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Action of Polymyxin B on Bacterial Membranes: Phosphatidylglycerol- and Cardiolipin-Induced Susceptibility to Polymyxin B in *Acholeplasma laidlawii* B

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To identify the polymyxin receptor molecules in the membranes of living microorganisms, fusion of intact *Acholeplasma laidlawii* B with lipid vesicles was investigated according to the procedure of Grant and McConnell (1973). The naturally polymyxin-resistant *A. laidlawii* B was treated with phospholipid vesicles prepared from purified phospholipids of the polymyxin-susceptible *Salmonella typhimurium* G30. *A. laidlawii* B absorbed between 15 and 45% of its own lipid content of the added tritium-labeled phospholipids without loss of viability. Association with the acidic components phosphatidylglycerol and cardiolipin produced a 10- to 30-fold increase in polymyxin susceptibility, which was not obtained with egg-phosphatidylcholine and mixed phosphatidylcholine-phosphatidylethanolamine vesicles. The polymyxin-sensitized cells bound 12 times more radioactive antibiotic than resistant cells. The phosphatidylglycerol-induced susceptibility was abolished by serum fraction V (Cohn) proteins.

The release of trapped glucose from artificial liposomes by the membrane-specific polypeptide antibiotic polymyxin B has been attributed by Imai et al. (6) to the presence of acidic lipids such as phosphatidylglycerol, cardiolipin, or dicetylphosphate. This is in accordance with the observation that only acidic phospholipids, dissolved in chloroform, are able to extract the positively charged antibiotic from the aqueous into the organic phase (22, 25). Phosphatidylethanolamine, which is the main membrane lipid of polymyxin-susceptible, gram-negative bacteria, is not capable of causing a high transfer of polymyxin into an organic solvent (25). In contrast, Feingold et al. (2) suggested that phosphatidylethanolamine is an effective target for polymyxin B in phosphatidylethanolamine-dicetylphosphate vesicles. Despite the biochemical evidence, it has never been directly demonstrated which phospholipids constitute the polymyxin receptor sites in the membranes of living microorganisms.

To prove in vivo the old concept of Few (3) suggesting electrostatic polymyxin-phospholipid interactions, a bacterium resistant to polymyxin B but amenable at will to lipid variation would be useful. The work of Grant and McConnell (5) on the successful fusion of lipid vesicles with living cells of the polymyxin-resistant, cell wall-lacking *Acholeplasma laidlawii* B opened the way to investigation of the role of individual phospholipids as potential polymyxin receptor sites in viable bacteria.

We show in this study that significantly increased polymyxin susceptibilities are generated in *A. laidlawii* B by association with cardiolipin or phosphatidylglycerol, but not with phosphatidylethanolamine. We therefore postulate that the minor lipids cardiolipin and phosphatidylglycerol are the polymyxin receptor molecules in the plasma membrane of gram-negative bacteria. A preliminary report has been presented (J. Bader and M. Teuber, Abstr. Commun. 9th Meet. Fed. Eur. Biochem. Soc., Budapest 1974, p. 258).

MATERIALS AND METHODS

A. laidlawii B was donated by W. Bredt (Mainz, Germany), and *Salmonella typhimurium* G30 was donated by M. J. Osborn (Farmington, Conn.).

A. laidlawii B was routinely grown at 37 C without shaking in liquid tryptose medium supplemented according to Smith (19) after autoclaving with 0.5% sterile glucose and 20 ml of PPLO serum (Difco) per liter. Solid medium was prepared by addition of 1.25% agar (Oxoid no. 3), 1% PPLO serum, and 100 μg of penicillin G-sodium (Hoechst, Germany).

The defined medium for *A. laidlawii* B was the one described by Smith (20). If synthesis of carote-

noids was not desired, sodium acetate and coenzyme A were omitted.

The fatty acid-containing medium was prepared according to Razin et al. (14).

Delipidations of serum fraction V and tryptose were performed with acetone and chloroform, respectively, as indicated by Razin and Rottem (12) and McElhaney and Tourtellotte (8).

A. laidlawii cultures were started by inoculation with 1% (by volume) of fully grown cultures (20 h at 30 or 37 C).

Polymyxin B sulfate (sterile powder) was a gift of Pfizer GmbH (Karlsruhe, Germany). Radioactive [^{14}C]mono-*N*-acetyl derivatives were synthesized, isolated, and tested as previously reported (21, 24). Phospholipids were extracted from *S. typhimurium* G30 grown in standard bouillon at 37 C, with shaking, into early stationary phase (per liter of distilled water: 1 g of meat extract [Difco], 10 g of peptone [tryptic from meat; Merck] and 5 g of NaCl, pH 7.5), by using the method of Folch et al. (4). Phosphatidylglycerol and glycolipids of *A. laidlawii* B were extracted from tryptose-grown cells. Phosphatidylcholine was isolated and purified from fresh eggs as described by Papahadjopoulos and Miller (11). The individual phospholipids were further purified and isolated by thin-layer chromatography on kieselgel G plates (Merck no. 5717) according to Eder (1). They were identified by co-chromatography with authentic lipids purchased from Supelco Inc. (Bellefonte, Pa). ^{3}H-labeled lipids were obtained by addition of 250 μCi of [^{3}H]glycerol (Amersham-Buchler, 500 μCi/μmol) to the growth medium of *S. typhimurium* G30.

