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Single- and Multistep Resistance Selection Studies on the Activity of Retapamulin Compared to Other Agents against *Staphylococcus aureus* and *Streptococcus pyogenes*

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Retapamulin had the lowest rate of spontaneous mutations by single-step passaging and the lowest parent and selected mutant MICs by multistep passaging among all drugs tested for all *Staphylococcus aureus* strains and three *Streptococcus pyogenes* strains which yielded resistant clones. Retapamulin has a low potential for resistance selection in *S. pyogenes*, with a slow and gradual propensity for resistance development in *S. aureus*.

Pleuromutilins are a new class of antimicrobials which inhibit protein synthesis by interacting at a unique site on the 70S ribosome and demonstrate excellent in vitro activity against gram-positive and some gram-negative bacteria (2, 5, 9). This study used multi- and single-step passage studies to test the ability of retapamulin (Fig. 1), compared to those of mupirocin, fusidic acid (only against *Staphylococcus aureus* strains), cephalexin, erythromycin, linezolid, vancomycin, and quinupristin-dalfopristin, to select for resistance in 12 *Staphylococcus aureus* and 10 *Streptococcus pyogenes* strains.

S. aureus strains comprised three methicillin- and quinolone-susceptible, four methicillin- and quinolone-resistant, three vancomycin-intermediate, and two vancomycin-resistant (VRSA) strains. The 10 *S. pyogenes* strains comprised two macrolide-susceptible strains, two *erm*(B)-, three *mef*(A)-, and two *erm*(TR)-positive strains, and one strain with a mutation in the L4 ribosomal protein (68KGT insertion). Retapamulin and mupirocin powders were obtained from GlaxoSmithKline, Collegeville, Pa., and other drugs were obtained from their respective manufacturers. Initial MICs were determined by the CLSI agar dilution method (7).

Multipassage resistance selection was done as described previously (3, 6). Daily passages were continued until a more-than-four-fold increase in the MIC was found (minimum passage number, 14; maximum passage number, 50). If MICs of ≥32 μg/ml were found, subculturing in the presence of an antibiotic ceased. Prolonged selection for the full 50 days was conducted for three random staphylococcal strains and all three streptococcal strains showing retapamulin resistance development. The stability of acquired resistance was determined by MIC determination after 10 daily passages on drug-free agar.

The frequency of spontaneous single-passage mutations was determined as described previously (3, 6). Pulsed-field gel electrophoresis was used to confirm the identities of all parents and clones (3, 6).

Genes encoding the L3 ribosomal protein were amplified and sequenced from all parents and from clones for which an elevation in the retapamulin MIC was observed by the multipassage study. Eight *S. aureus* and seven *S. pyogenes* single-passage mutants were randomly selected for L3 analysis. The genes encoding ribosomal proteins L4 and L22 and domains II and V of 23S rRNA were amplified and sequenced for all *S. aureus* and *S. pyogenes* macrolide-resistant parents and randomly selected macrolide-resistant clones obtained by multipassage (10, 11). For five selected *S. aureus* fusidic acid-resistant clones and parent strains, sequencing analysis of the *fusA* gene, encoding the EF-G protein, was performed, and the presence of the *fusB* determinant was tested (8). The mechanism of mupirocin resistance was tested with five selected *S. aureus* and all resistant *S. pyogenes* clones by sequencing portions of the *ileS* gene, encoding isoleucyl-tRNA synthetase (IRS) (1).

The multipassage results with *S. aureus* are presented in Table 1. Retapamulin MICs rose from 0.03 to 0.125 μg/ml (parents) to 0.5 to 2 μg/ml after 14 to 20 days for 12/12 strains (3 strains chosen for prolonged selection for 50 days had MICs that rose to 4 to 16 μg/ml) (Table 1); quinupristin-dalfopristin MICs rose from 0.25 to 0.5 μg/ml (parents) to 2 to >4 μg/ml after 14 to 28 days for 12/12 strains; erythromycin MICs rose from 0.5 to 1 μg/ml to 16 to >64 μg/ml after 11 to 17 days for 5/5 strains; linezolid MICs rose from 2 to 4 μg/ml to 16 to >32 μg/ml after 11 to 46 days for 8/12 strains; cephalexin MICs rose from 2 to 8 μg/ml to 32 to 64 μg/ml after 4 to 18 days for 4/4 strains tested; mupirocin MICs rose from 0.25 to 32 μg/ml to 4 to >64 μg/ml after 4 to 14 days for 12/12 strains; and fusidic acid MICs rose from 0.5 to 8 μg/ml to 32 to >64 μg/ml after 4

