 0.4% ammonium molybdate, and 0.2 ml of Fiske-SubbaRow reagent were added. The solution was heated in a boiling water bath for 15 min. After cooling and adjustment to a final volume of 5.0 ml with distilled water, the absorbance of the solution was read at 810 nm. The standard used in this method was anhydrous KH_2PO_4. A range of 1 to 6 μg of phosphorus in the standard was used.

RESULTS AND DISCUSSION

Various quantities of extractable lipid were obtained, depending upon the strain and media used. In the Bizio strain, the contents of the extractable lipid from whole cells grown on inorganic and enriched media were 53.3 and 34.5%, respectively, compared with 19.5 and 8.1% from whole cells of 08 strain grown on the corresponding media (Table 1). Regardless of the types of media used, the quantity of extractable lipid from the whole cell of Bizio (antibiotic-resistant) was approximately three times more than that of the 08 strain (antibiotic-sensitive), although the amounts did vary when inorganic versus enriched media was used.

The phospholipid content in the total extractable lipid from whole cells was determined by both phosphorus determination and gravimetric methods after the neutral lipids were removed by acetone extraction. The amount of phospholipid in the extractable lipid was estimated by multiplying the phosphorus content by 25 (Table 2). The percentages of phospholipid in the total extractable lipid in Bizio and 08 strains were approximately the same (1.75%). Since the quantity of extractable lipid in the whole cell of Bizio was three to four times more than that of the 08 strain, it can be assumed that the Bizio strain contained at least three times as much phospholipid in the total

TABLE 1. *Percentage of extractable lipid from the whole cell of Serratia marcescens*

Types of medium[a]	Extractable lipid (%) from strain		Ratio of extractable lipid
	Bizio	08	(Bizio:08)
Inorganic	53.3	19.5	2.7:1
Enriched	34.5	8.1	4.2:1

[a] Alaupovic et al. (1); Tsang et al. (24).

TABLE 2. *Comparison of the percentage of phospholipid in whole cell and cell wall of resistant and sensitive strains of Serratia marcescens*

Fraction	Phospholipid (%)[a] in extractable lipid	Phospholipid (%)[b]	Ratio
Whole cell[c]			
Bizio resistant	1.78	0.95	2.78
08 sensitive	1.73	0.34	1.00
Cell wall[d]			
Bizio resistant		8.9	3.1
08 sensitive		2.9	1.0

[a] The % of phospholipid = % of phosphorus × 25.

[b] Phospholipid content was determined gravimetrically after neutral lipid was removed by acetone extraction.

[c] Both strains were grown on inorganic medium.

[d] Cell wall of the resistant strain was grown on inorganic medium. The cell wall of the sensitive strain was grown on enriched medium.

extractable lipid. This assumption was confirmed by a separate method. This was accomplished by determining phospholipid gravimetrically after the neutral lipids were removed by exhaustive acetone extraction. The ratio of the content of phospholipid from Bizio to that of 08 was 3:1 (Table 2). In separate experiments, the phospholipid in the cell wall of 08 and Bizio was determined by the same method. In this case, the phospholipids of Bizio and 08 were found to be 8 to 9% and 2 to 3%, respectively. Again, the ratio is approximately 3:1 (Table 2).

By means of one- and two-dimensional thin-layer chromatography on Silica Gel G, eight different major components were observed in the phospholipid fractions of both strains. The results are presented in Fig. 1 and 2. Lysophosphatidylethanolamine, phosphatidylserine, lecithin, phosphatidylglycerol, phosphatidylethanolamine, and polyglycerolphosphatides were identified (Table 3). In both cases, phosphatidylethanolamine was present as the major component. Other than that the pigment (prodigiosin) was an additional component identified in 08 strain, no major strain differences were observed. These results were consistent with those of Kates et al., who investigated the composition of lipids from *S. marcescens* Tem-

