

Interactions between the antimicrobial peptide, magainin 2, and *Salmonella typhimurium* lipopolysaccharides

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Using FT-IR spectroscopy, the effects of magainin 2 on the thermotropic behavior of LPS isolated from wild-type (SL3770) and LPS-mutant strains of *Salmonella typhimurium* are characterized and compared. The mutant strains include Ra (SL3749), polymyxin-sensitive Rb₂(s) (SH5014), polymyxin-resistant Rb₂(r) (SH5357) and Rc (HN202) LPS chemotypes, whose polysaccharide chains differ in length but possess an identical number of phosphorylation sites. In all cases, magainin 2 causes a concentration-dependent disordering of the LPS fatty acyl chains. Differences in disordering of LPS correlate more closely with the charge on the LPS molecule (determined by high-resolution ³¹P NMR) rather than with the length of the LPS sugar side chain, contradicting the currently accepted model for the interaction of cationic antibiotics with the Gram-negative cell envelope.

Magainin; Lipopolysaccharide; Phase change; FT-IR spectroscopy; ³¹P NMR spectroscopy; *Salmonella typhimurium*

1. INTRODUCTION

Recently, Macias et al. [1] demonstrated that *S. typhimurium* LPS mutants, which produce LPS molecules with an incomplete sugar side-chain, show a progressive loss of resistance to the antimicrobial activity of magainin 2 as the length of the LPS sugar side-chain decreases. The amino acid sequence of this cationic peptide is shown below:

G I G K F L H S A K K F G K A F V G E I M N S
1 5 10 15 20

Similar bactericidal activities have been noted for fractions of the granular contents from human and rat polymorphonuclear leukocytes containing a family of low molecular weight cationic peptides known as defensins [2–4] and the cationic bactericidal-permeability increasing (BPI) protein [5,6], which suggests that the molecular mechanisms of defensins, BPI protein and magainins may share common features. In addition, magainin 2 interacts strongly with *S. typhimurium* outer membranes [7] and wild-type LPS [8], disordering the hydrocarbon chains. These observations implicate LPS and the outer membrane as integral factors in the interaction between magainin and the Gram-negative cell envelope. Weiss et al. [5] proposed that the enhanced susceptibility of LPS- mutant strains to the bactericidal activity of BPI

protein arises from the accessibility of negatively charged residues on LPS molecules with deficient sugar side-chains. The presumption is that these charges are less effectively shielded from interactions with positively charged groups of cationic peptides in comparison to wild-type LPS, which has an extensive polysaccharide moiety (i.e. an umbrella effect).

In order to test this hypothesis and better understand the nature of the increased sensitivity of LPS mutants to the antimicrobial effects of magainin 2, peptide-induced changes in the thermotropic behavior of LPS isolated from wild-type *S. typhimurium* [8] and mutant strains that produce Ra (SL3749), polymyxin-sensitive Rb₂(s) (SH5014), polymyxin-resistant Rb₂(r) (SH5357) and Rc (HN202) LPS chemotypes are characterized by comparing temperature-dependent changes in the infrared spectrum. Although the polysaccharide chains of these LPS molecules differ in length, the number of phosphorylation sites among the variants is identical (see Fig. 1). Since the phosphorylation sites are heterogeneous and may contain either monophosphates or diphosphates, and may be free or esterified to either ethanolamine or arabinosamine [9], high resolution ³¹P NMR is used to delineate the LPS phosphorylation pattern and estimate the average charge per LPS molecule.

Magainin 2 disorders the fatty acyl chains of LPS from the mutant strains in a concentration-dependent fashion which does not depend upon the length of the LPS sugar side-chain, but correlates more closely with the level of charge on the LPS molecule. Similar results were observed for outer membrane-peptidoglycan complexes from wild-type and LPS-mutant organisms [7].

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These findings are inconsistent with the currently accepted model [5] describing the increased susceptibility of LPS mutants to the killing activity of cationic antibiotics.

