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Phenotypic Expression and Genetic Heterogeneity of Lincosamide Inactivation in *Staphylococcus* spp.

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We examined the resistance phenotype and the genetic basis of lincosamide modification in 25 clinical isolates of *Staphylococcus* spp. inactivating lincomycin and clindamycin. The strains were resistant to high levels of lincomycin but remained susceptible to clindamycin. However, MBCs and inoculum effects showed that the activity of clindamycin was impaired. The distribution in these strains of nucleotide sequences related to *linA* and *linA'*, the genes encoding lincosamide nucleotidylation in *Staphylococcus haemolyticus* BM4610 and *S. aureus* BM4611, respectively, was studied by dot blot hybridization. The genes responsible for lincosamide inactivation in *Staphylococcus* spp. were found to constitute a family of related sequences which are not species specific.

The lincosamide antibiotics lincomycin and clindamycin are useful in the treatment of infections due to anaerobes and gram-positive cocci (6). Resistance of staphylococci to these drugs usually involves alteration of the ribosome following N^6-dimethylation of an adenine residue in 23S rRNA (23). This resistance is associated with coresistance to macrolide and streptogramin B-type antibiotics, the so-called MLS phenotype (3, 4). Another type of resistance toward lincomycin has been reported that occurs in staphylococci of animal origin which resist by inactivating the antibiotic (5). We recently described a human clinical isolate, *Staphylococcus haemolyticus* BM4610, which is highly resistant to lincomycin (MIC, 64 μg/ml) and inactivates lincomycin and clindamycin (12). Lincosamide inactivation in this strain is due to synthesis of a 4-lincosamide *O*-nucleotidyltransferase, which catalyzes the nucleotidylation of the hydroxyl group in position 4 of lincomycin and clindamycin (A. Brisson-Noël, P. Delrieu, D. Samain, and P. Courvalin, manuscript in preparation). The nucleotide sequence of the plasmid gene *linA* responsible for this new phenotype in BM4610 has been determined (1). A 77-base-pair fragment internal to this gene was used as a probe in hybridization experiments to study the dissemination of *linA* in staphylococci resisting lincomycin by inactivation. *S. aureus* BM4611 did not hybridize to the probe and was studied further. Gene *linA'*, which encodes a 4-lincosamide *O*-nucleotidyltransferase in this strain, was sequenced and compared with *linA* (Brisson-Noël et al., in preparation). Both genes are 483 base pairs long but differ by 34 base substitutions, and the derived proteins differ by 14 amino acids. In this paper we study the phenotypic expression of resistance to lincosamides by inactivation and the distribution of *linA*, *linA'*, and related genes in clinical isolates of staphylococci.

MATERIALS AND METHODS

Bacterial strains. Strains of staphylococci highly resistant to lincomycin originated from four hospitals in Paris. They were screened for antibiotic resistance by the disk agar diffusion technique. Strains resistant to lincomycin and susceptible to spiramycin were selected to avoid the constitutive MLS phenotype. Bacteria were identified by the method of Kloos and Schleifer (10). *S. aureus* strains were isolated from abcesses, and coagulase-negative staphylococci were isolated from wound infections, intravenous catheters, and urine samples. The incidence of lincomycin resistance was 0.2% in *S. aureus* (2,100 strains screened), 4.6% in *S. epidermidis* (240 strains screened), and 8% in *S. cohnii* (50 strains screened). *S. haemolyticus* BM4610 harboring *linA* (1, 12), its cured derivative BM4610-1 (12), *S. aureus* BM4611 harboring *linA'* (Brisson-Noël et al., in preparation), and antibiotic-susceptible *S. aureus* RN450 (17) and 209P were included as controls.

Media. Brain heart infusion broth and agar (Difco Laboratories, Detroit, Mich.) were used as media. Antibiotic susceptibility tests were performed with Mueller-Hinton broth and agar (Diagnostics Pasteur, Marnes-la-Coquette, France). All incubations were done at 37°C.

Determination of MICs and MBCs. The method of Steers et al. (19) was used to determine the MICs of the antibiotics in solid medium. The MICs and MBCs (≥99.9% reduction in CFU per milliliter) of clindamycin were also determined by macrodilution in liquid medium (21) with an inoculum of approximately 5×10^5 CFU/ml. To study the effect of the size of the inoculum, clindamycin MICs in liquid medium were determined with the standard inoculum (5×10^5 CFU/ml) and with a 10^2-fold-higher inoculum (5×10^7 CFU/ml).

