CELL WALL PHOSPHOLIPID AND VIOMYCIN RESISTANCE IN RHIZOBIUM MELILOTI

C. R. MacKenzie and D. C. Jordan
Department of Microbiology, University of Guelph
Guelph, Ontario, Canada

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SUMMARY

Viomycin resistance in a mutant of Rhizobium meliloti was found to involve an accumulation of phospholipid in the cell envelope. ^{14}C-labelling revealed that this accumulation occurred at the expense of neutral lipids in the envelope. Viomycin was shown to form complexes with phospholipid under artificial conditions and it is suggested that phospholipid-viomycin interactions also occur within the surface layers of the cell, resulting in a decreased permeability toward the antibiotic in the resistant cells.

Acquisition of resistance to certain antibiotics in bacteria of the genus Rhizobium is often accompanied by a simultaneous loss in nitrogen fixing ability (1). Therefore, studies on such mutants are valuable in elucidating the mechanisms of antibiotic resistance and in understanding the phenomenon of symbiotic nitrogen fixation.

Previous work in this laboratory had shown that viomycin resistance in Rhizobium meliloti was due to a permeability barrier (2,3). Compositional studies were subsequently carried out on the cell envelopes of the viomycin-sensitive parent strain of this bacterium and of a viomycin-resistant mutant and the results of some of these studies are reported here.

MATERIALS AND METHODS

Bacterial Strains

The bacterial strains used were Rhizobium meliloti R21 vio-s, a viomycin sensitive strain (inhibited by 4-5 µg of viomycin / ml), and Rhizobium meliloti R21 vio-r (McK), a single-step mutant with

a low level of resistance to viomycin (inhibited by 16-18 µg of viomycin / ml).

Cultivation and Labelling of Cells

Cells were grown in a medium containing 0.5g K_2HPO_4, 0.2g $MgSO_4 \cdot 7H_2O$, 0.1g NaCl and 3.0g Difco yeast extract per litre of distilled water. ^{14}C-labelling of lipids was accomplished by the addition of 15 µCi $CH_3{}^{14}CO_2Na$ (1.15 mCi/mM) to each litre of medium and phospholipids were labelled in some experiments by the addition of 250 µCi $H_3{}^{32}PO_4$ per litre of medium.

Preparation of Cell Envelopes

Log-phase cells were suspended in distilled water and broken with a Ribi cell disrupter. Intact cells were removed by centrifugation at 5,000 g for 20 minutes and cell envelopes by centrifugation at 20,000 g for 20 minutes. The envelopes were washed extensively in distilled water and freeze-dried. For amino acid and carbohydrate analyses they were purified of plasma membrane by digestion with trypsin, pepsin and DNAse (2).

Amino Acid and Carbohydrate Analyses

Cell envelope hydrolysates were analyzed with a Beckman 120 amino acid analyzer. Total reducing sugar in the envelopes was determined by a submicro method and total hexoseamine by the Elson-Morgan reaction. Individual sugars were examined by thin-layer chromatography.

Extraction and Chromatography of Lipids

Five mg quantities of dried envelopes were suspended in 5 mls of distilled water and extracted for 30 minutes with 15 mls of chloroform-methanol (2:1, v/v). The entire lipid extracts were either taken to dryness in liquid scintillation vials or spotted on

silica gel thin-layer plates. Solvent systems of chloroform-methanol-butanol-acetic acid-water (90:60:40:20:20 , v/v) and hexane-diethyl ether-acetic acid (90:10:1, v/v) were used for the separation of phospholipids and neutral lipids respectively. Spots were made visible by exposure to iodine vapours, then scraped from the plates and placed in liquid scintillation vials for radioactivity determination.

Viomycin-phospholipid Interactions

Interaction studies were carried out using the procedure of Lesslauer et al. (4). The phospholipid was obtained by extraction of whole cells with chloroform-methanol (2:1, v/v) followed by removal of neutral lipids from the extract by thin-layer chromatography. Samples (15-20 mg) of phospholipid were sonicated in 10 mls of water and 5 mls of 95% ethanol, then 5-10 mg viomycin sulfate (Parke, Davis and Company, Detroit, Mich.) and 14 mls of hexane (British Drug Houses) were added. The mixture was shaken for two hours and the hexane layer examined for the presence of viomycin using a Beckman DB spectrophotometer.

RESULTS AND DISCUSSION

The carbohydrate composition of the cell envelope was found to be similar in both strains and only minor differences in amino acid composition were observed.

Visual observation of thin-layer plates indicated an accumulation of phosphatidylcholine and phophatidylethanolamine (the two major lipid components) in the resistant strain. ^{14}C-labelling confirmed this observation and provided a measure of the relative amounts present (Table 1). The resistant cells' envelopes contained approximately 30 % more phospholipid than those of the sensitive strain and this excess phospholipid appeared to be

Table 1

A comparison of the incorporation of $CH_3{}^{14}CO_2Na$ into the cell envelope lipids of viomycin resistant and sensitive strains of Rhizobium meliloti R21.

Lipid	cpm/5 mg dry weight of envelopes	
	Resistant Cells	Sensitive Cells
Total	28,600	22,600
Neutrals	200	1,400
phosphatidyl-ethanolamine	8,900	7,400
phosphatidyl-choline	7,200	5,000

synthesized at the expense of neutral lipids. No qualitative differences were observed between the lipid extracts of the two strains. These quantitative differences in lipid composition are apparently confined to the cell envelopes since they are much reduced in whole cell extracts. ^{32}P-labelling of cells confirmed the accumulation of phospholipid in the cell envelope of the resistant cells and also showed an accumulation in purified cell walls.

Viomycin, which is completely insoluble in hexane, was made soluble by the addition of phospholipid. The hexane layer of the interaction mixture had a UV spectrum which was almost identical to that of an aqueous solution of viomycin (Figure I) and no change in the maximum absorption peak at 268 nm was observed. This finding was interpreted as indicating complexing of the phospholipid and the antibiotic.

Viomycin is a strongly basic cyclic polypeptide with a molecular weight of 600-650. Phospholipids are known to interact with basic proteins (4) and it appears that this may be a general

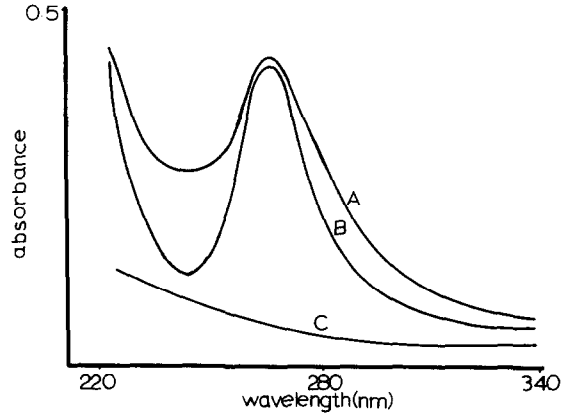

Figure 1. Ultraviolet spectra of hexane layer of viomycin-phospholipid interaction mixture (curve A), aqueous solution of viomycin sulfate (curve B) and solution of phospholipid in hexane (curve C).

property of such proteins. Tyrocidine B, a cationic cyclic polypeptide, readily penetrates artificial lipid membranes with such penetration involving an initial adsorption of the antibiotic on the surface of the membrane (5,6). Viomycin resistance in the mutant studied here is thought to be due to binding of the antibiotic in the cellular envelope because of the accumulation in this structure of excess phospholipid.

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