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# Effect of Plasmid RP1 on Phase Changes in Inner and Outer Membranes and Lipopolysaccharide from *Acinetobacter calcoaceticus*: A Fourier Transform Infrared Study†

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ABSTRACT: The successful transfer of the resistance plasmid RP1 into the Gram-negative bacterium *Acinetobacter calcoaceticus* resulted in increased resistance of this microorganism to the antibiotics kanamycin and tetracycline. Microorganisms harboring the RP1 plasmid showed altered fatty acid composition in the lipopolysaccharide fraction and increased outer membrane permeability compared to organisms without the plasmid. Thermotropic gel to liquid crystal lipid phase changes were detected in both inner and outer membranes and purified lipopolysaccharide by Fourier transform infrared spectroscopy. The phase transition temperatures observed in the outer membranes and isolated lipopolysaccharide of the plasmid-containing cells were significantly higher than those of the plasmid-free organisms, while little difference was observed for the inner membranes. The plasmid-induced decrease in outer membrane fluidity may play a mediating role in the mechanisms of antibiotic resistance and susceptibility to host immune cells in Gram-negative microorganisms.

Phase changes in bacterial membranes have been detected by a variety of physical and spectroscopic techniques, such as calorimetry (Steim et al., 1969) and NMR (Smith, 1979), ESR[1] (Davis et al., 1985), and fluorescence (Tecoma et al., 1977) spectroscopy. The structure of the *Acholeplasma laidlawii* plasma membrane has been studied intensively since this organism possesses no cell wall and the fatty acid composition of its membrane lipids can be easily modified by including appropriate fatty acid supplements in the growth

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[1] Abbreviations: ESR, electron spin resonance; FT-IR, Fourier transform infrared; KDO, 2-keto-3-deoxyoctonate; LPS, lipopolysaccharide; PMN, polymorphonuclear leukocyte; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

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medium [for a recent review, see McElhaney (1984)]. Much less is known, however, concerning the molecular architecture of Gram-negative bacterial membranes, especially the outer membrane in which LPS is concentrated on the exterior surface. Some evidence exists to support the notion that, in certain Gram-negative organisms, the outer membrane is less fluid than the inner membrane (Shukla et al., 1980); however, a detailed description of the motional characteristics of the hydrophobic interior of the outer membrane of these organisms is lacking.

In the past few years, FT-IR spectroscopy has emerged as a useful tool for the study of temperature-dependent structural changes in lipid bilayers and biological membranes in an aqueous environment (Cameron et al., 1979; Blazyk & Rana, 1987; Cameron & Dluhy, 1986). By analyzing the symmetric methylene C–H stretching band of the lipid hydrocarbon chains as a function of temperature, changes in lipid fluidity can be detected. A shift in the position of the band toward higher frequency indicates an increase in the percentage of gauche conformers (and hence a more nonlinear geometry), while an increase in bandwidth correlates with greater translational and rotational motion of the methylene groups. Phase changes have been detected in *A. laidlawii* plasma membranes by this technique (Casal et al., 1980).

The antibacterial potential of PMN depends in part upon phagocytosis and the subsequent destruction of the phagocytized bacteria by subcellular granule contents that are delivered to the phagosome during degranulation. The role of the Gram-negative bacterial membrane in this interaction with PMN during phagocytosis and the intracellular destruction of the bacteria has been recently reviewed (Spitznagel, 1983). The relative resistance or sensitivity of target Gram-negative bacteria to the antimicrobial potential of PMN granule contents has been shown to be influenced by pili (Punsalang & Sawyer, 1973), LPS (Modrzakowski & Spitznagel, 1979; Hodinka & Modrzakowski, 1983; Weiss et al., 1983, Shafer et al., 1984), and lipid composition of the outer membrane (Modrzakowski & Paranavitana, 1981). We have observed changes in resistance to phagocytosis and sensitivity to the nonoxidative bactericidal activity of isolated rat PMN granule extract on *Acinetobacter calcoaceticus* HO1-N upon acquisition of the resistance plasmid RP1; in particular, a PMN protein fraction rich in low molecular weight cationic peptides shows a marked increase in bactericidal potency for $RP1^+$ organisms (Loeffelholz & Modrzakowski, 1987). The introduction of this resistance plasmid into *A. calcoaceticus* is postulated to have altered the outer membrane character of the target microorganism.

