4-Amino-4-deoxy-\(\text{L}\)-arabinose in LPS of enterobacterial R-mutants and its possible role for their polymyxin reactivity

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Abstract: The content of 4-amino-4-deoxy-\(\text{L}\)-arabinopyranose (L-Arap4N) and the phosphate substitution pattern of the LPS of various strains from \(\text{Salmonella minnesota}\), \(\text{Yersinia enterocolitica}\) and \(\text{Proteus mirabilis}\) was determined by GC/MS, HPLC and \(^{31}\)P-NMR. These data allowed us to examine the possible role of these components for the polymyxin B-binding capacity of LPS and for the minimal inhibiting concentration (MIC) and the minimal bactericidal concentration (MBC) of polymyxins B and E towards the respective R-mutants. Contrary to other investigated Re-, Rd- and Rc-mutants of \(\text{S. minnesota}\), strain R595 (Re-mutant) showed about a 90% substitution of the ester-linked phosphate-group with L-Arap4N, whereas the L-Arap4N content of the other \(\text{S. minnesota}\) strains amounted to 17–25%. Neither the binding capacity of LPS to polymyxin B, determined by a bioassay, nor the MIC- and MBC-values of the R-mutants were significantly affected by this alteration. Similar results were obtained after using the temperature-dependent changes in the L-Arap4N-content and phosphate substitution pattern of \(\text{Y. enterocolitica}\) 75R. In order to explore the relevant polymyxin B binding site, lipid A samples with or without substitution of their ester-linked phosphate group were prepared and subjected to the polymyxin-binding assay. The results obtained so far indicated that the inner core bound L-Arap4N, detected in all resistant strains investigated, may play a decisive role in the decreased binding of polymyxin B, responsible for the bacterial resistance towards polymyxin(s).

Key words: Polymyxin B and E; LPS; 4-Amino-\(\text{L}\)-arabinose; Bacterial resistance, Endotoxin inhibitor; Minimal inhibition concentration

Introduction

Polymyxins, a group of cyclic, polycationic, amphiphilic peptide antibiotics, exhibit a rather selective activity against Gram-negative bacteria [1]. Due to the five positive charges of their diamino-butyric-acid residues, polymyxins show a high affinity to negatively charged surface structures of the cell envelope such as LPS, capsular antigens, or phospholipids by electrostatic interactions [2]. It has been well established that an effective binding of polymyxin to the LPS of the OM is
essential for its bactericidal activity [3-5]. Besides its importance as an antibiotic, polymyxin B (PmxB) is known as a potent endotoxin (LPS)-antagonist [6]. It has been shown that PmxB inhibits LPS-induced synthesis of the macrophage tumor necrosis factor-α [7], as well as the release of interleukin-1 from human monocytes by interaction with the polyanionic lipid A/Kdo-region [8]. There is strong evidence that the affinity to LPS is dependent on the chemical structure of the lipid A/Kdo region. 4-Amino-4-deoxy-L-arabinopyranose (L-Arap4N), as a substituent of the ester-linked phosphate-group of lipid A, was suggested to play an important role for the PmxB-resistance of, e.g., Proteus mirabilis strains. Contrary to its parent strain, P. mirabilis R4/O28, a Rc-type mutant, has been shown to lack 4-amino pentose completely [5,9]. This alteration resulted not only in an increased binding of polymyxin B to isolated LPS but also rendered the mutant sensitive to polymyxins [5]. These results confirmed earlier studies with the pmrA-mutant of Salmonella typhimurium whose LPS showed a decreased binding of this antibiotic, due to a four- to six-fold higher substitution of the ester-linked phosphate-group in lipid A by L-Ara.p4N. This mutant was shown to be resistant to the bactericidal action of polymyxin [10].

In several strains of P. mirabilis, Providencia rettgeri, Morganella morganii and Serratia marcescens, all showing a natural resistance to polymyxins, L-Ara.p4N was not only detected as an (almost) quantitative substituent of the ester-linked phosphate group in lipid A but also, at least in the strains tested, as a constituent of the inner core region [11]. In the LPS of the R-mutant P. mirabilis R45/1959 1-Ara.p4N was proven to be attached to Kdo by a (1 → 8) linkage [12].

It has not previously been investigated whether it is the lipid A-phosphate-bound 1-Ara.p4N, or the Kdo-bound 1-Ara.p4N, or even both types, which are responsible for the decreased binding of polymyxins, leading to a resistance towards these antibiotics. Schindler and Osborn [13] suggested the importance of the carboxyl-group of the Kdo-residues while other authors claimed that the non-substituted ester-bound phosphate group is the preferential polymyxin B-binding site of LPS [14,15].

