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Abstract

In the light of occurrence of bacterial strains with multiple resistances against most antibiotics, antimicrobial peptides that interact with the outer layer of gram-negative bacteria, such as polymyxin (PMX), have recently received increased attention. Here we present a study of the interactions of PMX-B, -E and -M with lipopolysaccharide (LPS) from a deep-rough mutant strain of E. coli. A method for efficient purification of biosynthetically produced LPS using RP-HPLC in combination with ternary solvent mixtures was developed. LPS was incorporated into a membrane model, dodecylphosphocholine (DPC) micelles, and its interaction with polymyxins was studied by heteronuclear NMR spectroscopy. Data from chemical shift mapping using isotope-labeled LPS or labeled polymyxin, as well as from isotope-filtered NOESY experiments, reveal the mode of interaction of LPS with polymyxins. Using MD calculations the complex of LPS with PMX-B in the presence of DPC micelles was modelled using restraints derived from chemical shift mapping data and intermolecular NOEs. In the modelled complex the macrocycle of PMX is centered around the phosphate group at GlcN-B, and additional contacts from polar sidechains are formed to GlcN-A and Kdo-C, while hydrophobic sidechains penetrate the acyl chain region.
INTERACTIONS OF LIPOPOLYSACCHARIDE AND POLYMXYXIN STUDIED BY NMR SPECTROSCOPY

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In the light of occurrence of bacterial strains with multiple resistances against most antibiotics, antimicrobial peptides that interact with the outer layer of gram-negative bacteria, such as polymyxin (PMX), have recently received increased attention. Here we present a study of the interactions of PMX-B, -E and -M with lipopolysaccharide (LPS) from a deep-rough mutant strain of E. coli. A method for efficient purification of biosynthetically produced LPS using RP-HPLC in combination with ternary solvent mixtures was developed. LPS was incorporated into a membrane model, dodecylphosphocholine (DPC) micelles, and its interaction with polymyxins was studied by heteronuclear NMR spectroscopy. Data from chemical shift mapping using isotope-labeled LPS or labeled polymyxin, as well as from isotope-filtered NOESY experiments, reveal the mode of interaction of LPS with polymyxins. Using MD calculations the complex of LPS with PMX-B in the presence of DPC micelles was modelled using restraints derived from chemical shift mapping data and intermolecular NOEs. In the modelled complex the macrocyle of PMX is centered around the phosphate group at GlcN-B, and additional contacts from polar sidechains are formed to GlcN-A and Kdo-C, while hydrophobic sidechains penetrate the acyl chain region.

Keywords: LPS, glycolipids, polymyxin, structural biology, NMR spectroscopy

Cellular membranes segregate the interior of cells from their surroundings and therefore are crucial to maintain cells as autonomously functioning systems (1). The chemical constituents of outer membranes from mammalian cells and bacteria are fundamentally different (2). The mammalian outer membranes are largely formed by phospholipid bilayers, whereas additional coating structures are present covering these in bacteria. In gram-positive bacteria a thick peptidoglycan layer is built around the phospholipid bilayer. In gram-negative bacteria, the peptidoglycan structure is much thinner and coated by an additional phospholipid-containing bilayer, whose outer leaflet is mainly composed of lipopolysaccharides (LPS) (3). LPS are high-molecular-weight, strongly negatively charged molecules, that for smooth LPS can be divided in three regions: the lipid A portion of LPS inserts into the phospholipidic membrane and in many gram-negative bacteria consists of a di-glucosamine diphosphate with 5 to 7 fatty acid chains extending to one side of the disaccharide. The lipid A is appended to a region (the inner core) of 8-12 variable sugars (including the negatively charged 3-deoxy-D-manno-oct-2-ulosonate (Kdo) units) and 3-8 phosphate residues. To the inner core is covalently associated the O-antigen, an oligosaccharide chain of variable length and chemical composition, depending on the exact type of LPS.

Sepsis caused by gram-negative bacteria is a serious source of mortality in many clinical cases, accounting for approximately 200,000 deaths in the US annually (see David (4) and references therein). The primary trigger for sepsis was identified as LPS, and LPS-neutralizing agents are therefore valuable therapeutics. Antimicrobial peptides against gram-negative bacteria can interfere with the integrity of this LPS layer. PMX-B is considered as the “gold-standard” for LPS-sequestering agents. Polymyxin-B, -M and –E are characterized by a heptapeptide ring and a fatty acid tail (Fig. 1). These highly cationic decapeptides contain six diaminobutyric acid (Dab) residues, a macrocyclic ring involving residues 4 to 10, and an acyl chain coupled to the N terminus. Severe toxic side effects have
limited their usage to treatments against bacteria resistant against most other antibiotics such as *Pseudomonas aeruginosa*. The interaction of LPS with various antimicrobial peptides has been the subject of a number of studies (4-15). In some of these, the conformation of the LPS-bound peptides was established using transferred NOE effects, and the complex between LPS and the peptides was established by docking the trNOE-derived peptide conformer to LPS (11), whose coordinates were taken from the crystal-structure of FhuA-bound LPS (16).