For the preparation of lipid vesicles according to Grant and McConnell (5), 20 to 24 μmol of phospholipids on phosphate bases was dissolved in chloroform and mixed with 170,000 counts/min of the corresponding labeled phospholipid. The solvent was removed under nitrogen and reduced pressure at 37 C. The remaining lipid layer was dispersed in 2 ml of 0.14 M NaCl-0.05 M sodium phosphate buffer, pH 7, by (i) 10-min shaking on a Whirlimix with three glass beads (3 mm) at 30 C and (ii) 30-min sonications in 30-s steps at 3-min intervals (Branson sonifier step 4). The resulting almost clear, but opalescent, vesicle preparations were stored and stable for several weeks at 4 C.

Protein was determined by the procedure of Lowry et al. (7), and radioactivity was determined in a toluene-based scintillation liquid with a Beckman LS100 spectrometer or, in some instances, a Geiger-Mueller gas-flow counter.

For the association of *A. laidlawii* B with lipid vesicles, the cells were grown at 37 C in tryptose broth to a cell density of 2×10^{9} to 4×10^{9}/ml, centrifuged for 10 min at 13,000 $\times$ *g* and room temperature, and gently resuspended (by using a Pasteur pipette) in 0.5% (by volume) prewarmed (37 C) 0.14 M NaCl-0.05 M sodium phosphate buffer, pH 7.0. A 50-μl amount of this cell suspension was incubated at 37 C for 30 min with 50 μl of phospholipid vesicles. The following incubation with polymyxin B was started by addition of 1 ml of polymyxin solution in phosphate buffer (pH 7). The resulting viable counts (colony-forming units [CFU]) were enumerated, after suitable dilutions, with tryptose broth by spotting three parallel 10-μl fractions onto tryptose agar. The number of colonies was determined with a microscope at 15-fold magnification after 3 days of incubation at 37 C.

The incorporation of [^{14}C]lysine was measured with 0.2 ml of vesicle- and polymyxin-treated cell suspensions by addition of 0.3 ml of phosphate buffer and 0.5 ml of modified defined medium and incubation for 3 h at 37 C. The reaction was stopped after centrifugation (40,000 $\times$ *g*, 10 min at 0 C) by addition of 0.1 ml of phosphate buffer and 0.5 ml of 5% trichloroacetic acid. The precipitated material at 0 C was collected on cellulose nitrate filters (Sartorius, Göttingen, Germany; pore size, 0.45 μm) and counted in a liquid scintillation spectrometer. The modified medium contained 0.1 μCi of [^{14}C]lysine (150 μCi/μmol; Amersham-Buchler) instead of L-lysine (0.5 ml). NaCl, Na_2HPO_4, and sodium acetate were omitted. The content of $MgCl_2 \cdot 7H_2O$ was reduced to 20 mg/liter. Serum albumin fractionV was substituted by 1% PPLO serum. All other substances were concentrated twofold to account for dilution with the cell suspension. The incorporation of [^{14}C]uracil was measured under the same conditions with 0.3 μCi of [^{14}C]uracil (61 μCi/μmol), substituting uridine.

N-[^{14}C]acetyl-serum albumin fraction V was prepared by reaction of 2 ml of a 10% delipidated fraction V in 0.5 ml of saturated aqueous sodium bicarbonate with 10.4 μmol of [1-^{14}C]acetic anhydride (24 μCi/μmol). After 30 min at 0 C, the reaction mixture was dialyzed for 24 h at 4 C against 0.05 M sodium phosphate-0.14 M NaCl, pH 8. One milligram of protein contained 88,800 counts/min as checked in a gas-flow counter. Forty percent of the protein amino groups had been blocked by this acetylation, as determined by reaction with dinitrofluorobenzene. To determine the binding to *A. laidlawii* B, 50 μl of cell suspension in phosphate buffer was incubated with 50 μl of 1% [^{14}C]serum fraction V for 15 min at 37 C. The suspension was diluted 1:10 with buffer, and the cells were sedimented by a 4-min centrifugation at 2,500 $\times$ *g* and washed once by the same procedure with buffer. Sedimented radioactivity was counted as above.

RESULTS

The following studies were carried out with phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin. The phospholipids had been extracted and purified from *S. typhimurium* G30. The polymyxin susceptibility of this strain has been characterized (23, 24). Only the acidic compounds phosphatidylglycerol and cardiolipin were potent in neutralizing the antibiotic effects of polymyxin B against growing cells of *S. typhimurium* in a stoichiometric manner: four molecules of phosphatidylglycerol or two molecules of cardiolipin inactivated

