

**Effect of Polymyxin B Nonapeptide on Daptomycin Permeability
and Cell Surface Properties in *Pseudomonas aeruginosa*,
Escherichia coli, and *Pasteurella multocida***

CHARLES M. MORRIS^a, ABRAHAM GEORGE^b, W. WILLIAM WILSON^b
and FRANKLIN R. CHAMPLIN^{a,*}

Departments of Biological Sciences^a and Chemistry^b, Mississippi State University,
P.O. Drawer GY, Mississippi State, MS 39762 U.S.A.

(Received for publication August 22, 1994)

The present study was carried out to determine if sensitization of Gram-negative bacteria to the polyanionic antibiotic daptomycin by cationic molecules can be explained on the basis of decreased cell surface charge in order to better understand intrinsic resistance. Turbidimetric assessments of batch cultural growth kinetics revealed the outer membrane permeabilizer polymyxin B nonapeptide sensitized *Pseudomonas aeruginosa* and *Escherichia coli* to the hydrophobic probe novobiocin, whereas little or no sensitization was observed for two surface hydrophobicity variants of *Pasteurella multocida*. Polymyxin B nonapeptide and daptomycin synergistically inhibited growth of *P. aeruginosa* only. A hydrocarbon adherence assay revealed permeabilizing concentrations of polymyxin B nonapeptide increased cell surface hydrophobicity of *P. aeruginosa* and the hydrophobic *P. multocida* variant, while *E. coli* and the hydrophilic *P. multocida* variant remained unaffected. Measurement of cellular electrophoretic mobility showed polymyxin B nonapeptide permeabilization of *P. aeruginosa* to daptomycin occurred concomitantly with a significant decrease in cell surface charge, while no such sensitization occurred in organisms which failed to undergo polymyxin B nonapeptide-induced surface charge decreases. These data suggest that sensitization of Gram-negative bacteria to polyanionic lipopeptides by growth in the presence of polycationic outer membrane permeabilizers such as polymyxin B nonapeptide is dependent on diminution of overall cell surface charge and polarity, thereby allowing outer cell envelope permeation.

*Pseudomonas aeruginosa*¹⁾, *Escherichia coli*²⁾, and *Pasteurella multocida*³⁾ possess typically Gram-negative ultrastructure in that their cytoplasmic membrane and peptidoglycan components are surrounded by an outer membrane which functions in most such organisms as a diffusion barrier to lipophilic molecules⁴⁾. However, we have found that with the exception of the polyanionic lipopeptide daptomycin⁵⁾, the outer membrane of naturally-occurring *P. multocida* strains is permeable to structurally-diverse hydrophobic antimicrobial agents⁶⁾. That the basis for Gram-negative resistance to daptomycin is of a nonspecific nature and involves outer membrane impermeability is strongly suggested by its exclusively Gram-positive antibacterial spectrum⁷⁾, despite possessing a primary mechanism of action which appears to involve the calcium-dependent depletion of cytoplasmic membrane energization⁸⁾ and inhibition of amino acid transport⁹⁾.

We have previously speculated that in view of the general nature of cell envelope permeability to lipophilic molecules in *P. multocida*, intrinsic daptomycin resistance may be due to electrostatic repulsion between the negatively-charged outer membrane surface and the

anionic antibiotic, thereby precluding their association⁶⁾. LAKEY and LEA¹⁰⁾ have suggested the inability of daptomycin analog A21978C to cause hemolysis may be due to the negatively-charged surface of erythrocytes. The assessment of net bacterial cell surface charge can be accomplished in a precise manner by determining cellular electrophoretic mobility¹¹⁾.

CHAMPLIN and HART⁵⁾ found polymyxin B (PMB) disruption of the outer membrane sensitized *P. multocida* P-1581, but not *P. aeruginosa* PAO1 or *P. multocida* ATCC 11039, to daptomycin, thereby suggesting the ability of the outer membrane to exclude daptomycin does not always require normal ultrastructural integrity. Polymyxin B nonapeptide (PMBN) is much less toxic to Gram-negative bacteria than PMB, yet retains the ability to permeabilize outer membranes^{12,13)}. PETERSON *et al.*¹⁴⁾ showed that the polycationic PMB binds to lipopolysaccharide, thereby rigidifying it by altering its overall charge and perturbing the packaging arrangement of outer membrane components. PMBN lacks the PMB fatty acid tail while retaining its electrostatically positive nature. We employed PMBN in an effort to sensitize *P. aeruginosa*, *E. coli*, and *P. multocida* strains to

daptomycin reasoning this compound might mitigate the negative charge of the cell surface while disorganizing the outer membrane such that daptomycin would penetrate in a manner not masked by inherent toxicity of the permeabilizer itself.