Inactivation of MLS antibiotics. Inactivation of MLS antibiotics was screened by the test described by Gots (7) and modified as follows. The test strains were streaked on the surfaces of plates containing *Micrococcus luteus* ATCC 9341 as the indicator organism and concentrations of MLS antibiotics slightly higher than the MICs of these antibiotics for *M. luteus*. Inactivation of the antibiotic in the culture medium by the test organism allowed growth of the indicator organism in the surrounding medium.

Preparation of DNA. Cells from 1.5 ml of an overnight broth culture were harvested, suspended in 100 μl of 0.05 M Tris hydrochloride (pH 8.0)–0.01 M EDTA–25% sucrose containing lysostaphin (0.25 mg/ml), and incubated at 37°C

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TABLE 1. MICs of various MLS antibiotics against staphylococcal strains

Organism	MIC (μg/ml)[a]								
	ERY	OLE	SPI	JOS	LIN	CLI	PRI I	PRI II	PRI
S. aureus 209P	0.12	0.5	0.5	0.5	0.5	0.06	4	1	0.25
S. haemolyticus BM4610-1	0.12	0.5	0.5	0.5	0.25	0.06	8	1	0.25
S. haemolyticus BM4610	0.12	0.5	0.5	0.5	64	0.12	8	1	0.25
S. aureus BM4611	>128	>128	0.5	0.5	32	0.25	8	2	0.5
Staphylococci[b]	0.12–0.25	0.25–1	2–4	0.5–2	16–128	0.06–0.5	8–16	2–8	0.25–1
Staphylococci[c]	>128	>128	2–4	0.5–2	16–128	0.06–0.5	8–16	2–8	0.25–1

[a] Abbreviations: ERY, erythromycin; OLE, oleandomycin; SPI, spiramycin; JOS, josamycin; LIN, lincomycin; CLI, clindamycin; PRI I, pristinamycin factor I; PRI II, pristinamycin factor II; PRI, pristinamycin.
[b] Nineteen strains highly resistant to lincomycin only.
[c] Six strains highly resistant to lincomycin and to erythromycin and oleandomycin (inducible MLS resistance).

for 1 h. The resulting protoplasts were lysed by phenol-chloroform extraction, and total DNA was recovered in the supernatant after centrifugation. Aliquots of this crude extract were denaturated for 10 min at 100°C before being spotted on nitrocellulose membranes.

Construction of probes and hybridizations. Fragments of DNA to be used as probes were cloned in M13mp10 (15) replicative-form DNA and transfected in *Escherichia coli* JM101 (14), and single-stranded recombinants were purified and labeled (9) with [α-^{32}P]dATP. Probes were hybridized to DNA immobilized on nitrocellulose filters in 50% formamide at 37°C; the filters were washed in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 37°C.

Enzymes and chemicals. Restriction endonuclease *Sau*3A and exonuclease BAL 31 (Amersham Corp., Arlington Heights, Ill.) were used as recommended by the manufacturer. Lysostaphin was from Sigma Chemical Co., St. Louis, Mo. [α-^{32}P]dATP, triethylammonium salt, was from the Radiochemical Centre, Amersham, England. The antibiotics were provided by the following companies: clindamycin and lincomycin, The Upjohn Co., Kalamazoo, Mich.; erythromycin, Roussel-Uclaf, Paris, France; josamycin, Spret-Mauchant, Gennevilliers, France; oleandomycin, Pfizer Inc., New York, N.Y.; and spiramycin and pristinamycin factor I, factor II, and complex, Rhône-Poulenc, Vitry-sur-Seine, France.

RESULTS

Properties of the strains studied. The 25 strains of staphylococci highly resistant to lincomycin were members of five species: *S. epidermidis* (11 strains), *S. haemolyticus* (5 strains), *S. aureus* (3 strains), *S. cohnii* (4 strains), and *S. hominis* (2 strains). The susceptibility of the strains to 21 antibiotics was tested by the disk agar diffusion technique (data not shown). All strains, except one *S. cohnii* isolate, were also resistant to one or several antibiotics. For every species, the strains appeared unrelated, since they had different antibiotic resistance phenotypes and/or were isolated in different hospitals.

MLS resistance. The MICs of MLS antibiotics for the staphylococcal strains are shown in Table 1. All the strains were resistant to high levels of lincomycin (MIC, 16 to 128 μg/ml) by inactivation, as demonstrated by microbiological techniques (7, 12). *S. aureus* 209P and BM4610-1, susceptible to MLS antibiotics and used as controls, did not inactivate the antibiotics. None of the commercially available macrolides (erythromycin, oleandomycin, josamycin, and spiramycin) or streptogramins (pristinamycin factor I, factor II, and complex) was inactivated. The strains remained fully susceptible to these drugs, except for seven strains, including *S. aureus* BM4611, which exhibited a complex phenotype resulting from the combination of inducible MLS resistance and lincomycin inactivation.