The emphasis of this work was to obtain experimental data on the LPS-PMX complex, thereby allowing a detailed understanding of the interacting moieties. Since LPS in the outer membrane of *E. coli* cells is integrated into a phospholipid bilayer, it was studied while integrated into phospholipid micelles to better mimic the natural environment. In our studies we used LPS from the deep rough mutant D31m4 of *E. coli* (Re-LPS). Biosynthetic production of the latter and its isolation and purification from the membrane-fraction of the corresponding cells was described in literature. However, to facilitate purification by RP-HPLC, the phosphate groups were methylated (17). The modified LPS was investigated in detail by NMR (18). Since interactions with the charged phosphate groups were proposed to be important for binding antimicrobial peptides, we developed an HPLC-based method that allows purification of LPS in its natural (non-methylated) form (Fig. 1). $^{15}$C,$^{15}$N-labeled LPS from the deep rough mutant strain was isolated and purified to chemical homogeneity. Extensive use of heteronuclear solution NMR techniques allowed characterization of LPS embedded in DPC micelles, and facilitated the study of its interactions with polymyxins from different organisms. The interaction studies relied on chemical shift mapping techniques and isotope-filtered NOEs, and allowed direct probing for the interaction sites.

Experimental Procedures:

**Materials.** $^{15}$NH$_4$Cl was purchased from Spectra Isotopes (Columbia, USA), perdeuterated DPC-d38 (99%-d), and D$_2$O were ordered from Cambridge Isotope Laboratories (Andover, Massachusetts, USA). Methyl-5-doxylstearic acid was bought from Aldrich (Buchs, Switzerland). The Re-LPS producing strain D31m4 was purchased from the *E. coli* Genetic Resource Center, New Haven. The PMX-M producing strain *Paenibacillus kobensis* M was obtained from Prof. J.C. Vederas and PMX-B and -E were purchased from Sigma Aldrich Laboratories.

**Production of $^{15}$C-labeled LPS from the *E. coli* strain D31m4.** Cells from the D31m4 strain of *E. coli* were grown at 37°C to an OD of around 1.0 at 600 nm on minimal medium M9 using 4 g $^{15}$C glucose and 1 g $^{15}$N ammonium chloride supplemented with 100 mg of Trp, His and Pro per liter.

After harvest cells were resuspended in 50 ml of ice-cold water and pelleted down. To the pellet a minimum amount of cold water was added such that a thick paste was formed. LPS together with other components was precipitated through addition of 90 ml ice-cold methanol and centrifuged at 8000 g. The pellet was resuspended in 90 ml ice-cold acetone, homogenized and centrifuged again, followed by another acetone washing step. The lyophilized cells (ca. 0.7 g) were taken up in 50 ml of a phenol:chloroform:petroleum ether (4:10:16; v:v) solvent mixture and centrifuged at 9200 g, after which most of the LPS was contained in the supernatant. The remaining pellet was extracted once more to increase the yield in LPS. The supernatant was concentrated under a nitrogen stream and 2 ml of water were added dropwise to the concentrate. A waxy precipitate was formed followed by three cycles of washing with methanol and subsequent centrifugation. Thereafter the pellet was dried and lyophilized, after which it could only be resuspended in water using repetitive additions of small amounts of water followed by sonication. Solubilization was improved upon adding aqueous 0.1M EDTA in the first portions. The resulting solution was centrifuged at 20000 g overnight and then lyophilized.

Chromatography used the following solvents: solvent A (methanol:chloroform:water 57:12:31 v/v/v) and solvent B (methanol:chloroform 29.8:70.2 v/v). The lyophilized pellet after resuspension in the mixture of solvent A and aqueous EDTA was directly loaded onto the HPLC column. For optimal purification of LPS a gradient system involving ternary solvent mixtures was used consisting of solvent A in 10mM NH$_4$Cl and solvent B in 50mM NH$_4$Cl. LPS (6 mg) was dispersed in a two phase system formed from 0.8 ml of solvent A and 0.2 ml of 0.1 M aqueous EDTA pH = 7 and loaded directly onto the RP-C8 column. Chromatographic
separation was achieved using the following gradient of solvents A and B: 2 CV of 2% B, 3 CV (2-17% B), 3.5 CV (17-27% B). UV detection was impossible and hence fractions were lyophilized and their content checked by MALDI-TOF using 6-aza-2-thiothymine as the matrix. Elution of the desired LPS occurred around 20-23% of solvent B.

**Production of \(^{13}\text{C, }^{15}\text{N}-labeled PMX-M from *P. kobensis* M** - The producer strain, *Paenibacillus kobensis* M, was grown aerobically at 30 °C on tryptic soy agar (TSA). A 1-liter batch of M9 medium was inoculated with a 10-ml *P. kobensis* M preculture (1% inoculum). After a total growth time of 16-24 h at 30°C with shaking (200 rpm), the cells were removed by centrifugation (1 hr, 10,000 rpm) and the supernatant was then passed through a Amberlite XAD-16 column. After washing with 30% ethanol, active peptide was then eluted with 70% isopropanol, which was adjusted to pH 2 (pH meter reading) with 12N HCl.