Resultant data provide further evidence that intrinsic daptomycin resistance in Gram-negative bacteria is in fact outer membrane mediated and normally intact structure is not essential to the ability of the outer membrane to preclude its permeation. Moreover, sensitization to polyanionic lipopeptides such as daptomycin by polycationic permeabilizers is dependent on the ability of the compounds to sufficiently decrease the net negative electrostatic charge of the outer membrane surface such that antibiotic association with and diffusion through the outer cell envelope can occur.

Materials and Methods

Bacterial Strains and Inocula Preparation

P. aeruginosa PAO1, *E. coli* ATCC 25922, *P. multocida* ATCC 11039 (hydrophilic cell surface variant)¹⁵⁾, and *P. multocida* P-1581 (hydrophobic cell surface variant)¹⁶⁾ were obtained⁶⁾ and maintained under cryoprotective conditions¹⁶⁾ as described elsewhere. Stationary-phase inocula for all experiments were taken from starter cultures consisting of 20 ml of Mueller-Hinton broth (MHB; Difco Laboratories, Detroit, MI) supplemented with Ca^{2+} (50 $\mu\text{g}/\text{ml}$ as CaCl_2 ; MHB-Ca) in 125-ml growth flasks after overnight incubation (15~18 hours) at 37°C with rotary aeration at 180 rpm in a model G24 Environmental Incubator Shaker (New Brunswick Scientific Co. Inc., Edison, NJ). MHB-Ca was prepared by aseptically adding 5 ml of filter-sterilized (0.22- μm Acrodisc disposable filter assemblies; Gelman Sciences, Inc., Ann Arbor, MI) CaCl_2 solution (3.68 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ per 100 ml of H_2O) to one liter of sterile MHB.

MIC Determination

Stock solutions of the outer membrane permeabilizer PMBN (Boehringer Mannheim Biochemicals, Indianapolis, IN), and the antibiotic probes novobiocin sodium salt (control; Sigma Chemical Co., St. Louis, MO) and daptomycin (test; Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, IN) were prepared in MHB-Ca, filter sterilized, and stored at 4°C for no more than seven days. Daptomycin potency was examined periodically by determining the MIC for daptomycin-susceptible *Streptococcus agalactiae* 090 (Dr. S. B. PRUETT, Mississippi State University, Mississippi State, MS). MICs of all permeabilizer and probe molecules were determined using a standard macrobroth dilution method¹⁷⁾ as modified for use in this laboratory¹⁶⁾ with MHB-Ca employed as diluent. Each dilution tube was inoculated with one volume of an early exponential-phase batch

MHB-Ca culture (*ca.* 2.5×10^5 cfu/ml final viable cell density) cultivated as described below.

Experimental Batch Culture Conditions

Batch cultures were grown in 125-ml flasks containing 50 ml of MHB-Ca each (flask: volume ratio of 2.5) after inoculation with stationary-phase starter cultures to an initial optical density of approx. 0.05 at 550 nm (OD_{550} ; Spectronic 20D Optical Spectrophotometer; Milton Roy, Co., Rochester, NY). Cultures were incubated at 37°C with rotary aeration until early exponential phase (OD_{550} of 0.10), at which time 2.5-ml aliquots were dispensed into test culture tubes (18 \times 150 mm; Kimax) containing 2.5 ml of MHB-Ca (control), or MHB-Ca supplemented with sublethal concentrations of PMBN, novobiocin, daptomycin, PMBN plus novobiocin, or PMBN plus daptomycin and incubated as before in order to determine the effect of growth in the presence of PMBN on outer cell surface permeability, hydrophobicity, and electrostatic charge.