In this paper, we have used FT-IR spectroscopy to monitor the effects of the plasmid RP1 on the thermotropic phase transitions observed in isolated inner and outer membranes and purified LPS from *A. calcoaceticus*.

## Materials and Methods

*Bacteria and Culture Conditions. A. calcoaceticus* HO1-N was maintained on BBL Trypticase soy agar. For membrane isolation, bacteria were grown on BBL Trypticase soy broth at 37 °C to logarithmic growth (optical density of 0.5–0.6 at 540 nm). The resistance plasmid RP1 was transferred via conjugation in Trypticase soy broth (Miller, 1972). *Pseudomonas aeruginosa* PA067 (histidine minus) served as the donor strain. Equal volumes of donor and recipient overnight broth cultures were combined in a test tube and incubated at 37 °C for 30 min. Aliquots were plated on acetate minimal agar with kanamycin (Sigma, 20 μg/mL) as the selective agent. Plasmid DNA was isolated from crude cell lysates and analyzed on agarose gels (Kado & Liu, 1981). Expression of RP1 was confirmed by determining the minimal inhibitory concentrations of tetracycline and kanamycin in Trypticase broth. The conjugated strain was continually monitored for expression of RP1 by testing for antibiotic sensitivity with the disc diffusion method (Bauer et al., 1966).

*Isolation of Membranes and Purification and Analysis of LPS.* Bacterial membranes were isolated by the procedure of Hancock and Nikaido (1978). All steps were conducted at 4 °C. Midlog-phase bacteria were washed once in 30 mM Tris, pH 8, and resuspended in the same medium containing 20% sucrose (w/v). Ribonuclease, deoxyribonuclease, and egg white lysozyme (Sigma) were added to final concentrations of 0.5, 0.5, and 0.1 mg/mL, respectively. The cell suspension was passed through a French press cell twice at 15000 psi and centrifuged at 1000*g* for 10 min to remove cell debris. The supernatant was layered over a discontinuous sucrose gradient consisting of 5 mL of 60% sucrose (w/v) over 5 mL of 70% sucrose (w/v) in 30 mM Tris, pH 8, and was centrifuged at 160000*g* in a Beckman SW40Ti rotor for 3 h. The two resulting bands were removed, pelleted, and washed in distilled water. Purity of the separated membranes was confirmed by assaying for the outer membrane, KDO (Waravdekar & Saslaw, 1959).

LPS was purified according to the phenol–water method of Westphal and Jann (1965). This preparation contained less than 6% nucleic acid as determined by absorbance measurements at 260 nm. LPS was examined for RP1-mediated morphological differences by SDS–PAGE. Since changes in polysaccharide content have been shown to affect the electrophoretic migration of LPS (Hitchcock & Brown, 1983), purified LPS and whole-cell lysates treated with proteinase K were electrophoresed on 14% acrylamide gels (Laemmli, 1970) and visualized with silver stain. Lipid A and glucosamine were isolated from LPS that was hydrolyzed in 0.1 N acetic acid for 4 h (Galanos et al., 1971). Lipid A content was determined gravimetrically while glucosamine was measured by the procedure of Rondle and Morgan (1955). Content of simple carbohydrates (reducing sugars not containing amino groups) in LPS was quantitated by the phenol–sulfuric acid procedure of Dubois et al. (1956).

Fatty acid methyl esters were prepared from LPS by acid hydrolysis followed by treatment with $BF_3$–methanol (Keleti & Lederer, 1974) and were identified and quantitated by gas chromatography on a 10% Silar 5CP (Alltech Associates) column by comparison to known standards.

