ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 2006, p. 2038–2041 Vol. 50, No. 6
0066-4804/06/$08.00+0 doi:10.1128/AAC.01574-05

DNA Microarray for Detection of Macrolide Resistance Genes

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Received 10 December 2005/Returned for modification 26 January 2006/Accepted 31 March 2006

A DNA microarray was developed to detect bacterial genes conferring resistance to macrolides and related antibiotics. A database containing 65 nonredundant genes selected from publicly available DNA sequences was constructed and used to design 100 oligonucleotide probes that could specifically detect and discriminate all 65 genes. Probes were spotted on a glass slide, and the array was reacted with DNA templates extracted from 20 reference strains of eight different bacterial species (*Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus aureus*, *Staphylococcus haemolyticus*, *Escherichia coli*, and *Bacteroides fragilis*) known to harbor 29 different macrolide resistance genes. Hybridization results showed that probes reacted with, and only with, the expected DNA templates and allowed discovery of three unexpected genes, including *msr*(SA) in *B. fragilis*, an efflux gene that has not yet been described for gram-negative bacteria.

Resistance to macrolides and related antibiotics (*m*acrolides- *l*incosamides-*s*treptogramins [MLS]) is of great concern because these drugs are commonly used to treat many different infectious syndromes and because this resistance is spreading among gram-positive and gram-negative bacteria, including strains isolated from life-threatening infections such as pneumonia, sepsis, endocarditis, and meningitis. Different classes of genes coding for MLS resistance have been described, and their nucleotide sequences are available in public databases (22). Although macrolide resistance is present worldwide, patterns and mechanisms of resistance may vary widely in different geographic areas, leading to different therapeutic strategies for infective syndromes, such as community-acquired pneumonia (15, 16, 19).

Detection of single bacterial genes (e.g., antibiotic resistance genes or species-specific genes) in diagnostics and in epidemiological studies is typically carried out by PCR, whereas DNA microarrays have been developed to perform a large number of different hybridization experiments simultaneously on a single membrane or glass substrate. They are well-suited to comprehensively investigate and quantitatively compare the expression levels of a large number of genes, but they can also be easily used in qualitative studies to detect selected DNA sequences (7, 8, 21). To assist epidemiological studies on the genetics of macrolide resistance in clinical isolates, a method based on DNA microarrays was developed to comprehensively assess the presence of MLS genes in bacterial genomes.

MATERIALS AND METHODS

Database construction and probe design. The sequences of MLS resistance genes were retrieved from public databases and comparatively analyzed to avoid redundancy. The file containing the selected sequences in multi-FASTA format (http://www.compbio.ox.ac.uk/faq/format_examples.shtml) was used to generate a database to be searched by Array Designer 2.0 software (Premier Biosoft, Palo Alto, CA). Probes, 40 to 60 nucleotides in size, with a melting temperature of 83 ± 1°C, were designed to specifically target each gene of the database. Oligonucleotide probes generated by the software were checked for homology to unrelated sequences present in public databases, and, when possible, two probes for each gene were designed for the array.

Construction of microarray slides. Oligonucleotide probes were synthesized by MWG Biotech (Munich, Germany), with a C_6 amino linker to allow better

TABLE 1. Bacterial strains

Strain (plasmid[s])	Gene(s)	Source (reference)
Streptococcus pyogenes A200	*erm*(TR)	H. Seppala (25)
Staphylococcus aureus N315 (pN315)	*erm*(A)	T. Ito (14)
S. aureus BM4611	*erm*(C)[a], *lnu*(A)[b]	P. Courvalin (6)
S. aureus BM3093 (pIP680)	*vat*(A), *vgb*(A), *vga*(A)	N. El Solh (12)
S. aureus BM12392 (pIP1714)	*vgb*(B), *vat*(C)	N. El Solh (2)
S. aureus BM12235 (pIP1633)	*vga*(B), *vat*(B), *vga*(A)*v*[a]	N. El Solh (1)
Staphylococcus haemolyticus BM4610 (pIP855)	*lnu*(A)[c]	P. Courvalin (5)
Enterococcus faecium A41	*vat*(E-3), *erm*(B)	N. Woodford (26)
E. faecium UW1965	*vat*(E), *erm*(B)	G. Werner (27)
Enterococcus faecalis JH2-2 (pAM401)	*erm*(B), *lsa*	This laboratory (28)
E. faecalis JH2-2 (pAMβ1)	*erm*(B), *lsa*	This laboratory (18)
Escherichia coli DH1 (pVA891)	*erm*(B), *mac*(A), *mac*(B)	This laboratory (17)
E. coli DH5α (pTZ3519)	*mph*(A), *mac*(A), *mac*(B)	N. Noguchi (20)
E. coli DB10 (pAT421)	*vat*(D), *mph*(A), *mac*(A), *mac*(B)	P. Courvalin (24)
E. coli BM2506 (pTZ3721 and pTZ3723)	*mph*(B), *erm*(B), *mac*(A), *mac*(B)	P. Courvalin (13)
E. coli BM2570 (pIP1527)	*ere*(B), *erm*(B), *mac*(A), *mac*(B)	P. Courvalin (3)
E. coli BM8463 (pIP1810)	*vga*(A)v, *mac*(A), *mac*(B)	N. El Solh (11)
Bacteroides fragilis V503 (pVA503)	*erm*(FU), *msr*(SA)[a]	M. C. Halula (10)
Streptococcus pneumoniae PN150	*mef*(E), *mel*	A. Pantosti (9)
S. pneumoniae MF4	*mef*(A), *msr*(D)	This laboratory (23)

