DISRUPTION OF THE *ESCHERICHIA COLI* OUTER MEMBRANE PERMEABILITY BARRIER BY IMMOBILIZED POLYMYXIN B

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One of the apparent roles of the outer membrane system in gram-negative bacteria is to function as a selective permeability barrier. A number of antibiotics active against gram-positive bacteria are relatively ineffective against gram-negative bacteria presumably because of the implied barrier function of the outer membrane. This interpretation has been strengthened by studies demonstrating synergism between outer membrane perturbing agents such as EDTA or polymyxin B and specific antibiotics. In the case of polymyxin B, it is not totally clear that synergism with other antimicrobials is due to disruption of the outer membrane permeability barrier or to interactions with the inner membrane. In order to resolve this question, polymyxin B was covalently attached to agarose in order to limit interactions with the outer surface of *E. coli*. These studies demonstrate that immobilized polymyxin B acts synergistically with bacitracin, rifampicin, or lysozyme. It is proposed that synergistic effects exhibited by polymyxin B are due to its interaction with the outer membrane system.

The functions of the outer membrane system in gram-negative bacteria have not been totally defined although there is evidence for its permeability and osmotic barrier function.[1,2] The impermeability of outer membranes with respect to certain antibiotics and lysozyme has been demonstrated by synergism between outer membrane perturbing agents and lysozyme or specific antibiotics[1,3~7]. Polymyxin has been shown to disrupt the structure of the outer membranes[8,9] and selectively stimulate the release of periplasmic proteins from gram-negative bacteria[10]. Many antibiotics active against gram-positive bacteria are much less active against gram-negative bacteria, persumably because they are excluded by the outer membrane. The activities of tetracyclines, amphotericin B and chloramphenicols against gram-negative bacteria are accentuated by polymyxin B[4,5,9]. Similarly, lysozyme cannot normally reach the peptidoglycan layer of gram-negative bacteria unless the outer membrane structure is perturbed by EDTA complexation of divalent cations[6]. Polymyxin B can substitute for EDTA and a combination of this antibiotic and lysozyme has been used to prepare spheroplasts[7]. It has been proposed that the synergistic effects observed with polymyxin B are due to disruption of the outer membrane permeability barrier by the peptide[5,7]. Although this interpretation is certainly reasonable, it is likely that free polymyxin B can penetrate to inner layers of the cell envelope introducting a degree of uncertainty for the interpretation of these synergistic effects.

We have recently reported that polymyxin B covalently attached to agarose inhibits the respiration and growth of gram-negative bacteria but not gram-positive bacteria[11]. It was concluded that the activity of immobilized polymyxin was due to disruption of the outer membrane structure. In this report, synergism between polymyxin-agarose and several antibiotics is reported. It is also shown that spheroplasts can be formed by lysozyme in the presence of polymyxin-agarose. Since immobilization of polymyxin B restricted interactions to the outer surface of *Escherichia coli*, these results support the

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previous proposal that polymyxin synergism is due to disruption of outer membrane structure.

Materials and Methods

E. coli SC 9251 was supplied by the Squibb Institute of Medical Research. The growth minimum inhibitory concentration of free polymyxin B for SC 9251 is 1.2 μg/ml. Polymyxin B, bacitracin, rifampicin and lysozyme were purchased from Sigma. Affi-gel 10 was purchased from Bio-Rad. *E. coli* were grown in synthetic media consisting of 50 mM potassium phosphate, 0.12 mM $MgCl_2$, 1 mM $(NH_4)_2SO_4$, 20 mM sorbitol and 0.1% casein hydrolysate at pH 7.0. Minimal salts media consisted of 40 mM K_2HPO_4, 20 mM KH_2PO_4, 0.41 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 1.7 mM sodium citrate, and 10 mM sorbitol at pH 7.0.