In the present study the lipid A/Kdo regions of strains with very similar lipid A structures were investigated which, however, showed natural or temperature-dependent differences in their incorporation of L-Ara.p4N [16]. The influence of the amount of L-Ara.p4N on both, the minimal inhibitory concentration of polymyxin B on the mutant strains and the affinity of pmxB to isolated LPS were investigated. Further, a comparison of the LPS-binding capacities of native LPS, native lipid A and lipid A after splitting off the phosphate bound 1-Ara.p4N was made.

Material and Methods

Bacterial strains and lipopolysaccharides
Salmonella minnesota R mutants R595, R613, R3, R7 and E. coli K-12 W3100 were obtained from the strain collection of the Max-Planck-Institut für Immunobiologie (Freiburg i.Br., FRG). The R-mutant strains of Proteus mirabilis R45/1959 and R110/1959 were from the collection of the Institute of Microbiology (University of Łódź, Łódź, Poland). Yersinia enterocolitica Ye75R was a kind gift of Dr. K. Wartenberg, (Institut für Klinische Mikrobiologie, Universität Erlangen, FRG). All bacteria were cultivated under aerobic conditions at 37°C in antibiotic medium No. 3 (Difco, Detroit). Additionally, Y. enterocolitica 75R (O:3) was grown at 10°C and 22°C (using the same medium). LPS from most Salmonella-strains was generously provided by Dr. S. Schlecht (Max-Planck-Institut für Immunobiologie, Freiburg i.Br., FRG) and LPS from Y. enterocolitica 75R was obtained from Dr. K. Wartenberg. All LPS were isolated as described earlier [17]. Prior to the LPS/lipid A-polymyxin B-binding studies the lipopolysaccharides were electrodialysed, as described previously [18].

Antibiotics
Polymyxin B and E of the highest available purity were obtained from Sigma (Deisenhofen, FRG).
**Determination of the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC)**

MIC of polymyxin B and E was determined in liquid antibiotic medium No. 3 (Difco, Detroit) [5]. Overnight cultures of the strains were diluted 1/100 with fresh medium, and 0.5 ml of these suspensions were added to 0.5 ml of two-fold serial sterile dilutions of polymyxin B and E in antibiotic medium No. 3 (sterilization was performed by Millipore filtration). The mixture was incubated for 12 h at 37°C. Y. enterocolitica 75R was additionally incubated with the antibiotic for 24 h at 22°C and for 6 days at 10°C. MIC was defined as the lowest concentration of the antibiotic which showed no visible growth.

MBC was determined on agar plates of antibiotic medium No. 3. Samples (200 µl) of those cultures which showed no growth after the expected time were inoculated on agar plates. The lowest concentration of the antibiotic which showed no visible growth after an incubation for 24 h at 37°C was documented as MBC [5].

The determinations of MIC and MBC were repeated at least three times.

**Lipid A preparation**

Lipid A was precipitated from isolated LPS by hydrolysis either with 0.1 M sodium acetate buffer (pH 4.5, 2.5 h, 100°C) [19], or with 0.02 M HCl (10 min, 100°C) [16]. After centrifugation (10 000 × g, 30 min), the precipitates were washed twice with water (20°C and 40°C), then with ice-cold ethanol and finally with ice-cold acetone. For obtaining purified lipid A preparations, the samples were further cleaned by extraction with CH₂Cl₂/water (1:1; v/v).

To check whether the splitting of the ketosidic linkage between the polysaccharide portion and the lipid A was complete, the Kdo content of both LPS and lipid A was determined with the thiobarbituric acid assay.

**Detection of lipid A bound 4-amino-4-deoxy-L-arabinose**

Lipid A bound l-Ara p4N was liberated from LPS (5 mg) by hydrolysis with 0.02 M HCl for 10 min at 100°C) [16]. After centrifugation (10 000 × g, 10 min) the supernatant was passed over a filter (Millipore, 0.2 µm) and lyophilized. For qualitative analysis high voltage paper electrophoresis (HVPE) [20] was carried out using a pyridine/acetic acid/water (10:4:86; v/v/v) solvent of pH 5.3 (3 kV, 70–90 min). The spots were stained either with ninhydrin or with alkaline silver nitrate.

l-Ara p4N was characterized by GC/MS of its alditol acetate as described previously [22] and was quantified by HPLC (reversed phase chromatography, Pico-Tag-HPLC-system, Waters, Eschborn, FRG) after liberation and derivatisation with phenylisothiocyanate to the corresponding phenylthiocarbamoyl-derivatives. Since no authentic standard was available, l-Ara p4N was isolated by preparative high voltage paper electrophoresis from P. mirabilis R45/1959 LPS (whose ester-linked phosphate-group shows a quantitative substitution with l-Ara p4N [21]) by elution of the corresponding area (m<sub>Glcn</sub> = 1.18) on the electropherogram with 0.01 M HCl. The isolated sugar was subjected to HPLC analysis as the reference substance after confirming its chemical nature by GC/MS analysis [22]. In each case, quantification was carried out by comparing the peak areas on the HPLC chromatograms with that of P. mirabilis R45/1959.