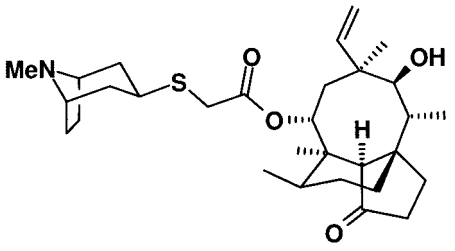

FIG. 1. Chemical structure of retapamulin.

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TABLE 1. Multipassage resistance selection results with *S. aureus*

Strain	Antibiotic[a]	Initial MIC (μg/ml)	Selected resistance		Retest MIC after 10 antibiotic-free subcultures								Detected resistance mechanism[b]
			MIC	No. of passages	REP	MUP	FA	CEP	ERY	LIN	VAN	Q/D	
SA040	REP	0.125	2	16	2	0.5	0.5	4	0.5	8	2	0.5	L3 mutations (G152D, D159Y)
	MUP	0.25	4	14	0.125	2	0.5	2	2	4	2	0.25	IRS mutation (G593V)
	FA	1	32	6	0.125	0.25	32	4	2	4	1	0.125	EF-G mutation (P404R)
	CEP	4	64	8	0.06	0.25	0.5	32	8	4	2	0.25	NT
	ERY	1	32	11	0.125	0.5	0.5	4	64	8	1	0.5	L4 mutations (Q67K, G69E)
	LIN	4	32	11	0.25	0.25	1	4	2	>32	2	0.25	—
	QD	0.25	2	24	0.125	0.5	0.5	4	16	8	2	2	NT
SA099	REP	0.06	0.5	14	0.5	0.25	0.5	4	8	8	1	0.5	—
	MUP	0.25	32	11	0.125	16	0.5	8	2	4	2	0.25	IRS mutations (G591D, G593D)
	FA	0.5	32	14	0.125	0.25	32	4	1	4	2	0.25	EF-G mutation (R464C)
	CEP	4	32	6	0.125	0.5	1	32	4	4	1	0.25	NT
	ERY	1	16	14	0.125	0.25	0.5	8	8	4	2	0.5	—
	LIN	4	>32	31	1	0.25	0.5	4	1	>32	2	0.5	—
	QD	0.25	2	14	0.125	0.25	0.5	4	8	4	2	2	NT
SA104	REP	0.125	1	20	1	0.5	0.5	32	4	8	1	0.5	L3 mutation (G152D)
	MUP	0.5	4	14	0.125	2	0.5	64	2	4	1	0.25	IRS mutation (Q709R)
	FA	0.5	32	4	0.125	0.5	32	32	2	4	1	0.125	EF-G mutation (F437Y)
	ERY	0.5	>32	17	0.125	0.25	0.25	64	>32	4	1	0.25	—
	LIN	4	32	23	0.25	0.25	0.5	64	2	32	2	0.25	—
	QD	0.25	2	20	0.125	0.25	0.5	4	32	4	1	4	NT
SA138	REP	0.06	1	19	0.5	0.25	1	>64	>32	8	1	0.5	L3 mutation (G152D)
	REP	0.06	16	50	16	NT	NT	NT	NT	NT	NT	NT	L3 mutations (G152D, D159Y)
	MUP	0.25	32	14	0.06	32	0.5	64	>32	4	2	0.25	NT
	FA	0.5	32	6	0.06	0.25	64	>64	>32	4	1	0.25	NT
	QD	0.25	2	16	0.125	0.5	0.5	>64	>32	4	2	1	NT
SA238	REP	0.06	0.5	20	0.5	0.25	0.5	64	2	4	1	0.25	L3 mutation (G144D)
	MUP	0.25	64	4	0.125	32	0.5	64	2	4	4	0.25	IRS mutation (V588F)
	FA	0.5	32	4	0.125	0.25	32	64	2	4	2	0.25	EF-G mutation (G452C)
	ERY	0.5	>64	14	0.06	0.5	1	>64	>64	4	2	0.5	L4 mutations (G69A, T70P)
	LIN	4	>32	22	0.25	0.25	0.5	64	2	32	2	0.25	—
	QD	0.25	2	25	0.125	0.25	1	>64	16	4	2	2	NT
SA248	REP	0.06	1	14	2	0.25	0.5	>64	>32	8	0.5	0.5	L3 mutations (G152D, D159Y)
	MUP	0.25	8	14	0.06	8	1	64	>64	4	0.5	0.25	NT
	FA	1	32	5	0.125	0.25	32	>64	>32	4	0.5	0.5	NT
	QD	0.25	4	14	0.25	0.5	0.5	>64	>32	8	1	8	NT
SA262	REP	0.06	1	16	2	0.25	0.5	>64	>32	4	2	1	L3 mutation (S153Y)
	MUP	0.25	>64	9	0.125	>64	0.5	>64	>32	4	2	1	NT
	FA	1	32	4	0.125	0.25	32	>64	>32	4	1	1	NT
	LIN	4	>32	22	2	0.25	0.5	64	>32	32	2	2	NT
	QD	0.5	>4	14	0.5	0.25	0.5	>64	>32	8	2	8	—
SA505	REP	0.06	0.5	16	0.5	0.25	0.5	4	1	4	4	0.5	L3 mutation (G152D)
	REP	0.06	4	50	4	NT	NT	NT	NT	NT	NT	NT	L3 mutations (G152D, D159Y)
	MUP	0.25	32	11	0.125	32	0.5	2	2	4	4	0.5	IRS mutation (I604N)
	FA	1	64	4	0.125	0.25	64	2	1	4	4	0.25	EF-G mutation (G452S)
	CEP	2	64	18	0.125	0.5	0.5	64	2	2	4	0.25	NT
	ERY	0.5	64	13	0.125	0.25	1	>64	64	4	2	0.5	—
	QD	0.25	2	18	0.125	0.25	0.5	4	4	4	8	1	NT
SA506	REP	0.06	0.5	15	0.5	0.5	0.5	>64	>32	2	2	0.5	L3 mutation (S158L)
	MUP	0.25	8	14	0.06	16	1	64	>32	4	8	1	NT
	FA	0.5	64	4	0.125	0.25	64	>64	>32	2	8	0.5	NT

