

## Resistance Studies with Daptomycin

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**We studied the in vitro emergence of resistance to daptomycin using three methods: spontaneous resistance incidence, serial passage in the presence of increasing drug concentrations, and chemical mutagenesis. No spontaneously resistant mutants were obtained for any organism tested ( $<10^{-10}$  for *Staphylococcus aureus*,  $<10^{-9}$  for *Staphylococcus epidermidis*,  $<10^{-9}$  for *Enterococcus faecalis*,  $<10^{-9}$  for *Enterococcus faecium*,  $<10^{-8}$  for *Streptococcus pneumoniae*). Population analysis demonstrated that bacterial susceptibility to daptomycin is heterogeneous. Assay results were sensitive to calcium concentration and culture density, both of which can affect apparent resistance rates. Stable *S. aureus* mutants were isolated by both serial passage in liquid media and chemical mutagenesis. The daptomycin MICs for these isolates were 8- to 32-fold higher than for the parental strain. Many mutants with high MICs ( $>12.5$   $\mu\text{g/ml}$ ) had significant growth defects but did not display phenotypes typical of *S. aureus* small colony variants. The voltage component ( $\Delta\psi$ ) of the bacterial membrane potential was increased in three independent resistant isolates. In vivo data showed that some daptomycin-resistant mutants had lost significant virulence. For other mutants, the degree of in vitro resistance was greater than the change in in vivo susceptibility. These results suggest that infection with some daptomycin-resistant organisms may still be easily treatable.**

Daptomycin is a novel lipopeptide antibiotic with potent bactericidal activity in vitro against most clinically important gram-positive pathogens, including vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus*, glycopeptide-intermediate *S. aureus*, coagulase-negative staphylococci, and penicillin-resistant *Streptococcus pneumoniae* (12, 19, 25, 26, 28). The mechanism of action, while not yet fully elucidated, appears to involve disruption of bacterial plasma membrane function (1–5). Daptomycin was efficacious in phase 2 clinical trials in skin and soft tissue infection and bacteremia (28). Additional phase 2 and 3 clinical studies are ongoing.

Results from in vitro studies and clinical trials indicate that resistance to daptomycin is rare (16, 28; P. Courvalin, personal communication; G. W. Kaatz, personal communication). However, one group has reported relatively high resistance rates in vitro (18). In an effort to resolve this discrepancy, we determined spontaneous resistance rates and analyzed technical factors influencing those rates. In addition, the possible utility of resistant mutants in pinpointing daptomycin's mechanism of action prompted us to isolate and analyze resistant organisms.

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### MATERIALS AND METHODS

**Strains, media, and antibiotics.** Bacteria were propagated at 37°C. Staphylococci were grown in Mueller-Hinton broth (MHB; Becton, Dickinson, Cockeysville, Md.), enterococci in brain heart infusion (Becton, Dickinson), and *S. pneumoniae* in Todd-Hewitt broth plus 5% horse serum. MIC testing was performed according to NCCLS guidelines for broth microdilution (23) except that all cultures were grown at 37°C. In addition, daptomycin MICs were determined using MHB supplemented with 50 mg of  $\text{Ca}^{2+}$  per liter (MHBC). Modifications

for determining nisin MICs were made as previously described (27). Vancomycin, ampicillin, gentamicin, and nisin were purchased from Sigma Chemical Company (St. Louis, Mo.). Complete defined media was obtained from JRH Biosciences (Lenexa, Kans.).

**Heterogeneity assay.** Heterogeneous susceptibility was measured based on methods developed by de Lencastre et al. (8). Overnight cultures were plated at dilutions (made in MHBC) ranging from  $10^0$  to  $10^{-7}$  on Mueller-Hinton agar (MHA) or MHA plus 50 mg of  $\text{CaCl}_2$  per liter containing twofold dilutions of daptomycin over the range 0.125 to 64  $\mu\text{g/ml}$ .