**Detection of Kdo-bound 4-Amino-4-deoxy-L-arabinose**

For a qualitative detection of Kdo-bound l-Ara p4N as the 8-O-(4-amino-4-deoxy-β-l-arabinopyranosyl)-3-deoxy-manno-octulosonic acid disaccharide [12], high voltage paper electrophoresis (HVPE) was performed. After hydrolysis of LPS (15 mg) with 0.1 M sodium acetate buffer (pH 4.5, 2.5 h, 100°C), followed by removal of the precipitated lipid A-moiety by centrifugation (10 000 × g), the supernatant was passed over a filter and put to HVE-analysis under the conditions described above. Kdo-bound l-Ara p4N was detected on electropherograms by a simultaneous staining with thiobarbituric acid, ninhydrin and alkaline silver nitrate.

**Phosphate substitution pattern**

The phosphate substitution patterns of the different LPS-types were investigated by ³¹P-NMR
on a Bruker WM 300 spectrometer operating in the Fourier-transform mode at 121.51 mHz. Chemical shifts were measured relatively to 85% phosphoric acid as an external $^{31}$P-source (0.00 ppm) [23]. Samples were dissolved in 5 mM EDTA and 2% sodium deoxycholate (Sigma) in $D_2O$.

**Binding of polymyxin B to isolated LPS**

The binding of polymyxin B to isolated LPS was investigated by a bioassay [5]. The ability of isolated LPS to inhibit the bactericidal activity of polymyxin B was used for quantifying the binding of the antibiotic to LPS.

To 0.25 ml of a serial dilution of isolated LPS (0–240 nmol/ml) in 0.9% NaCl, 0.25 ml of 20 nmol/ml solution of polymyxin B in 0.9% NaCl was added. After incubation for 1 h at 40°C, 4.5 ml of a 1/100 diluted overnight culture of the indicator strain, *E. coli* K-12 W3100 (O.D. approx. 0.15–0.2) was added. After a further incubation for about 1.5 h (O.D. without antibiotic should be about four times higher than the O.D. of the starting culture), bacterial growth was immediately stopped with 100 μl of a 37%
formaldehyde solution. The effect of the co-incubated isolated LPS on the bactericidal activity of polymyxin B was determined by measuring the O.D. at $\lambda = 580$ nm.

**Results**

**Determination of 4-amino-1-arabinose**

Qualitative and quantitative determination of lipid A-bound $\text{L}$-$\text{Ara}p4\text{N}$ was carried out by HVPE and HPLC after a mild acid hydrolysis (100°C, 10 min, 0.02 M HCl) of the different LPS's.

Up to five different spots could be visualized on the electropherograms after ninhydrin staining: alanine and lysine (most probably originating from the peptidoglycan), $\text{EtN-P}$ ($m_{\text{GlcN}} = 0.04$), $\text{EtN}$ ($m_{\text{GlcN}} = 2.09$) and $\text{L-Ara}p4\text{N}$ ($m_{\text{GlcN}} = 1.18$; characteristic brownish spot).

In order to quantitatively explore the substitution of the ester-linked phosphate group in lipid A with $\text{L-Ara}p4\text{N}$, we first isolated the amino pentose from LPS of *P. mirabilis* R45/1959, sub-
jecting the isolated substance to both GC/MS- and HPLC-analyses in order to obtain a standard for the quantification of $\text{L-Ara}p4\text{N}$. The mass spectrum of the isolated N-acetylated, NaBD$_4$-reduced and then peracetylated substance showed a fragmentation pattern identical to that of 4-amino-4-deoxy-arabinitol, as described in earlier studies [22]. HPLC analysis of the isolated substance resulted in a single clearly defined main peak (elution time 9.3 min). The quantitative detection of $\text{L-Ara}p4\text{N}$ in the structurally known LPS's of the *S. minnesota* strains R613 (Re-mutant), R7 (Rd-mutant) and R5 (Rc-mutant) revealed amounts of 0.17–0.25 mol $\text{L-Ara}p4\text{N}$/mol LPS, whereas the LPS of the Re-mutant *S. minnesota* R595 showed a much higher amount of incorporated $\text{L-Ara}p4\text{N}$ (0.89 mol/mol LPS) (Table 1).