2. MATERIALS AND METHODS

Salmonella typhimurium wild-type SL3770 (Sm), SL3749 (Ra), polymyxin-sensitive SH5014 (Rb₂(s)), polymyxin-resistant SH5357 (Rb₂(r)) and HN 202 (Rc) strains were maintained on Trypticase soy agar (BBL Microbiology), grown overnight at 37°C in Trypticase soy broth (BBL Microbiology) and diluted 150-fold into fresh media. The cells were cultured at 37°C to the late-log phase, harvested and washed with distilled H₂O, followed by acetone, then dried in vacuo and stored below 0°C until used. LPS was extracted as the natural salt form from the dried cells [8] using the method of Galanos et al. [10]. Lyophilized LPS was added to D₂O and incubated above the phase transition temperature until the grayish-white LPS precipitate disappeared, to ensure that the various LPS chemotypes were homogeneously dispersed and fully hydrated. The LPS concentration was determined using phosphorus as a marker by the method of Chen et al. [11].

Magainin 2 was synthesized by solid-phase peptide synthesis using Merrifield resin and t-Boc amino acids by Dr. Milind Deshpande (Laboratory for Rational Drug Design, Boston University Medical Center). The peptide was purified by gel filtration and HPLC, and its purity was determined by amino acid analysis and bioassay. Aliquots from a stock solution of magainin 2 in D₂O (approx. 2 mg/ml) were used to prepare samples for FT-IR experiments. Protein content was quantitated using the Pierce BCA protein assay with bovine serum albumin as the standard.

FT-IR spectroscopy, data processing and analysis of spectral parameters of LPS and LPS-magainin 2 complexes were performed as previously described [8,12]. LPS fatty acid methyl esters were prepared by heating 10 mg of LPS in 5 ml of freshly prepared 1% methanolic sulfuric acid overnight at 70°C. After the addition of 5 ml H₂O, the fatty acid methyl esters were extracted with petroleum ether, dried under a stream of N₂, dissolved in redistilled hexane and stored below 0°C until analyzed [13]. Gas chromatography was performed using a Hewlett Packard 5890 A gas chromatograph equipped with a flame ionization detector. Fatty acid methyl esters were separated using a 1 µm × 15 m Supelco SPB-1 column and identified by comparison of retention times with those obtained from a bacterial fatty acid methyl ester standard (Supelco).

High-resolution ³¹P NMR spectra of LPS (50–70 mg) dispersed in 2 ml of 50% D₂O/50% H₂O containing 20 mg/ml sodium dodecyl-sulfate and 10 mM EDTA, were used to determine the average number of phosphorus atoms per LPS molecule, as described previously [7]. Representative ³¹P NMR spectra of Rb₂(s) and Rb₂(r) LPS at pH 7.4 are shown in Fig. 2. The signals located between 0 and 5 ppm result from both monophosphate monoesters and diesters; the broad signal near -5 ppm arises from the β-phosphorus of diphosphate monoesters, and those observed between -10 and -13 ppm correspond to both the β-phosphorus of diphosphate diesters and the α-phosphorus of both diphosphate monoesters and diesters [8]. Monophosphate diester signals were identified by the relatively limited pH dependence of their chemical shift values [8]. Since the peak areas are proportional to the number of phosphorus atoms, and the diphosphate monoester β-phosphorus atoms correspond to the signal at -5 ppm, the relative number of diphosphate diester α- and β-phosphorus atoms was calculated by subtracting the area of the signal at -5 ppm from the total area of the signal located between -10 and -13 ppm. It is therefore possible to determine the proportion of each type of phosphorus atom in the LPS molecules even though the identity of the individual peaks in the -10 to -13 ppm region remains ambiguous.

