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Outer Membrane Permeability Barrier to Azithromycin, Clarithromycin, and Roxithromycin in Gram-Negative Enteric Bacteria

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Mutations which severely affect the function of the outer membrane of *Escherichia coli* and *Salmonella typhimurium* (*lpxA* and *firA* mutations of lipid A synthesis and *rfaE* mutation of the lipopolysaccharide inner-core synthesis) were found to decrease the MICs of erythromycin, roxithromycin, clarithromycin, and azithromycin by factors of 32 to 512, 32 to 1,024, 64 to 512, and 16 to 64, respectively. The sensitization factors for three other hydrophobic antibiotics (rifampin, fusidic acid, and mupirocin) ranged from 16 to 300. The outer membrane permeability-increasing agents polymyxin B nonapeptide (3 μg/ml) and deacylpolymyxin B (1 μg/ml) sensitized wild-type *E. coli* to azithromycin by factors of 10 and 30, respectively. Quantitatively very similar sensitization to the other macrolides took place. Polymyxin-resistant *pmrA* mutants of *S. typhimurium* displayed no cross-resistance to azithromycin. *Proteus mirabilis* mutants which were sensitized to polymyxin by a factor of ≥300 to ≥1,000 had a maximal two- to fourfold increase in sensitivity to azithromycin. These results indicate that azithromycin and the other new macrolides use the hydrophobic pathway across the outer membrane and that the intact outer membrane is an effective barrier against them. Furthermore, the results indicate that azithromycin, in contrast to polymyxin, does not effectively diffuse through the outer membrane by interacting electrostatically with the lipopolysaccharide.

The outer membrane (OM) of gram-negative bacteria acts as a relatively effective permeability barrier against all hydrophobic antibiotics, including erythromycin (8, 9). Therefore, erythromycin has only very limited use in the treatment of gram-negative infections. On the other hand, the newer macrolides clarithromycin and azithromycin have increased activity against several gram-negative bacterial species (4, 7, 10). This could suggest that they penetrate the OM better than erythromycin. The present communication assesses the role of the OM as a resistance factor to these antibiotics by using (i) bacterial mutants which have drastically altered OM permeability barrier function (mutants supersusceptible to hydrophobic antibiotics and mutants resistant or supersusceptible to polycations) as well as (ii) agents which permeabilize the OM.

OM-defective, antibiotic-supersusceptible mutants included those which produce a heptose-deficient lipopolysaccharide (LPS) (strain D21f2 of *Escherichia coli* and strain SL1102 of *Salmonella typhimurium* [17, 18]) and those which are thermosensitive for growth and defective in the synthesis of the lipid A component of the LPS (the *firA* mutants of *E. coli* and *S. typhimurium* as well as the *lpxA* mutant of *E. coli* [3, 16, 17]). The MICs were determined by broth microdilution method in L broth as described previously (16). Erythromycin base, rifampin, and fusidic acid (Na salt) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Roxithromycin, clarithromycin, azithromycin, and mupirocin were kind gifts from Roussel Uclaf (Paris, France), Abbott (Queensborough, Kent, United Kingdom), Pfizer (Groton, Conn.), and SmithKline Beecham (Worthing, West Sussex, United Kingdom), respectively.

All the mutants proved to be extremely susceptible to the macrolides tested (Table 1). A very low azithromycin MIC (0.06 μg/ml) was recorded for four of the five mutants studied; in all these cases, the mutation decreased the MIC of azithromycin by a factor of 64. In the fifth mutant, the corresponding sensitization factor was 16. Even larger factors were occasionally observed for other macrolides. The most susceptible permeability mutant (*lpxA2*) was sensitized to roxithromycin, erythromycin, and clarithromycin by factors of 1,024, 512, and 512, respectively. Regression analysis of the results of Table 1 revealed that the MICs of erythromycin correlated well with those of other macrolides (r^2 values of 0.99, 0.98, and 0.91 with roxithromycin, clarithromycin, and azithromycin, respectively).

Furthermore, the susceptibility of the OM permeability mutants to all macrolides paralleled very closely their susceptibility to other hydrophobic antibiotics, such as rifampin, fusidic acid, and mupirocin (Table 1). The correlation of fusidic acid MICs with those of each macrolide was good (r^2 values of 0.89, 0.86, 0.94, and 0.86 with erythromycin, roxithromycin, clarithromycin, and azithromycin, respectively). These results suggest that azithromycin and the other new macrolides use the hydrophobic pathway across the OM, as does erythromycin and the other hydrophobic antibiotics. In other words, the intact OM, where this pathway is largely closed, is an effective barrier against all these macrolides.

