Characterization of Erythromycin-Resistant Isolates of *Staphylococcus aureus* Recovered in the United States from 1958 through 1969

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We tested 16 erythromycin-resistant clinical isolates of *S. aureus*, recovered from patients hospitalized in the United States from 1958 to 1969, for the presence of *ermA*, *ermB*, and *ermC* by using PCR. Fifteen of 16 isolates contained at least one copy of *ermA*; the remaining isolate, which was also clindamycin resistant, contained *ermB*. Eight of the 15 isolates harboring *ermA*, all of which were inducible, contained a single copy of the gene in the chromosome, while the remaining seven isolates had two copies of the gene. *ermB* was plasmid encoded and mediated constitutive resistance to erythromycin.

Erythromycin resistance genes are widely disseminated among many species of bacteria; over a dozen resistance determinants have been described (1, 11, 34). In *Staphylococcus aureus*, erythromycin resistance is usually due either to ribosomal modification by 23S rRNA methylases mediated primarily by *ermA*, *ermB*, or *ermC* or to active efflux of the antimicrobial agent by an ATP-dependent pump mediated by *msrA* (10, 14, 27, 29). *ermA* is most often harbored on the transposon Tn*554*, which also encodes spectinomycin resistance (8, 18, 22, 33), while *ermB* is often associated with transposon Tn*551* and the penicillinase plasmid, pI258 (21, 28). The *ermC* gene, which appears to be rare in strains isolated prior to 1970, is normally located on small plasmids ranging in size from 2.4 to 5 kb (12, 13, 27). All of the *erm* determinants confer cross-resistance to macrolides, lincosamides, and streptogramin B agents (MLS$_B$ phenotype) (1, 11, 19).

Several reports have characterized erythromycin resistance determinants in *S. aureus* isolates recovered prior to 1970 in Denmark (35, 36) or during later periods in the United States or the United Kingdom (11, 15, 33), however, no reports to our knowledge describe the epidemiology of erythromycin resistance in *S. aureus* isolates from the United States in the period immediately after erythromycin was introduced into clinical practice. Therefore, the goal of this study was to characterize the genes encoding erythromycin resistance in strains of *S. aureus* isolated from patients in the United States from 1958 to 1969.

Eighteen isolates of *S. aureus* obtained from patients in the United States prior to 1970 were recovered from freezers at the Centers for Disease Control and Prevention. The organisms were identified by using standard biochemicals (17); bacteriophage results were those previously determined at the Centers for Disease Control and Prevention (3, 4). Antimicrobial susceptibility testing was performed by broth microdilution using cation-adjusted Mueller-Hinton (MH) broth (Becton Dickinson Microbiology Systems, Cockeysville, Md.) (26). Spectinomycin susceptibility was tested by dispensing 5 μl of a

0.5 McFarland standard suspension of the isolate onto MH agar plates containing 300 μg of spectinomycin per ml (33).

Two of the *S. aureus* isolates were resistant to penicillin and tetracycline only, while 15 were resistant to erythromycin, penicillin, spectinomycin, and tetracycline (Table 1). The remaining isolate (isolate 65-20) manifested high-level erythromycin and clindamycin resistance in addition to penicillin and tetracycline resistance; this was the only erythromycin-resistant isolate that was susceptible to 300 μg of spectinomycin per ml. All 18 isolates were susceptible to oxacillin.

The induction of erythromycin resistance was tested by disk diffusion by using standard erythromycin (15 μg) and clindamycin (2 μg) disks (25) as described by Jenssen et al. (15). All of the isolates except 65-20 showed blunting of the clindamycin zones in the disk screening test, indicating induction. Strains 65-20, RN1551 (constitutive *ermA* control; provided by Gregory Stone, Abbott Park, Ill.), and RN11 (*ermB*) (28) were clindamycin resistant. To confirm the disk results, strains were induced in MH broth with erythromycin for 90 min and then tested for erythromycin resistance by agar dilution on MH agar with a final inoculum size of 5×10^4 CFU/spot. The control strain *S. aureus* ATCC 29213 remained susceptible to erythromycin (MIC, <0.5 μg/ml), while strain 1206 (inducible strain containing *ermA*; provided by Bernard Weisblum, Madison, Wis.) and the four representative isolates 65-8, 65-1322, 68-397, and 58-480 showed an increase in erythromycin MIC of five or more doubling dilutions, rising from 2 to >64 μg/ml.