Sensitivity to actinomycin D (Sigma) was used to assess the permeability of the outer membrane since resistance of Gram-negative bacteria to actinomycin D results from the inability of the drug to cross the outer membrane (Nikaido & Nakae, 1979). Organisms grown in Trypticase soy broth ($10^3$ cells/mL) were treated with actinomycin D (50 μg/mL) and incubated at 37 °C for 60 min. Aliquots (including controls) were plated in duplicate on Trypticase soy agar. Viability was expressed as the percentage of colony-forming units in the treated cells as compared to controls.

*FT-IR Spectroscopy and Data Analysis.* Membrane or LPS samples were pelleted by centrifugation, resuspended in $D_2O$, and repelleted. The sample (containing about 10% dry weight) was contained in a thermoelectrically temperature-controlled 25 μm thick liquid FT-IR cell with $CaF_2$ windows, as previously described (Blazyk & Rana, 1987). The accuracy of the temperature control and measurement is about ±0.5 deg while the precision is about ±0.1 deg. Automated infrared spectroscopic measurements were performed on a Mattson

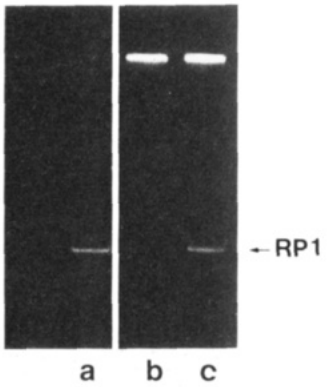


FIGURE 1: Agarose gel electrophoresis of bacterial DNA: (a) RP1 isolated from *P. aeruginosa* PA067 (donor strain); (b) *A. calcoaceticus* HO1-N; (c) *A. calcoaceticus* HO1-N (RP1$^+$).

Sirius 100 FT-IR spectrometer equipped with a mercury–cadmium–telluride detector. A total of 250 interferograms were coadded and Fourier transformed with triangular apodization and one level of zero filling to produce a spectrum with a resolution of 4 cm$^{-1}$, and data points encoded each 2 cm$^{-1}$. The peak position was calculated as the center of area of the top five data points of the peak (Cameron et al., 1982). The signal to noise ratio of the spectra was estimated at 500–1000. The error associated with the center of gravity measurements of these spectra is about ±0.1 cm$^{-1}$ (Cameron et al., 1982). The bandwidth at three-quarter height was determined after subtraction of the gently sloping base line.

## Results

*Confirmation of Plasmid Transfer and Effect on Properties of the Outer Membrane and Isolated LPS.* Successful transfer of the plasmid RP1 to *A. calcoaceticus* was demonstrated by the appearance of plasmid DNA in agarose gels (shown in Figure 1) and an increased resistance to kanamycin and tetracycline, two antibiotics to which resistance is conferred by RP1. The minimum inhibitory concentrations of kanamycin and tetracycline were 390 and 475 μg/mL, respectively, in RP1$^+$ organisms, and less than 10 μg/mL for each antibiotic in RP1$^-$ organisms. An analysis of LPS composition showed no significant variation in percent composition of lipid A, total carbohydrate, KDO, or glucosamine between the two strains. Moreover, the electrophoretic behavior of LPS from the two strains was nearly identical (data not shown), indicating the lack of major structural alterations in the O-specific polysaccharide side chains.

On the other hand, sizable differences in fatty acid composition of the isolated LPS were observed. The major fatty acid constituents of the RP1$^-$ LPS were β-hydroxylaurate (~50%) and β-hydroxymyristate (~30%). In the RP1$^+$ LPS, a large decrease in β-hydroxylaurate (to ~5%) and a slight decrease in β-hydroxymyristate (to ~20%) were found, with corresponding increases in *n*-alkanoyl chains (particularly palmitate and stearate). In addition, acquisition of the plasmid was associated with a decrease in resistance to actinomycin D, with only 10 ± 2% viability compared to 46 ± 5% viability for the organism lacking the plasmid, revealing a significant increase in the permeability of the RP1$^+$ outer membrane.