	LIN	2	16	46	0.5	0.25	0.25	64	>64	16	4	1	NT
	QD	0.25	2	24	0.125	0.25	0.25	>64	>32	2	4	2	NT
SA508	REP	0.125	2	14	2	0.5	8	8	>32	2	8	0.5	L3 mutation (S158L)
	MUP	0.5	32	12	0.125	32	4	8	>64	2	4	0.25	NT
	FA	8	>64	4	0.25	0.5	>64	8	>32	2	8	0.25	NT
	CEP	8	32	4	0.125	0.5	8	16	>32	2	4	0.25	NT
	LIN	2	16	45	1	0.25	8	4	>32	16	4	0.5	NT
	QD	0.25	2	28	0.25	1	16	8	>32	2	4	2	NT
SA509	REP	0.06	2	14	2	32	0.5	>64	>32	4	2	1	L3 deletion (153S) and mutation (D159Y)
	MUP	32	>64	4	0.06	>64	0.5	>64	>32	4	>256	0.5	NT
	FA	0.5	64	4	0.06	32	64	>64	>32	2	2	0.5	NT
	LIN	2	16	28	1	32	0.5	>64	>32	32	1,024	1	NT
	VAN	2,048	2,048	7 (no change)	0.06	32	0.5	>64	>32	4	1,024	0.5	NT
	QD	0.5	4	18	0.06	>64	1	>64	>32	4	2	4	NT
SA510	REP	0.03	0.5	15	0.5	0.25	0.5	>64	>32	4	64	0.5	—
	REP	0.03	16	50	8	NT	NT	NT	NT	NT	NT	NT	L3 mutation (G155R)
	MUP	0.25	16	14	0.06	16	0.5	>64	>32	4	>64	0.5	NT
	FA	1	>64	4	0.06	0.125	>64	>64	>32	4	64	0.5	NT
	VAN	32	2,048[c]	13	0.06	0.125	0.5	>64	>32	4	1,024	0.5	NT
	QD	0.25	4	14	0.125	0.25	1	>64	>32	4	2	4	NT