Covalent Attachment of Polymyxin B to Agarose

Polymyxin B was attached to agarose beads using Affi-Gel 10[11]. Affi-Gel 10 is an agarose derivative containing 10 Å long spacer arms terminated by carboxy-N-hydroxy-succinimide activated esters. Polymyxin B was attached to the agarose through an amide bond between the spacer arm and δ-amino groups of diaminobutyric acid residues in polymyxin B. Affi-Gel 10 was added to a 15 molar excess of polymyxin B dissolved in 10 mM $NaHCO_3$, pH 7.2 and allowed to react for 12 hours at 4°C. The beads were then washed on a column with 1 M NaCl, distilled water, then batch washed with 6 M guanidine-HCl followed by distilled water again. The polymyxin-agarose was dried by successive dehydrations through ethanol, anhydrose methanol and dioxane. The polymyxin-agarose beads were tested for bleed-off of free polymyxin by the methods previously described[11]. Free polymyxin was not released in the presence of *E. coli*.

Determination of Growth Minimum Inhibitory Concentrations

Antimicrobial activities were determined in shaker culture. Prior to innoculation, 0.2 mg/ml polymyxin-agarose was allowed to rehydrate in the media for 2 hours. The flasks were then inoculated with approximately 10^6 cells/ml, allowed to grow for one hour and the second antibiotic, bacitracin or rifampicin (in methanol solution) was added. The concentrations of methanol used had no effect on the growth of the bacteria. After 24 hours incubation, growth of the cultures was determined by light scattering using a Klett spectrophotometer.

Determination of Spheroplast Formation by Polymyxin-Agarose and Lysozyme

A culture of 100 ml *E. coli* SC 9251 was grown to mid-log phase in nutrient media at 37°C, harvested by centrifugation at 4,000 × *g*, washed once with minimal salts and resuspended in 20 ml of the same. For spheroplast lysis determination, 1.5 ml of the cell suspension was added either to 8.5 ml distilled water, distilled water containing 1.0 mg/ml rehydrated polymyxin-agarose, or 20 μg/ml polymyxin B. Lysozyme was added to the appropriate samples and the amount of cell lysis monitored by light scattering using a Klett spectrophotometer.

Spheroplast formation was also detected by the change in morphology of the cells as a result of the above treatments. Photomicrographs were taken of control cells and polymyxin-agarose and lysozyme treated cells suspended in either 20% sucrose or distilled water.

Results

Synergism between Polymyxin-Agarose and Bacitracin or Rifampicin

E. coli SC 9251 was quite sensitive to free polymyxin B (minimum inhibitory concentration of 1.2 μg/ml) but relatively insensitive to either bacitracin or rifampicin (Table 1). Bacitracin at concentrations greater than 200 μg/ml had no effect on the growth of this strain. However, in the presence of 0.2 mg/ml polymyxin-agarose, bacitracin completely inhibited *E. coli* SC 9251 growth at 25 μg/ml. Growth curves illustrating synergism between bacitracin and polymyxin-agarose are given in Fig. 1. At a concentration of 0.2 mg/ml, polymyxin-agarose caused a two-hour growth lag as previously

Fig. 1. The effect of polymyxin-agarose and bacitracin on the growth of *E. coli* SC 9251. Synthetic media was inoculated with 5×10^6 cells/ml at time zero and treated with zero or 200 μg/ml bacitracin, 0.2 mg/ml polymyxin-agarose, or 20 μg/ml bacitracin and 0.2 mg/ml polymyxin-agarose.

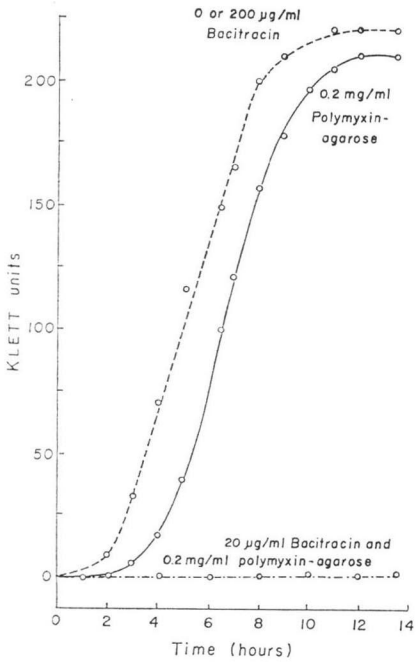


Table 1. Synergism between bacitracin or rifampicin and polymyxin-agarose

Antibiotic	Growth[a]	
	without polymyxin-agarose	with polymyxin-agarose 0.2 mg/ml[b]
No addition	210	210[c]
Bacitracin		
5 μg/ml	200	200
10	210	210
25	208	18
50	210	19
100	210	19
200	210	19
Rifampicin		
5	180	15
10	180	15
25	185	15
50	170	15
100	150	15
200	15	15