[a] Gene found by microarray analysis and confirmed by sequencing (this work).
[b] GenBank accession no. J03947.
[c] GenBank accession no. M14039.

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TABLE 2. Probes for ribosomal methylation genes

Gene	GenBank accession no.	Probe	Position (nucleotides)
erm(A)	AP003129	013	56198–56252
		014[a]	56494–56547
erm(B)	Y00116	017	362–405
		123	615–662
erm(C)	Y17294	019	818–877
erm(C)	Y09003	020	546–606
erm(33)	AJ313523	021[b]	286–348
erm(D)	M29832	095	1062–1107
erm(D)	M77505	088	1009–1072
erm(F)	M14730	152	585–634
erm(FU)	M62487	091	754–796
		092	910–959
erm(G)	M15332	089	793–857
erm(GM)	AB014481	090	662–725
erm(H)	M16503	077	525–560
		078	365–399
erm(K)	AB024564	085	1296–1339
		086	1103–1146
erm(K)	M77505	087	1371–1419
erm(M)	AF462611	083	217–251
		084	892–927
erm(Q)	L22689	082	626–687
erm(T)	AF310974	081	1419–1480
erm(TR)	AF002716	015	368–417
		016	684–740
erm(X)	AF411029	079	1731–1776
erm(X)	AF338706	080	1293–1336
erm(34)	AY234334	148	913–949
		147	818–863
erm(35)	AF319779	094	271–334
erm(38)	AY154657	134	199–233
		135	136–174

[a] Reacts also with *erm*(33) (AJ313523).
[b] Reacts also with *erm*(C) (Y09003) and *erm*(C) (Y17294).

TABLE 3. Probes for efflux genes

Gene	GenBank accession no.	Probe	Position (nucleotides)
mef(A)	AF227520	008	4168–4221
		010	4205–4251
mef(E)	AF376746	001[a]	1561–1609
		012	2265–2319
msr(D)	AF227520	027[b]	5416–5460
mel	AF376746	028	2829–2877
msr(SA)	AB013298	031[c]	1530–1582
		138	513–574
msr(A)	AF167161	142	4228–4293
msr(A)	X52085	141	471–536
lmr(A)	X59926	075	318–352
		076	1208–1244
car(A)	M80346	100	424–462
lmr(C)	X79146	073	33240–33206
mac(A)	AE016758	114	72526–72565
mac(A)	AE009478	115	3649–3690
		116	4344–4383
mac(A)	AE016866	117	46881–46920
		118	47318–47357
mac(B)	AB071146	119	1615–1656
		120	835–877
mac(B)	AE016866	125	48789–48828
		126	49664–49704
mre(A)	U92073	103	304–349
		104	696–741
ole(C)-*orf5*	AL939112	057	2835–2871
		058	2486–2520
tlr(C)	M57437	056	277–311
var(M)	AB035547	054	2690–2724
		055	2840–2874
vga(A)	M90056	040	1712–1764
		041	1637–1693
vga(A)v	AF186237	039	5242–5293
vga(B)	U82085	036	1547–1604
		037	1943–2006
lsa	AE016955	130	196532–196585

[a] Reacts also with *mef*(A) (AF227520).
[b] Reacts also with *mel* (AF376746).
[c] Reacts also with *msr*(A) (AF167161) and *msr*(A) (X52085).

binding to the slide. Epoxy-modified glass slides (Pan-Epoxy slides; MWG Biotech) and a four-head pin ring spotting apparatus (GMS 417 arrayer; Genetics MicroSystems, Woburn, MA) were used. Probes were spotted in at least three replicates at a concentration of 30 pmol/μl in 20% dimethyl sulfoxide and 0.1% Tween 20. Resulting spots had a diameter of 80 to 120 μm.