[a] Abbreviations: REP, retapamulin; MUP, mupirocin; FA, fusidic acid; CEP, cephalexin; ERY, erythromycin; LIN, linezolid; VAN, vancomycin; and Q/D, quinupristin-dalfopristin.
[b] —, mechanism not detected; NT, not tested.
[c] Increase in MIC of more than fourfold (MIC = 256) obtained after two passages.

TABLE 2. Multipassage resistance selection results with *S. pyogenes*

Strain	Antibiotic[a]	Initial MIC (μg/ml)	Selected resistance		Retest MIC after 10 antibiotic-free subcultures							Detected resistance mechanism[b]
			MIC	No. of passages	Retapamulin	Quin/Dalfo	Cephalexin	Erythromycin	Linezolid	Mupirocin	Vancomycin	
2094	Retapamulin	0.016	0.125	26	0.125	0.5	0.5	4	1	0.25	0.5	—
	Quin/Dalfo	0.25	2	38	0.016	0.5	1	8	4	0.25	0.5	—
	Erythromycin	4	>64	4	0.03	0.5	1	16	2	0.25	0.5	NT
	Mupirocin	0.25	32	12	0.016	0.25	1	4	2	16	0.5	—
2042	Erythromycin	4	32	4	0.03	0.125	1	16	1	0.125	0.5	NT
	Mupirocin	0.125	>32	9	0.03	0.125	1	8	1	>32	0.5	—
2011	Erythromycin	16	>64	33	0.03	0.125	0.5	>64	1	0.125	0.5	NT
	Mupirocin	0.125	2	14	0.03	0.125	0.5	16	1	2	0.5	—
1714	Retapamulin	0.03	0.25	44	0.125	0.25	0.5	16	2	0.25	0.5	—
	Erythromycin	8	64	7	0.03	0.125	0.5	16	2	0.25	0.5	NT
	Mupirocin	0.25	>32	14	0.03	0.125	0.5	16	2	>32	0.5	—
2393	Erythromycin	16	>64	33	0.03	0.125	0.5	16	1	0.06	0.5	NT
	Mupirocin	0.06	1	14	0.03	0.125	0.5	16	2	0.5	0.5	—
2132	Erythromycin	0.06	0.5	24	0.03	0.125	0.5	0.5	1	0.125	0.5	—
	Mupirocin	0.06	4	14	0.016	0.125	0.5	0.06	0.5	4	0.5	—
2022	Erythromycin	0.125	1	50	0.03	0.125	0.5	1	2	0.125	0.5	NT
	Mupirocin	0.25	>2	20	0.016	0.125	0.5	0.125	1	2	0.5	—
237	Erythromycin	1	8	50	0.03	0.5	0.5	8	2	0.125	0.5	—
	Mupirocin	0.25	32	13	0.03	0.25	1	1	2	16	0.5	—
2368	Mupirocin	0.06	1	14	0.016	0.125	0.5	>64	1	0.5	0.5	—
2620	Retapamulin	0.016	0.125	48	0.125	0.125	0.5	>64	1	0.125	0.5	L3 mutation (N149K)
	Mupirocin	0.06	1	14	0.016	0.125	0.5	>64	1	0.5	0.5	—

[a] Quin/Dalfo, quinupristin-dalfopristin.
[b] —, mechanism not detected; NT, mechanisms not tested.