**Serial-passage mutagenesis.** On day 1, MHBC containing daptomycin at 0.25, 0.5, 1, or 2 times the MIC was inoculated with *S. aureus* (strain Sa42) from a single colony. Cultures were incubated overnight at 37°C with shaking. From the highest concentration that supported growth, cultures were diluted 1:10,000 into fresh media plus daptomycin at twofold dilutions. This process was continued for 21 days or until three successive cultures failed to show any decrease in susceptibility. Putative mutants were colony purified for three generations on MHA, prior to further characterization.

**Chemical mutagenesis.** *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG; Sigma) mutagenesis was performed using methods developed by Miller (21) and Chatterjee (7). Mid-exponential cultures of *S. aureus* (ca.  $3 \times 10^8/\text{ml}$ ) were washed twice with 0.03 M phosphate buffer (pH 7.0) and resuspended in 0.05 M Tris-acetate (pH 6.0)–10 mM  $\text{MgCl}_2$  (TAM). Then, 50  $\mu\text{g}$  of MNNG per ml was added, and cells were incubated at 37°C to achieve 50% killing (30 min). Bacteria were washed three times with TAM, incubated overnight at 37°C in MHBC, and plated on selective media for 48 h. Putative mutants were purified on MHA prior to further characterization. To ensure that all mutants were independent, only one mutant was isolated from each selection plate.

**Virulence titration and daptomycin protection studies.** For virulence titrations, five CD-1 female mice per group (Charles River Laboratories, Wilmington, Mass.) were inoculated intraperitoneally (i.p.) with 10-fold dilutions ranging from  $10^5$  to  $10^8$  CFU in phosphate-buffered saline (PBS) containing 6% hog gastric mucin (Sigma) or PBS-mucin only. The 50 and 100% lethal doses ( $\text{LD}_{50}$  and  $\text{LD}_{100}$ ) were calculated based on the number of mice surviving after 7 days. For daptomycin protection studies, mice were inoculated i.p. with two times the  $\text{LD}_{100}$ . Daptomycin was dissolved in PBS and administered subcutaneously immediately and at 4 h postinfection. The 50% protective dose ( $\text{PD}_{50}$ ) was calculated via the method of probits (9) from the number of mice surviving after 7 days.

**Membrane potential measurements.** Accumulation of the membrane-permeant anion tetraphenyl phosphonium bromide (TPP) was used to measure the voltage component of the bacterial membrane potential ( $\Delta\psi$ ) (15, 17, 20). Overnight cultures of *S. aureus* were diluted 1:1,000 into fresh MHBC, grown for 2 h at 37°C, and then shifted to room temperature and grown for an additional 2 h. Samples were collected for protein determination via the Coomassie protein

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assay (Pierce Chemical, Rockford, Ill.). Four 4-ml aliquots were withdrawn from the remaining culture. One was treated with 8% butanol for 10 min prior to labeling to produce a background value. Cells were labeled with 10  $\mu\text{M}$  [ $^3\text{H}$ ]TPP (29 Ci/mmol; Moravsek Biochemicals, Brea, Calif.) for 10 min, then filtered through GF/C filters (Whatman, Mardstone, United Kingdom). Filters were washed with 0.15 M NaCl and dried; the radioactivity was determined in a liquid scintillation counter. The Nernst equation,  $\Delta\psi = [-(RT/F)\ln([TPP^+]_{in}/[TPP^+]_{out})]$ , and a 4.2- $\mu\text{l}$  intracellular volume per mg of protein (determined as previously described [24]) were used to calculate  $\Delta\psi$ .

## RESULTS AND DISCUSSION

**Spontaneous resistance testing.** Previous studies of daptomycin resistance have yielded conflicting results, with rates varying by up to 3 orders of magnitude for some species (16, 18, G. W. Kaatz, personal communication; P. Courvalin, personal communication; protocol summary report no. B8B FP 2001 [Lilly Research Laboratories, Greenfield, Ind. 1986]) To resolve this discrepancy, we carried out additional resistance studies. Initially, we isolated colonies on MHA containing daptomycin at concentrations above the MIC. However, these isolates were not resistant. They failed to grow when restreaked to MHA containing daptomycin and, following purification on MHA, yielded MICs identical to those for the parent strain. The appearance of these colonies indicates that susceptibility to daptomycin is heterogeneous, as has been described for methicillin and vancomycin (6, 13, 14). This is demonstrated in Fig. 1A. Population heterogeneity may contribute to differences in apparent resistance rates.