Outer Membrane Perturbation Assay

The ability of novobiocin or daptomycin to synergistically inhibit the growth of test organisms in the presence of PMBN was assessed on the basis of turbidimetric growth kinetic measurements using the method of CHAMPLIN and HART⁵⁾.

Cell Surface Hydrophobicity Assay

Cell surface hydrophobic properties of control and PMBN-treated cells were assessed on the basis of the degree to which they partitioned into *n*-hexadecane¹⁸⁾. Cells from late exponential-phase test cultures (OD_{550} of approximately 0.50) were pooled, harvested by centrifugation at $10,000 \times g$ and 4°C for 10 minutes, washed with one volume of cold PPMS buffer (6.97 g of K_2HPO_4 , 2.99 g of KH_2PO_4 and 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ per liter of glass-distilled water; pH 7.12), and suspended in room temperature PPMS buffer to an OD_{550} of 0.50. Cell surface hydrophobicity was measured as described previously¹⁶⁾.

Cell Surface Charge Assay

Cell surface electrostatic charge properties of control and PMBN-treated cells were assessed by measuring electrophoretic mobility of cultures in terms of zeta potential¹¹⁾ as determined on the basis of light scattering analysis using a Coulter DELSA 440 (Coulter Corporation, Hialeah, FL). Late exponential-phase test cultures (OD_{550} of *ca.* 0.50) were placed in an ice bath until ready for analysis.

Statistical Analysis

Numerical data were analyzed for statistical significance by employing the Student *t* test and the two tailed table to determine P values. P values less than 0.01 and 0.05 indicate differences between means are statistically significant at the 99% and 95% confidence

levels, respectively.

Results

Susceptibility to Outer Membrane Permeabilizers and Antibiotic Probes

The MICs of permeabilizer and probe molecules for all test organisms can be seen in Table 1. The greater than ten-fold decrease in MIC exhibited by daptomycin for *S. agalactiae* in MHB-Ca²⁺ confirms the Ca²⁺ dependency of its mechanism of action reported elsewhere for various Gram-positive bacteria^{7,8}. The decision was made to incorporate Ca²⁺ in all experimental media in order to optimize the bacteriocidal properties of daptomycin, thereby increasing the sensitivity of the cell envelope permeability assay. Data obtained using the hydrophobic cell surface variant *P. multocida* P-1581 reveal that, unlike the parent compound PMB, PMBN is nonlethal in the presence of added Ca²⁺. All test organisms were clearly resistant to both PMBN and daptomycin. In contrast, only *E. coli* ATCC 25922 and *P. aeruginosa* PAO1 were resistant to novobiocin, while *P. multocida* strains ATCC 11039 and P-1581 were susceptible as previously reported⁶.

Effect of PMBN on Outer Membrane Permeability Properties

We have previously employed turbidimetric assessments of cultural growth kinetics to determine that PMB is able to nonspecifically permeabilize the outer membrane of *P. multocida* P-1581, thereby sensitizing it to daptomycin in MHB⁵. A similar degree of PMB-induced sensitization to daptomycin was obtained more

recently in MHB containing Ca²⁺ at the concentration employed for the present study (unpublished data). The nonbacteriocidal PMBN¹³ was selected for subsequent use as a permeabilizing agent under these conditions after it was found to render *P. aeruginosa* PAO1 susceptible to novobiocin, while poly-L-lysine, EDTA, and diaminoacetone exhibited only slight to moderate synergy with novobiocin under these conditions¹⁹.

Control experiments were performed to establish the degree to which PMBN is able to nonspecifically permeabilize the outer membranes of test organisms as judged on the basis of sensitization to the normally excluded hydrophobic antibiotic novobiocin. As can be seen in Fig. 1, growth in the presence of PMBN significantly sensitized *P. aeruginosa* PAO1 and *E. coli* ATCC 11039 to novobiocin, while little or no synergy was observed in the *P. multocida* variants. It should be noted that growth of the *P. multocida* variants was consistently less in the presence of both PMBN and

Fig. 1. Growth of test organisms in the presence of PMBN and/or NOV. Each value represents the mean of three to six independent determinations.