*Phase Changes in the Inner and Outer Membranes of RP1$^-$ and RP1$^+$ Strains of A. calcoaceticus.* The peak positions of the symmetric methylene C–H stretching bands of inner and outer membranes from RP1$^-$ and RP1$^+$ organisms as a function of temperature are shown in Figure 2. For the inner membranes (Figure 2A), an increase in the slope of the curves between 20 and 25 °C indicates that the lipid fatty acyl chains

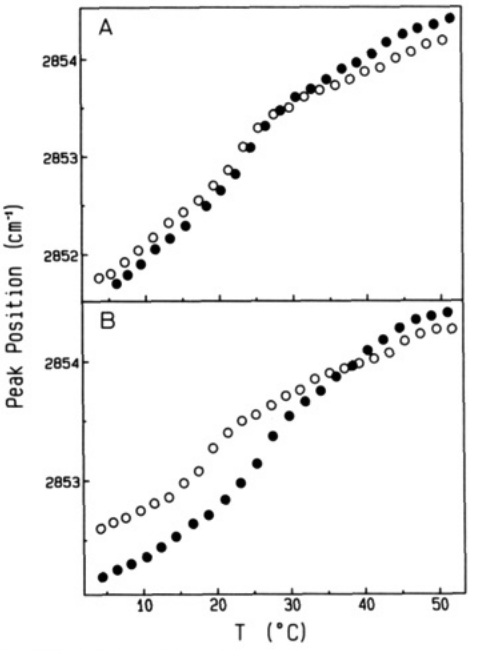


FIGURE 2: Effect of plasmid RP1 on the temperature dependence of the symmetric methylene C–H stretching band in *A. calcoaceticus* inner and outer membranes. The change in frequency as a function of temperature is shown for (A) inner membranes and (B) outer membranes from RP1$^-$ (○) and RP1$^+$ (●) organisms.

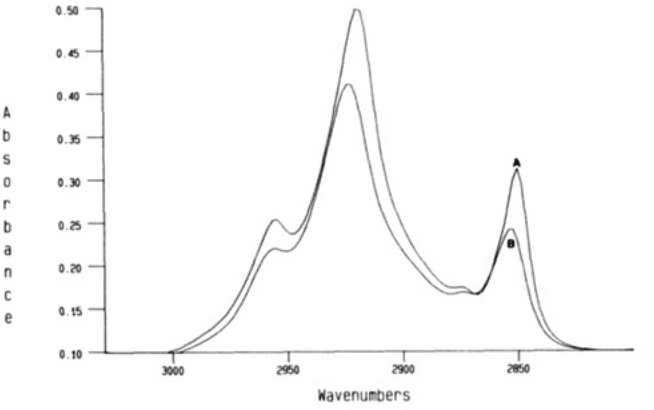


FIGURE 3: Temperature dependence of the infrared C–H stretching region between 3030 and 2800 cm$^{-1}$ for RP1$^-$ LPS. The upper (A) and lower (B) spectra were measured at 6.2 and 50.7 °C, respectively. The two antisymmetric bands are observed near 2920 cm$^{-1}$ (methylene) and 2960 cm$^{-1}$ (methyl), while the two symmetric bands occur near 2850 cm$^{-1}$ (methylene) and 2875 cm$^{-1}$ (methyl).

are becoming more disordered (as a result of a gel to liquid crystal phase change in the membrane lipids) over this temperature interval. Very little difference in the transition temperature range of the inner membranes from the RP1$^-$ and RP1$^+$ organisms is observed.

In the case of the outer membranes (Figure 2B), the phase transition temperature for the RP1$^-$ strain (near 20 °C) is below that of the RP1$^+$ strain (about 25–30 °C), suggesting that the lipids in the outer membrane of the RP1$^+$ organism are significantly less fluid between 20 and 30 °C as compared to those of the RP1$^-$ organism. Since the outer membrane contains both phospholipids and LPS and since the LPS is concentrated on the external face of the outer membrane, a comparison of the motional characteristics of LPS isolated from each strain was undertaken.