Template DNA extraction, labeling, and hybridization. Genomic DNA was extracted from a 10-ml bacterial culture harvested in exponential phase, according to a published protocol (4). For staphylococci, 20 U of lysostaphin was added to the lysis solution. One microgram of template DNA, in a reaction volume of 25 μl, was labeled with the fluorescent cytosine analog Cy5 (Amersham Biosciences, Piscataway, NJ) by random priming using 40 U of Klenow DNA polymerase, with a Cy5/dCTP ratio of 1. Ten microliters of the labeled DNA was brought to a volume of 14 μl in hybridization buffer (3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 30 mM HEPES, pH 8, 0.3% sodium dodecyl sulfate, 5× Denhardt's solution), containing tRNA of *Saccharomyces cerevisiae* (Sigma, St. Louis, Mo.) at 1.5 mg per ml. After 2 min of denaturation at 100°C and 10 min at room temperature, the 14-μl mix was layered on the slide and hybridized for 1 h at 55°C. Slides were washed twice for 5 min in 2× SSC-0.1% sodium dodecyl sulfate at 65°C and then twice for 5 min in 1× SSC at room temperature and twice for 5 min in 0.2× SSC.

Data analysis. Microarray slides were read using a GMS 428 array scanner (Genetics MicroSystems, Woburn, MA). Data were acquired using GenePix Pro 5.0 software (Axon Instruments, Union City, CA) and managed with Microsoft Excel. For each spot, median pixel intensity was assessed, and background signal was subtracted. To control for congruity of results obtained with replicate spots of a probe, the mean fluorescence intensity and the standard deviation (intraprobe standard deviation) was calculated for each probe. If, for a probe, the intraprobe standard deviation was higher than the mean fluorescence intensity, hybridization results were considered negative. The standard deviation of the mean fluorescence intensity of all probes of the microarray was also calculated. A probe was considered positive when its fluorescence intensity was higher than the mean fluorescence intensity of all probes plus 1 standard deviation.

Bacterial strains. We hybridized total DNA from 20 bacterial strains carrying reference MLS resistance genes (Table 1).

RESULTS AND DISCUSSION

Probes for macrolide resistance genes. A database which included 65 nonredundant macrolide resistance genes published in GenBank was selected (Tables 2 to 4). Genes were identified by accession number, since in some cases two or more genes with different sequences share the same name. One hundred oligonucleotide probes were designed and spotted on the microarray slide to allow differential detection of the 65 selected MLS genes. Probes for ribosomal methylation genes and their positions in the coding sequence are reported in Table 2, probes for efflux genes in Table 3, and probes for genes coding for esterases, nucleotidyltransferases, phosphotransferases, acetyltransferases, and hydrolases in Table 4.

Microarray hybridization. Microarray slides were tested by hybridizing DNA templates extracted from 20 strains belonging to eight different species and known to harbor 29 different MLS genes (Table 1). All of the probes designed to be specific

TABLE 4. Probes for genes coding for esterases, nucleotidyltransferases, phosphotransferases, acetyltransferases, and hydrolases

Gene product	Gene	GenBank accession no.	Probe	Position (nucleotides)
Esterase	*ere*(A)	AY183453	098	3173–3216
			099	3049–3091
	ere(A-2)	AF099140	096	1362–1406
			097	177–223
	ere(B)	X03988	022	772–827
Nucleotidyltransferase	*lnu*(A)	J03947	069	939–987
	lnu(A)	M14039	072	457–510
	lnu(B)	AJ238249	067	281–324
	lnu(B)-like	AJ293027	065	5830–5770
			066	5501–5448
Phosphotransferase	*mph*(A)	U36578	143	1004–1042
			144	1117–1151
	mph(B)	D85892	063	1685–1729
			064	2019–2064
	mph(C)	AB013298	059	2497–2554
			060	2514–2556
	mph(C)	AF167161	061	5883–5925
			062	5866–5923
Acetyltransferase	*vat*(A)	L07778	052	634–680
	vat(B)	U19459	050	408–459
			051	260–317
	vat(C)	AF015628	048	1661–1703
			049	1595–1641
	vat(D)	L12033	046	563–614
			047	362–420
	vat(E)	AF139725	045[a]	430–476
			044	74–120
	vat(E-3)	AY008284	042	7–52
Hydrolase	*vgb*(A)	M20129	128	1221–1277
			127	899–950
	vgb(B)	AF015628	035	908–953
			034	1016–1068

[a] Reacts also with *vat*(E-3) (AY008284).

for the 29 MLS genes reacted with the predicted DNA templates, allowing validation of a total of 48 probes (Table 5). Three unexpected results were also obtained: (i) the DNA of *Bacteroides fragilis* V503 reacted with probe msrSA-31, (ii) the DNA of *Staphylococcus aureus* BM12235 reacted with probe vgaAv-39, and (iii) the DNA of *S. aureus* BM4611 reacted with probes ermC-19 and ermC-20 (Table 5).