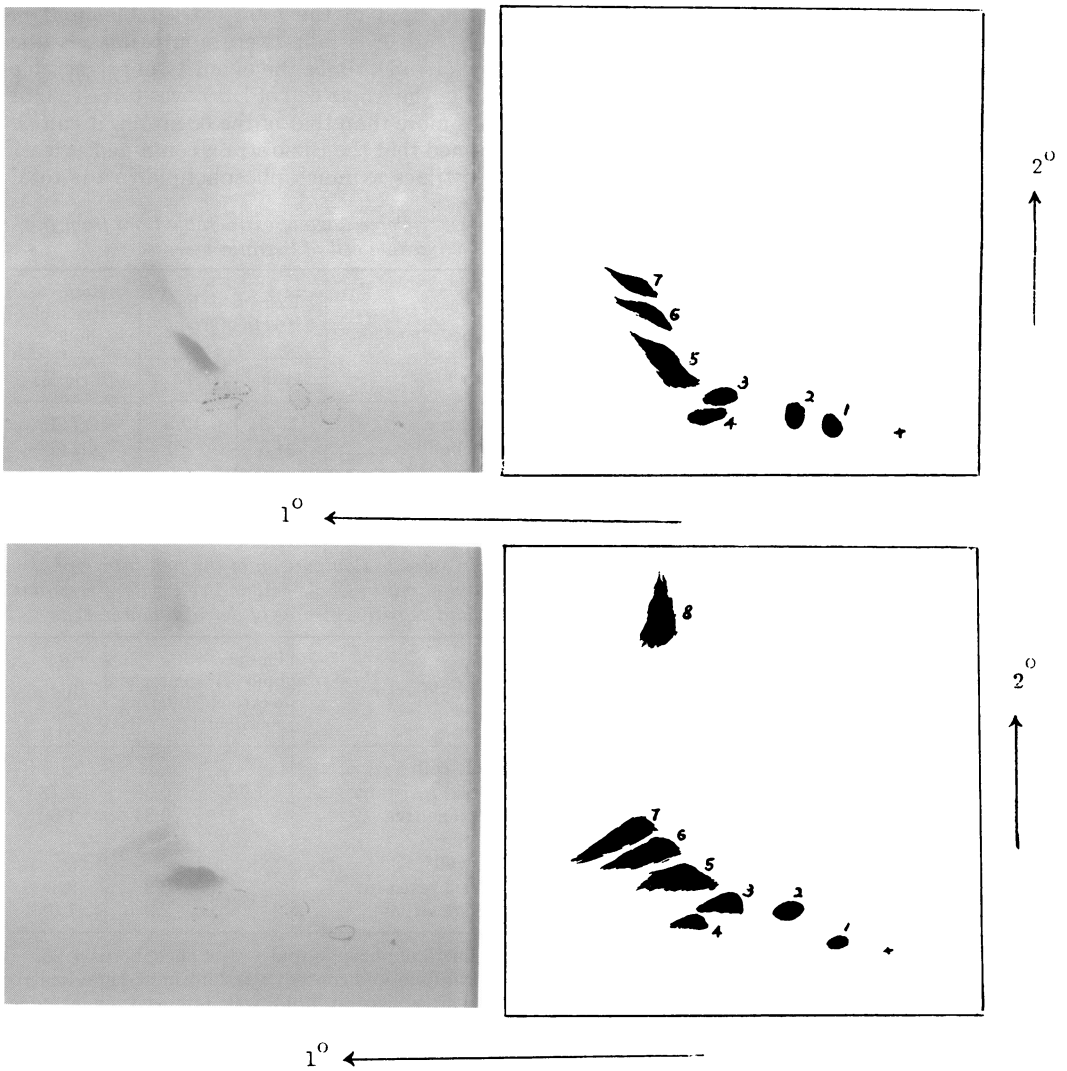

FIG. 1. *Two-dimensional thin-layer chromatograms of phospholipid from Serratia marcescens Bizio (top) and 08 (bottom) strains. Solvent system: 1st dimension, chloroform-methanol-water (65:25:4, v/v/v), 2nd dimension, chloroform-methanol-water (80:20:2, v/v/v). Stain, molybdenum blue.*