(A) *P. aeruginosa* PAO1; Symbols: ○, control; ●, PMBN (10 μg/ml); □, NOV (256 μg/ml); and ■, PMBN (10 μg/ml) and NOV (256 μg/ml). (B) *E. coli* ATCC 25922; Symbols: ○, control; ●, PMBN (8 μg/ml); □, NOV (4 μg/ml); and ■, PMBN (8 μg/ml) and NOV (4 μg/ml). (C) *P. multocida* ATCC 11039; Symbols: ○, control; ●, PMBN (128 μg/ml); □, NOV (1 μg/ml); and ■, PMBN (128 μg/ml) and NOV (1 μg/ml). (D) *P. multocida* P-1581; Symbols: ○, control; ●, PMBN (10 μg/ml); □, NOV (0.5 μg/ml); and ■, PMBN (10 μg/ml) and NOV (0.5 μg/ml).

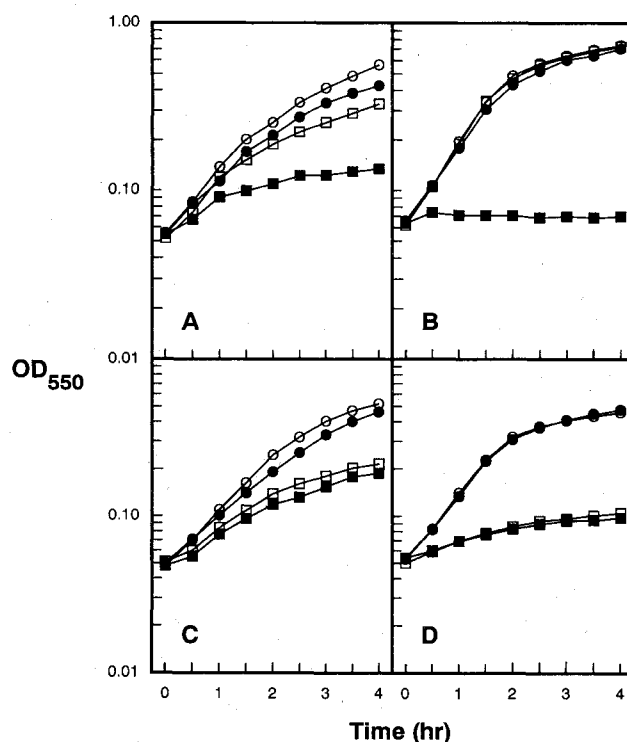


Table 1. Susceptibility of organisms to polymyxin B, polymyxin B nonapeptide, novobiocin, and daptomycin as determined by broth dilution assay in MHB-Ca.

Organism	MIC (μg/ml) ^a			
	PMB	PMBN	NOV	DAP
<i>P. aeruginosa</i> PAO1	ND	>256.0	>256.0	>256.0
<i>E. coli</i> ATCC 25922	ND	>256.0	128.0	>256.0
<i>P. multocida</i> ATCC 11039	ND	>256.0	4.0	>256.0
<i>P. multocida</i> P-1581	2.0	>256.0	2.0	>256.0
<i>S. agalactiae</i> 090	ND	ND	ND	0.25 (8.0) ^b

^a Each value was obtained from at least three replicate two-fold serial dilutions using a macrobroth dilution assay in MHB supplemented with Ca²⁺ (50 μg/ml). Abbreviations: PMB, polymyxin B; PMBN, polymyxin B nonapeptide; NOV, novobiocin; DAP, daptomycin; ND, not determined.

^b Assay performed in MHB without Ca²⁺ supplementation.

novobiocin than that seen with novobiocin alone in each of up to six independent determinations. It therefore appears likely that a slight, yet reproducible, potentiation of novobiocin growth inhibition does occur for this organism in the presence of PMBN. Data in Fig. 2 reveal PMBN also sensitized *P. aeruginosa* PAO1 to

daptomycin, while all other organisms remained unaffected.