The temperature-dependent incorporation of $\text{L-Ara}p4\text{N}$ in the LPS of *Y. enterocolitica* Ye75-strains, first described by Lakshmi et al. [16], offered another possibility to compare the effect of the presence of this amino pentose on the interaction between LPS and polymyxins. The

<table>
<thead>
<tr>
<th>Strain</th>
<th>Chemo-type</th>
<th>Amount of $\text{L-Ara}p4\text{N}$ * (mol/mol LPS) **</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella minnesota</em> R595</td>
<td>Re</td>
<td>0.89</td>
</tr>
<tr>
<td><em>Salmonella minnesota</em> R613</td>
<td>Re</td>
<td>0.17</td>
</tr>
<tr>
<td><em>Salmonella minnesota</em> R3</td>
<td>Rd$_2$</td>
<td>0.25</td>
</tr>
<tr>
<td><em>Salmonella minnesota</em> R7</td>
<td>Rd$_1$</td>
<td>0.25</td>
</tr>
<tr>
<td><em>Yersinia enterocolitica</em> 75R</td>
<td>10°C</td>
<td>Re</td>
</tr>
<tr>
<td><em>Yersinia enterocolitica</em> 75R</td>
<td>22°C</td>
<td>Re</td>
</tr>
<tr>
<td><em>Yersinia enterocolitica</em> 75R</td>
<td>37°C</td>
<td>Re</td>
</tr>
<tr>
<td><em>Escherichia coli</em> K-12 D21f2</td>
<td>Re</td>
<td>0.00</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> R45/1959</td>
<td>Re</td>
<td>1.00</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> R110/1959</td>
<td>Ra</td>
<td>0.92</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> R4/O28</td>
<td>Re</td>
<td>0.00</td>
</tr>
</tbody>
</table>

* Quantification was carried out by HPLC analysis.
** Molecular weight of LPS was calculated from the known chemical structures of the defined R-mutants.
results of the quantitative HPLC-analysis indicated that the amount of L-Arap4N in the LPS of Y. enterocolitica 75R, grown at low temperatures (10°C and 22°C), is about ten times higher than that of the same strain cultivated at high temperature (37°C) (Table 1).

Quantification of L-Arap4N in LPS of the polymyxin-resistant strain P. mirabilis R110/1959 (Ra-mutant) yielded a similarly high content as found with the LPS of the Re-mutant P. mirabilis R45/1959, whereas L-Arap4N was neither detectable in the LPS of the PmxB-sensitive strain P. mirabilis R4/O28, nor in the LPS of the control strain E. coli K-12 D21f2 (Re-mutant).

Kdo-linked L-Arap4N was qualitatively detected, possibly also as the 8-O-(4-amino-4-deoxy-β-L-arabino)-3-deoxy-D-manno-octulosonic acid-disaccharide by HVPE after mild hydrolysis (100°C, 0.1 M sodium acetate buffer, pH 4.5, 2.5 h), and after staining with mild alkaline silver nitrate and with periodate/thiobarbituric acid (cherry-red spots with MGIcN = 0.17). Only the LPS's of P. mirabilis mutants R45/1959 (Re), R13/1959 (Rc) and R110/1959 (Ra) revealed the presence of L-Arap4N linked to Kdo in the inner core region, whereas investigation of the LPS of the strains from S. minnesota (R595, R613), Ye75R and P. mirabilis R4/028 indicated a lack of the Kdo-bound L-Arap4N.

Phosphate-substitution pattern

The phosphate substitution patterns of the two Re-mutants of S. minnesota, R595 and R613, as well as that of Y. enterocolitica Ye75R, cultivated at 22°C and at 37°C were investigated by 31P-NMR (Figs. 1 and 2).

The remarkable differences in the amount of L-Arap4N, released by 0.02 M HCl-hydrolysis, observed with the two Re-mutants of S. minnesota, corresponded well with the differences found in the 31P-NMR-spectra of these two mutants. Signal b in the spectrum of S. minnesota R595, showing a chemical shift value of about -2.0 ppm, was identified as the ester-linked phosphodiester group, since an increase of the pH from 7.4 to 9.1 did not lead, as expected for phosphomonoesters, to a change in its chemical shift. Signals c, d, f and g also did not show pH-dependent chemical shifts. Due to their chemical shift values, at -10 to -12 ppm, as well

Fig. 1. 31P-NMR spectra of the LPS from (A) Salmonella minnesota R595, pH 7.4, and (B) Salmonella minnesota R613, pH 7.2. The spectra were recorded in the presence of 5 mM EDTA and 2% DOC in 2-3 ml D2O with chemical shifts relatively to an external standard (85% phosphoric acid) at 25°C (0.00 ppm).
as due to their relative intensities, these four signals must originate from $^{31}\text{P}$-$^{31}\text{P}$-coupling and are thus identified as pyrophosphodiester, representing the glycosidic phosphate-group (which seems to be almost 100% substituted with EtN-P). Signal a, with about 1/10 of the intensity of signal b, is the only signal with a pH-dependent chemical shift in the $^{31}\text{P}$-NMR-spectrum of \textit{S. minnesota} R595. This signal is therefore assumed to represent the ester-linked phosphate groups ($\sim 10\%$) not being substituted by L-Arap4N.

