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Detection of Agents That Alter the Bacterial Cell Surface

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Proteus mirabilis is not killed by polymyxin B, normal serum, or sodium deoxycholate. Exposure to polymyxin B renders the cells susceptible to killing by the latter two agents. The data suggest that this synergism is due to polymyxin B-induced surface changes. The results point out an inadequacy of existing methods of screening for antibiotics; they fail to detect agents which, while showing no in vitro effect on growth, may alter a resistant organism so that it becomes more susceptible to other antimicrobials or host defense mechanisms. A method is described which can be used to detect such cell surface-modifying agents.

Some agents which have little or no effect on the growth of bacteria in vitro can alter the surface properties of the organisms and in so doing alter antimicrobial susceptibility. For example, serum-resistant *Escherichia coli*, when grown in the presence of subinhibitory concentrations of diphenylamine, are killed by exposure to serum (3). Polymyxin B-resistant *Proteus mirabilis*, after a brief exposure to polymyxin B, show marked changes in surface properties; the cells are killed by sodium deoxycholate (DOC) and tris(hydroxymethyl)-aminomethane and become extremely osmotically fragile unlike wild-type ortanisms (10). Recent reports (2, 7, 11) on polymyxin-serum synergy against gram-negative organisms also suggest polymyxin-mediated surface changes.

These data suggest the existence of antimicrobial agents which have little effect on in vitro growth but alter the cell such that its in vivo survival may be affected. Agents acting in this way may prove useful in antibiotic therapy but, unless specifically sought, they will be missed by the usual antibiotic screening methods employing in vitro inhibition of growth.

This paper describes a rapid method of screening for such antibiotics. The surface modifying agent employed is polymyxin B. A serum-resistant *P. mirabilis* is the test organism.

MATERIALS AND METHODS

The origin, maintenance, and cultural conditions, including the growth medium (LB broth) for the *P. mirabilis* strain used, have been described (10). To minimize swarming on solid media, the surface of the agar was well dried before use and the plates were incubated at 30 C. Polymyxin B (Aerosporin, Burroughs Wellcome & Co.) was used at concentration of 20 μg/ml in liquid media and 100 μg/ml in solid media. Serum was obtained and stored as described earlier (3). It was used at a final concentration of 30% in liquid media.

For determining serum sensitivity in liquid media, cultures of growing cells in LB broth were diluted into normal saline buffered with potassium phosphate (0.05 M; pH 7.2). Polymyxin B and/or serum (heated to 56 C for 30 min or unheated) were added as required by the experiment. After incubation for 1 h at 37 C, 0.1 ml of the culture was plated on LB agar plate and the colonies were counted after overnight incubation at 30 C.

For demonstration of the interaction of polymyxin B and serum or polymyxin B and DOC on solid medium, plates of LB agar with and without polymyxin B or DOC were swabbed using a suspension of cells containing 4×10^5 to 5×10^5 cells/ml. The plates were incubated at 37 C for 2 h. One drop of serum or DOC (0.5% solution) was placed on the agar surface and the plates were then placed at 30 C for overnight incubation.

RESULTS

Polymyxin B or serum alone had little effect on the viability of *P. mirabilis*. However, a combination of the two proved to be bactericidal, killing over 97% of the cells (Table 1).

To clarify further the combined effect of polymyxin B and serum, organisms growing in LB broth were exposed to one or the other agent for 30 min. The cells were then diluted 10,000-fold into buffered normal saline. The polymyxin B-treated cells were then exposed to serum and the serum-treated cells exposed to polymyxin B for 1 h at 37 C. The results in Table 2 show that polymyxin B-treated cells were killed by serum, whereas, with the reverse sequence, the viability of the cells was unaffected. Similar results

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were obtained if serum was replaced by DOC (10).

The effect of polymyxin B on the bacteria lasted for several generations even after removal of the antibiotic (Table 3). After exposure to polymyxin B, the culture was diluted 10,000-fold and the cells were exposed to serum at intervals. Even after 4 h (about eight generations) of growth in antibiotic-free (2×10^{-3} μg/ml) medium, the cells were still sensitive to killing by serum. Serum could be replaced by DOC without affecting results (10).