As for BM4610 (12), although clindamycin was also inactivated, the strains appeared susceptible to this antibiotic when tested by disk agar diffusion (data not shown) and by dilution in solid (Table 1) or liquid (Table 2) medium. However, the bactericidal activity of clindamycin was impaired, since its MBCs were 4- to 32-fold higher than that against the susceptible strains BM4610-1 and 209P (Table 2). When determined with a 10^2-fold-higher inoculum in liquid medium, the MICs of clindamycin were approximately 10 times higher against the resistant strains than against the susceptible reference strains (Table 2).

Construction of intragenic probes. The sequences of genes *linA* (1) and *linA'* (Brisson-Noël et al., in preparation) have been determined. Comparison of the sequences obtained allowed the construction of three probes (Fig. 1). A 77-base-pair *Sau*3A DNA fragment of *linA* (*linA* probe) was intended to be specific for *linA*, whereas two fragments generated by BAL 31 exonuclease and intragenic to *linA'* were supposed to hybridize to *linA'* only (*linA'* probe) or to *linA*, *linA'*, and related genes (*linA*-like probe), respectively. The specificity of these DNA fragments towards the two genes was predicted by calculating the melting temperature of homo- or heteroduplex DNA with the theoretical formula (2, 13)

$$T_m = 69.3 + 0.41G - 500/L - 0.7F - M$$

TABLE 2. Activity of clindamycin against staphylococcal strains

Strain	Inoculum of 5 × 10^5/ml			MIC (μg/ml) in liquid medium for inoculum of 5 × 10^7/ml
	MIC (μg/ml)		MBC (μg/ml)	
	Solid medium	Liquid medium		
S. aureus 209P	0.06	0.12	4	0.25
S. haemolyticus BM4610-1	0.06	0.12	4	0.25
S. haemolyticus BM4610	0.12	0.25	64	2
S. aureus BM4611	0.25	0.5	64	4
Staphylococci (25 strains)	0.06–0.5	0.12–0.5	16–128	1–4

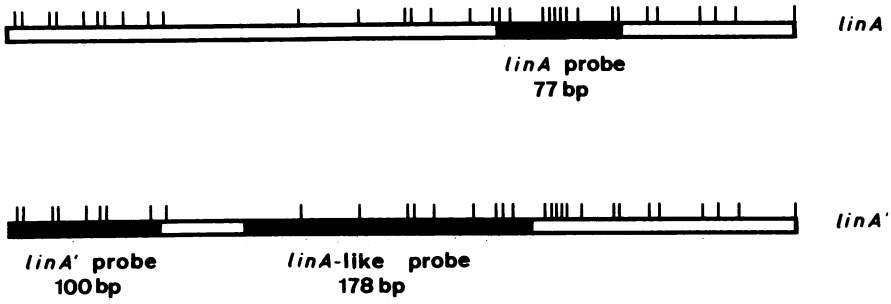

FIG. 1. *linA*, *linA'*, and *linA*-like probes. The probes are depicted by solid boxes. Vertical bars indicate base substitutions between genes *linA* and *linA'*.

where G represents the guanine-plus-cytosine content (%G+C) of the probe, L is the length (in base pairs) of the probe, F is the percentage of formamide in the hybridization solution, and M is the percentage of mismatched base pairs between probe and target DNA. Hybridization between probe and target DNA occurs at temperatures below the melting temperature T_m. The characteristics of the probes are summarized in Table 3. The T_m values indicate that the probes should display the expected specificities at 37°C in 50% formamide.

Hybridization. The presence of *linA*, *linA'*, or related sequences in the strains of staphylococci inactivating lincosamides was tested by dot blot hybridization (Fig. 2; Table 4). Homology with the *linA*-like probe was detected in the 25 strains studied. Fifteen (including *S. aureus*, *S. haemolyticus*, *S. epidermidis*, and *S. hominis*) hybridized to the *linA* probe and seven (including *S. aureus*, *S. epidermidis*, and *S. cohnii*) hybridized to the *linA'* probe. The remaining three *S. cohnii* strains showed homology with the *linA*-like probe only. We did not find any strain hybridizing to both *linA* and *linA'* probes. No homology was observed between the probes and the control susceptible strains. The specificity of the probes was as predicted, since total DNA of *S. haemolyticus* BM4610 hybridized to the *linA* probe, total DNA of *S. aureus* BM4611 hybridized to the *linA'* probe, and both DNAs hybridized to the *linA*-like probe.