3. RESULTS AND DISCUSSION

Both the ³¹P NMR spectra and pH dependence of the NMR signals of Sm [8], Ra, Rb₂(s) and Rc LPS are

quite similar [14], showing that altering the length of the LPS sugar side-chain has no major effect on the general phosphorylation pattern or the microenvironment of the phosphorylation sites, although small differences in the relative areas of the ³¹P NMR signals are observed. The composition of the phosphorylation sites determined by ³¹P NMR is summarized in Table I. Monophosphate monoesters are the predominant substituent. Diphosphate mono- and diesters occur at roughly the same frequency, with the exception of Rb₂(r) LPS, which contains only trace levels of diphosphate monoesters and elevated levels of mono- and diphosphate diesters. The loss of the O-antigen side chain is accompanied by a slight increase in the negative charge of Ra and Rc LPS, while Rb₂(s) LPS has about the same charge as wild-type LPS. The reduced charge of Rb₂(r) LPS is a consequence of charge neutralization by etha-

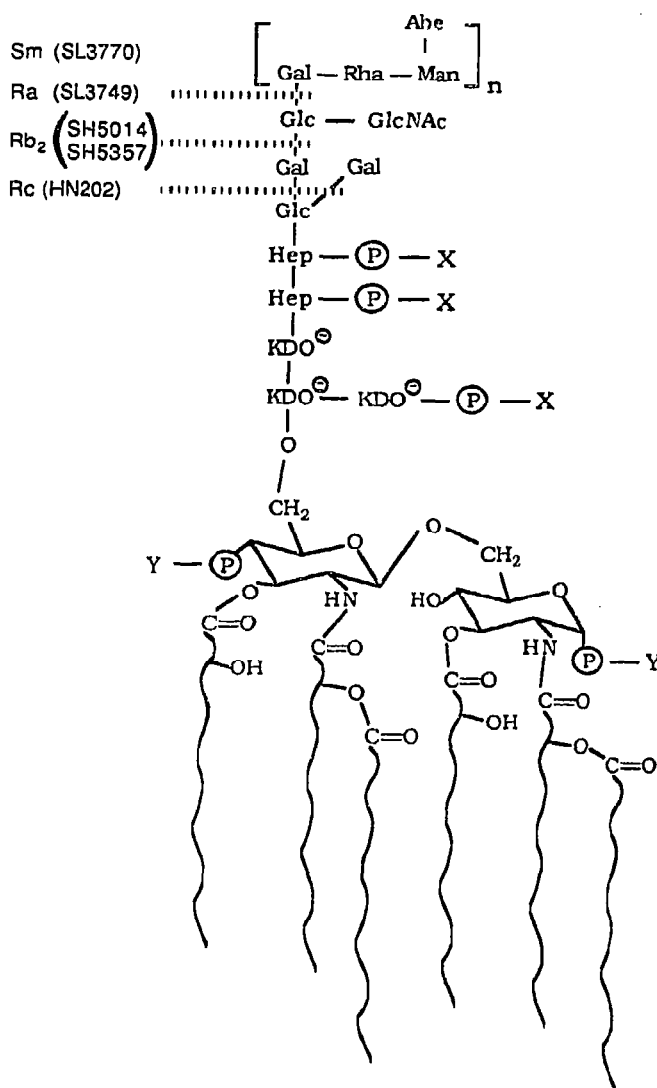


Fig. 1. Structure of wild-type and LPS-mutant *S. typhimurium* LPS molecules (KDO, 2-keto-3-deoxyoctonate; Hep, heptose; Glc, glucose; Gal, galactose; GlcNAc, N-acetylglucosamine; Rha, rhamnose; Man, mannose; Abe, abequose). Ethanolamine or arabinosamine may be substituted at the positions labeled X and Y.

nolamine and/or arabinosamine substituents, as reflected by an average charge per phosphate residue of about -0.6 compared to a value of -1.0 for the other chemotypes, in agreement with Peterson et al. [4].

Temperature-dependent changes in the frequency of the symmetric methylene C-H stretching band for aqueous dispersions of *S. typhimurium* Ra and Rc LPS as a function of magainin 2 concentration are shown in Fig. 3. The position of this band is indicative of the geometry of the lipid fatty acyl chains (i.e. the relative number of trans vs. gauche conformers). A shift to higher frequency correlates with the introduction of more gauche conformers and thus more disorder in the chains. Aqueous dispersions of LPS undergo a thermotropic gel-to-liquid crystal phase change. In the absence of magainin, the midpoint of this phase change (T_m) is 46°C for Ra LPS and 30°C for Rc LPS (see Fig. 3). For Sm, Rb₂(s) and Rb₂(r) LPS, the T_m values are 35 , 36 and 29°C , respectively. The fatty acid composition of the five LPS chemotypes is not appreciably different (Table II). Thus, the differences in T_m values most likely result from changes in the carbohydrate portion of the LPS molecules.