*Fluidity of LPS from RP1$^-$ and RP1$^+$ Strains of A. calcoaceticus.* The complete C–H stretching region for RP1$^-$ LPS at two different temperatures (one below and one above the phase transition temperature) is shown in Figure 3. At low

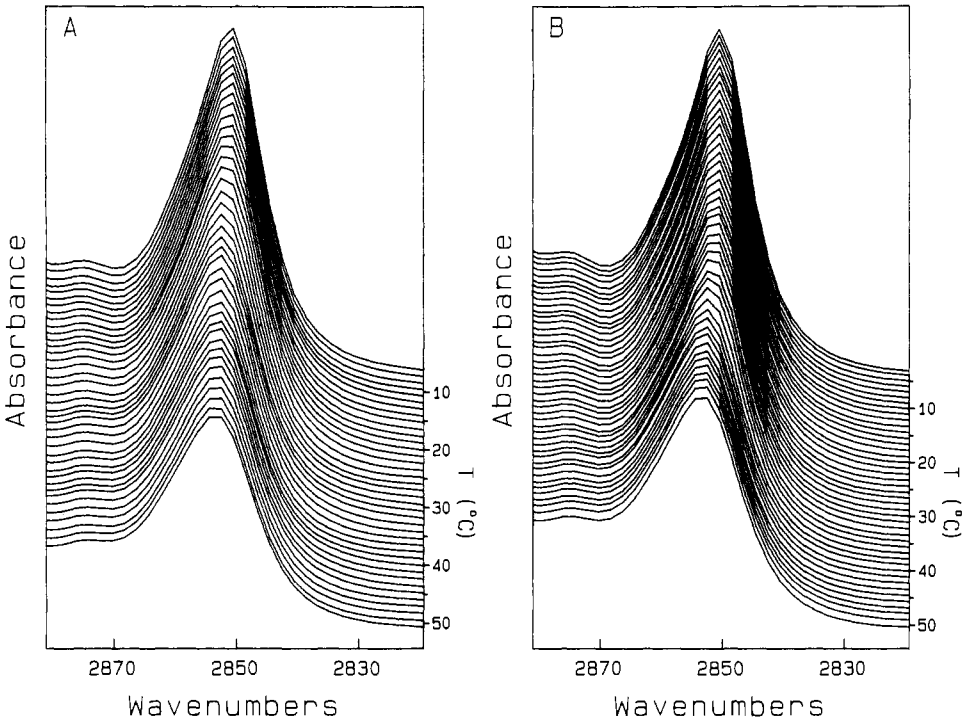


FIGURE 4: Infrared spectra between 2820 and 2880 $cm^{-1}$ for LPS extracted from *A. calcoaceticus*. Effect of temperature between 5 and 50 °C on the symmetric methylene C–H stretching band of LPS from (A) $RP1^-$ and (B) $RP1^+$ organisms.

temperature, the bands are sharp and occur at relatively lower frequency. During the phase change, the peaks broaden and shift toward higher frequency. The symmetric methylene C–H stretching band between 2820 and 2880 $cm^{-1}$ of LPS from each strain was analyzed as a function of temperature (Figure 4). For LPS from the $RP1^-$ organism (Figures 4A and 5), the shift in peak position is maximal in the spectra between 20 and 30 °C. On the other hand, for the LPS extracted from the plasmid-containing organisms (Figures 4B and 5), the maximal shift occurs at a much higher temperature (near 35 °C) and over a smaller temperature range. Changes in bandwidth appear at an earlier stage during the phase transition than changes in frequency and go through a maximum during the transition (Figure 5). At 20 °C, the hydrocarbon chains of $RP1^-$ LPS are highly mobile, while those of the $RP1^+$ LPS do not approach the same level of mobility until the temperature is above 30 °C.