Figure 1 (a and b) shows the results of a method used for demonstrating polymyxin B-serum or polymyxin B-DOC synergy on solid medium. On LB agar after heavy inoculation, growth was confluent even where a drop of serum, heated serum, or 0.5% DOC solution was placed. On LB agar containing polymyxin B, a drop of serum or DOC produced a zone of zero, or very few colonies after similar inoculation; heated serum had no growth inhibitory effect. There was considerable swarming by the organism on LB agar; on polymyxin B-containing agar, however, swarming was completely inhibited. This is another piece of evidence that polymyxin B alters the cell surface.

TABLE 1. *Synergistic action of polymyxin B and normal serum on P. mirabilis*[a]

Treatment	Viable counts/ml × 10^3
None	1.28
Polymyxin B[b]	1.16
Serum[c]	0.94
Polymyxin B and serum	0.03
Polymyxin B and heated serum[c]	1.24

[a] Cells growing in LB broth were diluted into normal saline-potassium phosphate buffer (0.05 M; pH 7.2) to approximately 10^3 cells/ml. After the indicated additions, the tubes were incubated at 37 C for 1 h. Samples (0.1 ml) were then plated on LB agar.
[b] 20 μg/ml.
[c] 30% (final concentration).

TABLE 2. *Sequential action of polymyxin B and normal serum on P. mirabilis*[a]

Treatment		Viable cells/ml × 10^3
First	Second	
Polymyxin		1.7
Polymyxin	Serum	0.02
Serum		2.1
Serum	Polymyxin	1.9

[a] Cells growing in broth were exposed to polymyxin B (20 μg/ml) or serum (30%) for 30 min. After 10,000-fold dilution into buffered normal saline, the polymyxin B-exposed cells were treated with serum and the serum-exposed cells with polymyxin B for 1 h. Samples (0.1 ml) were plated for viable counts.

TABLE 3. *Duration of the effect of polymyxin B on P. mirabilis*[a]

Time (h)	Viable cells/ml		Survival (%)
	Before exposure to serum	After exposure to serum	
0	5.5×10^3	9×10^1	1.7
1	1×10^4		
2	8.5×10^4	1×10^2	0.1
3	2.7×10^5	1.4×10^3	0.5
4	1.3×10^6	3.1×10^4	2.4
5	2.5×10^6	1.6×10^6	64

[a] Cells growing in LB broth were exposed to polymyxin B (20 μg/ml) for 30 min. After 10,000-fold dilution into LB broth (0 h), samples were taken at hourly intervals and viable counts performed before and after exposure to normal serum for 1 h at 37 C.

The synergistic action of polymyxin B and DOC could be reproduced on solid media with the antimicrobials reversed. Bacteria were spread on LB agar with or without DOC (0.5%). A drop of polymyxin B (0.1 mg/ml) was placed on the agar and the plate was incubated. As shown in Fig. 2, no colonies developed on DOC-containing agar where the polymyxin B drop was placed.

DISCUSSION

This and an earlier paper (10) clearly show that an antibiotic, although having little or no effect on the viability or growth of an organism, can markedly alter the properties of the cell. *Proteus* species are resistant to polymyxin B; after addition of high levels of polymyxin B, the cells continue to grow with little or no effect on the rate of growth. The antibiotic, however, modifies the cells such that they develop sensitivity to other agents; the cells are killed by DOC and tris(hydroxymethyl)aminomethane (10) and, as shown here, are killed by serum to which they were previously resistant.

The data show that synergy between polymyxin B and serum is due to polymyxin B-induced changes since the cells are killed by serum only after prior exposure to polymyxin B. The mechanism by which polymyxin B modifies the cells is not known. The fact that treated cells are killed by surface active agents, DOC and tris(hydroxymethyl)aminomethane, and are osmotically fragile (10) suggests that outer aspects of the cell surface are involved. The nature of the bactericidal action of serum on