All fractions were assessed for antimicrobial activity using a well plate assay. The contents of the active fraction were applied to a Superdex peptide 10/300 column (Amersham Biosciences). Fractions were collected for 3 column volumes with pure MilliQ Water and each assayed for activity. All active fractions were pooled, concentrated and applied as 20% isopropanol solutions to C18 reverse-phase HPLC. Complete purification required two separate steps of C18-HPLC. The first separation used a gradient of water/isopropanol (0.1% trifluoroacetic acid), from 20% to 50% isopropanol, and the second step a water/methanol gradient (0.1% trifluoroacetic acid), from 45% to 85% methanol. PMX-M eluted at around 55%. Finally, 8 - 10 mg of pure PMX-M was obtained as slightly yellowish powder from a 1-liter culture and its chemical nature verified by ESI-MS (exp. mass: 1224.73 Da, theoret. mass: 1223.57 Da).

During all steps of expression and purification antimicrobial activity was monitored by inhibition of growth of an indicator strain. Agar plates were prepared by inoculating molten TSA (40 g/liter) with a culture of the indicator organism *E. coli* (1.0% inoculum). Small wells (app 4.6 mm diameter) were made in the seeded agar plates and 50-µl of filtered culture supernatant were added to the wells. Plates were incubated at 30°C, and the growth of the indicator organism was visible after approx. 3 h.

**NMR spectroscopy** - LPS samples used for assignment purposes contained approx. 1 mM LPS, 300 mM d15-N,N-methylaniline in 40 mM d13-C-acetate buffer, pH=5.8. Resonance positions required very small changes to adapt to \([^{13}\text{C, }^{15}\text{N},^{1}\text{H}]-\text{HSQC}\) spectra in 40 mM acetate buffer at pH 4.4. All interaction studies were performed in 40 mM acetate buffer, 300 mM d15-N,N-methylaniline in D2O or H2O/D2O 9/1 pH=4.4. Measurements of interactions between LPS and PMX by chemical shift mapping observing LPS resonances utilized a 350 µM solution of \(^{15}\text{N},^{13}\text{C}\)-labeled PMX-M and equimolar unlabeled peptides. Chemical shift changes in PMX-M were monitored on a 200 µM solution of \(^{15}\text{N},^{13}\text{C}\)-labeled PMX-M and equimolar unlabeled Re-LPS. No further salt was added in the measurements except for initial attempts to optimize conditions for \(^{15}\text{N},^{1}\text{H}\)-HSQC spectra in the PMX-M—Re-LPS sample (vide infra). In order to satisfy requirements for better sensitivity higher concentrations of doubly labeled LPS (500µM) were used in the isotope-filtered NOESY experiments (200ms mixing time). Due to the moderate dissociation constant the experiment in fact monitored transferred NOEs, and therefore 3-fold excess of unlabeled PMX-E or PMX-B (2mM solutions) were used with conditions of pH, detergent and temperature otherwise identical to those of the shift mapping studies.

Spectra were recorded on Bruker AV-600 or AV-700 NMR spectrometer at T=310K. Proton and carbon chemical shifts were calibrated to DSS, and nitrogen shifts were referenced indirectly to liquid NH3 (19). The spectra were processed using the Bruker Topspin2.0 software and transferred into CARA (21) or SPARKY(22) programs for further analysis.

For chemical shift assignments of \(^{13}\text{C, }^{15}\text{N},^{1}\text{H}\)-labeled LPS 2D versions of 3D double- and triple-resonance experiments were recorded. In general, experiments used coherence selection schemes via pulsed-field gradients (23) and sensitivity-enhancement building blocks (24, 25) whenever possible. For assignments of the carbon spin systems in the lipid chains and the sugar units (H)CCCH experiments recorded with 4 and 12 ms DIPSI-2 C-C mixing cycles were used. Linkage of the lipid chains onto the glucosamine parts of lipid A was achieved via correlations with the amide nitrogens using HNCA and HN(CO)CA experiments. To
distinguish the two Kdo units key NOEs derived from a $^{13}$C-resolved NOESY were exploited. Assignment of all resonances of polymyxin was done using HN(CO)CACB (26), HNCACB (27) and (H)CCH experiments (28, 29) analogous to the procedure used for proteins. Because of the small size of the peptide 2D versions were recorded with a total of less than 12 h measuring time for acquiring all spectra. Assignments of polymyxin-B and –E were based on assignments from PMX-M adjusted by using additional 2D heteronuclear spectra.

In the spin label experiments, a 0.5 mM solution of LPS was separated into two aliquots, and to one of these 5-doxystearate methyl ester was added so that the final concentration corresponded to approximately one spin-label per micelle. Signal intensities from the two corresponding constant-time $[^{13}\text{C},^{1}\text{H}]-\text{HSQC}$ were extracted and the ratio of signal intensities from the samples with and without spin label was calculated.

**Molecular dynamics calculations-** All calculations were performed within the program GROMACS (30). Briefly, coordinates of LPS were adapted from the pdb entry 1QFF and coordinates of polymyxin B were built using the program Ghemical (31). Parameters and topologies of PMX-B and LPS as well as partial charges of PMX-B for GROMACS were established based on data from PRODRG server (32) and the GROMOS 53a6 forcefield (33). Partial charges of LPS were assigned for a protonation state corresponding to approximately one spin-label per micelle. Signal intensities from the two corresponding constant-time $[^{13}\text{C},^{1}\text{H}]-\text{HSQC}$ were extracted and the ratio of signal intensities from the samples with and without spin label was calculated.