As previously observed for *S. typhimurium* outer membrane-peptidoglycan complexes [7] and Sm LPS [8], magainin 2 induces a concentration-dependent disordering of the LPS fatty acyl chains over the entire temperature interval examined (Fig. 3). As the peptide level is increased, the LPS hydrocarbon chains become progressively more disordered, and the cooperativity of the observed phase change is reduced considerably. At even higher ratios of magainin to LPS, the phase change is abolished and LPS hydrocarbon chains are extremely fluid even at the lowest temperature monitored (approx. 5°C). Similar results were observed for the Rb₂(s) and

Rb₂(r) LPS chemotypes. It should be noted that the disordering of the fatty acyl chains is not static, but involves an increase in the fluidity of the hydrophobic interior of the LPS aggregates, since concomitant increases in band-width, which indicate greater motional freedom of the fatty acyl chains, accompany the magainin-induced frequency shifts.

The ability of magainin 2 to disorder the LPS fatty acyl chains as a function of the phase state of LPS is examined in Fig. 4. In all cases, disordering is more pronounced in the gel rather than the liquid crystal phase, as judged by the frequency shifts induced by magainin 2 at a T_m of 15°C below T_m (gel state) and 10°C above T_m (liquid crystal state) for each LPS. If the O-antigen side chain were able to protect the negatively charged groups of Sm LPS from interaction with magainin 2, the fluidizing effect of the peptide should be considerably diminished for Sm LPS compared to the other mutants. This is clearly not the case since Sm LPS is disordered to the same or greater extent as all of the mutant LPS, with the possible exception of the Ra strain at low peptide concentrations.

Electrostatic forces are critical in determining the extent of magainin-LPS interactions. The degree of disordering

Table I

Phosphate structure of LPS from wild-type and mutant strains

Strain	Chemo-type	% Occurrence				(Average)		
		P _m ^a	P _d ^b	PP _m ^c	PP _d ^d	P/LPS ^e	Charge/ P ^f	Charge/ LPS ^g
SL3770	Sm	47	14	21	20	7.0	-1.04	-7.3
SL3749	Ra	42	6	29	24	7.7	-1.05	-8.2
SH5014	Rb ₂ (s)	51	2	17	28	7.3	-1.01	-7.4
SH5357	Rb ₂ (r)	19	25	1	55	7.8	-0.58	-4.5
HN202	Rc	51	7	18	24	7.1	-1.10	-7.8

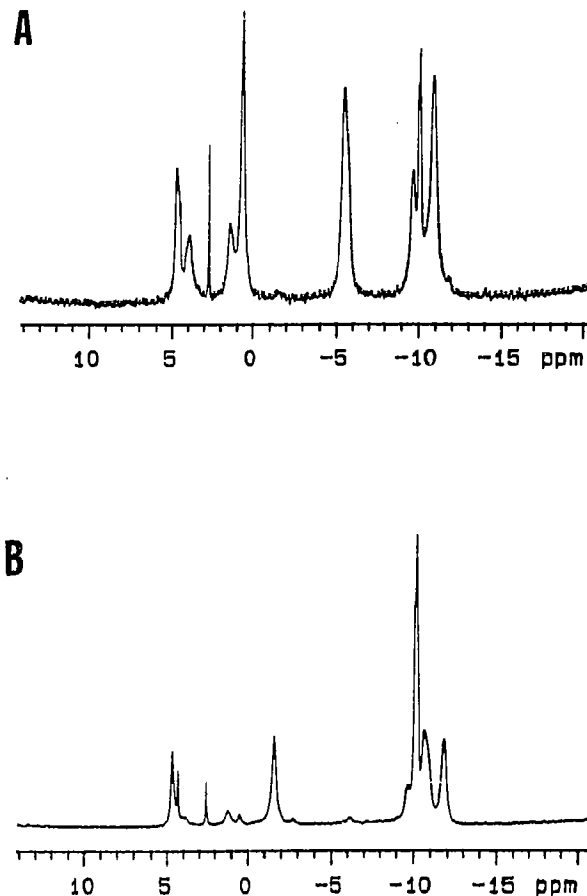
^aPhosphate monoesters^bPhosphate diesters^cDiphosphate monoesters^dDiphosphate diesters^eAverage number of phosphorus atoms per LPS molecule^fAverage charge per phosphorus atom on the LPS molecule^gAverage total charge of the phosphate residues on the LPS molecule (these values do not include the contribution from the KDO residues, which adds an extra -3 to the total charge on the LPS molecule)