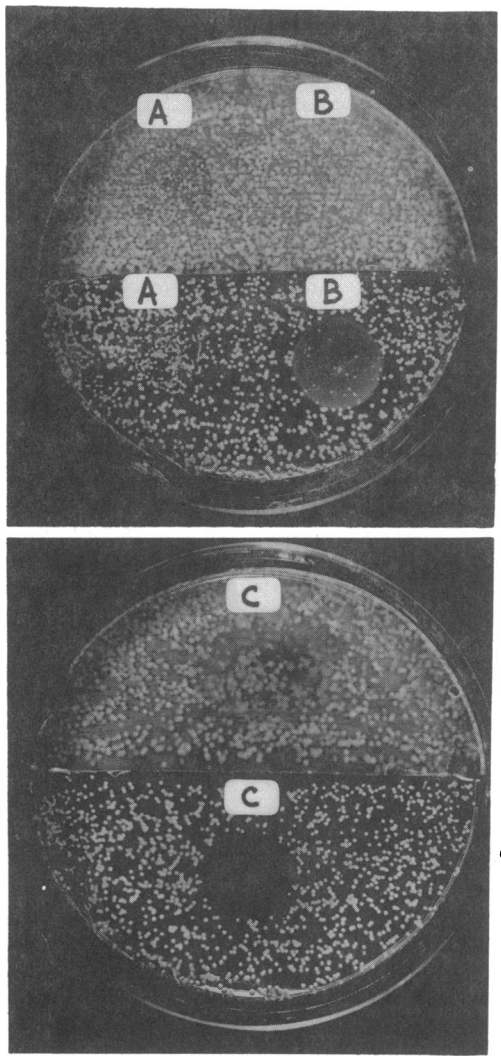

FIG. 1. *Polymyxin B-serum (a) and polymyxin B-DOC (b) synergy against P. mirabilis. Bacteria were spread on LB agar (upper halves of plates) and LB agar with 100 μg of polymyxin B/ml (lower halves of plates). One drop of heated serum (A), unheated serum (B), or 0.5% solution of DOC (C) was placed on agar. Plates were incubated at 30 C overnight.*

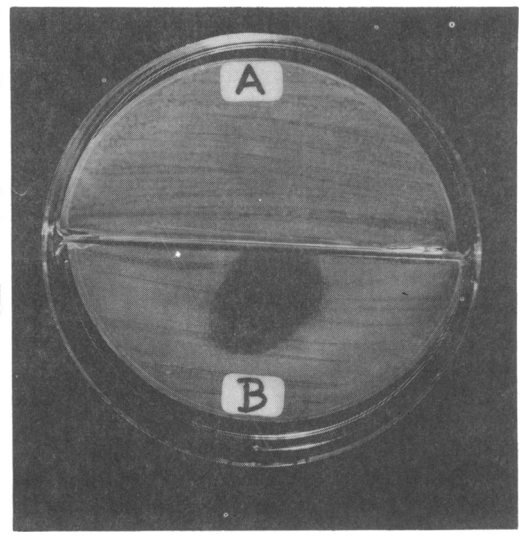

FIG. 2. *Polymyxin B-DOC synergy against P. mirabilis. Bacteria were spread on LB agar (A) and LB agar with 0.5% DOC (B). A drop of polymyxin B solution (0.1 mg/ml) was placed at A and B and the plate was incubated overnight at 30 C.*

sensitive organisms is not clearly understood but it is believed to act by disrupting the envelope of the cell. Since untreated *P. mirabilis* is resistant to DOC, tris(hydroxymethyl)aminomethane, or serum, it appears that polymyxin B-induced surface changes allow these agents to reach their target sites. Polymyxin is bactericidal because of its interaction with phospholipids of the inner bacterial membrane (6). It is also known to react with other biological anions such as ribonucleic acid and lipopolysaccharide (4, 5, 8, 9). Recently (1) formation of a complex between polymyxin and isolated lipopolysaccharide has been demonstrated and lipid A has been implicated as the binding site. It appears likely that such an interaction will alter permeability and structural characteristics of the cell surface, thus possibly explaining the polymyxin B-induced changes referred to earlier.

It has been previously pointed out (3) that antibiotics, which show no in vitro effect on a microorganism, might be of clinical use. Thus polymyxin B could alter a resistant organism in vivo so that the organism may become susceptible to host defense mechanisms. To detect such antimicrobial agents in vitro, modification of the existing screening methods is required. The method described in this study appears suitable for screening for antibiotics which alter the cell surface and, thereby, increase the susceptibility of the bacteria to host defenses or other antibiotics. For example, DOC-resistant organisms can be spread on solid medium with and without DOC and various antimicrobials to be examined can be tested simultaneously by placing drops of antimicrobial solutions on the agar surface. Compounds inhibiting growth only on the DOC-containing plates can then be examined further.

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