Pulsed-field gel electrophoresis (PFGE) of genomic DNA after digestion in situ with *Sma*I (3, 32) was performed to determine the genetic relatedness of the isolates. The PFGE patterns of all of the isolates were indistinguishable (Fig. 1) with the exception of isolate 67-331, which showed an additional band at approximately 400 kb (Fig. 1A, lane M). All of the isolates, including the erythromycin-susceptible isolates, belonged to the 80/81 bacteriophage group, including those from 1958 which were presumed to be from nosocomial outbreaks (23, 24). Three isolates, 65-1119, 65-1322, and 67-43, were typed as belonging to the 52/52A/80/81 complex, which is included in the 80/81 bacteriophage complex.

Using the PCR primers for *ermA*, *ermB*, and *ermC* described by Sutcliffe et al. (30, 31), we identified *ermA* in all 15 erythromycin-resistant isolates that showed an inducible pattern of expression (Table 1). The *ermB* determinant was present in the remaining isolate (65-20), which showed high-level erythromy-

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TABLE 1. Characteristics of erythromycin-resistant isolates

Strain	Isolation[a]		Gene/expression	ermA inserts[b]		Resistance pattern[c]
	Yr	State		No.	Size (kb)	
58-362	1958	Georgia		0		Pn Tc
58-431	1958	Georgia		0		Pn Tc
58-424	1958	Georgia	ermA/inducible	1	6.0	Er Pn Sp Tc
58-434	1958	Georgia	ermA/inducible	1	6.0	Er Pn Sp Tc
58-480	1958	Georgia	ermA/inducible	2	6.0–9.7	Er Pn Sp Tc
58-488	1958	Georgia	ermA/inducible	1	6.0	Er Pn Sp Tc
58-490	1958	Georgia	ermA/inducible	2	6.0–9.7	Er Pn Sp Tc
65-8	1965	Georgia	ermA/inducible	1	6.0	Er Pn Sp Tc
65-20	1965	Georgia	ermB/constitutive	0		Cc Er Pn Tc
65-1119	1965	Alabama	ermA/inducible	1	6.0	Er Pn Sp Tc
65-1322	1965	West Virginia	ermA/inducible	1	6.0	Er Pn Sp Tc
66-1752	1966	Arkansas	ermA/inducible	1	6.0	Er Pn Sp Tc
67-43	1967	West Virginia	ermA/inducible	1	6.0	Er Pn Sp Tc
67-331	1967	California	ermA/inducible	2	6.0–9.7	Er Pn Sp Tc
68-81	1968	New York	ermA/inducible	2	6.0–9.7	Er Pn Sp Tc
68-397	1968	Texas	ermA/inducible	2	6.0–7.0	Er Pn Sp Tc
69-172	1969	California	ermA/inducible	2	6.0–9.7	Er Pn Sp Tc
69-412	1969	Delaware	ermA/inducible	2	6.0–9.7	Er Pn Sp Tc

[a] Isolates from Georgia are presumed to be from the investigation reported in reference 24.
[b] Size of the EcoRI fragments that hybridized with the ermA DNA probe.
[c] Cc, clindamycin; Er, erythromycin; Pn, penicillin; Tc, tetracycline; Sp, spectinomycin.

cin and clindamycin resistance that was constitutively expressed. The ermC gene was not present in any of the isolates tested. We did not test for the presence of msrA or other erythromycin resistance genes.