Cell Surface Hydrophobicity

The relative abilities of control and PMBN-treated cells to associate with *n*-hexadecane were examined in order to determine if PMBN-induced permeabilization is accompanied by a concomitant change in overall cell surface hydrophobicity. Growth in the presence of PMBN at concentrations employed in attempts to permeabilize outer membranes to daptomycin caused significant increases in cell surface hydrophobicity of *P. aeruginosa* PAO1 and the hydrophobic *P. multocida* variant P-1581 (Table 2), even though only the former organism was sensitized to the antibiotic (Fig. 2). No significant differences in hydrophobicity properties were detected between treated and untreated *E. coli* ATCC 29522 and *P. multocida* ATCC 11039.

Cell Surface Charge

In order to determine the effect of PMBN-induced permeabilization on cell surface charge, zeta potential values reflective of the degree of net surface electronegativity were determined for test organisms grown in the absence and presence of the permeabilizer. *P. aeruginosa* PAO1 underwent a significant decrease in overall negative cell surface charge as a result of being cultured in the presence of PMBN (Table 2). In contrast, PMBN exerted no significant effect on the cell surface charge properties of *E. coli* ATCC 29522, *P. multocida* ATCC 11039, or *P. multocida* P-1581.

Discussion

The exclusively Gram-positive antibacterial spectrum of daptomycin suggests the intrinsic resistance exhibited by Gram-negative organisms is nonspecific and most likely involves outer membrane impermeability. Failure of the outer membrane to function as a barrier to the

Fig. 2. Growth of test organisms in the presence of PMBN and/or DAP. Each value represents the mean of three to four independent determinations.

(A) *P. aeruginosa* PAO1; Symbols: ○, control; ●, PMBN (10 µg/ml); □, DAP (400 µg/ml); and ■, PMBN (10 µg/ml) and DAP (400 µg/ml). (B) *E. coli* ATCC 25922; Symbols: ○, control; ●, PMBN (8 µg/ml); □, DAP (400 µg/ml); and ■, PMBN (8 µg/ml) and DAP (400 µg/ml). (C) *P. multocida* ATCC 11039; Symbols: ○, control; ●, PMBN (128 µg/ml); □, DAP (400 µg/ml); and ■, PMBN (128 µg/ml) and DAP (400 µg/ml). (D) *P. multocida* P-1581; Symbols: ○, control; ●, PMBN (10 µg/ml); □, DAP (400 µg/ml); and ■, PMBN (10 µg/ml) and DAP (400 µg/ml).

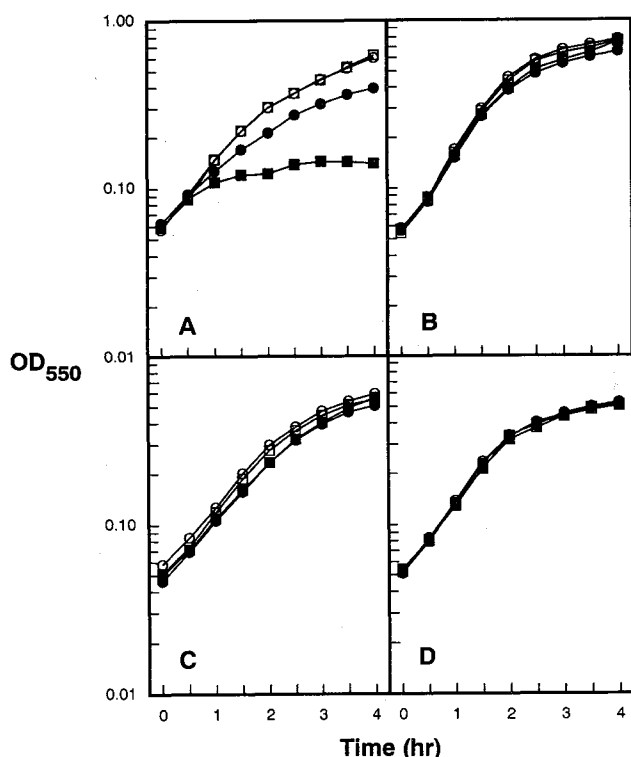


Table 2. Effect of PMBN permeabilization on cell surface hydrophobicity and surface charge.