Fig. 2. High-resolution ^{31}P NMR spectra of *S. typhimurium* LPS dispersed in 50% D_2O /50% H_2O containing 20 mg/ml sodium dodecyl-sulfate and 10 mM EDTA at pH 7.4. (A) Rb₂(s) chemotype, (B) Rb₂(r) chemotype.

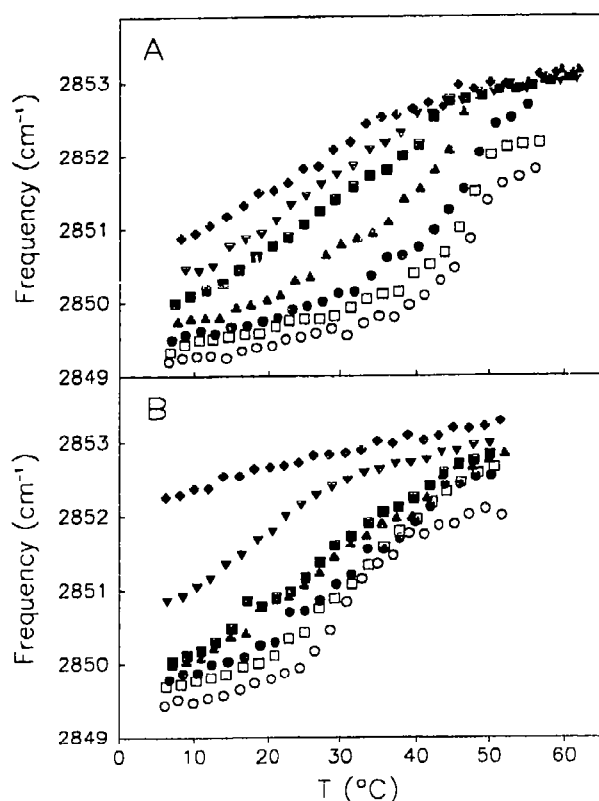


Fig. 3. Temperature dependence of the frequency of the symmetric methylene C-H stretching band for (A) Ra and (B) Rc *S. typhimurium* LPS at magainin/LPS molar ratios of: 0 (○), 0.15 (□), 0.22 (●), 0.31 (▲), 0.53 (■), 0.63 (▼) and 1.54 (◆) for Ra LPS; and 0 (○), 0.14 (□), 0.21 (●), 0.28 (▲), 0.36 (■), 0.48 (▼) and 1.43 (◆) for Rc LPS.

dering of Rb₂(r) LPS by magainin 2 is markedly less than that observed for the other LPS strains. The phosphorylation sites on the Rb₂(r) LPS polysaccharide and lipid A moieties are more heavily esterified by ethanolamine and arabinosamine, resulting in a much lower net

Table II
Fatty acid composition of LPS

Strain	LPS chemo-type	Composition (% weight)				
		12:0 ^a	14:0 ^b	14:0 (β-OH) ^c	14:1 ^d	16:0 ^e
SL3770	Sm	12.8	17.4	55.8	5.5	6.9
SL3749	Ra	10.7	17.3	62.0	6.0	3.8
SH5014	Rb ₂ (s)	15.4	19.3	59.9	2.0	2.4
SH5357	Rb ₂ (r)	11.7	19.3	59.6	2.7	6.0
HN202	Rc	10.3	14.4	61.6	10.4	1.2

The average deviation for all values is less than 5%.