DNA-DNA hybridization studies of total genomic DNA digested with the restriction enzyme EcoRI were performed with an ermA-specific probe labeled with digoxigenin (5, 36). Since EcoRI does not cleave the DNA probe used for detection of ermA, each band observed after hybridization represents one insert of ermA. These assays demonstrated that 8 of 15 isolates
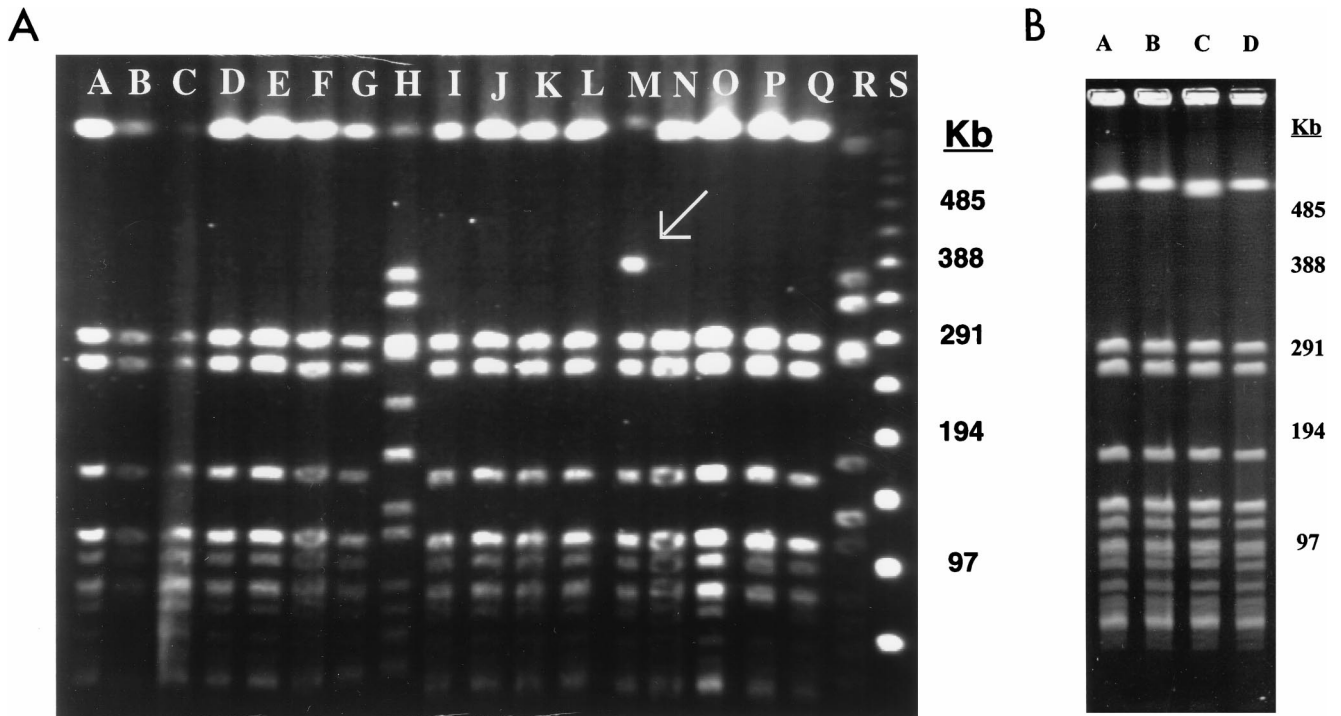