Production and purification of $^{13}$C-labeled LPS- LPS from the deep rough E. coli mutant D31m4 was isolated from the membrane fraction of cells grown on minimal medium containing $^{13}$C-glucose and $^{15}$N-NH$_4$Cl as the sole carbon and nitrogen sources, respectively. After PCP extraction and further purification using published protocols (3, 36) the yield was ca. 129 mg/l of culture. Remaining impurities were removed by RP-HPLC using a ternary solvent mixture. In this procedure the solvent system was carefully adapted to form a single phase over the whole gradient range of solvent A (methanol:chloroform:water) and solvent B (methanol:chloroform) at RT. Importantly, any mixture of these two solvent systems is relatively close to a two-phase system, and this condition proved to have favorable properties for dissolving LPS. MALDI-TOF spectra of LPS before and after this HPLC purification step are depicted in Fig. 2. Sufficient quantities (40 mg) of chemically pure LPS for the NMR studies could be produced from 1 L of culture using this protocol. As demonstrated in Fig. S4 in the Supp. Mat. this method is capable of separating pyrophosphate from the monophosphate derivatives.

**Assignment of LPS and polymyxin resonances-** Chemical shift mapping (37) or NOE-based methods (38-40) can be used to study biomolecular interactions (see also (41-43)). Both methods potentially deliver information on interacting moieties but require assignments of chemical shifts. The best chemical shift dispersion is usually available in heteronuclear shift correlation spectra (e.g. $[^{15}\text{N},^{1}\text{H}]-\text{HSQC}$ or $[^{13}\text{C},^{1}\text{H}]-\text{HSQC}$ spectra). Importantly, these experiments still work well in the presence of the increased line-widths that are usually present in systems that are stably anchored into phospholipids micelles. In addition, as was unfortunately the case in some of our applications, additional exchange broadening occurred upon complex formation. To probe integration of LPS into DPC micelles and to study its interaction with peptides we decided to label it with $^{13}$C and $^{15}$N isotopes and

**Results:**

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use the corresponding HSQC spectra for chemical shift mapping.

To assign all signals in the constant-time \(^{13}\text{C},^{1}\text{H}\)-HSQC spectrum (44), 3D (H)CCH-TOCSY spectra (28, 29) were used for assignment of spin systems (Fig. 3). The C,H-plane of the HNCA (45, 46) and HN(CO)CA (45) experiments were used to establish scalar connectivities between terminal carbons of the fatty acid chains and C-2 of the glucosamine moieties. Unsubstituted hydroxymyristoyl (HM) can be distinguished from lauroxymyristoyl (LM) and thereby helps to differentiate between GlcN-A from GlcN-B. Due to chemical shift degeneracies it was impossible to assign chains of the fatty acids (myristoyl of myristoxy-myristoyl and lauroyl of lauroxymyristoyl). The two Kdo units were linked and thereby distinguished from each other using several key NOEs in the 3D \(^{13}\text{C}\)-NOESY spectra. The unique chemical shift of the C3 moiety of Kdo is located in a region separated from all other sugar resonances, and was used as a starting point for sequential assignment. Only the methylene group C3 of Kdo-C is expected to receive an NOE from H(C6) of GlcN-B. This assignment was additionally supported by the fact that H(C6) and H(C7) of Kdo-C displayed an NOE to H(C3) of both Kdo units, which is unlikely to be the case for H(C6) of Kdo-D. The \(^1\text{H},^{13}\text{C}\) and \(^{15}\text{N}\) chemical shifts of LPS in DPC micelles are reported in Table 1.

**Topology of LPS and polymyxin insertion into the DPC micelle** - To probe whether LPS properly inserts into the DPC micelles, and whether the sugar moieties really protrude into the aqueous phase, the micelle-integrating spin label methyl 5-doxylstearate was used. The paramagnetic moiety of the spin label resides in the headgroup region (47,48), and signal reductions in the proton-carbon correlation maps indicate proximity of the corresponding C-H moiety to the water-micelle interface. The results from the spin-label experiment are depicted in Fig. 4. Strongest attenuations of signals are observed for C,H moieties of terminal acyl chain carbons, that are close to the GlcN sugars. Much less pronounced attenuations are observed for signals from the GlcN moieties, and the Kdo units are essentially not affected. The data demonstrate that LPS inserts into the DPC micelles such that the amide moieties of LPS are located in the headgroup region, the acyl chains are inserted into the micelle interior, and the carbohydrate units are exposed on the surface of the micelles.

We additionally probed for the topology of the polymyxin-micelle complex using the micelle-integrating spin-label methyl 5-doxylstearate as described in the previous section. Notably PMX-B, -E and -M differ only for residues in position 6 and 7 (see legend of Fig.1). Largest attenuations were observed for the amide moieties of the first residue, and for residues 6 and 7 in PMX-B and -E, the latter two present hydrophobic or aromatic residues (Table S13), revealing that polymyxins are attached via the N-terminal lipid chain and the apolar residues to the micelle. Note the stronger attenuations for position 7 in PMX-B and –E compared to PMX-M underlining the importance of hydrophobic residues for partitioning into the water-membrane interface.