a) All cultures inoculated with *E. coli* SC 9251 (5×10^6 cells/ml). Growth of the cultures was monitored after 14 hours by light scattering and reported as Klett units.
b) Dry polymyxin-agarose was allowed to swell for 2 hours in the media prior to innoculation.
c) Growth was preceded by a 2.0-hour lag period.

reported[11] but in a 14-hour period bacterial growth was comparable to untreated controls or cultures treated with 200 μg/ml of bacitracin. As shown in Fig. 1, the combination of polymyxin-agarose and 20 μg/ml of bacitracin completely inhibited *E. coli* growth for at least 14 hours. Thus, the activity of bacitracin was enhanced at least 10 fold in the presence of polymyxin-agarose. Similar results were obtained with rifampicin (Table 1). The minimal inhibitory concentration of rifampicin against *E. coli* SC 9251 was in the range of 100 to 200 μg/ml. In the presence of polymyxin-agarose, rifampicin completely inhibited *E. coli* growth at 5 μg/ml. Underivatized agarose beads or hydrolyzed Affi-Gel 501 did not show synergism with these antibiotics.

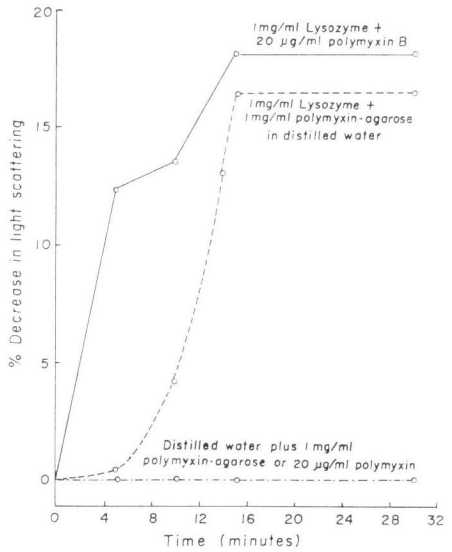
The concentrations of polymyxin-agarose employed in these experiments may seem quite high since free polymyxin shows synergism with various antibiotics at much lower concentrations[4,5]. However, the polymyxin-agarose beads contained approximately 30 μg of covalently attached polymyxin per 0.2 mg of polymyxin-agarose. Furthermore, if it is assumed that the bacteria can only interact with the surface of the beads, then only a minor percentage of the polymyxin was available for interaction with the bacteria. It is not possible to make accurate estimates for the amount of polymyxin B accessible on the outer surface of the beads. However, the beads were approximately 100 μm in diameter and the length of the arm to which polymyxin was attached was 10 Å. It can be estimated from these dimensions that no more than 1% of the bound polymyxin was accessible on the surface.

Spheroplast Formation by Lysozyme in the Presence of Polymyxin-Agarose

Spheroplast formation from *E. coli* by lysozyme requires the presence of EDTA[6]. Metal ion