one molecule of polymyxin B (25). Whereas phosphatidylglycerol from *A. laidlawii* B revealed the same phenomenon, *A. laidlawii*-glycolipids, egg-phosphatidylcholine, and *Salmonella*-phosphatidylethanolamine did not influence the antibiotic properties of polymyxin B. Since the phospholipid-polymyxin reaction was performed in growth medium in the presence of polymyxin-susceptible bacteria, the experiment proved that the precipitated lipid-antibiotic complex was not bactericidal but stable enough to prevent transfer of active antibiotic from the complex onto susceptible bacteria. If this specific behavior of acidic phospholipids in vitro has anything to do with the polymyxin susceptibility of the plasma membrane in living bacteria, the proposed fusion experiments using isolated phospholipids and polymyxin-resistant *A. laidlawii* B cells should generate polymyxin susceptibility just by application of phosphatidylglycerol or cardiolipin. As a preliminary to this experiment, Table 1 describes the quantitative adsorption of individual lipid vesicles to *A. laidlawii* B. This bacterium could absorb 13.5 to 45% of its own lipid content from the added lipid vesicles. In comparison, a value of about 50% has been reported by Grant and McConnell (5) for the same organism and dipalmitoyl phosphatidylcholine. These values can differ somewhat, depending on age and storage conditions of single vesicle preparations. However, as indicated in the last column in Table 1, polymyxin susceptibility was only produced from vesicles containing phosphatidylglycerol or cardiolipin.

Figure 1 shows that normal *A. laidlawii* B was very resistant to a 30-min exposure to up to 600 μg of polymyxin B per ml. When grown in the defined medium containing coenzyme A and acetate, the cells were completely resistant; when grown in the complex tryptose broth, 500 μg of polymyxin caused a 50% reduction in CFU. With or without cholesterol, a 40% decrease in CFU was observed in the defined medium without coenzyme A and acetate at 600 μg of polymyxin per ml. In contrast, *A. laidlawii* B associated with phosphatidylglycerol for 30 min exhibited a 50% CFU reduction with as little as 25 μg of antibiotic per ml. This 20-fold increase in polymyxin B susceptibility was evident over the entire range of the dose-response curve.

To assure that this phosphatidylglycerol-dependent, increased polymyxin susceptibility was not caused by aggregation of many still viable microorganisms yielding an apparent (but not true) drop in CFU, it was necessary to investigate other parameters of cell viability.

TABLE 1. *Absorption of lipid vesicles by A. laidlawii B*[a]

Composition of vesicles	Source of lipids[b]	^3H label in:	μg of phospholipid bound per mg of *A. laidlawii* lipid	Polymyxin susceptibility
PG	S	PG	245	+
CL	S	CL	270	+
PC	E	PE	135	−
PE/PC[c]	S/E	PE	365	−
PE/PG[c]	S/S	ND[d]	ND	+
CL/PC[c]	S/E	CL	310	+
PG	A	PG	450	+
PG/GL[e]	S/A	PG	225	−

[a] Cells suspended in phosphate buffer (950 μg [dry weight]/ml, containing 170 μg of lipids) were incubated with 4 μmol of phospholipid vesicles per ml for 30 min at 37 C. The vesicles were labeled with the indicated [^3H]phospholipids from *S. typhimurium*. Cells were separated from remaining vesicles by 4-min centrifugation at 2,500 × *g*, washed once with buffer, and counted. Cell-bound lipid was calculated from the known specific activity and the amount of radioactivity in the sediment and supernatant. PG, Phosphatidylglycerol; CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; GL, glycolipid.

[b] S, *S. typhimurium;* E, egg; A, *A. laidlawii* B.

[c] 1:1 mixture based on phosphate content.

[d] ND, Not determined.

[e] 1:1 mixture based on dry weight.

The inhibition by polymyxin B of protein and ribonucleic acid synthesis in *A. laidlawii* B fused with phosphatidylglycerol is demonstrated in Fig. 2. The slightly higher polymyxin susceptibility of the control cells in these experiments is due to the prolonged 3-h incubation time in the presence of antibiotic to obtain sufficient incorporation of radioactive precursors.

The dependence of increased polymyxin susceptibility on the composition of added lipid vesicles indicated in Table 1 is quantitatively documented in Fig. 3 and 4. Only phosphatidylglycerol, cardiolipin, and mixtures thereof with phosphatidylcholine or phosphatidylethanolamine showed this effect. Phosphatidylcholine alone and together with phosphatidylethanolamine did not induce increased antibiotic susceptibility. Though phosphatidylglycerol mixed with phosphatidylethanolamine yielded high susceptibility this was not obtained when phosphatidylglycerol was mixed with a glycolipid fraction from *A. laidlawii* B. Since phosphatidylethanolamine was inefficient, together with phosphatidylcholine being inefficient it-