Experimental factors that increase population heterogeneity could increase apparent resistance rates. The activity of daptomycin is calcium dependent (10), and the levels of free calcium in MHA are variable (23). We determined the effect of calcium levels on population heterogeneity. As shown in Fig. 1B, the addition of 1 mM  $\text{CaCl}_2$  to the test agar results in homogeneous susceptibility and eliminates the appearance of falsely resistant colonies. Differences in calcium levels may have contributed to past differences in spontaneous resistance rates. Consistent with this idea, it has previously been demonstrated that resistance rates in liquid media increase as calcium levels decrease (29).

Based on the studies described above, we performed all resistance testing by plating overnight cultures on MHA supplemented with 1 mM  $\text{CaCl}_2$  and daptomycin at 8 times the MIC. Eight laboratory (American Type Culture Collection, Rockville, Md.) and eight clinical isolates (provided by D. Snyderman, New England Medical Center, Boston, Mass.) were tested. No spontaneously resistant mutants were obtained, yielding resistance rates of  $<10^{-10}$  for *S. aureus* ( $n = 4$  strains tested),  $<10^{-9}$  for *Staphylococcus epidermidis* ( $n = 4$ ),  $<10^{-9}$  for *Enterococcus faecalis* ( $n = 4$ ),  $<10^{-9}$  for *E. faecium* ( $n = 2$ ), and  $<10^{-9}$  for *S. pneumoniae* ( $n = 2$ ). These values support earlier reports of low resistance rates (16; G. W. Kaatz, personal communication; P. Courvalin, personal communication). As described above, earlier reports of higher rates of spontaneous resistance (18) may reflect variations in calcium levels in the test media and the effects of population heterogeneity.

**Serial passage and chemical mutagenesis.** Two methodologies were employed to obtain daptomycin-resistant *S. aureus*. One mutant (Sa278) was obtained via 21-day serial passage in liquid culture. (Pulsed-field gel electrophoresis fingerprinting confirms that Sa278 is a derivative of the parent strain [data

