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Macrolide Antibiotic-Mediated Downregulation of MexAB-OprM Efflux Pump Expression in *Pseudomonas aeruginosa*▽

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Macrolide antibiotics modulate the quorum-sensing system of *Pseudomonas aeruginosa*. We tested the effect of macrolide antibiotics on the cell density-dependent expression of the MexAB-OprM efflux pump and found that 1.0 μg/ml (MIC/6.25) of azithromycin suppressed the expression of MexAB-OprM by about 70%, with the result that the cells became two- to fourfold more susceptible to antibiotics such as aztreonam, tetracycline, carbenicillin, chloramphenicol, and novobiocin.

Long-term administration of a sub-MIC level of macrolide antibiotics has been used empirically to treat chronic respiratory infection caused by *Pseudomonas aeruginosa* (8, 13–15), though *P. aeruginosa* is intrinsically resistant to macrolides. The observation was interpreted to mean that macrolides perturb the production of cell density-dependent virulence factors via the quorum-sensing (Q-S) circuits (12, 27, 29, 30). The Q-S circuits in *P. aeruginosa* are composed of the *las* and *rhl* systems, which regulate the production of virulence factors, e.g., elastase and protease and biofilm formation (3, 9, 21, 30).

The *P. aeruginosa* genome carries several xenobiotic efflux pumps, and among them, MexAB-OprM plays a major role in antibiotic extrusion and resistance (5, 6, 19, 20, 24). The MexAB-OprM pump consists of the subunits MexB, OprM, and MexA (19, 20, 24), and their roles are the substrate-recognizing energy-transmitting subunit (7, 10, 18), the adapter protein (2, 6, 11) connecting MexB and OprM, and the antibiotics discharge duct protein (1, 19, 24), respectively. Thus, antibiotics are trapped by MexB, transferred to the OprM cavity, and eventually discharged to the external milieu with the aid of MexA (1, 2, 7, 11, 18). Expression of MexAB-OprM increases in a cell density-dependent manner, suggesting that expression may be linked to the Q-S circuit(s) (16, 22, 31). However, it is not known if low-level macrolide affects the expression of MexAB-OprM and, if so, how it influences antibiotic susceptibility of the cells. Thus, we investigated this issue in this study.

Relevant properties of the *P. aeruginosa* strains used are as follows: PAO4290 is a laboratory prototype strain (32); TNP090 is a derivative of PAO4290 that carries a chromosomal *mexB*::*xylE* fusion construct producing catechol 2,3-dioxygenase (Cat-2,3-diO) in place of MexB (16); TNP091, TNP092, and TNP093 are the derivatives of TNP090, which lack *lasI* and *rhlI* and both *lasI* and *rhlI*, respectively (16). TNP030 carries a mutation in *mexR*, producing an elevated level of MexAB-OprM (25). TNP077 lacks chromosomal *mexAB-oprM* (17). The amount of MexAB-OprM expressed was quantified as the reporter enzyme activity of Cat-2,3-diO. An overnight culture of the TNP090 strain (*las*$^+$ *rhl*$^+$) was diluted 100,000-fold (or 10,000-fold) with fresh Luria-Bertani broth containing 1 μg/ml of azithromycin (AZM) or without the drug, adjusting the A_{600} to 3×10^{-5} (or 3×10^{-4}). The flasks were rotated at 200 rpm at 37°C for the desired period of time. Cells were harvested by centrifugation at 5,000 × *g* for 15 min. Cat-2,3-diO activity was determined, as reported earlier (28), in the cell extracts, prepared by passage through a French pressure cell at 130 MPa three times and obtaining the supernatant from centrifugation at 13,000 × *g* for 20 min. One unit was defined as 1 nmol of substrate hydrolysis per min per mg of protein. Macrolide antibiotics did not interfere with the Cat-2,3-diO assay in the concentration range used. MICs of antibiotics were determined by the agar dilution method using Mueller-Hinton agar in the presence and absence of 1.0 μg/ml of AZM, which is 1/6.25 the MIC of AZM (MIC/6.25), in the respective strains. The cell number was adjusted to either 5×10^3 cells or 150 CFU/5 μl/spot.