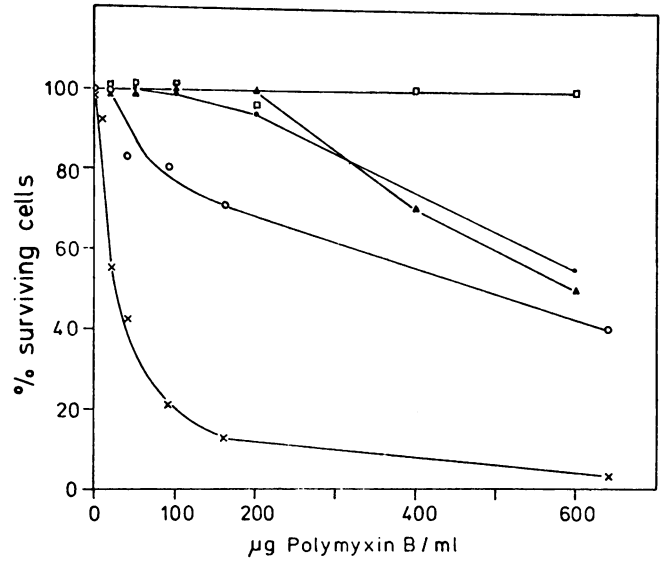

FIG. 1. *Susceptibility of A. laidlawii B (5 × 10⁸ cells/ml) to polymyxin B. The bacteria harvested by centrifugation were treated for 30 min at 37 C in phosphate buffer (pH 7) with antibiotic. Symbols: (●) cells grown in defined medium; (▲) cells grown in defined medium supplemented with 13 μg of cholesterol per ml; (□) cells grown in defined medium supplemented with 40 μg of coenzyme A and 100 μg of acetate per ml; (○) cells grown in tryptose broth and incubated prior to polymyxin treatment for 30 min at 37 C in 0.14 M NaCl-0.05 M sodium phosphate buffer (pH 7); (X) cells grown in tryptose broth and incubated as the preceding sample, but in the presence of 4 μmol of phosphatidylglycerol in the form of vesicles.*

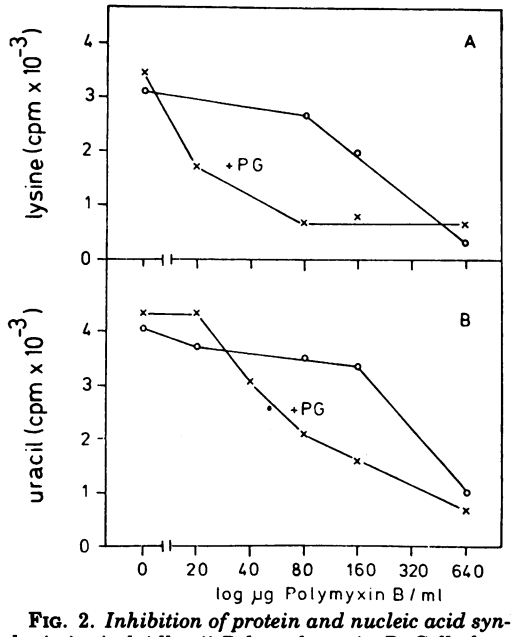

FIG. 2. *Inhibition of protein and nucleic acid synthesis in A. laidlawii B by polymyxin B. Cells harvested from tryptose broth were treated with phosphatidyl glycerol as indicated in Fig. 1. Action of polymyxin was measured in defined medium containing radioactive precursors after 3 h of incubation at 37 C.*

self, it is concluded that phosphatidylethanolamine is not the active partner in the mixture with phosphatidylglycerol, because this lipid and cardiolipin yielded active preparations with phosphatidylcholine. Unfortunately, phosphatidylethanolamine is unable to form lipid vesicles alone. This property has also hampered a direct approach in the cited liposome experiments of Imai et al. (6).

If fusion with phosphatidylglycerol or cardiolipin is actually the reason for the increased polymyxin susceptibility of *A. laidlawii* B, the kinetics of gain in polymyxin susceptibility should follow the one of association between lipid vesicles and bacteria. This behavior is shown in Fig. 5. The fast uptake of lipids, and the parallel increase in polymyxin susceptibility within a few minutes, favor a physical absorption process, not a biochemical uptake of lipids due to growth of the bacteria The large amount of lipid taken up points to an uptake of whole vesicles by fusion events, as described by Grant and McConnell (5). The absorption of single lipid molecules by accidental contacts between cells and vesicles also seems unlikely for the same reason.

The absorption of vesicles was a function of vesicle concentration, as demonstrated for cardiolipin in Fig. 6. Up to a concentration of