## Discussion

Plasmids, extrachromosomal elements of DNA, have been shown to induce a variety of alterations in the cell envelope of Gram-negative bacteria that are responsible for changes in the sensitivity of the organisms to various antimicrobial agents (Foster, 1975) and host immune functions (Lian & Pai, 1985). Plasmids therefore can be valuable tools for studying the structural basis of bacterial resistance mechanisms. Plasmid RP1 is of particular interest since it confers multiple antibiotic resistance (Grinstead et al., 1972) and possesses broad host range (Olsen & Shipley, 1973). It has been shown that the acquisition of plasmid RP1 by *P. aeruginosa* is associated with changes in lipid, cation, LPS, and peptidoglycan content (Kenward et al., 1978). A genetic region of RP1 designated *irp*, which provides *Escherichia coli* with nonenzymatic intrinsic resistance to antibiotics, has been identified and is believed to code for alterations to the cell envelope (Lian & Pai, 1985).

The outer membrane is the site of interactions between the Gram-negative cell and its environment. LPS, a major component of the exterior surface of the outer membrane, plays an important role in interactions with antibiotics (Nikaido, 1976) and antimicrobial functions of host immune cells. Changes in the outer membrane constituents may influence these interactions and, hence, the virulence of affected bacteria. We have demonstrated here that the fatty acyl composition of LPS in *A. calcoaceticus* is significantly altered by the presence of the plasmid RP1. While such changes in LPS

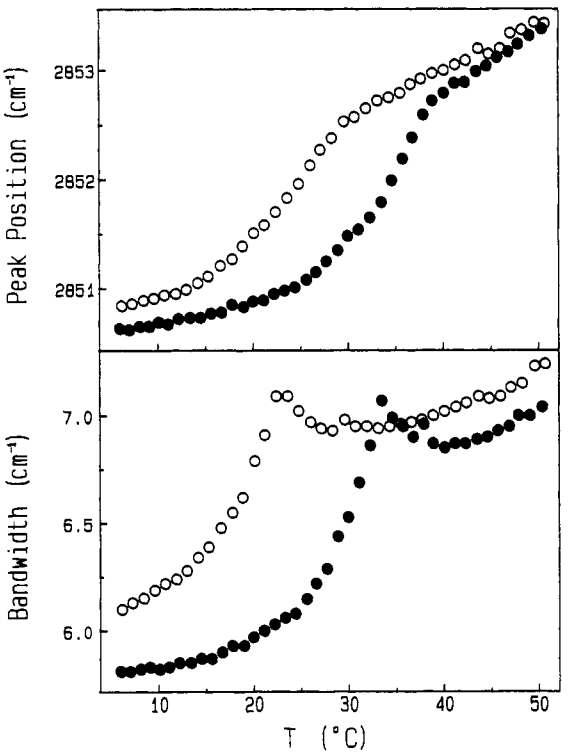


FIGURE 5: Temperature dependence of spectral parameters of the symmetric methylene C–H stretching band of LPS extracted from *A. calcoaceticus*. Changes in the peak position and bandwidth as a function of temperature for the spectra in Figure 4 are shown for LPS from $RP1^-$ (○) and $RP1^+$ (●) organisms.

structure are known to affect the sensitivity of bacteria to oxygen-independent bactericidal activity, the molecular mechanisms responsible for these effects remain unclear.

Using FT-IR spectroscopy, we have shown that the presence of the plasmid RP1 mediates substantial changes in the temperature-dependent phase behavior of the outer membrane and extracted LPS of *A. calcoaceticus*. The observed plasmid-induced decrease in outer membrane fluidity correlates well with phase changes in the isolated LPS. The decrease in $\beta$-hydroxy fatty acid content in RP1$^+$ LPS may account for this change in fluidity since the carbohydrate portion of the LPS molecules is not greatly affected by the introduction of the plasmid. These structural alterations in LPS might affect the interactions between PMN bactericidal proteins and the cell envelope. Further spectroscopic investigations of membranes and purified components from organisms whose lipid composition is regulated by growth on defined carbon sources are now under way and should help to clarify the correlation between outer membrane fluidity and resistance to both antibiotics and antimicrobial functions of PMN.

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