TNP090 carrying *mexB*::*xylE* showed a low-level MIC of AZM at 6.25 μg/ml, due to impaired MexAB-OprM; otherwise, the level was 100 μg/ml. Thus, most experiments were conducted in the presence and absence of MIC/6.25 (1.0 μg/ml) of AZM, as determined by the agar dilution method with 5×10^3 cells. Overnight precultures grown without AZM expressed 95.2 and 82.1 units of Cat-2,3-diO (Fig. 1). These precultures were diluted with a 100,000-fold volume of fresh medium (calculated by an A_{600} of 3×10^{-5}) to minimize the carryover of MexAB-OprM-positive cells, and the cells were grown for 4.5 h for drug-free culture and 5.5 h for AZM-containing culture to reach an A_{600} of 0.025. Cat-2,3-diO activity was 44.3 and 30.9 units for cultures in the presence and absence of AZM, respectively. The enzyme activity in cells without drug was 66.5, 167.0, and 179.6 units at 4.5 plus 2 h, plus 4 h, and plus 6 h, respectively. The results clearly showed

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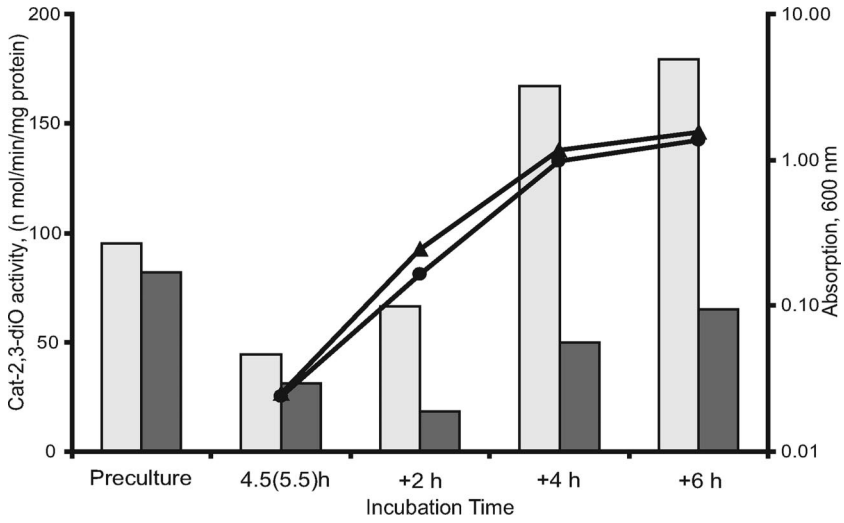

FIG. 1. Effect of sub-MIC level of azithromycin on the growth-phase-dependent expression of MexAB-OprM. An overnight culture of the TNP090 (*las*$^+$ *rhl*$^+$) mutant was diluted 100,000-fold as mentioned in the text, and the flasks were rotated at 200 rpm at 37°C for the desired period of time. Cells in the drug-free culture were harvested as the A_{600} reached ~0.03 in about 4.5 h and at 5.5 h for the 1-μg/ml AZM culture. Similarly, the culture was harvested at 4.5 (or 5.5) plus 2 h, plus 4 h, and plus 6 h. The amount of MexAB-OprM expressed was quantified as the activity of the reporter enzyme, Cat-2,3-diO. The values shown are representative results of repeated experiments. Gray columns, Cat-2,3-diO in the drug-free culture; dark-gray columns, Cat-2,3-diO in the 1-μg/ml AZM culture; filled triangle, $A_{600}{}^{1\text{ cm}}$ of the drug-free culture; filled circle, $A_{600}{}^{1\text{ cm}}$ of the 1-μg/ml AZM culture.

