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Clinical Strain of *Staphylococcus aureus* Inactivates and Causes Efflux of Macrolides

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Searching through a collection of 124 *Staphylococcus aureus* clinical strains, we found one isolate, strain 01A1032, that inactivates 14- and 16-membered macrolides. The products of inactivation were purified from supernatant fluids of cultures exposed to erythromycin for 30 h and were found to be identical to products of inactivation from *Escherichia coli* strains that encode either an EreA or EreB esterase. Further, strain 01A1032 was shown to be resistant to azithromycin, a 15-membered macrolide, by an alternate mechanism, efflux. Thus, strain 01A1032 harbors determinants encoding an esterase activity that hydrolyzes 14- and 16-membered macrolides and a macrolide efflux system.

Macrolide resistance can occur by several mechanisms (30, 31, 49). Clinically, the predominant form of resistance is target modification mediated by one or more *erm* genes (29, 31) encoding a 23S rRNA methylase. The addition of two methyl residues (26, 49, 50) to a highly conserved adenine residue in domain V, the peptidyl transferase center, of 23S rRNA leads to a conformational change in the ribosome, rendering the strain resistant to most macrolides, lincosamides, and streptogramin B compounds (17); phenotypically, this resistance pattern is known as macrolide-lincosamide-streptogramin B (MLS) resistance. The expression of MLS resistance can be inducible or constitutive and is unrelated to the class of the *erm* determinant (29, 48, 50).

Resistance to MLS antibiotics due to inactivation (30) has been described for a number of clinically important organisms, including *Staphylococcus aureus* (which produces a streptogramin A *O*-acetyltransferase [33], a streptogramin B hydrolase [32], or a lincosamide *O*-nucleotidyltransferase [10, 13, 28]), *Staphylococcus haemolyticus* (which produces a lincosamide *O*-nucleotidyltransferase [10, 28]), and *Escherichia coli* (which produces an EreA [1, 3, 6, 40, 42] or EreB [3, 4] esterase or a macrolide 2′-phosphotransferase [23, 39, 41]). Coagulase-negative staphylococcal strains appear to have an MsrA-dependent efflux system that has specificity for 14- and 15-membered macrolides and type B streptogramin molecules but not for lincosamide antibiotics (the macrolide sensitivity [MS] phenotype) (16, 27, 38, 43–45). Further, macrolide-producing organisms such as *Streptomyces ambofaciens* (a spiramycin producer), *Streptomyces thermotolerans* (a carbomycin producer), and *Streptomyces fradiae* (a tylosin producer) have macrolide-resistant genes that appear to encode efflux determinants (46). The sequences are homologous to MsrA and other members of a superfamily of related proteins that contain two conserved ATP-binding sites and are involved in transport (19). Although not all mechanisms have been found in all species, it is clear that reservoirs of these resistance mechanisms are present and that a horizontal spread of the different types of resistance can occur in nature (12).

In a recent study of macrolide resistance in five different species of coagulase-negative staphylococci (16), *ermC* was found to be the most widely distributed resistance mechanism (occurring in 50.6% of strains), either alone or in combination with a second *erm* determinant. *ermA* or *ermB* was present in a minority of the strains—5.9 and 7.2% of strains, respectively—whereas 33% of the isolates contained *msrA*.

Janosi et al. (20) found that 25.6% of the macrolide-resistant *S. aureus* strains isolated from 27 geographical regions in Hungary over a 12-year period manifested the MS phenotype (these authors refer to the phenotype as PMS for partial macrolide sensitivity). One strain was shown to have a 63-kDa membrane protein not present in erythromycin-sensitive strains (36); recently, 31 amino acids that compose the N terminus of the membrane protein were found to be identical to those of MsrA (35). A New Jersey hospital study (22) did not find any erythromycin-resistant *S. aureus* strains with the MS-resistant phenotype but did find that 7.3% of the erythromycin-resistant coagulase-negative staphylococci have this phenotype.

Epidemiological studies with 428 erythromycin-resistant *S. aureus* strains collected from 1959 to 1988 in Denmark revealed that *ermA* was solely responsible for erythromycin resistance until 1971 (51), with the *ermC* determinant becoming dominant between 1984 and 1988. Interestingly, there were six strains in which neither *ermA* nor *ermC* was found. Four of these six strains were resistant to erythromycin only (putative MS phenotype), while two of these strains had a constitutive MLS-resistant phenotype.

We initiated studies to characterize the macrolide resistance mechanisms in our collection of erythromycin-resistant *S. aureus* strains. The majority of these strains contained one or more *erm* genes; however, one strain, 01A1032, had an MS phenotype. The characterization of strain 01A1032 revealed the presence of both an efflux resistance determinant (*msrA*) (55) and an inactivating activity (54). We report that the product of erythromycin inactivation in *S. aureus* 01A1032 is identical to that of *E. coli* strains containing either *ereA* or *ereB* and that macrolide efflux in strain 01A1032 appears to be mediated by an Msr component system.