Organism	Percent adherence to hexadecane ^a			Zeta potential ^b		
	Control	Treated ^c	P value	Control	Treated ^c	P value
<i>P. aeruginosa</i> PAO1	-0.68 ± 0.38	8.12 ± 0.68	<0.05	-24.50 ± 1.82	-13.18 ± 2.03	<0.01
<i>E. coli</i> ATCC 25922	2.30 ± 1.45	0.60 ± 1.93	>0.05	-30.15 ± 4.35	-30.36 ± 4.74	>0.01
<i>P. multocida</i> ATCC 11039	-0.69 ± 0.49	0.35 ± 0.67	>0.05	-26.65 ± 5.85	-22.91 ± 7.67	>0.01
<i>P. multocida</i> P-1581	30.43 ± 4.77	58.20 ± 5.24	<0.05	-10.07 ± 4.31	-9.34 ± 8.57	>0.01

^a Percent decrease in turbidity of standardized cell suspension after mixing with 1,000 µl of *n*-hexadecane. Each value represents the mean of three-to-four independent determinations.

^b Zeta potential of standardized cell suspension expressed in mV. Each value represents the mean of four-to-six independent determinations.

^c Cells grown in the presence of PMBN at the concentrations used in attempts to sensitize respective organisms to NOV and DAP.

diffusion of antimicrobial agents can be examined by sensitization of bacteria to otherwise excluded molecules as assessed on the basis of turbidimetric measurements of altered cultural growth kinetics^{5,20}. CHAMPLIN and HART⁵) employed this method to determine the outer membranes of *P. aeruginosa* PAO1, *P. multocida* ATCC 11039 (a hydrophilic cell surface variant), and *P. multocida* P-1581 (a hydrophobic cell surface variant) were rendered permeable to the hydrophobic antibiotic novobiocin by growth in the presence of sublethal concentrations of either EDTA or PMB. Furthermore, PMB sensitized *P. multocida* P-1581 to daptomycin. The failure to sensitize *P. aeruginosa* PAO1 and *P. multocida* ATCC 11039 to daptomycin under conditions wherein their outer membranes were otherwise permeabilized to novobiocin suggests the ability of the outer cell envelope to retard daptomycin diffusion does not require normally intact structure. That *P. multocida* hydrophobic variant P-1581 was rendered susceptible to daptomycin by PMB under conditions which sensitized all organisms to novobiocin supports the hypothesis that intrinsic daptomycin resistance is a result of the permeability barrier function of the outer membrane. Furthermore, otherwise permeabilized outer membrane surfaces may retain sufficient residual negative charge to preclude association with the polyanionic daptomycin due to electrostatic repulsion in certain cases. PMB treatment caused no detectable changes in cell envelope lipid composition or cell surface charge (as judged using electrostatic interaction chromatography) of the *P. multocida* strains, thereby leaving strain variation with regard to susceptibility to PMB-daptomycin synergy unexplained. However, this study was hampered by the facts that permeabilizing concentrations of PMB are very close to bacteriocidal levels¹³) and a much more reliable method for measuring cell surface charge is available¹¹).

The net negative surface charge of Gram-negative bacteria is due in large part to the presence of lipopolysaccharide in the outer leaflet of the outer membrane²¹). The negative surface properties of *P. multocida* serotype A variants, as represented in this study by strain ATCC 11039, are most likely enhanced by capsular material which is composed largely of hyaluronic acid²²). The relative cell surface hydrophobicity of *P. multocida* variants has been shown to be related to the degree to which they are encapsulated and the hyaluronidase removal of capsular material is accompanied by a concomitant enhancement of hydrophobic properties¹⁵).

We reasoned it should then be possible to sensitize *P. aeruginosa* and *E. coli* (both having outer membranes refractory to hydrophobic molecules), and further sensitize *P. multocida* (which has an outer membrane permeable to hydrophobic molecules) to daptomycin by exposing them to the nonbacteriocidal outer membrane permeabilizer PMBN in the presence of added Ca^{2+} . Different amounts of synergy between PMBN and novobiocin for the various organisms indicate that PMBN compromises the ability of their respective outer

membranes to function as permeability barriers to lipophilic molecules to different degrees. That *P. aeruginosa* PAO1 was rendered susceptible to daptomycin by PMBN-induced outer membrane permeabilization is strongly supportive of the hypothesis that intrinsic daptomycin resistance in Gram-negative bacteria is a function of the outer membrane.