FIG. 1. (A) Analysis of SmaI restriction fragments of erythromycin-resistant isolates of S. aureus analyzed by PFGE. Lane A, 58-424; lane B, 58-434; lane C, 58-480; lane D, 58-488; lane E, 58-490; lane F, 65-8; lane G, 65-20 (ermB); lane H, 8325 (erm-negative control); lane I, 65-1119; lane J, 65-1322; lane K, 65-1752; lane L, 67-43; lane M, 67-331, lane N, 68-81; lane O, 68-397; lane P, 69-172; lane Q, 69-412; lane R, RN3944 (ermA control); lane S, lambda ladder. (B) Analysis of SmaI restriction fragments of erythromycin-susceptible and erythromycin-resistant isolates of S. aureus analyzed by PFGE. Lane A, 58-362 (erythromycin susceptible); lane B, 58-424 (erythromycin resistant); lane C, 58-431 (erythromycin susceptible); lane D, 58-434 (erythromycin resistant).

harboring *ermA* contained a single insert on an *Eco*RI fragment of 6.0 kb (Table 1). The other seven isolates contained two chromosomal inserts, one of which was always on a 6.0-kb fragment, and the others were located on fragments of either 9.7 kb (six isolates) or 7.0 kb (one isolate). The presence of multiple inserts was confirmed in repeated experiments, ruling out the possibility of partial digestion products. Conversely, *ermB* was located on a 28-kb plasmid on an *Eco*RI fragment of approximately 13 kb as determined by agarose gel electrophoresis (data not shown). This is consistent with the size of the *Eco*RI fragment of pI258 that encodes *ermB*, which is 12.8 kb.

Although resistance to erythromycin in *S. aureus* was reported shortly after this antimicrobial agent was introduced into clinical practice in the 1950s (7, 16), reports of erythromycin resistance in *S. aureus* isolates belonging to the 52/52A/80/81 complex, previously known as the 80/81 complex, are rare (2, 9, 20, 23, 24). Exactly how these strains acquired erythromycin resistance is unknown, but transposons are likely to have played a role since the *ermA* genes were all located in the chromosome and *ermA* is known to be associated with Tn*554* (8, 33). Although Tn*554* has a single, specific insertion site in the chromosome, secondary sites have been identified (18), and patterns similar to those reported here have been noted by Westh et al. (36). Our data, although limited, support the assertion of Westh et al. that *ermA* was the major erythromycin resistance determinant in *S. aureus* prior to 1970. However, based on recent reports, it appears that *ermC* has replaced *ermA* as the most prevalent erythromycin resistance determinant in *S. aureus* in Denmark and elsewhere (11, 15, 33, 35).

Given the variability of the phage typing patterns, the effect of lysogeny on PFGE patterns, and the 12-year time period over which the isolates were collected, we expected to observe significant variation in the PFGE profiles of the erythromycin-resistant isolates, particularly since oxacillin-susceptible isolates of *S. aureus* have been shown to have a multitude of PFGE patterns (6). However, all of the isolates, including the erythromycin-susceptible isolates, had virtually identical PFGE patterns. This, and the varying patterns of the *ermA* chromosomal insertions, suggest that transposons carrying *ermA* entered this *S. aureus* clone on multiple occasions. Although transduction cannot be ruled out as an additional mechanism of gene spread, we feel that this explanation is less likely given the specificity of the Tn*554* insertion sites (18).

Eady et al. reported that human isolates of *S. aureus* containing *ermB* are rare (11). Thus, we were surprised to find this gene among our isolates. The first *ermB* gene was identified on transposon Tn*551* on plasmid pI258 and was subsequently detected on a variety of other plasmids (35). Why *ermB* has not been more widely disseminated among *S. aureus* strains remains unclear.

In summary, we have shown that erythromycin-resistant isolates of *S. aureus* belonging to the 52/52A/80/81 complex recovered from 1958 to 1969 from patients in the United States can harbor either an *ermA* or *ermB* gene. Although the mode of entry of the resistance gene into the *S. aureus* isolates is unclear, these isolates appear to have acquired resistance on multiple occasions in multiple locations in the United States, suggesting that the selective pressure to develop resistance was greater during this early period of antimicrobial use than previously appreciated (1, 8, 34).

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