In sharp contrast to LPS of \textit{S. minnesota} R595, the phosphomonoester signals represented about 90% of the phosphate signals in the spectra of the \textit{S. minnesota} R613 LPS (Fig. 1B). Only signal d in the spectrum of the R613-mutant did not show the pH-dependent chemical shift. This signal is assumed to represent the 0.17 mol/mol LPS (data from HPLC analysis) of the 1-Arap4N-substituted ester-linked phosphate group of \textit{S. minnesota} R613 (Table 1).

The big differences in the 1-Arap4N-content of the LPS from \textit{Y. enterocolitica} 75R, cultivated at 22°C (0.77 mol/mol LPS) and at 37°C (0.08 mol/mol LPS), were also reflected in the $^{31}\text{P}$-NMR-spectra of these two LPS (Fig. 2A–D). The spectrum of the 37°C LPS showed only a single signal with a low intensity (signal c at $\delta = -2.0$ ppm), being not pH-dependent (Fig. 2A, B). Due to their $\delta$-values and their pH-dependency, the signals d–g were interpreted as the four signals typical for a pyrophosphomonoester with $^{31}\text{P}$-$^{31}\text{P}$-coupling, whereas the signals a and b very likely to have originated from phosphomonoesters.

In the spectrum of the LPS of \textit{Y. enterocolitica} 75R, cultivated at 22°C (Fig. 2C, D), the main signals d and e (with $\delta = -1.9$ at pH 7.3) could be recognized as phosphodiester. These two signals with high intensities showed, contrary to all other phosphomonoester and pyrophosphomonoester signals, no pH-dependent chemical shifts (Fig. 2C, D). This also corresponded well with the ten-fold increased 1-Arap4N-content of LPS from the 22°C-culture.

### Table 2
Determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of polymyxin B and E of several \textit{Enterobacteriaceae}-strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Polymyxin B</th>
<th>Polymyxin E (colistin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC ((\mu\text{g/ml}))</td>
<td>MBC ((\mu\text{g/ml}))</td>
</tr>
<tr>
<td>\textit{Salmonella minnesota} R595</td>
<td>0.5 (\pm) 0.2</td>
<td>1.0 (\pm) 0.3</td>
</tr>
<tr>
<td>\textit{Salmonella minnesota} R613</td>
<td>1.0 (\pm) 0.2</td>
<td>1.5 (\pm) 0.3</td>
</tr>
<tr>
<td>\textit{Salmonella minnesota} R5</td>
<td>0.5 (\pm) 0.1</td>
<td>1.5 (\pm) 0.4</td>
</tr>
<tr>
<td>\textit{Salmonella minnesota} R7</td>
<td>0.8 (\pm) 0.3</td>
<td>1.0 (\pm) 0.3</td>
</tr>
<tr>
<td>\textit{Yersinia enterocolitica} 75R 10°C</td>
<td>0.9 (\pm) 0.1</td>
<td>5.0 (\pm) 1.0</td>
</tr>
<tr>
<td>\textit{Yersinia enterocolitica} 75R 22°C</td>
<td>0.8 (\pm) 0.3</td>
<td>1.0 (\pm) 0.4</td>
</tr>
<tr>
<td>\textit{Yersinia enterocolitica} 75R 37°C</td>
<td>0.8 (\pm) 0.2</td>
<td>1.5 (\pm) 0.3</td>
</tr>
<tr>
<td>\textit{E. coli} K-12 D21f2</td>
<td>0.6 (\pm) 0.2</td>
<td>0.6 (\pm) 0.3</td>
</tr>
<tr>
<td>\textit{Proteus mirabilis} R110/1959</td>
<td>&gt; 1250$^1$</td>
<td>&gt; 1250$^1$</td>
</tr>
<tr>
<td>\textit{Proteus mirabilis} R4/028</td>
<td>5$^1$</td>
<td>18$^1$</td>
</tr>
</tbody>
</table>

$^1$ Results taken from Kaca et al. [5].
wards polymyxin B and E from the *S. minnesota* mutants R613, R5 and R7. The MIC of polymyxin B of all strains was between 0.5–1.0 μg/ml. Similar results were obtained when comparing the MIC’s and MBC’s of *Y. enterocolitica* 75R which showed big differences in L-Arap4N-incorporation and in the phosphate substitution pattern of their LPS, isolated from cultivations obtained at different culture temperatures. These strains showed growth temperature-independent MIC’s of polymyxin B and E of about 1.0 μg/ml.

As a control the polymyxin-resistant *P. mirabilis* mutant R45/1959 was used, whose MIC- and MBC-values were > 2000 μg/ml for polymyxins B and E.