**Interactions of LPS with various types of polymyxins** - As a result of exchange processes and the limited lifetime of the PMX-LPS complexes the number of intermolecular NOEs was insufficient to establish the nature of the molecular contacts. Therefore, a hybrid approach was chosen instead that included i) chemical shift mapping data to probe for moieties involved in forming intermolecular contacts both in LPS as well as in PMX, ii) measurement of intermolecular NOEs using isotope-filtered NOESY experiments and iii) restrained MD-simulations of the complex of Re-LPS and PMX-B in the presence of DPC micelles, followed by selection of a subset of conformers that are compatible with the experimental data.

For chemical shift mapping purposes we initially used very simplified model compounds of LPS derived from α-(D)-glucose (Fig. 1). (49) Considering the proposed importance of phosphate moieties for binding it was not surprising that compound 3 did not display any interactions with PMX-B, but also compound 4, that contains the presumably important phosphate group, did not cause any changes in the spectra upon addition of PMX-B, indicating that very little or no interaction exists.

Chemical shift mapping experiments using Re-LPS were performed by mixing either \(^{13}\text{C},^{15}\text{N}\)-labeled LPS and unlabeled PMX-M or \(^{13}\text{C},^{15}\text{N}\)-labeled PMX-M and unlabeled Re-LPS in order to localize the binding site within LPS or PMX-M, respectively. Unfortunately, broad lines were observed for resonances of PMX-M,
indicating the presence of exchange processes and complicating assignments (vide infra). In contrast, resonances of Re-LPS in the complex were much narrower allowing a more detailed analysis of the shift changes such that the binding site is experimentally better defined in LPS. The chemical shift changes of LPS carbon and proton frequencies upon adding PMX-B,-E or -M are mapped onto the structure in Fig. 5. In general, the largest changes were observed for all observable atoms of GlcN-B. Interestingly, resonances from C1 and C2 of GlcN-A were affected only very little (Fig. S3A and S3C). In addition, resonances from Kdo-C display significantly larger changes than those of Kdo-D, with C-3 of Kdo-C being shifted by the largest extent (see Fig. S3F). Moreover, large changes are additionally observed for the alpha (Fig. S3G), beta and gamma (Fig. S3H) positions of the fatty acid chains, in particular for those of HM2 (note that the second branch of MM and LM cannot be assigned due to resonance overlap). The Cα change for HM1 is less pronounced. The alpha position of HM4 was apparently more affected than of HM3. The chemical shift changes on the beta position were smaller and clear only for HM4.

A comparison of chemical shift changes of Re-LPS upon complexation with PMX-B, PMX-E and PMX-M may serve to identify differences in their binding modes. Chemical shift changes are almost identical at positions C-4, C-5 of GlcN-B as well as on position C-3 of Kdo-C for PMX-B and PMX-E (Fig. S3E and S3F), and very similar changes occur for the alpha position of HM2. These regions are most likely in contact with the conserved parts of polymyxins. However, significant changes were observed for C-3 of GlcN-B and moderate differences can be seen at positions of C-3, C-5 and C-6 of GlcN-A and at position C-1 of GlcN-B (Fig. S3B). Addition of PMX-M results in line-broadening for resonances of C-4 and C-5 of GlcN-B, as well as for C-3 of Kdo-C, and in a chemical shift change for C-6 of Kdo-C. We attribute this observation to a slightly different binding mode of PMX-M, which results in different contacts to Kdo-C.

A similar analysis for resonances of PMX-M to localize the binding site within the peptide was complicated by the above-described exchange-broadening. Nevertheless, line-widths in the [13C,1H]-HSQC spectra of PMX-M are moderate such that interactions can be detected by shift mapping methods although some resonances remained significantly broadened and some of the shifted resonances cannot be assigned unambiguously. The results shall only be briefly summarized here: largest chemical shift changes occur for Cα resonances of residues 4, 5, 6, 8 and 9 of polymyxin-M, the members of the macrocyclic ring. Interestingly, comparably small differences were observed for resonances from the lipid chain or from Dab-1, Thr-2 or Dab-3. The fact that only residues from the macrocycle experience large changes indicates that the heptapeptide ring of PMX binds to LPS. Our data additionally support the view that the interaction is primarily electrostatic (50): [15N,1H]-HSQC spectra of PMX-M initially used to screen conditions displayed multiple peaks and very broad lines. However, in the presence of 20 mM MgCl2 electrostatic interactions were largely screened and a good-quality [15N,1H]-HSQC of PMX-M was obtained with peak positions close to those in the absence of LPS.

Interactions between Re-LPS and polymyxin were additionally directly detected by measuring intermolecular NOEs between unlabeled PMX-E or -B and [13C,15N]-labeled LPS using isotope-filtered NOESY experiments (see Table 2 and Fig. S7). Interestingly, in the case of PMX-B, NOEs between side chain protons of Phe-6 or Leu-7 and the lipid chains of LPS are detected (see Fig. S7). A number of NOEs between protons of the α-system of Phe-6 and the lipid chains are observed, in particular to Cγ of HM2 and Cα of HM3. The fact that PMX-B or PMX-E form many contacts with LPS that involve side chains of residues 6 and 7 indicates that the hydrophobic nature of these side chains may be important for orienting polymyxin in the complex with LPS. Unfortunately, larger line-widths in the LPS—PMX-M complex precluded the measurement of isotope-filtered NOESY spectra in that case.