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MATERIALS AND METHODS

Chemicals. Erythromycin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), sodium arsenate, and all protein translation reagents except tRNA and radiolabeled lysine were purchased from Sigma Chemical Co. (St. Louis, Mo.). Dithiothreitol (DTT) and tRNA were purchased from Boehringer Mannheim (Indianapolis, Ind.). The streptogramin B analog CP-37277 was a fermentation product isolated from *Actinoplanes auranticolor* ATCC 31011 (11) at Pfizer Central Research (Groton, Conn.). Azithromycin (14), 4″-epiazithromycin (8), azithromycin 11,12 carbonate (47), isoazithromycin (53), erythromycin A 11,12 carbonate (47), and clarithromycin 11,12 carbonate (7) were prepared as described in the above-indicated references at Pfizer Central Research. Erythromycin (A, B, and D) and oleandomycin were fermentation products produced at Pfizer Central Research. Clarithromycin (Biaxan; Abbott) was purchased from Northeast Medical Products (Old Saybrook, Conn.). Spiramycin, josamycin, and rosaramicin were obtained from Sigma Chemical Co. Brain heart infusion (BHI) medium and antibiotic disks containing either clindamycin or erythromycin were purchased from Difco Laboratories (Detroit, Mich.). BHI and blood agar plates were purchased from Remel Microbiology Products (Lenexa, Kans.). PCR reagents were supplied by the following manufacturers. All DNA primers were purchased from GenoSys Biotechnologies (The Woodlands, Tex.), DNA polymerase was supplied by Perkin-Elmer (Danbury, Conn.), 10× deoxynucleotide mixture was supplied by Gibco BRL (Gaithersburg, Md.), and $MgCl_2$ solution was supplied by Sigma Chemical Co. ReadySafe cocktail was purchased from Beckman (Fullerton, Calif.). Potassium phosphate, sodium chloride, and organic solvents were purchased from Fisher Scientific (Fair Lawn, N.J.).

Radiochemicals. [*N-methyl*-^{14}C]erythromycin (55 mCi/mmol) was purchased from DuPont, NEN Research Products (Boston, Mass.). 9-Deoxo-9a-aza-9a-[^{14}C]*methyl*-9a-homoerythromycin A ([^{14}C]azithromycin, >98% purity, 14.0 mCi/mmol) was prepared by Pfizer Nagoya New Products Development Center (Nagoya, Japan). Radiolabeled aqueous formaldehyde (DuPont, NEN Research Products) was used to methylate the 9a-aza site of the precursor 9-deoxo-9a-homoerythromycin A (9) by a modified Clarke-Eschweiler procedure. [^{14}C]lysine (312 mCi/mmol) was purchased from Amersham (Arlington Heights, Ill.). MicroScint 20 was purchased from Packard (Meriden, Conn.).

Bacterial strains. The majority of staphylococcal strains (124 isolates) were obtained from hospitals in Connecticut, Massachusetts, Illinois, California, Virginia, and Washington between 1987 and 1994. Eight *S. aureus* strains were collected between the years 1969 and 1978. *Staphylococcus epidermidis* S1187 (45) and *E. coli* XL-1 Blue containing the 1.9-kb *Hin*dIII fragment of *msrA* (44) were obtained by courtesy of Anne Eady (University of Leeds). *E. coli* BM694 (pAT63) (*ereA*$^+$ on pBR322-derived plasmid) (42) and BM694(pAT72) (*ereB*$^+$ on pUC8-derived plasmid) (4) were a gift from Patrice Courvalin (Institut Pasteur, Paris, France). An erythromycin-sensitive *S. aureus* strain from the Pfizer collection, 01A0052, was used in experiments to determine residual macrolide antibacterial activity. Two erythromycin-resistant *S. aureus* control strains were used: strain 01A0110 (Pfizer strain collection), which contains *ermA* and *ermB* determinants, and strain SK983 (RN1786 containing pSK270, obtained from Saleem Kahn, University of Pittsburgh School of Medicine), which contains an inducible *ermC* determinant on pSK270.

Strain characterization. All strains were screened for MLS resistance by an agar disk diffusion assay. BHI broth was inoculated with several colonies and incubated overnight with shaking at 37°C. The cultures were diluted 1:10 in phosphate-buffered saline and swabbed onto BHI agar plates. Clindamycin (2 μg), erythromycin (15 μg), and CP-37277 (25 μg) disks were placed on the plates approximately 12 mm apart. The plates were incubated overnight at 37°C.

Antibiotic inactivation. Strains were grown overnight in BHI containing 5 μg of erythromycin per ml with shaking at 37°C. After harvesting by centrifugation, the cells were resuspended to 1/10 of the original volume in 0.1 M potassium phosphate buffer, pH 7.0. A 96-well microtiter plate containing test compounds (with final concentrations of either 40 or 400 μg/ml) was prepared, and 200 μl of the concentrated cell suspension was added per well. The plates were incubated with gentle shaking at 37°C, and aliquots were removed at 24 and 48 h. Following centrifugation, a portion of the supernatant (25 μl) was spotted onto sterile disks and placed onto BHI agar plates swabbed with an erythromycin-sensitive strain, *S. aureus* 01A0052. The plates were incubated overnight at 37°C. Zone sizes were compared with zone sizes produced when the antibiotic was incubated with either medium alone or with *S. aureus* 01A0052.