Next, the effect of two potent OM permeability-increasing agents, polymyxin B nonapeptide (PMBN, lot F7; Farmos Group Ltd., Turku, Finland) and deacylpolymyxin B (DAPB; a kind gift from Yukio Kimura, Mukogawa Women's University, Nishinomiya, Japan), was tested. Both are known to sensitize at low concentrations (0.3 to 3 μg/ml)

TABLE 1. Susceptibility of OM-defective mutants to macrolides and to other hydrophobic antibiotics

Organism and strain	Relevant genotype	MIC[a] (μg/ml) of:				Fold increase[b] in susceptibility to:						
		Erythromycin	Roxithromycin	Clarithromycin	Azithromycin	Erythromycin	Roxithromycin	Clarithromycin	Azithromycin	Rifampin	Fusidic acid	Mupirocin
E. coli												
SM105	Wild type	64	128	32	4							
SM101	*lpxA*	0.125	0.125	0.06	0.06	512	1,024	512	64	64	128	30
CDH23-213	*firA*	0.5	1	0.25	0.06	128	128	128	64	128	64	30
D21f2	*rfa*[c]	1	2	0.5	0.06	64	64	64	64	64	64	30
S. typhimurium												
SH5014	Wild type	64	128	64	4							
SH7622	*firA*	2	4	1	0.25	32	32	64	16	132	256	100
SL1102	*rfaE*[c]	1	2	1	0.06	64	64	64	64	16	256	300

[a] MICs were determined at 28°C for SM101 and CDH23-213 and at 37°C for the other strains in L broth.
[b] Values are the approximate ratio between the MIC for the wild-type control strain and that for the mutant strain.
[c] Encodes heptose-deficient LPS.

wild-type enteric bacteria to erythromycin and other hydrophobic antibiotics by factors of 10 to 1,000 (12), and as shown in Table 2, they sensitized the wild-type *E. coli* (IH3080 [14]) to each of the new macrolides as well. PMBN (3 μg/ml) and DAPB (1 μg/ml) decreased the MICs of azithromycin by factors of 10 and 30, respectively. Quantitatively very similar sensitization to roxithromycin and clarithromycin as well as to the control (erythromycin) also took place. These results give further credence to the view that the OM markedly limits the penetration of azithromycin.

Why then is azithromycin more active than erythromycin against bacteria such as *E. coli* and *S. typhimurium*? Azithromycin hardly diffuses significantly better than erythromycin through the unspecific and hydrophilic porin channels (1), even though this has not yet been tested. On the other hand, erythromycin, roxithromycin, and clarithromycin are monobasic whereas azithromycin is a dication. Since for *E. coli* SC9252 (the polymyxin-resistant variant described by Meyers et al. [6] the polymyxin B MIC is 64-fold higher and the azithromycin MIC is 4-fold higher but the erythromycin MIC is only 2-fold higher than the MICs for its parent strain, it has been suggested (1, 2) that azithromycin interacts with the acidic LPS and penetrates the OM by virtue of this interaction, in analogy to the pentacationic antibiotics of the polymyxin family.

The hypothesis was reevaluated by studying the azithromycin susceptibility of the well-characterized polymyxin-resistant mutants of *S. typhimurium*, mutated at the *pmrA* locus. These mutants are resistant to a number of cationic agents because of an alteration in their LPS which makes the LPS less anionic (for a review, see reference 12). Polymyxin resistance was found to reduce the susceptibility to azithromycin very little, if at all (Table 3). Whereas the polymyxin MICs (polymyxin B sulfate; Sigma) for both *pmrA* mutants were 30-fold higher than the MICs for their parent strains, the azithromycin MICs were not higher (SH6497) or only twofold higher (SH7426). Also, polymyxin-susceptible mutants of the deep rough *Proteus mirabilis* R45 strain (LPS chemotype Re, i.e., heptose-deficient LPS) were included in this study (Table 3). Even though wild-type *P. mirabilis* is known to be very resistant to azithromycin (7, 10), heptose-deficient R45 was susceptible. Its mutants, which were sensitized to polymyxin by a factor of ≥300 to 1,000, displayed a maximal two- to fourfold increase in susceptibility to azithromycin and a twofold increase in susceptibility to erythromycin (Table 3). When all the results shown in Table 3 were combined, the difference between the MICs of erythromycin and azithromycin was 16- to 32-fold for all polymyxin-resistant strains and 32-fold for all polymyxin-susceptible strains. This could suggest that the enhanced activity of azithromycin over that of erythromycin may not be mediated by any putative electrostatic binding to LPS.

TABLE 2. MICs of macrolides for wild-type *E. coli* (IH3080) in the absence and presence of OM permeability-increasing agents[a]

Macrolide	MIC (μg/ml) with OM permeability-increasing agent[b]:		
	None	PMBN	DAPB
Erythromycin	>100	10	1
Roxithromycin	>100	10	3
Clarithromycin	30	3	0.3
Azithromycin	10	1	0.3

[a] As determined by a synergistic 18-h growth inhibition assay (15) in L broth.
[b] PMBN (3 μg/ml); DAPB (1 μg/ml).