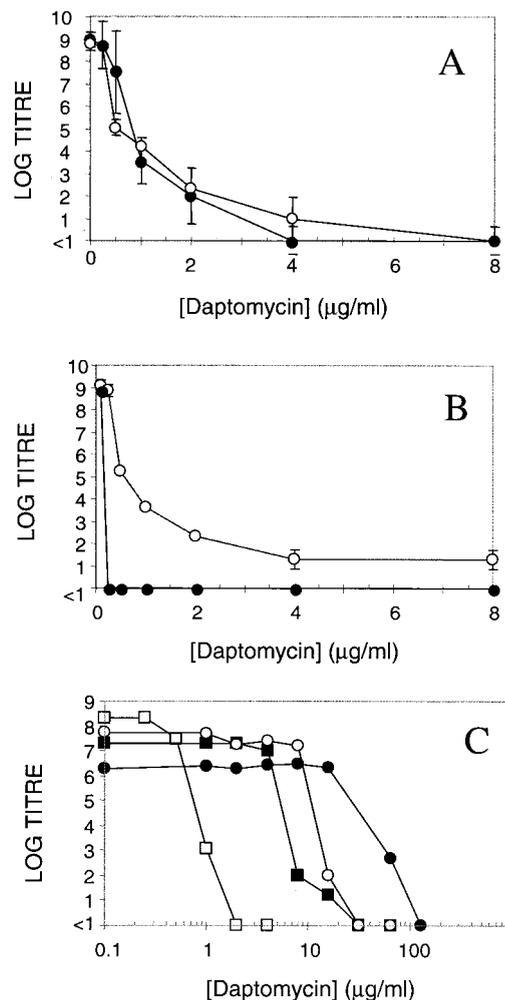


FIG. 1. Susceptibility to daptomycin is heterogeneous in *S. aureus*. (A) Susceptibility is heterogeneous in both laboratory (Sa42) and clinical (Sa675) isolates ( $\bullet$ , Sa42;  $\circ$ , Sa675). Data are mean values  $\pm$  the standard deviation. (B) Heterogeneous susceptibility in Sa42 is affected by calcium ( $\circ$ , MHA [MIC = 1  $\mu\text{g/ml}$ ];  $\bullet$ , MHA plus 50 mg of  $\text{Ca}^{2+}$  per liter [MIC = 0.3  $\mu\text{g/ml}$ ]). Data are mean values  $\pm$  the standard deviation. (C) Heterogeneous susceptibility is observed in daptomycin-resistant mutants ( $\square$ , Sa42 [parent];  $\blacksquare$ , Sa278 [class 1];  $\circ$ , Sa295 [class 2];  $\bullet$ , Sa291 [class 3]). Representative data are shown.

not shown]). Eleven additional independent mutants (Sa284 to Sa293 and Sa295) with varied colony morphology, growth phenotypes, and antibiotic resistance were isolated via chemical mutagenesis. The phenotypes of all mutants are summarized in Table 1. MIC increases were relatively modest (8- to 32-fold), consistent with the findings of previous serial passage experiments (18, 22). Daptomycin resistance is stable during repeated passage in the absence of continued drug selection (data not shown).

Mutants were grouped into three classes based on their growth phenotypes and antibiotic susceptibility ( $n \geq 3$  for all values). Class 1 mutants (including Sa278) exhibited normal growth rates and were cross-resistant to the peptide antibiotic nisin. Class 2 mutants had reduced growth rates on MHA, poor or no growth in complete defined media (CDM), and no antibiotic cross-resistance. Class 3 mutants showed severe growth

TABLE 1. Growth phenotypes and MICs of mutants obtained via serial passage or chemical mutagenesis<sup>a</sup>

Strain	Class	Growth phenotype on:				MIC ( $\mu\text{g/ml}$ )				
		Liquid media (OD <sub>600</sub> )		Solid media (colony size)		Dapto	Vanc	Amp	Nisin	Gent
		MHB	CDM	MHA	SCV					
Sa42	WT	6.05	6.6	+++	+++	0.78	0.78	6.25	3.125	0.78
Sa278 <sup>b</sup>	1	5.93	7.87	+++	+++	12.5	1.56	6.25	25	0.78
Sa284	1	4.97	6.38	+++	+++	6.25	1.56	6.25	25	0.78
Sa285	2	1.23	NG	++	++	6.25	0.78	3.13	25	0.39
Sa286	3	0.63	NG	+	+	6.25	0.39	ND	6.25	0.78
Sa287	1	3.89	6.59	+++	+++	6.25	1.56	6.25	25	1.56
Sa288	1	5.34	8.17	+++	+++	6.25	0.78	ND	25	0.78
Sa289	3	0.69	0.45	+	+	12.5	0.39	ND	3.125	ND
Sa290	3	0.91	NG	+	+	12.5	0.39	ND	ND	1.56
Sa291	3	0.71	NG	+	+	25	0.39	1.56	3.125	0.78
Sa292	3	1.37	NG	+	+	12.5	0.78	ND	ND	0.78
Sa293	3	0.43	NG	+	+	12.5	0.78	ND	ND	1.56
Sa295	2	1.28	NG	++	++	12.5	1.56	6.25	6.25	<0.1

<sup>a</sup> Abbreviations: OD<sub>600</sub>, optical density at 600 nm; SCV, MHA plus hemin, thiamine, and menadione; Dapto, daptomycin; Vanc, vancomycin; Amp, ampicillin; Gent, gentamicin; WT, wild type; NG, no growth, 16 h; ND, not determined.