Inactivation was also determined with radiolabeled erythromycin. Five milliliters of BHI containing 0.02 μg of erythromycin per ml was inoculated with an overnight culture to an optical density at 600 nm (OD_{660}) of 0.05 and allowed to grow to an OD_{660} of 0.35. [^{14}C]erythromycin (0.2 μg/ml) was added, and the tubes were allowed to shake at 37°C overnight. At various times after the addition of the radiolabeled substrate, a 1-ml aliquot was centrifuged to remove cells and the supernatant was adjusted to pH 8.5 with 1 M KOH. One milliliter of hexane-ethyl acetate (1:1) was added to the vial containing the basified supernatant fluid, and the material was shaken for 15 min at room temperature. After two layers formed, 0.1 ml of the upper phase was removed, added to six ml of scintillation fluid, and mixed vigorously. Radioactivity was determined by liquid scintillation counting with a Packard 1500 Tri-Carb scintillation analyzer. The loss of counts from the upper phase indicated inactivation of erythromycin.

Purification and characterization of the products of erythromycin inactivation. To produce sufficient quantities of the products from the inactivation of erythromycin, the bioassay conditions were scaled upward. Typically, 1 liter each of *S. aureus* 01A1032, *S. aureus* 01A0052, *E. coli* BM694(pAT63), and *E. coli* BM694(pAT72) were grown in BHI. After harvesting by centrifugation, the cells were resuspended in 200 ml of 0.1 M potassium phosphate buffer, pH 7.0. Erythromycin (200 mg in 5 ml of ethanol) was added to the cell suspension, and the resultant mixture was incubated at 37°C for 30 h. The isolation of inactivated erythromycin from the cell-free supernatant followed the procedure of Barthélémy et al. (6), with slight modifications (Fig. 1). The supernatant fluid (final pH, ~5.5 to 6.5) was adjusted to pH 8.5 with saturated potassium carbonate solution and extracted with chloroform (2 × 100 ml). The aqueous layer was neutralized to pH 7.0 with 6 N HCl and repeatedly extracted with *n*-butanol (4 × 70 ml). The *n*-butanol extract, after evaporation, gave the crude acid, the hydrolysis product from erythromycin (structure III in Fig. 1; hereafter, Roman numerals refer to the structures in Fig. 1). To aid characterization, the crude acid was methylated with diazomethane generated from the basic decomposition of 1-methyl-3-nitro-1-nitrosoguanidine (2). The resulting methyl ester (structure IV) was purified by column chromatography (silica gel, with a solvent mixture of $CHCl_3$-CH_3OH-NH_4OH [89:10:0.5] as eluent) and characterized by ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, and fast atom bombardment mass spectrometry.

Macrolide efflux by actively growing cells. Efflux assays were done by monitoring the uptake of radiolabeled erythromycin into cells growing exponentially. Thirty milliliters of BHI containing 0.02 μg of erythromycin per ml was inoculated with an overnight culture of the desired strain to an OD_{660} of 0.05. After the OD_{660} reached 0.35, the culture was exposed to CCCP (25 μM) or arsenate (10 mM) for 10 min and then 0.2 μg of [^{14}C]erythromycin or [^{14}C]azithromycin per ml was added. Samples (4.0 ml) were removed at designated times and filtered through Whatman GF/C glass microfiber filters previously wetted with 0.9% sodium chloride and 1 μg of erythromycin per ml. Filters were washed twice with the sodium chloride-erythromycin mixture (a total of 20 ml) and dried under a heat lamp for 60 min. Radioactivity was determined by liquid scintillation counting.

PCR. PCR amplification of DNA was performed in a final volume of 20 μl as described by Mabilat et al. (34). Primers were added at a final concentration of 0.25 μM, and the PCR cycle consisted of a denaturation step (95°C for 3 min) followed by 35 amplification cycles (elongation at 72°C for 90 s, denaturation at 93°C for 30 s, and annealing at 55°C for 2 min). Primers based on the published sequences of *msrA* (44) and *msrB* (38) were 5′-GCAAATGGTGTAGGTAA GACAACT-3′ and 5′-ATCATGTGATGTAAACAAAAT-3′. Boiled bacterial colonies were used as the source of crude DNA. Five to 20 colonies growing on blood agar plates were selected and suspended into 1 ml of preheated Tris buffer, pH 7.6, containing 10 mM EDTA and boiled for 1 h (18). The tubes were placed on ice and vortexed lightly. The boiled cell mass was diluted 1:10 in autoclaved H_2O, and 2 μl was used per PCR reaction mixture.