The inability of PMBN to sensitize the remaining organisms to daptomycin, while also failing to decrease their respective cell surface electronegativity, supports the hypothesis that sensitization by PMB and PMBN is dependent on their abilities to obviate electrostatic repulsion between the cell surface and the antibiotic. The increase in cell surface hydrophobicity which occurred concomitantly with the PMBN-induced permeabilization of the *P. aeruginosa* PAO1 outer membrane to daptomycin is consistent with a lessening of cell surface charge as the result of PMBN binding to the negatively-charged cell surface. That PMBN sensitization of *P. aeruginosa* PAO1 occurred concomitantly with a dramatic decrease in cell surface charge relative to control cells, whereas no sensitization occurred for organisms wherein PMBN failed to affect the overall cell surface charge, confirms the notion that the residual negative charge remaining on otherwise permeabilized outer membranes may preclude daptomycin permeation due to electrostatic repulsion.

The failure of PMBN at relatively high concentrations to cause significant permeabilization of the *P. multocida* strains to novobiocin, while significant permeabilization of *E. coli* was obtained at a concentration comparable to that used by VAARA and VAARA¹³) to permeabilize *Salmonella typhimurium*, suggests that PMBN is not an effective outer membrane permeabilizer for certain non-enteric organisms, particularly in the presence of elevated divalent cation levels. This conclusion is supported by the failure of larger concentrations of PMBN (51.2 $\mu\text{g/ml}$) to markedly further sensitize *Zymomonas mobilis* to novobiocin (unpublished data). Alternatively, the inherent susceptibility of naturally-occurring strains of *P. multocida* and *Z. mobilis* to hydrophobic molecules may simply mask further permeabilization of the outer membrane by PMBN.

It is interesting to note that hydrophobic *P. multocida* variant P-1581 was rendered susceptible to daptomycin with PMB⁵) and more hydrophobic by PMBN (Table 2), yet did not become less electronegative (Table 2) or susceptible to daptomycin (Fig. 2) as a consequence of growth in the presence of PMBN. Efforts are presently underway to determine if the presence of serotype A encapsulation, which covers an otherwise hydrophobic outer surface in *P. multocida*¹⁵) might be responsible for strain variation with regard to susceptibility to the effects of PMB and PMBN on cell surface properties.

Acknowledgments

This study was supported by a research grant to F.R.C. from

Lilly Research Laboratories. We are grateful to Lilly Research Laboratories for providing daptomycin, Ms. M. B. HUGHLETT for assistance with cell surface hydrophobicity assays, and Dr. T. R. SHRYOCK for critically reviewing the manuscript.