<sup>b</sup> Obtained via serial passage techniques.

defects in MHB and CDM, poor or no pigmentation on MHA, and no antibiotic cross-resistance. Poorly growing mutants (classes 2 and 3) were not rescued by the addition of menadione, hemin, and thiamine to MHA and were susceptible to gentamicin, suggesting that they were not classic *S. aureus* small colony variants. All classes of mutants retained the heterogeneous susceptibility of the parental strain (Fig. 1C) and exhibited homogeneous susceptibility in the presence of 1 mM Ca<sup>2+</sup> (data not shown). Daptomycin remained bactericidal at 8 times the MIC against all classes of mutants (data not shown). None of the mutants demonstrated cross-resistance to vancomycin or ampicillin, a finding consistent with known differences in the mechanism of action of these drugs.

**In vivo studies.** Selected mutants were tested for virulence in an acute murine IP infection model (Table 2). Sa278 and its daptomycin-sensitive parent had comparable LD<sub>50</sub> values, consistent with similar growth rates in vitro. The PD<sub>50</sub> for the resistant strain was <5-fold higher than that of the parent. This contrasts with a 16-fold increase in daptomycin MIC for Sa278 (verified by repeated testing [ $n = 40$ ]) and suggests that some in vitro resistant strains will still be susceptible in vivo. The LD<sub>50</sub> for a class 2 mutant was >20-fold higher (i.e., no animals died at the highest inoculum tested), meaning that a PD<sub>50</sub> could not be determined. This loss of virulence may be a consequence of the growth defects observed in vitro; alternatively, this strain may be defective in the production of critical virulence factors. Whether these defects are a consequence of the acquisition of daptomycin resistance remains to be determined.

TABLE 2. MIC, LD<sub>50</sub>, and PD<sub>50</sub> values for selected strains<sup>a</sup>

Strain	Class	MIC ( $\mu\text{g/ml}$ )	LD <sub>50</sub> (CFU)	PD <sub>50</sub> (mg/kg)
Sa42	WT	0.78	$4 \times 10^6$	0.22
Sa278	1	12.5	$1.3 \times 10^6$	1.03
Sa295	2	12.5	$>10^8$	ND

<sup>a</sup> Abbreviations: WT, wild type; ND, not determined.

**Membrane potential.** Daptomycin's mechanism of action is not clearly defined. Earlier research has suggested two likely targets: dissipation of the bacterial membrane potential (1) and inhibition of lipoteichoic acid synthesis (4, 5). We measured the membrane potential in the wild type and in selected class 1 mutants via the uptake of the permeant anion tetraphenyl phosphonium. As shown in Table 3, the voltage difference across the cytoplasmic membrane of daptomycin-resistant mutants is increased compared to the parent strain. This may be consistent with the membrane potential as the primary target. Higher levels of daptomycin may be necessary to fully dissipate the membrane potential in these mutants, accounting for their higher MICs. This may also account for their cross-resistance to nisin, which is known to dissipate membrane potential.

**Conclusion.** Antibiotic resistance is a growing worldwide problem, requiring the development of novel agents to combat resistant organisms. The low spontaneous resistance rates, limited increases in MICs during serial passage, and ease of treatment of resistant isolates are encouraging for the clinical development of daptomycin. The low resistance potential in vitro may provide daptomycin with a low clinical resistance rate, prolonging the drug's utility. The higher resistance rates in one earlier report may be attributable to variances in experimental protocols. Preliminary analysis of resistant mutants supports alteration of membrane potential as a mechanism of action for daptomycin.

TABLE 3.  $\Delta\psi$  in class 1 mutants<sup>a</sup>

Strain	Class	Trial 1		Trial 2	
		$\Delta\psi$ (mV)	% Change	$\Delta\psi$ (mV)	% Change
Sa42	WT	$-112 \pm 3$		$-127 \pm 18$	
Sa278	1	$-162 \pm 7$	45	$-174 \pm 20$	37
Sa284	1	$-147 \pm 8$	31	$-173 \pm 13$	36
Sa287	1	ND		$-161 \pm 6$	27

<sup>a</sup> Membrane potential was determined as described in the text. Data are the average of duplicate samples.

<sup>b</sup> Abbreviations: WT, wild type; ND, not determined.

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