In vitro protein translation. *S. aureus* 01A1032 (6 liters) was grown to late log phase in minimal A medium (37) supplemented with 10% BHI and 5 μg of erythromycin per ml at 37°C. The cells were harvested by centrifugation and washed with 10 mM Tris acetate (pH 8.0)–14 mM magnesium acetate–1.0 M potassium acetate–1 mM DTT. After a second wash (in the same buffer, but with 60 mM potassium acetate), the cell pellets were frozen at −70°C. When needed, cells were thawed and resuspended in 1% of the original volume of lysis buffer (10 mM Tris acetate [pH 8.0], 20 mM magnesium acetate, 60 mM potassium acetate, 1 mM DTT). Lysostaphin (80 U/ml) was added, and the cell suspension was incubated at 37°C for 45 min. After the addition of DNase (10 μg/ml), the suspension was placed on ice for 15 min and the DTT concentration was adjusted to 3.0 mM. The cell lysate was centrifuged at 30,000 × *g* for 30 min. The upper three-fourths of the supernatant was dialyzed (Spectra/Por membrane; molecular weight cutoff, 12,000 to 14,000) against 4.0 liters of 10 mM Tris acetate (pH 8.0)–14 mM magnesium acetate–60 mM potassium acetate–1 mM DTT for 4 h with two changes of buffer. The dialysate was subjected to ultracentrifugation for 4 h at 150,000 × *g*. The supernatant (S150 fraction) was quickly frozen in a dry ice-ethanol bath and stored at −70°C. The pellet, containing ribosomes, was resuspended in dialysis buffer and subjected to ultracentrifugation. The washed pellet was resuspended in dialysis buffer to an OD_{260} of 670, quickly frozen in 0.1-ml aliquots, and stored at −70°C.

Poly(A)-directed protein synthesis was performed in a final volume of 100 μl containing 45 mM Tris acetate (pH 8.0), 55 mM potassium acetate, 30 mM ammonium acetate, 15 mM magnesium acetate, 16 mg of polyethylene glycol 6000 per ml, 1.5 mM DTT, 400 μg of tRNA per ml, 20 mM phosphoenolpyruvate, 30 μg each of pyridoxine, NAD, flavin adenine dinucleotide, and folinic acid, 10 μg of *p*-aminobenzoic acid per ml, 2.3 mM ATP, 0.6 mM each CTP, GTP, and UTP, 13 μM [^{14}C]lysine, the S150 fraction (300 μg of protein), and ribosomes (0.025 μl). All of the components were prepared as a supermixture and chilled on ice. Dilutions of erythromycin and azithromycin were prepared in the wells of a microtiter plate. Poly(A) was heat denatured at 95°C for 5 min, cooled on ice for 5 min, and added to the microtiter plate at a final concentration of 500 μg/ml. The reaction was started with the addition of the supermixture. The plate was gently shaken for 45 min at 37°C. The reaction was terminated with the addition of 150 μl of cold 5% trichloroacetic acid–0.05% sodium tungstate–0.01% lysine. After overnight refrigeration, the plate was filtered with a Packard Unifilter 96 Harvester onto a GF/B filter plate previously wetted with 5% tri-

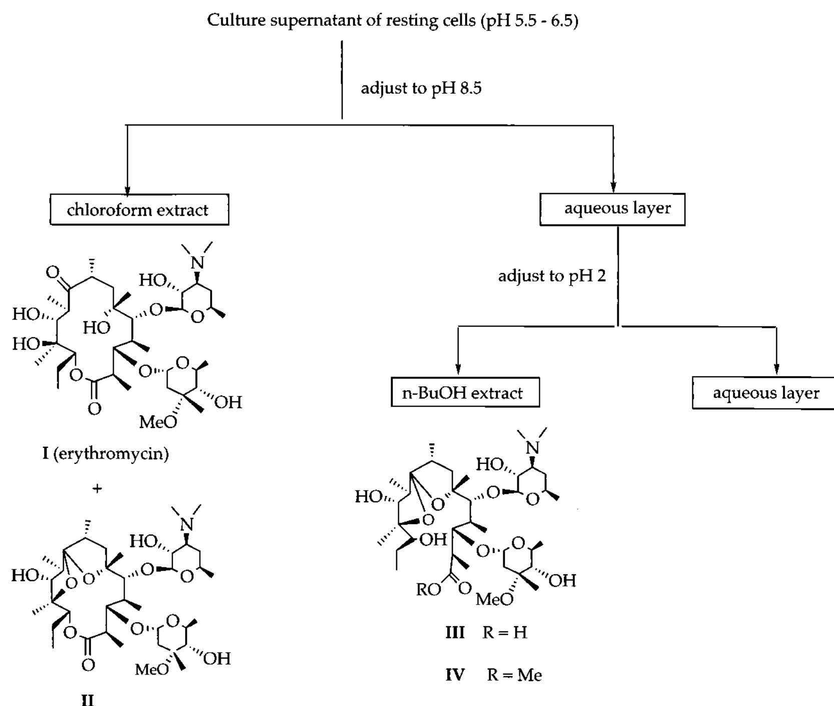

FIG. 1. Scheme for the isolation of the hydrolysis products of erythromycin. n-BuOH, *n*-butanol; Me, methyl.

chloroacetic acid–0.05% tungstate–0.01% lysine. The filter plate was washed three times with 5% trichloroacetic acid–0.05% tungstate and then washed once with 100% ethanol. After drying for 2 min under Unifilter 96 Harvester vacuum, 60 μl of MicroScint 20 scintillation fluid was added per well and the plate was sealed. Radioactivity was determined by liquid scintillation counting with a Packard TopCount microplate scintillation counter.