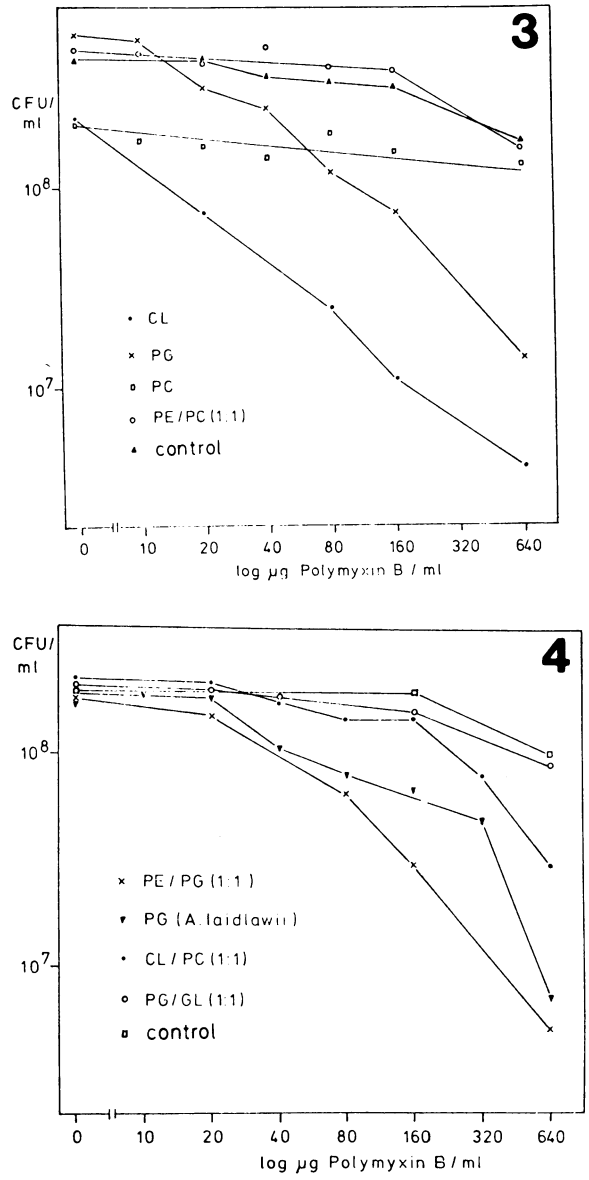

FIG. 3 and 4. *Influence of the composition of lipid vesicles on induced polymyxin susceptibility in A. laidlawii B. Treatment with vesicles and polymyxin was as in Fig. 1. CL, Cardiolipin; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; GL, glycolipid from A. laidlawii. Vesicle concentration was 4 µmol of lipid per ml during fusion.*

0.4 µmol of cardiolipin phosphate per ml and 950 µg of bacteria (dry weight), almost complete absorption of vesicles occurred. Above this point, saturation of *A. laidlawii* B with lipid was obtained. Figure 6 proves, in addition, that the lipid-induced polymyxin susceptibility parallels the absorption isotherms.

It has been shown by Newton (9) and in our laboratory (24) that binding of polymyxin to membranes does occur during bactericidal action. Figure 7 depicts the time course of binding of polymyxin B to phosphatidylglycerol-treated *A. laidlawii* B, which is exactly the reciprocal of the number of surviving cells.

If fusion of polymyxin-resistant *A. laidlawii* B with phosphatidylglycerol yields negatively charged membrane areas consisting of the externally added lipid as polymyxin receptor

sites, further growth of the bacteria without phosphatidylglycerol should lead to a dilution of the phosphatidylglycerol area and, consequently, to a diminished polymyxin susceptibility. We therefore investigated the polymyxin susceptibility of phosphatidylglycerol-treated *A. laidlawii* B after transfer into a fresh growth medium. The full resistance was restored within 5 min of incubation at room tempera-

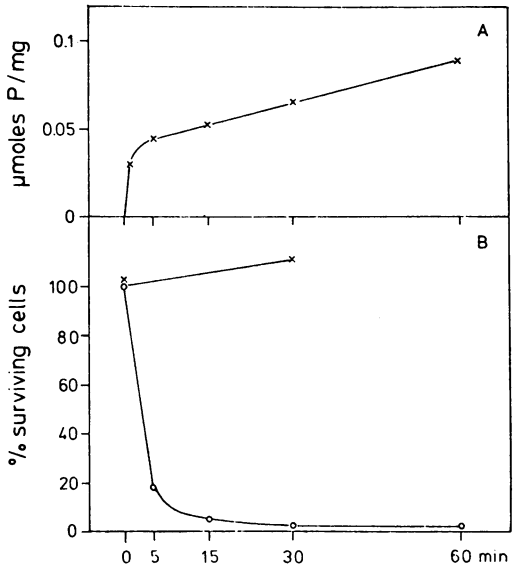

FIG. 5. *Time-dependent uptake of phosphatidylglycerol by A. laidlawii B compared to polymyxin susceptibility. A 950-µg amount of bacteria (dry weight) in phosphate buffer was treated with 4 µmol of phosphatidylglycerol per ml at 37 C. At the indicated time intervals, part of the sample was diluted to about 5 × 10⁸ viable cells/ml and incubated with 80 µg of polymyxin B per ml for 30 min at 37 C. The remaining was centrifuged at 2,500 × g. Phosphatidylglycerol sedimenting with the cells was determined by its tritium label. Symbols: (X) control cells; (O) phosphatidylglycerol cells.*

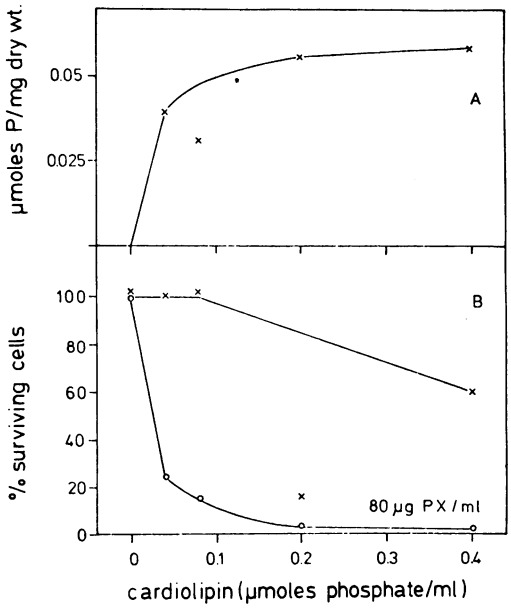

FIG. 6. *Concentration-dependent absorption of cardiolipin by A. laidlawii B compared to polymyxin susceptibility. Incubation with cardiolipin was for 30 min at 37 C; all other conditions were as described in Fig. 5. (A) Absorption isotherms for cardiolipin; (B) polymyxin susceptibility without phospholipid (X) and with phospholipid (O).*