TABLE 3. Single-step-passage mutation frequencies for retapamulin and comparators in 12 *S. aureus* strains

Selecting drug	No. of strains tested	MIC range (μg/ml)	Range of spontaneous mutation frequencies at:	
			4× MIC	8× MIC
Retapamulin	12	0.03–0.25	5.0×10^{-6}–$<1.0 \times 10^{-9}$	$<5.0 \times 10^{-8}$–$<1.0 \times 10^{-9}$
Cephalexin	3	2–4	1.3×10^{-6}–2.0×10^{-7}	1.3×10^{-6}–3.0×10^{-8}
Erythromycin	5	0.5–1	2.2×10^{-6}–4.4×10^{-7}	2.2×10^{-6}–$<2.5 \times 10^{-9}$
Fusidic acid	12	2–8	$>1.5 \times 10^{-4}$–2.6×10^{-6}	7.5×10^{-5}–1.2×10^{-6}
Linezolid	12	2–4	3.9×10^{-7}–$<1.1 \times 10^{-9}$	$<3.3 \times 10^{-9}$–$<1.1 \times 10^{-9}$
Mupirocin	11	0.25–0.5	1.3×10^{-6}–6.7×10^{-9}	1.2×10^{-6}–6.7×10^{-10}
Quinupristin-dalfopristin	12	0.25–0.5	2.2×10^{-6}–$<1.4 \times 10^{-9}$	6.0×10^{-7}–$<1.0 \times 10^{-9}$
Vancomycin	10	1–8	$<1.0 \times 10^{-6}$–$<1.0 \times 10^{-9}$	$<1.0 \times 10^{-6}$–$<1.0 \times 10^{-9}$

to 14 days for 12/12 strains. Subculturing in the presence of vancomycin raised the vancomycin MIC of the Hershey VRSA strain from 32 to 2,048 μg/ml after 5 days (4). Among the retapamulin-selected clones, two with raised retapamulin MICs showed cross-resistance (defined as an ≥8-fold increase in MIC) to erythromycin. Five of eight strains with raised linezolid MICs had cross-resistance to retapamulin, and one quinupristin-dalfopristin-resistant clone had a raised retapamulin MIC.

The results of multipassage studies with *S. pyogenes* are presented in Table 2. Retapamulin MICs rose from 0.016 to 0.03 μg/ml (parent) to 0.125 to 0.25 μg/ml (clones) for 3/10 strains after 26 to 48 days, and quinupristin-dalfopristin MICs rose from 0.125 to 0.25 μg/ml (parent) to 2 μg/ml in 38 days for 1 strain. Erythromycin [*erm*(B)-positive strains excluded] MICs rose from 0.06 to 16 μg/ml to 0.5 to >64 μg/ml for 8/8 strains after 4 to 50 days, and mupirocin MICs rose from 0.06 to 0.25 μg/ml to 1 to >32 μg/ml after 9 to 20 days for 10/10 strains (Table 2). No cross-resistance was observed.

Pulsed-field gel electrophoresis confirmed that all selected strains were identical or closely related.

Changes in the L3 protein occurred in 10/12 *S. aureus* strains and also in strains SA138, SA505, and SA510 after prolonged selection for 50 days. Five strains had double mutations in L3, with retapamulin MICs of 1 to 16 μg/ml, and eight strains had single substitutions, with retapamulin MICs ranging from 0.5 to 16 μg/ml (Table 1). Of three *S. pyogenes* clones with raised retapamulin MICs of 0.125 to 0.5 μg/ml, one strain had an altered L3 protein (Table 2). Among selected macrolide-resistant *S. aureus* strains, two strains had double alterations in the L4 protein, with MICs raised from 1 to 32 and 0.5 to >64 μg/ml. In the other three *S. aureus* and two *S. pyogenes* macrolide-resistant strains, no changes in the L4 or L22 protein or 23S rRNA were observed (Tables 1 and 2). In five fusidic acid-resistant *S. aureus* clones, two had changes in the EF-G protein associated with MIC increases from 1 to 64 and 0.5 to 32 μg/ml (Table 1). No strain had *fusB.* Five mupirocin-resistant *S. aureus* clones had changes in the IRS protein, and no changes were observed in *S. pyogenes* IRS (Tables 1 and 2).