RESULTS

Phenotypic analyses of *S. aureus* strains. By the agar disk diffusion assay, 124 strains of staphylococci were evaluated for inducible or constitutive MLS resistance or MS resistance. The blunting of the zones around clindamycin or streptogramin B indicated inducible resistance to these compounds (29, 31), whereas constitutive resistance was indicated by no zones to clindamycin (lincosamide) and CP-37277 (a streptogramin B analog) and either a small zone or no zone to erythromycin. Sixty-three percent of the strains were constitutively MLS resistant, while 36% were inducibly MLS resistant. Only one isolate, strain 01A1032, was found to have an MS phenotype, in that it was resistant to erythromycin, inducibly resistant to streptogramin B, and susceptible to clindamycin.

Strain 01A1032 inactivates erythromycin. All of the strains were evaluated for their abilities to inactivate erythromycin by both a bioassay and a radiolabeled assay employing [^{14}C]erythromycin and the differential extraction of culture supernatants (see Materials and Methods for details). Strain 01A1032 was the only strain that appeared to inactivate erythromycin by either assay. Although no early inactivation could be demonstrated in the bioassay, the more sensitive radiolabeled assay showed that significant inactivation occurs after 2 h (33% inactivation) (Fig. 2). Inactivation of erythromycin by 01A1032 is virtually complete after 14 h of incubation, whereas erythromycin remains intact when incubated in medium (BHI), with an erythromycin-sensitive strain (strain 01A0052) or with a strain that contains both *ermA* and *ermB* methylases (strain 01A0110).

Identification of inactivated erythromycin by strain 01A1032. By the protocol described in Materials and Methods, intact erythromycin I and the spiroketal of erythromycin (structure II in Fig. 1; also see Table 1) were found exclusively in the chloroform layer of the culture supernatant of *S. aureus* 01A0052, the erythromycin-susceptible control strain. In the cases of *S. aureus* 01A1032, *E. coli* BM694(pAT63), and *E. coli* BM694 (pAT72), very little or nothing was recovered in the chloroform layer. The major product was enzymatically modified erythromycin obtained solely from the butanol extract. The crude product (from the *n*-butanol extract) isolated from *S. aureus* 01A1032 was identical to the inactivation product obtained from *E. coli* strains containing either the EreA or EreB

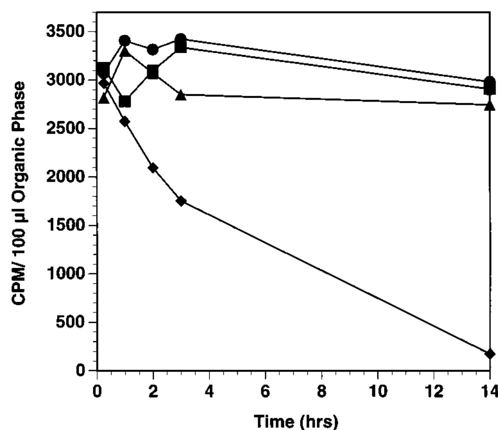

FIG. 2. Inactivation of erythromycin by *S. aureus* 01A1032. [^{14}C]erythromycin was incubated with BHI broth (■), an erythromycin-sensitive *S. aureus* strain, 01A0052 (●), a constitutive MLS-resistant *S. aureus* strain, 01A0110 (▲), or *S. aureus* 01A1032 (◆). The amount of intact erythromycin was monitored by tracing the depletion of the radiolabeled erythromycin from the organic phase.

TABLE 1. Measures of hydrolysis products from *E. coli* and *S. aureus* erythromycin inactivation

Strain	Esterase[a]	MIC of erythromycin (μg/ml)	Yield (%) of isolated product[b]:	
			I and II (in chloroform)	III (in *n*-butanol)
E. coli BM694(pAT63)	*ereA*	>500	NC[c]	NC
E. coli BM694(pAT72)	*ereB*	>500	0	63
S. aureus 01A0052	−	0.2	61	0
S. aureus 01A1032	+	100	0	28

[a] +, esterase activity present; −, no detectable esterase.
[b] Yields of III were calculated on the basis of the purified methyl ester IV. Roman numerals refer to the structures in Fig. 1.
[c] NC, not calculated. The acid product was not derivatized to its methyl ester; therefore, no accurate yield can be calculated. However, from the mass recovery and spectral analyses, III is the major product while I and II are present in minor amounts.

esterase, as indicated by proton NMR. Its molecular weight by fast atom bombardment mass spectroscopy (*m/e*, 735 [MH^+]) and its R_f value by thin-layer chromatography are in agreement with the data from a previous study (6) for structure III (Fig. 1).