**Preparation of LPS/lipid A with differences in phosphate substitution**

One aim of this study was to obtain LPS/lipid A samples from the Re-mutants R595 of *S. minnesota* (polymyxin sensitive) and R45/1959 of *P. mirabilis* (polymyxin resistant) with different phosphate substitution patterns and to determine their affinity towards polymyxin. Different hydrolysis conditions were chosen, after which the purified LPS/lipid A-samples were investigated for

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**Fig. 2.** $^{31}$P-NMR spectra of *Yersinia enterocolitica* 75R-LPS, (A) 37°C-LPS at pH 9.0; (B) 37°C-LPS at pH 6.8; (C) 22°C-LPS at pH 9.0; (D) 22°C-LPS at pH 7.3. For recording conditions see Fig. 1.
their content in \( \text{t-Ara} \text{p4N} \) (by HPLC) and in Kdo (by the thiobarbituric acid assay) and for their phosphate substitution pattern by \(^{31}\text{P}\)-NMR-spectroscopy.

The 'selective' release of \( \text{t-Ara} \text{p4N} \) of the ester-linked phosphate group with 0.02 M HCl (10 min, 100°C) led in both Re-LPS samples to nearly complete lipid A precipitations since, using the thiobarbituric acid assay, the amount of Kdo of the prepared LPS samples was \(< 10\%\) of the original Re-LPS. The \(^{31}\text{P}\)-NMR spectrum of this preparation from \textit{S. minnesota} R595 showed a complete conversion of the ester-linked and the glycosidically linked phosphate diesters to monoesters, indicating a complete lack of the phosphate substituents \( \text{t-Ara} \text{p4N} \) and EtN. Milder hydrolysis conditions, used to obtain LPS samples with a complete core but without phosphate-bound \( \text{t-Ara} \text{p4N} \), failed however: hydrolysis with 0.05-0.005 M HCl (37°C, 16 h) [24] resulted in a decreased liberation of the ester-linked \( \text{t-Ara} \text{p4N} \), whereas the content of the released Kdo remained unaffected. This indicated that the ketosidic linkage between Kdo and lipid A is more labile to diluted HCl than the ester linkage between \( \text{t-Ara} \text{p4N} \) and the lipid A-bound phosphate-group.

\(^{31}\text{P}\)-NMR-spectra of the lipid A from both \textit{Salmonella} Re-mutants investigated here, showed, however, a rather high stability of the phosphodiester linkages (more than 75\%) during hydrolysis with 0.1 M sodium acetate buffer (data shown in Table 3).

According to these results three different types of LPS/lipid A were available for the polymyxin B-binding studies: native LPS (nLPS), lipid A (after 0.02 M HCl hydrolysis) with 100\% phosphomonoesters (lipid A-P-OM), and lipid A with more than 75\% diesters (lipid A-P-t-Ara p4N) (Table 3).

### Polymyxin B binding to LPS

The binding of polymyxin B to the isolated LPS/lipid A was studied by a bioassay in which the inhibition of the bactericidal activity of polymyxin B by isolated LPS with an indicator strain (\textit{E. coli} K-12 W3100) was measured [10]. The assay was performed with native LPS, lipid A-P-OH and with lipid A-P-t-Ara p4N obtained from the different strains (Fig. 3a–f).

Native LPS from all the polymyxin B-sensitive strains investigated showed similar polymyxin B-binding capacities. The necessary nLPS concentrations for a rather complete inhibition of the bactericidal activity of 10 nmol/ml polymyxin B varied between 15 nmol/ml – 40 nmol/ml. Despite the big differences in the phosphate substitution pattern and the \( \text{t-Ara} \text{p4N} \) content, the polymyxin B-binding capacities of the native LPS of the Re-mutants of \textit{S. minnesota} (R595 and