DISCUSSION

Resistance to high levels of lincomycin by inactivation is spread among numerous species of staphylococci (Table 4). We have examined the resistance phenotype and the genetic basis of lincosamide modification in 25 strains of staphylococci inactivating lincomycin and clindamycin. Although all strains inactivated the two drugs, the in vitro bacteriostatic activity of lincomycin was abolished, whereas that of clindamycin was apparently unaffected (Table 1). However, two observations showed that the activity of clindamycin was impaired: the MBCs of the drug against inactivating strains were significantly higher than those against susceptible strains, and the MICs in liquid medium increased drastically when a stronger inoculum was used (Table 2). Moreover, when introduced into *E. coli* or *Bacillus subtilis*, genes encoding lincosamide inactivation confer high-level resistance to both antibiotics (1; Brisson-Noël et al., in preparation). The reason(s) for the differential expression of resistance in the original hosts against the two antibiotics and for the similar resistance in heterologous systems remains unexplained.

Two genes, *linA* and *linA'*, encoding a 4-lincosamide *O*-nucleotidyltransferase in *S. haemolyticus* BM4610 and *S. aureus* BM4611, respectively, has been sequenced (1; Brisson-Noël et al., in preparation). Probes specific for *linA*, *linA'*, and *linA*-like genes have been constructed (Fig. 1; Table 3) and used in dot blot hybridizations against the 25 clinical isolates of staphylococci inactivating lincosamides (Fig. 2; Table 4). Homology with the *linA*-like probe designed to hybridize to *linA*, *linA'*, and related sequences was detected in all the strains studied. Of these strains, 15 hybridized with the *linA* probe, 7 hybridized with the *linA'* probe, and 3 *S. cohnii* strains did not share homology with either probe. It seems, therefore, that other classes of genes specifying lincosamide detoxification that are similar but not identical to *linA* and *linA'* exist in nature. The *lin* genes, which appear to form a sequence continuum, obviously derive from a common ancestor. The classes are not species specific, since *linA* and *linA'* were present in *S. aureus* and *S. epidermidis*, but no strain was found to harbor both genes (Table 4). Differences in levels of lincomycin resistance (Table 1) did not correlate with hybridization classes (Table 4). The hybridization results confirmed that the probes were adequately designed. As expected, *linA* and *linA'* probes were highly specific, whereas the *linA*-like probe not only hybridized with all the strains harboring *linA* or *linA'* but also allowed the detection of other sequences related to these genes. The theoretical approach used, which requires comparative sequence data, allows the construction of probes with different and predictable specificities.

DNA probes have proved useful in the study of the distribution of certain resistance genes and in the detection

TABLE 3. Characteristics of probes used

Probe	*linA* gene			*linA'* gene		
	% Homology	T_m (°C)[a]	Hybridization[b]	% Homology	T_m (°C)[a]	Hybridization[b]
linA (77 bp)	100	42	+	87	28	−
linA' (100 bp)	90	30	−	100	40	+
linA-like (178 bp)	95	38	+	100	43	+

[a] Melting temperature of DNA duplexes (calculated as described in Results).
[b] Hybridization in 50% formamide at 37°C.