When erythromycin (200 mg) was incubated with an erythromycin-sensitive strain, *S. aureus* 01A0052, 125 mg (61% yield) of the spiroketal of erythromycin and intact erythromycin were obtained from the chloroform extract and no modified erythromycin was found in the butanol solution (Table 1). Erythromycin spiroketal II (Fig. 1), found in the chloroform phase with intact erythromycin I (Fig. 1), was formed through acid degradation of erythromycin (25, 52) as a result of the drop in pH (5.5 to 6.5) of the culture medium during the course of incubation. As a control, erythromycin was incubated in the same medium in the absence of bacteria. Erythromycin spiroketal II and intact erythromycin I (at ratio of 3/1) were recovered quantitatively from the chloroform extract. No modified erythromycin was found in the butanol solution. These results confirm that erythromycin spiroketal II is formed in an acidic environment and that enzymatic activity from *S. aureus* 01A1032 is responsible for the hydrolysis of the macrocyclic nucleus, yielding structure III.

For further characterization, the acid products recovered after the inactivation of erythromycin by *E. coli* BM694(pAT72) and *S. aureus* 01A1032 were derivatized to their methyl esters. When a portion (92 mg) of the crude product III (598 mg, which includes the initial weight of erythromycin plus the additional weight from particulates) isolated from the incubation of erythromycin (500 mg) with *E. coli* BM694(pAT72) was treated with diazomethane (CH_2N_2) in ethyl ether, methyl ester IV (55 mg) was obtained after chromatographic purification. Thus, the yield of inactivation (the formation of IV) is 63%, assuming that methylation was quantitative. Similarly, 45 mg from a total of 283 mg of crude product III isolated from the *S. aureus* 01A1032 incubation mixture provided 9 mg of IV, resulting in a 28% product yield from inactivating erythromycin. The spectrometric data (^{1}H NMR, R_f value by thin-layer chromatography) confirm that the products from these two bacterial cultures are identical and are in agreement with structure IV (Fig. 1).

By thin-layer chromatography, compound IV gave an R_f value of 0.50 (silica gel, with a solvent mixture of $CHCl_3$-CH_3OH-NH_4OH [89:10:0.5]), whereas that of structure III was 0. As expected, the acid ester is less polar than its parent acid.

For comparison, ^{13}C NMR spectra of erythromycin I and erythromycin spiroketal II were also measured. The ^{13}C NMR spectrum of IV indicated the absence of a ketone carbon (C-9 of erythromycin). In II, a spiroketal carbon (-O-C-O-) is seen at 113.2 ppm (C-9, quaternary), with C-9 appearing at 116.0 ppm. An extra methoxy-carbon of IV (C-1, -CO-OCH_3) was observed at 51.5 ppm in addition to 3″-OCH_3 at 49.3 ppm, whereas structure II has only one methoxy-carbon (3″-OCH_3 at 49.5 ppm). A single peak at 3.64 ppm in the ^{1}H NMR spectrum of IV was assigned to the methyl protons of -CO-OCH_3. The mass spectroscopic analysis of IV was as follows: by fast atom bombardment high-resolution mass spectrometry, *m/e* was 748.4845 (M^+ plus H, $C_{38}H_{70}NO_{13}$ requires 748.4828).

It should be noted that under the conditions of our protocol, the spiroketal of the acid (III) was the only inactivation product obtained. Barthélémy et al. found another product (at pH 8) in addition to compound III (6). However, at the lower pH, the exclusive formation of the product (III) is in accordance with their observations.

The lower yield of the inactivation product with *S. aureus* 01A1032 may be a reflection of a weak esterase activity. Alternatively, our conditions may not be optimal for the expression of the esterase in *S. aureus* 01A1032.

***S. aureus* 01A1032 inactivates 14- and 16-membered macrolides.** With a bioassay format, the substrate specificity of *S. aureus* 01A1032 was explored and compared with the substrate specificity of *E. coli* strains containing either *ereA* or *ereB*. All three strains inactivate all the 14-membered macrolides tested: erythromycin A, erythromycin B, erythromycin D, erythromycin A 11,12 carbonate, oleandomycin, clarithromycin, and clarithromycin 11,12 carbonate (data not shown). However, *S. aureus* 01A1032 did not inactivate azithromycin or 4″-epiazithromycin, 15-membered macrolides, whereas the two *E. coli* strains did (Table 2). Interestingly, the 11,12-carbonate derivative of azithromycin is a substrate for the esterases in all three strains while azithromycin's 8a-*N* regioisomer, isoazithromycin, is not a substrate for any of the esterases. Strain 01A1032 inactivated spiramycin, josamycin, and rosaramicin, all of which are 16-membered macrolides, providing a distinguishing pheno-

TABLE 2. Inactivation of 15-membered macrolides

Compound	—X–Y—	R_1	R_2	C-4″	Inactivation status[a] by		
					E. coli EreA	*E. coli* EreB	*S. aureus* 01A1032
Azithromycin	—N(Me)—C(H)(H)—	H	H	α-OH	+	+	−
4″-Epiazithromycin	—N(Me)—C(H)(H)—	H	H	β-OH	+	+	−
Azithromycin 11,12-carbonate	—N(Me)—C(H)(H)—	—C(=O)—		α-OH	+	+	+
Isoazithromycin	—C(H)(H)—N(Me)—	H	H	α-OH	−	−	−

[a] +, inactivation; −, no inactivation.