Restrained molecular dynamics calculations - To obtain a first picture of the complex formed between LPS and PMX-B we have performed a MD calculation in which LPS was docked to PMX-B using NOE-derived upper distance limits (Fig. 6) in the presence of a DPC micelle. In the complex backbone amide moieties of the peptide macrocycle form contacts with the phosphate group of GlcN-B. Residues from one hydrophobic site - D-Phe-6 and Leu-7 - are in proximity to the fatty acid chains of LPS. Leu-7 makes additional contacts with the GlcN-B...
moiety. The amino group of Dab-8 is involved in electrostatic contacts with the phosphate group at GlcN-A. Amino groups of Dab-9 and -3 are possibly forming electrostatic interactions with the carboxyl group of Kdo-C. The backbone of residues Dab-1 to Dab-3 is located in the acyl region of the branched fatty acids originating from GlcN-B. To summarize most contacts in this model structure are made with GlcN-B and the adjacent atoms of GlcN-A, as well as with parts of Kdo-C. The model structure is supported by the chemical shift mapping data and the intermolecular NOEs. In addition, interacting moieties of LPS that make contacts common with all polymyxin variants are in contact with conserved parts of these peptides (residues 1 to 4 and 8 to 10), while those sites, that differ in their interaction with the different peptides form contacts with non conserved residues.

Discussion:

In this work we have used polymyxins of different types and a combination of NMR spectroscopy and restrained molecular dynamics to study their interactions with LPS from the deep rough mutant E. coli strain. To our knowledge this work for the first time presents experimental data on the interacting moieties in a membrane mimetic environment.

The conformation of LPS-bound PMX-B and -E was elucidated previously using transfer-NOE techniques by Pristovsek (11). Therein, the LPS-bound peptides assume envelope-like bent cycles that separate the two hydrophobic residues 6 and 7 from the charged Dab residues 4, 5, 8 and 9. The conformation from the transfer-NOE derived structure was then docked onto a lipid A model. In the resulting complex the lipid chain forms transient contacts with acyl chains A and B from lipid A. Moreover, the two phosphate groups are in close contact with the γ-NH₂ groups of Dab residues 1/5 and 8/9. Their data highlight the importance of electrostatic interactions between phosphate groups and amino groups of polymyxin as well as hydrophobic interactions of the lipid chains with Phe or Leu residues. Martin et al. have studied the structure of LPS-bound PMX-M (19) which displayed a chair-like conformation, in which the side chain of Dab-4 or -8 and Leu-6 or Thr-7 point into the opposite directions in a fashion similar to that proposed for PMX-B and -E (19). Pristovsek determined the structure of a LPS-bound synthetic fragment of the LALF protein (10) revealing a hairpin-type fold, again characterized by spatial separation of hydrophobic and cationic residues. In another study Bhunia et al. investigated binding of melittin to LPS micelles (7). Cationic residues have also been postulated to critically contribute to binding of LPS to proteins such as MD-2 (51) or FhuA (16, 52).

Herein we set out to determine interactions between LPS and polymyxins in more detail. Considering that interacting chemical moieties have not been experimentally identified with confidence so far we reasoned that these studies should use chemically defined environments in a setup that mimics natural conditions. To achieve chemical homogeneity we developed a novel chromatographic separation procedure that does not require methylation of phosphates. To facilitate isotope labeling biosynthetic LPS was used. LPS from the deep rough mutant of E. coli presents a simple system containing all chemical moieties believed to be important for the interaction, and in the absence of phospholipids has been assigned previously using homonuclear 2D NMR techniques (17, 18). LPS in gram-negative bacteria is embedded in a phospholipid bilayer, and therefore the system was studied in DPC micelles. Our data reveal that LPS inserts into the micelle via integration of the acyl chains into the micelle interior, and that the GlcN and Kdo portions are fully exposed in the aqueous compartment. Using 13C isotope labeling we have been able to fully assign LPS while integrated into DPC micelles. In the latter environment spectra with reasonable line widths and resolution for LPS can be recorded. Spectroscopic features of peptide resonances of PMX were less favorable but, in combination with restrained MD calculations, allowed studying interactions in sufficient detail.