### Table 3

Chemical characterization of the lipid A/Kdo-region of native LPS and released lipid A after hydrolysis under different conditions from \textit{Salmonella minnesota} R595 (PmxB-sensitive) and \textit{Proteus mirabilis} R45/1959 (PmxB-resistant)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hydrolysis conditions</th>
<th>Kdo-content * (mol/mol LPS)</th>
<th>Substitution of the ester linked phosphate group *</th>
<th>Substitution of the glycosidic phosphate group *</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Salmonella minnesota} R595</td>
<td>untreated</td>
<td>2.63</td>
<td>89% ( \text{t-Ara} \text{p4N} )</td>
<td>100% EtN-P</td>
</tr>
<tr>
<td></td>
<td>0.02 M HCl</td>
<td>0.18</td>
<td>&lt; 10% ( \text{t-Ara} \text{p4N} )</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>0.1 M sodium acetate</td>
<td>0.07</td>
<td>&gt; 75% ( \text{t-Ara} \text{p4N} )</td>
<td>&gt; 75% EtN-P</td>
</tr>
<tr>
<td></td>
<td>pH 4.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Proteus mirabilis} R45/1959</td>
<td>untreated</td>
<td>2.02</td>
<td>100% ( \text{t-Ara} \text{p4N} )</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>0.02 M HCl</td>
<td>0.40</td>
<td>&lt; 10% ( \text{t-Ara} \text{p4N} )</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>0.1 M sodium acetate</td>
<td>0.13</td>
<td>&gt; 75% ( \text{t-Ara} \text{p4N} )</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>pH 4.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Kdo was determined with the thiobarbituric acid assay; phosphate substitution pattern was recorded with \(^{31}\text{P}\)-NMR.
Fig. 3. Polymyxin B binding capacities of non-treated LPS (nLPS), lipid A-P-OH and lipid A-P-4-Ara4N from different pmxB-sensitive and -resistant gram-negative strains. The binding was quantified with a bioassay in which the inhibitory action of LPS or lipid A on the bactericidal action of pmxB on an indicator strain (E. coli K12 D21f2, Re-mutant) was performed. For quantification, changes of the O.D. (λ = 580 nm) were measured after incubating the indicator strain with the preincubated (1 h, 37°C) pmxB/LPS-mixtures for 1.5 h).
R613), as well as those of \textit{Y. enterocolitica} 75R, cultivated at 22°C and 37°C, were very similar (Fig. 3a–c). Significantly higher concentrations (> 150 nmol/ml) of native LPS were necessary for the same effect when the LPS originated from a polymyxin B-resistant strain of \textit{P. mirabilis} (R45/1959 and R110/1959, which both showed a similar polymyxin B-binding capacity) (Fig. 3f). \textit{P. mirabilis} R4/O28, however, a polymyxin-sensitive strain, was able to bind polymyxin B as strongly as the other polymyxin-resistant strains investigated.

Comparing the different polymyxin B-binding capacities of nLPS and lipid A-P-l-Ara p4N from \textit{S. minnesota} R595 and \textit{Y. enterocolitica} 75R (22°C), at least a double concentration of lipid A-P-OH was necessary for an almost complete inhibition of the bactericidal action of 10 nmol/ml polymyxin B. In both cases the binding capacity of lipid A-P-l-Ara p4N was less than that of lipid A-P-OH. The LPS and lipid A preparations investigated with no or only a little incorporation of l-Ara p4N (\textit{E. coli} K-12 D21l2, \textit{Y. enterocolitica} 75R (37°C), \textit{S. minnesota} R613 and \textit{P. mirabilis} R4/O28) revealed similar results: in each case the native LPS showed a significantly higher activity than the corresponding lipid A-P-OH in the bioassay.

The polymyxin-resistant strain \textit{P. mirabilis} R45/1959 not only differed from the polymyxin-sensitive strains because of its decreased binding capacity of native LPS to polymyxin B, but the lipid A-P-OH preparation of this strain also showed a higher affinity to polymyxin B than nLPS, whereas lipid A-P-l-Ara p4N represented the preparation with the least inhibition of the bactericidal activity of polymyxin B in the bioassay (Fig. 3e).

\textbf{Discussion}

In the present study an attempt was made to elucidate the influence of the phosphate substitution pattern of lipid A, especially the role of the phosphate-linked l-Ara p4N in the interaction of isolated LPS with polymyxin B.

The extent of this interaction did not only concern the bactericidal action of polymyxin, but is also of importance for the well-established endotoxin-neutralizing activity of polymyxins [6]. Polymyxins belong to a group of polycationic substances which are able to permeabilize the outer membrane of Gram-negative bacteria for other substances (e.g. for hydrophobic antibiotics or lysozyme), which normally are unable to pass the permeation barrier [25]. In each case an effective binding of the two substances is necessary for the complete exertion of the respective activity of polymyxins. Since polymyxins are nephro-toxic [26], their clinical application is limited, although clinical trials with polymyxin B as endotoxin-inhibitor have already been successfully carried out [27]. Since the endotoxin inhibiting capacity by polymyxin can be completely replaced by less toxic polycationic substances, such as polymyxin-nonapeptide [28] or even by the bactericidal/permeability increasing protein (BPI) [29], produced by polymorphonuclear neutrophils, polymyxins are useful commercially available model substances.

In order to explore the Kdo/lipid A region for binding of polymyxins we first tested LPS structures with differences in only this region. Surprisingly, the Re-mutant \textit{S. minnesota} R595 showed an approx five times higher incorporation of l-Ara p4N in its LPS than another Re-mutant R613 derived from the same wild-type strain. \textsuperscript{31}P-NMR-investigations confirmed this result, showing a ratio of phosphodiester/phosphomonoester of about 10:1 for LPS of strain R595 and an inverse ratio of about 1:10 for the R613-mutant. Since the lipid A moiety of all \textit{S. minnesota} strains share identical basal structures [30], two LPS samples were thus available which differed only in their content in l-Ara p4N and thereupon in their phosphate substitution pattern. Similar results were obtained when the temperature-dependent incorporation of l-Ara p4N in LPS of \textit{Y. enterocolitica} 75R was quantitatively compared with the corresponding change in the phosphate substitution pattern. The differences in the l-Ara p4N-content, as well as in the ratio of phosphodiester/phosphomonoester (according to the \textsuperscript{31}P-NMR-spectra) of their LPS, namely of
bacteria cultivated at 22°C and 37°C, were even higher than those for the two Re-mutants of *S. minnesota*.