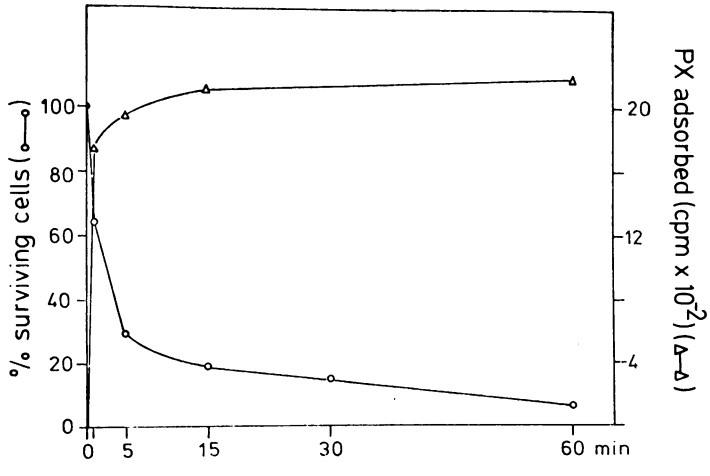

FIG. 7. *Time-dependent binding and action of [¹⁴C]mono-N-acetyl-polymyxin B (80 µg/ml) in A. laidlawii B fused with phosphatidylglycerol. Cell-bound radioactivity was determined in the sediment after rapid centrifugation at 2,500 × g.*

ture. This excludes dilution of polymyxin-susceptible membrane areas by growth processes. Since *A. laidlawii* B needs serum fraction V proteins present in all used growth media (20) as an indispensible growth factor, the influence of these proteins on the new polymyxin resistance was studied. Serum fraction V indeed induced a concentration-dependent decrease of the phosphatidylglycerol-caused polymyxin susceptibility (Fig. 8). Complete resistance was achieved at 0.5% serum fraction V, the usual content in the defined growth medium. Cytochrome *c*, which is also absorbed by *A. laidlawii* B (15), had no effect. Table 2 shows that the binding capacities of *A. laidlawii* B for serum fraction V were twice as high in bacteria associated with acidic phospholipids as in those treated with neutral phospholipids. Since binding of polymyxin B to polymyxin-susceptible, phosphatidylglycerol-treated cells was correlated with the bactericidal action (Fig. 7), the polymyxin-binding capacities of serum fraction V-incubated, polymyxin-resistant but formerly -sensitive cells should be greatly reduced. This result has been obtained (Fig. 9).

In our hands, 1 mg of *A. laidlawii* cells (dry weight) corresponded to 5×10^{10} CFU (viable cells). Assuming a molecular weight of 1,300 for the [^{14}C]mono-*N*-acetyl-polymyxin B derivative, the following amounts of antibiotic were bound per milligram of dry bacteria at the first point of saturation in Fig. 9 (25 μg of polymyxin/ml): 40.5 μg in phosphatidylglycerol-treated bacteria, but only 7.5 μg after addition of 0.5% serum fraction V to the same preparation, compared to 3.4 μg in the polymyxin-resistant control cells. This would be 385,000, 70,000, and 31,000 antibiotic molecules, respectively, bound per single cell. In phosphatidylglycerol-treated *A. laidlawii* B the molar ratio of absorbed polymyxin to absorbed phosphatidylglycerol (about 50 nmol/mg [dry weight]; see Fig. 5) is then about 0.62. This ratio is 0.25 in vitro, as mentioned at the beginning. Therefore, the absorbed external phosphatidylglycerol cannot be the only binding component in these cells. We assume that polymyxin can enter polymyxin-susceptible bacteria and complex in the cytoplasm with nucleic acids, as recently suggested for *S. typhimurium* (18).

TABLE 2. *Binding capacities of phospholipid-associated A. laidlawii B for serum fraction V*[a]

Added phospholipid vesicles	Polymyxin susceptibility	μg of *N*[^{14}C]acetyl-serum fraction V bound/mg (dry weight) of bacteria
None	−	0.83
PE/PG	+	1.80
CL	+	1.95
PC/PE	−	1.21
PC	−	1.02

[a] Experimental details are described in Materials and Methods. See Table 1 for abbreviations.

As in artificial liposomes, the polymyxin susceptibility of phosphatidylglycerol-associated bacteria was not severely inhibited by divalent cations (Fig. 10), because only a small fraction (~10%) of the whole cell population showed some protection by 10^{-2} M magnesium chloride.