that the MexAB-OprM transcriptional level increased in a cell density-dependent manner. Production of Cat-2,3-diO in the presence of 1.0 μg/ml of AZM at 5.5 h plus 2 h, plus 4 h, and plus 6 h of culture was −30.9, −73, −70.2, and −63%, respectively, relative to the values in the drug-free culture. These results clearly demonstrated that a MIC/6.25 of AZM suppressed the production of the MexAB-OprM pump. Similar experiments were conducted using the TNP091, TNP092, and TNP093 cells lacking *lasI* and *rhlI* and both *lasI* and *rhlI*, respectively, at 9 h of culture, which is equivalent to an A_{600} of ~1.5 or early stationary growth phase, and their Cat-2,3-diO activity levels appeared to be 99, 90, and 100%, respectively, of those in TNP090 (data not shown), suggesting that macrolide-mediated suppression of MexAB-OprM expression is unlikely to rely on the LasI and LasR circuits.

Since the sub-MIC level of AZM caused low-level expression of MexAB-OprM, it is conceivable that the cells grown in the presence of low-level AZM became more susceptible to the pump-substrate antibiotics than the cells grown in the AZM-free culture. Thus, we determined the MIC of several antibiotics in the presence of 16 μg/ml (MIC/6.25) of AZM in the PAO4290 cells producing the wild-type level of MexAB-OprM. The MICs of chloramphenicol, aztreonam, tetracycline, carbenicillin, and novobiocin in plates containing 16 μg/ml of AZM were 1/4, 1/2, 1/4, 1/4, and 1/4, respectively, of that in the drug-free culture (Table 1). The MIC of imipenem, which is not the substrate of the MexAB-OprM efflux pump, appeared the same in the presence and absence of AZM, as expected. The MICs of ceftazidime and ofloxacin, which are poor substrates for the MexAB-OprM pump (32), were indistinguishable in the presence and absence of AZM. AZM exerted no significant effect on the MICs of antibiotics in the mutant

TABLE 1. MICs of antibiotics in the presence and absence of AZM[a]

Strain	Genotype	Antibiotic MIC (μg/ml)[b]							
		CHL	ATM	TET	CAR	NOV	IPM	CAZ[c]	OFX[c]
PAO4290 without AZM	*leu-10 argF10 aph-9004 FP*	25	3.12	12.5	50	800	0.78	1.56	0.78
PAO4290 with AZM		6.25	1.56	3.13	12.5	200	0.78	1.56	0.78
TNP030 without AZM	*mexR* derivative of PAO4290	200	12.5	50	200	>1200	0.78	3.13	>1.56
TNP030 with AZM		200	12.5	50	200	>1200	0.78	3.13	>1.56
TNP077 without AZM	Δ*mexA* Δ*mexB* Δ*oprM* derivative of PAO4290	0.39	0.1	0.39	0.78	12.5	0.78	0.39	0.05
TNP077 with AZM		0.39	0.1	0.39	1.56	12.5	0.78	0.39	0.05

[a] MICs of various antibiotics were determined by the agar dilution method using Mueller-Hinton agar in the presence and absence of 1.0 μg/ml of AZM. The cell number was adjusted to 150 CFU/5 μl/spot.

[b] CHL, chloramphenicol; ATM, aztreonam; IPM, imipenem; TET, tetracycline; CAR, carbenicillin; NOV, novobiocin; CAZ, ceftazidime; OFX, ofloxacin.

[c] MICs of CAZ and OFX in the Δ*mexA* and Δ*mexB* strains are only twofold lower than in the strain producing a wild-type level of MexAB-OprM expression, and that in the Δ*oprM* strain is four- to eightfold lower (32). This gap is due to the fact that OprM collaborates not only with MexAB but also with other efflux pump subunits such as MexCD and MexXY.

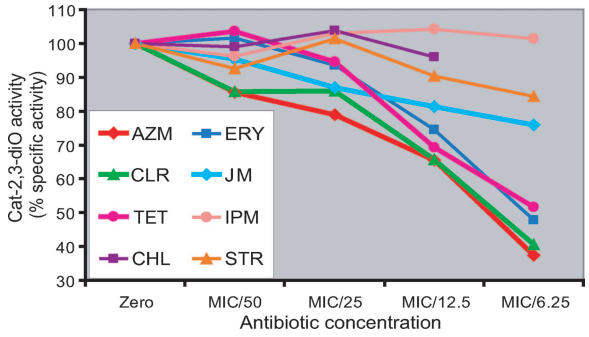

FIG. 2. Effect of various antibiotics at sub-MIC levels on the expression of MexAB-OprM. Precultures of the TNP090 cells grown overnight were diluted 10,000-fold with 300 ml of fresh medium, and the flasks were rotated at 200 rpm for 9 h (until A_{600} of ~1.5, which is early stationary growth phase) at 37°C. Preparation of cell extracts and the assay for the Cat-2,3-diO activity were similar to that described in the text. The Cat-2,3-diO activity was expressed relative to that in the drug-free culture. Antibiotic concentration was expressed relative to the MIC of the respective antibiotics in TNP090. The cells grew poorly in the presence of MIC/6.25 of tetracycline (TET). The values shown are representative results of repeated experiments. AZM, 6.25 μg/ml; CLR, clarithromycin (12.5 μg/ml); TET, 0.78 μg/ml; CHL, chloramphenicol (1.56 μg/ml); ERY, erythromycin (12.5 μg/ml); JM, josamycin (200 μg/ml); IPM, imipenem (0.78 μg/ml); STR, streptomycin (0.78 μg/ml).

lacking MexAB-OprM, TNP077, as expected. The MICs of antibiotics in the *mexR* mutant TNP030, which overproduces MexAB-OprM, were identical in cultures grown in the presence and absence of AZM. The reason for this is not known.

The effects of several antibiotics at MIC/50 through MIC/6.25 was examined. The Cat-2,3-diO activities in the presence of 0.125, 0.25, 0.5, and 1 μg/ml of AZM were lowered retrogressively to 85, 78, 65, and 37% compared with that of the respective drug-free cultures (Fig. 2). The results demonstrated that AZM suppressed the production of MexAB-OprM in a concentration-dependent manner. Analogous experiments were carried out using the 14-membered macrolides erythromycin and clarithromycin and confirmed that these macrolides exerted an effect similar to that of AZM. A 16-membered macrolide, josamycin, showed only a marginal effect. Tetracycline at 0.016, 0.03, 0.06, and 0.125 μg/ml led to Cat-2,3-diO production at levels of 103, 94, 69 and 51%, respectively. On the other hand, streptomycin and chloramphenicol showed only a marginal effect on the Cat-2,3-diO production. Imipenem exerted no detectable effect.

An unanswered question is whether macrolide-mediated suppression of the MexAB-OprM expression is linked to the Q-S system(s). This study revealed that at least the Las and Rhl systems are unlikely to be involved in the macrolide-mediated suppression of MexAB-OprM. On the other hand, the suppression was canceled in the *mexR* mutant. Our earlier study reported that the expression of *mexAB-oprM* was upregulated by the Q-S mediator C4-homoserine lactone (26). Therefore, the macrolide-mediated downregulation of MexAB-OprM expression is different from the Rhl-mediated upregulation; yet, the possible involvement of the Q-S system could not be entirely ruled out because uncharacterized Q-S systems, such as quinolone quorum sensing, may exist (4, 23). Though low-level macrolide antibiotics might have directly inhibited protein synthesis and consequently lowered the production of MexAB-OprM, this action is less likely because chloramphenicol and streptomycin, both acting on protein synthesis machinery, exerted only a little effect. Further studies may be needed to clarify this issue.

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REFERENCES

1. **Akama, H., M. Kanemaki, M. Yoshimura, T. Tsukihara, T. Kashiwagi, H. Yoneyama, S. Narita, A. Nakagawa, and T. Nakae.** 2004. Crystal structure of the drug discharge outer membrane protein, OprM, of *Pseudomonas aeruginosa*. J. Biol. Chem. **279:**52816–52819.
2. **Akama, H., T. Matuura, S. Kashiwagi, H. Yoneyama, S. Narita, T. Tsukihara, A. Nakagawa, and T. Nakae.** 2004. Crystal structure of the membrane fusion protein, MexA, of the multidrug transporter in *Pseudomonas aeruginosa*. J. Biol. Chem. **279:**25939–25942.
3. **Brooun, A., S. Liu, and K. Lewis.** 2000. A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilm. Antimicrob. Agents Chemother. **44:**640–646.
4. **Calfee, M.-W., J. P. Coleman, and E.-C. Pesci.** 2001. Interference with Pseudomonas quinolone signal synthesis inhibits virulence factor expression by *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **98:**11633–11637.
5. **Daigle, D. M., L. Cao, S. Fraud, M. S. Wilke, A. Pacey, R. Klinoski, N. C. Strynadka, C. R. Dean, and K. Poole.** 2007. Protein modulator of multidrug efflux gene expression in *Pseudomonas aeruginosa*. J. Bacteriol. **189:**5441–5451.
6. **Dinh, T., I. T. Paulsen, and M. H. Saier, Jr.** 1994. A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of gram-negative bacteria. J. Bacteriol. **176:**3825–3831.
7. **Eda, S., H. Maseda, and T. Nakae.** 2003. An elegant means of self-protection in Gram-negative bacteria by recognizing and extruding xenobiotics from the periplasmic space. J. Biol. Chem. **278:**2085–2088.
8. **Equi, A., I. M. Balfour-Lynn, A. Bush, and M. Rosenthal.** 2002. Long term azithromycin in children with cystic fibrosis: a randomised placebo-controlled crossover trial. Lancet **360:**978–984.
9. **Favre-Bonte, S., T. Kohler, and C. van Delden.** 2003. Biofilm formation by *Pseudomonas aeruginosa*: role of the C4-HSL cell-to-cell signal and inhibition by azithromycin. J. Antimicrob. Chemother. **52:**598–604.
10. **Guan, L., M. Ehrmann, H. Yoneyama, and T. Nakae.** 1999. Membrane topology of the xenobiotic-exporting subunit, MexB, of the MexA,B-OprM extrusion pump in *Pseudomonas aeruginosa*. J. Biol. Chem. **274:**10517–10522.
11. **Higgins, M. K., E. Bokma, E. Koronakis, C. Hughes, and V. Koronakis.** 2004. Structure of the periplasmic component of a bacterial drug efflux pump. Proc. Natl. Acad. Sci. USA **101:**9994–9999.
12. **Howe, R. A., and R. C. Spencer.** 1997. Macrolides for the treatment of *Pseudomonas aeruginosa* infections? J. Antimicrob. Chemother. **40:**153–155.
13. **Jaffe, A., J. Francis, M. Rosenthal, and A. Bush.** 1998. Long-term azithromycin may improve lung function in children with cystic fibrosis. Lancet **351:**420.
14. **Kudoh, S., H. Kimura, K. Uetake, et al.** 1984. Clinical effect of low-dose long-term administration of macrolides on diffuse panbronchiolitis. Jpn. J. Thorac. Dis. **22:**254. (In Japanese.)
15. **Kudoh, S., T. Uetake, K. Hagiwara, et al.** 1987. Clinical effects of low-dose longterm erythromycin chemotherapy on diffuse panbronchiolitis. Nippon Kyobu Shikkan Gakkai Zasshi **25:**632–642. (In Japanese.)
16. **Maseda, H., I. Sawada, K. Saito, H. Uchiyama, T. Nakae, and N. Nomura.** 2004. Enhancement of the MexAB-OprM efflux pump expression by a quorum-sensing autoinducer and its cancellation by a regulator, MexT, of the MexEF-OprN efflux pump operon in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **48:**1320–1328.
17. **Maseda, H., H. Yoneyama, and T. Nakae.** 2000. Assignment of the substrate-selective subunits of the MexEF-OprN multidrug efflux pump of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **44:**658–664.
18. **Murakami, S., R. Nakashima, E. Yamashita, T. Matsumoto, and A. Yamaguchi.** 2006. Crystal structures of a multidrug transporter reveal a functionally rotating mechanism. Nature **2006:**173–179.
19. **Nakae, T., E. Yoshihara, and H. Yoneyama.** 1997. Multiantibiotic resistance caused by active drug extrusion in hospital pathogens. J. Infect. Chemother. **3:**173–183.
20. **Nikaido, H.** 1996. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science **264:**221–229.
21. **Nouwens, A. S., S. A. Beatson, C. B. Whitchurch, B. J. Walsh, H. P. Schweizer, J. S. Mattick, and S. J. Cordwell.** 2003. Proteome analysis of extracellular proteins regulated by the las and rhl quorum sensing systems in *Pseudomonas aeruginosa* PAO1. Microbiology **149:**1311–1322.
22. **Pearson, J. P., C. van Delden, and B. H. Iglewski.** 1999. Active efflux and

diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signal. J. Bacteriol. **181:**1203–1210.
23. **Pesci, E. C., J. B. J. Milbank, J. P. Pearson, S. Mcknight, A. S. Kende, E. P. Greenberg, and B. H. Iglewski.** 1999. Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **96:**11229–11234.
24. **Poole, K.** 2001. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. J. Mol. Microbiol. Biotechnol. **3:**255–263.
25. **Saito, K., H. Yoneyama, and T. Nakae.** 1999. nalB-type mutations causing the overexpression of the MexAB-OprM efflux pump are located in the *mexR* gene of the *Pseudomonas aeruginosa* chromosome. FEMS Microbiol. Lett. **179:**67–72.
26. **Sawada, I., H. Maseda, T. Nakae, H. Uchiyama, and N. Nomura.** 2004. A quorum-sensing autoinducer enhances the mexAB-oprM efflux-pump expression without the MexR-mediated regulation in *Pseudomonas aeruginosa*. Microbiol. Immunol. **48:**435–439.
27. **Schuster, M., C. P. Lostroh, T. Ogi, and E. P. Greenberg.** 2003. Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. J. Bacteriol. **185:**2066–2079.
28. **Schweizer, H. P.** 1993. Two plasmids, X1918 and Z1918, for easy recovery of the xelE and lacZ reporter genes. Gene **134:**89–91.
29. **Sofer, D., N. Gilboa-Garber, A. Belz, and N. C. Garber.** 1999. Subinhibitory erythromycin represses production of *Pseudomonas aeruginosa* lectins, autoinducer and virulence factors. Chemotherapy **45:**335–341.
30. **Tateda, K., T. J. Standiford, J. C. Pechere, and K. Yamaguchi.** 2004. Regulatory effects of macrolides on bacterial virulence: potential role as quorum-sensing inhibitors. Curr. Pharm. Design. **10:**3055–3065.
31. **Whitehead, N. A., A. M. Bernard, H. Slater, N. J. Simpson, and G. P. Salmond.** 2001. Quorum-sensing in Gram-negative bacteria. FEMS Microbiol. Lett. **25:**365–404.
32. **Yoneyama, H., A. Ocaktan, M. Tsuda, and T. Nakae.** 1997. The role of *mex*-gene products in antibiotic extrusion in *Pseudomonas aeruginosa*. Biochem. Biophys. Res. Commun. **233:**611–618.