References

- 1) GILLELAND, H. E., Jr. & L. B. FARLEY: Adaptive resistance to polymyxin in *Pseudomonas aeruginosa* due to outer membrane impermeability mechanism. *Can. J. Microbiol.* 28: 830~840, 1982
- 2) LEIVE, L.; S. TELESITSKY, W. G. COLEMAN, Jr. & D. CARR: Tetracyclines of various hydrophobicities as a probe for permeability of *Escherichia coli* outer membranes. *Antimicrob. Agents Chemother.* 25: 539~544, 1984
- 3) BROGDEN, K. A. & R. B. RIMLER: Lysates of turkey-grown *Pasteurella multocida*: examination of vaccine preparations by electron microscopy. *Am. J. Vet. Res.* 43: 304~309, 1982
- 4) NIKAIDO, H.: Outer membrane of *Salmonella typhimurium*: transmembrane diffusion of some hydrophobic substances. *Biochim. Biophys. Acta.* 433: 118~132, 1976
- 5) CHAMPLIN, F. R. & M. E. HART: Cell envelope impermeability to daptomycin in *Pseudomonas aeruginosa* and *Pasteurella multocida*. *Curr. Microbiol.* 21: 367~372, 1990
- 6) HART, M. E. & F. R. CHAMPLIN: Susceptibility to hydrophobic molecules and phospholipid composition in *Pasteurella multocida* and *Actinobacillus lignieresii*. *Antimicrob. Agents Chemother.* 32: 1354~1359, 1988
- 7) ELIOPOULOS, G. M.; S. WILLEY, E. REISZNER, P. G. SPITZER, G. CAPUTO & R. C. MOELLERING, Jr.: In vitro and in vivo activity of LY146032, a new cyclic lipopeptide antibiotic. *Antimicrob. Agents Chemother.* 30: 532~535, 1986
- 8) ALBORN, W. E., Jr.; N. E. ALLEN & D. A. PRESTON: Daptomycin disrupts membrane potential in growing *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 35: 2282~2287, 1991
- 9) ALLEN, N. E.; W. E. ALBORN, Jr. & J. S. HOBBS, Jr.: Inhibition of membrane potential-dependent amino acid transport by daptomycin. *Antimicrob. Agents Chemother.* 35: 2639~2642, 1991
- 10) LAKEY, J. H. & E. J. A. LEA: The role of acyl chain character and other determinants on the bilayer activity of A21978C an acidic lipopeptide antibiotic. *Biochim. Biophys. Acta.* 859: 219~226, 1986
- 11) VAN DER MEI, H. C.; A. J. LÉONARD, A. H. WEERKAMP, P. G. ROUXHET & H. J. BUSSCHER: Surface properties of *Streptococcus salivarius* HB and nonfibrillar mutants: measurement of zeta potential and elemental composition with X-ray photoelectron spectroscopy. *J. Bacteriol.* 170: 2462~2466, 1988
- 12) OHEMENG, K. A.; C. F. SCHWENDER, K. P. FU & J. F. BARRETT: DNA gyrase inhibitory and antibacterial activity of some flavones. *Bioorg. Med. Chem. Lett.* 3: 225~230, 1993
- 13) VAARA, M. & T. VAARA: Polycations sensitize enteric bacteria to antibiotics. *Antimicrob. Agents Chemother.* 24: 107~113, 1983
- 14) PETERSON, A. A.; R. E. W. HANCOCK & E. J. MCGROARTY: Binding of polycationic antibiotics and polyamines to lipopolysaccharides of *Pseudomonas aeruginosa*. *J. Bacteriol.* 164: 1256~1261, 1985
- 15) THIES, K. L. & F. R. CHAMPLIN: Compositional factors influencing cell surface hydrophobicity of *Pasteurella multocida* variants. *Curr. Microbiol.* 18: 385~390, 1989
- 16) DARNELL, K. R.; M. E. HART & F. R. CHAMPLIN: Variability of cell surface hydrophobicity among *Pasteurella multocida* somatic serotype and *Actinobacillus lignieresii* strains. *J. Clin. Microbiol.* 25: 67~71, 1987
- 17) FINEGOLD, S. M. & W. J. MARTIN: Determination of susceptibility of bacteria to antimicrobial agents; assay of antimicrobial agents. In *Bailey and Scott's Diagnostic Microbiology*, 6th ed. pp. 532~557, The C. V. Mosby Co., St. Louis, 1982
- 18) ROSENBERG, M.; D. GUTNICK & E. ROSENBERG: Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol. Lett.* 9: 29~33, 1980
- 19) WATT, J. M.; C. M. MORRIS, A. GEORGE, W. W. WILSON & F. R. CHAMPLIN: Effect of polymyxin B nonapeptide on cell surface charge of permeabilized gram-negative bacteria. *Abstracts of Papers of Annu. Meet. South Central Branch Am. Soc. Microbiol.*, No. 42, p. 18, 1993
- 20) TSUCHIDO, T. & M. TAKANO: Sensitization by heat treatment of *Escherichia coli* K-12 cells to hydrophobic antibacterial compounds. *Antimicrob. Agents Chemother.* 32: 1680~1683, 1988
- 21) STENDAHL, O.; L. EDEBO, K.-E. MAGNUSSON, C. TAGESSON & S. HJERTÉN: Surface-charge characteristics of smooth and rough *Salmonella typhimurium* bacteria determined by aqueous two-phase partitioning and free zone electrophoresis. *Acta Path. Microbiol. Scand. Sect. B.* 85: 334~340, 1977
- 22) CARTER, G. R. & S. W. RUNDELL: Identification of type A strains of *Pasteurella multocida* using staphylococcal hyaluronidase. *Vet. Rec.* 96: 343, 1975