The differences detected in the 1-ArA p4N-content of the LPS's from *S. minnesota* R595 and R613, as well as from those of *Y. enterocolitica* 75R, cultivated at 22°C and 37°C, had neither an effect on the inhibitory concentrations of polymyxins B and E, nor did they affect the binding capacities of polymyxins to the isolated LPS. In both cases MIC- and MBC-values of polymyxins, as well as the affinity of isolated LPS to polymyxin B, were of a similar extent and differed very clearly from corresponding values of the polymyxin-resistant strains of *P. mirabilis* R45/1959 or R110/1959. Consequently, these results indicated that a high content of 1-ArA p4N, bound to the ester-linked phosphate group of lipid A, cannot be the only structural prerequisite for a decreased binding of polymyxins, and hence for an increase of the corresponding strains in both the MIC and MBC.

Comparative binding studies between native LPS, lipid A-P-1-ArA p4N and lipid A-P-OH of all investigated polymyxin B-sensitive strains, revealed the following order of their binding capacity: native LPS > lipid A-P-OH > lipid A-P-1-ArA p4N. These results indicate that the presence of the core region of the LPS may apparently play a much more important role in the binding of polymyxin B than the presence of 1-ArA p4N bound to the ester-linked phosphate-group of lipid A. In the case of the two Re-mutants from *S. minnesota* the core region is composed only of two or three Kdo residues. Our data support the assumption that the carboxyl groups of Kdo represent the high affinity binding site in the lipid A/Kdo region, as first described by Schindler and Osborn [13].

In order to explore which other components, beside lipid A-bound 1-ArA p4N, could be involved in the decreased binding of the antibiotic and in the phenomena of bacterial resistance to polymyxins, we attempted to find common structural details in the LPS typical for polymyxin-resistant strains. It had been reported that in strains of *Proteae* and of *Serratia marcescens*, all showing high resistance towards polymyxins, 1-ArA p4N was not only present in a linkage to the lipid A-bound phosphate group, but was additionally present also in the core region [11]. In our study, the core-linked 1-ArA p4N, assumed to be also the 8-O-(4-amino-4-deoxy-β-L-arabino)-3-deoxy-β-manno-octulosonic acid-disaccharide was identified in the lipopolysaccharides of the *P. mirabilis* strains R13/1959, R110/1959 and R45/1959 (all polymyxin-sensitive). It would be of considerable interest to test the LPS of other polymyxin B-resistant strains, such as *Serratia marcescens*, *Morganella morganii* or *Providencia rettgeri* also for the presence of this or a structurally related disaccharide present in the inner core region.

The influence of the presence of Kdo-linked 1-ArA p4N might not only be due to the electrostatic repulsion between the positive charges of 1-ArA p4N and polymyxin B, but also to the sterical availability of the carboxyl-groups of the Kdo units. It has been reported that the lipid A backbone is orientated in a 45° angle relative to the outer membrane surface and that the resulting free space is filled with Kdo residues of the inner core region which may then lead to an exposure of free carboxyl groups of the Kdo residues [31,32]. This model confirms the assumption that these carboxylic-groups represent, probably together with ester-linked lipid A-phosphate group, the primary binding site of polycationic molecules such as polymyxins. The 1-ArA p4N-units linked to the ester-linked phosphate group as well as assumingly to one of the Kdo units might then interfere with the polymyxin binding to this specific site. This assumption is also supported by a comparison of the polymyxin B-binding capacity of native LPS and lipid A from the polymyxin B-resistant strain of *P. mirabilis* R45/1959: the affinity of lipid A-P-1-ArA p4N to polymyxin B was approx. in the same range as those from the corresponding lipid A structures of *S. minnesota* R595 and *Y. enterocolitica* 75R, whereas native LPS of *P. mirabilis* R45/1959 bound five to ten times less of the antibiotic. This indicated that, in polymyxin B-resistant strains, the polymyxin B affinity is not or only slightly modulated by the Kdo residues – most probably due to the presence of 1-ArA p4N in this region. Very recently, Din et al. [33] reported on the interatomic dis-
stances between 1-P and 4'-P and the carboxylic (C) groups of Kdo1 and Kdo2 in the energetically most favourable conformation of a Re-type LPS (E. coli K-12 D31m4). The distances between 1-P and Kdo1-C, as well as Kdo2-C is much larger than that of the 4'-P to these two Kdo carboxylic groups. In our discussion, we therefore concentrated only on the substitutions of the ester-linked 4'-P, which, together with Kdo, forms a structural element of high charge density [33].

References