S. typhimurium is resistant to polymyxin B below 12 C (24). This point can be varied in *Escherichia coli* deficient in unsaturated fatty acid synthesis by growth in oleic acid- or palmitelaidic acid-containing media (Bader and

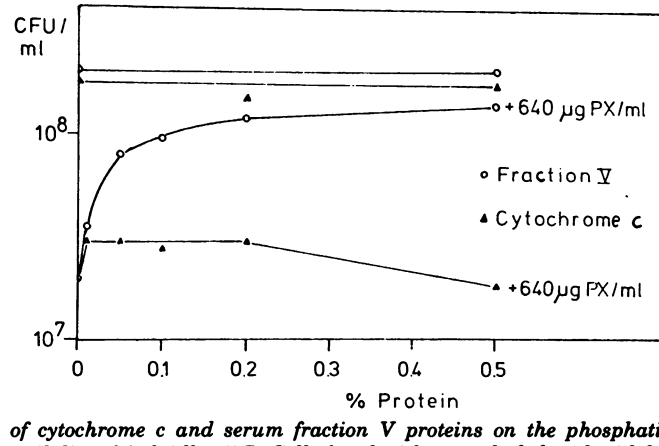

FIG. 8. *Influence of cytochrome c and serum fraction V proteins on the phosphatidylglycerol-induced polymyxin (PX) susceptibility of A. laidlawii B. Cells fused with 4 μmol of phosphatidylglycerol as described in Fig. 1 were incubated for 5 min at room temperature with the proteins. The following polymyxin treatment was for 30 min at 37 C.*

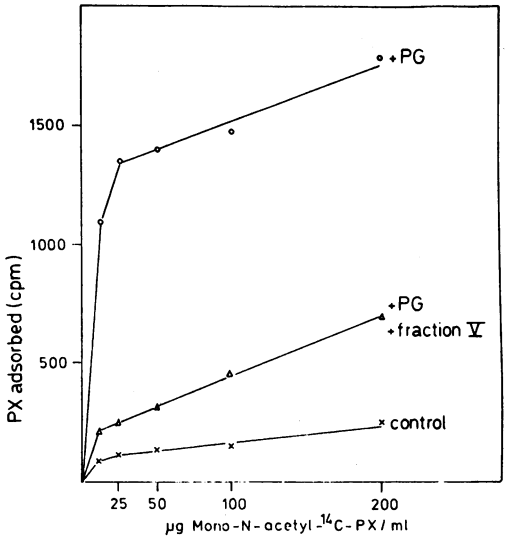

FIG. 9. *Binding isotherms of [14C]mono-N-acetyl-polymyxin B in polymyxin-susceptible and -resistant A. laidlawii B. PG, Cells fused with phosphatidylglycerol; PG + fraction V, cells fused with phosphatidylglycerol but incubated afterwards with 0.5% serum fraction V; control cells were incubated in phosphate buffer only. Incubation with radioactive polymyxin was with 8 × 10⁸ cells/ml for 30 min at 37 C. Cell-bound radioactivity was determined as in Fig. 7. One nanomole of polymyxin B = 2,680 counts/min.*

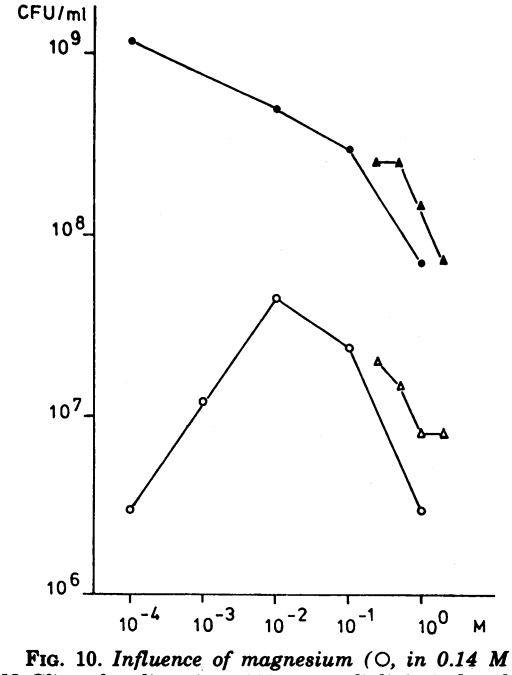

FIG. 10. *Influence of magnesium (○, in 0.14 M NaCl) and sodium ions (△) on cardiolipin-induced polymyxin susceptibility in A. laidlawii B. Before addition of 640 µg of polymyxin B per ml, the fused cells were treated for 2 min with the indicated ion concentration. Filled symbols, control without polymyxin; open symbols, CFU after incubation with polymyxin for 30 min at 37 C.*

Teuber, in preparation). *A. laidlawii* B also exerts some degree of polymyxin resistance at low temperature (Fig. 11). Oleic acid-grown cells show a break in the survival curve at 10 C, and palmitelaidic acid-grown bacteria show a break at 20 C, in accordance with a more or less rigid hydrocarbon chain of these fatty acids (10). These points are lowered in cells associated with *Salmonella* lipids. The results imply an important role of membrane fluidity in the polymyxin susceptibility of microorganisms.