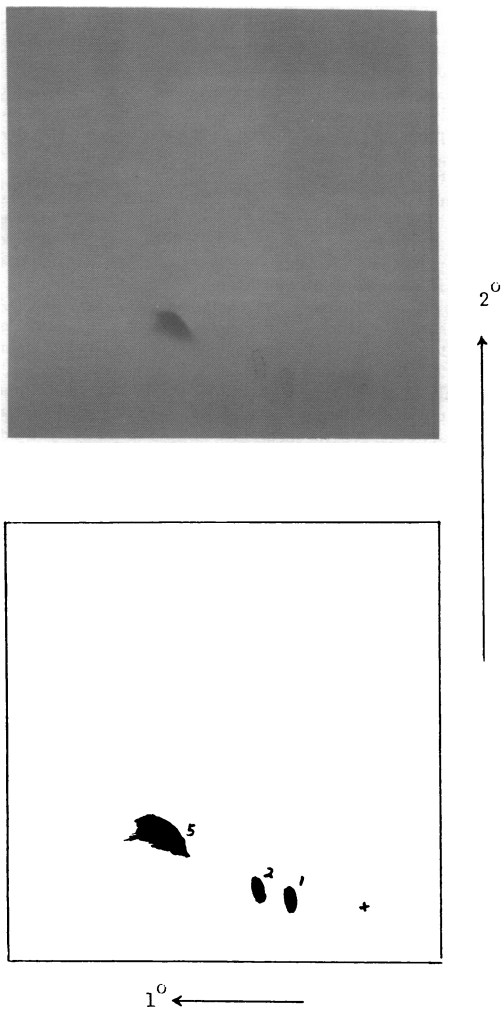

FIG. 2. *Two-dimensional thin-layer chromatograms of phospholipid from Serratia marcescens Bizio strain. Solvent system: 1st dimension, chloroform-methanol-water (65:25:4, v/v/v); 2nd dimension, chloroform-methanol-water (80:20:2, v/v/v). Stain, ninhydrin.*

ple University strain (17). In the same study, they found that the older cells (pigmented) and the younger cell (nonpigmented) of the same strain contained approximately the same amount of extractable lipid (6–10%), with 38% in the pigmented cells and 44% in the nonpigmented cells being phospholipids. It was concluded that despite the presence of pigment (prodigiosin) in the physiologically older cells, there was no significant difference in the total extractable lipid as well as phospholipid content.

In our experiments, the yield of extractable lipid from the antibiotic-sensitive, pigmented 08 strain ranged from 8.1 to 19.5%, whereas those of the Bizio strain ranged from 34.5 to 53.3%, depending on the culture media (Table 1). The value (8.1%) of total extractable lipid in the 08 strain is reminiscent of the result (6–10%) reported by Kates et al. However, the amount of extractable lipid present in the antibiotic-resistant, nonpigmented Bizio strain was at least three times higher than that of Kates et al.

According to the studies of Norrington and James (21) and others (13, 15, 20, 27), the total extractable lipid in antibiotic-resistant strains was usually two to five times higher than that of the antibiotic-sensitive ones (Table 4). In our case, we found in the antibiotic-resistant strain (Bizio) that the amount of total extractable lipid and the phospholipid was three times as

TABLE 3. *Qualitative results of phospholipid in two strains of Serratia marcescens*

Spot[a] number	Ninhydrin stain	Molybdenum blue stain	Identification
1	+	+	Lysophosphatidylethanolamine[b]
2	+	+	Phosphatidylserine
3	–	+	Lecithin
4	–	+	Phosphatidylglycerol
5	+	+	Phosphatidylethanolamine
6	–	+	Polyglycerolphosphatide[b]
7	–	+	Unknown
8	–	–	Pigment (08 only)

[a] Spot number as indicated in Fig. 1 and 2.
[b] Kates et al. (17).

TABLE 4. *Lipid content of various antibiotic-sensitive and antibiotic-resistant bacteria*

Bacteria	Antibiotic resistance	Extractable lipid in cell wall		Phospholipid in extractable lipid	
		%	Ratio	%	Ratio
Streptococcus pyrogenes[a]	Resistant (tetracycline)	12.6	2.5		
	Sensitive (tetracycline)	4.9	1.0		
Pseudomonas aeruginosa[b]	Resistant (polymyxin)	28.5	1.7	95.3	2.8
	Sensitive (polymyxin)	16.5	1.0	33.3	1.0
Rhizobium meliloti[c]	Resistant (vimomycin)				1.3
	Sensitive (vimomycin)				1.0

[a] Data from Norrington and James (21).
[b] Data from Brown and Watkins (4).
[c] Data from MacKenzie and Jordan (20).

much as that in the antibiotic-sensitive 08 strain. This datum compares favorably with the reported results of Norrington and others.

In recent years, the structure and function of the relationship of cell wall components to bacterial antibiotic resistance have been studied extensively (24). Studies by a number of investigators (5, 6, 19) have suggested that increased synthesis of lipid may be a factor in the resistance of bacteria to antibacterial substances (2, 5, 6, 19, 27). More recently, the alteration of fatty acid composition of gram-negative bacteria was noted as a consequence of antibiotic resistance (9). The increase of lipid synthesis in the cell wall and alteration of fatty acid composition may create a permeability barrier for uptake of antibiotics by the outer membrane (lipopolysaccharide-lipoprotein complex) or by the cytoplasmic membrane, or both. In any event, the role of phospholipid in these systems is immensely important. The coenzymatic role of phospholipid in the biosynthesis of lipopolysaccharide and cell wall components is well documented (10, 11, 18). In addition to the permeability factor for the transport of antibiotics, the role of phospholipids as a cofactor for the enhancement of any degradative enzymatic activity toward antibiotics should also be considered.

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