complexation by EDTA has been shown by LEIVE to result in release of lipopolysaccharide from outer membranes[11]. Lysozyme cannot penetrate through the outer membrane unless its structure is modified by EDTA treatment. TEUBER has demonstrated that spheroplasts can also be formed by lysozyme in the presence of free polymyxin B[7]. It was concluded that polymyxin modified the structure of the outer membranes allowing lysozyme to reach the peptidiglycan layer. Therefore, *E. coli* SC 9251 was treated with lysozyme, polymyxin-agarose, or a combination of lysozyme and polymyxin-agarose and the samples were examined under a phase contrast light microscope. Lysozyme or polymyxin-agarose alone had no effect on *E. coli* morphology or osmotic sensitivity. A combination of the two greatly increased the adhesiveness of the cells resulting in pronounced clumping of bacteria. This is a characteristic property of spheroplasts formed either with EDTA and lysozyme[6] of polymyxin B and lysozyme. Spheroplasts are osmotically fragile and are readily lysed when transferred to distilled water. Lysis can be monitored by a measurable decrease in light scattering. *E. coli* SC 9251 was treated with lysozyme, polymyxin-agarose, lysozyme and polymyxin-agarose or lysozyme and polymyxin B and then suspended in distilled water (Fig. 2). Only the latter two preparations showed a decrease in light scattering when suspended in distilled water. Lysis of bacteria treated with lysozyme and polymyxin-agarose occurred with 10 minutes after transfer into distilled water. The extent of lysis was approximately the same for bacteria treated with free or immobilized polymyxin although the kinetics for lysis were somewhat slower with immobilized polymyxin B.

Fig. 2. Osmotic sensitivity of *E. coli* SC 9251 treated with lysozyme and polymyxin-agarose. *E. coli* SC 9251 prepared as described in methods was suspended in distilled water, distilled water plus 1 mg/ml lysozyme, distilled water plus 1 mg/ml polymyxin-agarose, or distilled water plus 20 μg/ml polymyxin; 1 mg/ml lysozyme plus 1 mg/ml polymyxin-agarose in distilled water; or 1 mg/ml lysozyme plus 20 μg/ml polymyxin B in distilled water.



Discussion

It is well documented that polymyxin B binds to and disrupts the structure of outer membranes[8,9,12]. Evidence supporting this conclusion includes changes in the morphology of cells treated with polymyxin B detected by electron microscopy[8] and the release of periplasmic proteins stimulated by polymyxin B[10]. TEUBER has directly measured binding of radioactive polymyxin B to outer membranes[12] and changes in the structure of isolated outer membranes induced by polymyxin B have been detected by fluorescence techniques[13]. In addition, polymyxin B has been shown to bind quite strongly to lipopolysaccharide[14] and phospholipids[13] which are both important structural elements of outer membranes. However, the outer membrane structural changes caused by polymyxin B have not been defined in detailed molecular terms.

Synergism between polymyxin B and antibiotics or lysozyme has been interpreted in terms of the barrier function of outer membranes and disruption of its structure by the antibiotic[4,5,9]. However, free polymyxin can penetrate into inner domains of the cell including the cytoplasmic membrane.

Therefore, it was not absolutely clear that synergism between polymyxin B and other agents was due to outer membrane structural damage or to indirect effects resulting from interactions with the inner membrane. In this report, we have observed synergism between polymyxin B covalently attached to agarose and bacitracin, rifampicin, or lysozyme. The minimum inhibitory concentrations of bacitracin or rifampicin for *E. coli* SC 9251 were normally in excess of 100 μg/ml. In the presence of immobilized polymyxin, minimum inhibitory concentrations decreased at least ten fold. Synergism between immobilized polymyxin B and lysozyme was also observed. Since the antibiotic was attached to agarose beads by a 10 Å spacer arm, contact was necessarily limited to the outer surface of *E. coli*. These results therefore support the proposal that synergism between polymyxin B and various antibiotics is due to disruption of the outer membrane permeability barrier.

It is interesting to note that free and immobilized polymyxin B share a number of common features. Both forms of the antibiotic appear to disrupt the outer membrane permeability barrier. In addition, free and immobilized polymyxin B inhibit the respiration of gram-negative bacteria and polymyxin resistant *E. coli* strains were also resistant to immobilized polymyxin B[11]. These data, taken collectively, suggest that the primary site of polymyxin action against gram-negative bacteria may actually be at the outer membrane rather than the inner membrane as previously assumed. The results reported in this study clearly support the implied barrier function of outer membranes and illustrate that immobilization of some antibiotics is a powerful tool for studying the structure and function of bacterial membranes. It should be noted that immobilized penicillin has also been used to study interactions between the antibiotic and the D-alanine carboxypeptidase in bacterial membranes[15].

Acknowledgements

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