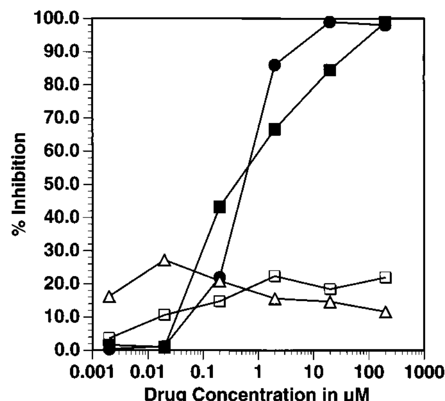

FIG. 3. Susceptibility of translation to erythromycin and azithromycin. An in vitro translation system was reconstituted with purified ribosomes and S150 fractions from either *S. aureus* 01A1032 (closed symbols) or SK983 (open symbols). The sensitivity of each system to erythromycin (■, △) or azithromycin (●, □) was determined.

type from the *E. coli* esterase-producing strains that did not hydrolyze these representative 16-membered macrolides (data not shown).

Identification of *msrA* in strain 01A1032. Strain 01A1032 does not inactivate azithromycin, but the azithromycin MIC (>100 μg/ml) indicated that this strain was resistant to the 15-membered macrolide. To determine if resistance to azithromycin was mediated by a change in a ribosomal protein or an alteration in rRNA, ribosomes and S150 fractions from *S. aureus* 01A1032 were isolated. Reconstitution of these two components yielded a translation system that was fully susceptible to inhibition by erythromycin or azithromycin (their 50% inhibitory concentrations were 0.4 and 0.55 μM, respectively) (Fig. 3). Translation mediated by components from strain SK983, an *S. aureus* strain that contains *ermC* on a resident plasmid, was resistant to erythromycin and azithromycin (with 50% inhibitory concentrations of >200 μM). Thus, it appeared that strain 01A1032 did not have any mutations at the level of the ribosome that resulted in erythromycin or azithromycin resistance.

The efflux of erythromycin and azithromycin have been described in coagulase-negative staphylococci with an MS phenotype. To verify if *S. aureus* 01A1032 had an efflux determinant, we assessed the uptakes of radiolabeled erythromycin and azithromycin into intact, exponentially growing cells. This strain could import the macrolides maximally only when the cells were pretreated with either 25 μM CCCP (Fig. 4) or 10 mM arsenate (data not shown). These results suggest that strain 01A1032 contains an efflux determinant that recognizes both erythromycin and azithromycin as substrates.

To determine if strain 01A1032 contains a macrolide efflux system that uses an *msrA*-like determinant, PCRs using oligonucleotide primers designed to *msrB* (38) and to an identical sequence in the carboxy-terminal half of *msrA* (44) were performed. The primer set gave the expected 0.4-kb PCR product with DNA from strain 01A1032, consistent with an *msrA*-like gene being present in this strain (data not shown). Further, primers that encompassed nearly the entire coding region of *msrA* confirmed the presence of *msrA* in the *S. aureus* strain (data not shown). The absence of an *erm* gene was confirmed with degenerate primers (5) designed to detect *ermA*, *ermB*, *ermC*, and *ermG* (data not shown).

DISCUSSION

Macrolide-inactivating enzymes have been detected in clinical and macrolide-producing strains and include enzymes that modify macrolides via phosphorylation (23, 39, 41) or glycosylation (21, 24) of the 2′-OH in the desosamine moiety. Inactivation by esterases has been described for gram-negative members of the family *Enterobacteriaceae* (3, 4, 6, 40, 42). The lactone ring of erythromycin A readily undergoes hydrolysis catalyzed by either the EreA or EreB esterase. Although there have been no reports of esterase activity in gram-positive organisms, EreB has the GC content and codon preference of a gram-positive strain (4). We therefore reasoned that an esterase capable of hydrolyzing macrolides might be present in some strains of erythromycin-resistant staphylococci. By using assays that detect erythromycin inactivation, one of 124 strains of *S. aureus* was found to inactivate 14- and 16-membered macrolides. Products isolated from the inactivation of erythromycin by *S. aureus* 01A1032 revealed that they were identical to the products obtained from strains of *E. coli* containing an EreA or EreB esterase. Thus, strain 01A1032 is the first gram-positive strain to be described with an inactivation determinant for macrolides and, more specifically, an esterase.

The esterase activity of *S. aureus* 01A1032 has a substrate specificity different from those of the previously described *E. coli* EreA and EreB esterases. The gram-positive esterase predominantly hydrolyzes 14- and 16-membered macrolides, whereas the gram-negative esterases hydrolyze 14- and 15-membered macrolides exclusively. However, when azithromycin is