TABLE 3. MICs of azithromycin and erythromycin for the polymyxin-resistant *S. typhimurium* mutants and polymyxin-susceptible *P. mirabilis* mutants

Strain	Relevant characteristic(s)	Reference	MIC (μg/ml) of:		
			Polymyxin B	Azithromycin	Erythromycin
S. typhimurium SH6482	*pmrA*$^+$	11	0.3	4	128
S. typhimurium SH9178	*pmrA*$^+$	11	0.3	2	64
S. typhimurium SH6497	*pmrA125*	11	10	2	64
S. typhimurium SH7426	*pmrA163*	11	10	4	64
P. mirabilis R45	*rfa*	5	≥300	4	64
P. mirabilis IH1301	*rfa*, PM-S[a]	13	0.3	2	64
P. mirabilis IH1304	*rfa*, PM-S	13	1	1	32

[a] PM-S, polymyxin-susceptible mutant of *P. mirabilis* R45.

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REFERENCES

1. **Farmer, S., Z. S. Li, and R. E. W. Hancock.** 1992. Influence of outer membrane mutations on susceptibility of *Escherichia coli* to the dibasic macrolide azithromycin. J. Antimicrob. Chemother. **29:**27–33.
2. **Hancock, R. E. W.** 1991. Bacterial outer membranes: evolving concepts. Specific structures provide gram-negative bacteria with several unique advantages. ASM News **57:**175–182.
3. **Hirvas, L., P. Koski, and M. Vaara.** 1991. Identification and sequence analysis of the gene mutated in the conditionally lethal outer membrane permeability mutant SS-C of *Salmonella typhimurium*. EMBO J. **10:**1017–1023.
4. **Kirst, H. A., and G. D. Sides.** 1989. New directions for macrolide antibiotics: structural modifications and in vitro activity. Antimicrob. Agents Chemother. **33:**1413–1418.
5. **Kotelko, K.** 1986. Proteus mirabilis: taxonomic position, peculiarities of growth, components of the cell envelope. Curr. Top. Microbiol. Immunol. **129:**181–215.
6. **Meyers, E., W. L. Parker, and W. E. Brown.** 1974. EM49: a new polypeptide antibiotic active against cell membranes. Ann. N.Y. Acad. Sci. **235:**493–501.
7. **Neu, H. C., N. X. Chin, G. Saha, and P. Labthavikul.** 1988. Comparative in vitro activity of the new oral macrolide azithromycin. Eur. J. Clin. Microbiol. Infect. Dis. **7:**541–544.
8. **Nikaido, H., and M. Vaara.** 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **49:**1–32.
9. **Nikaido, H., and M. Vaara.** 1987. Outer membrane, p. 7–22. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
10. **Retsema, J., A. Girard, W. Schelkly, M. Manousos, M. Anderson, G. Bright, R. Borovoy, L. Brennan, and R. Mason.** 1987. Spectrum and mode of action of azithromycin (CP-62,993), a new 15-membered-ring macrolide with improved potency against gram-negative organisms. Antimicrob. Agents Chemother. **31:** 1939–1947.
11. **Vaara, M.** 1981. Increased outer membrane resistance to ethylenediaminetetraacetate and cations in novel lipid A mutants. J. Bacteriol. **148:**426–434.
12. **Vaara, M.** 1992. Agents that increase the permeability of the outer membrane. Microbiol. Rev. **56:**395–411.
13. **Vaara, M.** Unpublished data.
14. **Vaara, M., P. Viljanen, T. Vaara, and P. H. Mäkelä.** 1984. An outer membrane-disorganizing peptide PMBN sensitizes *E. coli* strains to serum bactericidal action. J. Immunol. **132:**2582–2589.
15. **Viljanen, P., H. Matsunaga, Y. Kimura, and M. Vaara.** 1991. The outer membrane permeability-increasing action of deacylpolymyxins. J. Antibiot. **44:**517–523.
16. **Vuorio, R., and M. Vaara.** 1992. The lipid A biosynthesis mutation *lpxA2* of *Escherichia coli* results in drastic antibiotic supersusceptibility. Antimicrob. Agents Chemother. **36:**826–829.
17. **Vuorio, R., and M. Vaara.** 1992. Mutants carrying lethal mutations in outer membrane genes *omsA* and *firA* (*ssc*) are phenotypically similar, and *omsA* is allelic to *firA*. J. Bacteriol. **174:**7090–7097.
18. **Wilkinson, R. G., P. Gemski, Jr., and B. A. D. Stocker.** 1972. Non-smooth mutants of *Salmonella typhimurium*: differentiation by phage sensitivity and genetic mapping. J. Gen. Microbiol. **70:**527–554.