The results of single-passage studies with *S. aureus* (4× MIC and 8× MIC) can be seen in Table 3. Resistance frequencies ranging from 2× MIC to 8× MIC in *S. aureus* were as follows: for retapamulin, 6.7×10^{-5} to $<1.0 \times 10^{-9}$; for mupirocin, 1.0×10^{-5} to 6.7×10^{-10}; for fusidic acid, $>1.5 \times 10^{-4}$ to 1.2×10^{-6}; for cephalexin, 4.0×10^{-6} to 3.0×10^{-8}; for erythromycin, 2.2×10^{-6} to $<2.5 \times 10^{-9}$; for linezolid, 2.5×10^{-5} to $<1.1 \times 10^{-9}$; for vancomycin, $<1.0 \times 10^{-6}$ to $<1.0 \times 10^{-9}$; and for quinupristin-dalfopristin, 7.5×10^{-4} to $<1.0 \times 10^{-9}$.

In single-step passage studies with *S. pyogenes* (Table 4), single-step *S. pyogenes* mutation frequencies at concentrations from 1× MIC to 8× MIC were as follows: for retapamulin, 3.3×10^{-4} to $<1.7 \times 10^{-10}$; for mupirocin, 7.0×10^{-5} to $<1.2 \times 10^{-10}$; for cephalexin, 2.5×10^{-7} to $<3.3 \times 10^{-10}$; for erythromycin, $<1.2 \times 10^{-4}$ to $<2.9 \times 10^{-10}$; for linezolid, $<1.4 \times 10^{-9}$ to $<3.3 \times 10^{-10}$; for vancomycin, $<1.7 \times 10^{-5}$ to $<1.4 \times 10^{-10}$; and for quinupristin-dalfopristin, $<3.3 \times 10^{-5}$ to $<2.5 \times 10^{-10}$.

Of eight selected single-step-passaged *S. aureus* clones tested for L3 alterations, two had substitutions, either S158L or S161Y. No alteration in L3 protein was observed in seven randomly selected *S. pyogenes* strains.

Retapamulin had a lower frequency of spontaneous resistance against *S. aureus* than all other drugs tested except linezolid. For *S. pyogenes*, retapamulin had the lowest single-step resistance frequency compared to the other compounds. In our studies, retapamulin had the lowest MICs by multipassage for *S. aureus* and *S. pyogenes*. Although clones with increased retapamulin MICs were obtained with all 12 *S. aureus*

TABLE 4. Single-step-passage mutation frequencies for retapamulin and comparators in 10 *S. pyogenes* strains

Selecting drug	No. of strains tested	MIC range (μg/ml)	Range of spontaneous mutation frequencies at:	
			4× MIC	8× MIC
Retapamulin	10	0.016–0.03	$<8.3 \times 10^{-10}$–$<1.7 \times 10^{-10}$	$<8.3 \times 10^{-10}$–$<1.7 \times 10^{-10}$
Cephalexin	10	0.5–1	$<1.2 \times 10^{-9}$–$<3.3 \times 10^{-10}$	$<1.2 \times 10^{-9}$–$<3.3 \times 10^{-10}$
Erythromycin	8	0.06–16	$<6.7 \times 10^{-5}$–$<7.7 \times 10^{-10}$	$<1.2 \times 10^{-8}$–$<2.9 \times 10^{-10}$
Linezolid	10	1–2	$<1.4 \times 10^{-9}$–$<3.3 \times 10^{-10}$	$<1.4 \times 10^{-9}$–$<3.3 \times 10^{-10}$
Mupirocin	10	0.06–0.25	1.5×10^{-6}–$<1.0 \times 10^{-9}$	1.0×10^{-6}–$<1.2 \times 10^{-10}$
Quinupristin-dalfopristin	10	0.125–0.25	$<3.3 \times 10^{-9}$–$<2.5 \times 10^{-10}$	$<3.3 \times 10^{-9}$–$<2.5 \times 10^{-10}$
Vancomycin	10	0.5–1	$<3.3 \times 10^{-9}$–$<1.4 \times 10^{-10}$	$<2.0 \times 10^{-9}$–$<1.4 \times 10^{-10}$