DISCUSSION

Acholeplasma and *Mycoplasma* species are able to vary their membrane composition by incorporation of exogenous fatty acids and cholesterol (14), synthesis of carotenoids (13), uptake of exogenous proteins (15), and fusion with

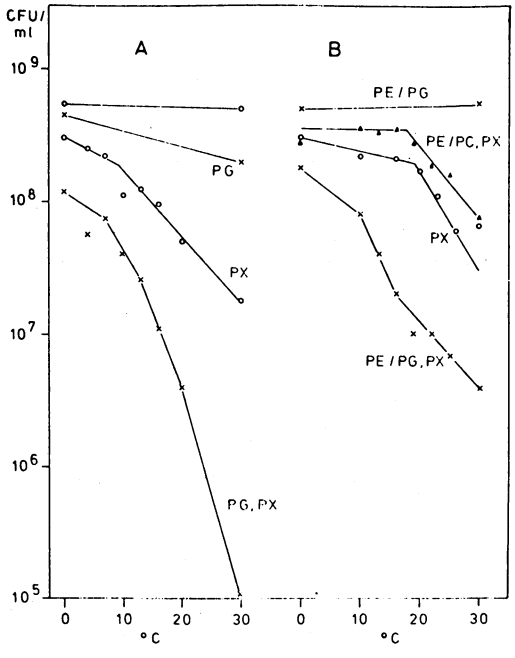

FIG. 11. *Effect of fatty acid supplementation on polymyxin susceptibility of A. laidlawii B at different temperatures. (A) Cells grown with oleic acid; (B) cells grown with palmitelaidic acid. Fusion with vesicles was as in Fig. 3 and 4; treatment with polymyxin (PX, 420 µg/ml) was for 30 min at the indicated temperature. After the polymyxin treatment the cell suspensions were diluted with tryptose broth adjusted to the previous incubation temperature.*

phospholipid vesicles (5). The reason for the unusual polymyxin resistance of these cell wall-lacking bacteria, however, has not been investigated. Two factors could contribute to this resistance.

(i) Polymyxin cannot adsorb to the cell membrane because serum fraction V proteins present in all growth media are adsorbed to the cell surface covering and protecting the lipid bilayer. Evidence for such a surface protein sheet has been provided by Rottem and Razin's laboratory (16) by the observation that membrane phospholipids of *Mycoplasma hominis* were only attacked by phospholipase C after treatment with proteolytic enzymes. Similarly, Schiefer and co-workers could only agglutinate *M. hominis* cells with phosphatidylglycerol-specific antibodies if the cells had been subjected to a proteolytic digestion (17).

(ii) The phosphatidylglycerol molecules are asymmetrically localized in the inner leaflet of the lipid bilayer of the membrane and therefore inaccessible for polymyxin B. Alternatively, the *Acholeplasma* glycolipids prevent interaction of the antibiotic with phosphatidylglycerol in an unknown mechanism.

Irrespective of these considerations, however, fusion of *A. laidlawii* B with phospholipid vesicles would generate membrane areas differing from the original state by the amount, nature, and distribution of lipids. Our data confirm the observation of Grant and McConnell (5) that large amounts of lipid can be absorbed by *A. laidlawii* B from added lipid vesicles without loss of viability. This absorption was fast and concentration dependent. It led to an increased polymyxin susceptibility if vesicles containing phosphatidylglycerol or cardiolipin were employed. No polymyxin susceptibility was produced with phosphatidylethanolamine, the main phospholipid of the gram-negative enterobacteria, or with phosphatidylcholine. It is not clear why vesicles composed of phosphatidylglycerol and *A. laidlawii* B-glycolipids did not induce polymyxin susceptibility.

Although *A. laidlawii* associated with lipid vesicles was still able to divide and form colonies on solid media, these preparations were more sensitive to mechanical stress such as centrifugation and resuspension. For this reason, fusion experiments were terminated, if possible, by dilution before addition of polymyxin B.

According to the electron-spin resonance data of Grant and McConnell (5), the spin-labeled dipalmitoyl phosphatidylcholine was distributed by lateral diffusion in the membrane of *A. laidlawii* B, with some remaining phosphatidylcholine patches. This lateral diffusion could be the reason why the polymyxin concentrations necessary to kill the phosphatidylglycerol-associated *A. laidlawii* B were about 10 times higher than the minimum inhibitory concentrations for susceptible enteric bacteria.

Though we used colony-forming ability as a very sensitive method to test the polymyxin action on *A. laidlawii*, polymyxin indeed induced destruction of the selective permeability barrier. This was demonstrated by the fact that nicotinamide adenine dinucleotide-dependent, cytoplasmic lactic dehydrogenase could be measured with external reduced nicotinamide adenine dinucleotide in the presence of polymyxin B with much higher specific activities than with intact bacteria (Bader, unpublished data). This "activation" of internal enzymes is typical for membrane-damaged bacteria (26).

From the cited literature and our own data, we therefore propose the following model for the polymyxin resistance of mycoplasmas. The mycoplasma membrane is covered with protein, possibly taken up from serum fraction V in the growth media. This protein leaflet, perhaps together with glycolipids, hinders the cationic polymyxin B from binding to the anionic phosphatidylglycerol of the mycoplasma membrane. Fusion of intact mycoplasmas with phospholipid vesicles dilutes the protein coat and generates surface areas representing the outer leaflet of a lipid bilayer. If this lipid bilayer is composed of acidic phosphatides, the membrane becomes susceptible to polymyxin B. As soon as these membrane areas are again coated with protein by addition of serum fraction V, the gained polymyxin susceptibility is lost. It remains to be established why incorporation of polymyxin B into a membrane destroys the selective permeability of this membrane.

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