Identification of additional MLS genes in control strains. Microarray data indicating the presence of unexpected MLS genes in control strains were confirmed by DNA sequencing of the entire open reading frame, using templates obtained by PCR, as previously described (23). In *B. fragilis* strain V503, carrying the methylase gene *erm*(FU), sequence data indicated the concomitant presence of an efflux gene identical to *msr*(SA) (100% identity at the DNA level) of *S. aureus* (GenBank accession no. AB013298). The *msr*(SA) gene is considered typical of *Staphylococcus* spp. and has never been found in gram-negative bacteria. In *S. aureus* strain BM12235, carrying the major facilitator streptogramin efflux gene *vga*(B) and the streptogramin acetyltransferase gene *vat*(B), it was possible to identify also the presence of *vga*(A)v, an ATP-binding transporter gene which is commonly associated with *vga*(B) and *vat*(B) (11, 12). DNA sequence analysis showed that *vga*(A)v of BM12235 was essentially identical (99% identity at the DNA level) to *vga*(A)v of *S. aureus* BM3327 (GenBank accession no. AF186237). In *S. aureus* strain BM4611, carrying the lincomycin nucleotidyltranferase gene *lnu*(A), an associated methylase gene of the *erm*(C) class was found, with up to 90% identity at the nucleotide level with several *erm*(C) genes present in GenBank.

Conclusions. This work provides detailed information for construction of a simple and powerful tool to investigate the

TABLE 5. Hybridization results[a]

Organism	Strain (plasmid[s])	Positive probe(s)[b]
Bacteroides fragilis	V503 (pVA503)	ermFU-91, ermFU-92, msrSA-31*
Escherichia coli	BM8463 (pIP1810)	vgaAv-39, macA-114, macB-119, macB-120
	DH5α (pTZ3519)	mphA-143, mphA-144, macA-114, macB-119, macB-120
	HB101 (pVA891)	ermB-17, ermB-123, macA-114, macB-119, macB-120
	DB10 (pAT421)	vatD-46, vatD-47, mphA-143, mphA-144, macA-114, macB-119, macB-120
	BM2506 (pTZ3721 and pTZ3723)	mphB-63, mphB-64, ermB-17, ermB-123, macA-114, macB-119, macB-120
	BM2570 (pIP1527)	ereB-22, ermB-17, ermB-123, macA-114, macB-119, macB-120
Enterococcus faecalis	JH2-2 (pAMβ1)	ermB-17, ermB-123, lsa-130
	JH2-2 (pAM401)	ermB-17, ermB-123, lsa-130
Enterococcus faecium	A41	vatE3-42, vatE-45, ermB-17, ermB-123
	UW1965	vatE-44, vetE-45, ermB-17, ermB-123
Staphylococcus aureus	BM12235 (pIP1633)	vgaB-36, vgaB-37, vatB-50, vatB-51, vgaAv-39*
	BM12392 (pIP1714)	vgbB-34, vgbB-35, vatC-48, vatC-49
	BM3093 (pIP680)	vgaA-40, vgaA-41, vatA-52, vatA-53, vgbA-127, vgbA-128
	N315	ermA-13, ermA-14
	BM4611	lnuA-69, ermC-19*, ermC-20*
Staphylococcus haemolyticus	BM4610 (pIP855)	lnuA-72
Streptococcus pyogenes	A200	ermTR-15, ermTR-16
Streptococcus pneumoniae	MF4	mefA-8, mefA-10, mefE-1, msrD-27
	PN150	mefE-12, mefE-1, mel-28, msrD-27

[a] Target genes and positive probes are indicated.
[b] Probes are identified by the gene name and a number (see Tables 2 through 4). *, new findings.

genetic basis of macrolide resistance in bacterial isolates. Careful analysis of DNA sequences deposited in public databases allowed compilation of a list of 65 bacterial genes encoding resistance to macrolides and related drugs. Oligonucleotide DNA microarrays designed to detect these 65 genes in bacterial genomes were produced and used to test a collection of strains carrying well-characterized MLS genes. Results provided both (i) validation of the microarray chip and (ii) proof of concept that the microarray approach is effective in detecting associations of MLS genes not necessarily inferred by the resistance phenotype. Unlike other DNA microarrays developed to detect the most common resistance genes (8, 21), this one, by its comprehensive approach, is well-suited for surveillance studies specific for MLS resistance, where characterization of the resistance genotype is sought. This DNA microarray could significantly contribute to molecular epidemiology studies by allowing simultaneous testing for the presence of known MLS genes and in particular could help to define and understand the clustering of different MLS genes in genetic elements and genomes.

ACKNOWLEDGMENTS

We thank Annalisa Pantosti for advice and all researchers listed in Table 1 for kindly providing control strains.

The work was funded in part by grants from Istituto Superiore di Sanità, from the University of Siena (PAR), and from MIUR (FIRB, RBAU01X9TB).

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