^aLauric acid

^bMyristic acid

^c3-Hydroxymyristic acid

^d*cis*-Tetradecanoic acid

^ePalmitic acid

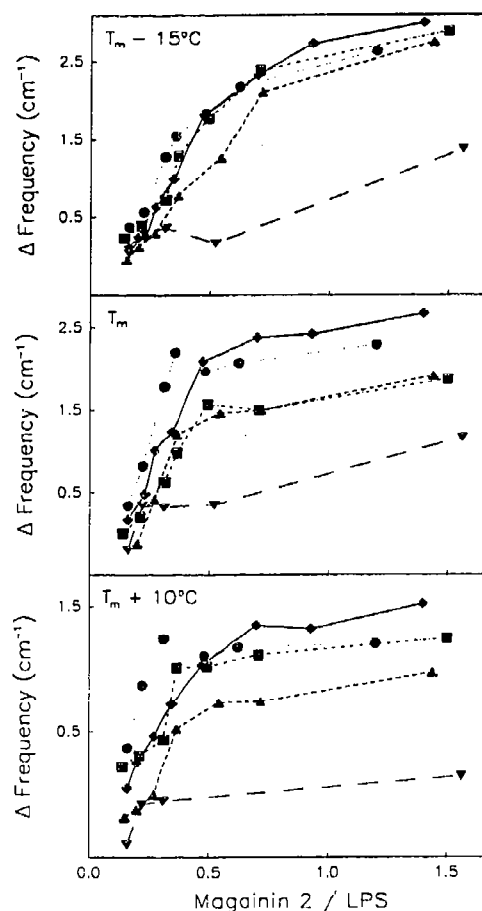


Fig. 4. Frequency change of the symmetric methylene C-H stretching band for Sm (◆), Ra (●), Rb₂(s) (▲), Rb₂(r) (▼) and Rc (■) *S. typhimurium* LPS as a function of magainin 2 concentration for the gel state ($T_m - 15^\circ\text{C}$), the transition midpoint (T_m) and the liquid crystal state ($T_m + 10^\circ\text{C}$). Typical average deviations are 0.1 cm⁻¹.

charge on the molecule (Table I). Peterson et al. [15] observed a decreased affinity for polymyxin B in LPS isolated from the polymyxin-resistant mutants of *S. typhimurium* and *E. coli* containing elevated mono- and disphosphate diester substituents. In the liquid crystal state, the degree of disordering of the mutant LPS chemotypes by magainin 2 is $Ra \approx Rc > Rb_2(s) \gg Rb_2(r)$ (see Fig. 4), which correlates well with the charge on the LPS molecules. The negatively charged sites on the LPS molecules in these aqueous dispersions evidently are readily accessible to the peptide. On the other hand, in the gel state, Rb₂(s) and Rc LPS are disordered to about the same extent as Ra LPS in the presence of magainin. Ra LPS may be more tightly packed in the gel state than the other types of LPS since its T_m is at least 10° higher than the T_m values for any of the other LPS molecules, suggesting that some negative charges on Ra LPS may be partially masked, especially at low magainin concentrations.

Above T_m , Sm LPS is disordered to a greater extent than its Ra counterpart at high peptide levels, in spite of its lower net negative charge; however, Ra LPS is

more sensitive to the fluidizing effects of magainin at low peptide levels. The presence of the O-antigen side chain on Sm LPS may enhance the magainin-induced disordering through steric interactions which increase the intermolecular distance between adjacent LPS molecules as peptide molecules bind to anionic sites on the lipid. The observation that the addition of exogenous wild-type *S. typhimurium* LPS inhibits the antimicrobial activity of polymorphonuclear leukocyte granular contents more effectively than mutant LPS chemotypes has been attributed to direct interactions between granular components and the O-antigen sugars [16]. The results presented here do not support a significant role for the O-antigen side chain in binding cationic peptides. Enhanced resistance of wild-type *S. typhimurium* does not arise from either binding of the peptide directly to the O-antigen sugars or protection of anionic sites on LPS by an O-antigen umbrella, but most likely results from a more stable outer membrane which is less easily penetrated by cationic peptides. The large volume of the Sm LPS polysaccharide group may help to maintain the integrity of the outer membrane despite the disorder induced by the peptides in the hydrophobic core.

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