The chemical shift-mapping data (Fig. 5), the intermolecular NOEs (Table 2) and the model derived from molecular dynamics restrained by these experimental data (Fig. 6, S9) now support the structure of the PMX-B LPS complex as proposed by Pristovsek et al. (11) in most but not all of its details. In their complex the macrocycle of PMX-B covers the GlcN disaccharide unit, and hydrophobic sidechains of polymyxin form contacts with the lipid chains from LPS. A similar complex topology was proposed for the interaction of LPS with PMX-M (19). Such an arrangement is facilitated by the amphiphilic nature of PMX, in which
hydrophobic and hydrophilic side chains point into opposite directions. A similar separation of hydrophobic and hydrophilic moieties in LPS helps to orient the two molecules with respect to each other through hydrophobicity matching. Interestingly, in a phospholipid environment such effects are even amplified, because similar requirements influence binding of polymyxin to phospholipids surface or to LPS. Accordingly, Leu or Phe residues, for which favorable energies for partitioning into the membrane interior or the water-membrane interfacial region have been measured (53), form contacts with the $\alpha$, $\beta$, and $\gamma$ acyl chain carbons of LPS, as evident from both large changes in the chemical shift mapping experiments as well as from the corresponding intermolecular NOEs. In PMX-M position 7 is occupied by Thr, a much more polar residue. According to the data from this work, PMX-M may be slightly differently oriented, possibly forming somewhat stronger contacts with the Kdo units thereby compensating for loss of interaction energy with the acyl chains. In the PMX-M—LPS complex electrostatic or polar interactions involving the Dab residues of polymyxin and the carbohydrate moieties of GlcN-B or Kdo-C and to a smaller extent, Kdo-D dominate, while in the case of PMX—B or —E additional hydrophobic interactions from side chains of Leu-7 are likely to contribute to binding. While our work confirmed the contacts made with moieties of the GlcN units proposed in the model complex from Pristovsek (11) it is different in that it emphasizes the importance of contacts to Kdo units, in particular to Kdo-C.

Different models have been proposed for the mechanism of action of polymyxin. Shai et al. have proposed that binding of polymyxin to the core part of LPS, lipid A, results in disturbance of the LPS-phospholipid bilayer destroying the integrity of the outer membrane, and possibly leading to pore formation (9,13). The orientation of LPS in the phospholipid micelles as measured using spin labels has demonstrated that the carbonyl group region of the LPS lipid chains is located in the headgroup region. Binding of polymyxin to LPS therefore places the amphiphilic peptide in a similar position compared to binding to pure phospholipid micelles. Accordingly, similar mechanisms for membrane permeabilization are plausible.

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Tables

Table 1: Carbon and proton chemical shifts of LPS from the deep-rough mutant of *E. coli*, 0.35mM LPS in 300mM DPC, pH= 4.4; 40mM acetate, T=310K

| Sugar moieties | GlcN-A | GlcN-B | Kdo-C | Kdo-D |
|---------------|--------|--------|-------|-------|
| H 1           | 5.346  | 4.681  |       |       |
| H 2           | 3.928  | 3.861  |       |       |
| H 3           | 5.143  | 5.072  | 1.900 | 2.104 |
| H 3           |       |        | 1.937 | 1.731 |
| H 4           | 3.689  | 3.879  | 4.086 | 4.022 |
| H 5           | 4.040  | 3.654  | 4.079 | 3.989 |
| H 6           | 4.018  | 3.409  | 3.647 | 3.550 |
| H 6           | 3.826  | 3.734  |       |       |
| H 7           |        |        | 3.869 | 3.920 |
| H 8           |        |        | 3.872 | 3.926 |
| H 8           |        |        | 3.599 | 3.697 |
| C 1           | 95.56  | 104.41 |       |       |
| C 2           | 54.05  | 55.61  |       |       |
| C 3           | 75.43  | 76.03  | 35.33 | 36.50 |
| C 4           | 68.63  | 74.75  | 70.58 | 68.00 |
| C 5           | 73.32  | 76.19  | 66.63 | 68.35 |
| C 6           | 70.50  | 64.73  | 73.66 | 74.63 |
| C 7           |        |        | 71.89 | 71.77 |
| C 8           |        |        | 65.50 | 65.32 |

| Lipid chains | LM | MM |
|--------------|----|----|
|               | L  | HM2| M  | HM1| HM-4| HM-3 |
| HA           | 2.306*| 2.562| *| 2.643| 2.312| 2.383 |
| HA           | 2.585| *| 2.670| 2.366| 2.433 |
| HB           | 1.563*| 5.283| *| 5.128| 3.890| 3.910 |
| HC           | 1.210*| 1.519| *| 1.548| 1.303| 1.383 |
| HC           | 1.559| *| 1.601| 1.380| 1.471 |
| HD           |    |    | 1.281| 1.392| |
| CA           | 36.51*| 43.15| *| 41.10| 45.38| 44.18 |
| CB           | 27.34*| 72.37| *| 72.50| 69.77| 69.61 |
| CG           | 31.50*| 37.18| *| 36.25| 38.30| 40.30 |
| CD           |    |    | 27.98| 28.08| |

* Resonances that cannot be distinguished between L and M because of overlap
Table 2: Intermolecular NOEs as detected in $^{13}$C-filtered NOESY experiments performed with $^{13}$C-labeled LPS and unlabeled PMX-B and –E. 0.5mM LPS, 2mM PMX, 300mM DPC, 40mM acetate, pH=4.4, 310K. The strength of the NOE is classified as weak (“w”), medium (“m”) or strong (“s”).