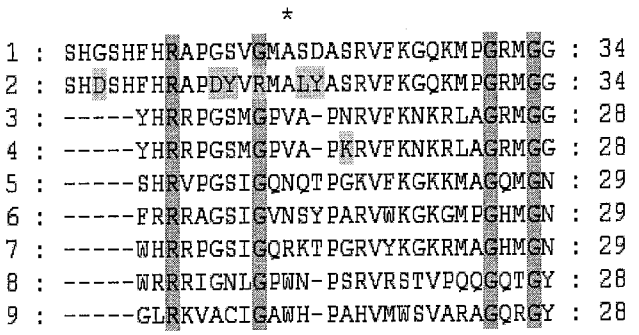

FIG. 2. Mutations found in *S. aureus* and *S. pyogenes* multi- and single-passage mutants with reduced susceptibilities to retapamulin. The figures shows an alignment of various ribosomal protein L3 sequences in the regions flanking the mutations. The positions of the characterized mutations in *S. aureus* and *S. pyogenes* mutants are highlighted in a shade of gray; position 149 in *E. coli* and analogous positions in other sequences crucial for developing resistance are indicated with an asterisk; and positions of amino acid identity are highlighted in dark gray. Sequences: 1, parental *S. aureus* strain starting from amino acid position 142; 2, selected *S. aureus* mutant starting from position 142; 3, parental *S. pyogenes* strain starting from position 137; 4, selected *S. pyogenes* mutant starting from position 137; 5, *E. coli* starting from position 139; 6, *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* starting from position 139; 7, *Deinococcus radiodurans* starting from position 134; 8, *Haloarcula marismortui* starting from position 232; and 9, *Saccharomyces cerevisiae* starting from position 245.

strains, the highest retapamulin MIC was only ≤2 μg/ml. While no genetic evidence could be found, cross-resistance between linezolid-, quinupristin-dalfopristin-, and erythromycin-resistant clones and clones with raised retapamulin MICs may represent the ribosomal targeting of all four drugs, albeit at different target sites (8).

Previous investigations (2, 9) have revealed that pleuromutilin resistance develops in a slow, stepwise manner associated with a change of Asn to Asp at position 149 in ribosomal protein L3 (*Escherichia coli* numbering). Our study revealed few mutations which affected retapamulin susceptibility, and these mutations appeared in a very conservative region whose amino acid residues are very well preserved in different organisms (Fig. 2). *S. aureus* multipassage mutants had double and single amino acid substitutions or no alterations in the L3 protein (Table 1). Double and single mutations were associated with MICs of 1 to 2 μg/ml and 0.5 to 2 μg/ml, respectively. Three clones selected for prolonged selection for 50 days (parent strains SA138, SA505, and SA510) yielded mutants with retapamulin MICs ranging from 4 to 16 μg/ml, each with an additional mutation in L3 (Table 1). This suggests that resistance development in retapamulin is a slow, multistep process and that mutations accumulate gradually in the presence of drug pressure, similar to the case for tiamulin (2). Kosowska and coworkers also observed that prolonged selection led to high rates of resistance development for linezolid, quinupristin-dalfopristin, and telithromycin (6). It is unknown how the other comparators in this study would perform if subjected to similar prolonged selection. Only one strain of three multipassage *S. pyogenes* clones had an amino acid substitution in L3 (Table 2).

Mechanisms of resistance for macrolides and fusidic acid (Tables 1 and 2) have been described before. For mupirocin, the new mutations G591D, G593D, and I604N are within the essential binding site, but the significance of the Q709R mutation is unclear (Table 1).

Our results suggest that retapamulin has a low potential for resistance development which is less than or comparable to that of mupirocin and fusidic acid, two topical agents commonly used for the treatment of uncomplicated *S. aureus* and *S. pyogenes* skin and soft tissue infections. This hypothesis requires validation by experimental and clinical testing.

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