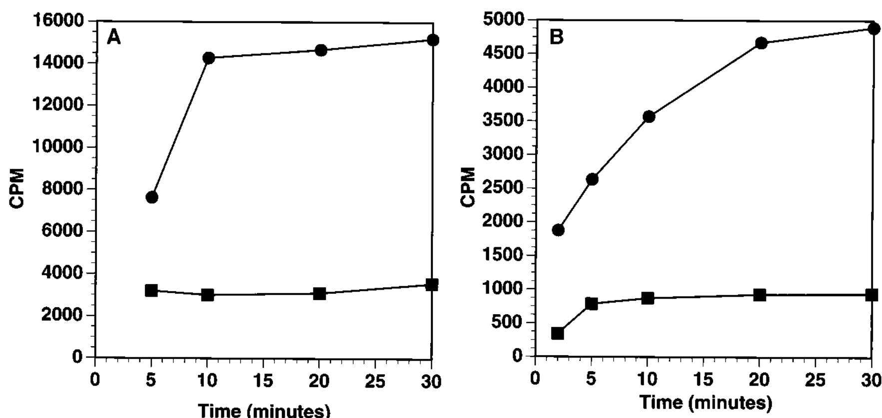

FIG. 4. Uptake and efflux of [^{14}C]erythromycin (A) and [^{14}C]azithromycin (B) by *S. aureus* 01A1032 in the presence (●) or absence (■) of 25 μM CCCP.

modified to its 11,12-carbonate analog, a more promiscuous substrate results; this derivative appears to be inactivated by the esterase from *S. aureus* as well as by the enteric esterases. Resistance to azithromycin in strain 01A1032 appears to be mediated by another mechanism, namely, efflux.

The deduced amino acid sequence of one of the efflux determinants from coagulase-negative staphylococci, *msrA*, reveals the presence of two ATP-binding motifs separated by a long Q-linker, an interdomain region comprising hydrophilic and hydrophobic amino acids in the characteristic spacing found in prokaryotic multidomain proteins (19, 44). The two ATP-binding domains of MsrA are homologous to the highly conserved ATP-binding cassettes of a family of transport-related proteins in prokaryotes and eukaryotes described as ABC (ATP-binding cassette) transporters (19, 44). Coagulase-negative staphylococci with an MS phenotype treated with dinitrophenol or arsenate differentially take up radiolabeled erythromycin (44); these results help to substantiate that MS strains contain an ATP-dependent efflux pump. The C-terminal domain of MsrA is required for erythromycin resistance, as deletion of the 42 C-terminal codons from a 1.9-kb cloned fragment from *S. epidermidis* results in the loss of the resistance phenotype (44). A strain of *Staphylococcus xylosus* that contains an erythromycin determinant (named *msrB*) 100% homologous to the carboxy-terminal ATP-binding cassette of MsrA has been described previously (38). Although this strain was not characterized for its uptake of macrolides, erythromycin resistance in the absence of susceptibility to lincomycin was transferable by the single open reading frame encoding a 21-kDa protein, MsrB. These results suggest that efflux may be mediated by a single ATP-binding domain; alternatively, if two motifs are requisite, the MsrB protein could satisfy the requirement if it functioned as a dimer.

Recently, two chromosomal genes in *S. aureus*, *stpC* and *smpC*, have been identified as components that may interact with an imported *msrA* gene from coagulase-negative staphylococci to form a functional efflux system for macrolides and streptogramin B (43). The StpC and SmpC proteins are 85 and 65% identical to StpA and SmpA, respectively, two gene products encoded by a region adjacent to *msrA* in the *S. epidermidis* plasmid from which the *msrA* determinant was originally cloned and sequenced (44). *stpA* and *stpC* encode ATP-binding proteins homologous to MsrA, while *smpA* and *smpC* encode hydrophobic transmembrane proteins, the putative pumps. Apparently, the presence of *stpC* and *smpC* on the chromosome of *S. aureus* RN4220, an erythromycin- and streptogramin B-sensitive cloning host, may substitute for the StpA and SmpA proteins encoded by the original plasmid conferring MS resistance. Presently, it is unclear what role each protein plays in the functional efflux system; however, it is clear that MsrA is needed to confer the MS phenotype.

Strain 01A1032 was the only strain in our collection to have an MS phenotype, reminiscent of the phenotype described in coagulase-negative staphylococci (and more recently described in an *S. aureus* strain of Hungarian origin; [35]), with an efflux determinant, *msrA*. Therefore, it seemed plausible that strain 01A1032 might contain an efflux determinant. Indeed, the strain was found to import more erythromycin and azithromycin when cells were pretreated with arsenate or CCCP. Also, translation mediated by ribosomes isolated from strain 01A1032 was completely susceptible to inhibition by erythromycin and azithromycin. Finally, strain 01A1032 was shown to contain an *msrA*-like gene with PCR primers specific for *msrA* and *msrB*. Therefore, it appears that strain 01A1032 is resistant to azithromycin, a 15-membered macrolide, and erythromycin, a 14-membered macrolide, by virtue of an ATP- and MsrA-dependent efflux pump.

It is unclear if the two phenotypes, macrolide inactivation by an esterase and efflux of erythromycin and azithromycin by an MsrA-dependent pump, are part of the same mechanism or two separate (and perhaps synergistic) mechanisms. Within the sensitivity of our assay conditions, erythromycin efflux could be separated from inactivation by an esterase-mediated hydrolysis. No hydrolysis was apparent during the time course of efflux (30 min) in strain 01A1032. Further, even with longer periods of exposure to concentrated cultures of strain 01A1032, azithromycin remained intact. However, final clarification of the phenotypes awaits the cloning and purification of the erythromycin-resistant determinant(s) in strain 01A1032.

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