| Polymyxin-B |       |       |       |       |
|-------------|-------|-------|-------|-------|
| HM4-H(Cα)   | Leu7-Hδ1 | w     | HM3-H(Cγ) | Phe6-Hε   | w     |
| HM4-H(Cβ)   | Leu7-Hδ1 | s     | HM3-H(Cδ) | Phe6-Hδ   | s     |
| HM4-H(Cδ)   | Leu7-Hδ1 | w     | HM3-H(Cδ) | Phe6-Hε   | s     |
| HM4-H(Cδ)   | Leu7-Hδ2 | s     | HM4-H(Cδ) | Phe6-Hδ   | s     |
| HM4-H(Cβ)   | Leu7-Hδ2 | s     | HM4-H(Cβ) | Phe6-Hδ   | s     |
| HM4-H(Cα)   | Leu7-Hδ2 | s     | HM3-H(Cβ) | Phe6-Hδ   | s     |
| HM4-H(Cβ)   | Leu7-Hβ2 | s     | HM4-H(Cα) | Phe6-Hδ   | s     |
| HM4-H(Cβ)   | Leu7-Hβ1 | w     | HM4-H(Cγ) | Phe6-Hδ   | s     |
| HM3-H(Cβ)   | Phe6-Hβ2 | w     | HM3-H(Cα) | Phe6-Hδ   | s     |
| HM1-H(Cβ)   | Phe6-Hβ2 | w     | HM3-H(Cβ) | Phe6-Hδ   | s     |
| HM2-H(Cα)   | Phe6-Hβ1 | w     | HM2-H(Cα) | Phe6-Hδ   | s     |
| HM4-H(Cα)   | DABA8-Hγ | s     | HM2-H(Cβ) | Phe6-Hε   | w     |
| HM4-H(Cβ)   | DABA8-Hγ | w     | HM4-H(Cα) | DABA8-HN  | w     |
| Polymyxin-E |       |       |       |       |
| HM4-H(Cα)   | Leu6-Hδ1 | m     | HM2-H(Cα) | Leu6-Hδ1 | s     |
| HM4-H(Cα)   | Leu7-Hβ | m     | HM2-H(Cα) | Leu6-HN  | m     |
| HM4-H(Cβ)   | Leu6-Hγ | m     | HM2-H(Cα) | Leu6-Hγ  | m     |
| HM4-H(Cβ)   | Leu7-Hβ | m     | HM2-H(Cβ) | Leu6-Hγ  | m     |
| HM4-H(Cβ)   | Leu7-Hδ1 | m     | HM1-H(Cγ) | Dab4-HN   | w     |
| HM4-H(Cβ)   | Leu7-Hγ | w     | HM1-H(Cγ) | Leu6-HN  | m     |
| HM4-H(Cβ)   | Leu7-HN | m     | HM3-H(Cα) | Leu6-Hδ1 | m     |
| HM4-H(Cδ)   | Leu7-Hβ | w     | HM3-H(Cβ) | Leu6-Hδ1 | m     |
| HM4-H(Cγ)   | Leu6-Hδ1 | s     | HM3-H(Cγ) | Leu6-Hδ1 | m     |
Figure Captions:

**Figure 1:** Chemical structures of LPS from the D31m4 *E. coli* strain (1), the two model compounds for LPS (2 and 3) and PMX-M (4). Note that in PMX-B residue D-Leu-6 is replaced by D-Phe and Thr-7 is replaced by Leu, and in polymyxin-E Thr-7 is replaced by Leu. In the text, the glycosidic residues of LPS are identified with the letters A-D, starting from the far right GlcN residue.

**Figure 2:** Comparison of the MALDI-TOF MS spectrum of commercial LPS with LPS from the deep rough D31m4 *E. coli* strain purified by the protocol that includes an additional HPLC purification step.

**Figure 3:** Assignment of $^{13}$C spin systems of a short stretch contained in Kdo-D from LPS using (H)CCH-TOCSY spectra. On the right the constant-time $[^{13}$C,$^1$H]-HSQC spectrum is shown, in the middle a strip from the (H)CCH-TOCSY taken at the proton frequency of H-(C4) of Kdo-D.

**Figure 4:** Left: Results from the spin-label experiments displayed on the structure of LPS. The size of the sphere is proportional to the remaining signal in the proton-carbon correlation map. Because signals from ends of the fatty acid chains are not resolved in the spectra, no encoding is shown for these atoms. Right: Sketch of LPS from the D31m4 strain when inserted into DPC micelles. The phospholipid headgroups are depicted as grey spheres.

**Figure 5:** Changes in $^1$H and $^{13}$C chemical shifts of resonances from LPS when adding PMX-B (A), PMX-E(B) and PMX-M(C). The size of the spheres is proportional to the observed differences. Deviations of peak positions were extracted from the $[^{13}$C,$^1$H]-HSQC spectra and computed according to $\Delta \delta = \sqrt{((\Delta \delta \text{ C})^2 + (10 \Delta \delta \text{ H})^2}$. Those atoms that are exchange-broadened beyond detection are depicted by green transparent spheres.
**Figure 6:** Left: Structure of the PMX-B—LPS complex as derived from the MD calculation. The covalent structure of PMX-B is depicted in yellow. Heavy atoms of LPS are drawn as van der Waals spheres, with lipid chains colored in ice-blue, GlcN carbons in dark blue, Kdo carbons in orange, and phosphorous atoms in white. DPC molecules are indicated by thin lines. Residue numbers of PMX-B are placed close to the corresponding sidechains.

Right: Representation showing only bonds of LPS and PMX-B. PMX-B is again drawn in yellow, with amino nitrogens of DAB residues in blue, while LPS bonds are drawn in ice-blue (lipids) or green (sugar parts).
Figures:

Fig. 1:

Fig. 2:

Fig. 3:
