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Fluorescence Assay for Studying the Ability of Macrolides To Induce Production of Ribosomal Methylase

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A screening assay to test the inducing capacity of macrolides by fusing the attenuator of the inducible *erm*(B) gene from *Streptococcus pneumoniae* HM28 with the *gfpmut1* gene has been designed. Fluorescence was detected under UV light around disks impregnated with inducer macrolides (erythromycin or azithromycin) but not with noninducer ketolides. Induction could be quantified by fluorometry.

Resistance to macrolide-lincosamide-streptogramin B antibiotics defining the so-called MLS phenotype is common in streptococci and enterococci (11). It is mediated by dimethylation of adenine 2058 in the ribosomal 23S rRNA target, which reduces the affinity between the antibiotic and the ribosome (17). The genes that encode 23S rRNA methylases are designated *erm* (erythromycin resistance methylase). In streptococci and enterococci, MLS resistance is generally encoded by genes belonging to the *erm*(B) group (13). Expression of MLS resistance may be inducible or constitutive, depending upon a regulatory region preceding the gene (10, 18). In contrast to the pattern of inducer macrolides for the staphylococcal gene *erm*(C), which is limited to 14- and 15-member ring macrolides, *erm*(B) is inducible by most members of the MLS group (10). Recently, a new class of macrolides, the ketolides, which are derivatives of clarithromycin or erythromycin A characterized by a 3-keto function instead of the cladinose moiety and an 11- or 12-carbamate extension, has been shown to be active against most streptococci resistant to erythromycin (1, 3, 7, 8; C. Agouridas, Y. Benedetti, A. Denis, O. Le Martret, and J. F. Chantot, Abstr. 35th Intersci. Conf. Antimicrob. Agents Chemother., abstr. F157, 1995). This activity was attributed, in part, to the lack of induction of MLS resistance by these antimicrobials (2, 3, 5, 19). We have fused the inducible attenuator of *erm*(B) with the green fluorescent protein reporter gene to provide a new fluorescence assay to easily detect the inducing capacity of macrolides and related antimicrobials.

Gene fusions. We have amplified by PCR a 741-bp fragment from plasmid pAT505 composed of the entire *gfpmut1* gene except the initiation codon (9) using oligonucleotides modified by insertion of *Sma*I and *Pst*I restriction sites (underlined): GFP1 (5′-GGA GAT ATC CCC GGG GGT AAA GGA GAA G-3′) and GFP2 (5′-GCA TGC CTG CAGTTA TTT GTA CAA TT-3′). The PCR product was digested with *Sma*I and *Pst*I, cloned in pUC18, and introduced into *Escherichia coli* DH10B cells by electrotransformation. A 384-bp fragment comprising the attenuator and the first 54 nucleotides of the *erm*(B) gene of *Streptococcus pneumoniae* HM28 inducibly resistant to MLS antibiotics (14) was amplified by PCR using oligonucleotides modified to include restriction sites *Eco*RI and *Sma*I (underlined): ermB1 (5′-CTT AGA AGA ATT C TT AAG AGT GTG-3′) and ermB2 (5′-TTA TTA TTT GCC CGG GTA CCT TTT C-3′). The amplification product was digested with the appropriate enzymes and cloned upstream from the truncated *gfpmut1* gene in pUC18. The fusion was electrotransformed into *E. coli* DH10B and subcloned in the *Eco*RI and *Sph*I restriction sites of the shuttle multicopy vector pAT28 (which confers spectinomycin resistance) (16) to generate plasmid pUV4. The hybrid plasmids were introduced by electrotransformation into *Staphylococcus aureus* RN4220. The transformants were plated onto media containing 180 μg of spectinomycin/ml and an inducing concentration of erythromycin (0.03 μg/ml). The fluorescent colonies were identified by UV (at 385 nm).

A constitutively expressed *gfpmut1* gene was also constructed by fusion of the *gfpmut1* gene with a 179-bp sequence upstream from the constitutive *erm*(B) gene of *Enterococcus faecalis* BM4110/pAMβ1 amplified by PCR with the ermB1 and ermB2 primers. The amplified fragment lacked the leader peptide sequence (12). The recombinant plasmid, pUV5, was electrotransformed into *S. aureus* RN4220 and used as a positive control.

MICs and induction experiments. MICs of antibiotics were determined by agar dilution using Mueller-Hinton medium (Bio-Rad, Marnes-la-Coquette, France) supplemented with 5% horse blood (4). The following antibiotics were provided by their manufacturers: telithromycin, RU 69874, and the 2-fluoroketolides HMR 3562 and HMR 3787 (Aventis, Romainville, France) (6). RU 69874 is structurally similar to telithromycin except for a replacement of the 3-keto function by a L-cladinose sugar. Erythromycin, spectinomycin, and amoxicillin were from Sigma-Aldrich (St. Quentin Fallavier, France), and azithromycin was from Pfizer (Orsay, France). Induction of resistance by antibiotics was further assessed by growth curves of uninduced or induced cells (at a concentration equal to 1/10 the MIC of the macrolides) in the presence or absence of challenging (just subinhibitory) concentrations of antibiotic as described earlier (15). Bacterial growth was followed by CO_2 production measured with the BacT/Alert 3D system (Organon Teknika Corp., Oklahoma City, Okla.). Growth curves

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TABLE 1. Induction of MLS resistance assessed by growth curve measurements against strains used in fusion experiments[c]

Strain	Antibiotic	MIC (μg/ml)	Lag phase time (in h) for	
			Uninduced culture	Induced culture (inducing concen [μg/ml])
S. pneumoniae HM28[a]	Erythromycin	256	22.3	11.5 (25)
	Telithromycin	0.03	13.2	13.7 (0.003)
	RU69874	2	43.9	20.8 (0.2)
E. faecalis BM4110/pAMβ1[b]	Erythromycin	8,000	9	8.2 (800)
	Telithromycin	32	22.5	22.1 (3.2)
	RU69874	16	11.8	11.7 (1.6)

[a] Challenge concentrations were 50, 0.006, and 0.4 μg/ml for erythromycin, telithromycin, and RU69874, respectively.
[b] Challenge concentrations were 1,600, 6.4, and 3.2 μg/ml for erythromycin, telithromycin, and RU69874, respectively.
[c] Results are means of two experiments.

were plotted and lag phases were calculated. All experiments were conducted twice.

Fluorescence induction assays. Fluorescence induction was detected qualitatively by the disk diffusion method (4). Disks impregnated with 15 μg of the various macrolides and ketolides were placed on inoculated blood agar plates which were incubated for 24 h at 37°C and observed under UV light. For quantitative assays, overnight *S. aureus* RN4220/pUV4 or *S. aureus* RN4220/pUV5 cultures grown in Trypticase soy broth were used to inoculate fresh medium at a dilution of 1: 25. After 3 h of incubation at 37°C under aeration, cells were added to Trypticase soy broth at 10^6 CFU/ml, containing increasing concentrations of antibiotics, and incubated for 1 h at 37°C. The cultures were then washed three times with phosphate-buffered saline, and cells were resuspended in phosphate-buffered saline at 10^6 CFU/ml. The fluorescence intensity was determined by spectrofluorometry performed with a Hitachi model F-1200 fluorescence spectrophotometer (Hitachi Co., Tokyo, Japan) at an excitation wavelength of 460 nm and an emission wavelength of 510 nm.

MLS resistance phenotypes. The MICs of and induction of resistance by erythromycin, telithromycin, and RU 69874 were determined for *S. pneumoniae* HM28 and *E. faecalis* BM4110/pAMβ1 (Table 1). Preinduced cells of *S. pneumoniae* HM28 challenged with erythromycin had a lag phase that was shortened, confirming that MLS resistance was inducible by this antimicrobial. As expected, expression of erythromycin resistance in *E. faecalis* BM4110/pAMβ1 was constitutive. Telithromycin did not appear to be an inducer for MLS resistance in *S. pneumoniae* HM28; in contrast, RU 69874 did reduce the lag phase, confirming the role of the L-cladinose residue in induction. Telithromycin was active only against the inducible strain *S. pneumoniae* HM28.

Induction of fluorescence by macrolides. The ability of erythromycin and ketolides to induce fluorescence was studied with fusion constructs. After exposure to UV light, agar plates spread with *S. aureus* RN4220/pUV4 exhibited fluorescence localized at the border of inhibition zones for disks containing erythromycin and spiramycin. Fluorescence was barely visible with telithromycin and HMR 3787; however, HMR 3562 in-

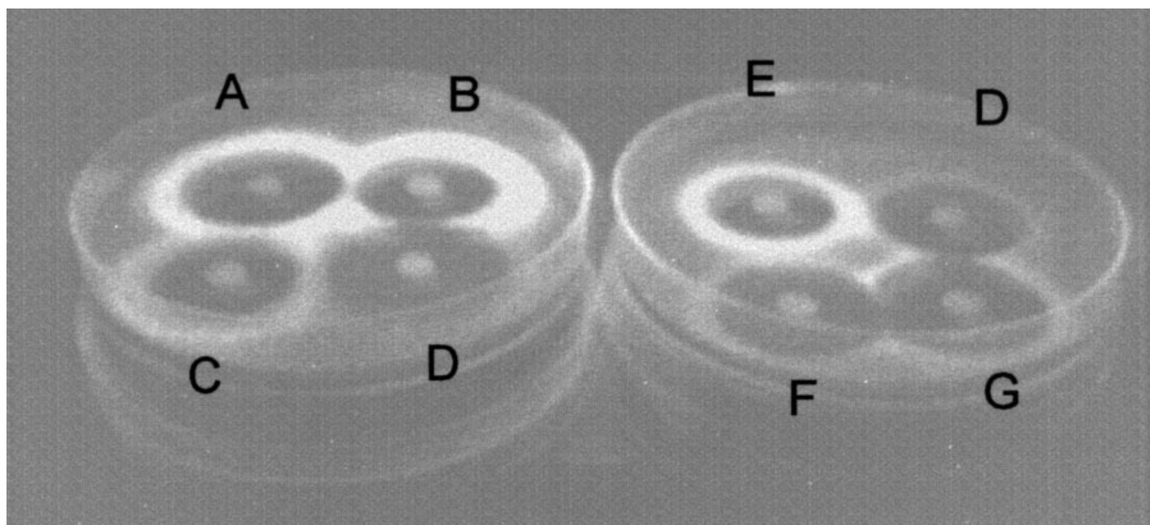

FIG. 1. Fluorescence induced by various antibiotics in *S. aureus* RN4220/pUV4. A bacterial suspension was spread onto blood agar plates, and disks containing macrolides were placed on the surface of the plates. After an incubation for 24 h at 37°C, plates were examined under UV light. A, erythromycin; B, azithromycin; C, spiramycin; D, telithromycin; E, RU 69874; G, HMR 3562; and F, HMR 3787.

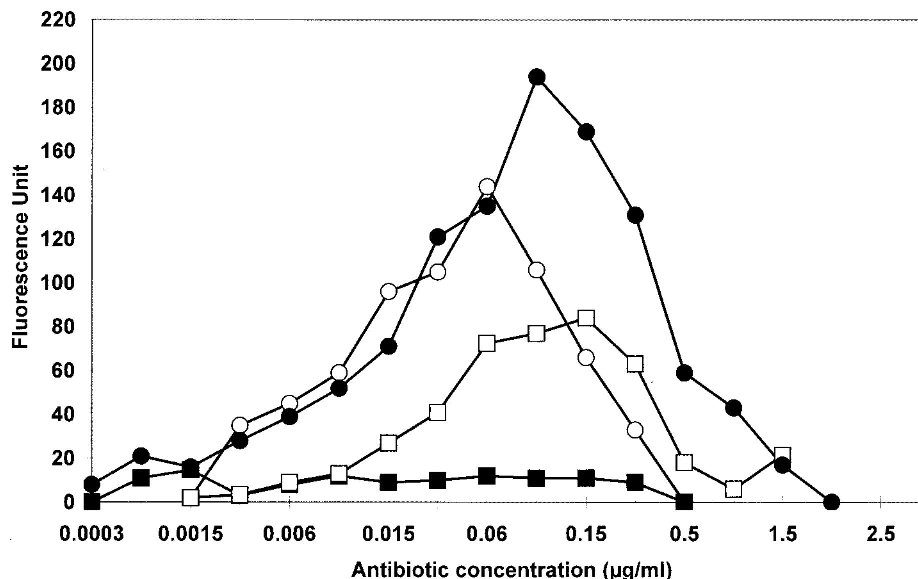

FIG. 2. Fluorometric detection of fluorescence induced by azithromycin (closed circles), erythromycin (open circles), RU 69874 (open squares), and telithromycin (closed squares) in *S. aureus* RN4220/pUV4. Fluorescence was measured in arbitrary fluorescence units.

duced a weak fluorescence (Fig. 1). Fluorescence was strongly expressed in the presence of RU 69874. The fluorescence of *S. aureus* RN4220/pUV5 was expressed in the absence of antibiotic and was not enhanced in the presence of erythromycin or spiramycin (data not shown).

Fluorescence expressed in the presence of erythromycin, azithromycin, telithromycin, RU 69874, and two fluoroketolides (HMR 3562 and HMR 3787) was quantified by spectrofluorometry (Fig. 2). For noninduced *S. aureus* RN4220/pUV4, no basal fluorescence could be detected, while cells of *S. aureus* RN4220/pUV5 expressed a fluorescence equal to approximately 90 U, irrespective of the presence or absence of a macrolide (data not shown). Azithromycin and erythromycin were strong inducers with fluorescence peaks reaching 194 U at 0.09 μg/ml and 144 U at 0.06 μg/ml, respectively. Fluorescence was detected at 0.02 to 0.1 times the MIC of the macrolides for *S. aureus* RN4220/pUV4, reaching a peak at nearly one-quarter to one-half the MIC and then decreasing rapidly at higher concentrations. By contrast, telithromycin (Fig. 2) and the 2-fluoroketolides HMR 3562 and HMR 3787 (data not shown) induced a very weak fluorescence. The fluorescence maxima were 14.6, 18.6, and 21.4 U for telithromycin, HMR 3562, and HMR 3787, respectively. As expected, RU 69874 induced marked fluorescence.

Therefore, the pattern of induction by the antibiotics was similar, as determined by fluorescence or growth curve experiments. However, the fluorescence assay was convenient and easy to use, since it did not require substrates or cofactors. Additionally, the reporter system also delineates viable cells (A. Lefort, M. Arthur, C. Vignes-Colombeix, C. Vissuzaine, D. Henin, C. Carbon, and P. Courvalin, Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 668, 2000). This assay can be used to study qualitatively or quantitatively the relationship between structure and activity of the macrolides, ketolides, or any inducer of methylase production.

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