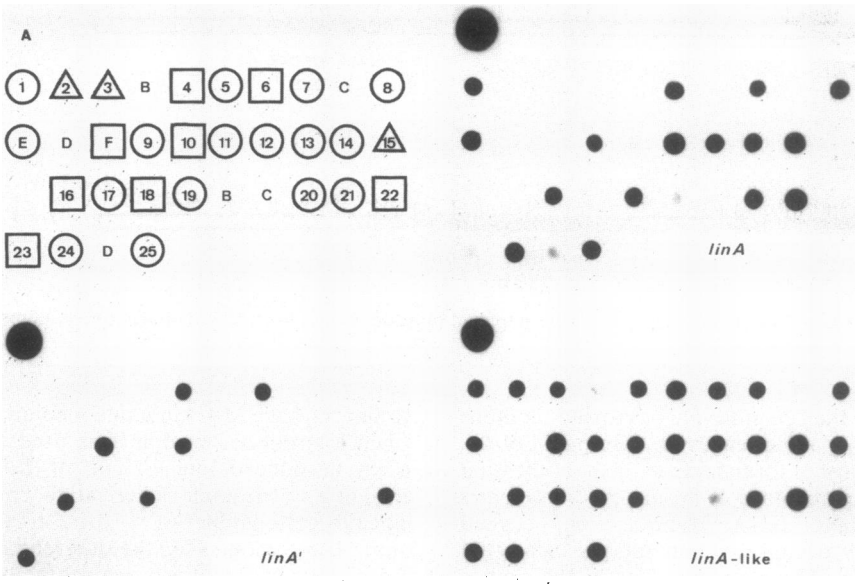

FIG. 2. Analysis of staphylococcal DNA by dot blot hybridization. Total DNA was transferred to nitrocellulose sheets and hybridized to the probes indicated. Upper left, Schematic representation of the sheets. ○, □, Positive results with *linA* and *linA'* probes, respectively; △, strains hybridizing with the *linA*-like probe only. Abbreviations: A, homologous reaction; B, RN450; C, 209P; D, BM4610-1; E, BM4610; F, BM4611.

of new resistance determinants (20). Gene-specific probes have also been applied to the detection of antibiotic resistance in clinical isolates. This approach was recently used to study the correlation between aminoglycoside resistance phenotypes and genes encoding aminoglycoside-modifying enzymes in 10^3 clinical isolates of gram-positive cocci (H. Ounissi, E. Derlot, C. Carlier, and P. Courvalin, Program Abstr. Am. Soc. Microbiol. Conf. Streptococcal Genet. 2nd, Miami Beach, Fla., abstr. no. 111, 1986). The agreement (>99%) between the hybridization results and the predicted genotypes was excellent, indicating that this technique is valid in this particular resistance system. However, the results obtained in the present study stress a prerequisite of the use of this technique in clinical microbiology, namely the existence of a gene homogeneity for a given resistance phenotype. The use of either *linA* or *linA'* probe would have led to an underestimation of the prevalence of lincosamide resistance in staphylococci. By contrast, the *linA*-like probe could detect all the strains inactivating lincosamides. This peculiar situation is due to the surprising variability of *lin* genes and is not found in other well-studied resistance gene systems. For the *erm* group (genes encoding rRNA methylases conferring MLS resistance), seven hybridization classes have been described (18; M. Arthur, A. Brisson-Noël, and P. Courvalin, J. Antimicrob. Chemother., in press). Nucleotide sequences vary greatly from one class to another but are extremely well conserved within a class. For example, the *ermC* genes in *S. aureus* (8), *S. epidermidis* (11), and *B. subtilis* (16) differ by 7 of 732 bases (0.99%) (Arthur et al., in press). A similar situation is observed with the 3'-aminoglycoside phosphotransferases. Genes encoding enzymes types I to V display no significant homology, but the *aphA-3* genes found in phylogenetically remote species such as *S. aureus*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, and *Campylobacter coli* (22) differ by 5 of 792 bases (0.6%). Comparatively, *linA* and *linA'*, which were isolated in the same bacterial genus, differ by 34 of 483 bases (7%). Gene classes are based on the fact that genes conferring resistance by the same mechanism and belonging to different classes do not cross-hybridize even under low-stringency conditions (Arthur et al., in press). Therefore, *lin* genes must be assigned to a single class comprising heterologous sequences.

TABLE 4. Hybridization classes of staphylococci resistant to lincomycin by inactivation (*S. haemolyticus* BM4610 and *S. aureus* BM4611 are included)

Species (no. of strains)	Hybridization with probe:		
	linA	*linA'*	*linA*-like
S. epidermidis (6)	+	−	+
S. haemolyticus (6)	+	−	+
S. aureus (2)	+	−	+
S. hominis (2)	+	−	+
S. epidermidis (5)	−	+	+
S. aureus (2)	−	+	+
S. cohnii (1)	−	+	+
S. cohnii (3)	−	−	+

These observations emphasize the need for two types of probes depending upon the goal of the study. Detection of resistance has to take into account the existence of gene classes and of gene variants within a given class. Thus, a universal probe would consist of a mixture of fragments specific for each class and as long as possible to overcome intraclass variability. In contrast, short probes, natural or synthetic, can be used to discriminate gene variants.

There are two pitfalls in the detection of lincosamide resistance of staphylococci. First, the strains appear susceptible to clindamycin. This might lead to erroneous therapy, since the drug is inactivated and has impaired bactericidal activity, a possibility which is being tested in animal models. Lincomycin as well as clindamycin should therefore be used in routine tests. Second, genes encoding this type of resistance display an unusual sequence variability, which has to

our knowledge not yet been described for other resistance gene systems.

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